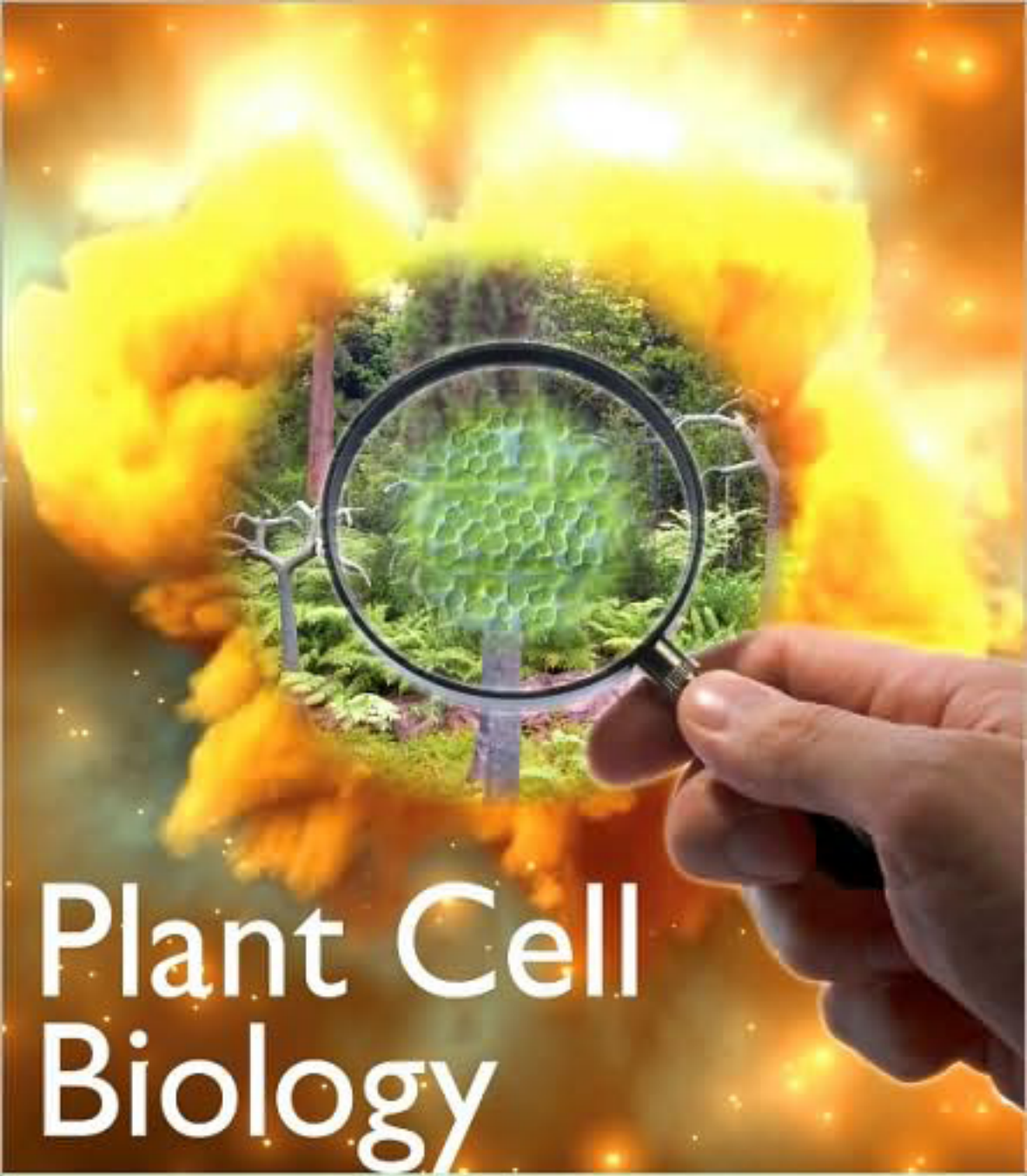

Plant Cell Biology

From Astronomy to Zoology

Randy Wayne

AP

Preface

This book is in essence the lectures I give in my plant cell biology course at Cornell University. Heretofore, the lecture notes have gone by various titles, including "Cell La Vie," "The Book Formerly Known as Cell La Vie," "Molecular Theology of the Cell," "Know Thy Cell" (with apologies to Socrates), "Cell This Book" (with apologies to Abbie Hoffman), and "Impressionistic Plant Cell Biology." I would like to take this opportunity to describe this course. It is a semester-long course for undergraduate and graduate students. Since the undergraduate biology majors are required to take genetics, biochemistry, and evolution as well as 1 year each of mathematics and physics, and 2 years of chemistry, I have done my best to integrate these disciplines into my teaching. Moreover, many of the students also take plant anatomy, plant physiology, plant growth and development, plant taxonomy, plant biochemistry, plant molecular biology, and a variety of courses that end with the suffix "-omics"; I have tried to show the connections between these courses and plant cell biology. Nonbotanists can find a good introduction to plant biology in Mauseth (2009) and Taiz and Zeiger (2006).

Much of the content has grown over the past 20 years from the questions and insights of the students and teaching assistants who have participated in the class. The students' interest has been sparked by the imaginative and insightful studies done by the worldwide community of cell biologists, which I had the honor of presenting.

I have taken the approach that real divisions do not exist between subject areas taught in a university, but only in the state of mind of the teachers and researchers. With this approach, I hope that my students do not see plant cell biology as an isolated subject area, but as an entrée into every aspect of human endeavor. One of the goals of my course is to try to reestablish the connections that once existed between mathematics, astronomy, physics, chemistry, geology, philosophy, and biology. It is my own personal attempt, and it is an ongoing process. Consequently, it is far from complete. Even so, I try to provide the motivation and resources for my students to weave together the threads of these disciplines to create their own personal tapestry of the cell from the various lines of research.

Recognizing the basic similarities between all living eukaryotic cells (Quekett, 1852, 1854; Huxley, 1893), I discuss both animal and plant cells in my course. Although the examples are biased toward plants (as they should be in a plant cell biology course), I try to present the best example to illustrate a process and sometimes the best examples are from animal cells. I take the approach used by August Krogh (1929); that is, there are many organisms in the treasure house of nature and if one respects this treasure, one can find an organism created to best illuminate each principle! I try to present my course in a balanced manner, covering all aspects of plant cell biology without emphasizing any one plant, organelle, molecule, or technique. I realize, however, that the majority of papers in plant cell biology today are using a few model organisms and "-omic" techniques. My students can learn about the successes gained though this approach in a multitude of other courses. I teach them that there are other approaches.

Pythagoras believed in the power of numbers, and I believe that the power of numbers is useful for understanding the nature of the cell. In my class, I apply the power of numbers to help relate quantities that one wishes to know to things that can be easily measured (Hobson, 1923; Whitehead, 1925; Hardy, 1940; Synge, 1951, 1970; Feynman, 1965; Schrödinger, 1996). For example, the area of a rectangle is difficult to measure. However, if one knows its length and width, and the relation that area is the product of length and width, the area can be calculated from the easily measurable quantities. Likewise, the circumference or area of a circle is relatively difficult to measure. However, if one measures the diameter and multiplies it by π, or the square of the diameter by $\pi/4$, one can easily obtain the circumference and area, respectively. In the same way, one can easily estimate the height of a tree from easily measurable quantities if one understands trigonometry and the definition of tangent.

My teaching was greatly influenced by a story that Hans Bethe told at a meeting at Cornell University commemorating the 50th anniversary of the chain reaction produced by Enrico Fermi. Bethe spoke about the difference between his graduate adviser, Arnold Sommerfeld, and his postdoctoral adviser, Enrico Fermi. He said that, in the

field of atomic physics, Sommerfeld was a genius at creating a mathematical theory to describe the available data. Sommerfeld's skill, however, depended on the presence of data. Fermi, on the other hand, could come up with theories even if the relevant data were not apparent. He would make estimates of the data from first principles. For example, he estimated the force of the first atomic bomb by measuring the distance small pieces of paper flew as they fell to the ground during the blast in Alamogordo. Knowing that the force of the blast diminished with the square of the distance from the bomb, Fermi estimated the force of the bomb relative to the force of gravity. Within seconds of the blast, he calculated the force of the bomb to be approximately 20 kilotons, similar to which the expensive machines recorded (Fermi, 1954; Lamont, 1965).

In order to train his students to estimate things that they did not know, Fermi would ask them, "How many piano tuners are there in Los Angeles?" After they looked befuddled, he would say, "You can estimate the number of piano tuners from first principles! For example, how many people are there in Los Angeles? One million? What percentage has pianos? Five percent? Then there are 50,000 pianos in Los Angeles. How often does a piano need to be tuned? About once a year? Then 50,000 pianos need to be tuned in a year. How many pianos can a piano tuner tune in a day? Three? Then one tuner must spend 16,667 days a year tuning pianos. But since there are not that many days in a year, and he or she probably only works 250 days a year, then there must be around 67 piano tuners in Los Angeles."

My students apply the power of numbers to the study of cellular processes, including membrane transport, photosynthesis, and respiration, in order to get a feel for these processes and the interconversions that occur during these processes between different forms of energy. My students apply the power of numbers to the study of cell growth, chromosome motion, and membrane trafficking in order to be able to postulate and evaluate the potential mechanisms involved in these processes, and the relationships between these processes and the bioenergetic events that power them. Becoming facile with numbers allows the students to understand, develop, and critique theories. "As the Greek origin of the word [theory] implies, the Theory is the true *seeing* of things—the insight that should come with healthy sight" (Adams and Whicher, 1949).

Using the power of numbers to relate seemingly unrelated processes, my students are able to try to analyze all their conclusions in terms of first principles. They also learn to make predictions based on first principles. The students must be explicit in terms of what they are considering to be facts, what they are considering to be the relationship between facts, and where they are making assumptions. This provides a good entrée into research, because the facts must be refined and the assumptions must be tested (East, 1923).

I do not try to introduce any more terminology in my class than is necessary, and I try to explain the origin of each term. Some specialized terms are essential for precise communication in science just as it is in describing love and beauty. However, some terms are created to hide our ignorance, and consequently prevent further inquiry, because something with an official-sounding name seems well understood (Locke, 1824; Hayakawa, 1941; Rapoport, 1975). In Goethe's (1808) "Faust Part One," Mephistopheles says: "For at the point where concepts fail. At the right time a word is thrust in there. With words we fitly can our foes assail." Francis Bacon (1620) referred to this problem as the "Idols of the Marketplace." Often we think we are great thinkers when we answer a question with a Greek or Latin word. For example, if I am asked, "Why are leaves green?" I quickly retort, "Because they have chlorophyll." The questioner is satisfied, and says "Oh." The conversation ends. However, chlorophyll is just the Greek word for green leaf. Thus, I really answered the question with a tautology. I really said "Leaves are green because leaves are green" and did not answer the question at all. It was as if I was reciting a sentence from scripture, which I had committed to memory without giving it much thought. However, I gave the answer in Greek, and with authority … so it was a scientific answer.

In "An Essay Concerning Human Understanding," John Locke (1824) admonished that words are often used in a nonintellectual manner. He wrote,

> *… he would not be much better than the Indian before-mentioned, who, saying that the world was supported by a great elephant, was asked what the elephant rested on; to which his answer was, a great tortoise. But being again pressed to know what gave support to the broad-backed tortoise, replied, something he knew not what. And thus here, as in all other cases where we use words without having clear and distinct ideas, we talk like children; who being questioned what such a thing is, which they know not, readily give the satisfactory answer, that it is something; which in truth signifies no more, when so used either by children or men, but that they know not what; and that the thing that they pretend to know and talk of is what they have no distinct idea of at all, and so are perfectly ignorant of it, and in the dark.*

Sometimes terms are created to become the shibboleths of a field, and sometimes they are created for political reasons, financial reasons, or to transfer credit from someone who discovers something to someone who renames it (Agre et al., 1995). Joseph Fruton (1992) recounted (and translated) a story of a conversation with a famous chemist in Honoré de Balzac's La Peau de Chagrin:

> *"Well, my old friend," said Planchette upon seeing Japhet seated in an armchair and examining a precipitate, "How goes it in chemistry?"*
>
> *"It is asleep. Nothing new. The Académie has in the meantime recognized the existence of salicine. But salicine, asparagine, vauqueline, digitaline are not new discoveries."*

"If one is unable to produce new things," said Raphael, "it seems that you are reduced to inventing new names."

"That is indeed true, young man."

I teach plant cell biology with a historical approach and teach "not only of the fruits but also of the trees which have borne them, and of those who planted these trees" (Lenard, 1906). This approach also allows them to understand the origins and meanings of terms; to capture the excitement of the moment of discovery; to elucidate how we, as a scientific community, know what we know; and it emphasizes the unity and continuity of human thought (Haldane, 1985). I want my students to become familiar with the great innovators in science and to learn their way of doing science (Wayne and Staves, 1998, 2008). I want my students to learn how the scientists we learn about choose and pose questions, and how they go about solving them. I do not want my students to know just the results and regurgitate those results on a test (Szent-Györgyi, 1964; Farber, 1969). I do not want my students to become scientists who merely repeat on another organism the work of others. I want my students to become like the citizens of Athens, who according to Pericles "do not imitate—but are a model to others." Whether or not my students become professional cell biologists, I hope they forever remain amateurs and dilettantes in terms of cell biology. That is, I hope that I have helped them become "one who loves cell biology" and "one who delights in cell biology" (Chargaff, 1986)—not someone who cannot recognize the difference between a pile of bricks and an edifice (Forscher, 1963), not someone who sells "buyology" (Wayne and Staves, 2008), and not someone who sells his or her academic freedom (Rabounski, 2006; Apostol, 2007).

Often people think that a science course should teach what is new, but I answer this with an amusing anecdote told by Erwin Chargaff (1986): "Kaiser Wilhelm I of Germany, Bismark's old emperor, visited the Bonn Observatory and asked the director: 'Well, dear Argelander, what's new in the starry sky?' The director answered promptly: 'Does your Majesty already know the old?' The emperor reportedly shook with laughter every time he retold the story."

According to R. John Ellis (1996),

It is useful to consider the origins of a new subject for two reasons. First, it can be instructive; the history of science provides sobering take-home messages about the importance of not ignoring observations that do not fit the prevailing conceptual paradigm, and about the value of thinking laterally, in case apparently unrelated phenomena conceal common principles. Second, once a new idea has become accepted there is often a tendency to believe that it was obvious all along—hindsight is a wonderful thing, but the problem is that it is never around when you need it!

The historical approach is necessary, in the words of George Palade (1963), "to indicate that recent findings and present concepts are only the last approximation in a long series of similar attempts which, of course, is not ended."

I teach my students that it is important to be skeptical when considering old as well as new ideas. According to Thomas Gold (1989),

New ideas in science are not always right just because they are new. Nor are the old ideas always wrong just because they are old. A critical attitude is clearly required of every scientist. But what is required is to be equally critical to the old ideas as to the new. Whenever the established ideas are accepted uncritically, but conflicting new evidence is brushed aside and not reported because it does not fit, then that particular science is in deep trouble—and it has happened quite often in the historical past.

To emphasize the problem of scientists unquestioningly accepting the conventional wisdom, Conrad H. Waddington (1977) proposed the acronym COWDUNG to signify the Conventional Wisdom of the Dominant Group.

In teaching in a historical manner, I recognize the importance of Thomas H. Huxley's (1853) warnings that "Truth often has more than one Avatar, and whatever the forgetfulness of men, history should be just, and not allow those who had the misfortune to be before their time to pass for that reason into oblivion" and "The world, always too happy to join in toadying the rich, and taking away the 'one ewe lamb' from the poor." Indeed, it is often difficult to determine who makes a discovery (Djerassi and Hoffmann, 2001). I try to the best of my ability to give a fair and accurate account of the historical aspects of cell biology.

My course includes a laboratory section and my students perform experiments to acquire personal experience in understanding the living cell and how it works (Hume, 1748; Wilson, 1952; Ramón y Cajal, 1999). Justus von Liebig (1840) described the importance of the experimental approach this way:

Nature speaks to us in a peculiar language, in the language of phenomena; she answers at all times the questions which are put to her; and such questions are experiments. An experiment is the expression of a thought: we are near the truth when the phenomenon, elicited by the experiment, corresponds to the thought; while the opposite result shows that the question was falsely stated, and that the conception was erroneous.

My students cannot wait to get into the laboratory. In fact, they often come in on nights and weekends to use the microscopes to take photomicrographs. At the end of the semester, the students come over to my house for dinner (I worked my way through college as a cook) and bring their best photomicrographs. After dinner, they vote on the twelve best, and those are incorporated into a class calendar. The calendars are beautiful and the students often make extra to give as gifts.

In 1952, Edgar Bright Wilson Jr. wrote in *An Introduction to Scientific Research*, "There is no excuse for

doing a given job in an expensive way when it can be carried through equally effectively with less expenditure." Today, with an emphasis on research that can garner significant money for a college or university through indirect costs, there is an emphasis on the first use of expensive techniques to answer cell biological questions and often questions that have already been answered. However, the very expense of the techniques often prevents one from performing the preliminary experiments necessary to learn how to do the experiment so that meaningful and valuable data and not just lists are generated. Unfortunately, the lists generated with expensive techniques often require statisticians and computer programmers, who are far removed from experiencing the living cells through observation and measurement, to tell the scientist which entries on the list are meaningful. Thus, there is a potential for the distinction between meaningful science and meaningless science to become a blur. I use John Synge's (1951) essay on vicious circles to help my students realize that there is a need to distinguish for themselves what is fundamental and what is derived.

By contrast, this book emphasizes the importance of the scientists who have made the great discoveries in cell biology using relatively low-tech quantitative and observational methods. But—and this is a big *but*—these scientists also treated their brains, eyes, and hands as highly developed scientific instruments. I want my students to have the ability to get to know these great scientists. I ask them to name who they think are the 10 best scientists who ever lived. Then I ask if they have ever read any of their original work. In the majority of the cases, they have never read a single work by the people who they consider to be the best scientists. This is a shame. They read the work of others … but not the best. Interestingly, they usually are well read when it comes to reading the best writers (e.g., Shakespeare, Faulkner, etc.).

Typically, the people on my students' lists of best scientists have written books for the layperson or an autobiography (Wayne and Staves, 1998). Even Isaac Newton wrote a book for the layperson! I give my class these references and encourage them to become familiar with their favorite scientists first hand. The goal of my lectures and this book is to facilitate my students' personal and continual journey in the study of life.

My goal in teaching plant cell biology is not only to help my students understand the mechanisms of the cell and its organelles in converting energy and material matter into a living organism that performs all the functions we ascribe to life. I also hope to deepen my students' ideas of the meaning, beauty, and value of life and the value in searching for meaning and understanding in all processes involved in living.

I thank Mark Staves and my family, Michelle, Katherine, Zack, Beth, Scott, my mother and father, and aunts and uncles, for their support over the years. I also thank my colleagues at Cornell University and teachers at the Universities of Massachusetts, Georgia, and California at Los Angeles, and especially Peter Hepler and Masashi Tazawa, who taught me how to see the universe in a living cell.

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Dedicated to President John F. Kennedy
for inspiring my generation to be courageous in the pursuit of science

Chapter 1

On the Nature of Cells

The world globes itself in a drop of dew. The microscope cannot find the animalcule which is less perfect for being little. Eyes, ears, taste, smell, motion, resistance, appetite, and organs of reproduction that take hold on eternity—all find room to consist in the small creature. So do we put our life into every act. The true doctrine of omnipresence is that God reappears with all His parts in every moss and cobweb.

— Ralph Waldo Emerson, "Compensation"

1.1 INTRODUCTION: WHAT IS A CELL?

In the introduction to his book, *Grundzüge der Botanik*, Matthias Schleiden (1842), often considered the cofounder of the cell theory, admonished, "Anyone who has an idea of learning botany from the present book, may just as well put it at once aside unread; for from books botany is not learnt" (quoted in Goebel, 1926). Likewise, I would like to stress that an understanding of plant cell biology, and what a plant cell is, comes from direct experience. I hope that this book helps facilitate your own personal journey into the world of the cell.

Exploring the world made accessible by the invention of the microscope, Robert Hooke (1665) discovered a regular, repeating structure in cork that he called a cell. The word *cell* comes from the Latin *celle*, which in Hooke's time meant "a small apartment, *esp*. one of several such in the same building, used e.g. for a store-closet, slave's room, prison cell; also cell of a honeycomb; … also a monk's or hermit's cell" (*Oxford English Dictionary*, 1933). Hooke used the word *cell* to denote the stark appearance of the air-filled pores he saw in the honeycomb-like pattern in the cork that he viewed with his microscope (Figure 1.1). Hooke's perspective of the emptiness of cells was propagated by Nehemiah Grew (1682), who compared the cells of the pith of asparagus to the froth of beer (Figure 1.2), and is still implied in words with the prefix *cytos*, which in Greek means "hollow place". Hooke, however, did realize that there might be more to a cell than he could see. He wrote,

> *Now, though I have with great diligence endeavoured to find whether there be any such thing in those microscopical pores of wood or piths, as the valves in the heart, veins and other passages of animals, that open and give passage to the contained fluid juices one way, and shut themselves, and impede the passage of such liquors back again, yet have I not hitherto been able to say anything positive in it; … but … some diligent observer, if helped with better microscopes, may in time, detect [them].*

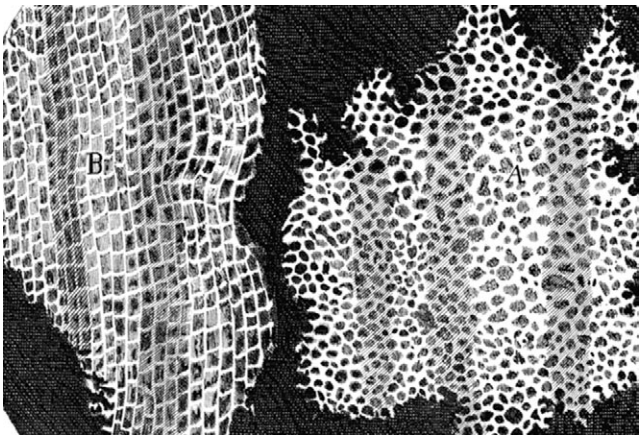


FIGURE 1.1 Cells of cork. (*Source:* From Hooke, 1665.)

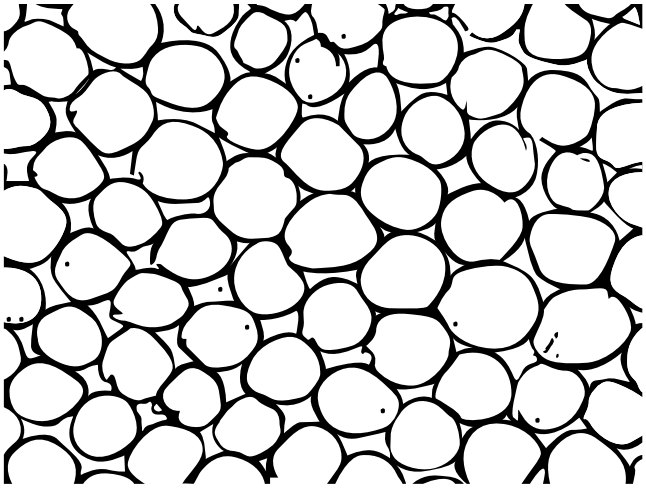


FIGURE 1.2 The cortical cells of a small root of asparagus. (*Source:* From Grew, 1682.)

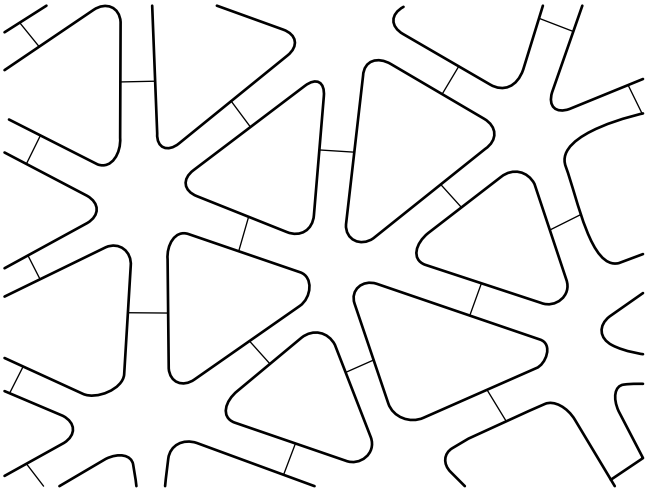


FIGURE 1.3 Stellate cells from the petiole of a banana. (*Source:* From von Mohl, 1852.)

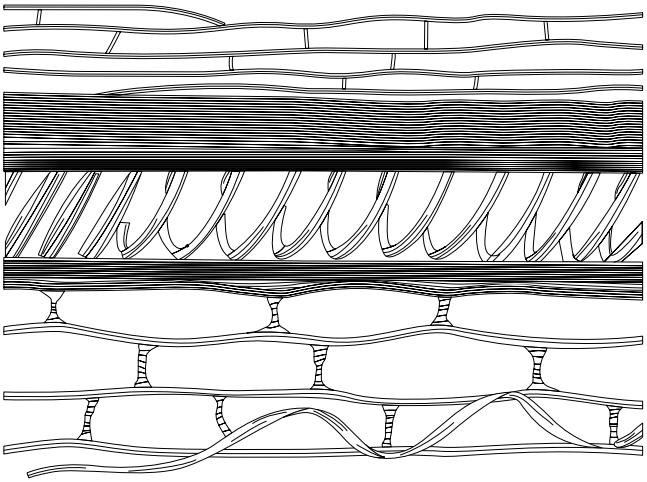


FIGURE 1.4 Spiral vessels, sap tubes, and cells of *Marantha lutea*. (*Source:* From deCandolle and Sprengel, 1821.)

Hugo von Mohl (1852) pointed out in *Principles of the Anatomy and Physiology of the Vegetable Cell*, the first textbook devoted to plant cell biology, that indeed plant cells are not vacuous when viewed with optically corrected microscopes, but contain a nucleus and "an opake, viscid fluid of a white colour, having granules intermingled in it, which fluid I call protoplasm." Von Mohl, echoing the conclusions of Henri Dutrochet (1824) and John Queckett (1852), further revealed through his developmental studies that cells have a variety of shapes (Figure 1.3) and give rise to all structures in the plant including the phloem and xylem. This was contrary to the earlier opinions of deCandolle and Sprengel (1821), who believed that there were three elementary forms in plants—dodecahedral-shaped cells, noncellular tubes, and noncellular spirals (Figure 1.4). By focusing on mature plants, deCandolle and Sprengel had not realized that the tubelike vessels and the spiral-like protoxylem developed from dodecahedral-shaped cells.

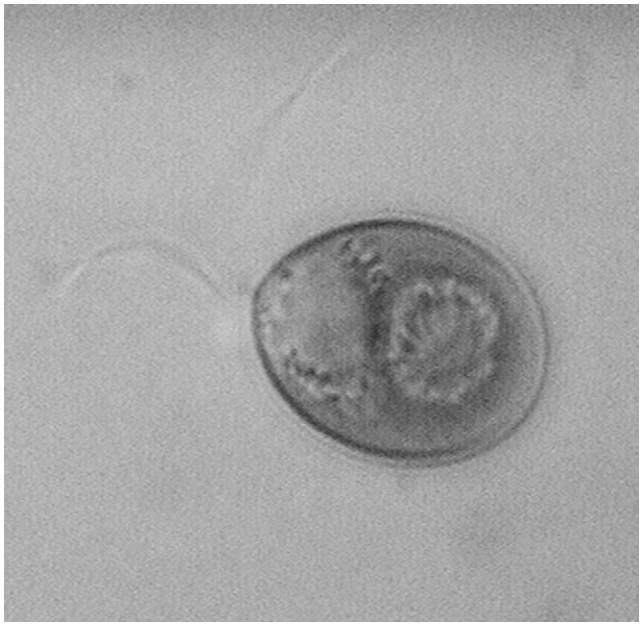


FIGURE 1.5 Photomicrograph of a swimming *Dunaliella* cell taken with Nomarski differential interference contrast optics.

To further emphasize the vitality of cells, von Mohl also stressed that cells were endowed with the ability to perform all kinds of movements.

In the world of the living cell, the only thing that is certain is change—movement occurs at all levels, from the molecular to the whole cell. While I was taught that plants, unlike animals, do not move, some plants can constantly change their position. Get a drop of pond water and look at it under the microscope. Watch a single-celled alga like *Dunaliella* under the microscope (Figure 1.5). See it swim? These plant cells are Olympic-class swimmers: they swim about 50 μm/s—equivalent to five body lengths per second. Not only can the cells swim, but they can also change their motile behavior in response to external stimuli. When a bright flash of light (from the sun or a photographic flash) strikes swimming *Dunaliella* cells, like synchronous swimmers, they all swim backward for about a half second. From this observation, even a casual observer will conclude that individual cells have well-developed sensory systems that can sense and respond to external stimuli (Wayne et al., 1991).

In contrast to *Dunaliella*, some cells, particularly those of higher plants, remain static within an immobile cell wall. Yet, if you look inside the cell, you are again faced with movement. You see that the protoplasm dramatically flows throughout a plant cell, a phenomenon known as *cytoplasmic streaming* (Kamiya, 1959). Look at the giant internodal cell of *Chara* (Figure 1.6). The cytoplasm rotates around the cell at about 100 μm/s. If you electrically stimulate the cell, the cytoplasmic streaming ceases instantly. As the neurobiologists say, the cell is excitable and responds to external stimuli. In fact, action potentials were observed in characean internodal cells before they were observed in the

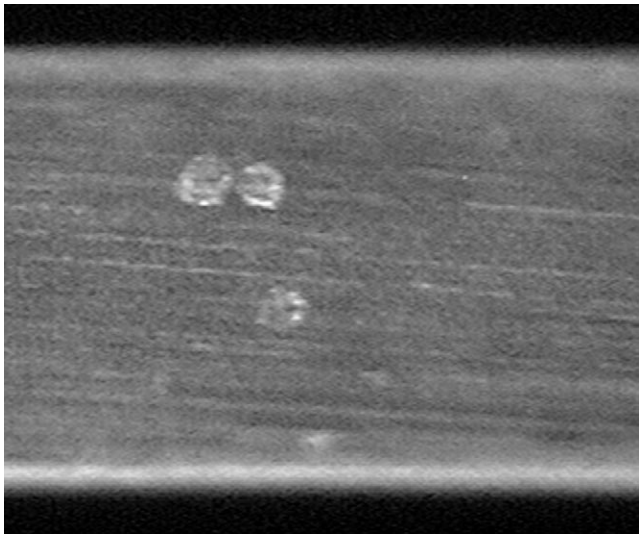


FIGURE 1.6 Photomicrograph of a portion of a giant internodal cell of *Chara* showing several nuclei being carried by cytoplasmic streaming.

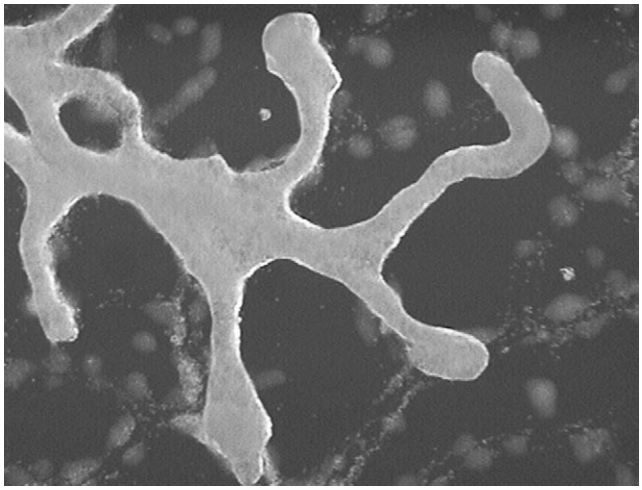


FIGURE 1.7 Dark-field photomicrograph of the slime mold *Physarum polycephalum.*

nerve cells of animals (Cole and Curtis, 1938, 1939). The events that occur between electrical stimulation and the cessation of streaming are relatively well understood, and I discuss these throughout the book.

Lastly, take a look at the large single-celled plasmodium of the slime mold *Physarum* (Figure 1.7; Coman, 1940; Kamiya, 1959; Carlisle, 1970; Konijn and Koevenig, 1971; Ueda et al., 1975; Durham and Ridgway, 1976; Chet et al., 1977; Kincaid and Mansour, 1978a,b; Hato, 1979; Dove and Rusch, 1980; Sauer, 1982; Dove et al., 1986; Bailey, 1997; Bozzone and Martin, 1998). Its cytoplasm streams at about 2000 μm/s. The force exerted by the streaming causes the plasmodium to migrate about 0.1 μm/s. Why does it move so slowly when streaming is so rapid? Notice that the cytoplasmic streaming changes direction in a rhythmic manner.

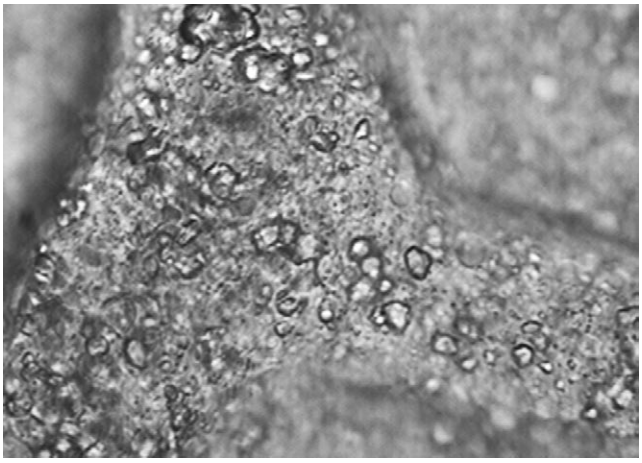


FIGURE 1.8 Bright-field photomicrograph of the streaming cytoplasm of the slime mold *Physarum polycephalum.*

The velocity in one direction is slightly greater than the velocity in the opposite direction. This causes the cell to migrate in the direction of the more rapid streaming. Since the plasmodium migrates toward food, the velocity of cytoplasmic streaming in each direction is probably affected by the gradient of nutrients. Nobody knows how this cell perceives the direction of food and how this signal is converted into directions for migration. Will you find out?

While looking at *Physarum*, notice that the protoplasm is not homogeneous, but is full of relatively large round bodies rushing through the cell (Figure 1.8). Is what you see the true nature of protoplasm, or are there smaller entities, which are invisible in a light microscope, that are also important in the understanding of cells? Edmund B. Wilson (1923) describes the power and the limitations of the light microscope in studying protoplasm:

> *When viewed under a relatively low magnification … only the larger bodies are seen; but as ... we increase the magnification … we see smaller and smaller bodies coming into view, at every stage graduating down to the limit of vision … which in round numbers is not less than 200 submicrons. … Such an order of magnitude seems to be far greater than that of the molecules of proteins and other inorganic substances. … Therefore an immense gap remains between the smallest bodies visible with the microscope and the molecules of even the most complex organic substances. For these reasons alone ... we should be certain that below the horizon of our present high-power microscopes there exists an invisible realm peopled by a multitude of suspended or dispersed particles, and one that is perhaps quite as complex as the visible region of the system with which the cytologist is directly occupied.*
>
> *We have now arrived at a borderland, where the cytologist and the colloidal chemist are almost within hailing distance of each other—a region, it must be added, where both are treading on dangerous ground. Some of our friends seem disposed to think that the cytologist should halt at the artificial boundary set by the existing limits of microscopical vision and hand over his inquiry to the biochemist and biophysicist with a*

farewell greeting. The cytologist views the matter somewhat differently. Unless he is afflicted with complete paralysis of his cerebral protoplasm he can not stop at the artificial boundary set up by the existing limits of microscopical vision.

Looking at the streaming plasmodium of *Physarum* inspires a sense of awe and wonder about life. How is that single cell able to sense the presence of the oatmeal flake and move toward it? How does it generate the force to move from within? What kind of endogenous timekeeper is in the cell that allows the streaming cytoplasm to move back and forth with the rhythm and regularity of a beating heart (*Time*, 1937, 1940)? We will explore these and other questions about living cells. However, in order to cross the "artificial boundaries" and comprehend the nature of the living cell, it is necessary to develop knowledge of mathematics, chemistry, and physics as well as cytology, anatomy, physiology, genetics, and developmental biology. The practice of cell biology that incorporates these various disciplines is still in its adolescent period and is "treading on dangerous ground." As in any developing science, observations and measurements contain a given amount of uncertainty or "probable error," and the exactness of the measurements, and thus the science, evolves (Hubble, 1954). Perhaps cell biology is at the stage thermodynamics was a century ago. Gilbert Newton Lewis and Merle Randall described the growth and development of thermodynamics in the Preface to their 1923 book, *Thermodynamics and the Free Energy of Chemical Substances*:

There are ancient cathedrals which, apart from their consecrated purpose, inspire solemnity and awe. Even the curious visitor speaks of serious things, with hushed voice, and as each whisper reverberates through the vaulted nave, the returning echo seems to bear a message of mystery. The labor of generations of architects and artisans has been forgotten, the scaffolding erected for their toil has long since been removed, their mistakes have been erased, or have become hidden by the dust of centuries. Seeing only the perfection of the completed whole, we are impressed as by some superhuman agency. But sometimes we enter such an edifice that is still partly under construction; then the sound of hammers, the reek of tobacco, the trivial jests bandied from workman to workman, enable us to realize that these great structures are but the result of giving to ordinary human effort a direction and a purpose.

Science has its cathedrals.

Cell biology is a young, vibrant, growing science, the beginnings of which took place in the early part of the 19th century when scientists, including Schleiden (1853), pondered what regular element may underlie the vast array of plant forms from "the slender palm, waving its elegant crown in the refreshing breezes … to the delicate moss, barely an inch in length, which clothes our damp grottos with its phosphorescent verdue." Schleiden felt that "we may never expect to be enabled to spy into the mysteries of nature, until we are guided by our researches to very simple relations … the simple element, the regular basis of all the various forms."

1.2 THE BASIC UNIT OF LIFE

Prior to 1824, organic particles or a vegetative force that organized organic particles were considered by some prominent scientists including Gottfried Leibniz, Comte de Buffon, and John Needham to be the basic unit of life (Roger, 1997). In fact, John Needham (1749) and John Bywater (1817, 1824) observed these living particles in infusions of plant and animal material that they placed under the microscope. Bywater observed that they writhed about in a very active manner and conjectured that the immediate source of the movement was thermal energy, which originated from the "particles of [sun]light which come in contact with the earth, and have lost their rapid momentum." Bywater considered sunlight to carry the vital force, and concluded "that the particles of which bodies are composed, are not merely inert matter, but have received from the Deity certain qualities, which render them actively instrumental in promoting the physical economy of the world."[1]

Henri Dutrochet (1824) emphasized the importance of the cell, as opposed to living particles or the whole organism, as the basic unit of life. Dutrochet came to this conclusion from his microscopical observations, by which he observed "plants are derived entirely from cells, or of organs which are obviously derived from cells." He extended his observations to animals, and concluded that all organic beings are "composed of an infinite number of microscopic parts, which are only related by their proximity" (quoted in Rich, 1926). More than a decade later, Dutrochet's cell theory was promoted by Schleiden and Schwann. Schleiden (1838), a botanist, wrote:

Every plant developed in any higher degree, is an aggregate of fully individualized, independent, separate beings, even the cells themselves. Each cell leads a double life: an independent one pertaining to its own development alone, and another incidental, in so far as it has become an integral part of a plant. It is, however, easy to perceive that the vital process of the individual cells must form the very first, absolutely indispensable fundamental basis.

Likewise, Schwann (1838), a zoologist, concluded that "the whole animal body, like that of plants, is thus composed of cells and does not differ fundamentally in its structure from plant tissue." Thanks to the extensive research, and active promotion by Schleiden and Schwann, by the end of the 1830s, Dutrochet's concept that the cell is the basic unit of all life became well established, accepted and extended to emphasize the interrelationships between cells. The expanded cell theory provided a framework to understand the nature of life as well as its origin and continuity.

[1]Robert Brown (1828, 1829) independently observed the movement of particles. However, Brown, in contrast to Bywater, did not consider the movement of the particles to be a sign of vitality or life, but just a physical process.

We often divide various objects on Earth into two categories: the living and the lifeless. Therefore, the investigation of cells may provide us with a method to understand the question, "What is life?" We often characterize life as something that possesses attributes that the lifeless lack (Beale, 1892; Blackman, 1906; Tashiro, 1917; Osterhout, 1924; Harold, 2001). The power of movement is a distinctive aspect of living matter, where the movement has an internal rather than an external origin. Living matter generates electricity. Living matter also takes up nutrients from the external environment and, by performing synthetic reactions at ambient temperatures, converts the inorganic elements into living matter. Living matter also expels the matter that would be toxic to it. The ability to synthesize macromolecules from inorganic elements allows growth, another characteristic of living matter. Living matter also contains information, and thus has the ability to reproduce itself, with near-perfect fidelity. Lastly, living matter is self-regulating. It is capable of sensing and responding to environmental signals in order to maintain a homeostasis (Cannon, 1932, 1941) or to adjust to new conditions by entering metastable states, or other states, in a process known as allostasis (Spencer, 1864; Emerson, 1954; Sapolsky, 1998).

The above-mentioned properties are characteristic of living things and their possession defines a living thing. Mathews (1916) notes, "When we speak of life we mean this peculiar group of phenomena; and when we speak of explaining life, we mean the explanation of these phenomena in the terms of better known processes in the non-living." There are entities like viruses that exhibit some but not all of the characteristics of life. Are viruses the smallest living organism as the botanist Martinus Beijerinck thought when he isolated the tobacco mosaic virus in 1898, or are they the largest molecules as the chemist Wendell Stanley thought when he crystallized the tobacco mosaic virus in 1935 (Stanley and Valens, 1961)? While the distinction between nonliving and living is truly blurred (Pirie, 1938; Baitsell, 1940), the cell in general is the smallest unit capable of performing all the processes associated with life.

For centuries, people believed that the difference between living and nonliving matter arises from the fact that living matter possesses a vital force, also known as the *vis vitalis*, a purpose, a soul, Maxwell's demon, a spirit, an archaeus, or an entelechy (Reil, 1796; Loew, 1896; Lovejoy, 1911; Ritter, 1911; Driesch, 1914, 1929; Waddington, 1977). According to the view of the "vitalists and dualists," the laws of physics and chemistry used to describe inorganic nature are, in principle, incapable of describing living things. By contrast, mechanists, materialists, mechanical materialists and monists believe that there is a unity of nature and a continuum between the nonliving and the living—and all things, whether living or not, are made of the same material and are subject to the same physical laws and mechanisms (Dutrochet, 1824; Bernard, 1865; Helmholtz, 1903; Koenigsberger, 1906; Rich, 1926; Brooks and Cranefield, 1959).

Mary Shelley (1818) wrote about the potential of the materialistic/mechanical view and the ethics involved in experimentation on the nature of life when she described how Victor Frankenstein discovered that life could emerge spontaneously when he put together the right combination of matter and activated it with electrical energy. In the materialist/mechanical view, living matter is merely a complex arrangement of atoms and molecules, performing chemical reactions and following physical laws. Thus, according to this view, the laws of chemistry and physics are not only applicable but also essential to the understanding of life (Belfast Address, Tyndall, 1898). Claude Bernard (1865) believed that "the term 'vital properties' is only provisional; because we call properties vital which we have not yet been able to reduce to physico-chemical terms; but in that we shall doubtless succeed some day." An understanding of the relationship between nonliving matter and living matter underlies the understanding of the relationship between the body and the soul, and the definition of personal identity, free will, and immortality (Dennett, 1978; Perry, 1978; Popper and Eccles, 1977; Eccles, 1979).

Thomas H. Huxley (1890) explains:

> *The existence of the matter of life depends on the pre-existence of certain compounds; namely, carbonic acid, water and ammonia. Withdraw any one of these three from the world, and all vital phenomena come to an end. They are related to the protoplasm of the plant, as the protoplasm of the plant is to that of the animal. Carbon, hydrogen, oxygen, and nitrogen are all lifeless bodies. Of these, carbon and oxygen unite, in certain proportions and under certain conditions, to give rise to carbonic acid; hydrogen and oxygen produce water; nitrogen and hydrogen give rise to ammonia. These new compounds, like the elementary bodies of which they are composed, are lifeless. But when they are brought together, under certain conditions they give rise to the still more complex body, protoplasm, and this protoplasm exhibits the phenomena of life.*
>
> *When hydrogen and oxygen are mixed in a certain proportion, and an electric spark is passed through them, they disappear, and a quantity of water … appears in their place. … At 32° Fahrenheit and far below that temperature, oxygen and hydrogen are elastic gaseous bodies. … Water, at the same temperature, is a strong though brittle solid. … Nevertheless, … we do not hesitate to believe that … [the properties of water] result from the properties of the component elements of the water. We do not assume that a something called "aquosity" entered into and took possession of the oxide of hydrogen as soon as it was formed. … On the contrary, we live in the hope and in the faith that, by the advance of molecular physics, we shall by and by be able to see our way clearly from the constituents of water to the properties of water, as we are now able to deduce the operations of a watch from the form of its parts and the manner in which they are put together.*
>
> *Is the case in any way changed when carbonic acid, water, and ammonium disappear, and in their place, under the influence of pre-existing living protoplasm, an equivalent weight of the matter of life makes its appearance? … What better philosophical status has "vitality" than "aquosity"?*

With a like mind, Edmund B. Wilson (1923) concluded his essay on "The Physical Basis of Life" by saying:

> *I do not in the least mean by this that our faith in mechanistic methods and conceptions is shaken. It is by following precisely these methods and conceptions that observation and experiment are every day enlarging our knowledge of colloidal systems, lifeless and living. Who will set a limit to their future progress? But I am not speaking of tomorrow but of today; and the mechanist should not deceive himself in regard to the magnitude of the task that still lies before him. Perhaps, indeed, a day may come (and here I use the words of Professor Troland) when we may be able "to show how in accordance with recognized principles of physics a complex of specific, autocatalytic, colloidal particles in the germ-cell can engineer the construction of a vertebrate organism"; but assuredly that day is not yet within sight. … Shall we then join hands with the neo-vitalists in referring the unifying and regulatory principle to the operation of an unknown power …? … No, a thousand times, if we hope really to advance our understanding of the living organism.*

In the spirit of E. B. Wilson as well as many others, we will begin our study of the cell by becoming familiar with its chemical and physical nature. During our journey, I will not take the extreme perspective of Edward O. Wilson (1998) that life can be reduced to the laws of physics, nor will I take the extreme perspective of the electrophysiologist Emil DuBois-Reymond (1872), who proclaimed that there are absolute limits to our knowledge of nature and moreover he would not try to find these limits using science ("*Ignoramus et ignorabimus*"). I will also not take the perspective offered by the Copenhagen School of Physics that blurs the distinction between living and nonliving when it states that until you observe a cell that has been kept from view, that cell is both living and dead according to the rules of quantum superposition. This view was ridiculed by Erwin Schrödinger in his story of the cat in a box (Gribbin, 1984, 1995). I will try to take a middle ground (Heitler, 1963), looking at the cell physico-chemically without losing sight of the miracle, value, and meaning of life (Bischof, 1996; Berry, 2000).

Max Planck wrote, "In my opinion every philosophy has the task of developing an understanding of the meaning of life, and in setting up this task one supposes that life really has a meaning. Therefore whoever denies the meaning of life at the same time denies the precondition of every ethics and of every philosophy that penetrates to fundamentals" (quoted in Heilbron, 1986). As discoveries made by cell biologists become techniques used by biotechnologists to create new choices for humanity, we realize that our own discoveries can have profound effects on the meaning of life.

1.3 THE CHEMICAL COMPOSITION OF CELLS

Living cells are made out of the same elements found in the inorganic world. However, out of the more than 100 elements available on Earth, cells are primarily made out of carbon, hydrogen, and oxygen (Mulder, 1849; see also Table 1.1). According to Lawrence Henderson (1917), it is the special physico-chemical properties of these elements and their compounds that allow life, as we know it, to exist.

TABLE 1.1 Atomic composition of the large spore cells of *Onoclea*

Element	Percent Dry Weight	nmol/mg Dry Weight	Atoms/ Cell
C	58.59	48,784	4×10^{15}
O	21.25	13,281	1×10^{15}
H	7.76	76,942	6×10^{15}
N	4.59	3277	2×10^{14}
P	0.82	255	2×10^{13}
K	0.70	179	1×10^{13}
S	0.53	164	1×10^{13}
Mg	0.34	140	1×10^{13}
Na	0.23	100	8×10^{12}
Ca	0.20	50	4×10^{12}
Cl	0.11	31	2×10^{12}
Co	0.04	7	5×10^{11}
Fe	0.02	4	3×10^{11}
Ni	0.01	2	2×10^{11}
Mn	0.01	1	8×10^{10}
Zn	0.01	1	8×10^{10}
Cu	0.01	1	8×10^{10}

Source: Wayne and Hepler (1985).

The vast majority of the oxygen and hydrogen in the cells exists in the cell as water, which provides the milieu in which the other chemicals exist (Ball, 2000; Franks, 2000). The large numbers of atoms of carbon, oxygen, hydrogen, nitrogen, sulfur, and phosphorous found in cells are for the most part combined into macromolecules. The macromolecular composition of a "typical" bacterial cell calculated by Albert Lehninger in his book *Bioenergetics* (1965) is shown in Table 1.2.

The cell uses these various macromolecules to build the machinery of the cell. A cell has various components that help it to transform information into structure; and it has various structures to help it convert mass and energy into work so it can maintain a homeostasis, move, grow, and reproduce. We will begin discussing the organization of the cell in Chapter 2. For now, let us get a sense of scale.

TABLE 1.2 Macromolecular composition of a bacterial cell

Chemical	Percent of Component	Number of Molecules Dry Weight per Cell
DNA	5	4
RNA	10	15,000
Protein	70	1,700,000
Lipid	10	15,000,000
Polysaccharides	5	39,000

Source: From Lehninger (1965).

Before we discuss the scale of living cells, let us discuss an experiment described by Irving Langmuir in order to get a feeling for the size of a macromolecule, for example, a lipid (Langmuir, 1917; Taylor et al., 1942; see also Appendix 1). When you place a drop (10^{-7} m^3) of lipid like olive oil on the surface of a trough full of water, the olive oil will spread out and form a monolayer. Since the lipid is amphiphilic, in that it has both a hydrophilic end (glycerol) and a hydrophobic or lipophilic end (the hydrocarbon derived from oleic acid), the hydrophilic glycerol end will dissolve in the water and the hydrophobic hydrocarbon end will stick into the air. We can use this observation to determine the size of the lipid molecules—but how?

If we know the volume of oil we started with and the area of the monolayer, we can estimate the thickness of the oil molecules. For example, Benjamin Franklin found that a teaspoonful[2] of oil covers a surface of about half an acre (Tanford, 1989). Since a teaspoonful of oil contains approximately 2×10^{-6} m^3 of oil and a half acre is approximately 2000 m^2, the thickness of the monolayer and thus the length of the molecule, obtained by dividing the volume by the area, is approximately 1 nm (Laidler, 1993).

Franklin never made this calculation, probably because at the time the concept of molecules had not been developed. However, now that we understand the molecular organization of matter, we can go even further in our analysis. For example, if we know the density (ρ) and molecular mass (M_r) of the oil (e.g., $\rho = 900$ kg/m^3 and $M_r = 0.282$ kg/mol for olive oil), we can calculate the number of molecules in the drop using dimensional analysis and Avogadro's number (6.02×10^{23} molecules/mol; Avogadro, 1837; Deslattes, 1980):

$$(2 \times 10^{-6}\ \text{m}^3)(900\,\text{kg m}^{-3})(0.282\,\text{kg mol}^{-1})^{-1} (6.02 \times 10^{23}\ \text{molecules mol}^{-1}) = 3.8 \times 10^{21}\ \text{molecules}$$

[2]For reference, one milliliter is one-millionth of a cubic meter, and one liter is one-thousandth of a cubic meter.

Since we know how many molecules we applied to the water and the area the oil takes up, we can calculate the cross-sectional area of each molecule. We obtain the cross-sectional area of each molecule (5.3×10^{-19} m^2) by dividing the area of the monolayer by the number of molecules in it. If we assume that the molecules have a circular cross-section, we can estimate their diameter ($2r$) from their area (πr^2). We get a diameter of approximately 0.8 nm. We can do the experiment more rigorously using pipettes and a Langmuir trough, but the answers are not so different.

It is amazing how much you can learn with a teaspoon and a ruler if you apply a little algebra! You have just deduced the size of a molecule from first principles using dimensional analysis! Lipids are important in the structure of cellular membranes. However, since membranes are exposed to aqueous solutions on both sides, the lipids form double layers also known as *bilayers*. Membranes are also composed of proteins that have characteristic lengths on the order of 5 nm. As I will discuss in Chapter 2, the diameters of proteins can be determined from studies on their rate of diffusion. Can you estimate the thickness of a membrane composed of proteins inserted in a single lipid bilayer?

1.4 A SENSE OF CELLULAR SCALE

In order to understand cells we must get a grasp of their dimensions, because, while there are many similarities between the living processes of cells and multicellular organisms like ourselves, of which we are most familiar, we will find that there are limits to the similarities between single cells and multicellular organisms that must be taken into consideration (Hill, 1926).

How small can a cell be? The lower size limit of a cell is determined by the minimal number and size of the components that are necessary for an autonomous existence. In order to live autonomously, a cell has to perform approximately 100 metabolic reactions involved with primary metabolism (e.g., the biosynthesis of amino acids, nucleotides, sugars, and lipids, as well as the polymers of these molecules) and transport. Therefore, about 100 different enzymes, with an average diameter of 5 nm, and the corresponding amount of substrate molecules must be present. In addition, one DNA molecule, 100 mRNA molecules, 20 tRNA molecules, and several rRNA molecules are needed to synthesize these enzymes. If we assume that there is one copy of each molecule, we can estimate the volume of the molecules and the water needed to dissolve them. In order to keep the enzymes together, the cell must have a limiting membrane. If we add the dimensions of a plasma membrane (10 nm thick) we find that the minimum cell diameter is about 65 nm. The smallest known organisms are *Rickettsia* (Bovarnick, 1955) and various mycoplasmas (Maniloff and Morowitz, 1972; Hutchison et al., 1999), which have diameters of approximately 100 nm.

TABLE 1.3 Relationship between surface and volume of a sphere

Radius (r, in m)	Surface Area (A, in m^2) ($4\pi r^2$)	Volume (V, in m^3) ($(4/3)\pi r^3$)	Surface-to-Volume Ratio (A/V, in m^{-1}) $= (3/r)$
0.1	0.126	0.0042	30.0
1	12.56	4.19	3.0
10	1256.64	4188.79	0.3
100	125663.71	4188790.21	0.03
1000	12566370.61	4188790205	0.003

There is a limit as to how big a cell can be. Assume that a cell is spherical. The surface area of a cell with radius r will be given by $4\pi r^2$ and its volume will be given by $(4/3)\pi r^3$. Thus, its surface to volume ratio will be $3/r$, and as the cell gets larger and larger, its surface to volume ratio will decrease exponentially. This limits the cell's ability to take up nutrients and to eliminate wastes (Table 1.3).

Some cells are very large. For example, an ostrich egg can be 10.5 cm in diameter. In this case, a large portion of the intracellular volume is occupied by the yolk. The yolk is "inert" relative to the cytoplasm. In the case of large plant cells, the vacuole functions as an inert space filler. Haldane (1985) illustrates the bridge between mathematics and biology beautifully in his essay "On Being the Right Size." In it he writes, "Comparative anatomy is largely the story of the struggle to increase surface in proportion to volume."

How long is a typical plant cell? While their lengths vary from a few micrometers in meristematic cells to 1.5 mm in root hairs and 25 cm in phloem fibers (Haberlandt, 1914; Esau, 1965; Ridge and Emons, 2000; Bhaskar, 2003), for the present we will assume that a typical plant cell is a cube where each side has a length of 10^{-5} m. Such a typical cell has a surface area of 6×10^{-10} m^2 and a volume of 10^{-15} m^3.

How much does a cell weigh? We can estimate its weight from "first principles." A cell is composed mostly of water, so let us assume that it is made totally out of water, which has a density (ρ) of 10^3 kg/m^3. Using dimensional analysis and multiplying the volume of the cell by its density, we see that the mass of the cell is 1×10^{-12} kg or 1 nanogram (Figure 1.9). Multiplying its mass by the acceleration due to gravity (g), we find that it weighs 9.8×10^{-12} N (or 9.8 pN). Since the actual density of the protoplasm is about 1015 kg/m^3, the weight of a single cell is 9.95 pN. Our approximation was not so bad, was it?

We often talk about the importance of pH in enzyme reactions and the energetics of cells. The pH is a measure of the concentration of protons, which are ionized hydrogen atoms. Concentration is a measure of the amount of a substance in moles divided by the volume. Usually we do not realize how small that volume is when we talk about cells. So, to get a feel for cellular volumes, let us calculate how many protons there are in a mitochondrion, an organelle that is involved in molecular free energy (E, in Joules [J]) transduction. A mitochondrion has a volume of approximately $(10^{-6}$ m$)^3$ or 10^{-18} m^3, a value that is about the size of a prokaryotic cell and one-thousandth the size of a typical eukaryotic cell.

Consider that the mitochondrion has an internal pH of 7. Since pH is $-\log [H^+]$, at pH 7 there are 10^{-7} mol H^+/l, which is equal to 10^{-4} mol/m^3. Now we will need to use Avogadro's number as a conversion factor that relates the number of particles to the number of moles of that particle. Now that all the units match, we will use dimensional analysis to calculate how many protons there are in the mitochondrion (Figure 1.10):

$$(10^{-4}\ \text{mol m}^{-3})\,(10^{-6}\ \text{m})^3 \\ (6.02 \times 10^{23}\ \text{protons mol}^{-1}) = 60\ \text{protons}$$

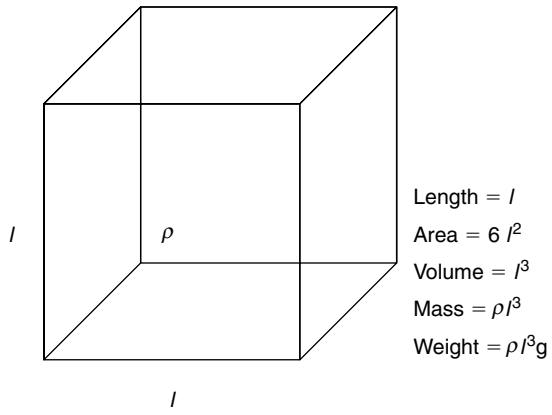


FIGURE 1.9 A geometrical model of a cell.

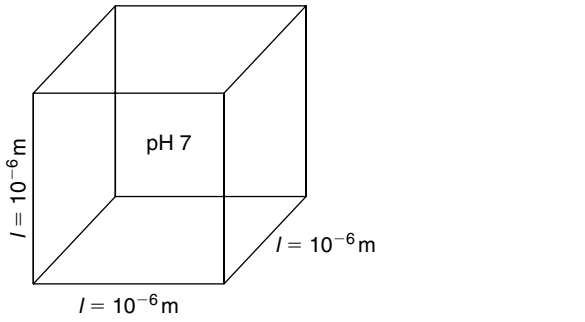


FIGURE 1.10 A calculation of the number of H^+ in a mitochondrion.

If the pH of the mitochondrion is raised to 8, how many protons are now in the mitochondrion?

$$(10^{-5}\ \text{mol m}^{-3})\,(10^{-6}\ \text{m})^3 \\ (6.02 \times 10^{23}\ \text{protons mol}^{-1}) = 6\ \text{protons}$$

Thus, 54 protons would have to leave the mitochondrion in order to raise the pH from pH 7 to pH 8. Interestingly, while it is common knowledge to every introductory biology student that energy conversion in the mitochondrion involves the movement of protons, have you ever realized how few protons actually move? Now we are beginning to understand the scale of the cell (Peters, 1929; McLaren and Babcock, 1959).

1.5 THE ENERGETICS OF CELLS

The molecular free energy (E, in J) is the cellular currency, and all cellular processes can be considered as free energy–transduction mechanisms that convert one form of free energy to another according to the First Law of Thermodynamics proposed by the physician Julius Robert Mayer and demonstrated by the brewer James Joule. That is, while energy can be converted from one form to another in various processes, it is conserved and thus cannot be created or destroyed (Joule, 1852, 1892; Grove et al., 1867; Maxwell, 1897; Lenard, 1933). In the words of James Joule (1843), "the grand agents of nature are, by the Creator's fiat, indestructible; and that whatever mechanical force is expended, an exact equivalent of heat is always obtained."

The Second Law of Thermodynamics states that the amount of energy available to do work is lessened to some degree by each conversion (Magie, 1899; Koenig, 1959; Bent, 1965). In the words of William Thomson (1852), "It is impossible, by means of inanimate material agency, to derive mechanical effect from any portion of matter by cooling it below the temperature of the coldest of the surrounding objects." While the original statements of the laws of thermodynamics have a spiritual overtone, we will assume that there is no vital force, and that no reactions can be greater than 100 percent efficient. Interestingly, this assumption was tested by Baas-Becking and Parks (1927) by calculating the free-energy efficiencies of autotrophic bacteria. They never found thermodynamic efficiencies greater than 100 percent, and concluded that the laws of thermodynamics apply to living systems.

That the First Law of Thermodynamics applies to living things should be of no surprise. Indeed, the First Law of Thermodynamics, like many other physical principles we will discuss throughout this book (e.g., Fick's Law, Poiseuille's Law, Brownian motion, sound waves involved in hearing, light waves involved in vision), have their roots in biological observations. Mayer, while spending the summer of 1840 in Java, noticed that the venous blood of the people there was bright red and not bluish, as it was in people of temperate regions. He concluded that the venous blood was so bright because less oxidation was needed to maintain the body temperature in hot climates compared with cold ones, and as a result, the excess oxygen remained in the venous blood. Mayer also realized that people not only generate heat inside their bodies, but outside as well by performing work, and he postulated that there is a fixed relationship between the amount of food oxidized and the total amount of heat generated by a body. He wrote: "I count, therefore, upon your agreement with me when I state as an axiomatic truth, that during vital processes, the conversion only and never the creation of matter or force occurs" (quoted in Tyndall, 1898).

Using a thermometer, James Joule observed that electrical energy, mechanical energy, and chemical energy produced heat, and then he developed the quantitative relationships between the different forms of energy in terms of the equivalent amount of heat generated. Energy is a particularly convenient measure to compare various seemingly unrelated things because energy, unlike force and velocity, is a scalar quantity and not a vector quantity. Thus, the difference in energy over time and space can be determined with simple algebra. Thus, we will typically convert measurements of force, the electric field, concentration, etc. into energy units (Joules) by using a number of coefficients that transform numbers with given units into numbers with energy units. These include g the acceleration due to gravity (9.8 m/s), R (the universal gas constant, 8.31 J mol^{-1} K^{-1}), k (Boltzmann's constant, 1.38×10^{-23} J/K), F (Faraday's constant, 9.65×10^4 C/mol), e (the elementary charge, 1.6×10^{-19} C), c (the speed of light, 3×10^8 m/s), h (Planck's constant, 6.6×10^{-34} J s), and N_A (Avogadro's number, 6.02×10^{23} molecules (or atoms)/mol). We will implicitly assume that the volume under consideration is defined, although we will see that this is not always so simple to do and that estimates of geometrical values provide a source of error because they are more difficult to estimate than one may initially think. We will also assume that all cells are at standard atmospheric conditions of 298 K and 0.1 MPa of pressure, and for all intents and purposes, the temperature and pressure remain constant. Using these assumptions, in later chapters, we will determine the minimum energy capable of performing mechanical work to move a vesicle, chromosome, or cell; osmotic work to move a solute; or biosynthetic work to form new chemical bonds.

The potential energy of a given mass equals the product of force and distance. The gravitational potential energy of a protoplast settling inside a static extracellular matrix can be converted into the potential energy of a stretched spring-like protein in the extracellular matrix if a helical, spring-like region of the protein is attached to both the plasma membrane and the extracellular matrix of the settling protoplast. Let us determine the potential energy of the falling of a protoplast. The potential energy equals *force* × *distance*, so if a cell that weighs 9.95×10^{-12} N falls 1 nm in a

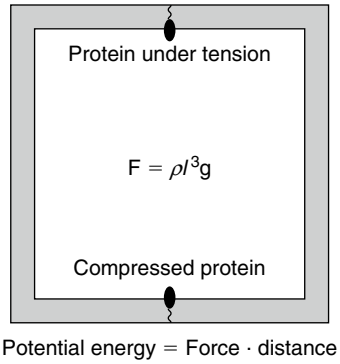


FIGURE 1.11 Potential energy of a protoplast in a gravitational field.

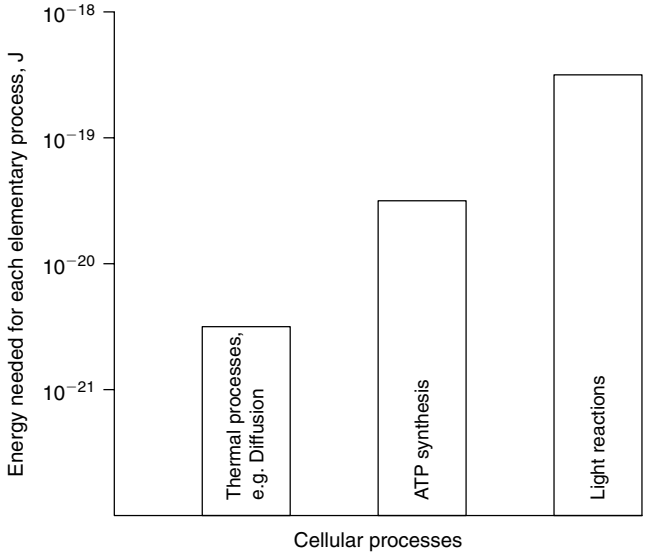


FIGURE 1.12 A comparison of the energetics of some cellular processes.

gravitational field (i.e., changes its position by -1 nm), it makes available 9.95×10^{-21} J of energy that can be used to do work. Some of the potential energy will be degraded as a result of friction, and thus the potential energy in the springlike protein will be somewhat less than the gravitational energy of the protoplast. The potential energy released by the falling protoplast is used for the perception of gravity (Figure 1.11; Wayne and Staves, 1997).

What are the minimum and maximum values for molecular free energies in cellular processes (Figure 1.12)? The unitary processes that utilize the greatest quantity of energy are typically light-activated processes. One such process is photosynthesis, which uses the radiant energy of sunlight to convert water and carbon dioxide to carbohydrates. The energy in a photon of light depends on its wavelength (λ) and is given by the equation: $E = hc/\lambda$. Photosynthesis utilizes both blue and red light. These colors represent photons with the highest and lowest energy contents, respectively. Since blue light has a wavelength of 450 nm and red light has a wavelength of 650 nm, the energy of a photon of blue and red light is 4.4×10^{-19} and 3.0×10^{-19} J, respectively. Since light-driven processes are high-energy reactions in cells, we might expect a typical single reaction to require or release free energy on the order of less than 4×10^{-19} J.

What is the minimum free energy that may be involved in a cellular reaction? The free energy generated by the collisions of molecules in the cell at the ambient temperature is approximately equal to kT, which is $(1.38 \times 10^{-23}$ J/K) (298 K) $= 4.1 \times 10^{-21}$ J at room temperature. An input of free energy lower than this cannot be utilized by a receptor in a cell to do work since the effect of such small energies will be overshadowed by random changes in the state of the receptor due to thermal collisions between the receptor and the water or lipid molecules that surround it.

The free energy of single reactions in a cell thus falls between 4×10^{-21} and 4×10^{-19} J. For a reference, let us look at adenosine triphosphate, a molecule involved in the activation of many molecules in the cell (Lipmann, 1941). The hydrolysis of one adenosine triphosphate (ATP) molecule liberates a maximum of 8×10^{-20} J of free energy, which, if coupled to other processes, is capable of doing work (Rosing and Slater, 1972; Shikama and Nakamura, 1973; Jencks, 1975). This is only an order of magnitude greater than the energy of thermal motion. Since many reactions that require an input of free energy (i.e., endergonic reactions) are coupled to the hydrolysis of ATP, many unitary, endergonic cellular reactions will require energies on the order of 8×10^{-20} J to proceed. I am calculating the free energies per molecule to stress the small number of molecules found in cells compared to the number found in experiments with ideal gases, and to help us visualize the possible mechanisms of cellular reactions. I am assuming that the average energy of any molecule is equal to the average energy of all the molecules. The free energy in a molecule is related to the free energy in a mole of molecules by Avogadro's number, since Boltzmann's constant, k, is equal to R, the universal gas constant, divided by N_A. Therefore, RT gives the free energy in a mole of molecules, and kT gives the free energy in one molecule.

1.6 ARE THERE LIMITS TO THE MECHANISTIC VIEW?

Many people have applied the laws of thermodynamics to cells. These laws are extremely helpful in all aspects of cell biology from calculating the permeability of molecules passing through the membrane to calculating the free energy liberated from the hydrolysis of ATP. Thermodynamics allows us to calculate equilibrium, affinity, and dissociation constants. Thermodynamics provides the boundary conditions, which the reactions of the cell must obey, independent of the detailed physical mechanisms. However, thermodynamics does not tell us anything about the mechanisms of the processes. In our everyday experience, kinetic theory and statistical mechanics provide a model to explain

TABLE 1.4 Relationship between number of molecules and statistical noise

Number of Molecules (n)	Noise ($n^{½}$)	Proportion of noise ($n^{½}/n$)
10^{20}	10^{10}	10^{-10}
10^{10}	10^{5}	10^{-5}
10^{6}	10^{3}	10^{-3}
10^{3}	31.6	0.03
10^{2}	10	0.1
30	5.5	0.18
10	3.16	0.32

thermodynamics (Clausius, 1879; Maxwell, 1897; Loeb, 1961; Jeans, 1962; Boltzmann, 1964; Brush, 1983; Garber et al., 1986; Schroeder, 2000). However, the assumptions that the models on which statistical mechanics are based may not be met in the cell (Schrödinger, 1944). According to Albert Szent-Györgyi (1960):

> *There is a basic difference between physics and biology. Physics is the science of probabilities. … Biology is the science of the improbable and I think it is on principle that the body works only with reactions that are statistically improbable. … I do not mean to say that biological reactions do not obey physics. In the last instance it is physics which has to explain them, only over a detour which may seem entirely improbable on first sight.*

According to Erwin Schrödinger (1944), there should be about 10^{20} molecules or ions present before the predictions based on the laws of statistical mechanics are accurate. The need for large numbers results from the fact that the statistical noise is equal to $\sqrt{n}$, where n is the number of molecules or ions (Table 1.4). That is, if there were on the average 1,000,000 molecules in a given sample volume, upon sampling that volume you may find between 999,000 and 1,001,000 molecules, and thus the relative error is 0.1 percent. Likewise, if there were on the average 100 molecules in a given sample volume, upon sampling you would find 90–110, and the relative error would be 10 percent. We can see from these calculations that the number of protons in a cell or mitochondrion is small compared to the number required for accurate predictions using statistical mechanics (Guye, n.d.). Even in the large spore cell of *Onoclea*, if we count all the atoms, there are 10,000 times too few to use reliably the laws of statistical mechanics.

Can we use statistical mechanics to understand cells? Yes and no. Perhaps it is possible that cells function on a statistical basis where the noise level is typically 10 percent. We should consider statistical mechanics to be a first approximation, since the assumptions on which it is based do not take into consideration the scale of a single cell. Furthermore, the cell is not just a reaction vessel, but a polyphasic system composed of a number of compartments, solid-state supports, and transport systems (e.g., membranes and cytoskeletal elements) that facilitate biochemical reactions in cells (Peters, 1929, 1937; Needham, 1936). Because of the complex structure and small numbers of atoms or molecules within each compartment, we may need a solid-state, quantum mechanical model to fully understand the nature of the living cell (Donnan, 1928, 1937). According to Niels Bohr (1950), mechanistic and vitalistic arguments are complementary and must be reconciled in order to understand life.

Perhaps you will discover a new set of laws that will better predict the processes that go on in cells. But first, learn the old laws—they have been very useful—but keep an open, skeptical, and inquisitive mind (Feynman, 1955, 1969).

Everyone must strike his or her own balance in reducing the complicated processes of life to the laws of physics and chemistry. This is well put in *The Taming of the Shrew* (Shakespeare, 1623), where Tranio says to Lucentio, "The mathematics and the metaphysics—Fall to them as you find your stomach serves you." In this book, I take a reductionist approach, although I appreciate other points of view (Clark, 1890; Stokes, 1891, 1893; Duncan and Eakin, 1981). The absurdity of blindly applying the laws of physics to complicated situations is well described by Needham (1930), in which he quotes Albert Mathews:

> *Adsorption is a physico-chemical term meaning the concentration of substances at phase-boundaries in heterogeneous systems. Dressing can be called a process of adsorption. Every morning when we dress, clothing which has been distributed throughout our environment—dispersed in the surrounding phase—concentrates itself at the surface of our bodies. At night the process is reversed. We might go on to express these events by a curve or isotherm, showing how the quantity adsorbed is a function of the amount in the room, how it usually proceeds to an equilibrium, how it is reversible and not accompanied by chemical change in the clothes, that it is specific in that certain clothes are adsorbed with greater avidity than others, that certain adsorbants (people) adsorb with greater avidity than others, or more so, and finally we could prove that the clothing moved into the surface film in virtue of the second law of thermodynamics and in consonance with the principle of Willard Gibbs.*

1.7 THE MECHANISTIC VIEWPOINT AND GOD

In general, there seems to be a war between science and religion (White, 1877, 1913; Draper, 1898), but this does not need to occur. In studying mechanisms, one must deconstruct the whole into its parts and determine the relationships between the parts as well as the relationships between the

parts and the whole. Each community has words or a word to describe "the whole." Throughout civilization, *Homo sapiens* have strived to live up to our specific epithet by struggling to understand the relationship between the parts and the whole in terms of understanding, among other things, our place in the universe, our relation to other people, our relationship to other species, and our relationship to the environment (Leopold, 1949). Science and religion have been guides throughout this struggle to understand (Power, 1664; Griffiths, 2008; Lerner and Griffiths, 2008; Wayne and Staves, 2008). Science and religion may be two sides of the same coin of understanding, each with a measure of truth, and each complementing the other. Herbert Spencer (1880) writes:

> *Assuming, then, that since these two great realities are constituents of the same mind and respond to different aspects of the same universe, there must be a fundamental harmony between them; we see good reason to conclude that the most abstract truth contained in religion and the most abstract truth contained in science must be the one in which the two coalesce. … Uniting these positive and negative poles of human thought, it must be the ultimate fact in our intelligence.*

It is often thought that a mechanistic viewpoint of nature excludes God. Philosophers have discussed the relationship between God and mechanics (Planck, 1932), and many scientists, including Kepler, Galileo, Boyle, Newton, Schleiden, Planck, Einstein, and Millikan, believed that the study of nature led to an understanding of God. For example, while imprisoned by the forces of the Inquisition, Galileo wrote (quoted in Gamow, 1988):

> *When I ask: whose work is the Sun, the Moon, the Earth, the Stars, their motions and dispositions, I shall probably be told that they are God's work. When I continue to ask whose work is Holy Scripture, I shall certainly be told that it is the work of the Holy Ghost, i.e. God's work also. If now I ask if the Holy Ghost uses words which are manifest contradictions of the truth as to satisfy the understanding of the generally uneducated masses, I am convinced that I shall be told, with many citations from all the sanctified writers, that this is indeed the custom that taken literally would be nothing but heresy and blasphemy, for in them God appears as a Being full of hatred, guilt and forgetfulness. If now I ask whether God, so as to be understood by the masses, had ever altered His works, or else if Nature, unchangeable and inaccessible as it is to human desires, has always retained the same kinds of motion, forms and divisions of the Universe, I am certain to be told that the Moon has always been round, even though it was long considered to be flat. To condense all this into one phrase: Nobody will maintain that Nature has ever changed in order to make its works palatable to men. If this be the case, then I ask why it is that, in order to arrive at an understanding of the different parts of the world, we must begin with the investigation of the Words of God, rather than of His Works. Is then the Work less venerable than the Word? If someone had held it to be heresy to say that the Earth moves, and if later verification and experiments were to show us that it does indeed do so, what difficulties would the church not encounter! If, on the contrary, whenever the Works and the Word cannot be made to agree, we consider Holy Scripture as secondary, no harm will befall it, for it has often been modified to suit the masses and has frequently attributed false qualities to God. Therefore I must ask why it is that we insist that whenever it speaks of the Sun or of the Earth, Holy Scripture is considered quite infallible?*

In this book, I will not base any mechanisms on the existence of God, and at the same time, I will not conclude that the discovery of a mechanism precludes the existence of a God.

1.8 WHAT IS CELL BIOLOGY?

First, let me define biology. According to G. R. Treviranus (1802), who along with J. B. Lamarck (1802) gave us the term *biology*: "The subject of our researches will be the different forms and phenomena of life, the conditions and laws under which this state occurs, and the causes which produce it. We shall designate the science which is occupied with these things as biology or the theory of life" (quoted in Driesch, 1914). By the end of the 19th century, the Roman Catholic priest, Jean Baptiste Carnoy (1884) stressed the importance of establishing a field of cellular biology to understand all aspects of biology. He envisioned cell biology as a multidisciplinary field, saying, "To be complete it is necessary to envision the cell from all of its facets, from the point of view of its morphology, its anatomy, its physiology and its biochemistry." By 1939, Lorande Woodruff wrote that when it comes to biology, the study of life, the cell has become "a sort of half-way house through which biological problems must pass, going or coming before they complete their destiny."

We will center our study of biology on the cell—the basic unit of life. We will try to understand the processes that contribute to our definition of life from first principles, that is, with the fewest assumptions possible (Northrop, 1931). In our search, we will use the techniques and tenets of biochemistry, biophysics, microscopy, immunology, physiology, genetics, and the various "-omics." By studying the basic unit of life, we will try to understand the nature of life and its unity.

Enjoy your search into the nature of the cell and remember what Albert Szent-Györgyi (1960) said about research: "The basic texture of research consists of dreams into which the threads of reasoning, measurement, and calculation are woven."

1.9 SUMMARY

Life consists of the ability to move and generate electricity; to take up nutrients and expel wastes; to perform chemical syntheses of organic molecules at ambient temperatures and pressures, and therefore grow; to reproduce itself with near-perfect fidelity; and to sense and respond to changes in the external environment in order to maintain itself. The cell is the lowest level of organization that has the ability to perform all these processes and thus is the basic unit of life (Table 1.5).

TABLE 1.5 Cell as the basic unit of life in context

Numbers and constants (mathematics)—*e*, π, −1, 0, 1, etc., k, h, c, G
Elementary particles (physics)—quarks, antiquarks, leptons
Free lifeless particles
Elements (chemistry)—H, C, N, O, P, S, etc.
Molecules—H_2O, CO_2, NO_3, PO_4, etc.
Minerals (mineralogy)—(e.g., clays, which are able to grow and reproduce themselves in an ionic solution)
Simple organic molecules (organic chemistry)—CH_4, NH_3, H_2S, HCN, etc. (organic chemistry)
$C(H_2O)$, C(OOH)C(HR)NH_3, fatty acids, adenine, etc.
Organic macromolecules (biochemistry, physical chemistry, molecular biology, genomics and other -omics)
—carbohydrates, proteins, lipids, nucleic acids

All the above levels of lifeless particles show passive translational motion (i.e., diffusion) and move passively in response to pressure and thermal gradients and electromagnetic and gravitational fields. Radiant energy causes a change in their electronic structure.

Viruses (proteins and nucleic acids; virology): Viruses are able to reproduce, adapt, and evolve in a living environment created by other organisms.
Cells—living particles (cell biology): Cells are able to take up nutrients, grow, synthesize compounds at body temperature and 1 atm of pressure, degrade compounds at body temperature and 1 atm of pressure, cause conversion of kinetically stable compounds into kinetically unstable compounds to be used as a ready supply of energy to perform endergonic reactions, expel wastes, regulate the biosynthetic and degradative processes, sense and respond to the environment in an adaptive manner, and move actively and reproduce with near-perfect fidelity to allow for the continuity of life as well as adaptation by natural selection.
Bacteria and Protoctists (single-celled prokaryotic and eukaryotic organisms): Able to perform all the functions of life (microbiology).
Colonies (psychology, invertebrate biology)
Multicellular organisms:
Animals, Fungi, Plants (zoology, mycology, botany, biology, anatomy, morphology, physiology, developmental biology, taxonomy, systematics, biogeography, biomechanics, biophysics, etc.)
Soul (neurobiology, behavior, psychology, psychiatry, philosophy, theology)
Mind (neurobiology, behavior, psychology, psychiatry, philosophy)
Thinking (neurobiology, behavior, psychology, psychiatry, philosophy)
Personality (neurobiology, behavior, psychology, psychiatry, philosophy)
Individuality (neurobiology, behavior, psychology, psychiatry, philosophy, political theory)
Spirituality (neurobiology, behavior, psychology, psychiatry, philosophy, theology)
Cells interact within an organism to make possible highly specialized cells, tissues, organs, and the processes that they perform.
Multiorganism level:
Relationship between the organism and other organisms (political science, ecology, psychology, sociology, philosophy, theology)
Relationship between the organism and the environment (political science, ecology, psychology, sociology, philosophy, theology)
Relationship between the organism and the universe (theology, astronomy, cosmology)
Each organism does not live in isolation. For example, it may be the predator and/or the prey. Or it may be a symbiont. It may be a member of a pioneer species, or it may come into an environment after the way is prepared, etc. And unbelievably, Homo sapiens *have the ability to know their place in the universe.*

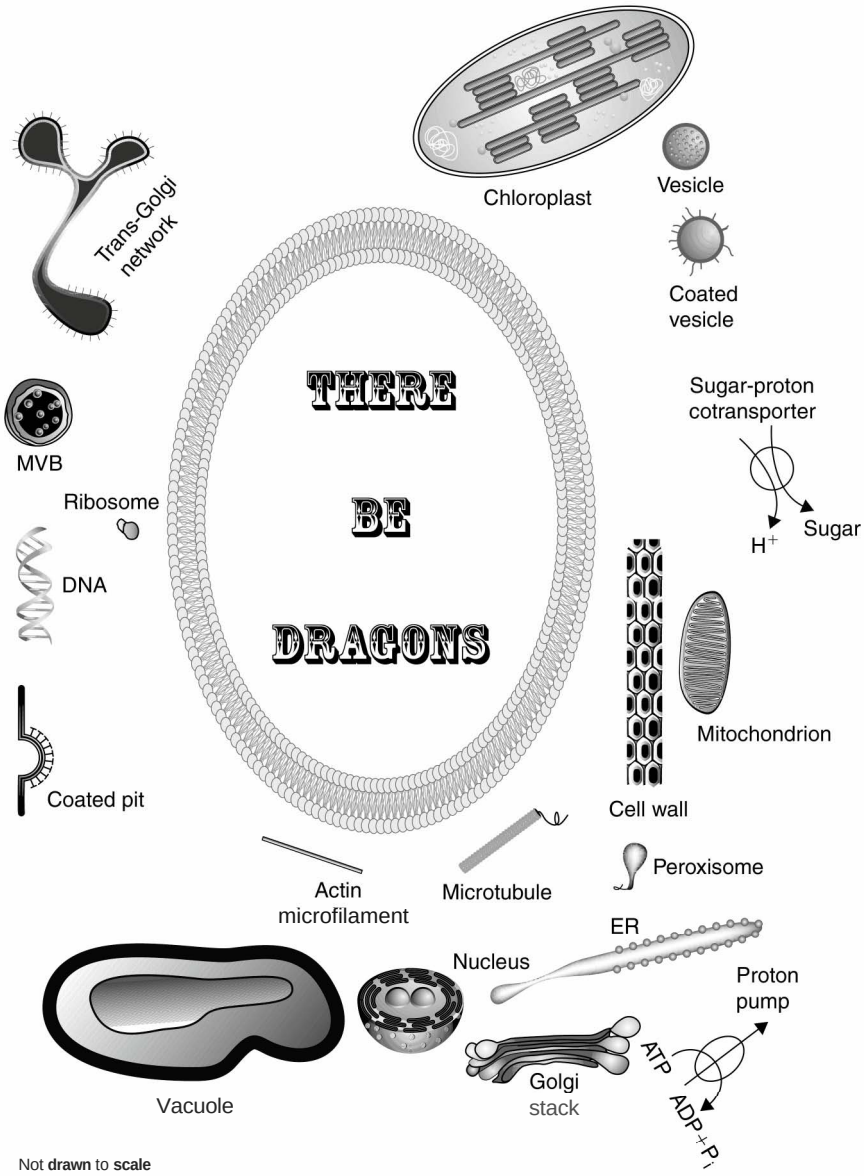


FIGURE 1.13 Build your own map of a cell. Make a copy of this figure and place the organelles in the cell as you learn about them. What are the similarities between cells? What are the differences? How do the organelles change their positions? Which organelles are derived from other organelles?

Our endeavor is to understand the vital processes that are made possible by cells from a physico-chemical standpoint. Your own personal map of the cell is provided in Figure 1.13. Throughout your journey through the cell, add the landmarks you discover. Keep in mind that the quantity, composition, and arrangement of any of the landmarks may change during cell development, following a change in the cell's environment, and as you travel from cell to cell. Develop an idea of which landmarks and which of their characteristics are fundamental, which are important in specialized systems, and which may be ephemeral.

1.10 QUESTIONS

1.1. What is life and how can the study of cells help us bring meaning and value to life?
1.2. How can mathematics help us understand living processes?

Chapter 3

Plasmodesmata

3.1 THE RELATIONSHIP BETWEEN CELLS AND THE ORGANISM

Cells in multicellular organisms are both autonomous and interdependent (Huxley, 1912; Canguilhem, 1969; Andrews, 2007). Biologists have argued about the relationship between cells and the organism—or the part to the whole—with as much passion as those who argue about the relationship of the individual to the state (Hobbes, 1651; Locke, 1690; Hume, 1748; Rousseau, 1762; Priestley, 1771; Lafayette and Jefferson, 1789; Stanton, 1848; Thoreau, 1849; Spencer, 1860; Roberts, 1938; Hamilton et al., 1961) or the individual to the rest of the world (Taylor, 2008). Proponents of the organismal theory of plant development and the cellular theory of plant development still argue vehemently about the respective importance of each level of organization in plant development, although, like many arguments, there are elements of truth in both views (Weiss, 1940; Kaplan and Hagemann, 1991; Kaplan, 1992; Baluska et al., 2004).

The organismal theory of plant development arose after botanists, including Charles-François Brisseau-Mirbel (1808) and Augustin deCandolle and Sprengel (1821), studied static sections of plants and concluded that there were three elementary components of plants: cells, tubes, and spirals. Consequently, the whole plant was considered the single most elementary form of vegetable life. By contrast, Dutrochet (1824) took a dynamic developmental approach and noticed that all the structures in plants, including the tubes and spirals, developed from cells. Dutrochet not only championed the view that the cell is the fundamental element in multicellular organisms, but also emphasized that cells were independent entities. Dutrochet (1824, in Buvat 1969) wrote,

> *I may repeat here what I have revealed previously about the organic texture of plants. We have seen that these organisms were entirely composed of cells, or of organs obviously derived from cells. We have seen that these hollow organs were simply contiguous, and held to each other by a cohesive force, but that such an assembly of cells did not really form one continuous tissue. Thus it seemed to us that an organic creature consists of an infinite number of microscopic components, which have no relationship to each other beyond that of being adjacent.*

The cell view was later supported by the evolutionary interpretation of the trends in both the plant and animal kingdoms to form more and more elaborate organisms. We see such trends vividly in the green algae where some organisms, like *Chlamydomonas*, are composed of only a single cell, while others are organized loosely into colonies that show no (e.g., *Gonium*) or minimal (e.g., *Volvox*) differentiation. Still others, for example, *Ulva*, are even differentiated into leaflike and rhizoidal tissues (Bold and Wynne, 1978). This phylogenetic series implies that multicellular organisms are *cell republics*, which result from the assemblage of a large number of independent units.

The organismal view was supported by Julius Sachs (1887), who wrote,

> *That plants consist of cells is now known to every well-informed man; yet the true meaning of the word cell may be quite clear to but a few, the less so since biologists themselves, even now, hold and discuss the most different opinions upon it. To many, the cell is always an independent living being, which sometimes exists for itself alone, and sometimes "becomes joined with" others—millions of its like in order to form a cell-colony, or, as Häckel has named it for the plant particularly, a cell republic. To others again, to whom the author of this book also belongs, cell-formation is a phenomenon very general, it is true, in organic life, but still only of secondary significance; at all events, it is merely one of the numerous expressions of the formative forces which reside in all matter, in the highest degree, however, in organic substances.*

T. H. Huxley (1853) also felt that cells "are not instruments, but indications—that they are no more the producers of the vital phenomena than the shells scattered in orderly lines along the sea-beach are the instruments by which the gravitative force of the moon acts upon the ocean. Like these, the cells mark only where the vital tides have been, and how they have acted." Anton de Bary put it more succinctly: "The plant forms cells, not cells the plant" (quoted in Barlow, 1982).

The organismal theory was further supported by Whitman (1894) and Lester Sharp (1934), who wrote in

his book, *Introduction to Cytology*, "The body is not an aggregation of elementary organisms, but a single organism which has evolved a multicellular structure." He noted that many plants, particularly the gymnosperms and *Paeonia*, pass through a coenocytic stage during early embryogenesis (Bierhorst, 1971), and indeed, the differentiation of the organism into cells is not necessary for complex development since there are large organisms, including *Caulerpa* and *Bryopsis*, that consist of only one cell, yet differentiate into leaflike, stemlike, and rootlike structures. However, Sharp went on to say:

> *The presence of cell partitions allows a more effective segregation of functionally specialized regions and a fuller play to those important physico-chemical processes which depend on surfaces and thin films for their action. Furthermore, it permits the development of larger plant bodies by furnishing an ideal basis for the more effective operation of turgor and for the deposition of supporting materials. . . . The evolution of higher organisms has unquestionably been very largely conditioned by the multicellular state, but we should think of such organisms primarily as highly differentiated protoplasmic individuals rather than cell republics.*

Is this old and ever-recurrent problem of cell theory versus organismal theory a moot question? According to Wilhelm Ostwald (1910), we can determine whether a question is moot by asking ourselves, "What would be the difference empirically if the one or the other view were correct?" I think that both theories have elements of truth that help understand plant development. I concur with the organismal view of multicellular organization and believe that it is erroneous to work on the assumption that an organism is only equal to the sum of its parts and has no greater level of organization and coordination.

Multicellular organisms have emergent properties that the individual cells themselves lack (Heitler, 1963). Even water has a higher level of organization and integration than the oxygen and hydrogen of which it is composed! A purely cellular view could hinder research on higher levels of integration. However, it will become clear as we continue our journey that a purely organismal view could lead to erroneous experimental results. Each organism is made up of many different cell types, each of which is surrounded by a differentially permeable membrane that determines the degree of autonomy of each cell. Some of these cells may be undergoing different processes at a given time than others. Thus, when breaking the organism up into its parts in order to understand it physiology, misleading results and unjust interpretations may occur unless one separates and studies individual cell types (Wayne, 1994). On the other hand, no cell in a multicellular organism is completely autonomous, and when we isolate cells, we must be aware of the mechanical, electrical, and chemical influences we are severing (Lintilhac, 1999; Roelfsema and Hedrich, 2002). Indeed, the enucleate sieve tube elements are completely dependent on their companion cells for a continuous supply of protein (Parthasarathy, 1974; Esau and Thorsch, 1985; Lough and Lucas, 2006).

In Chapter 2, I spoke about cells as if they existed in isolation, protected by the plasma membrane from an ever-changing and sometimes hostile environment. However, the cells in multicellular plants are not only physically touching, but often connected by small structures called plasmodesmata (singular, plasmodesma), which allow direct cell-to-cell communication (Tangl, 1879; Elsberg, 1883; Goebel, 1926; Ehlers and Kollmann, 2001; Oparka and Roberts, 2001; Roberts, 2005). Indeed, the presence of functioning plasmodesmata is correlated with the ability of cells to divide synchronously (Ehlers and Kollmann, 2000) and the loss of plasmodesmatal function is correlated with programmed cell death (Zhu and Rost, 2000). Moreover, as a result of the presence of plasmodesmata, the plasma membrane of one cell is continuous with the plasma membrane of the adjoining cell, thus forming a continuum of P-spaces, known as the *symplast*, and a continuum of E-spaces outside the plasma membrane, known as the *apoplast*.

The cells in multicellular animals are often connected by structures analogous to plasmodesmata, known as *gap junctions* (Sjöstrand et al., 1958; Revel and Karnovsky, 1967; McNutt and Weinstein, 1973; Cox, 1974). Intercellular connections between animal cells, 50–200 nm in diameter and several cell diameters long, are known as *cytonemes, nanotubular structures*, or *tunneling nanotubes* (TNT; Ramirez-Weber and Kornberg, 1999; Rustom et al., 2004). The plasma membranes of the connected cells are in direct communication and can be seen to exchange fluorescently labeled fusion proteins (Rustom et al., 2004).

3.2 DISCOVERY AND OCCURRENCE OF PLASMODESMATA

Eduard Tangl (1879) was the first person to observe connections between cells while observing with the light microscope the endosperm of a variety of plants (Figure 3.1). He serendipitously discovered these connections while investigating cell walls with organic dyes (Köhler and Carr, 2006). Tangl proposed that these connections were important for transport between cells. These intercellular connections, which pass through the surrounding extracellular matrix, came to be known as *plasmodesmata* (Meeuse, 1957). Plasmodesmata occur in all the major groups of plants from algae to higher plants, and although the structure of the plasmodesmata in all these groups is remarkably similar (Robards and Lucas, 1990) there is some variation at the microscopic (Franceschi et al., 1994; Botha et al., 2005) and nanoscopic (Beebe and Turgeon, 1991; Waigmann et al., 1997) levels. Evolutionary studies of plasmodesmata are ongoing (Cooke et al., 1997; Raven, 1997, 2005; Cooke and Graham, 1999; van Bel and Kesteren, 1999).

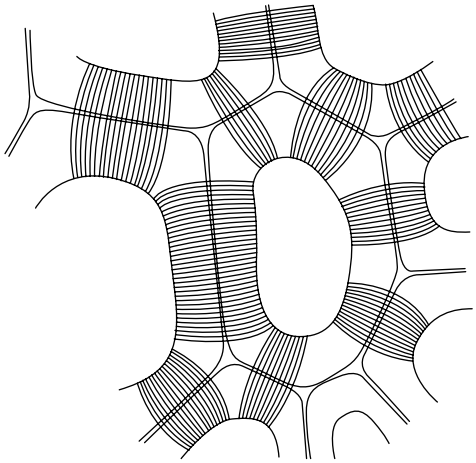


FIGURE 3.1 Intercellular connections (plasmodesmata) between endosperm cells of *Strychnos nuxvomica*. The neuromuscular poisons,

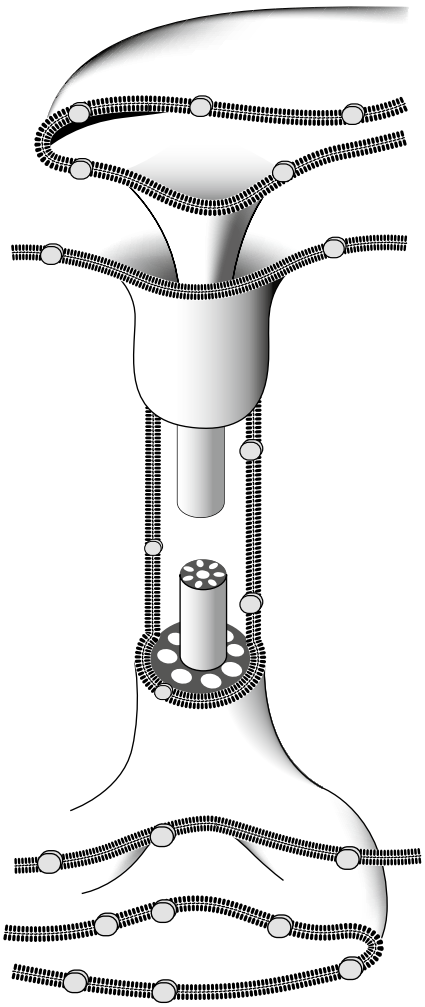


FIGURE 3.2 Diagram of a plasmodesmata showing the three-dimensional relationship between the ER, plasma membrane, and desmotubule. (*Source:* From Gunning and Overall, 1983.)

Plasmodesmata between two sister cells are typically formed during cytokinesis and are called *primary plasmodesmata*. However, plasmodesmata formation can take place between any two adjacent cells, forming new symplastic pathways. Plasmodesmata that are formed between two cells that are already separated by an extracellular matrix are called *secondary plasmodesmata*. In terms of primary and secondary plasmodesmata, plasmodesmatal formation in *Chara*, a genus of algae on the evolutionary line that gave rise to higher plants, is of interest. While *Chara zelanica* produces both primary and secondary plasmodesmata (Cooke et al., 1997), *Chara corallina* produces only secondary plasmodesmata (Franceschi et al., 1994). The secondary plasmodesmata may have different transport characteristics from the primary plasmodesmata in the same cell (Itaya et al., 1998).

The biogenesis of the primary plasmodesmata will be discussed in Chapter 19. Secondary plasmodesmata, however, begin their formation when the extracellular matrix thins in regions where the endoplasmic reticulum (ER) is abutting the plasma membrane. As the extracellular matrix dissolves in this localized area, the endoplasmic reticula of the two adjoining cells, as well as the bordering plasma membranes, fuse to form a plasmodesma (Kollmann and Glockmann, 1991). Both primary and secondary plasmodesmata are initially simple in structure, but can form complex structures through branching and/or fusion of exiting plasmodesmata or the fusion of established and newly formed plasmodesmata (Oparka et al., 1999; Elhers and Kollmann, 2001; Roberts et al., 2001; Faulkner et al., 2008).

There are generally between 1 and 15 plasmodesmata/μm^2, although as many as 39 plasmodesmata/μm^2 have been observed. Plasmodesmata can either be uniformly distributed around the cell or occur in aggregates. In a given cell, at a given time, the number and density of plasmodesmata are precisely determined (Tilney et al., 1990b). However, the density of plasmodesmata, their structure, and/or their unitary conductance can change over time (Palevitz and Hepler, 1985; Zambryski and Crawford, 2000; Kwiatkowska, 2003). At maturity, guard cells and tracheary elements lose all plasmodesmatal connections to neighboring cells (Wille and Lucas, 1984; Erwee et al., 1985; Palevitz and Hepler, 1985; Lachaud and Maurousset, 1996). The frequency of plasmodesmata is influenced by day length and cytokinin application (Ormmenese et al., 2006).

3.3 STRUCTURE OF PLASMODESMATA

Based on electron microscopic evidence, López-Sáez et al. (1966a) proposed a model for plasmodesmatal structure (Figure 3.2). Although this model has been contested (Gunning and Robards, 1976), it is still widely accepted (Overall et al., 1982; Hepler, 1982). Electron micrographs show that a plasmodesma is a cylindrical, membrane-lined, mostly aqueous canal that is 20–40 nm in diameter and can

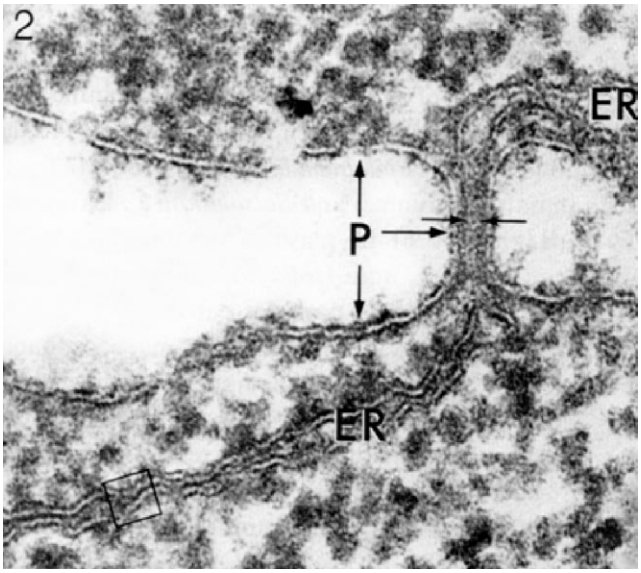


FIGURE 3.3 Longitudinal view of a plasmodesma in *Azolla pinnata* root cells. The arrow points to the desmotubule. ER, endoplasmic reticulum; P, plasma membrane. ×175,000. (*Source:* From Overall et al., 1982.)

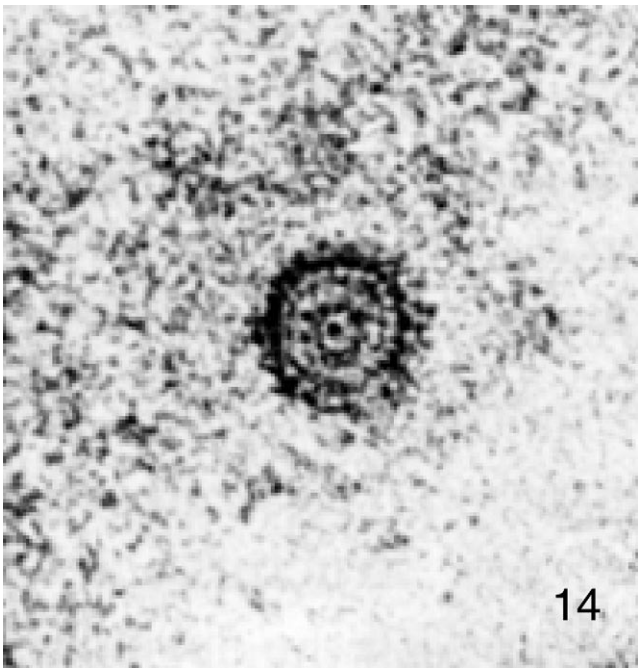


FIGURE 3.4 Transverse view of a plasmodesma in a lettuce root tip cell, ×210,000. (*Source:* From Hepler, 1982.)

be hundreds to thousands of nanometers long, depending on the thickness of the intervening extracellular matrix (Figure 3.3). In the center of the canal is a cylindrical structure. It was originally called the *axial component* and now is commonly called the *desmotubule* (Figure 3.4). The desmotubule is continuous with the endoplasmic reticulum (see Chapter 4). A cytoplasmic pathway, called the *cytoplasmic annulus*, surrounds the desmotubule and is continuous from cell to cell. The ends of the cytoplasmic annulus often seem to be constricted. These constrictions may regulate the flux of substances through the cytoplasmic annulus, although currently there is no evidence for this.

Electron microscopic images of plasmodesmata are shown in Figures 3.3 and 3.4. The plasma membrane shows up as a tripartite structure that is 7.2 nm wide, and the dense central rod is 1.4 nm in radius. The width of the pale ring that surrounds the dense central rod is 2.2 nm. This is consistent with the hypothesis that the desmotubule is made of the membrane of the endoplasmic reticulum without any lumen. The central rod represents the polar head groups of two oppressed inner leaflets of the ER membrane that are close-packed, and the clear ring represents the fatty acyl groups of the bilayer. The layer between the inner leaflet of the plasma membrane and the hydrocarbon ring of the desmotubule is called the *cytoplasmic annulus*. The cytoplasmic annulus appears as a densely stained region, approximately 4.5 nm wide, and shows some substructure. The lumen of the endoplasmic reticulum is not continuous within a plasmodesma between cells, as evidenced by the discontinuity in staining by a lumen-filling stain (Figure 3.5), as well as the lack of cell-to-cell transport of green fluorescent protein (GFP) that targeted the lumen of the ER (Oparka et al., 1999).

In transverse sections, the neck region often appears different from the rest of the plasmodesmata (Robards and Lucas, 1990). An extracellular ring of large particles appears to surround the outer part of the neck construction (Taiz and Jones, 1973; Olesen, 1979; Mollenhauer and Morré, 1987). It is possible that these extracellular particles regulate the size of the cytoplasmic annulus. However, the extracellular particles are not seen in rapidly freeze-fixed tissues, indicating that they may be wound-induced localized formations of callose, which under natural wounding conditions would serve to isolate the wounded cell (Ding et al., 1992b; Radford et al., 1998).

Freeze fixation followed by freeze substitution has allowed a more detailed knowledge of plasmodesmatal structure compared with chemical fixation, because with freeze fixation, the cells are killed and the structures are fixed within milliseconds. With chemical fixation, cells take several seconds to die due to the relatively slow penetration of chemicals compared to the rate in which heat can be dissipated (Mersey and McCully, 1978). Thus, during chemical fixation, there is sufficient time for wound processes to occur and for cellular structures to become modified (Buvat, 1969).

Freeze fixation is done by plunging a cell or small tissue into liquid propane. Then the vitrified water in the sample is removed with organic solvents. Then chemical fixatives are added to stabilize the cellular structures. The samples are then warmed to room temperature, embedded in plastic, sectioned, stained, and viewed with an electron microscope.

The general structure of plasmodesmata in a freeze-substituted tobacco leaf is similar to that seen in chemically

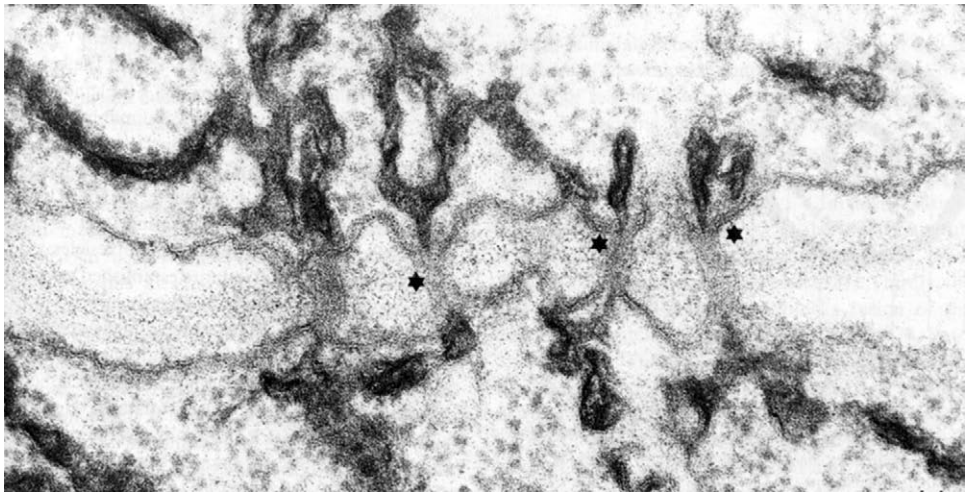


FIGURE 3.5 Longitudinal view of plasmodesmata in lettuce root tip cells. The lumen of the endoplasmic reticulum is stained with OsFeCN. The cisternal space is constricted where the endoplasmic reticulum enters the plasmodesmata (asterisks). ×100,000. (*Source:* From Hepler, 1982.)

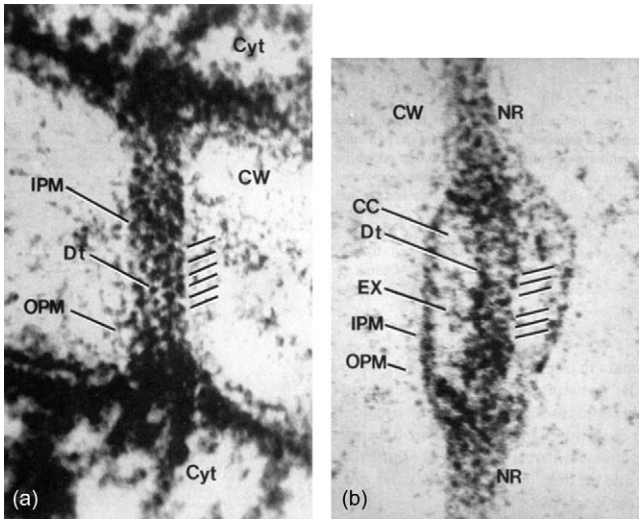


FIGURE 3.6 Longitudinal sections through plasmodesmata of tobacco cells that have been prepared by freeze fixation and freeze substitution: (a) a plasmodesma between phloem parenchyma cells; (b) a plasmodesma between a phloem parenchyma cell and a bundle sheath cell. IPM, inner leaflet of the plasma membrane; OPM, outer leaflet of the plasma membrane; Dt, desmotubule; CW, extracellular matrix; cyt, cytoplasm; NR, neck region; EX, spoke-like extensions; CC, central cavity. (*Source:* From Ding et al., 1992b.)

fixed materials. However, new details in the substructure can be seen (Ding et al., 1992b; Ding et al., 1999; see Figure 3.6). The inner leaflet of the plasma membrane running through the plasmodesmata appears to be lined with a series of helically arranged electron-dense particles. In addition, the outer leaflet of the ER that makes up the desmotubule is also lined with helically arranged electron-dense particles. The gaps between the particles on the plasma membrane inner leaflet and desmotubule seem to form the aqueous transport canals of the plasmodesmata. If so, the canals may not be straight, but helical as indicated by unlabeled lines in Figure 3.6. Compared with cell-to-cell diffusion through straight channels, diffusion from cell to cell through helical channels will take longer because the effective distance between the two cells will be longer.

A variety of intercellular connections that range from large simple holes to elaborate structures can be found in the fungi (Reichle and Alexander, 1965; Carroll, 1967; Brenner and Carroll, 1968; Carroll, 1972; Furtado, 1971; Beckett et al., 1974) and the red algae (Bold and Wynne, 1978). Each structure represents a compromise between cell individuality and the organismal whole.

3.4 ISOLATION AND COMPOSITION OF PLASMODESMATA

Pure and intact plasmodesmata can be isolated (Kotlizsky et al., 1992; Epel et al., 1996; Bayer et al., 2004). In order to isolate plasmodesmata, plants are frozen and pulverized to a fine powder. The powder is further homogenized in a buffer and passed through a nylon mesh that retains the plasmodesmata embedded in the extracellular matrix. The extracellular matrix fraction is then passed through a valve under pressure to shear the fraction into tiny fragments. These fragments, which contain the plasmodesmata, are collected by centrifugation at 600 g for 10 minutes.

The proteins of the plasmodesmata are then characterized by solubilizing them in sodium dodecyl sulfate (SDS) and subjecting them to polyacrylamide gel electrophoresis. While there are many polypeptides in the wall, one is of particular interest. It is a 26- to 27-kDa protein that cross-reacts with antibodies made against connexin (Meiners and Schindler, 1987, 1989; Meiners et al., 1991b; Yahalom et al., 1991), which is a component of the intercellular connections (i.e., gap junctions) of animal cells.

Yahalom et al. (1991), using immunolocalization electron microscopy, found that a connexin-like protein is present in the plasmodesmata along the entire length, including the cytoplasmic annulus and the neck region. Immunolocalization electron microscopy involves treating thin sections with an antibody that is specific for an antigen, which in this case is a connexin-like protein. After washing away the loosely bound antibodies,

the sections are treated with a secondary antibody attached to 12- to 15-nm particles of gold. This secondary antibody recognizes the primary antibody. The antigen can be localized because the electron-dense gold is precipitated nearby. A calcium-dependent protein kinase (Yahalom et al., 1998), centrin (Blackman et al., 1999), calreticulin (Baluska et al., 1999; Bayer et al., 2004), myosin (Radford and White, 1998; Reichelt et al., 1999), actin (White et al., 1994; Blackman and Overall, 1998), a reversibly glycosylated polypeptide (Sagi et al., 2005), a protein kinase (Lee et al., 2005), and a β-1,3-glucanase (Levy et al., 2007) have also been localized in the plasmodesmata. Other as yet unidentified proteins have been observed to be associated with plasmodesmata through proteomic analysis (Faulkner et al., 2005).

Plasmodesmatal proteins are being identified by fusing sequences that encode GFP with random stretches of cDNA, and then after transient expression, looking for those proteins that localize to the plasmodesmata (Escobar et al., 2003). Thomas et al. (2008) have discovered a protein that is capable of influencing the transport of GFP through the plasmodesmata and have discovered the amino acid sequence necessary to specifically target this plasmodesmatal protein to the plasmodesmata.

3.5 PERMEABILITY OF PLASMODESMATA

The fundamental significance of plasmodesmata is that they form a low-resistance pathway between two cells through which large hydrophilic molecules can travel faster than they would if they had to pass through the plasma membrane to leave a cell and through another plasma membrane to enter the next cell. In order to calculate the permeability coefficient of plasmodesmata, Goodwin et al. (1990) injected fluorescent dyes into cells of *Egeria* and measured the rate in which the dyes diffused into the next cell. They also calculated the permeability coefficient for the plasma membrane by measuring the rate in which the dye diffused into the cell from the extracellular medium. The permeabilities of the plasma membrane and plasmodesmata are shown in Table 3.1.

The plasmodesmata are approximately 10,000 times more permeable than the plasma membrane to the dyes with molecular masses less than 700 Da. For dye molecules greater than 1000 Da, the permeability coefficients of the plasmodesmata become indistinguishable from those of the plasma membrane.

The plasmodesmatal permeability coefficients (P) are obtained by assuming that the dyes move from cell 1 (C_1) to cell 2 (C_2) by diffusion during time t, and can thus be modeled by Runnström's (1911) modification of Fick's Law (see Chapter 2):

$$ds_2/(A\,dt) = -P(C_2 - C_1) \tag{3.1}$$

where A is the area between cell 1 and cell 2.

TABLE 3.1 Permeability coefficients of plasmodesmata and plasma membrane of *Egeria*

	Permeability Coefficient (m/s)	
Molecule (M_r)	**Plasmodesmata**	**Plasma Membrane**
6-CF (376)	112×10^{-8}	2.4×10^{-12}
FITC + glutamic acid (536)	15.5×10^{-8}	1.8×10^{-12}
FITC + glutamyl-glutamic acid (665)	11.4×10^{-8}	1.3×10^{-12}
FITC + hexaglycine (744)	0.66×10^{-8}	2.3×10^{-12}
FITC + leucyl diglutamyl-leucine (874)	0.009×10^{-8}	1.6×10^{-12}

Source: From Goodwin et al. (1990).

The volumes of the cells (V_1 and V_2) remain constant during the experiment. The amount of dye that diffuses into cell 2 is equal to the change in concentration (dC_2) in cell 2 times the volume (V_2) of cell 2. That is, since $ds_2 = V_2 dC_2$, then

$$(V_2/A)(dC_2/dt) = -P(C_2 - C_1) \tag{3.2}$$

and

$$(dC_2/dt) = -P(A/V_2)(C_2 - C_1) \tag{3.3}$$

After dividing both sides by $(C_2 - C_1)$ and multiplying both sides by dt, we get:

$$(dC_2)/(C_2 - C_1) = -P(A/V_2)dt \tag{3.4}$$

In order to calculate P, we must integrate the Eq. 3.4. To integrate easily, we must assume that P, A, V_2, and C_1 remain constant. Since we know that C_1 will decrease with time, we must do the experiment over short periods of time.

$$\int_{t=0}^{t=t} (dC_2)/(C_2 - C_1) = -P(A/V_2)\int_{t=0}^{t=t} dt \tag{3.5}$$

First, let us integrate the left side. Let $u = C_2 - C_1$, thus, if C_1 is constant, then $du = dC_2$ and $\int_{t=0}^{t=t} (dC_2)/(C_2 - C_1) = \int_{t=0}^{t=t} (du/u)$, which according to the Fundamental Theorem of Calculus is equal to $\ln(u_t/u_0)$, which after substitution is equal to $\ln[(C_2 - C_1)_t/(C_2 - C_1)_0]$.

Now let us integrate the right side:

$$-P(A/V_2)\int_{t=0}^{t=t} dt = -P(A/V_2)t \tag{3.6}$$

Thus,

$$\ln[(C_2 - C_1)_t/(C_2 - C_1)_0] = -P(A/V_2)t \quad (3.7)$$

At $t = 0$ and $C_2 = 0$, thus $(C_2 - C_1)_0 = (-C_1)_0$, and

$$\ln[(C_2 - C_1)_t/(-C_1)_0] = -P(A/V_2)t \quad (3.8)$$

If the experiment is done for short times and C_1 barely changes, and $(C_1)_0 = (C_1)_t$, then

$$\ln[(-C_2)_t/(C_1)_0 + 1] = \ln[1 - (C_2)_t/(C_1)_0] = -P(A/V_2)t \quad (3.9)$$

Since C_2, C_1, A, V_2, and t are all measurable quantities, we can calculate P from the slope of an experimentally derived curve that relates $-\ln[1 - (C_2)_t/(C_1)_0]$ to t. P is equal to the slope (in s^{-1}) times (V_2/A). The permeability of the plasmodesmata is influenced to some extent on the tissue preparation technique (Radford and White, 2001).

Dye movement experiments have been performed on filaments of soybean culture cells using fluorescence redistribution after photobleaching (FRAP; Baron-Epel et al., 1988b). With this technique, the hydrophobic form of carboxyfluorescein (i.e., carboxyfluorescein diacetate) is added to the external medium. The dye is passively taken up across the plasma membrane in the ester form. Esterases then cleave the hydrophilic portion of the dye from the hydrophobic acetates. The cell then glows from the dye. Then a laser beam bleaches the dye in one cell and the movement of dye into this cell from neighboring cells is monitored over time. A rate constant (1/time) is obtained from these data. The rate constant can be transformed into a permeability coefficient if we postulate that the rate (K) that the dye moves into the cell is proportional to the area (A) on two sides of the cell since the plasmodesmata are only on two sides of soybean culture cells. We must also assume that the rate in which the cell gets brighter is inversely proportional to the volume (V) of the cell. Last, we must define the permeability coefficient (P) as the conversion factor that relates the rate to the area and volume. Thus,

$$K = P(A/V) \quad (3.10)$$

Baron-Epel et al. (1988) obtained a rate of 0.0015 s^{-1}. Since for soybean culture cells, $A/V = 1.7 \times 10^5\,m^{-1}$, then $P = 0.9 \times 10^{-8}$ m/s, which is the ballpark of the values found by Goodwin et al. (1990) for *Egeria*.

The diameter of the aqueous canals of the plasmodesmata can be estimated from dye injection experiments by using dyes of various sizes. The diameters depend on the cell type tested. These experiments show that plasmodesmata can pass molecules that have a molecular mass of less than 376–800 Da in *Elodea* (Goodwin, 1983; Erwee and Goodwin, 1985), 700–800 Da in *Setcreasea* stamen hairs (Tucker, 1982), 850–900 Da in bundle sheath cells of C4 plants (Weiner et al., 1988) and molecules as large as 1090 Da in the nectary trichome cells of *Abutilon* (Terry and Robards, 1987; Fisher, 1999), and 20,000 Da in the internodal cells of *Nitella* (Kikuyama et al., 1992).

TABLE 3.2 Relationship between molecular mass and hydrodynamic radius

Molecular Mass (M_r, Da)	Hydrodynamic Radius (r_H) nm
100	0.26
200	0.35
400	0.51
700	0.76
800	0.85
900	0.93
1000	1.01
1090	1.09

Dye permeation experiments can help us determine the size of the plasmodesmatal canals since there is a direct relationship between molecular mass and the hydrodynamic radius for small organic molecules (Table 3.2). The hydrodynamic radius of a molecule can be determined from diffusion or viscosity measurements with molecules of known molecular mass (Schultz and Solomon, 1961).

The hydrodynamic radius (r_H) of a spherical molecule can be calculated from the Stokes-Einstein equation presented in Chapter 2, as long as one knows the diffusion coefficient of the molecule and the viscosity of the solution:

$$r_H = kT/(6\pi D\eta) \quad (3.11)$$

Using the measurements of the hydrodynamic radius determined by using the Stokes-Einstein equation and the molecular masses of the solutes, I have come up with the following empirical formula to express the relationship between the hydrodynamic radius (in nm) and the molecular mass (M_r, in Da):

$$r_H = 0.00083327\ (M_r) + 0.18 \quad (3.12)$$

Thus, the dye permeation studies indicate that the cytoplasmic annuli have size exclusion limits that typically vary between 0.7 and 4 nm, depending on the cell type. These estimates are compatible with what would be expected from structural studies. Movement through the plasmodesmata is not restricted to hydrophilic molecules. Hydrophobic molecules may also pass from cell to cell by translation through the lipid bilayers in the membranes

that make up the plasmodesmata (Baron-Epel et al., 1988b; Grabski et al., 1993; Fisher, 1999).

In order to test the influence of particular amino acid sequences on plasmodesmatal transport, the biolistic bombardment technique is used to quantify transport (Oparka and Boevick, 2005). With this transient expression technique, genes that are engineered to express proteins that are fluorescent, have various enzymatic or regulatory activities, and plasmodesmatal targeting sequences are shot into a cell using the gene gun. Once the protein encoded by the engineered gene is expressed, the movement of the fluorescent protein with the engineered sequences to neighboring cells is observed and quantified.

Classical electrophysiological techniques similar to those used to characterize the plasma membrane show that the plasmodesmata provide a high-conductance pathway for the movement of ions between cells (Spanswick and Costerton, 1967; Overall and Gunning, 1982; van Bel and Ehlers, 2005). The plasmodesmata have a specific conductance approximately 50 times greater than that of the plasma membrane (Spanswick, 1974b).

The permeability of plasmodesmata can be regulated. For example, Ding and Tazawa (1989) and Oparka and Prior (1992) have shown that pressure can regulate plasmodesmatal conductivity and Baron-Epel et al. (1988b) and Tucker (1990) have shown that increasing the intracellular Ca^{2+} concentration inhibits intercellular movement of dyes. Holdaway-Clark et al. (2000) have shown that elevated cytosolic concentrations of Ca^{2+} increase the resistance of the plasmodesmata, providing further evidence that the plasmodesmata close in response to Ca^{2+}. This is particularly interesting since the $[Ca^{2+}]$ outside the cell is typically high ($\sim 1\,mol/m^3$) while it is low in the cell ($\sim 10^{-4}\,mol/m^3$), thus a high intracellular $[Ca^{2+}]$ is a sign of a damaged cell (e.g., the plasma membrane is lysed). Thus, the decreased conductance of the plasmodesmata due to high Ca^{2+} may isolate a damaged cell from its healthy neighbors. External stimuli, including red light, can also influence plasmodesmatal conductance (Racusen, 1976). Plasmodesmatal permeability is also regulated by actin microfilaments (Ding et al., 1996) and can change during cell development (Gisel et al., 1999, 2001; Oparka and Turgeon, 1999; Ruan et al., 2001; Kim et al., 2005).

Plasmodesmatal permeability is not only regulated by physiological and developmental signals, but is also increased by some of the proteins that are trafficked through them. This discovery came from the study by plant virologists who wanted to know how globular viruses 18–80 nm in diameter, or helical or filamentous rods 10–25 nm in diameter and up to 2.5 μm in length, pass through plasmodesmata (Lazarowitz, 1999; Lazarowitz and Beachy, 1999). Some viruses, like the dahlia mosaic virus and cauliflower mosaic virus, somehow drastically modify the structure of the plasmodesmata, getting rid of the desmotubule and expanding the diameter of the cytoplasmic annulus to 60–80 nm. These two viruses are commonly found within the plasmodesmata in transmission electron micrographs, indicating that the viruses move through the plasmodesmata to attack the host everywhere.

By contrast, the tobacco mosaic virus (TMV) is never observed in plasmodesmata. It is possible that only the small RNA genome passes through the plasmodesmata so that the plasmodesmata structure is only minimally affected. Through genetic studies of a temperature-sensitive mutant of this virus, Nishiguchi et al. (1980) found the gene that coded for the ability of the virus to move through the plant. They found the gene by obtaining a mutant virus that was able to replicate at 32°C, but was unable to move through the plant at this temperature. However, the virus was able to also move through the plant, if the temperature was lowered to the permissive level of 22°C.

Leonard and Zaitlin (1982) discovered the protein involved in virus movement when they found that the in vitro translation products of the mutant and wild type differed only in one 30-kDa protein. They concluded that this protein is involved in virus movement. The genes of the wild type and mutant have been sequenced and they differ only in one amino acid at position 154. The wild type has serine, while the mutant protein has proline (Ohno et al., 1983). Tomenius et al. (1987) have used immunogold cytochemistry to localize the 30-kDa protein in infected tobacco leaves and find it in the plasmodesmata. The plasmodesmatal proteins that interact with the movement protein are being identified (Kishi-Kaboshi et al., 2005).

A breakthrough in plasmodesmata research occurred when Deom et al. (1987, 1990, 1991) combined techniques of plant biotechnology and virology to construct a chimeric gene that encoded the 30-kDa movement protein and introduced it into tobacco plants. This allowed the study of the function of the 30-kDa gene product in the absence of all the other TMV gene products. They found that the 30-kDa protein was associated with the extracellular matrix fraction (see Chapter 20). Furthermore, in a type of complementation study it was found that mutant viruses could move through the transgenic plant at nonpermissive temperatures.

Wolf et al. (1989, 1991) showed with dye movement experiments that while control tobacco plants have a size exclusion limit of ca. 750 Da for cell-to-cell transport, transgenic tobacco plants that are expressing the movement protein have a size exclusion limit between 9400 Da and 17,200 Da. Thus, the movement protein is capable of regulating the size of the plasmodesmatal canals. Wolf et al. (1989) postulated that the exclusion limit of control plants was approximately 0.73 nm, while the transgenic plants had a size exclusion limit of 2.4–3.1 nm. This is still too small to pass the 8×300-nm virus or its approximately 10-nm RNA. Thus, it is likely that the movement protein acts as a chaperone to facilitate the movement of the viral RNA through the plasmodesmata (Lucas et al., 1993; Wolf and Lucas, 1994; Ghoshroy et al., 1997).

Movement proteins can also facilitate the movement of viral DNA through the plasmodesmata. Plant cells injected with movement protein (from bean dwarf mosaic gemini virus) and fluorescently labeled viral DNA show that the movement protein causes the movement of viral DNA from cell to cell (Noueiry et al., 1994). By contrast, red clover necrotic virus movement protein enhances the movement of RNA, but not DNA (Fujiwara et al., 1993).

It is likely that specific amino acid sequences are necessary for proteins to bind to and pass through the plasmodesmata. This hypothesis is supported by the observation that a fusion protein made by combining the targeting sequence from the viral movement protein with the sequence for the GFP enhances the cell-to-cell movement of the GFP (Crawford and Zambryski, 2000; Zambryski and Crawford, 2000; Liarzi and Epel, 2005). The low activation energy for the transport of GFP and other proteins not normally targeted to the plasmodesmata through the plasmodesmata indicates that any conformational changes of the plasmodesmata necessary to allow the movement of this large molecule must be minimal (Schönknecht et al., 2008). The movement of other proteins through plasmodesmata may require the transported protein to unfold in order to enter the plasmodesmata and refold when they exit. Moreover, the movement of proteins through plasmodesmata may require the plasmodesmatal proteins to change their conformation in order to increase the size-exclusion limit. The folding and unfolding may be facilitated by molecular chaperone proteins, including heat shock proteins, protein disulfide isomerases, and peptidyl-proyl *cis-trans* isomerases.

The search for native plant polypeptides that interact with the plasmodesmata and facilitate the movement of themselves or other proteins through the plasmodesmata is ongoing. Some proteins specifically target themselves or other proteins to the plasmodesmata and others unfold the proteins so that they can fit through the plasmodesmata and refold them upon passage or interact directly with the plasmodesmata in order to increase the size-exclusion limit (Kragler et al., 2000; Zambryski and Crawford, 2000; Haywood et al., 2002; Kragler, 2005). Recently, Gottschalk et al. (2008) have shown that the chaperone peptidyl-proyl *cis-trans* isomerase, which is also known as cyclophilin, is able to increase the size-exclusion limit of the plasmodesmata between mesophyll cells so that a 10-kDa fluorescent dextran can pass from the injected cell to other cells.

In many plants, the concentration of sucrose is greater in the mesophyll cells where it is produced by photosynthesis than in the cells of the phloem. Consequently, the sucrose formed in the mesophyll cells is transported by diffusion through the plasmodesmata connecting the cells between the mesophyll and the phloem (Turgeon and Medville, 2004). In order to move by diffusion, in these plants, the sucrose concentration must be higher in the mesophyll cells than in the sieve tube elements. In many other plants, however, the concentration of sugar is greater in the sieve tube elements than in the mesophyll cells. In these cases, a mechanism must exist to actively load the sugar into the phloem (Roberts and Oparka, 2003). There are two major hypotheses to describe how sugar is transported into the phloem against its concentration gradient. Data obtained by Robert Turgeon show that it is not an either/or situation. Some plants use one mechanism for phloem loading, others use the second mechanism for phloem loading exclusively, and still others use additional mechanisms.

According to the canonical apoplastic hypothesis of phloem loading, sugars pass through plasmodesmata from the mesophyll cells until they reach the phloem. At this point, the plasmodesmata are occluded and thus the sugars are unloaded into the apoplast (Beebe and Evert, 1992). According to the apoplastic hypothesis, the sugar is then loaded into the phloem against its concentration gradient in an ATP-dependent manner (Geiger et al., 1973, 1974; Sovonick et al., 1974; Giaquinta, 1976; Maynard and Lucas, 1982). Specifically, the sugars are then taken up through the plasma membranes of the sieve tube element–companion cell complex by sucrose/H^+ symporters that use the free energy inherent in the electrochemical difference of protons across the membrane formed by the H^+-ATPase.

According to the canonical symplastic-loading hypothesis, the sugar stays within the symplast. The major problem with the symplastic-loading hypothesis is being able to explain how sugars can move by diffusion through plasmodesmata against their concentration gradient (Turgeon and Hepler, 1989; van Bel, 1989). Robert Turgeon and Ester Gowan (1990) and Turgeon (1991) propose that special cells in the phloem known as intermediary cells act as a "molecular size-discrimination trap." In this model, sucrose and galactinol synthesized by the photosynthesizing mesophyll cells diffuse down their concentration gradients through the plasmodesmata between the bundle sheath cells and the intermediary cells. At this point, an enzyme combines the two small molecules into the larger raffinose, which is too big to diffuse back through the plasmodesmata that are thinner on the intermediary cell side (Figure 3.7). In this way, small molecules diffuse down their concentration gradient to the phloem, where they are converted to raffinose. The raffinose, unable to move back into the bundle sheath cell,

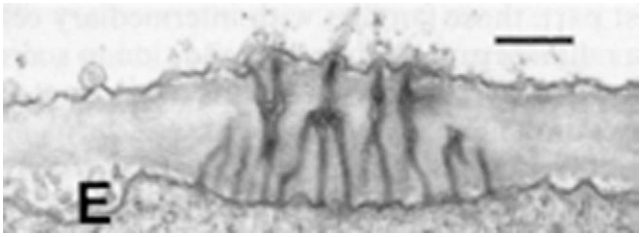


FIGURE 3.7 Plasmodesmata between an intermediary cell and a bundle sheath cell of *Alonsoa warcewiczii*. The portion of the plasmodesmata on the intermediary cell side is extensively branched and the branches are narrower than those on the bundle sheath cell side. Bar, 250 nm. (*Source:* From Turgeon et al., 1993.)

then diffuses into the sieve tubes (Turgeon and Beebe, 1991; Turgeon, 2000). This polymer trap model has also been used to explain oligofructan transport (Wang and Nobel, 1998).

The proteins needed by the sieve tube elements, which do not contain a nucleus, are synthesized in the companion cells. The proteins then pass from the companion cells through the plasmodesmata to the sieve tube elements (Fisher et al., 1992). The large cytoplasmic pathway through these plasmodesmata can be visualized by following the movement of GFP, which is a cylinder 2.1 nm in diameter and 4.2 nm long (Imlau et al., 1999). There is a protein in the companion cells that increases the permeability of the plasmodesmata so that proteins can move from the companion cells into the sieve tube elements. This protein is a plant homolog of the viral movement protein (Xoconostle-Cázares et al., 1999). It is a member of the cytochrome b_5 reductase family and must be processed by a protease in the companion cell before it can pass through the plasmodesmata to the sieve tube elements (Xoconostle-Cázares et al., 2000).

I opened this chapter by discussing whether the organism has a level of coordination that is greater than that of cells. While physical and readily diffusible hormonal factors certainly are important in communication within an organism (D'Arcy Thompson, 1959; Turing, 1992), plasmodesmata must also be important in integrating the parts with the whole (Goebel, 1926; Sharp, 1934). Research is just beginning on determining whether patterns of morphogenesis are related to the ability of plasmodesmata to transport certain macromolecules, including RNA and proteins, that are able to influence cell differentiation (Lucas et al., 1995; van der Shoot, 1995; Bergmans et al., 1997; Ding, 1998; Lucas, 1999; Zambryski and Crawford, 2000; Kim et al., 2001; Nakajima et al., 2001; Itaya et al., 2002; Haywood et al., 2002; Wu et al., 2002; Cilia and Jackson, 2004, 2005; Kim et al., 2003; Heinlein and Epel, 2004; Qi et al., 2004; Ryabov et al., 2004; Yoo et al., 2004; Zambryski, 2004; Heinlein, 2005; Kobayashi et al., 2005; Ding and Itaya, 2007; Zhong et al., 2007; Zhong and Ding, 2008).

3.6 SUMMARY

Plasmodesmata are structures that provide a pathway for the transport of information in the form of molecules from cell to cell. Along with other positional influences that determine development, the distribution and unitary conductance of plasmodesmata will determine the degree in which a given cell will act as an individual or as a member of the whole organism.

3.7 QUESTIONS

3.1. What is the evidence that the plasmodesmata provide a mechanism by which cells communicate with each other?
3.2. What are the mechanisms by which the plasmodesmata can facilitate cell-to-cell communication?
3.3. What are the limitations of thinking about the plasmodesmata as the sole mechanism of cell-to-cell communication?

Chapter 4

Endoplasmic Reticulum

4.1 SIGNIFICANCE AND EVOLUTION OF THE ENDOPLASMIC RETICULUM

In Chapter 2, I discussed cells as if their only membrane were the plasma membrane. Perhaps this is just what the precursors of the first eukaryotic cells were like. The plasma membrane of the precursor cell, like those of present-day prokaryotic cells, probably performed all of the membrane-dependent functions. It is likely that the precursor prokaryotic cell perhaps had a volume of $10^{-18}\,m^3$ and a surface-to-volume ratio of $10^6\,m^{-1}$, while a modern eukaryotic plant cell has a volume of $10^{-15}\,m^3$ or more and a surface-to-volume ratio of $10^5\,m^{-1}$ or less. That is, the volume of a eukaryotic plant cell is approximately one thousand times greater than the volume of the putative precursor. Since the surface-to-volume ratio decreases as the radius increases ($A/V = 3/r$ for spherical cells), it may have been impossible for a large eukaryotic cell to perform all the required membrane-dependent processes on the plasma membrane alone.

As larger cells evolved, the plasma membrane may have invaginated and pinched off, forming membrane-bound vesicles, a process that would maintain a high surface-to-volume ratio. The inside of such a vesicle is called the lumen and is topologically an E-space. Indeed, *Epulopiscium fishelsoni*, the largest prokaryote, has a highly invaginated plasma membrane (Angert et al., 1993, 1996; Bresler et al., 1998; Robinow and Angert, 1998). In eukaryotes today, the internal membranes, known collectively as the endomembrane system, are differentiated into the endoplasmic reticulum, the Golgi apparatus, and the vacuolar compartment along with all the adjoining membranes. Each compartment has its own function (Lunn, 2006).

The endoplasmic reticulum (ER) is a highly convoluted, netlike meshwork that extends throughout the cytoplasm (Staehelin, 1997). It is composed of a single membrane and constitutes more than half of the total membrane of the cell. It contributes to a surface-to-volume ratio of approximately $10^6\,m^{-1}$ in root cells and $10^7\,m^{-1}$ in tapetal cells (Gunning and Steer, 1996). I will discuss the endoplasmic reticulum in terms of how it complements the plasma membrane in performing transport activities, as well as how it is involved in the synthesis of many membranes, including the plasma membrane (Brandizzi et al., 2002b,c; Saint-Jore et al., 2002).

4.2 DISCOVERY OF THE ENDOPLASMIC RETICULUM

The introduction of electron microscopy into the study of cells opened up a whole new world that was approximately 100 times smaller than that which had been previously visualized. In 1945, Keith Porter, Albert Claude, and Ernest Fullam first observed a lacelike reticulum in cultured chick embryo cells (Figures 4.1–4.3). They used cultured

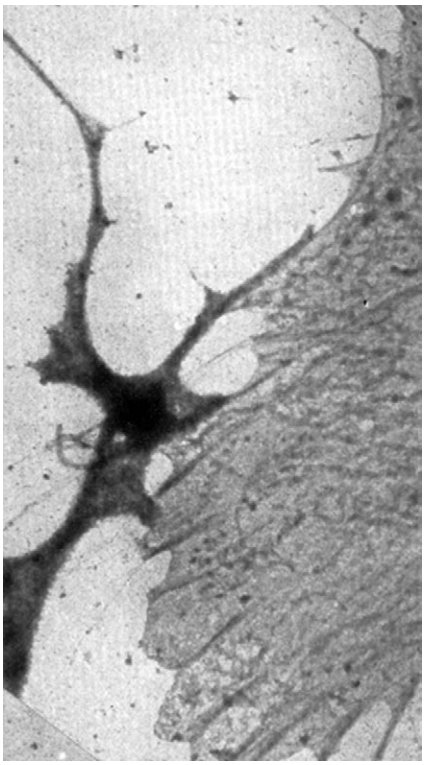


FIGURE 4.1 Cytoplasmic reticulum in a fibroblast-like cell cultured from chick embryo tissue. (*Source:* From Porter et al., 1945.)

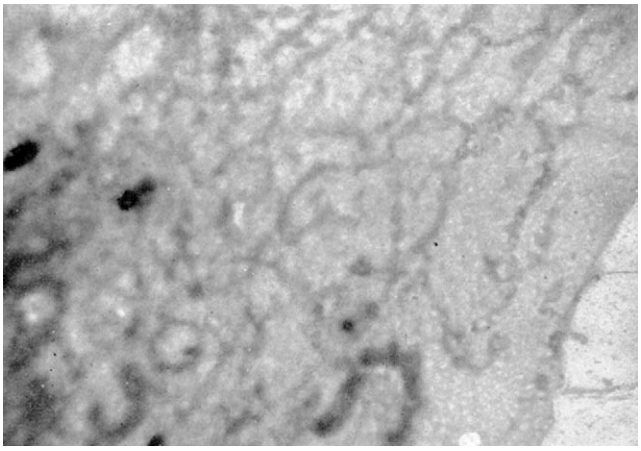


FIGURE 4.2 Lacelike reticulum in a cultured chick fibroblast cell that in places appears to be made up of chains of vesicles. (*Source:* From Porter et al., 1945.)

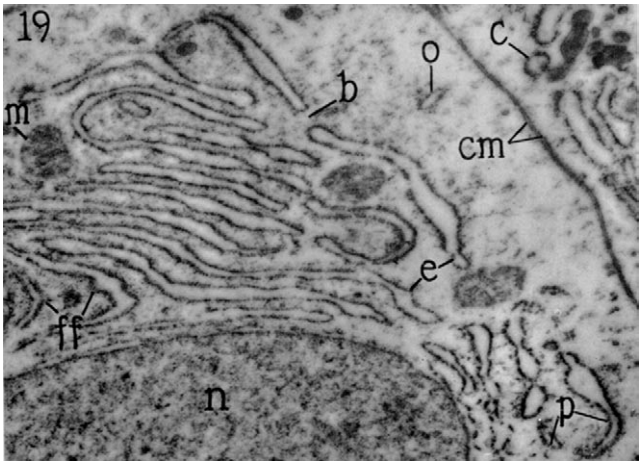


FIGURE 4.4 Electron micrograph of a thin section through the endoplasmic reticulum in a paratoid gland cell. (*Source:* From Palade and Porter, 1954.)

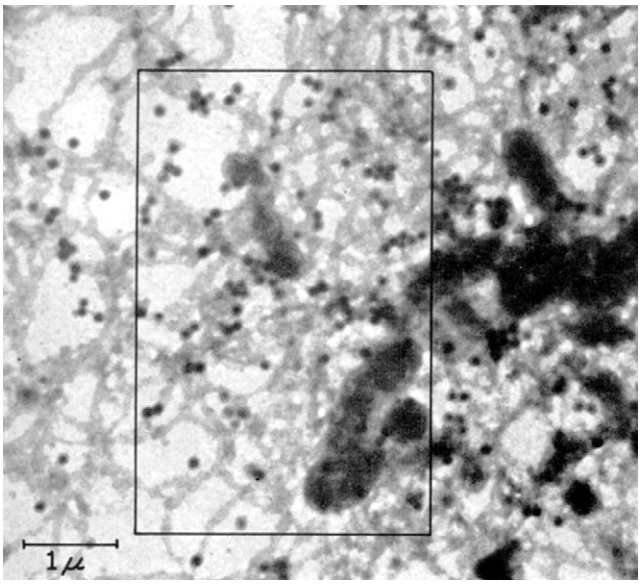


FIGURE 4.3 The endoplasmic reticulum of an epithelial tumor cell. The preparation was dried directly on a wire mesh and observed with the electron microscope. (*Source:* From Porter and Thompson, 1948.)

cells because they were thin enough to be penetrated by an electron beam. This was important since the ultramicrotome had not yet been invented. Imagine their excitement when they saw this beautiful lacelike structure revealed by the electron microscope. Approximately 100 years earlier, Félix Dujardin (1835, quoted in Buvat 1969) had described protoplasm viewed with a light microscope as a substance that has "absolutely no trace of any organization … neither fibres, nor membranes, nor any sign of cellular structure."

Immediately following the discovery of the lacelike reticulum, Albert Claude (1943a,b; 1946a,b; 1948) isolated it using the technique of differential centrifugation developed by Bensley and Hoerr (1934). For the first time morphology and biochemistry could be combined. Claude called the membranes he isolated *microsomes*, a term originally coined by Johannes Hanstein to mean the unidentified vesicles he saw in plant cells. Claude used *microsome* as a noncommittal term emphasizing only the size. Claude chemically analyzed the microsomes and found that they contained approximately 9 percent N, 2.5 percent P, 40–45 percent lipid, 0.75 percent S, 0.01 percent Cu, and 0.03 percent Fe. A few years later, the lacelike reticulum visible in the electron microscope was renamed the *endoplasmic reticulum* by Porter and Thompson (1948).

With the advent of the ultramicrotome and methacrylate embedding procedures, the ER was first seen with high resolution by George Palade and Keith Porter in 1954 (Figure 4.4). With thin sections, it was possible to see that the ER was composed of membranes that were 5.5 to 6.5 nm thick. Perhaps it was lucky that the lacelike reticulum had been discovered before the invention of the ultramicrotome, because it is possible that the three-dimensional arrangement of the endoplasmic reticulum may not have been deduced from 20- to 40-nm-thick sections (Palade, 1956). By 1956, Palade and Siekevitz began an integrated study combining electron microscopy and biochemistry, a combination that led to the award of the Nobel Prize to Palade (1975). Buvat and Carasso (1957) contributed to the notion that the ER was a fundamental part of the protoplasm of eukaryotic cells by showing that it is present in the cells of the plant kingdom as well as those of the animal kingdom.

4.3 STRUCTURE OF THE ENDOPLASMIC RETICULUM

The architecture of the ER is dynamic; it varies from cell to cell and changes throughout the cell cycle (Haguenau, 1958; Hepler, 1989). The form of the ER can be seen best by treating the cells with stains that fill the luminal space of the ER and consequently contrast the ER against the rest

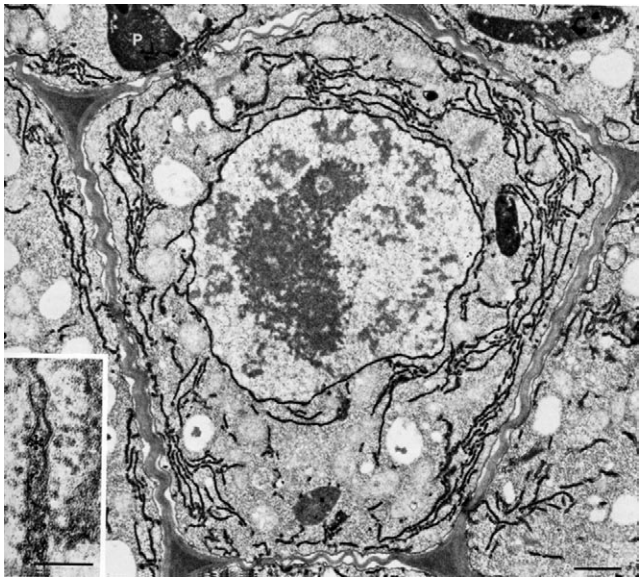


FIGURE 4.5 Electron micrograph of the endoplasmic reticulum of a lettuce root cell that has been fixed in OsFeCN. Bar, 1 μm. (*Inset*) High-magnification electron micrograph of a segment of endoplasmic reticulum showing that the inner leaflet (*) is stained more darkly than the outer leaflet. Bar, 100 nm. (*Source:* From Hepler, 1981.)

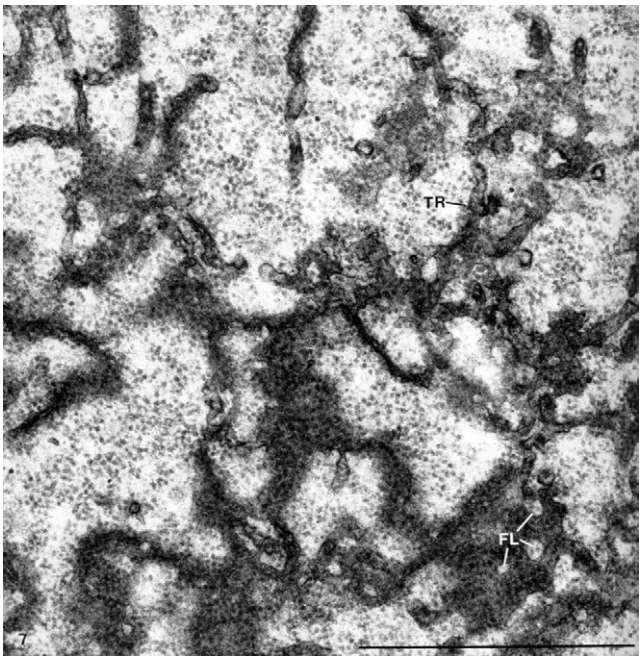


FIGURE 4.6 Electron micrograph of the endoplasmic reticulum of a lettuce root cell that has been fixed in OsFeCN. Notice the fenestrated lamellae (FL). The tubular elements (TRs) intergrade with the cisternal elements. Bar, 1 μm. (*Source:* From Hepler, 1981.)

of the cell (see Figure 4.5; Hepler, 1981; Stephenson and Hawes, 1986). The ER exists both as tubules and as lamellae, and some of the lamellae may have pores or fenestrations that are reminiscent of nuclear pores (see Chapter 16 and Figure 4.6). Focal arrays of ER can also be seen, and these may be the sites of active membrane growth (Hepler, 1981). Zheng and Staehelin (2001) call similar focal arrays *nodal ER* and suggest that the nodal ER in columella cells are involved in gravity sensing.

It is also possible to visualize the exquisitely delicate form of the ER in the light microscope (Url, 1964; Lichtscheidl and Url, 1990). The three-dimensional arrangement of ER is particularly clear after staining the cells with the lipophilic, anionic, fluorescent dye, $DiOC_6(3)$ (see Figure 4.7; Terasaki et al., 1984, 1986; Quader and Schnepf, 1986; Quader et al., 1987; Terasaki, 1989), ER-directed green fluorescent protein (Boevink et al., 1996; Hawes et al., 2001; Brandizzi et al., 2002a; Goodin et al., 2007), or other fluorescent proteins (Held et al., 2008).

There are various architectural classes of ER, which are interconnected. One class consists of thin, flat, variably sized cisternae that are connected by thin tubular elements that are approximately 100–400 nm in diameter. This form of *endo*plasmic reticulum, which has a lacelike appearance, is found in the thin cytoplasm adjacent to and parallel with the plasma membrane (Lancelle and Hepler, 1992). Ironically, it is found in the *ecto*plasm of plant cells! Another type of ER consists of bundles of long thin tubular elements that run away from or toward the nucleus through transvacuolar strands. A third class, rediscovered in green fluorescent protein (GFP)–transformed cells, but also

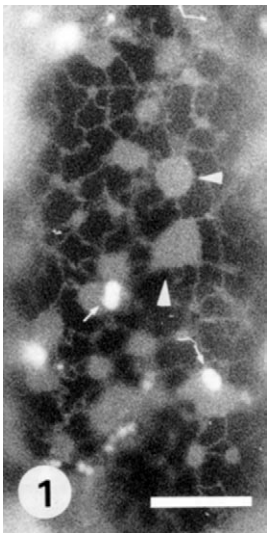


FIGURE 4.7 Fluorescence light micrograph of the endoplasmic reticulum in an onion bulb scale cell stained with $DiOC_6(3)$. The arrowheads indicate cisternal ER and the arrow indicates a mitochondrion. Bar, 20 μm. (*Source:* From Quader and Schnepf, 1986.)

found in wild-type cells, consists of fusiform bodies several micrometers long and a few micrometers wide (Bonnett and Newcomb, 1965; Hawes et al., 2001; Matsushima et al., 2003). The distribution of the ER is cell type specific and distinct forms of ER are found in various cells, including sieve tube elements (Sjolund and Shih, 1983; Schulz, 1992), the tip of *Chara* rhizoids (Bartnik and Sievers, 1988), and the statocytes of *Lepidium* (Hensel, 1987).

One advantage of light microscopy is that the ER can be observed in living cells and one can see that it is not a static organelle, but a dynamic one that exhibits constant movement and undergoes dramatic transformations (Goodin et al., 2007). For example, some tubules grow and shrink at a rate of about 10 μm/s while other sites do not move (Knebel et al., 1990). The shape of the ER is controlled by temperature, Ca^{2+} and pH (Quader, 1990; Quader and Fast, 1990). The shape and position also depend on cytoplasmic structures, known as microfilaments and microtubules, which are discussed in Chapters 10 and 11 (Quader et al., 1987; Lancelle and Hepler, 1988; Allen and Brown, 1988; Lee et al., 1989; Quader, 1990; Lichtscheidl et al., 1990; Knebel et al., 1990; Lancelle and Hepler, 1992; Liebe and Quader, 1994; Yokota et al., 2008).

4.4 STRUCTURAL SPECIALIZATIONS THAT RELATE TO FUNCTION

The first step in membrane biosynthesis begins on the ER, where the component proteins and lipids are synthesized. Proteins that are destined to become integral membrane proteins are synthesized on polyribosomes that are attached to the ER. Ribosomes, originally called *Palade's small particles*, are 15- to 20-nm complexes that are composed of ribonucleic acid and protein. They provide the workbench for protein synthesis, which will be discussed in Chapter 17. Since the ribosomes cover the P-surface of the ER, they give the ER a "rough" appearance, and these regions of the ER are called the *rough endoplasmic reticulum* or RER (see Figure 4.8; Palade, 1955). Cells that are active in secreting proteins are rich in RER, indicating that the RER is involved in protein synthesis. The proteins synthesized by the ribosomes that are attached to the ER are imported into the ER as they are synthesized. Since the protein is translocated into the ER as the linear mRNA sequence is being translated into a linear sequence of amino acids, the import of the nascent proteins is called the *cotranslational import*. While the majority of proteins that enter the ER are imported cotranslationally, some are synthesized on cytosolic ribosomes and enter the ER posttranslationally (Mueckler and Lodish, 1986).

Some regions of the ER lack ribosomes and appear smooth (Figure 4.9). These regions are called the *smooth endoplasmic reticulum* or SER. Cells that have abundant SER are specialized for lipid production, indicating that the SER may be responsible for lipid biosynthesis. The oil glands of *Arctium* or the stigmatic cells of *Petunia* have an extensive network of SER needed for the synthesis and secretion of lipophilic molecules (Konar and Linskins, 1966; Schnepf, 1969a,b,c). The SER of plant cells functions in detoxification much as it does in liver cells (Kreuz et al., 1996). There are regions of ER that are partly smooth

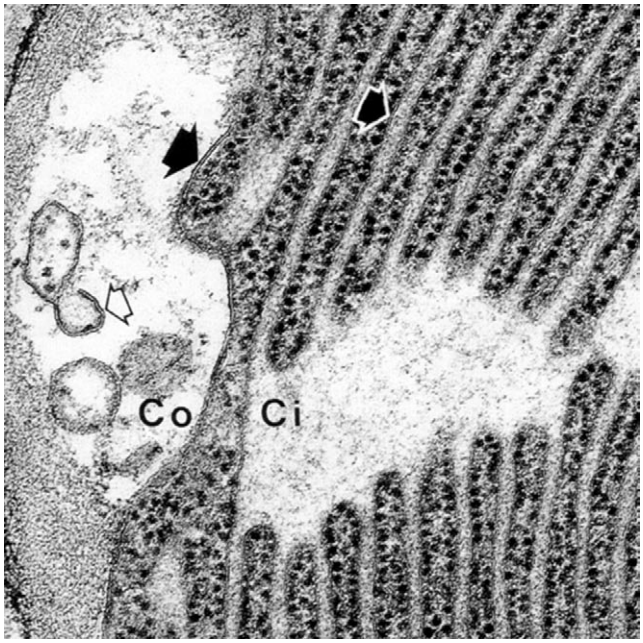


FIGURE 4.8 Rough endoplasmic reticulum in the trichomes of *Coleus blumei*. The ribosome-studded tubular endoplasmic reticulum is connected by a cisterna (Ci). The plasma membrane (black arrow) is thicker than the endoplasmic reticulum membrane (black-and-white arrow). ×75,000. (*Source:* From Gunning and Steer, 1996.)

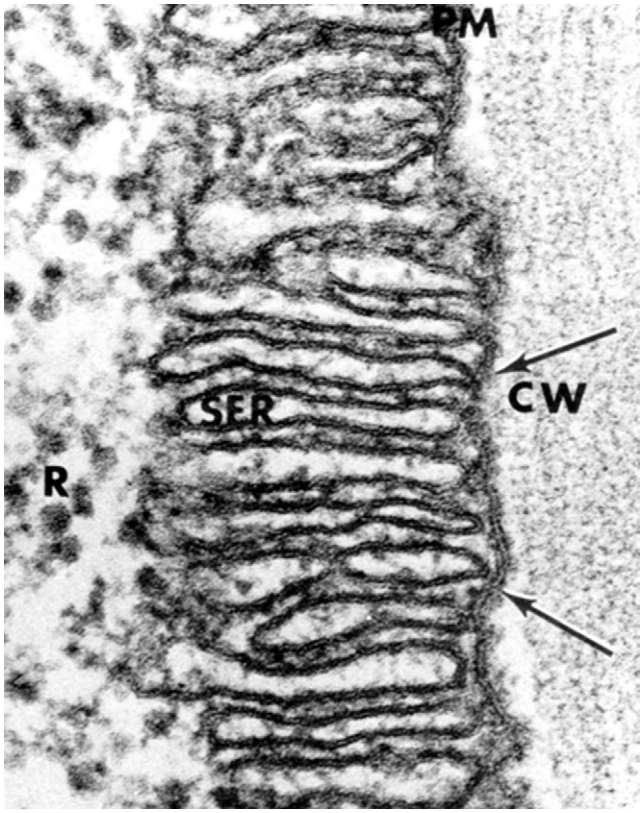


FIGURE 4.9 SER in the periphery of the sieve tube elements of *Streptanthus tortuosus*. CW, extracellular matrix; PM, plasma membrane; R, ribosomes. ×165,000. (*Source:* From Sjolund and Shih, 1983.)

and partly rough and are called *transitional elements* (Paulik et al., 1987, Morré et al., 1989a). Some transitional elements are specialized regions involved in producing the vesicles that transport newly synthesized proteins and lipids to the Golgi apparatus. Other transitional elements produce osmotically active lipid bodies and their associated proteins (Wu et al., 1997; Thompson et al., 1998; Murphy and Vance, 1999; Hsieh and Huang, 2004; Lersten et al., 2006). Such lipid bodies may serve as a novel source of biofuel (Chisti, 2007, 2008; Fortman et al., 2008; Li et al., 2008).

4.5 ISOLATION OF RER AND SER

The aleurone layer is a tissue that surrounds the endosperm in cereal grains, and has been a favorite material for the study of ER since it contains a large amount of ER. The ER in the cells of this tissue is involved in the synthesis and secretion of vast quantities of hydrolytic enzymes required to break down the storage products of the endosperm into the metabolites used by the beer industry (i.e., starch to maltose).

In order to isolate ER membranes, aleurone layers are homogenized, filtered through cheesecloth to remove the extracellular matrix, and then centrifuged at 100 g to remove the large organelles. The supernatant is then centrifuged (70,000 g; 2.5 h) on a discontinuous sucrose density gradient consisting of a 50 percent (w/w) sucrose cushion overlaid with 13 percent (w/w) sucrose. The microsomal membranes that accumulate between the 50/13 percent interface are collected and layered on a sucrose density gradient, and then centrifuged at 70,000 g for 14 h. The ER forms a defined peak at 30 percent sucrose. The isolation is done in the presence of the Mg^{2+}-binding agent ethylenediametetraacetic acid (EDTA) in order to "capture" both the RER and the SER in the same fraction (Lord, 1983; Bush et al., 1989a,b; Sticher et al., 1990).

Polyribosome binding to the endoplasmic reticulum requires Mg^{2+}. Since ribosome-studded ER is denser than ribosome-free ER, the RER membranes undergo a Mg^{2+}-dependent shift in their densities on sucrose density gradients. In the absence of Mg^{2+}, the ER forms a sharp band at 1.12 g/mL; in the presence of Mg^{2+}, the ER forms a broader band at 1.16 g/mL (Lord, 1983). Since the plasma membrane has a peak between 1.14 and 1.17 g/mL (Hall, 1983), the inclusion of EDTA to chelate the Mg^{2+} ions helps to isolate pure ER membranes from sucrose density gradients. The ER can also be isolated using aqueous two-phase partitioning. With this procedure, the ER membranes are preferentially accumulated in the lower phase (Walker et al., 1993; Gilroy and Jones, 1993).

During isolation of the ER, its presence and purity are determined with the help of marker enzymes. The ER contains a number of enzymes that are endemic to it. Some of these enzymes, including NADH- and NADPH-dependent cytochrome c reductases, are involved in oxidation-reduction reactions and can be readily assayed spectrophotometrically. Consequently, these enzymes are often used for marker enzymes (Martin and Morton, 1956). The ER also contains a number of cytochromes that can be identified spectrophotometrically by their difference spectra. The oxidized minus dithionite-reduced difference spectrum of ER membranes has peaks at 555, 527, and 410 nm, which are typical of cytochrome b_5.

4.6 COMPOSITION OF THE ER

The membrane and lumen of the endoplasmic reticulum contain proteins that are involved in lipid synthesis, protein synthesis, and processing, as well as ionic regulation. An auxin-binding protein also appears to be localized in the ER (Hesse et al., 1989; Inohara et al., 1989). Moreover, there are specific proteins that allow the attachment of the ribosomes to the ER. I will discuss some of these proteins individually.

The lipid composition of the endoplasmic reticulum is similar although not identical to the lipid composition of the plasma membrane (Philipp et al., 1967; Donaldson and Beevers, 1977; Coughlan et al., 1996). In fact, all membranes in the endomembrane system have a basic similarity related to their common origin and function as permeability barriers. The differences may result from specializations of the various membranes. However, unlike the case of yeast (Schneiter et al., 1999), it must be noted that the lipids of all the membranes from a single plant cell type of a single species have not yet been characterized. This observation, combined with the fact that the composition of the ER lipids varies depending on the environmental conditions (Holden et al., 1994), makes comparisons between different membranes somewhat tenuous (Table 4.1).

4.7 FUNCTION OF THE ENDOPLASMIC RETICULUM

4.7.1 Lipid Synthesis

The ER produces most of the lipids needed for membrane synthesis (Moore 1982, 1987; Chapman and Trelease, 1991a,b; Vance and Vance, 2008). One representative biosynthetic pathway involves the formation of phosphatidylcholine from a glycerol-3-phosphate molecule, a cytidine diphosphate-choline molecule, and two fatty acids that have been activated by coenzyme A (Lipmann, 1971). The A stands for acylation. Coenzyme A (CoA) is a derivative of the vitamin pantothenic acid and is involved in the activation of acyl groups. This activation process is necessary to make the acetic acid groups reactive enough to participate in fatty acid elongation and attachment to the glycerol-3-phosphate

TABLE 4.1 Lipid composition of endoplasmic reticulum membrane

Lipid	Onion	Castor Bean
Phospholipids	**(% of lipid phosphorus)**	
Phosphatidylcholine	30.3	45.3
Lysophosphatidylcholine	4.4	—
Phosphatidic acid	24.6	—
Phosphatidylethanolamine	21.4	28.7
Lysophosphatidylethanolamine	2.5	—
Phosphatidylinositol	7.4	13.1
Phosphatidylserine	2.1	2.3
Phosphatidylglycerol	6.0	3.6
Cardiolipin	1.3	2.5
Major Sterols	**(% wt. of sterols)**	
β-sitosterol	81.0	
Campesterol	6.6	
Fatty Acid Precursors of Phospholipids		**(%wt)**
16:0 stearic acid	32	
18:1 oleic acid	6	
18:2 linoleic acid	53	
18:3 linolenic acid	7	

Lysophospholipids contain a single acyl chain.
Source: *From Philipp et al. (1967) and Donaldson and Beevers (1977).*

molecule, which is produced in the cytosol by glycolysis (see Chapter 14). The fatty acids used in lipid synthesis are made in the plastids of plant cells and in the cytosol of animal cells. In order to initiate the synthesis of lipids on the ER, an acyl transferase combines the glycerol-3-phosphate with the two fatty acyl CoAs in a dehydration reaction to form phosphatidic acid, and releases two CoA molecules in the process (Figure 4.10). Subsequently a phosphatase cleaves the phosphate from phosphatidic acid, thus producing diacylglycerol. Then choline phosphotransferase catalyzes the exchange of choline phosphate from cytidinediphosphate-choline (CDP-choline) to diacylglycerol, thus producing phosphatidylcholine and cytidine monophosphate (CMP). Phosphatidylethanolamine and phosphatidylserine are synthesized in a similar manner.

In addition, phosphatidylethanolamine can be converted to phosphatidylcholine by a methylation reaction, and phosphatidylserine can be converted to phosphatidylethanolamine by a decarboxylation reaction. Exchange reactions also take place in which serine replaces the ethanolamine in phosphatidylethanolamine to form phosphatidylserine, or ethanolamine replaces the serine in phosphatidylserine to form phosphatidylethanolamine. There are numerous enzymes and pathways involved in synthesizing the various lipids. It would be wonderful to know why nature goes to such lengths to form the lipid bilayer.

It is not always the head group that is activated by CDP. In the case of phosphatidylinositol synthesis, CDP activates the diacylglycerol molecule, which then attaches to an inositol molecule to form phosphatidylinositol.

The addition of the phosphatidic acid to the membrane results in membrane growth. Each step in lipid biosynthesis occurs on the cytoplasmic leaflet of the ER membrane. If this kept up, a monolayer would be formed. However, a bilayer results, not just due to thermodynamics, but because the endoplasmic reticulum has head group–specific phospholipid translocators, which flip-flop the lipid across the membrane at a rate of $10^{-2}\,s^{-1}$. This means it takes a lipid approximately 10^2 s to be translocated across the membrane. The translocator-facilitated rate is 100–10,000 times greater than the rate of spontaneous flip-flops (10^{-4} – $10^{-6}\,s^{-1}$). Since there are more PC translocators than PE, PI, or PS translocators, the membrane remains asymmetric and PC is concentrated on the E-leaflet, while PE, PI, and PS are concentrated on the P-leaflet of the bilayer (Shin and Moore, 1990). The lipid translocators can be regulated through phosphorylation (Nakano et al., 2008).

4.7.2 Protein Synthesis on the Endoplasmic Reticulum

Special proteins have been found in the RER of animal cells that bind the large subunit of the ribosome and prevent the lateral movement of the ribosome to the SER. The RER has about 20 more types of polypeptides than the SER. Some of these polypeptides may be involved in ribosome anchoring; others may be involved in maintaining the shape of the flattened cisternae.

The mechanism of protein synthesis is discussed in Chapter 17. For now, let us accept the fact that ribosomes contain the means to synthesize proteins, which was demonstrated by measuring the incorporation of radioactive amino acids into proteins in the presence of isolated ribosomes. Comparative cytochemical studies in secretory cells suggested that the free ribosomes synthesize proteins that were used by the cell, while bound ribosomes synthesize secreted proteins (Siekevitz and Palade, 1960a,b). Subsequent biochemical work in vitro using free and ER-bound ribosomes that had been separated from each other, confirmed that the two populations of ribosomes produce different proteins (Figure 4.11). Moreover, the secretory proteins were probably inserted into the lumen of the ER, since experiments using isolated microsomes showed that the newly formed proteins were protected from proteolysis by the ER membrane in the absence, but not the presence,

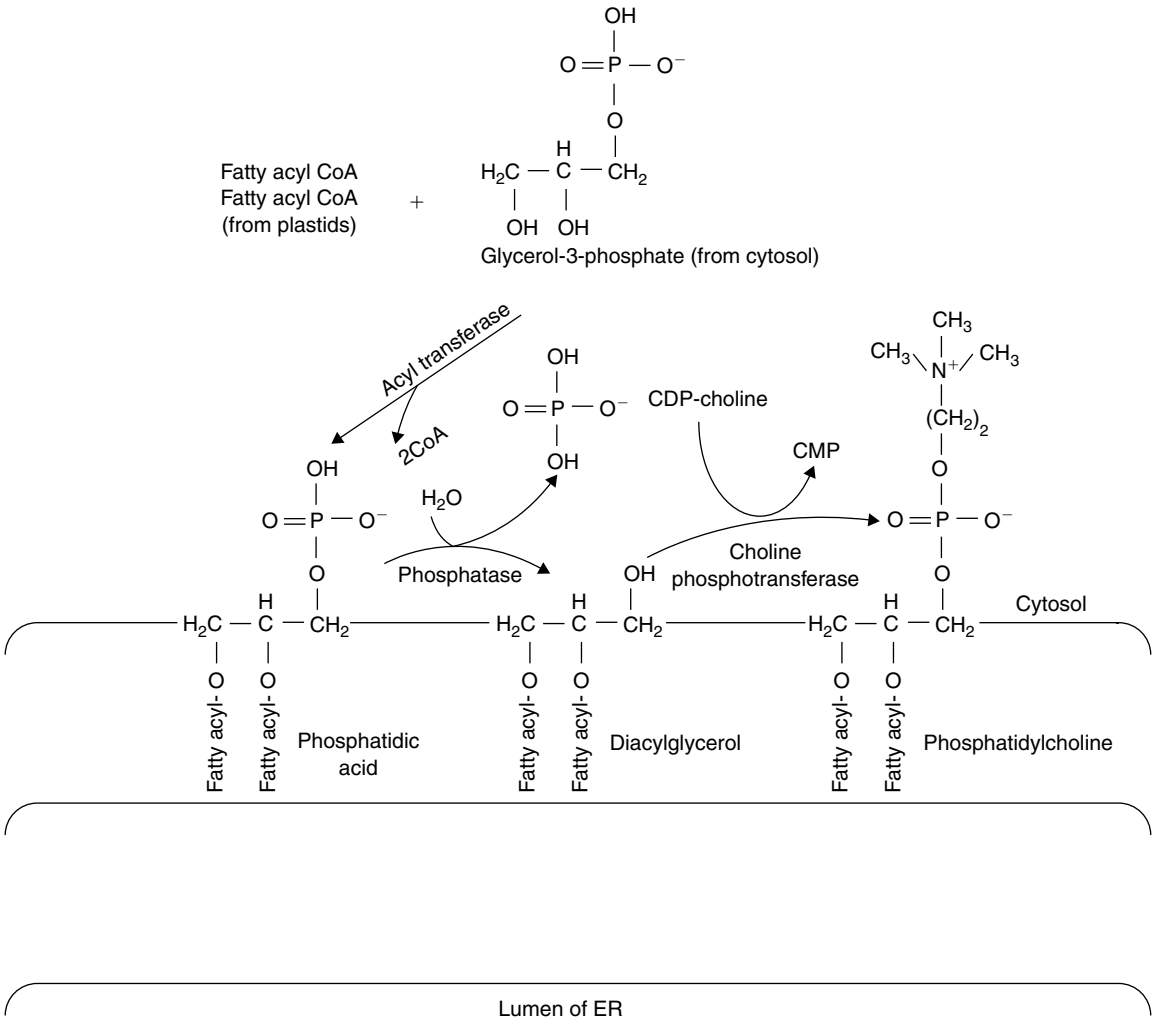


FIGURE 4.10 Lipid synthesis at the outer leaflet of the ER.

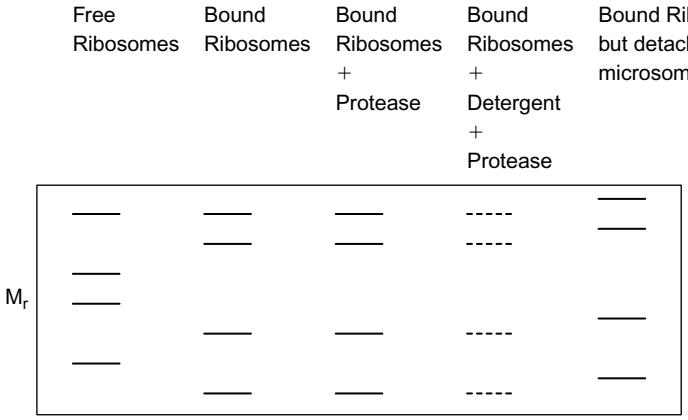


FIGURE 4.11 Diagram of the sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis gels that led to the signal hypothesis.

of a detergent (Takagi and Ogata, 1968; Redman, 1969; Hicks et al., 1969). It was also discovered that secreted proteins are synthesized as larger proproteins. The proproteins were found to be larger than the mature form.

It soon became apparent to Günter Blobel and his colleagues that since the RER exists in all cells, not just secretory cells, the observations made on secretory proteins might have a more general significance. But before they could understand the reason some ribosomes synthesize proteins on the ER, they repeated previous work on membrane-associated protein synthesis in vitro (Blobel and Potter, 1967a,b; Blobel and Sabatini, 1970, 1971; Sabatini and Blobel, 1970). By 1971,

Günter Blobel and David Sabatini proposed that in all cells, the mRNA of the proteins that will be synthesized on the RER, unlike those that are synthesized on free ribosomes, would prove to have a certain sequence at the 5′ end of a gene, which would result in a certain amino acid sequence at the amino-terminus. They predicted that this sequence would cause the ribosomes that have bound that particular mRNA to be delivered to the ER. Protein synthesis would then continue on the ER where the nascent polypeptide would be vectorially transported into the lumen and the signal peptide would be removed.

This proposal, which came to be known as the *signal hypothesis*, was directly tested by Blobel and Dobberstein (1975a,b). They found that ribosomes detached from microsomes produce longer proteins than do ribosomes in the presence of microsomal membranes (see Figure 4.11). They also found that proteins made in the absence of microsomes were degraded by an added protease, while those made in the presence of microsomes were protected, indicating that the newly synthesized proteins were in the ER lumen. They also found that the protein produced by free ribosomes had an amino-terminal leader peptide that was cleaved in the presence of microsomes to make a protein of the correct size, while the rest of the protein was still being synthesized. They named this ER-localized peptidase the *signal peptidase*. Reconstitution experiments using free ribosomes and mRNA that encodes a secreted protein showed that the mRNA has the information necessary to deliver the ribosome to the ER.

The importance of the signal sequence is dramatically shown in experiments in which the DNA that codes for this sequence is inserted in front of a DNA sequence that encodes a protein that is typically translated on free ribosomes. Instead of being translated on free ribosomes and ending up in the cytosol, the fusion protein is translated by bound ribosomes and inserted into the ER! Moreover, when recombinant DNA technology is used to delete the signal sequence from proteins typically synthesized on membrane-bound ribosomes, these proteins are synthesized on free cytosolic ribosomes. Much is known about the structure of signal peptides (von Heijne, 1990). They have a three-domain structure that includes an amino-terminal positively charged region that is 1–5 amino acids long, a central hydrophobic region that is 7–15 amino acids long, and a more polar carboxy-terminal domain that is 3–7 amino acids long. Beyond this pattern, there is no precise sequence conservation. Site-directed mutagenesis shows that the amino and central regions are required for translocation, while the carboxy region contains the sequence that is recognized by the signal peptidase and thus specifies the cleavage site. The amino acid sequences known as *molecular zip codes* are discussed in Chapter 17.

The signal hypothesis has been very powerful in providing a theoretical framework to understand how a given protein ends up in the appropriate organelle. According to the general theory of protein targeting and translocation (Blobel 1980; Simon and Blobel, 1991):

1. A protein that is translocated across or integrated into a distinct membrane must contain a signal sequence.
2. The signal sequence is specific for each membrane.
3. A signal sequence–specific recognition factor and its receptor on the correct membrane are needed for successful targeting.
4. Translocation across the membrane occurs through a proteinaceous channel.
5. The nascent protein has a series of amino acids that form an alpha helix, the outer surface of which is hydrophobic and functions as a start-transfer or stop-transfer sequence, depending on its position in the polypeptide.
6. If the protein is to be integrated into the membrane, a start-transfer or stop-transfer sequence in the polypeptide opens the protein-conducting channel and displaces the polypeptide from the aqueous environment of the channel into the lipid bilayer.

The signal peptide of a protein that is destined to be synthesized on the ER is guided into the ER by a signal recognition particle (SRP; see Figure 4.12; Ng and Walter, 1994). The SRP is composed of six different polypeptide chains bound to a single molecule of 7S RNA. Just as in ribosomes, here is another example where proteins and RNA function together in a complex. The SRP binds to the signal peptide as soon as it emerges from the ribosome. Actually the 54-kDa polypeptide of the SRP is methionine rich and forms a hydrophobic pocket and binds to the signal peptide (High and Dobberstein, 1991). The binding of the SRP to the nascent polypeptide somehow causes a halt in the synthesis of that protein, thus allowing time for the large subunit of the ribosome to bind to the ER. Protein synthesis is reinitiated once the SRP binds to an SRP receptor on the ER. This occurs because the SRP receptor displaces the SRP from the nascent polypeptide.

The association of the SRP with the nascent protein synthesized by the ribosome, and the association of the SRP receptor with the protein-translocating channel, requires guanosine triphosphate (GTP) (Mandon et al., 2003). The SRP receptor then brings the ribosome and its nascent SRP-binding polypeptide in contact with the protein-translocating channel (Walter, 1997). Cross-linking studies performed at various times following the interaction of the 54-kDa subunit of the SRP with the ribosome and ending with the binding of the ribosome to the protein-translocating channel have allowed the identification of a number of polypeptides involved in these processes (Takahashi et al., 2002).

In vitro studies where detergent-treated ER is depleted of the SRP receptor shows that the SRP receptor is essential for protein translocation across the ER membrane (Migliaccio et al., 1992). Both the SRP and the SRP receptor were originally identified as components needed to

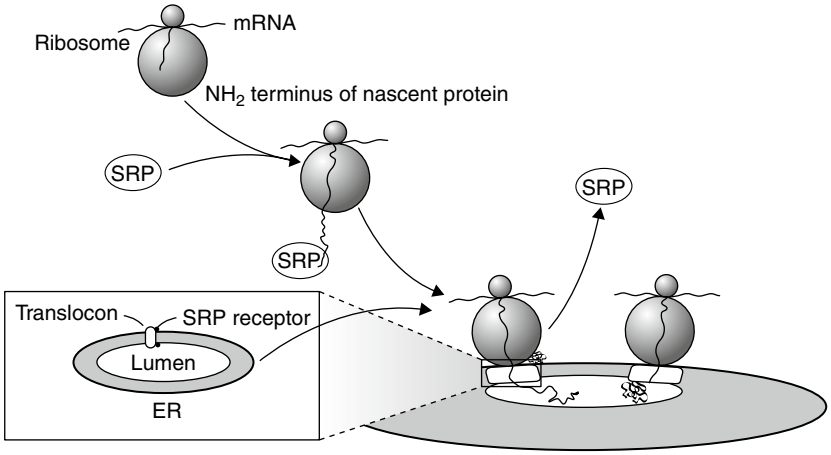


FIGURE 4.12 The delivery of a nascent protein to the ER.

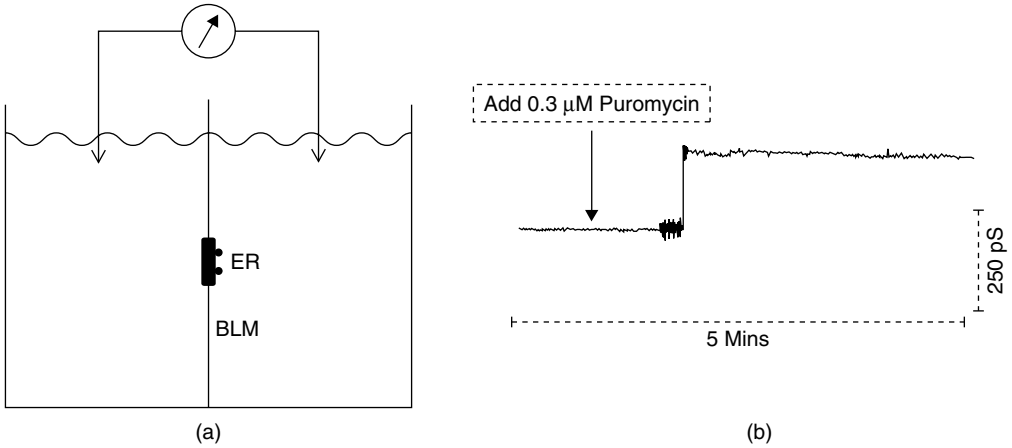


FIGURE 4.13 An electrophysiological experiment demonstrating a single protein-translocating channel revealed by the application of puromycin. The protein-translocating channel has a conductance of approximately 220 pS. This is much greater than the conductance of ion channels, the activation of which is shown by the small variations in the conductance trace. (*Source:* From Simon and Blobel, 1991.)

reconstitute in vitro protein translocation into the ER. Again, we see the importance of a functional assay, involving reconstitution to identify the proteins involved and their functions. The SRP receptor is an integral membrane protein that contains four polypeptide chains. The function of each polypeptide has been determined by reconstitution experiments (Görlich and Rapoport, 1993). Genetic studies of yeast mutants that are unable to secrete proteins have identified the secretory, or sec, genes that encode proteins involved in protein translocation into the ER. We are just beginning to find that plants use the same protein-targeting and -translocation mechanism (Thoyts et al., 1995; Beaudoin et al., 2000; Shy et al., 2001; Jang et al., 2005).

Exciting work has begun on determining the mechanism of how proteins can pass through the ER membrane (Simon, 1993, 2002; Schatz and Dobberstein, 1996). Simon and Blobel (1991) and Simon et al. (1989) have identified protein-translocating channels using electrophysiological techniques. They isolated vesicles of the RER and incorporated them into one side of a planera lipid membrane. They then applied an electrical potential (ψ) across the two sides of the bilayer and measured the resulting current (I). They calculated the conductance (G) of the protein-translocating channels using Ohm's Law ($G = -I/\psi$).

Initially, the conductance is approximately 0 pS. However, after adding 100 μM of puromycin, an adenosine derivative that uncouples a nascent polypeptide from its ribosome-bound peptidyl-tRNA, a large increase in conductance occurs. When a low concentration of puromycin is added, so that elongation of one chain is stopped at a time, discrete changes in conductance of 220-pS steps is seen (Figure 4.13). The conductance results from the fact that the nascent polypeptide no longer occludes the channel, and now K^+ can move through the channel and produce a current in response to the applied voltage. The protein-translocating channel probably remains open until the ribosome moves away from the membrane since the high-conductance

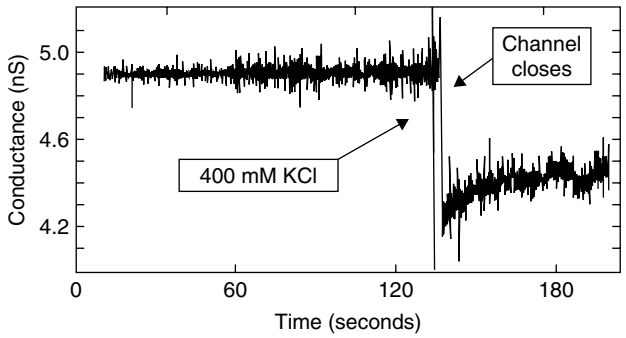


FIGURE 4.14 An electrophysiological experiment demonstrating the closure of one of four protein-translocating channels following the addition of 400 mM KCl, which probably washed off the ribosome on the channel that closed. (*Source:* From Simon and Blobel, 1991.)

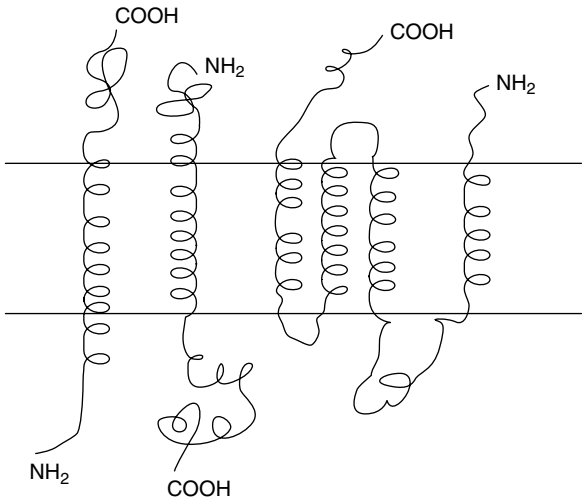


FIGURE 4.15 Membrane proteins in the ER with various orientations.

state is stable until the ribosomes are washed off with high salt (Figure 4.14). Electron microscopy of the protein-translocating channel indicates that the pore has a diameter of 4–6 nm (Hanein et al., 1996; Hamman et al., 1997).

Crowley et al. (1993, 1994) have created a great technique to monitor the polarity of the channel that the nascent polypeptide goes through. They incorporated a fluorescent probe into the nascent polypeptide. The probe was chosen so that its fluorescence lifetime depends on the environment immediately surrounding it. The lifetime is short when it is in an aqueous environment and long when it is in a lipid environment.

When attached to the signal sequence, the dye has a short fluorescence lifetime, indicating that the signal sequence goes through an aqueous channel. However, since the fluorescence cannot be eliminated by adding an aqueous-quenching agent, the aqueous pore is not continuous with the aqueous environment surrounding the membrane when the polypeptide is in the pore. Cross-linking studies show that the hydrophobic portion of the signal sequence is in contact with lipids in the bilayer during an early stage of protein insertion, indicating that the protein-translocating channel can open laterally, at least during an early stage of protein insertion (Martoglio et al., 1995).

How is an integral protein inserted into the ER membrane (High and Dobberstein, 1992)? The actual mechanism still needs to be elucidated, but we assume that a sequence of approximately 7–21 nonpolar amino acids are long enough to span the membrane and encourage the nascent polypeptide to partition out of the translocon and into the lipid bilayer, while a series of polar amino acids encourages the polypeptide to remain where the hydrophilic sequence begins—either on the cytosolic side or on the luminal side of the ER (Figure 4.15).

Proteins are synthesized from the amino-terminus to the carboxy-terminus. The first sequence of hydrophobic amino acids acts as a start-transfer sequence and the subsequent sequences of hydrophobic amino acid act alternately as stop-transfer and start-transfer sequences. If the membrane protein is to be inserted such that only the amino-terminus is to remain in the lumen, there must be a start-transfer sequence at the amino-terminus followed by a stop transfer sequence. If the carboxy-terminus is to remain in the lumen, the start-transfer sequence occurs distal to the amino-terminus and there is no stop-transfer sequence. In the case of multipass transmembrane proteins, there are many start-transfer as well as stop-transfer sequences that allow polypeptide chains to repeatedly pass the membrane, leaving loops in the cytoplasmic space and the luminal space (Singer, 1990).

While little is known about the how start-transfer and stop-transfer sequences function, in general they are probably determined by the hydrophobicity and charge of the amino acids (see Figure 4.16; High and Dobberstein, 1992). The chemical properties of the amino acid sequences may influence the partition of that segment of the polypeptide between the aqueous channel, the lipid bilayer, the cytosol, and the lumen. The start-transfer and stop-transfer sequences must influence the axial and lateral gating behavior of the protein-translocating channel. The gating is not only influenced by the amino acid sequence in the nascent protein that is going through the channel, but also by the adjoining transmembrane amino acid sequences. In addition, there are also long-range allosteric effects on translocon gating determined by the amino acid sequence still in the ribosome (Liao et al., 1997).

What allows the translocating protein to move through the pore? It probably reptates back and forth though the pore as a result of thermal energy (Simon et al., 1992). However, it is also possible that the energy and direction of the vectorial transport may be influenced by the binding of sugars to the nascent polypeptide (Nicchotta and Blobel, 1993), or the binding of other proteins, known as chaperonins, that help

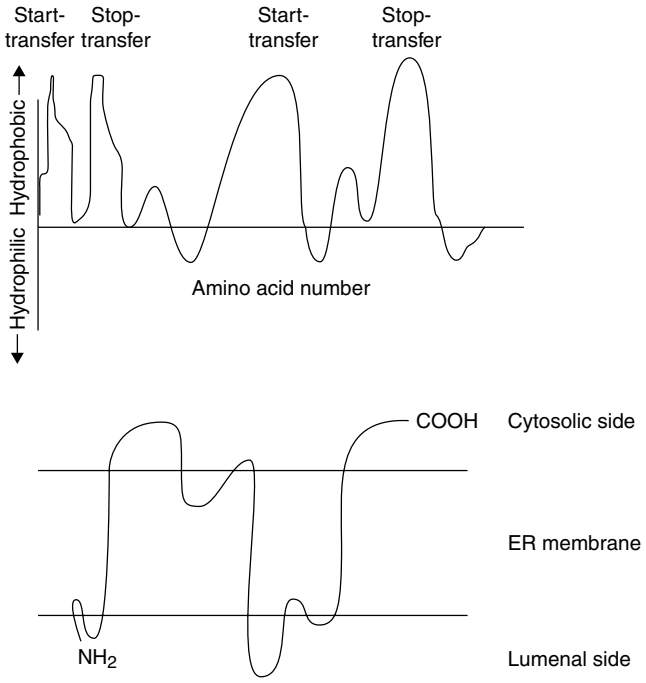


FIGURE 4.16 Comparison between a Kite-Doolittle plot, which characterizes the hydrophobicity of regions of a protein with the localization of those regions in the membrane.

fold a polypeptide into its mature conformation. A chaperonin, according to R. J. Ellis (1996),

> *… is a precise molecular analog of the human chaperone. The traditional role of the latter is to prevent incorrect interactions between pairs of human beings, without either providing the steric information necessary for their correct interaction or being present during their subsequent married life—but often reappearing at divorce and remarriage! So the term is a precise description of an essential function that we now know all cells require in order to increase the probability of correct macromolecular interactions.*

Some of the resident proteins in the lumen of the ER act as chaperonins (Coughlan et al., 1996). For example, BiP, which stands for *bi*nding *p*rotein, is an ER lumenal protein that is involved in protein folding, perhaps by acting as a detergent that helps solubilize the polypeptide so that it can be properly folded (Pelham, 1989; Jones and Bush, 1991; Fontes et al., 1991; Li et al., 1993; Anderson et al., 1994). BiP is an unusual protein since it utilizes ATP in the E-space. The proteins that will remain in the lumen of the ER have specific sequences that keep them in the ER so that they do not continue along the secretory pathway. Misfolded proteins that are not transported out of the ER are degraded in the ER itself as a type of quality control (Gant and Hendershot, 1993). The length of the membrane-spanning regions of some ER proteins determines whether those proteins will remain in the ER or travel through the secretory pathway to the Golgi bodies or plasma membrane (Brandizzi et al., 2002c).

4.7.3 Protein Glycosylation (Carbohydrate Synthesis)

Protein glycosylation takes place in the ER where a single type of oligosaccharide [(N-Acetylglucosamine)$_2$(mannose)$_9$(glucose)$_3$] is added to an amino group of asparagine (Faye et al., 1989). Oligosaccharides linked in this way are called *N*-linked oligosaccharides, since the oligosaccharides are added to the amino (NH_2) group of asparagines that are found in the following amino acid sequence N-X-S/T, where X stands for any amino acid except proline (Figure 4.17). Oligosaccharyl transferase is a membrane-bound enzyme that catalyzes the transfer of the entire oligosaccharide from dolichyl pyrophosphate to the asparagine group.

Dolichols are long-chain unsaturated alcohols made up of 16–21 isoprenoid units (-$CH_2C[CH_3]$=CH-CH_2−). Dolichols are found in the ER and the Golgi apparatus and serve as intermediates in the formation of oligosaccharides (Figure 4.18; Elbein, 1979). The dolichol that is in the ER membrane must be charged by ATP to make dolichyl phosphate. This process takes place on the cytosolic side of the membrane or in the P-space. Subsequently, two UDP-*N*-acetyl-glucosamines are added to form dolichol pyrophosphate (N-acetyl-glucosamine)$_2$, uridine monophosphate (UMP), and uridine diphosphate (UDP). In cells, these nucleoside phosphates are specific for transferring sugar molecules much like coenzyme A is specific for transferring acyl groups. Tunicamycin is a specific inhibitor of this step, and thus a good way of testing the importance of glycosylation in a given process.

Five GDP-mannoses are added to the N-acetyl-glucosamine and five GDPs are released. At this point, the lipid-sugar complex flips to the other side of the membrane where it faces the lumen. From this point on, four mannose and three glucose residues are added to the oligosaccharide from dolichyl-P-mannose and dolichyl-P-glucose, which were originally formed on the cytosolic surface of the ER membrane and then flipped across into the lumenal side. Before the newly formed glycoprotein leaves the ER, one mannose and three glucose residues are cleaved from it leaving a high-mannose glycoprotein. Here, it is important to realize that not all membrane transporters are proteins—this is a case where a sugar-phosphate is transported by a polymer of isoprenoid units.

4.7.4 Calcium Regulation

The endoplasmic reticulum, like the plasma membrane, helps to control the ionic composition of the cytosol. Like the plasma membrane, the endoplasmic reticulum

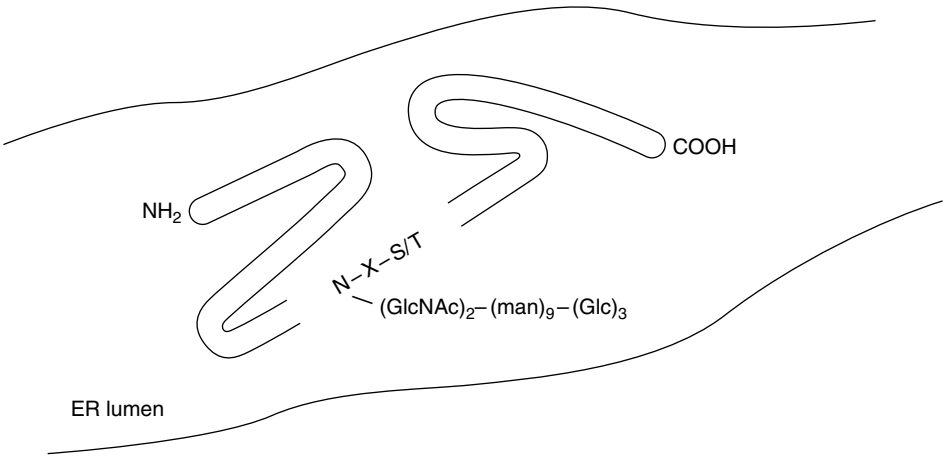


FIGURE 4.17 Synthesis of the carbohydrate group of *N*-linked glycoproteins in the membrane.

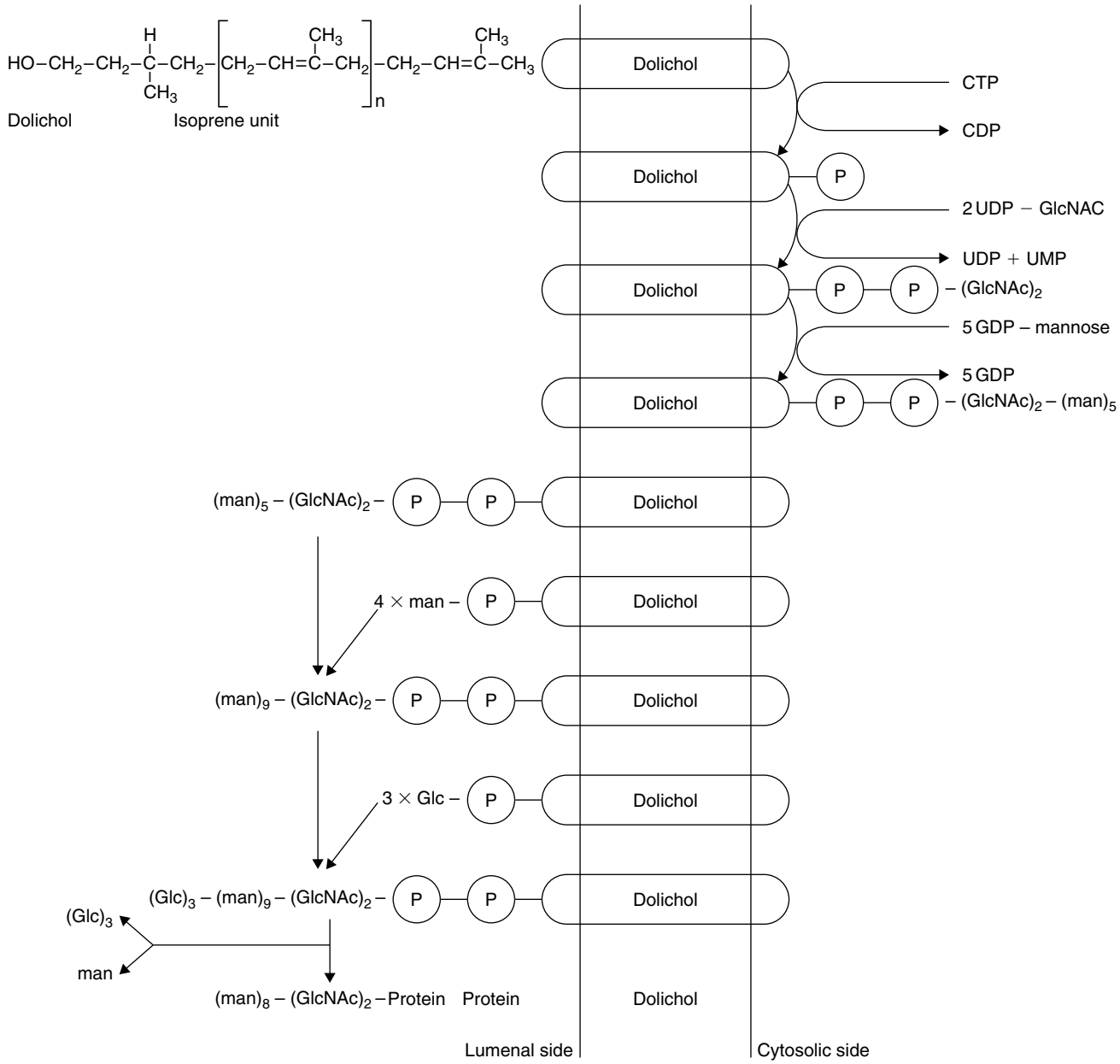


FIGURE 4.18 Attachment of carbohydrate groups to the asparagine residue of a nascent polypeptide by oligosaccharide transferase.

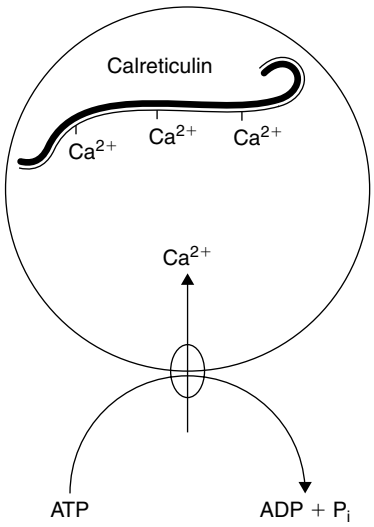


FIGURE 4.19 A right-side-out ER vesicle showing the orientation of a Ca^{2+}-ATPase and the calcium-binding protein, calreticulin, in the E-space.

has a Ca^{2+} pumping ATPase that pumps Ca^{2+} from the cytosol, which is a P-space into the lumen, which is an E-space (Figure 4.19; Buckhout, 1984; Bush and Sze, 1986; Giannini et al., 1988). The properties of the endoplasmic reticulum–localized Ca^{2+}-ATPase are analyzed essentially the same way that the H^+-ATPase of the plasma membrane is studied. One way to measure Ca^{2+} pumping activity is to challenge purified and intact, right-side out ER vesicles with radioactive Ca^{2+} and initiate uptake by adding an energy source (e.g., ATP). Then the rate of uptake is measured per mg protein by capturing the membranes on a filter. Using such an assay, Williams et al. (1990) found that the ER Ca^{2+}-ATPase is almost identical to the plasma membrane–localized Ca^{2+}-ATPase except that the plasma membrane-bound ATPase can use guanosine triphosphate (GTP) as a substrate, while the ER one cannot. The ER Ca^{2+}-ATPase, like the plasma membrane Ca^{2+}- and H^+-ATPases, is inhibited by vanadate and dicyclohexylcarbodiimide (DCCD). Ca^{2+} uptake into the endoplasmic reticulum of barley aleurone cells requires 0.07 mol/m^3 ATP and 0.0005 mol/m^3 Ca^{2+} for half-maximal activity (Bush et al., 1989a).

The sarcoplasmic reticulum of muscle cells is a highly developed form of ER that specializes in storing and releasing the Ca^{2+} necessary for muscle contraction and relaxation. It contains a low-affinity, high-capacity Ca^{2+} binding protein called *calsequestrin* in its lumen. The affinity constant of calsequestrin for Ca^{2+} is approximately 1 m^3/mol and it can bind approximately 50 Ca^{2+} ions per 51-kDa molecule (Ebashi, 1985). Calsequestrin also occurs in the endoplasmic reticulum of plant cells (Krause et al., 1989; Chou et al., 1989).

Calreticulin, another low-affinity, high-capacity Ca^{2+}-binding protein, also occurs in the ER of plant cells (Opas et al., 1996). What evidence should we look for to determine whether calreticulin or calsequestrin are integral membrane proteins, or localized in the lumen? First, they can be isolated and purified from osmotically shocked microsomes without detergents; second, the purified protein always stays in the hydrophilic phase and never goes into the hydrophobic phase during two-phase partitioning; third, immunogold labeling shows that the protein is localized in the lumen; last, the protein is protected by the ER and not degraded when the ER is treated with trypsin, a protease that cannot cross membranes.

How would you measure the binding capacity and affinity of a Ca^{2+} binding protein? Put the protein in a dialysis membrane surrounded by solutions with various concentrations of Ca^{2+}. At equilibrium, remove the protein, measure the amount of protein spectrophotometrically and the Ca^{2+} content of the protein using atomic absorption spectrophotometry, and then determine how many moles of Ca^{2+} are bound to each mole of protein at each Ca^{2+} concentration. Then plot this ratio versus the Ca^{2+} concentration. The Ca^{2+} concentration that gives the half-maximal saturation of the protein is an estimate of the dissociation constant (K_d, in M) of the protein for Ca^{2+}. The dissociation constant is the reciprocal of the affinity constant (K_a, in M^{-1}) of the protein for Ca^{2+}. The capacity and affinity of typical lumenal Ca^{2+}-binding proteins are 20–50 mol Ca^{2+}/mol protein and 1 m^3/mol, respectively (Ebashi, 1985; Macer and Koch, 1988; Michalak et al., 1992). The Ca^{2+} are typically bound to the negatively-charged, acidic amino acids.

We are now ready to learn a general principle. In a particular location, if an enzyme has the function for which it is named, then the substrate in its local environment, particularly around the active site, must be present in concentrations around the enzyme's binding constants (K_m, $1/K_a$ or K_d) for that substrate. That is, we can use the values of the binding constants to estimate the concentrations of molecules and ions in various compartments in the cell. This assumes that the binding constants were determined in intact enzymes, under the correct physiological conditions. Given the binding constants found for the proteins involved in Ca^{2+} regulation, we can assume that the Ca^{2+} concentration in the lumen of the ER (E-space) is approximately 1 mol/m^3 and the Ca^{2+} concentration in the cytosol (P-space) is approximately 10^{-4} mol/m^3. As I will discuss in Chapter 12, Ca^{2+} is a cytotoxin, and this metastable, nonequilibrium distribution must be strongly regulated or cell death will result.

4.7.5 Phenylpropanoid and Flavonoid Synthesis

The phenylpropanoid pathway is part of the plant aromatic pathway, and one of the major enzymes of this pathway (cinnamate 4-hydroxylase) is embedded in the ER as part of a multienzyme complex known as a metabolon (Wagner and Hrazdina, 1984; Hrazdina and Wagner, 1985; Hrazdina and Jensen, 1992; Burbulis and Winkel-Shirley, 1999;

Winkel-Shirley, 2002; Winkel, 2004). The phenylpropanoid pathway begins with the amino acid phenylalanine, which is the end product of the shikimic acid pathway. Phenylalanine is the precursor for the synthesis of phytoalexins, which are involved in defense mechanisms; flavonoids, which in part cause the colors of plants that attract pollinators; lignin, which is the major molecule participating in the incrustation and stiffening of walls; and coumarin, the molecule that gives us the smell of freshly cut grass.

The enzymes in the branch of the phenylpropanoid pathway involved in flavonoid synthesis (chalcone synthase and malonyl-CoA:4 coumaroyl-CoA malonyltransferase) have been localized in the ER fraction (Hrazdina et al., 1987). Furthermore, immunocytochemistry with colloidal gold particles shows that the enzymes are associated with the cytosolic leaflet of the ER membrane. The flavonoids synthesized by these enzymes are stored in the vacuole (see Chapter 7).

4.8 SUMMARY

The ER functions in maintaining a surface-to-volume ratio in large cells of approximately $10^6\,m^{-1}$ and thus duplicates many of the transport functions of the plasma membrane, particularly those involved with Ca^{2+} transport. The ER also serves as the workbench of the cell for building itself and other membranes, and thus is endowed with the enzymes necessary for lipid synthesis and the ribosomes necessary for protein synthesis. We have learned that proteins that are synthesized on the ER go there because they contain a signal peptide. As we will see, the signal hypothesis describes a general mechanism of how specific proteins are targeted to each organelle.

4.9 QUESTIONS

4.1. How is the endoplasmic reticulum similar to the plasma membrane, and how is it different?
4.2. Why is the surface-to-volume ratio important in biology?
4.3. How is the endoplasmic reticulum involved in the synthesis of the plasma membrane?

Chapter 5

Peroxisomes

5.1 DISCOVERY OF MICROBODIES

Microbodies are usually considered to be single membrane–enclosed organelles approximately 0.2–1.5 μm in diameter. While they are often round in thin sections, they can also appear ellipsoidal or dumb bell shaped (Frederick et al., 1968; Gruber et al., 1972). As discussed later, they may actually form a peroxisomal reticulum. Microbodies have a limiting membrane, which is approximately 6.5 nm thick and surrounds a matrix that can appear amorphous, granular, fibrillar, or paracrystalline (Vigil, 1983).

While microbodies were first seen in electron micrographs by Rhodin (1954), who coined the term *microbody*, Rouiller and Bernhard (1956) presented the first widely available pictures of microbodies in liver cells (Figure 5.1). Approximately a decade later, Christian de Duve and his coworkers isolated microbodies from rat liver cells as a contaminant of the mitochondrial fraction (Baudhuin et al., 1965). In plants, microbodies were first isolated from castor bean seedlings by Breidenbach and Beevers (1967) and from spinach leaves by Tolbert et al. (1968). The papers written by the pioneers in microbody research are very exciting because biochemists and electron microscopists were meeting at the borderlands of their sciences and providing cellular biology with the depth originally envisioned by Jean Baptiste Carnoy when he coined the term *cellular biology* in 1884. Now biochemists could "see" the organelles that contained the enzymes of interest and electron microscopists could assign a function to the structures and significance to the relative positions of organelles.

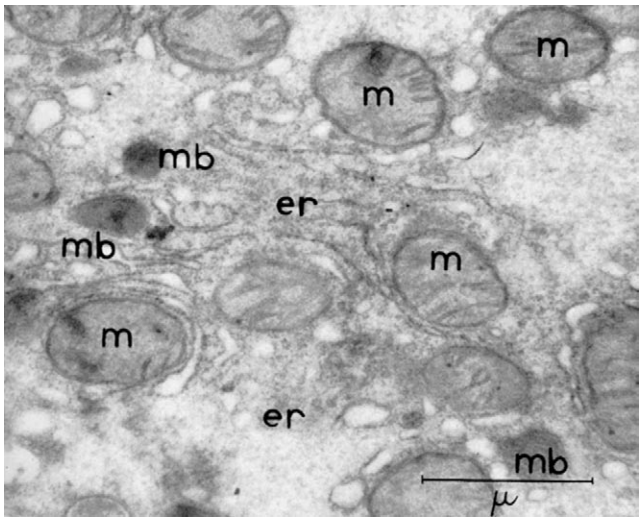


FIGURE 5.1 Microbodies (mb) in the cytoplasm of a rat liver cell. er, ergastoplasm; m, mitochondria. (*Source:* From Rouiller and Bernhard, 1956.)

De Duve and Baudhuin (1966) did not like the term *microbody* because it was so general and strictly morphological, but they did not want to name it something else until they knew more about its true function. These were the days when objects were given functional names only after the functions were understood. However, after learning more about the function of microbodies, they named them *peroxisomes*. They defined the peroxisome as a single membrane–enclosed organelle that contains at least one oxidase that forms the toxic molecule H_2O_2, as well as catalase, an enzyme that breaks the H_2O_2 down into nontoxic oxygen and water. Since peroxisomes contain catalase, they are easily identified in electron microscopical sections that have been stained with diaminobenzidine, since this agent forms an electron-dense deposit in the presence of catalase (Vigil, 1970). Functioning peroxisomes are necessary for normal plant growth and development (Zolman et al., 2001; Hu et al., 2002; Zolman and Bartel, 2004; Woodward and Bartel, 2005) and may be involved in auxin metabolism (Zolman et al., 2007) and the synthesis of jasmonic acid (Cruz Castillo et al., 2004).

5.2 ISOLATION OF PEROXISOMES

In order to isolate peroxisomes, the tissue is homogenized and filtered to remove the extracellular matrix. Then the filtrate is centrifuged at about 500 g for 10 minutes to remove the nuclei, starch, plastids, and fat. The fat-free supernatant is then centrifuged at 10,000 g for 20 minutes to get the

mitochondrial fraction, which contains the peroxisomes. The resuspended pellet is layered on a linear sucrose gradient (30–60% sucrose), and centrifuged at 62,000 g for 4–5 hours. The peroxisomes show up as a very dense (1.25 g/mL) fraction (Vigil, 1983), which indicates that they are high in protein.

The peroxisomes can be further fractionated by osmotic shock. The membrane can be separated from the matrix by placing the peroxisomes into a dilute buffer and then recentrifuging them at 100,000 g for 60 minutes to pellet the membranes. The matrix proteins remain in the supernatant.

5.3 COMPOSITION OF PEROXISOMES

Almost all peroxisomes contain catalase, but depending on their function, they have a variety of oxidases, which are discussed in the next section. Porins have been identified as part of the peroxisomal membrane (Corpas et al., 2000).

Although the phospholipid content of peroxisomes is particularly high, the lipid composition is similar to that of the endoplasmic reticulum (ER) and plasma membrane (Donaldson and Beevers, 1977; Donaldson et al., 1972, 1981; Chapman and Trelease, 1991a). Again, the similar composition reflects similar function, and the rationale for the differences is not yet known. The lipid compositions of peroxisomes are given in Table 5.1.

5.4 FUNCTION OF PEROXISOMES

Peroxisomes participate in a diverse set of biochemical reactions, including H_2O_2-based respiration, the β-oxidation of fatty acyl chains, the initial reactions in ether glycerolipid biosynthesis, cholesterol and dolichol synthesis, the glyoxylate cycle, photorespiration, jasmonic acid synthesis, alcohol oxidation, transaminations, purine and polyamine catabolism, ureide anabolism, nitric oxide synthesis, and the formation of gly betaine, an osmoprotectant (Frederick et al., 1975; Tolbert, 1981; Huang et al., 1983; Masters and Crane, 1995; Minorsky, 2002; Emanuelsson et al., 2003; Lin et al., 2004; Reumann, 2004; Reumann et al., 2004). Interestingly, the β-oxidation of fatty acyl chains was first found to occur in the peroxisomes of castor bean cells (Cooper and Beevers, 1969) and only later found in the peroxisomes of animal cells (Lazarow and de Duve, 1976). Two of the pathways, including the β-oxidation and the glycolate pathways, are discussed in the following sections in order to demonstrate where marker enzymes fit into the picture and to see how the various organelles cooperate in the realization of complete biochemical pathways. I also show that duplication or functional redundancy exists in cells in that many of the same enzymes (e.g., malate dehydrogenase and citrate synthase) exist in more than one organelle.

TABLE 5.1 Lipid composition of the castor bean peroxisomal membrane

(mol%) of Lipid Phosphorus in Membrane	
Phospholipid	
Phosphatidylcholine	51.4
Phosphatidylethanolamine	27.2
Phosphatidylinositol	9.0
Phosphatidylserine	1.5
Phosphatidylglycerol	2.7
Cardiolipin	2.3
Other	2.0

Source: *From Donaldson and Beevers (1977).*

5.4.1 β-Oxidation

In general, β-oxidation is involved in the formation of sucrose from fatty acyl chains and is very important during the germination of oil-rich seeds and spores (DeMaggio et al., 1980; Hayashi et al., 2001) and in heterotrophic cells living on a lipid food source (Binns et al., 2006). In these cases, the peroxisomes are found in close proximity with lipid bodies and mitochondria (Figure 5.2). Peroxisomes can and do move in cells, apparently to where they need to be. In animal cells, they tend to move along microtubules (Rapp et al., 1996), while in plant cells they tend to move along microfilaments (Collings et al., 2002; Mano et al., 2002; Mathur et al., 2002; Jedd and Chua, 2002). Catalase, isocitrate lyase, and malate synthase are some of the enzymes that function in β-oxidation and are used as marker enzymes for peroxisomes.

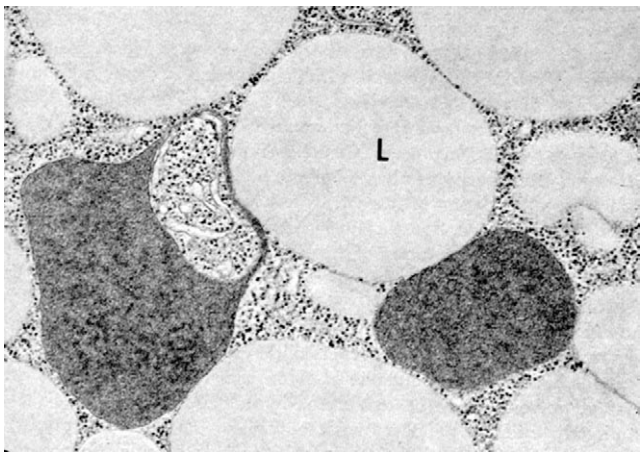


FIGURE 5.2 Peroxisome (glyoxysome) next to lipid bodies (L) in a tomato cotyledon cell. ×29,000. Notice how the peroxisome on the left encloses a mitochondrion. (*Source:* From Frederick et al., 1975.)

Peroxisomes in fatty cells contain lipases that break down the stored lipids into their constitutive fatty acyl chains. Fatty acyl CoA enters peroxisomes by way of an ABC transport protein (Zolman et al., 2001; Footitt et al., 2002; Hayashi et al., 2002; Theodoulou et al., 2005). The fatty acyl chains then undergo β-oxidation, a series of reactions that lead to the breakdown of long fatty acyl chains into many acetyl-CoA molecules (Figure 5.3). CoA is involved in the activation and transfer of acetate groups between molecules (Lipmann, 1971). The enzymes involved in β-oxidation include fatty acyl–CoA synthetase and fatty acyl–CoA oxidase. The fatty acyl–CoA oxidase generates hydrogen peroxide. The hydrogen peroxide would be deadly to the cell, but it is broken down by catalase to $½O_2 + H_2O$, both of which are nontoxic. Subsequently, the acetyl-CoA molecules formed during β-oxidation are joined by malate synthase to glyoxylic acid molecules to make malic acid. This is one way acetyl CoA enters the glyoxylic acid cycle.

The malic acid is oxidized to oxaloacetic acid by malate dehydrogenase. Another acetyl CoA is attached to the oxaloacetic acid by citrate synthase to form citric acid. The citric acid is converted to isocitric acid by aconitase. Then isocitrate lyase splits the isocitric acid into glyoxylic acid, which is used to recharge the glyoxylic acid cycle, and succinic acid, which leaves the peroxisome. In essence, the glyoxylic acid cycle in the peroxisome converts two acetic acid molecules into one succinic acid molecule.

The succinic acid moves from the peroxisome to the mitochondrion, where it is converted to malic acid. The malic acid may exit the mitochondrion on the same transporter that allows the entrance of succinic acid so that a 1:1 stoichiometry is maintained. The malic acid that leaves the mitochondrion is converted to oxaloacetic acid in the cytosol by malate dehydrogenase. Then the oxaloacetic acid is converted into phosphoenolpyruvic acid by phosphoenolpyruvate carboxykinase. In seeds, the phosphoenolpyruvic acid

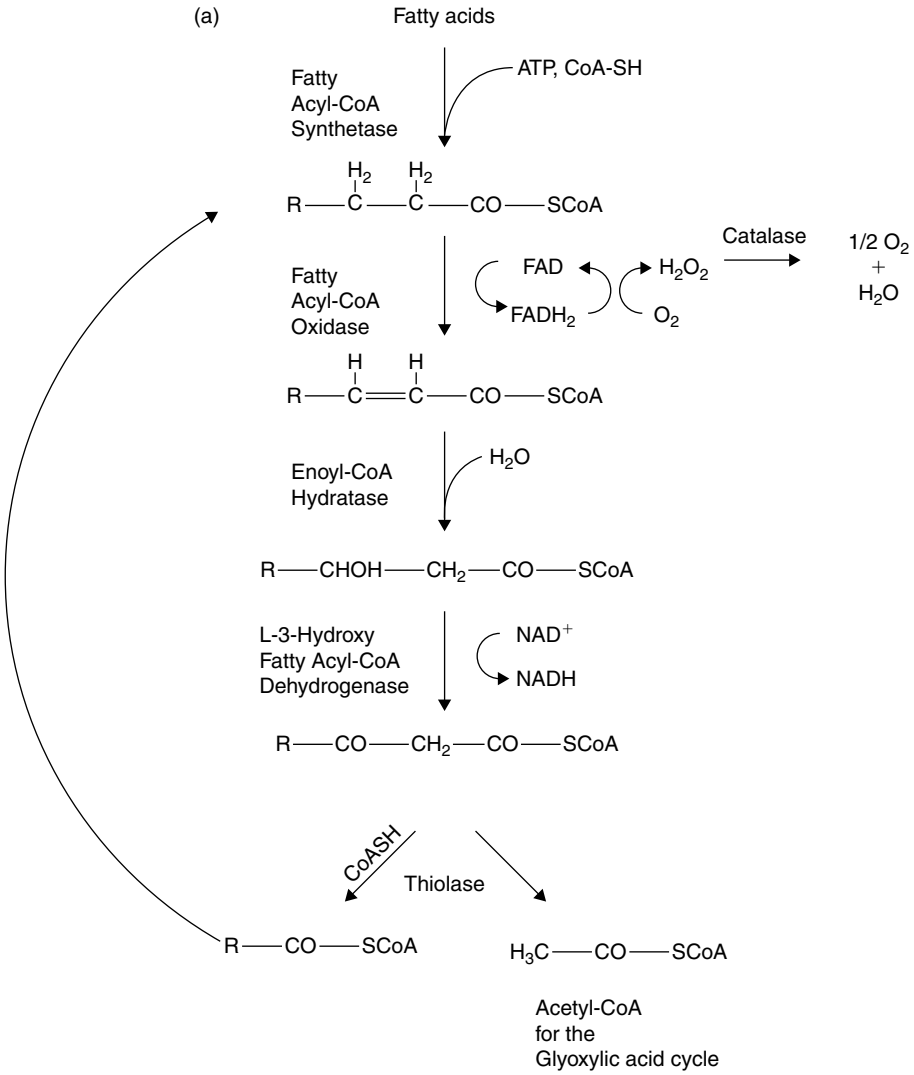


FIGURE 5.3 Pathway for the breakdown of fatty acyl chains: (a) β-oxidation of fatty acyl chains, (b) the glyoxylic acid cycle. (Continued)

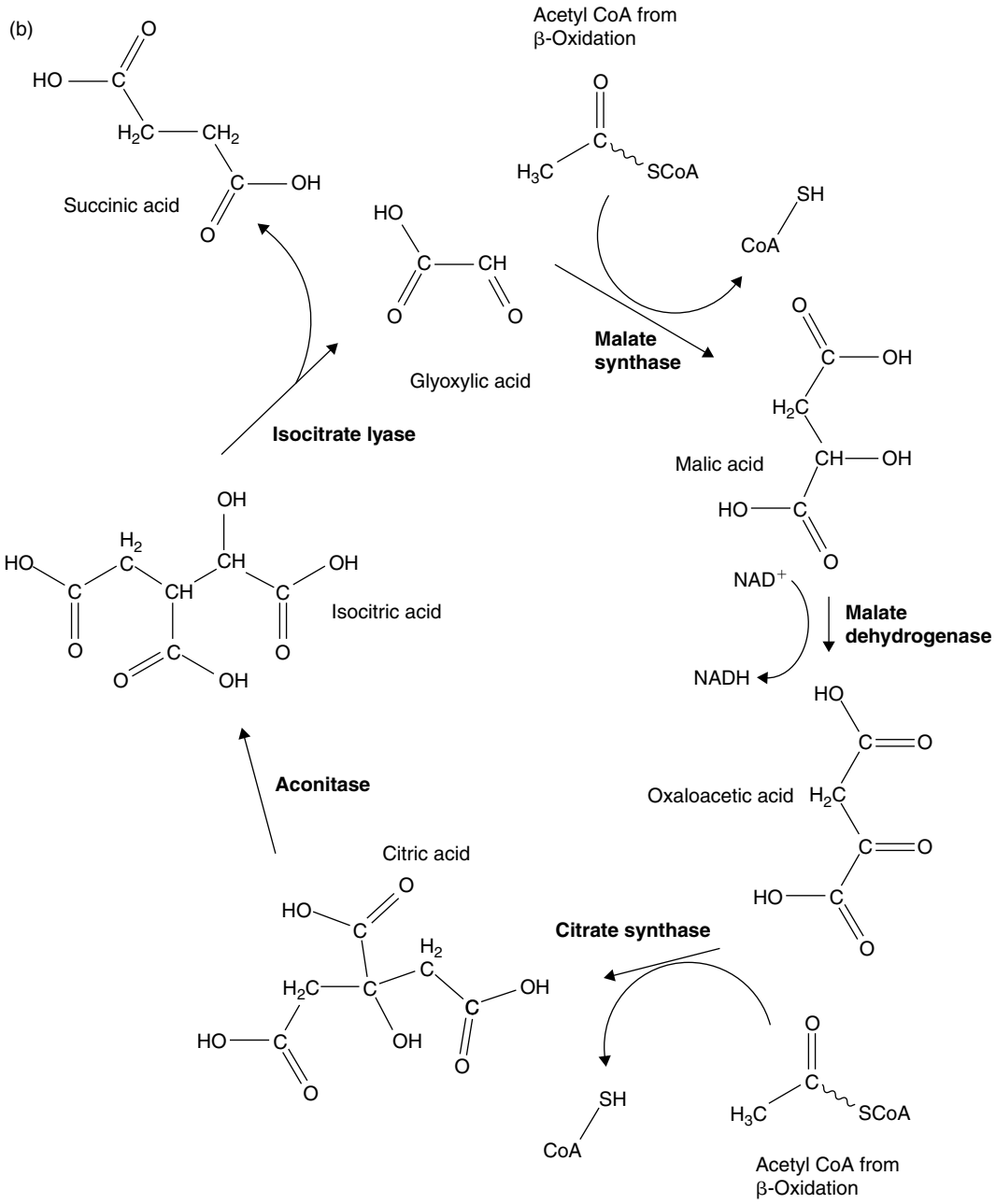


FIGURE 5.3 (Continued)

is converted to glucose via the gluconeogenesis pathway. Since glucose is a reducing sugar, which makes it highly reactive, it must be converted to a relatively nonreactive form like sucrose or starch. The hydrophilic sucrose molecules are then translocated out of the seed where they nourish the growing regions of the plant.

5.4.2 Photorespiration

John Decker (1955, 1959) discovered a light-dependent respiratory pathway in plants that came to be known as *photorespiration*. His discovery was not widely accepted, because recognition of photorespiration would mean that scientists who studied photosynthesis would have to reinterpret much of their data (Zelitch, 2001). In fact, Decker's name is barely mentioned in the photorespiration literature (Goldsworthy, 1976). Photorespiration turned out to be real and it involves the capture of glycolic acid produced in the chloroplast (Ludwig and Canvin, 1971; Tolbert, 1971; Chollet and Ogren, 1975; Zelitch, 1964, 1971, 1975, 2001; Ogren 2003). Glycolic acid is formed from 2-phosphoglycolic acid, one of the products formed when ribulose bisphosphate carboxylase-oxygenase (i.e., rubisco) catalyzes the addition of oxygen instead of carbon dioxide to

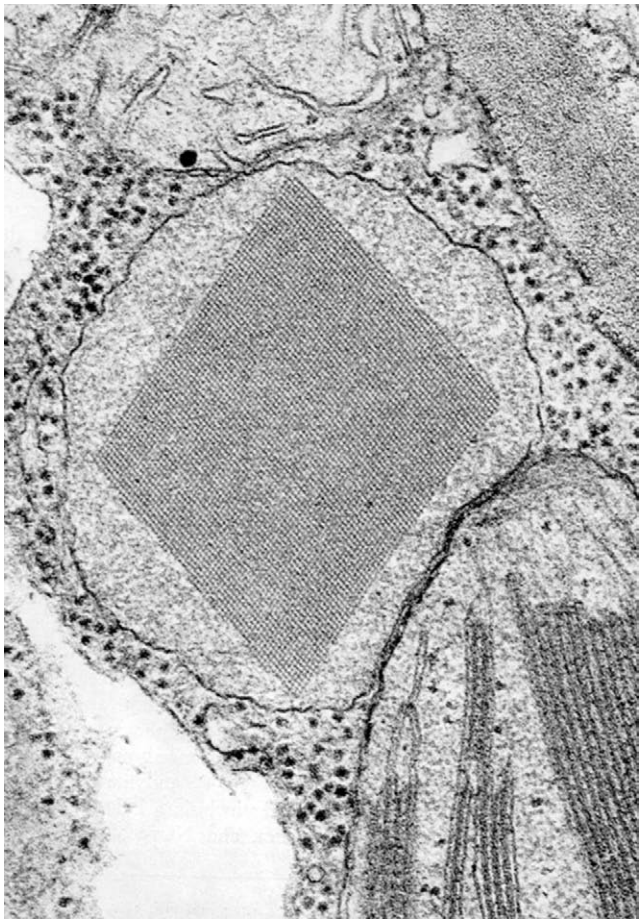


FIGURE 5.4 Peroxisome next to a chloroplast and a mitochondrion in a tobacco leaf mesophyll cell. ×109,000. (*Source:* From Frederick et al., 1975.)

ribulose bisphosphate (see Chapter 13). Thus, photorespiration is the cause of the "Warburg Effect," which is the apparent inhibition of photosynthesis by oxygen discovered by Otto Warburg (1920). In green leaves, the enzymes involved in photorespiration are found in the peroxisomes and the peroxisomes are found in close proximity to the chloroplasts and mitochondria (Frederick and Newcomb, 1969; see Figure 5.4). Catalase, serine:glyoxylate aminotransferase, and hydroxypyruvate reductase are three important enzymes involved in photorespiration (Figure 5.5).

The 2-phosphoglycolic acid formed by rubisco is dephosphorylated to glycolic acid. The glycolic acid diffuses to the peroxisome where it is converted to glyoxylic acid and then to glycine. The enzyme that causes the formation of glyoxylic acid from glycolic acid is glycolate oxidase. This reaction also produces hydrogen peroxide, which is then broken down by catalase. The glyoxylic acid is converted to glycine by glutamate:glyoxylate aminotransferase. An aminotransferase is an enzyme that exchanges amino groups from two amino acids to two α-keto acids. Peroxisomes contain aminotransferases that convert glyoxylate to glycine. At the same time, some of the aminotransferases convert glutamate to α-ketoglutarate, serine to hydroxypyruvate, aspartate to oxaloacetate, or alanine to pyruvate. The glycine formed by the aminotransferase enters the cytosol where it can participate in protein synthesis. The glycine can also be taken up by the mitochondria where two molecules of glycine and one molecule each of water and NAD^+ are converted to serine, NADH, NH_3, and CO_2. If the serine moves back to the peroxisome, it is converted to hydroxypyruvic acid by serine:glyoxylate aminotransferase (at the same time another glyoxylic acid is converted to glycine; Raghavendra et al., 1998). The hydroxypyruvic acid is then converted to glyceric acid by hydroxypyruvate reductase. The glyceric acid then moves back to the chloroplast, where it is phosphorylated. The phosphorylated form then enters the Calvin cycle in order to participate in starch metabolism. In this way, three out of four atoms of carbon lost by the chloroplast as two glycolic acid molecules are recycled to the chloroplast as a single molecule of the three-carbon glyceric acid (Berry et al., 1978).

In order to deduce the pathways involved in β-oxidation and photorespiration, experiments using radioactive carbon compounds are performed in vivo and in vitro to determine the temporal sequence in which intermediates become labeled. Radioactive labeling experiments also provide information about the rates that carbon moves through the pathway. These rates are then compared with the rates of each enzyme reaction estimated from the maximal velocity of that reaction (v_{max}) and the concentration of substrate [S] that is needed to achieve the half-maximal rate (K_m; Zelitch and Ochoa, 1953; Zelitch, 1953, 1955). The rate or velocity of an enzyme reaction (v) is estimated with the following equation that will be derived in Chapter 12.

$$v = (v_{max})/[(K_m/[S]) + 1] \quad (5.1)$$

In order to estimate the extent in which each enzyme reaction is rate-limiting, the flux through the pathway is tested in the absence and presence of inhibitors of each enzyme (Zelitch, 1957, 1959, 1965, 1966, 1974). Lastly, the energetics of each reaction is determined to make sure that the proposed pathway is consistent with the laws of thermodynamics (Anderson and Beardall, 1991).

It is also possible to do all these experiments in mutant or transformed plants that lack the normal enzymes presumed to be necessary for the reaction pathways. In fact, confirmation of the photorespiratory pathway was the first use of *Arabidopsis* as a model system (Somerville and Ogren, 1979, 1980, 1982; Somerville, 1986, 2001; Ogren, 2003). The confirmation was accomplished by screening chemically induced mutants for their ability to grow in 1 percent carbon dioxide but not in natural air, which contains 0.03 percent carbon dioxide. Since the oxygenase activity of rubisco would be low in a high carbon dioxide environment, the activity of the photorespiratory pathway would also be low and consequently the growth of photorespiratory mutants would not differ significantly compared to the growth of wild type plants under high carbon dioxide but would differ under low carbon dioxide conditions. Presumably, the plants that could only grow only in high carbon dioxide would

have a defect in the photorespiratory pathway and when these mutants were returned to air, they would not be able to grow well since the photorespiratory pathway would not be able to recycle the lost photosynthetic carbon. Genes in the mutants that coded for altered nonfunctional enzymes were characterized by determining which ^{14}C-labeled photorespiratory intermediate accumulated just as the plants treated with photorespiratory inhibitors were characterized.

It would be exquisite to know, in a variety of cells, how each step regulates the flow of carbon through the various pathways. In order to know this, we would need to know the concentrations of substrates at the active site of each enzyme, the concentration of the enzymes and their regulators, as well as the permeability coefficients of the peroxisomal and other organelle membranes for each molecule transported across them, or the v_{max}, K_m, and stoichiometry of each transport protein for the various substrates (Reumann et al., 1998). It would also be good to know the rates of the other reactions that compete for the same substrates (e.g., glycine is utilized in protein synthesis as well as in photorespiration). We must also know how numerous each organelle that participates is in a given pathway, their surface areas and volumes, and the distance between them. Then we will be able to visualize the flux of carbon molecules from organelle to organelle and truly understand the relationship between biochemistry and cell biology.

Kebeish et al. (2007) have altered the flux of glycolate from the chloroplast to the peroxisomes by transforming

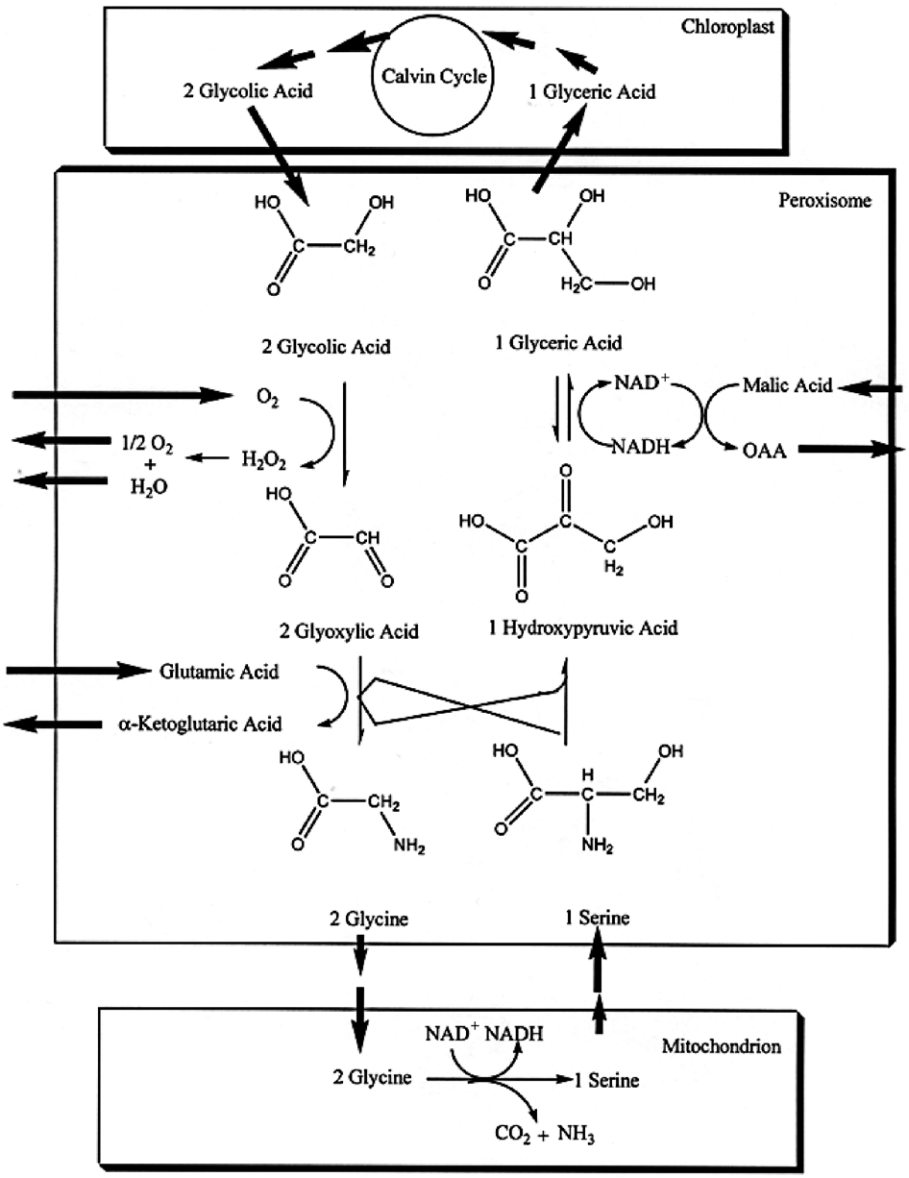


FIGURE 5.5 The glycolic acid pathway. Glycolic acid is converted to glyoxylic acid by glycollate oxidase. The breakdown of H_2O_2 to $½O_2$ and H_2O is catalyzed by catalase. Glyoxylic acid is converted to glycine by glutamate:glyoxylate aminotransferase. The conversion of serine to hydroxypyruvic acid is catalized by serine:glyoxylate aminotransferase. The conversion of hydroxypyruvic acid to glyceric acid is catalized by hydroxypyruvate reductase.

Arabidopsis plants with bacterial genes that encode glycolate dehydrogenase, glyoxylate carboligase, and tartronic semialdehyde reductase (Leegood, 2007; Sarwar Khan, 2007; Peterhänsel et al., 2008). The inserted genes also have a chloroplast-targeting sequence so that the enzymes they encode end up in the stroma of the chloroplast. In the transformed plants, the flux of glycolate from the chloroplast to the peroxisome is reduced and the chloroplastic glycolate is converted directly to glycerate. This results in faster-growing plants that produce more biomass and soluble sugars. When applied to crop plants, such technology could increase the yield of plants without diminishing their taste. While the photorespiratory pathway is usually considered to be wasteful of fixed carbon and thus a detriment to plants, it is also beneficial in promoting nitrate assimilation (Rachmilevitch et al., 2004) and reducing photoinhibition of photosystem II (see Chapter 13; Somerville and Ogren, 1979; Kozaki and Takeba, 1996; Takahashi et al., 2007). By altering the expression of the genes introduced by Kebeish et al. (2007), one could titrate the flux of glycolate from the chloroplast to the peroxisome, and thereby possibly maximize the benefits and minimize the demerits of the photorespiratory pathway in the peroxisomes.

5.5 RELATIONSHIP BETWEEN GLYOXYSOMES AND PEROXISOMES

The organelle to which Rhodin gave the name *microbody* has been called *cytosome, phragmosome, spherosome*, and *unidentified cytoplasmic organelle* by morphologists (Huang et al., 1983). When microbodies were first isolated from castor beans, they were given the name *glyoxysomes* because they contained the enzymes of the glyoxylate cycle. However, later it was found that they contain catalase as well as an H_2O_2-generating oxidase and fit de Duve's definition of a peroxisome.

The fate of castor bean glyoxysomes changes dramatically during greening. At the beginning of germination, the glyoxysomes function to convert fat to carbohydrate via β-oxidation and the glyoxylate cycle. However, after greening, the cotyledons make carbohydrate through photosynthesis and utilize the photorespiratory pathway in peroxisomes to capture carbon lost due to the binding of O_2 to rubisco. Before the cotyledons completely green, enzymes involved in both the glyoxylate cycle and glycolic acid pathway coexist as detected with enzymatic assays (Figure 5.6).

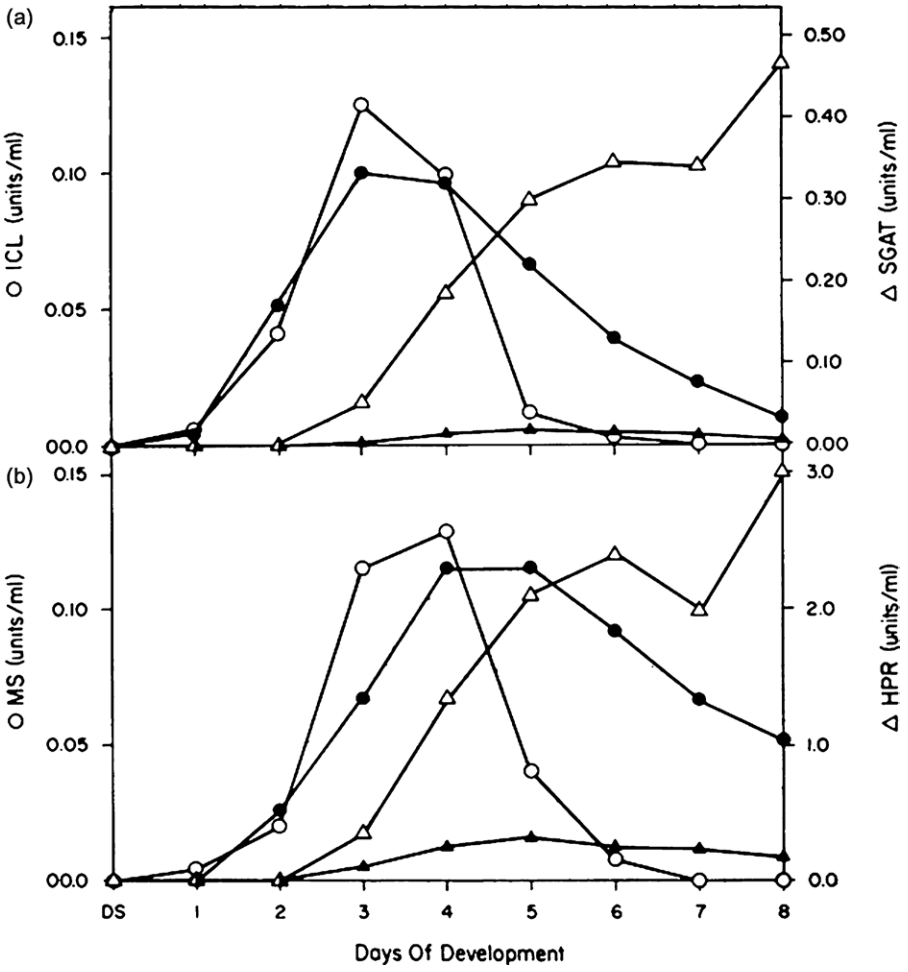


FIGURE 5.6 The activities of glyoxysomal and peroxisomal enzymes in cotyledons of light-grown (empty symbols) and dark-grown (filled symbols) plants. (*Source:* From Titus and Becker, 1985.)

Initially it was proposed that there were two separate microbody populations present during the transition: one containing the glyoxylate-cycle enzymes and the other containing the glycolic acid–pathway enzymes (McGregor and Beevers, 1969; Kagawa et al., 1973; Kagawa and Beevers, 1975). However, Gruber et al. (1970) proposed that the transition occurred within one population (Trelease et al., 1970). This was confirmed by Titus and Becker (1985) using immunoelectron microscopy with antibodies attached to two sizes of protein A gold (Figures 5.7–5.9). One size of colloidal gold

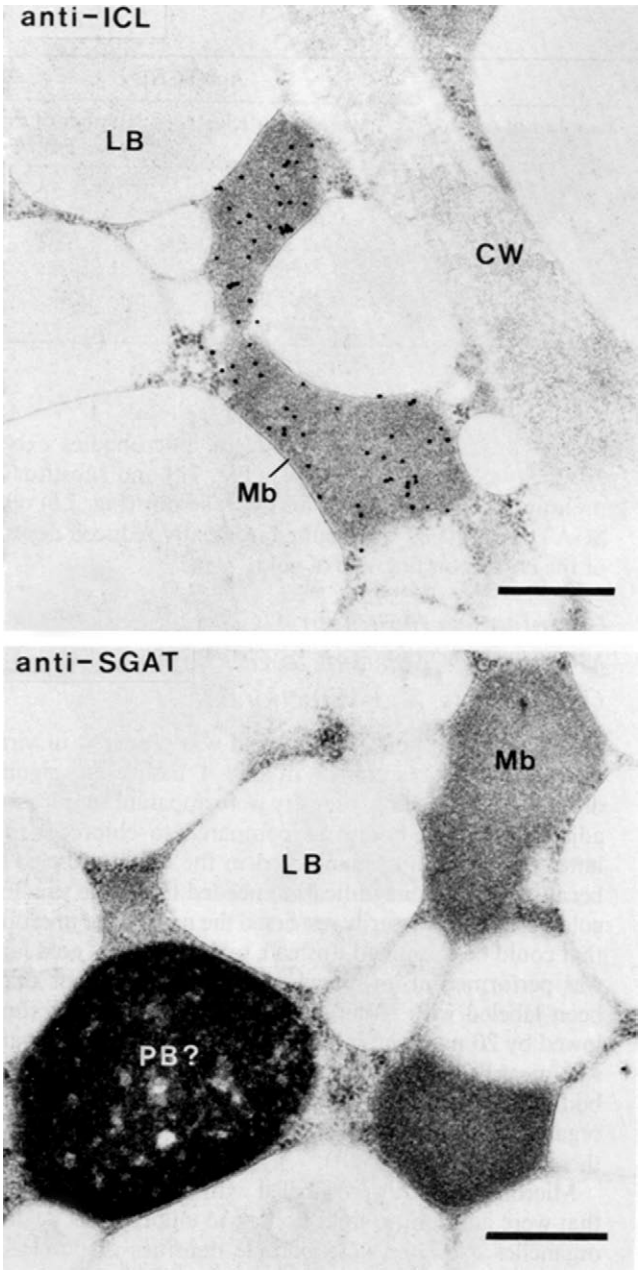


FIGURE 5.7 Cell of a 2-day-old cucumber cotyledon stained with colloidal gold particles conjugated to an antibody directed against isocitrate lyase (ICL) but not by antibodies directed against serine:glyoxylate aminotransferase (SGAT). Bar, 0.5 μm. (*Source:* From Titus and Becker, 1985.)

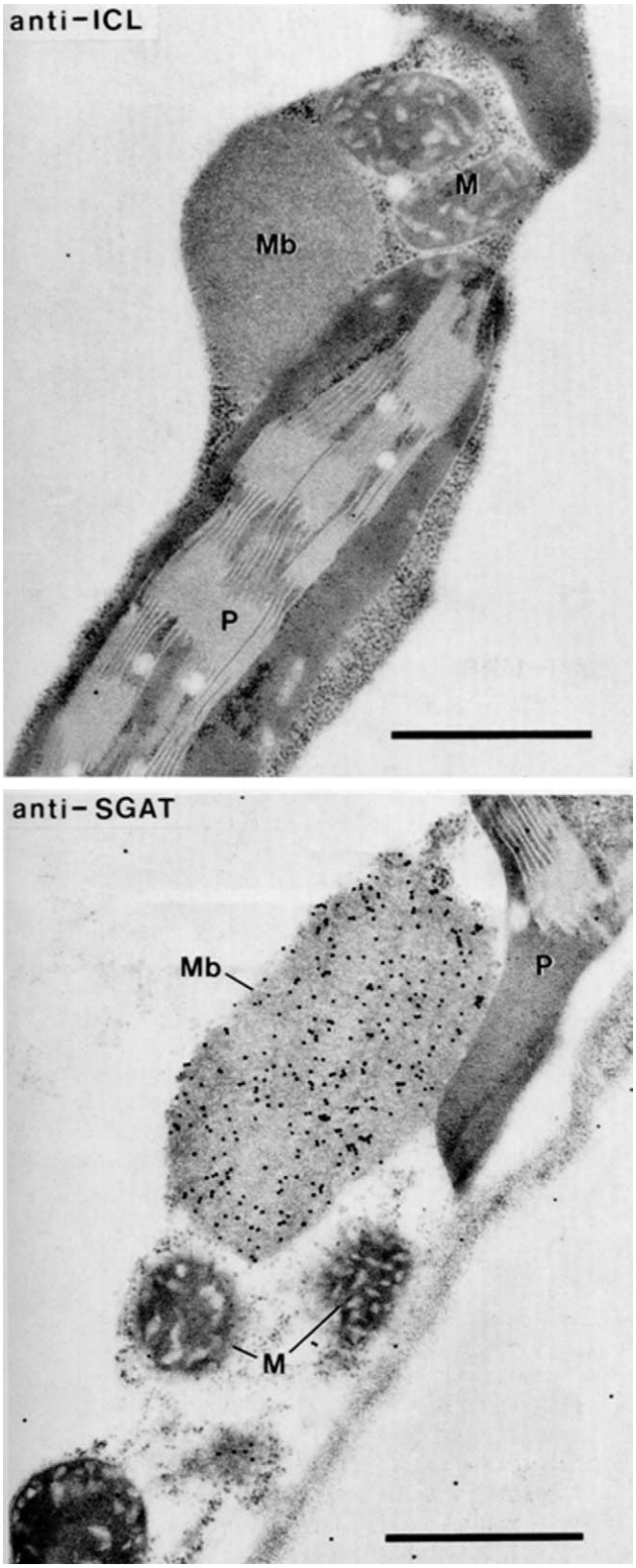


FIGURE 5.8 Cell of an 8-day-old cucumber cotyledon stained with colloidal gold particles conjugated to an antibody directed against serine:glyoxylate aminotransferase (SGAT) but not by antibodies directed against isocitrate lyase (ICL). Bar, 1 μm. (*Source:* From Titus and Becker, 1985.)

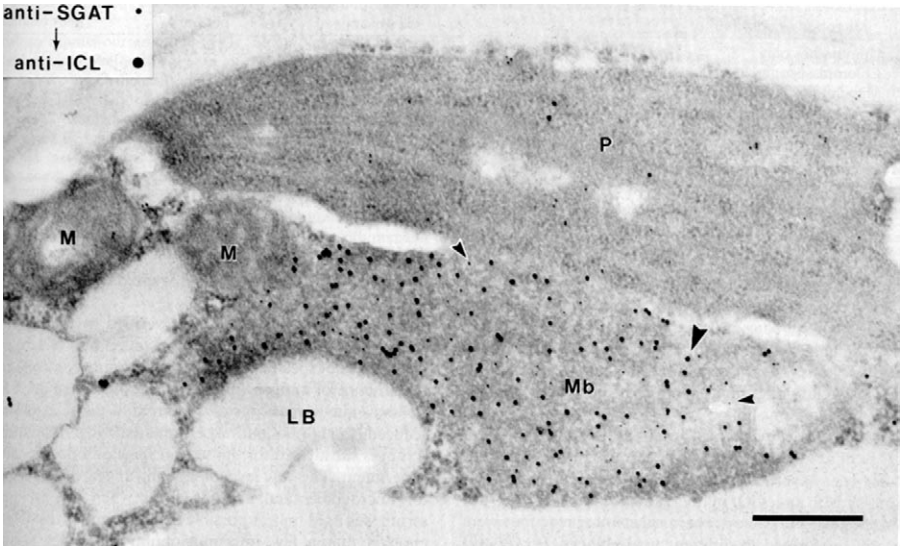


FIGURE 5.9 Cell of a 4-day-old cucumber cotyledon stained with 20-nm colloidal gold particles conjugated to an antibody directed against isocitrate lyase (large arrowhead), and 10-nm colloidal gold particles conjugated to an antibody directed against serine:glyoxylate aminotransferase (small arrowhead). Bar, 1 μm. (*Source:* From Titus and Becker, 1985.)

was attached to antibodies directed against typical glyoxysomal enzymes (isocitrate lyase and malate synthase) and the other size was attached to antibodies directed against typical peroxisomal enzymes (serine:glyoxylate aminotransferase and hydroxypyruvate reductase). They showed that while only glyoxysomal enzymes are present in the microbodies during early stages of development and only peroxisomal enzymes are present in the microbodies during later stages of development, both types of enzymes are present within the same organelle during the transition. This work and similar work by Sautter (1986) using watermelon cotyledons support the one-population hypothesis, where the glyoxysomes turn into peroxisomes during development and they are really one and the same organelle that can have two specialized functions.

Interestingly, during leaf senescence that accompanies the beautiful autumn colors, peroxisomes transform back to glyoxysomes; that is, green leaves have peroxisomes with high catalase and hydroxypyruvate activities while senescent leaves have peroxisomes with high malate synthase and isocitrate lyase activities (Nishimura et al., 1986,1993). The peroxisomes in senescing cells may be responsible for recycling the fatty acyl chains of leaf cells back to the plant in translocatable form (i.e., sugars). They have been given the name *gerontosomes* by Vincentini and Matile (1993).

5.6 METABOLITE CHANNELING

How do metabolites move through the various pathways? Membranes are usually considered the only cellular structure involved in the compartmentalization of the cell and the enzymes enclosed by the membranes are thought to be in solution. However, according to Hrazdina and Jensen (1992):

> *It has been both convenient and productive to treat cells as if they were simply a bag full of enzymes where reactions take place by chance encounter of substrate molecules with enzymes. However, improved methodology is now generating a basis for suggesting the existence of a strict spatial organization of enzymes in metabolic pathways.*

Prior to the isolation of urease by James Sumner (1926), it was universally believed that proteinaceous colloids provided the scaffolding for nonproteinaceous enzymes (Pauli, 1907; Willstätter, 1927). Perhaps it was reasonable to think that proteins only acted as structural entities, since at that time they were known to be the major constituent of hair, nails, horns, and hooves. Furthermore, it was believed that proteins were not high–molecular mass molecules, but aggregates of polypeptides, and further purification of any high–molecular mass entity would result in a pure protein with a molecular mass of 5–17 kDa (Fischer, 1923; Svedberg, 1937). Sumner's (1933) conclusion that enzymes were structurally independent proteins with high molecular masses was revolutionary in the 1930s. However, once this idea took hold, the best biochemists, not wanting to waste their clean thoughts on dirty enzymes, customarily purified proteins to homogeneity and then began to characterize their activity in vitro.

While this approach has contributed substantially to our understanding of cell metabolism, unfortunately, it has also had an undesirable side effect. That is, it led to the belief that proteins in vivo are spatially independent of one another, and the substrates and products diffuse to and away from the enzyme as they do in the test tube.

However, remember that in enzyme assays, the solutions are rapidly stirred so that the substrate concentration around the active site does not become depleted due to the limited speed of diffusion. Thus, while it was accepted that proteins could have a primary, secondary, tertiary, and quaternary structure, thoughts of a quintinary structure, where successive proteins in a complex form a real unit, was forbidden (Ovádi, 1991; Kühn-Velten, 1993; Mathews, 1993; Cascante et al., 1994). Thus, any observed protein–protein interactions that could potentially channel the product of one enzyme to the next enzyme in the pathway was considered an artifact of isolation. However, to paraphrase George Baitsell (1940), may it not be possible that the matrix of the peroxisome is a protein crystal in which a number of proteins are solidified into an ultramicroscopic pattern to perform the function of the organelle? Even in the inorganic world, there are many familiar examples of supramolecular structures, where the whole has different and emergent properties than the parts. I have already discussed T. H. Huxley's comments on the properties of water, which differ markedly from those of its constituents, oxygen and hydrogen. Irving Langmuir (1916) wrote, "it had been taken for granted that crystals were built up of molecules. But … it is clear that in crystals of this type [NaCl] the identity of the molecules is wholly lost, except in so far as we may look upon the whole crystal as composing a *single molecule*."

Recent work suggests that in some cases, the protein matrix in the cell (e.g., cytoskeleton) or an organelle (e.g., peroxisomal matrix) may provide a structure on which some enzymes reside, so that the enzymes form a highly efficient supramolecular structure in which the product of one enzyme is immediately channeled to another enzyme of which it is a substrate (Heupel and Heldt, 1994; Reumann et al., 1994; Reumann, 2000). It is also possible that the structure is composed either entirely or in part by the enzymes themselves.

Heupel et al. (1991) isolated peroxisomes from spinach leaves using Percoll–density gradient centrifugation. They then assayed six peroxisomal enzymes for activity in the presence and absence of detergent (Triton X-100). In the case of malate dehydrogenase, hydroxypyruvate reductase, serine:glyoxylate aminotransferase, catalase, and glutamate:glyoxylate aminotransferase, the enzyme activity of each enzyme was higher after Triton X-100 treatment than before (Figure 5.10). Significantly, the activity of the first enzyme in the pathway, glycolate oxidase, shows no increase in activity following detergent treatment, indicating that its active site is always exposed to its substrate. The increase in enzyme activity following detergent treatment is known as the latency of the enzyme. Since the detergent permeabilizes membranes so that the substrates can enter the peroxisomes, the barrier to the diffusion of the substrate is usually thought to be due exclusively to the membrane.

However, Heupel et al. (1991) broke the membrane by osmotically shocking the peroxisomes, and found that even after the membrane was lysed, the enzymes still showed

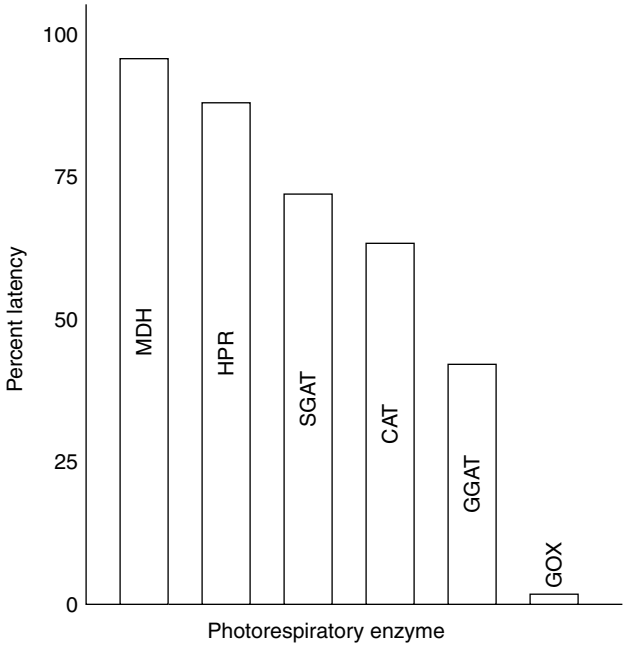


FIGURE 5.10 The latency of various photorespiratory enzymes. (*Source:* Data from Heupel et al., 1991.)

increased activity when treated with Triton X-100, indicating that the diffusion barrier may not be due exclusively to the membrane but may also be due to the specific positioning of enzymes into a complex. They concluded that Triton X-100 increased the availability of the active site of the enzyme to the substrate by solubilizing the enzymes from the complex. They believe that in vivo, this multiprotein organization allows the movement of the product of one enzyme directly to the active site of the next enzyme of which it is a substrate, and prevents competing substrates from getting to the active site (Figure 5.11). The ordered arrangement of enzymes leads to a process known as metabolite channeling.

Now let us look at the whole pathway. Heupel and Heldt (1994) measured the synthesis of glyceric acid from glycolic acid, glutamic acid, serine, and malic acid and found that it is inhibited in detergent-treated peroxisomes compared with intact or osmotically shocked peroxisomes (Figure 5.12). Likewise, the synthesis of glycine from glycolic acid, glutamic acid, and serine in detergent-treated peroxisomes occurs at a much-reduced rate compared to either intact or osmotically shocked peroxisomes. They concluded that detergent treatment solubilized the enzymes so that the substrates had to diffuse long distances to the next enzyme in the pathway and there was no longer metabolite channeling. According to Einstein's (1906) random-walk equation

$$t = x^2/2D \tag{5.2}$$

the time (t) it takes a molecule with a diffusion coefficient $D = [kT/(6\pi r_h \eta)]$ to diffuse from one place to another is proportional to the square of the distance (x). Thus, the rate

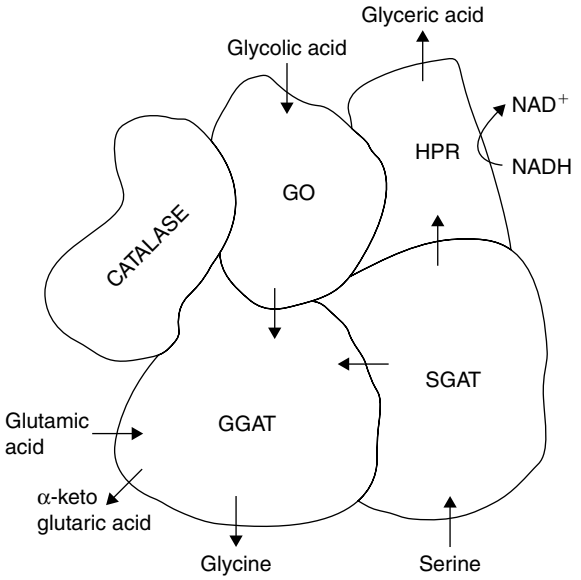


FIGURE 5.11 Schematic arrangement of photorespiratory enzymes involved in metabolite channeling. The active sites of many of the enzymes are blocked by protein–protein interactions. For example, glyoxylic acid can only get to the active site of GGAT by entering the complex at GO as glycolic acid, being converted to glyoxylic acid, and exiting GO as glyoxylic acid. In this way, glyoxylic acid is concentrated at the active site of GGAT, where its concentration will be close to the K_m of GGAT for glyoxylic acid. However, the average concentration of glyoxylic acid in the peroxisome will be low. Even with high concentrations of glyoxylic acid, the activity of GGAT will be low because the active site is not available. The active site becomes available following detergent treatment and the enzyme activity increases. GO, glycolate oxidase; GGAT, glutamate: glyoxylate aminotransferase; SGAT, serine:glyoxylate aminotransferase; HPR, hydroxypyruvate reductase.

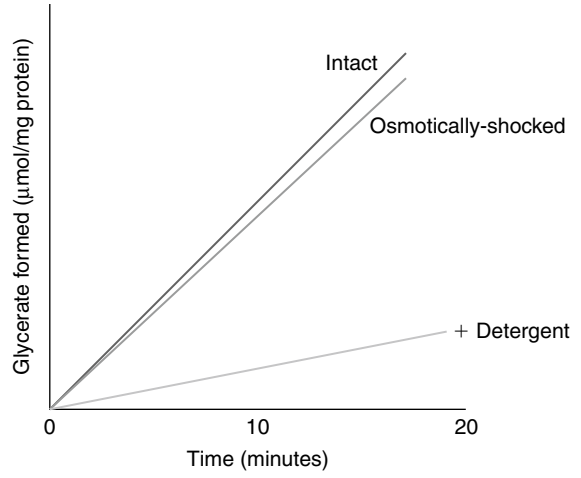


FIGURE 5.12 Time course of glycerate formation in intact, osmotically shocked, and detergent-treated peroxisomes.

of a diffusion-limited reaction will be 10,000 times faster if a substrate only has to diffuse 1 nm instead of 100 nm. If an enzymatic reaction is slow and not limited by diffusion, then metabolite channeling will not be helpful. However, if the enzyme reactions are limited by diffusion, metabolite channeling can be important. Consequently, the reduced rate of the metabolic pathway in detergent-treated peroxisomes is strong evidence for the importance of metabolite channeling by multienzyme complexes.

Interestingly, in intact or osmotically shocked peroxisomes, the intermediate, glyoxylic acid, is barely detectable ($<1\,\mu M$). However, in detergent-lysed peroxisomes, glyoxylic acid accumulates to high levels. This is because in detergent-lysed peroxisomes, the concentration of glyoxylic acid at the active site of the enzyme that converts glyoxylate to glycine (glutamate:glyoxylate aminotransferase) is too low to be transformed by the enzyme. In order for this enzyme to work, the concentration of glyoxylic acid must be approximately $150\,\mu M$ at the active site. Presumably, in intact or osmotically shocked peroxisomes, the concentration of glyoxylic acid at the active site is approximately $150\,\mu M$, and the enzyme actively converts glyoxylic acid to glycine. We are beginning to understand the relationships between local concentrations and enzyme affinities so that we can interpret the measured fluxes through biochemical pathways at the level of molecular dimensions (1–10 nm)[3].

We can deduce the presence and/or contribution of metabolite channeling through multienzyme complexes based on the following criteria:

1. The flux through an entire pathway is faster when the enzymes are in a complex rather than when they are separate.
2. In multienzyme complexes that depend on metabolite channeling, the local concentration of an intermediate may be very high while the overall concentration is low.
3. It should be possible to reconstitute a multienzyme complex from the component enzymes and regain the rapid flux due to metabolite channeling.

5.7 OTHER FUNCTIONS

The peroxisomes are multifunctional organelles that are capable of adapting to various cell types by adding or deleting enzymes involved in a variety of pathways (Baker and Graham, 2002). In animal cells, and perhaps some plant cells too, the peroxisomes are important in the catabolism of purines. The peroxisomes in nitrogen-fixing nodules may be specialized for ureide formation (Huang et al., 1983). In some fungi, they also participate in the biosynthesis of antibiotics. Using immunogold cytochemistry and cell fractionation, van den Bosch et al. (1992) have shown that the final enzyme involved in penicillin biosynthesis is localized in the peroxisomes of *Penicillium chrysogenum*. The peroxisomes of plants contain a Ca^{2+}-dependent nitric oxide synthase (Barroso et al., 1999; del Rio et al., 2002) and a sulfite oxidase (Eilers et al., 2001; Nakamura et al., 2002).

5.8 BIOGENESIS OF PEROXISOMES

When I was learning cell biology in the 1970s and 1980s, I was taught that peroxisomes are formed directly from the ER by a budding process. This was based on electron micrographs made by Novikoff and Shin (1964; see Figure 5.13). They interpreted these micrographs to reveal connections between the peroxisomes and the endoplasmic reticulum. Their interpretation was based on the work of Higashi and Peters (1963), which showed that newly synthesized catalase is found in the ER fraction. Gonzalez (1982) and Gonzalez and Beevers (1976) later obtained similar results in plants. However, with the introduction of in vitro translation techniques, something seemed amiss with the interpretation that the peroxisomes formed from the budding of the ER (Beevers, 1979; Tolbert, 1981; Kindl, 1982a,b; Vigil, 1983; Trelease, 1984; Lazarow and Fujiki, 1985). That is, if the peroxisomal proteins were synthesized on the ER, they should have a signal peptide, and thus the protein formed in vitro in the absence of microsomes should have a greater molecular mass than those synthesized in vivo. However, it was found that peroxisomal enzymes, including isocitrate lyase, glycolate oxidase, bifunctional enoyl-CoA hydratase/β-hydroxyacyl-CoA dehydrogenase, and catalase, are synthesized in vitro in a cell-free system at the same size that they are found in vivo (Frevert et al., 1980; Yamaguchi and Nishimura, 1984).

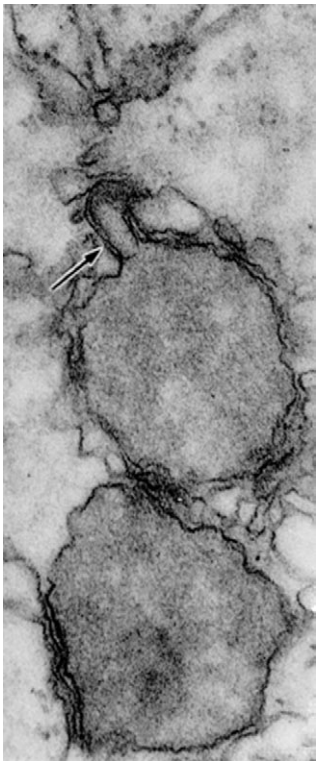


FIGURE 5.13 Microbody that appears to be continuous with the smooth endoplasmic reticulum (arrow). ×56,000. (*Source:* From Novikoff and Shin, 1964.)

The majority of the peroxisomal peptides is produced on cytosolic ribosomes and lack ER-specific signal peptides (Walk and Hoch, 1978; Riezman et al., 1980; Kruse et al., 1981; Lord and Roberts, 1982; Gietl, 1990). Thus, how can the peroxisomes be produced by budding off of the ER? They cannot! Then how can we reinterpret the data that support the budding hypothesis?

First, perhaps as a consequence of their ability to form multienzyme complexes, peroxisomal proteins form aggregates that artifactually cosediment with ER membranes (Kruse and Kindl, 1983). Second, serial sections of cells show that peroxisomes actually exist as a "peroxisomal reticulum" (Gorgas, 1984; Ferreira et al., 1989; see Figure 5.14) and the so-called attachments to the ER are interconnections between the peroxisomes themselves. At this point, it is worthwhile to remind ourselves that a single electron micrograph provides a static two-dimensional view of a dynamic three-dimensional cell. Thus, if we want to make three-dimensional interpretations, we should reconstruct images from serial sections, and if we wish to make dynamic interpretations, we should fix many cells at various points of time. The time interval between when we fix each sample should be at least half as long as the time resolution we would like to achieve in understanding the biological process in question. This sampling theorem is known as the Nyquist Theorem (Horowitz and Hill, 1989).

It is important to realize that while it is convenient to represent a three-dimensional structure in two dimensions, we must not think of a cell or organelle as being two dimensional. This is the lesson that Jacobius van't Hoff taught organic chemists in the past century when he introduced the field of stereochemistry to explain the mechanism of stereoisomerism. Indeed, Hermann Kolbe thought van't Hoff was crazy for introducing a three-dimensional aspect to chemicals, the structure of which could be written on a two-dimensional piece of paper. Kolbe wrote about his

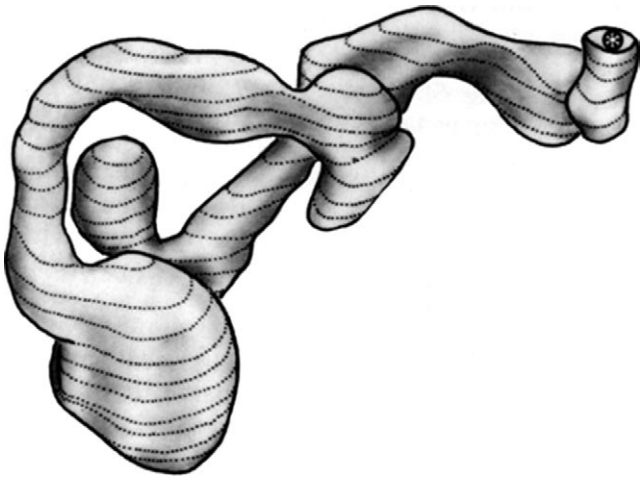


FIGURE 5.14 Three-dimensional reconstruction of a peroxisome from serial sections. (*Source:* From Gorgas, 1984.)

distaste for van't Hoff and his stereochemistry in no uncertain terms (van't Hoff, 1967)! We must remember that cells live in a three-dimensional world, and consequently, we must fight the temptation to look at the cell as an inhabitant of "Flatland" (Square, 1899).

If peroxisomes do not come from the ER, where do they come from? Lazarow and Fujiki (1985) and Lazarow (2003) propose that peroxisomes originate from preexisting peroxisomes. Peroxisome growth occurs by the incorporation of new protein and lipid into preexisting peroxisomes. Peroxisomes, like plastids and mitochondria, seem to increase in number by fission (Dinis and Mesquita, 1994) mediated by a dynamin-like protein (Koch et al., 2003; Li and Gould, 2003; Mano et al., 2004; see Chapters 13 and 14).

How do proteins get into the peroxisomes? As we will discuss in Chapter 17, the peroxisomal proteins do not have the ER signal peptide, but they do have another specific amino acid sequence that targets proteins to the peroxisome (Trelease et al., 1996; Mullen, 2002; Reumann, 2004). Isolated peroxisomes take up polypeptides that contain a peroxisomal targeting sequence. The peroxisomal targeting sequence is either SKL (ser-lys-leu) on the carboxy-terminal end of matrix proteins (Gould et al., 1990; Keller et al., 1991) or arg-leu/gln/ile-X5-his-leu on the amino-terminus (Gietl, 1990). This indicates that there may be multiple translocator pathways (Olsen and Harada, 1995). Interestingly, the targeting sequence for isocitrate lyase and malate synthase is the same for both peroxisomes and glyoxysomes, indicating that the functions of these organelles are not determined by protein targeting but by the synthesis of their constituent proteins (Olsen et al., 1993).

Once some peroxisomal proteins bind to a receptor (Wolins and Donaldson, 1994), their import is enhanced by chaperonins and requires energy in the form of ATP (Imanaka et al., 1987; Presig-Müller et al., 1994). Interestingly, some polypeptides, which do not contain any targeting sequences, are brought into the peroxisome as oligomers (Lee et al., 1997; Flynn et al., 1998; Kato et al., 1999). In fact, proteins containing the SKL targeting sequence will even bring 4- to 9-nm gold particles into the peroxisome (Walton et al., 1995). The proteins that function in the transport of proteins across the peroxisomal membrane have been given the name peroxins (Pool et al., 1998a,b; Tugal et al., 1999; Lopez-Huertas et al., 1999; Nito et al., 2007). It seems that a protein translocator in the peroxisomal membrane, unlike those in the chloroplast or mitochondrial membranes (see Chapters 13, 14, and 17), can transport proteins in their native form. This indicates that a peroxisomal protein translocator has a relatively large aqueous pore. Such a pore is possible in a membrane, like the peroxisomal membrane that does not maintain an electrical membrane potential difference. Such a large ungated pore would be incompatible in a membrane that must maintain an electrical potential difference.

Just when the "independent growth and division" model of peroxisome biogenesis seemed to win over the "ER-vesiculation" model, Robert Mullen (2002) and coworkers (Mullen et al., 1999, 2002; Mullen and Trelease, 2000; Lisenbee et al., 2003; Titorenko and Mullen, 2006) discovered that ascorbate peroxidase, a peroxisomal membrane protein, is posttranslationally inserted into the ER. This step requires ATP and chaperonins. The protein is localized in a distinct region of the ER. Inhibition of ER vesicle blebbing by brefeldin A prevents the movement of this protein into peroxisomes. Thus, it seems that some of the cytosolically synthesized proteins required for peroxisome biogenesis enter the peroxisome directly, while others, including ascorbate peroxidase, and a peroxin (Hoepfner et al., 2005), enter the peroxisome indirectly by means of specialized ER-derived vesicles. More and more data are being amassed that show a precursor-product relationship for peroxisomal membrane proteins beginning in the ER, suggesting that the peroxisome, like the other endomembranes, is a derived organelle, synthesized from the ER (Titorenko and Rachubinski, 1998; Hoepfner et al., 2005; Kunau, 2005; Schekman, 2005; Titorenko and Mullen, 2006). The conclusion that peroxisomes are derived from the ER is supported by the discovery that yeast mutants that are defective in their ability to transport secretory proteins from the ER do not produce visible peroxisomes.

There may be some truth to both the ER-vesiculation model and the independent growth and division model. It is always wise to know the history of a subject, because a synthesis of that subject requires putting together the thesis and the antithesis. Usually the accepted theory at any given time (the thesis) is only partly true, and the unaccepted theory at that time (the antithesis) is only partly false. Interestingly enough, the thesis of one generation often becomes the antithesis of the next. In all cases, the synthesis comes from combining the truth from both theories. At the risk of sounding too skeptical, a full resolution of the peroxisome controversy will have to await identification of the source or sources of all the peroxisomal proteins and a conformation or a refutation of the existence of a non-ER, vital peroxisomal element that can exist as a protoperoxisome (Lazarow, 2003).

Peroxisomes have a very limited capacity to synthesize lipids (Ballas et al., 1984; Chapman and Trelease, 1991a), so how do they get their lipids (Raychaudhuri and Prinz, 2008)? While some lipids may come directly from the ER membranes that vesiculate to form peroxisomes (Titorenko and Mullen, 2006), phospholipid exchange proteins (Dowhan, 1991; Cleves et al., 1991) may also be responsible for delivering lipids to the peroxisomal membrane. The exchange protein can extract a phospholipid from the ER membrane and then bury the phospholipid inside itself. Phospholipid exchange proteins are water-soluble proteins that diffuse through the cytoplasm until they bump into another membrane. Then they release the phospholipids into the new bilayer. It is thought that exchange proteins randomly distribute phospholipids throughout the cytoplasmic membranes

in a Robin Hood–like manner. Such a random exchange will lead to the net transfer of phospholipids from the phospholipid-rich ER (or lipid bodies) to the phospholipid-poor membranes (Abdelkader, 1973; Tanaka and Yamada, 1979; Crain and Zilversmit, 1980; Yaffe and Kennedy, 1983; Dawidowicz, 1987; Bishop and Bell, 1988; Chapman and Trelease, 1991b). It is also possible that some of the lipids come from the ER already assembled into the peroxisomal membrane (Titorenko and Mullen, 2006).

5.9 EVOLUTION OF PEROXISOMES

Peroxisomes are present in all eukaryotes except the *Archaezoa* (Cavalier-Smith, 1987) and may have evolved endosymbiotically (see Chapter 15; de Duve, 1991). Alternatively, peroxisomes, like the ER, may have evolved from invaginations of regions of other membranes that contained the enzymes involved in what are now considered peroxisomal pathways (de Duve, 1969; Hoepfner et al., 2005). There are similarities between the functions of peroxisomes and mitochondria. Both organelles are capable of using O_2. In general, it seems that the mitochondria have taken over the functions of the peroxisomes. In some cases, however, it seems like the peroxisomes have taken over the functions of the mitochondria.

The peroxisomes in the cells of various phyla exhibit both morphological and biochemical diversity. Perhaps the range of innovations possible for peroxisomes is best seen in the green algae. In this class, which gave rise to the higher plants, there is evidence that generally throughout evolution, the peroxisome has become more important as an organelle by taking over some of the functions of the mitochondrion (Stabenau, 1992). Of course, evolution is not linear and there is also evidence that in some phyla the peroxisomes have become a more vestigial organelle, giving much of its biochemical capacity back to the mitochondria (de Duve, 1969; Stabenau, 1992). We must keep in mind that cells are dynamic not only over cellular time scales, but throughout geological time scales. Taking this into consideration, cytologically oriented systematists have used the presence of glycolate oxidase in the peroxisome or glycolate dehydrogenase in the mitochondria as characters used for classification purposes in the green algae (Stewart and Mattox, 1975; Betsche et al., 1992).

5.10 SUMMARY

We have learned about the structure and function of peroxisomes, and that these organelles are multifunctional. In some cases, their function varies from cell to cell within an organism or temporally within a cell. Peroxisomes are not static organelles, but appear to have changed during the evolution of plants and animals. We have also learned about the concept of metabolite channeling, the importance of serial sectioning in interpreting electron micrographs, and the fact that a synthesis may require putting together the truths of the accepted and unaccepted theories.

5.11 QUESTIONS

5.1. How do the functions of peroxisomes change throughout the life of a cell?
5.2. What is metabolic channeling and what are its advantages?
5.3. What is the evidence that peroxisomes are autonomous organelles, and what is the evidence that they are derived from the ER?

Chapter 6

Golgi Apparatus

6.1 DISCOVERY AND STRUCTURE OF THE GOLGI APPARATUS

In the late 19th century, cytologists began to see that the cytoplasm was not homogeneous, but contained previously unknown and invisible internal structures or "formed elements" of which the identity could be recognized by their characteristic staining patterns. In 1898, Camillo Golgi visualized a netlike reticulum of fibrils in the Purkinje cells of the owl, *Strix flammea* (Figure 6.1). He could see this *internal reticular apparatus*, as he called it, because it reduced silver and thus became blackened and visible against the rest of the cytoplasm. The silver- or osmium-stained internal reticular apparatus was dubbed the *Golgi-Holmgren canals* by Santiago Ramón y Cajal (1937), and was later redubbed the *Golgi apparatus*.

In vertebrates, the Golgi apparatus usually appears morphologically as a fibrous network, while in invertebrates and plants it appears as separate elements. According to Kirkman and Severing (1938),

> *The Golgi apparatus appears to be the most protean of all cytoplasmic structures—it has been described as a fibrous reticulum, network, ring or cylinder, a very irregular fenestrated plate, a more or less incomplete hollow sphere, vesicle, or cup, a collection of small spheres, rodlets and platelets or discs, a series of anastomosing canals, a group of vacuoles, and a differentiated region of homogeneous cytoplasm crossed by irregular interfaces.*

As a consequence of its polymorphous appearance as well as its change in chemical composition and stainability throughout the life cycle of a cell, the Golgi apparatus has been given many names, including the paraflagellar apparatus, the dictyosomes, the canaliculi of Holmgren, the fluid canaliculi, the trophospongium, and the osmiophilic platelets. However, Bowen (1926), Duboscq and Grassé (1933), Wilson and Pollister (1937), and many others showed the morphological and/or functional homology between these organelles and suggested that they all be called the Golgi apparatus.

In an attempt to determine the homologies between the newly discovered organelles of plant and animal cells, Robert Bowen (1928) began to characterize cytologically the osmiophilic platelets in a variety of plant cells, including the root-tip cells of barley and bean. He observed ring-like or disc-shaped structures that blackened selectively with osmic acid, and tentatively concluded that these structures were homologous with the Golgi apparatus of animal cells (Figure 6.2). However, he cautioned that this identity rested only on morphological grounds and staining characteristics, and that it would be important to test whether the osmiophilic discs had the same secretory function as the Golgi apparatus in animal cells. Since the staining reactions for the Golgi apparatus were selective, but not specific, interpretations on the reality of the Golgi apparatus based on staining remained controversial up through the 1960s (Guilliermond, 1941; Bourne, 1942, 1951, 1962, 1964; Worley, 1946; Palade and Claude, 1949; Bensley, 1951; Baker, 1957; Dalton, 1961; Beams and Kessel, 1968; Buvat, 1969).

In the mid-1950s, the Golgi apparatus in animal cells was shown with the electron microscope to be a real membranous structure with a distinct architecture and not just a chemical substance. This provided a means to distinguish unequivocally the Golgi apparatus from all the other cellular organelles (Sjöstrand and Hanzon, 1954; Paley and

FIGURE 6.1 The internal reticular apparatus of Purkinje cells of an owl. (*Source:* From Golgi, 1898.)

Palade, 1955; Dalton and Felix, 1956). The Golgi apparatus in plant cells was observed with the electron microscope by Hodge and coworkers in 1956, although they did not recognize it as such. They called it the *cytoplasmic lamellae*, and proposed that the cytoplasmic lamellae participated in the formation of endoplasmic reticulum (ER) since the cytoplasmic lamellae looked like membrane factories where vesicle fusion was taking place. Despite the strong and persistent claims of the plant cytologist Alexandre Guilliermond (1941) that the Golgi apparatus did not exist in plants, Keith Porter (1957), E. Perner (1957), and Roger Buvat (1957) demonstrated the reality of the Golgi apparatus with the electron microscope and concluded once and for all that plant cells, like animal cells, have a Golgi apparatus. Indeed, in optically favorable material like the *Chara* rhizoid, it is possible to unequivocally identify the Golgi apparatus in living plant cells (Bartnik and Sievers, 1988) as it is in living animal cells (Brice et al., 1946; Oettlé, 1948). The Golgi apparatus can be easily visualized in living plant cells that have been transformed with fluorescent proteins fused to a resident Golgi apparatus protein (Boevink et al., 1998; Nebenführ et al., 1999, 2000; Brandizzi et al., 2002a,b; Saint-Jore et al., 2002; Neumann et al., 2003; daSilva et al., 2004; Zheng et al., 2004).

Perroncito (1910) noticed the Golgi apparatus split up into a number of elongated pieces during cell division and named each piece the *dictyosome*. Nowadays, each separate stack of Golgi membranes is called a Golgi stack or dictyosome (Mollenhauer and Morré, 1966). For the sake of uniformity among animal and plant cell biologists, I will refer to each separate stack as a Golgi stack as opposed to referring to it as a dictyosome. Although there can be between 0 and 25,000 Golgi stacks per cell (Rosen, 1968), there are typically hundreds (Satiat-Jeunemaitre and Hawes, 1994; Nebenführ et al., 1999). Golgi stacks are particularly abundant in secretory cells in plants as they are in animals (Bowen, 1926, 1927, 1929) and may be absent in dry seeds (Fahn, 1979).

The architecture of the Golgi apparatus, which consists of all the Golgi stacks in the cell, varies from cell to cell and throughout the life of the cell (Whaley et al., 1959, 1960; Manton, 1960; Bonneville and Voeller, 1963; Noguchi and Kakami, 1999). While the Golgi stacks may seem to be separate, electron microscopy of thick (1 μm) sections shows that the stacks may also be connected together in a three-dimensional Golgi reticulum (Rambourg and Clermont, 1990; Figure 6.3). The Golgi apparatus is thus differentiated into a compact zone, called the *traditional Golgi stack*, and a noncompact zone that connects the stacks. Such a noncompact zone can be called the *cis-Golgi network* when it is associated with the forming face of the Golgi stack, and the *trans-Golgi network* when it is associated with the maturing face. The Golgi apparatus may contain more or less differentiated cis- or trans-Golgi networks (Figure 6.4).

In systematics and evolution, we learn over and over that nature mocks human categories (Bergson, 1911). In the systematics of organelles, we learn the same lesson again. That is, while the Golgi stack can be unambiguously identified, the identities of the membranes associated with the outskirts of the Golgi stacks are less certain. The associated

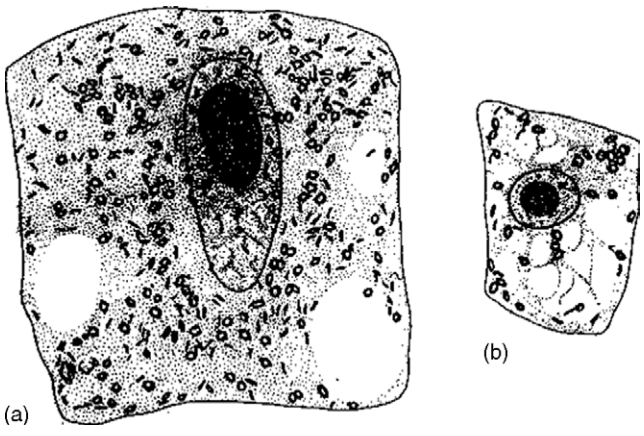


FIGURE 6.2 The osmiophilic platelets in (a) a large cell from the central core of a barley root tip and (b) a cell from the root tip of a kidney bean. (*Source:* From Bowen, 1927.)

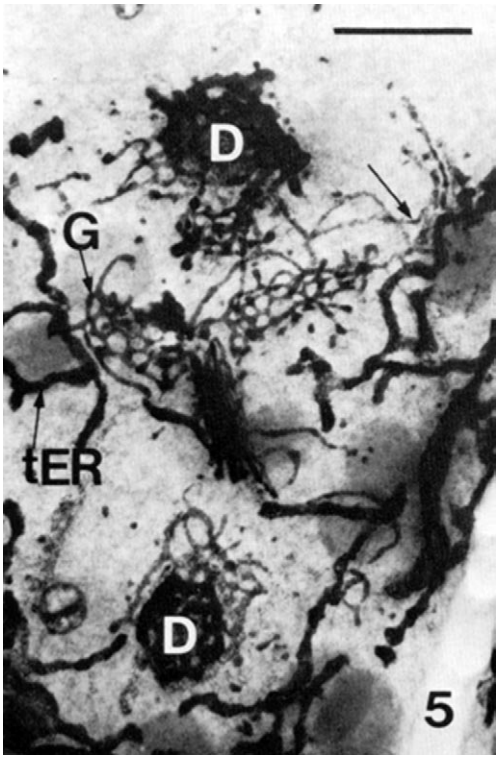


FIGURE 6.3 Electron micrograph of a vascular parenchyma cell of mung bean stained with zinc iodide-osmium tetroxide showing the tubular connections between the cisterna (D) of a Golgi stack and the tubular endoplasmic reticulum (tER). The fine tubules nearest the Golgi stack are approximately 10- to 20-nm thick. Bar, 500 nm. (*Source:* From Harris and Oparka, 1983.)

membranes have been given a variety of names, including the *cis-Golgi network* (CGN), the trans-Golgi network (TGN), the *Golgi-ER-lysosomal* continuum (GERL), the prevacuolar compartment (PVC), the *ER-Golgi intermediate compartment* (ERGIC), the *partially coated reticulum* (PCR), and the *vesicular-tubular cluster* (VTC). The degree of membrane elaboration probably reflects the developmental and/or functional state of the cell, and perhaps even the taxon to which the organism belongs (Robinson, 2003). In any case, the reification of the organellar status of these membranes is at the same stage that the reality of the currently accepted organelles was in the past. After techniques are developed to unambiguously identify these membranes morphologically and cytochemically, and their specific functions are shown in cell-free systems following cell fractionation, a consensus on their individuality and uniqueness will be reached.

Each Golgi stack is a flattened disc about 1 μm in diameter and 0.25 μm long. A Golgi stack typically consists of a stack of 4–7 flattened membranes or cisternae, although more than 20 cisternae may be present (Mollenhauer et al., 1983; Kiss et al., 1990; Staehelin et al., 1990; Mollenhauer et al., 1991; Zhang and Staehelin, 1992). Each cisterna is separated from the others in the stack by a minimal space of 10–15 nm. Parallel fibers, called *intercisternal elements*, about 3–6 nm in diameter, exist between the cisternae (Mollenhauer, 1965; Turner and Whaley, 1965). The cytosolic region that surrounds the Golgi stack and the trans-Golgi network is devoid of ribosomes. Staehelin and Moore (1995) call this structurally specialized region the *Golgi matrix*. Application of brefeldin A to cells causes the resorption of the Golgi stacks into the endoplasmic reticulum, and removal of brefeldin A results in the regeneration of well-defined Golgi stacks within 90 minutes (Langhans et al., 2007).

Not all Golgi apparati have their cisternae in stacks. The Golgi apparatus in the red alga, *Cyanidioschyzon merolae*, consists of only one or two cisternae (Okuwaki et al., 1996). Likewise, in the cells of many filamentous fungi, the Golgi apparatus appears as a single tubule known as a *Golgi equivalent* (Hoch and Staples, 1983; Roberson and Fuller, 1988; Bourett and Howard, 1996). The Golgi equivalents are clearly homologous with the Golgi apparatus since they are associated with secretory vesicles. The Golgi apparatus of the budding yeast *Pichia* consists of four cisternae (Mogelsvang et al., 2003).

6.2 POLARITY OF THE GOLGI STACK

The Golgi stack is an organelle that exhibits polarity (Shannon et al., 1982). It has two distinct faces: the forming or cis-face and the maturing or trans-face (Figures 6.5 and 6.6). The cis-face is often, but not always, associated with the transition elements of the ER, or in many algae, the

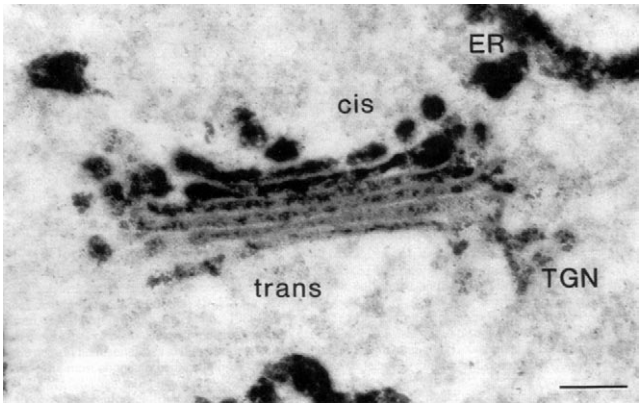


FIGURE 6.5 Electron micrograph of a Golgi stack in a cortical cell of a clover root stained with zinc iodide–osmium. TGN, trans-Golgi network. Bar, 100 nm. (*Source:* From Moore et al., 1991.)

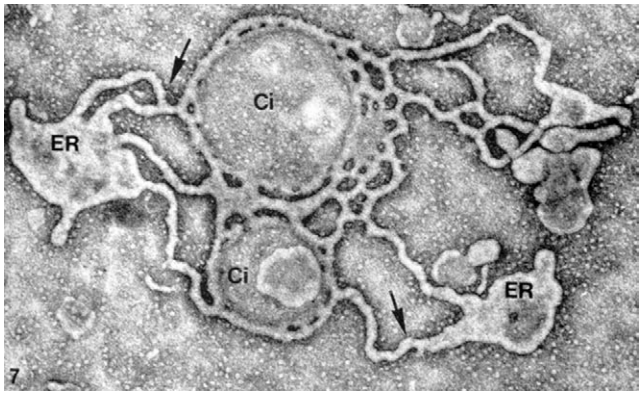


FIGURE 6.4 Electron micrograph of a negatively stained Golgi stack isolated from root-tip cells. The cisternae (Ci) are interconnected with each other and with the ER. ×55,000. (*Source:* From Mollenhauer and Morré, 1976b.)

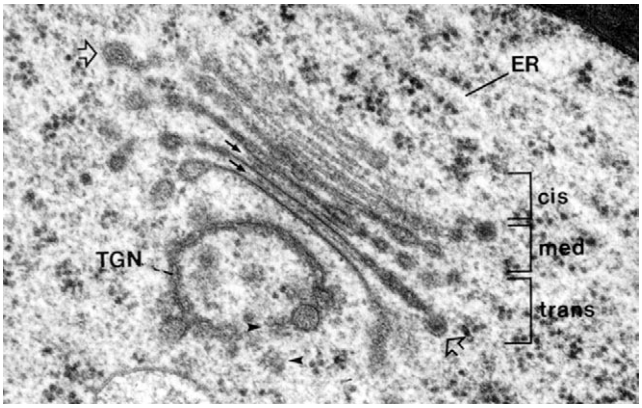


FIGURE 6.6 Electron micrograph of a Golgi stack in a columella cell of tobacco. Note the lightly stained *cis*-cisternae with wide lumena; the medial-cisternae contain darkly staining contents; and the *trans*-cisternae have tightly appressed membranes and very thin lumena. ×65,000. (*Source:* From Staehelin et al., 1990.)

nuclear envelope (Massalski and Leedale, 1969; Mollenhauer and Morré, 1976b; Robinson, 1980; Shannon et al., 1982). The cis-face is composed of many fenestrations that can be seen clearly in zinc iodide–osmium tetroxide–fixed cells (Dauwaulder and Whaley, 1973). The fenestrations are about 50 nm in diameter. Small vesicles 50 nm in diameter appear between the transition elements of the ER and the cis-face of the Golgi stack. It appears as if these vesicles bleb off from the transition ER and fuse with the Golgi apparatus. The fenestrations in the cis-face of the Golgi apparatus may represent the fusion of these vesicles with the cisterna on the cis-face. Plants transformed with green fluorescent protein (GFP) reveal the close and dynamic association between the ER and the cis-face of the Golgi apparatus (Boevink et al., 1998, 1999; Batoko et al., 2000). Tubular connection may also connect the ER, the cis-Golgi network, and the Golgi stack (Mollenhauer and Morré, 1976).

Cytohistological staining gives further evidence of polarity. For example, under certain conditions, osmium tetroxide–zinc iodide precipitates heavily in the lumen of the ER and the cis-face of a Golgi stack, but only minimally in the trans-face of the Golgi stack (Dauwalder and Whaley, 1973). Thus, Bowen (1928) probably observed the forming face of the Golgi in the light microscope. The cisternae of the Golgi stack also show different degrees of staining from the cis-side to the trans-side in terms of the cytohistological localization of various enzymes. Thiamine pyrophosphatase (TPPase), ITPase (inositol triphosphatase), and ATPase are localized on the trans-face, while CMPase, NADPase, and β-glycerolphosphatase are localized in vesicles emerging from the center of the trans-face (Dauwaulder and Whaley, 1973; Domozych, 1989; Rambourg and Clermont, 1990; Staehelin et al., 1990). GFP fused to α-1,2 mannosidase typically shows up in the cis-face of the Golgi stack (Nebenführ et al., 1999).

Another sign of polarity that is visible in conventional electron micrographs is that the cisternae become flatter from the cis-face to the trans-face. The flatness of the cisternae may depend on the intercisternal fibers since the number of these fibers increases from the cis-face to the trans-face (Turner and Whaley, 1965; Mollenhauer, 1965; Hawkins, 1974; Alley and Scott, 1977; Kristen, 1978). The membranes of the cisternae also increase in thickness from 5.6–6.4 nm at the cis-face to 6.4–9.1 nm at the trans-face (Morré and Mollenhauer, 1976).

The Golgi stack maintains its polarity while membranes are continually flowing through it (Pelham, 2001; Pelham and Rothman, 2000; Beznoussenko and Mironov, 2002; Marsh and Howell, 2002; Storrie and Nilsson, 2002; Nebenführ, 2003). In some cases, discussed below, it is thought that whole cisternae move through the Golgi stack, and thus the membranous and lumenal components of each cisterna show a temporal pattern of polarity (Mogelsvang et al., 2003). However, there is also evidence that each cisterna maintains its position in the stack and transfers membranes and contents either by making direct membranous contacts between adjacent cisternae via membrane tubulization, or by a process that involves vesicle blebbing and fusing (Morré and Keenan, 1994, 1997; Staehelin and Moore, 1995). In the latter two cases, the polarity would be exclusively spatial. It is not currently possible to unequivocally determine which model is correct without doing time-resolved, three-dimensional studies.

Once the membranous or lumenal components reach the trans-face of the Golgi stack, they become part of a tubular reticulum called the *trans-Golgi network*, where they eventually bleb off for the last time and go to various destinations, including the plasma membrane and the vacuolar compartment (Grove et al., 1970; Dauwalder et al., 1969). There is evidence that not all traffic through a Golgi stack is anterograde from the cis-face to the trans-face. Consistent with the original proposal by Hodge et al. (1956) there is also retrograde movement from the trans-face to the cis-face (see discussion in Chapter 8). While in growing plant cells, it is likely that there is a net movement of membranous and lumenal components from the cis-face through the central or medial region to the trans-face. In stationary state cells, the movement in opposite directions throughout the endomembrane system must be balanced.

Four kinds of proteinaceous coats are associated with the Golgi apparatus of plants, and it is thought that these coat proteins facilitate the blebbing and fusion of membranes. Coat protein, or Coatamers (COP I and COP II), appear predominantly on the transition elements of the ER, the transition vesicles, and on the cis- and medial-cisternae of the Golgi stacks. COP II is involved in the transfer of vesicles from the ER to the cis-Golgi complex and/or the cis-Golgi, while COP I is involved in the transfer of vesicles from the cis-Golgi and/or the cis-Golgi complex to the ER, as well as from one Golgi cisterna to another in either direction (Pelham, 1994; Schekman and Orci, 1996; Pimpl et al., 2000; Philipson et al., 2001; Robinson et al., 2007: Kang and Staehelin, 2008).

On the trans-Golgi network, buds are coated with either a clathrin coat or a lacelike coat. It is thought that the clathrin-coated vesicles originating from the trans-Golgi network are targeted to become part of the vacuolar compartment, while the lacelike-coated vesicles from the trans-Golgi network are destined to go to the plasma membrane.

6.3 ISOLATION OF THE GOLGI APPARATUS

Intact Golgi stacks can be isolated from plant cells by homogenizing the tissue in the presence of a stabilizing agent like 0.3 percent glutaraldehyde. The homogenate is filtered to remove the extracellular matrix and then centrifuged at low speed (100 g for 10 minutes) to remove the nuclei and plastids. The supernatant is recentrifuged at high speed (100,000 g

for 60 minutes) to concentrate the microsomal membranes, which are then resuspended and layered on a discontinuous sucrose density gradient. After centrifugation at 100,000 g for 60 minutes, the Golgi are found at the 1.03- to 1.077 M sucrose interface, which is equivalent to a density between 1.132 and 1.138 g/mL (Green, 1983). Thus, the density of the Golgi membranes is intermediate between the density of the ribosomeless membranes of the ER and the density of the plasma membrane. D. James Morré (1987) has developed a technique that uses free-flow electrophoresis to separate the isolated Golgi stacks into cis-, medial-, and trans-fractions. The Golgi-derived vesicles can also be isolated (van Der Woude et al., 1971; Hasegawa et al., 1998).

6.4 COMPOSITION OF THE GOLGI APPARATUS

The lipids of the Golgi apparati isolated from soybean stems and rat liver cells are similar. Interestingly, the relative lipid composition of the Golgi membranes is intermediate between that of the ER and that of the plasma membrane (Keenan and Morré, 1970; Morré and Ovtracht, 1977). Soybean Golgi contains approximately (percent of total membrane phosphorous) 30 percent phosphatidylcholine, 20 percent phosphatidylethanolamine, 10 percent phosphatidylinositol, and 1 percent phosphatidylserine.

The marker enzymes for the Golgi apparatus include latent inosine diphosphatase (IDPase) and a number of glycosyl transferases, including glucan synthase I and UDPG: sterol glucosyl transferase (Green, 1983). Nucleotide monophosphatase and diphosphatase have also been isolated from Golgi membranes (Staehelin and Moore, 1995; Gupta and Sharma, 1996). David Gibeaut and Nicholas Capita (1993, 1994) have succeeded in getting isolated Golgi apparati to synthesize natural polysaccharides.

6.5 FUNCTION OF THE GOLGI APPARATUS

While the debate on the reality of the Golgi apparatus was going on, it was surmised by believers that the Golgi apparatus was involved in secretion since, of all the cells studied, it was most highly developed in gland cells (Bowen, 1926, 1927, 1929). In plant and animal cells, the Golgi apparatus is involved in the processing of secretory as well as other glycoproteins. In plant cells, the Golgi apparatus also participates in the secretion of a variety of extracellular materials, including fucose-rich mucilage by root cap cells, wall-degrading enzymes during abscission, hydrolases that degrade the food reserves during germination, and digestive enzymes in insectivorous plants (Northcote and Pickett-Heaps, 1966; Dauwalder and Whaley, 1973; Sexton and Hall, 1974; Sexton et al., 1977; Robinson, 1980; Cornejo et al., 1988; Jones and Robinson, 1989; Roy and Vian, 1991). The Golgi apparatus is also involved in an internal secretory pathway that ends in the vacuolar compartment (see Chapter 8).

6.5.1 Processing of Glycoproteins

Northcote and Pickett-Heaps (1966) discovered, in radioautographic studies, that the Golgi apparatus is the principal site of glucose incorporation in root cells. In plants, 80 percent of the glycosylation reactions result in the biosynthesis of complex polysaccharides and 20 percent are involved with processing glycoproteins (Driouich et al., 1993a). The glycoproteins begin as polypeptides synthesized on the rough ER. There, oligosaccharides, containing 14 sugar residues, are attached to the amino groups of certain asparagine residues. Subsequently, one mannose and three glucose residues are removed by glucosidases I and II and mannosidase, leaving a high-mannose glycoprotein (see Figure 4.17 in Chapter 4). Once the glycoprotein reaches the Golgi apparatus, the oligosaccharides may be further processed by various glycosylases to form complex glycoproteins. Glycosylations that take place in the lumen of the ER or in the Golgi apparatus result in soluble glycosylated proteins that reside in the E-space or membrane proteins that are glycosylated on the regions exposed to the E-side.

A mutant of *Arabidopsis* (cge), which lacks N-acetyl glucosaminyl transferase I in the Golgi apparatus, is unable to process the N-linked glycoproteins (von Schaewen et al., 1993). When this mutant is transformed with a human cDNA that encodes this enzyme, the transformed mutant plant is capable of processing the N-linked glycoproteins (Gomez and Chrispeels, 1994). Interestingly, the mutants are identical to the wild-type plants, indicating that glycosylation of plant proteins may not be functional but fortuitous just because they pass through the Golgi apparatus (von Schaewen et al., 1993). While the function of some glycosylation reactions are understood (Höftberger et al., 1995), the functions of many remain a mystery!

In the Golgi apparatus, some sugars are also attached to the hydroxyl (OH) groups of serine or threonine. This is called O-linked glycosylation and is also catalyzed by glycosyl transferases. The O-linked glycosylation reactions necessary to form the arabinogalactan-rich proteins found in the extracellular matrix take place in the Golgi apparatus (Showalter, 1993).

6.5.2 Synthesis of Carbohydrates

In plants, the major secretory products of almost all cells are complex polysaccharides, including pectins and hemicelluloses, that are secreted into the extracellular matrix (Conrad et al., 1982; Crosthwaite et al., 1994). The Golgi apparati of plant cells secrete complex polysaccharides during cell plate formation (Hepler and Newcomb, 1967; Northcote

et al., 1989; Rancour et al., 2002), and once the cell plate is completed, the Golgi-derived vesicles continue supplying material to the extracellular matrix of the primary- and secondary-cell walls. Not only do the vesicles supply complex polysaccharides, but they may also contain glycoproteins as well as enzymes involved in wall formation and/or loosening (Schnepf, 1969a; Ray et al., 1976; Robinson et al., 1976a; Fry, 1986; Haigler and Brown, 1986; Moore and Staehelin, 1988; Moore et al., 1991; Paredez et al., 2006). Likewise, proteoglycans that are destined to reside in the extracellular matrix of animal cells are synthesized in the Golgi apparatus.

The complex polysaccharides found in the extracellular matrix of plant cells are often branched and are composed of more than 12 different monosaccharides, indicating that many enzymes, perhaps several hundred glycosyl transferases, may be necessary for their synthesis (Tezuka et al., 1992; Gibeaut and Carpita, 1993). Each enzyme may be capable of attaching a specific sugar at a specific position to make a given bond type. These enzymes are differentially localized within the Golgi apparatus and the Golgi-derived vesicles as shown by immunocytohistochemistry at the electron microscopic level (Brummel et al., 1990; Moore et al., 1991; Zhang and Staehelin, 1992; Fitchette-Lainé et al., 1994).

Why are the glycosylation enzymes differentially localized within a Golgi stack? Moore et al. (1991) have surmised that there are two ways of having error-free synthesis of complex carbohydrates in the Golgi apparatus. That is, if the enzymes involved in the synthesis of each complex polysaccharide were extremely specific, then there would not be a need for these enzymes to be localized in any special manner. Alternatively, if the enzymes involved in the synthesis of complex carbohydrates were less specific, then specific complex carbohydrates could be synthesized by segregating the glycosylation enzymes into separate cisternae of the Golgi apparatus or into separate Golgi stacks. Moore et al. (1991), using immunocytochemistry, find that the synthesis of different complex carbohydrates occurs in different cisternae of a Golgi stack. Using antibodies that recognize various epitopes of certain polysaccharides, Zhang and Staehelin (1992) have shown which cisternae are involved in putting specific sugars on glycoproteins, xyloglucans, and rhamnogalacturonans in suspension cells (see Chapter 20).

The synthesis of hemicelluloses, including xyloglucans, takes place in the Golgi apparatus (Zhang and Staehelin, 1992; Lynch and Staehelin, 1992; Staehelin et al., 1992). Many of the enzymes required for hemicellulose synthesis, including glucosyl, xylosyl, fucosyl, and arabinosyl transferases, have been localized in the Golgi apparatus (Ray et al., 1969; Gardiner and Chrispeels, 1975; Green and Northcote, 1978; James and Jones, 1979; Ray, 1980; Hayashi and Matsuda, 1981; Camirand et al., 1987). Using a battery of monoclonal antibodies, including Anti-XG, which recognizes the β-1,4-linked glucosyl backbone of xyloglucan, and CCRC-M1, which recognizes the terminal fucosyl residue of the trisaccharide side chain of xyloglucan, it was determined that the synthesis and modification of xyloglucans take place exclusively in the trans-Golgi cisternae and the TGN (Staehelin et al., 1992; Zhang and Staehelin, 1992).

By contrast, the backbone of pectins is initiated in the cis-Golgi cisternae and extended, and methyl-esterified in the medial-Golgi cisternae, and the side chains are added in the trans-Golgi cisternae. This spatial localization is inferred from observations that monoclonal antibodies like PGA/RG-I, which recognizes the esterified PGA/RG-I transition region, stains the cis-Golgi cisternae; JIM 7, which recognizes methylesterified PGA, stains the medial-Golgi cisternae; and CCRC-M2 and CCRC-M7, which recognize the side chains of RG-I, stains the trans-Golgi cisternae and the trans-Golgi network (Zhang and Staehelin, 1992; Staehelin et al., 1992).

The pathway of movement through a Golgi stack associated with the synthesis and packaging of various complex polysaccharides appears to differ depending on the polysaccharide. Consistent with this observation, monensin, an Na^+/H^+ ionophore that inhibits Golgi sorting at or near the trans-face (Boss et al., 1984), inhibits the movement of xyloglucan but not pectins through the Golgi apparatus.

6.5.3 Transport of Sugars

All sugars must be activated before they are reactive enough to participate in the glycosylation reactions. They become activated after becoming bound to nucleotide diphosphates in the cytosol. Since these are relatively large polar molecules, the membranes of the Golgi apparatus must contain the transporters (dolichols or proteins) to transfer the nucleotide-activated sugars into the lumen (Chanson et al., 1984; Ali and Akazawa, 1985; Ali et al., 1985; Chanson and Taiz, 1985; Gogarten-Boekels et al., 1988).

6.6 THE MECHANISM OF MOVEMENT FROM CISTERNA TO CISTERNA

Perhaps the production of scales in the alga *Pleurochrysis* is an ideal system to visualize a specific type of cisterna-to-cisterna movement through the Golgi apparatus (see Figures 6.7–6.11; Brown, 1969; Brown et al., 1979). This alga is covered by distinctive scales that are composed of cellulose microfibrils and pectins. The formation and secretion of these scales can be followed through the maturing Golgi cisternae, where they begin formation in a dilated polymerization center and end as a secreted exocytotic vesicle. The scales have an identical structure within the last cisternae as they do in the extracellular matrix. These observations indicate that in the case of *Pleurochrysis*, the individual cisterna move through the stack from the cis-face to the trans-face.

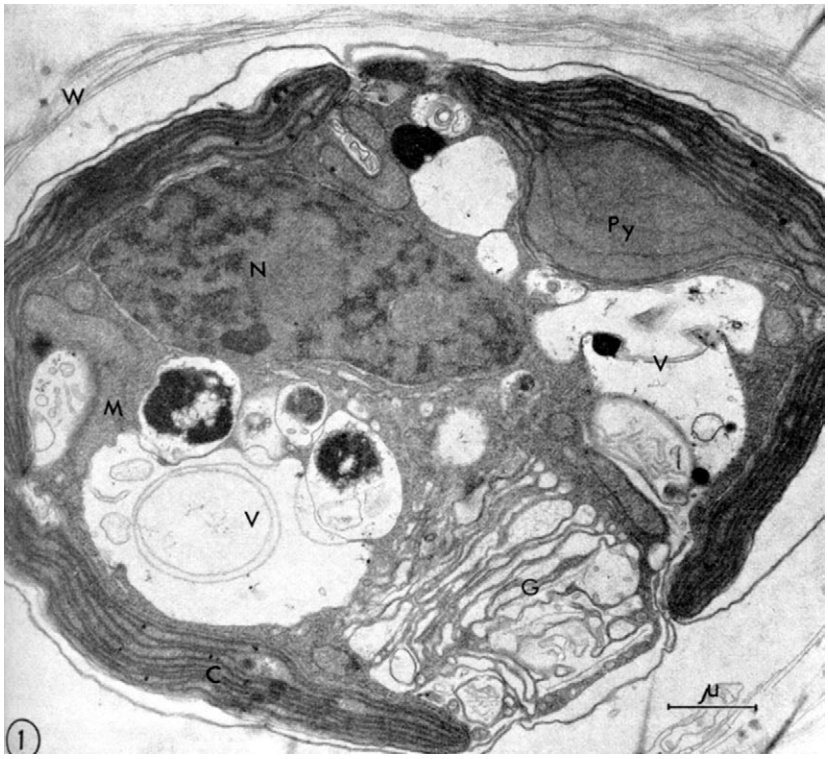


FIGURE 6.7 Electron micrograph of *Pleurochrysis scherffelii* showing a prominant Golgi stack, G. V, vacuole; N, nucleus; Py, pyrenoid; W, extracellular matrix. (*Source:* From Brown et al., 1970.)

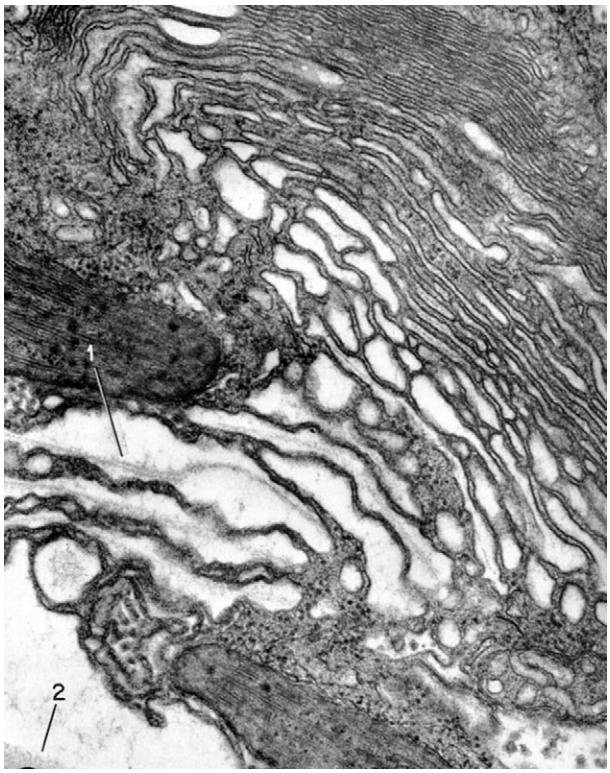


FIGURE 6.8 Medial section through a Golgi stack of *Pleurochrysis scherffelii* that contains a scale (1) destined to end up in the extracellular matrix (2). The cis-face of the Golgi stack is compact and the trans-face is inflated. ×64,800. (*Source:* From Brown, 1969.)

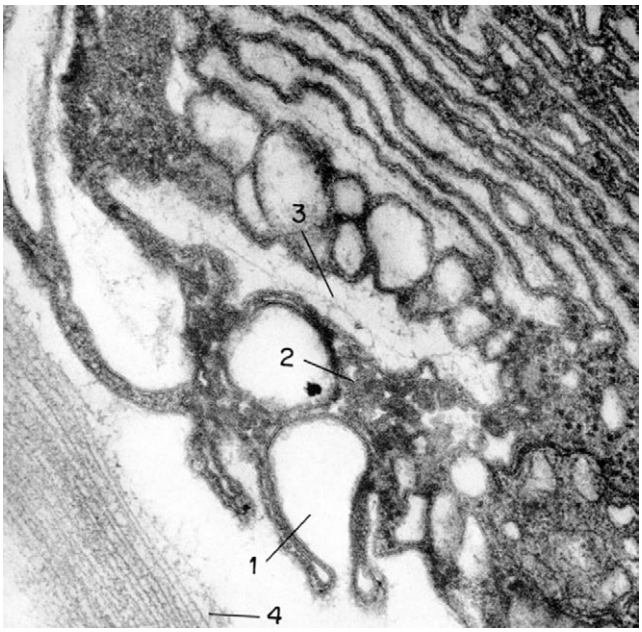


FIGURE 6.9 A Golgi stack of *Pleurochrysis scherffelii*. One cisterna (3) has a scale inside its lumen. Another cisterna (1) has probably just released a scale. Other cisternae (2) have previously deposited their scales. Arrow 4 points to the assembled laminate wall. ×89,600. (*Source:* From Brown, 1969.)

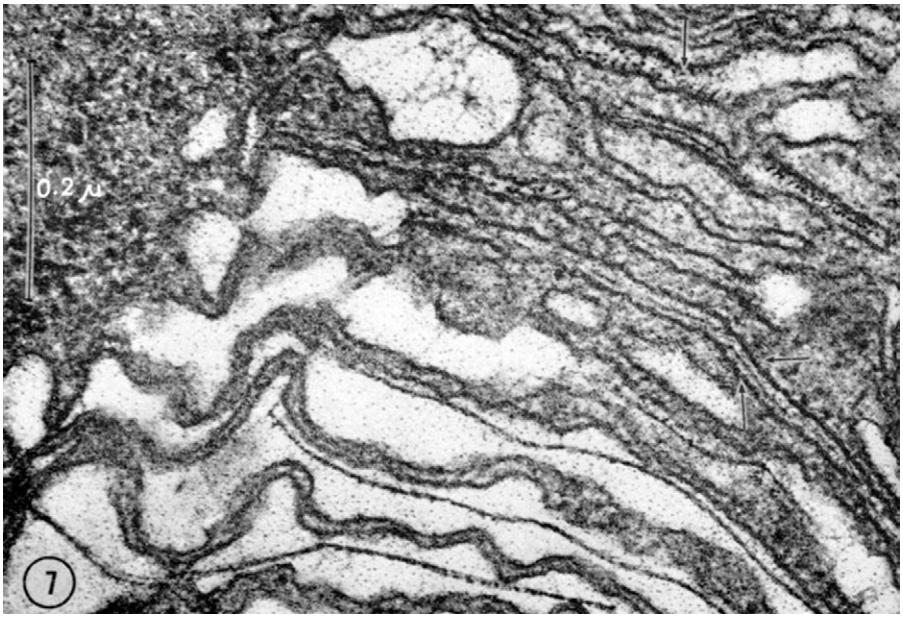


FIGURE 6.10 Electron micrograph showing the progressive maturation of scales in the Golgi stack of *Pleurochrysis scherffelii*. (*Source:* From Brown et al., 1970.)

The secretion of scales can be viewed with the light microscope; one scale is secreted every minute. Since there are about 30 cisternae per Golgi stack, each Golgi stack must turn over every 30 minutes according to the following calculation:

$$\frac{1\ \text{min}}{1\ \text{scale}} \times \frac{1\ \text{scale}}{1\ \text{cisternae}} \times \frac{30\ \text{cisternae}}{\text{Golgi stack}} = \frac{30\ \text{min}}{\text{Golgi stack}} \quad (6.1)$$

Three-dimensional tomography of serial freeze-fixed electron micrographic sections of the Golgi apparatus of the budding yeast *Pichia pastoris* indicates that the Golgi cisternae form at the cis-face from the fusion of COP II–coated vesicles derived from the transition ER. The cis-cisternae progressively mature into the medial-cisternae, the trans-cisternae, and trans-Golgi network cisternae. The trans-Golgi network cisterna eventually dissociates from the Golgi apparatus, becomes free in the cytoplasm, and gives rise to or receives clathrin-coated vesicles. Since Mogelsvang et al. (2003) see no connections between the cisternae, they believe that each cisterna matures and moves through the stack as it does in *Pleurochrysis*. However, Mogelsvang et al. also have not yet captured the formation of the cis-cisterna from the COP II–coated vesicles. When these elegant spatial studies are complemented with time-resolved studies, we will clearly know the mechanism of cisterna-to-cisterna movement in this organism. The cisternal progression model of intra-Golgi transport also appears to be sufficient to explain the movement of aggregates of procollagen in animal cells (Bonfanti et al., 1998; Nebenführ, 2003). However, intercisternal movements of membrane vesicles and connections by membrane tubules may also be important in intercisternal transport.

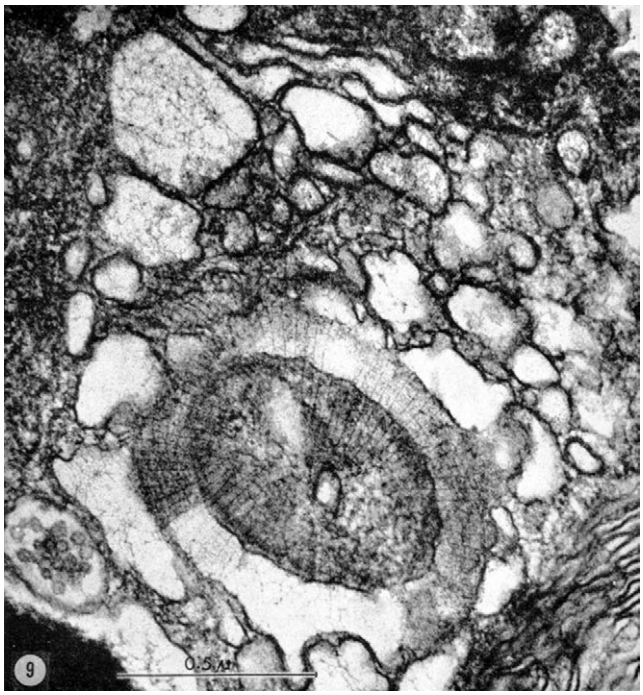


FIGURE 6.11 Electron micrograph showing a grazing section of a scale in the distal-most cisterna of the Golgi stack of *Pleurochrysis scherffelii*. (*Source:* From Brown et al., 1970.)

While interested in the transport of proteins from the ER to the Golgi apparatus in animal cells, James Rothman

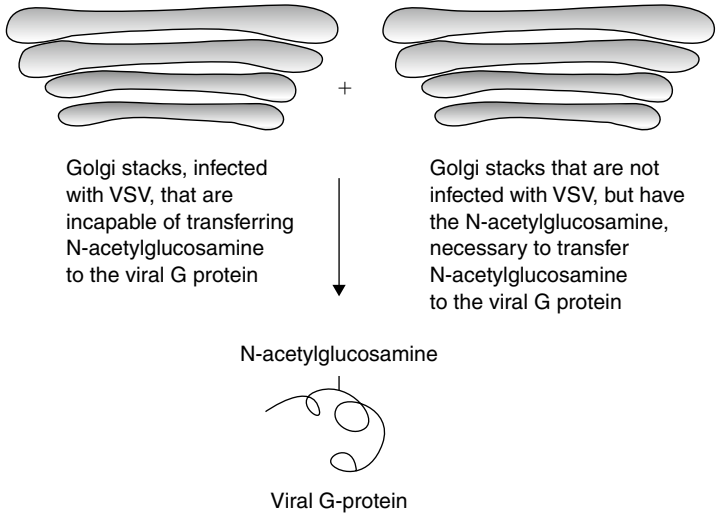


FIGURE 6.12 Mutant CHO cells were infected with VSV, which produces a membrane protein known as viral G protein. The mutant cells were missing an N-acetylglucosamine transferase and were incapable of transferring N-acetylglucosamine to the viral G protein. When Golgi stacks isolated from these cells was mixed with Golgi stacks isolated from uninfected wild-type cells, the viral G protein was glycosylated, indicating that their transport between the Golgi cisternae can occur.

(1992) serendipitously discovered that movement of membrane proteins between the cisternae of different Golgi stacks is also possible. Fascinated by George Palade's (1975) work on proteins that were synthesized on the ER and transported through the secretory pathway (see Chapter 8), Rothman (1992) wanted to know, "How could a membrane deform itself to pop out a vesicle? How could such a vesicle choose to fuse with the correct membrane? … And, how can all of this be organized in time and in space so as to allow the cytoplasm to maintain and propagate its membrane compartments?" Rothman decided to find the answer to these questions by developing a cell-free, membrane-transfer system that has now become the model for developing all cell-free systems for the study of membrane transfer.

Fries and Rothman (1980) developed a cell-free system using Chinese hamster ovary (CHO) cells (Figure 6.12). A batch of mutant cells was infected with the vesicular stomatitis virus (VSV), which produces an abundant membrane protein called *viral G protein*. The mutants were missing an N-acetylglucosamine transferase, and thus their Golgi apparati were incapable of transferring N-acetylglucosamine to the viral G protein. Fries and Rothman mixed a homogenate of these cells with one from an uninfected wild type to see if the viral G protein would pinch off of the ER of the infected cells and move to the Golgi apparatus of the uninfected cells and become glycosylated with N-acetylglucosamine. It did! However, in order to make the experiments more exacting, Fries and Rothman reduced the time in which they pulse-labeled the proteins in the infected cell to determine the time it took for the viral G protein to move from the ER to the Golgi apparatus. In this way, they could make sure that they were obtaining homogenates from the infected mutant cells while the viral G protein was still in the ER.

Unhappily, when they redid the membrane-transfer experiment under conditions when they were sure that the viral G protein was starting in the ER, they found that the viral G protein was not processed by the wild-type Golgi apparatus, indicating that they had not yet developed the conditions necessary for ER-to-Golgi transfer. However, if they waited 10 minutes and used homogenates from the infected mutant cells in which the labeled protein had already moved to the Golgi apparatus, they found glycosylation of the protein by the wild-type Golgi apparatus! This indicated that there must be vesicular and/or tubular pathways to move material from cisterna to cisterna. While Rothman favors the view that vesicles are involved, the published electron micrographs are consistent with both the vesicular and tubular hypotheses (Morré and Keenan, 1997). Glycosylation of the viral G protein also occurs in a similar manner *in vivo* when wild-type cells are fused with infected mutant cells (Rothman et al., 1984b).

The biochemical and morphological work on mammalian cells has been complemented by genetic studies done by Randy Schekman on yeast cells. A number of temperature-sensitive mutants known as *sec* mutants have been found that at high temperature are defective in their ability to *sec*rete (Schekman, 1996). There are many secretory mutants and each mutant is blocked in a specific part of the secretory pathway. Using the classic double-mutation technique developed by Mitchell and Houlahan (1946) to determine the sequence of gene products necessary for the synthesis of adenine, Novick et al. (1981) determined the order of gene products necessary for secretion. Moreover, they transfected

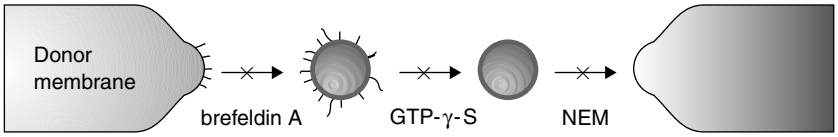


FIGURE 6.13 Diagram of GTP-dependent vesicular transport between two membranes, indicating possible sites of inhibitor action.

the temperature-sensitive mutants with wild-type DNA and then sequenced the genes that encode the proteins involved in the secretory pathway that allowed the yeast to secrete at high temperatures. Interestingly, many of these genes coded for GTP-binding proteins (Salminen and Novick, 1987; Goud and McCaffrey, 1991).

Work on the mammalian and yeast systems came together when Vivek Malhotra added GTP-γ-S, a nonhydrolyzable analog of GTP, to the mammalian cell-free system and found that GTP-γ-S inhibited membrane exchange between the cisternae of the Golgi apparatus (see Figure 6.13; Rothman, 1992). Moreover, this treatment caused the accumulation of vesicles the coats of which could be extracted by washing the isolated vesicles with 0.25 M KCl. In this way, many proteins could be isolated, including ADP ribosylation factor (ARF), which is a GTP-binding protein, coat proteins (α, β, γ, and δ COP), N-ethyl maleimide-sensitive fusion protein (NSF), soluble NSF attachment proteins (SNAPs), and SNAP receptor proteins (SNAREs).

These biochemical studies were combined with morphological and pharmacological studies to determine the function of each protein (Orci et al., 1986, 1989). For example, treatment with GTP-γ-S caused the accumulation of coated vesicles while treatment with N-ethyl maleimide (NEM) caused the accumulation of naked vesicles. When the two drugs are added together, the coated vesicles accumulated, indicating that the coated vesicles give rise to the naked vesicles. These experiments suggest that GTP hydrolysis by the ADP ribosylation factor is necessary for the removal of the coat prior to vesicle fusion and the N-ethyl maleimide-sensitive fusion protein is required for fusion. By contrast, brefeldin A, which binds to the ADP ribosylation factor, prevents vesicle formation, indicating that this protein is important for vesicle budding. The intercisternal transfer of membranes also requires adenosine triphosphate (ATP) as well as guanosine triphosphate (GTP) (Balch et al., 1984a,b; Rothman et al., 1984a). Work on intercisternal transport on multicellular plants is only beginning with the identification of genes and gene products involved in intercisternal transport in particular cell types (Staehelin and Moore, 1995; Sanderfoot et al., 2000; Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud et al., 2003; Pratelli et al., 2004; Uemura et al., 2004; Sutter et al., 2006; Lipka et al., 2007; Matheson et al., 2007; Min et al., 2007; Robinson et al., 2007; Sanderfoot, 2007; Zhang et al., 2007; Bassham and Blatt, 2008; Nielsen et al., 2008).

The identification of the genes and their proteins involved in the flow of membranes from cisterna to cisterna has been facilitated by finding yeast mutants that are defective in secretion. Many of these *sec* mutations act directly on the Golgi apparatus. The mutants defective in a given aspect of Golgi-mediated secretion are then transformed with plant DNA that putatively encodes a protein that acts in Golgi transport. If the mutant yeast strain is rescued by transfection with a given plant DNA sequence, then that sequence is assumed to code for a protein involved in the Golgi-mediated secretory system of that plant. The functions of many plant proteins have been discovered in this manner (Bassham and Raikhel, 2000; Bassham et al., 1995; Conceicao et al., 1997; d'Enfert et al., 1992; Zheng et al., 1999, 2004; Neumann et al., 2003; daSilva et al., 2004).

There is an enormous diversity in each class of proteins that facilitates movement between membranous compartments and the genes that encode them. The function of a given homolog is determined by visualizing secretion microscopically in a given plant cell that has been transiently transformed using the gene gun with an engineered copy of a gene that encodes a protein that functions in the secretory system. Such experiments show that specific homologs of a secretory protein function in the transport of proteins between given organelles, in a given direction, in a given cell type, during a given stage of development, or in response to a given environmental stimulus (Cheung et al., 2002; Goncalves et al., 2007). In this regard, secretion in unicellular organisms like yeast provides only a first-order approximation for the more complicated secretory processes that take place in the cells that make up multicellular plants.

6.7 POSITIONING OF THE GOLGI APPARATUS

The Golgi apparatus is a remarkably mobile organelle that can utilize the actomyosin system for its movement in plant cells (Boevink et al., 1998; Nebenführ et al., 1999; Nebenführ and Staehelin, 2001) and is closely associated with the endoplasmic reticulum export sites (ERESs; daSilva et al., 2004), which are probably synonymous with the transition ER.

The cause of the geographical position of the Golgi apparatus and the Golgi-derived vesicles in the cell is a wonderful puzzle. As a consequence of the importance of cell polarity

in many fascinating processes on plant and cell growth and development, plants serve as ideal organisms for studying the position of the Golgi apparatus and the Golgi-derived vesicles. Many plant and fungal cells have a specialized type of polarized growth called *tip growth* where Golgi-derived vesicles are targeted to a single locus in the growing cell (Sievers, 1963; Rosen et al., 1964; Rosen, 1968; Grove et al., 1970; Franke et al., 1972; Bartnik and Sievers, 1988; Steer and Steer, 1989). Moreover, plant organs and presumably the cells within them also show differential growth in response to gravity and light. Thus, it is possible that differential growth results from the differential distribution of the Golgi stacks or Golgi-derived vesicles on opposite sides of the cell (Shen-Miller and Miller, 1972; Shen-Miller and Hinchman, 1974). Differential positioning of the Golgi apparatus or Golgi-derived vesicles may also result in the remarkable annular, spiral, scalariform, and reticulate secondary wall thickenings that occur in tracheary elements (Bierhorst, 1971).

6.8 SUMMARY

The Golgi apparatus plays a central role in the flow of membranes, proteins, and carbohydrates through the cell. It is a factory with many loading docks involved in bringing in raw materials, sending back defective parts, delivering processed materials to the plasma membrane and vacuolar compartments, and retrieving recycled merchandise. Perhaps its protean morphology results from the fact that it is the obligate intermediate between the endoplasmic reticulum on the one side and the cell surface and the vacuolar compartment on the other side, and may constantly adjust to the demands of the rest of the secretory pathway. Indeed, determining the precise boundaries of this pivotal organelle with respect to the rest of the organelles involved in the secretory pathway is reminiscent of the recurrent problem of determining the relationships of the parts to the whole.

6.9 QUESTIONS

6.1. What is the function of the Golgi apparatus?
6.2. How does its structure reflect its function?
6.3. How may its polarity affect its function?
6.4. How might the structure and function of the Golgi apparati differ in a growing cell, in a cell in steady state, and in a senescing cell?

Chapter 7

The Vacuole

7.1 DISCOVERY OF THE VACUOLE

Most plant cells contain a conspicuous central region that appears empty in the light microscope (von Mohl, 1852). This region, which includes a transparent, or rarely colored, watery substance known as the cell sap, is called the *vacuole*, a term that comes from the Latin word for "empty." The large central vacuole can take up approximately 95 percent of the protoplast volume, although in typical higher plant cells, it takes up approximately 60 percent or more (Winter et al., 1994; see Figure 7.1). A large central vacuole is not limited to plant cells. According to Bensley (1951), a large central vacuole is also found in the cells of the flagellate *Noctiluca*, the ciliate *Trachelius*, the ectoderm and endoderm of the Coelenterates, and in the Heliozoa.

Vacuoles were first observed in protozoa. The contractile vacuoles or "stars" of many protozoa were seen by Lazzaro Spallanzani (1776), although he mistook them for respiratory organs (see Zirkle, 1937). These "stars" were named *vacuoles* by Félix Dujardin (1841). Although the optically structureless cell sap had been observed by botanists for years, the term *vacuole* was first applied to plant cells by Matthias Schleiden in 1842 when he distinguished the vacuole from the rest of the protoplasm (Zirkle, 1937).

The cell sap is surrounded by a differentially permeable membrane as determined from osmotic studies done by Hugo de Vries on *Tradescantia* epidermal cells and many other cell types (1884a,b, 1885, 1888a,b). In these studies, he noticed that the cell walls bulged when the cells were placed in pure water. As he increased the concentration of solutes in the external solution, the walls relaxed, and, at higher concentrations of solutes, he observed that the violet-colored vacuole shrank. De Vries concluded that a membrane must surround the cell sap in order for the vacuole to behave as an osmometer. He coined the term *tonoplast* to designate the membrane that surrounded the cell sap. The tonoplast was so named because he thought that it was the regulator of turgor, also known as tonicity, in the cell (de Vries, 1910). He mistakenly believed that the tonoplast was differentially permeable but the plasma membrane was not, and consequently, only the tonoplast regulated turgor.

De Vries (1885) also thought that the tonoplast was an autonomous self-replicating particle in the cell. However, Wilhelm Pfeffer (1900–1906) showed that vacuoles are not autonomous but form *de novo* during phagocytosis. Nowadays it is possible to observe vacuoles develop in evacuolated (Hörtensteiner et al., 1992) or vacuoleless protoplasts (Davies et al., 1996). Since the tonoplast is neither

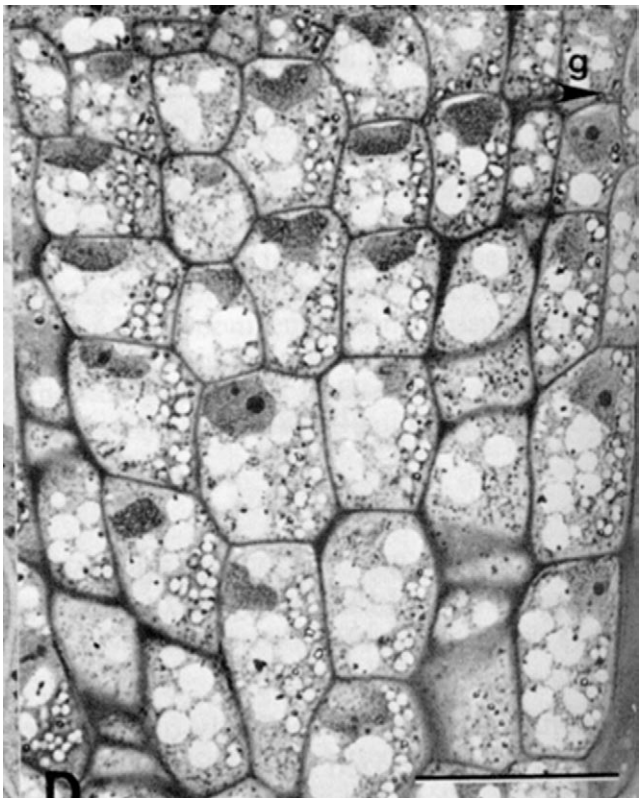


FIGURE 7.1 The vacuoles are a prominent component in the columella cells of this agravitropic mutant of barley. Bar, 50 μm. (*Source:* From Moore, 1985.)

self-replicating nor the primary site of turgor regulation, I will use the term *vacuolar membrane* to denote the differentially permeable membrane that surrounds the cell sap, as suggested by Pfeffer (1886).

7.2 STRUCTURE, BIOGENESIS, AND DYNAMIC ASPECTS OF VACUOLES

Although a few meristematic cells, including the apical cell in *Osmunda* and *Lunularia*, as well as the cambial initials in higher plants have prominent vacuoles (Bailey, 1930; Sharp 1934), the vacuole is inconspicuous in most meristematic cells (Porter and Machado, 1960). The development of the vacuole can be followed in the light microscope. For example, Pensa (see Guilliermond, 1941) looked at the development of the vacuolar system in the cells of the teeth of young, living rose leaflets (Figure 7.2). The vacuolar systems in these cells are easy to observe since the vacuoles are filled with anthocyanin. In the youngest cells at the tip, the vacuoles appear as numerous, tiny filamentous elements. In slightly older cells, these filamentous elements appear to swell. Eventually, in the mature cells at the base, the swollen elements fuse into larger vacuoles and eventually form a large central vacuole. Dangeard (1919) gave the name *vacuome* or *vacuolar system* to all the vacuoles contained in the cell during all its phases of development.

A similar vacuolar development can be seen in maturing barley root cells stained with neutral red, a vital stain that is preferentially taken up into acidic compartments (Figure 7.3). Other good examples of vacuolar development include the epidermal cells of young leaves and the hairs on the sepals of *Iris germanica*, the glandular hairs on the leaflets of walnut, and the leaves of *Anagallis arvensis*. By contrast, the vacuolar system of *Elodea canadensis* never goes through a filamentous stage, but starts as small spherical vacuoles that later fuse into a large central vacuole (Guilliermond, 1941).

A more recent study of vacuolar development has been done by Palevitz and O'Kane (1981) and Palevitz et al. (1981) using the autofluorescent vacuole found in the developing guard cells of *Allium cepa*. They find that the vacuoles of young guard mother cells are globular. As the guard mother cells develop, the vacuole is transformed into a reticulum of interlinked tubules and small chambers. The tubules are approximately 100–500 nm in diameter. In the guard mother cell, the network continually undergoes changes in shape and remains reticulate during the division that gives rise to the two guard cells. The reticulate networks persist through the early stages of guard cell differentiation and then they are transformed into two large globular vacuoles, one in each guard cell (Figure 7.4). The dynamics of the vacuolar compartment have also been confirmed using various vacuolar proteins fused to green fluorescent protein (GFP; Flückiger et al., 2003; Hicks et al., 2004; Zouhar et al., 2004).

The developmental pattern seen in vacuoles can be reversed under certain physiological conditions. For example, Charles Darwin (1897) noticed that the vacuole of the tentacle of the carnivorous plant *Drosera rotundifolia*, which is filled with anthocyanin, appears to break up after the leaf is stimulated by an insect. (Actually, Darwin

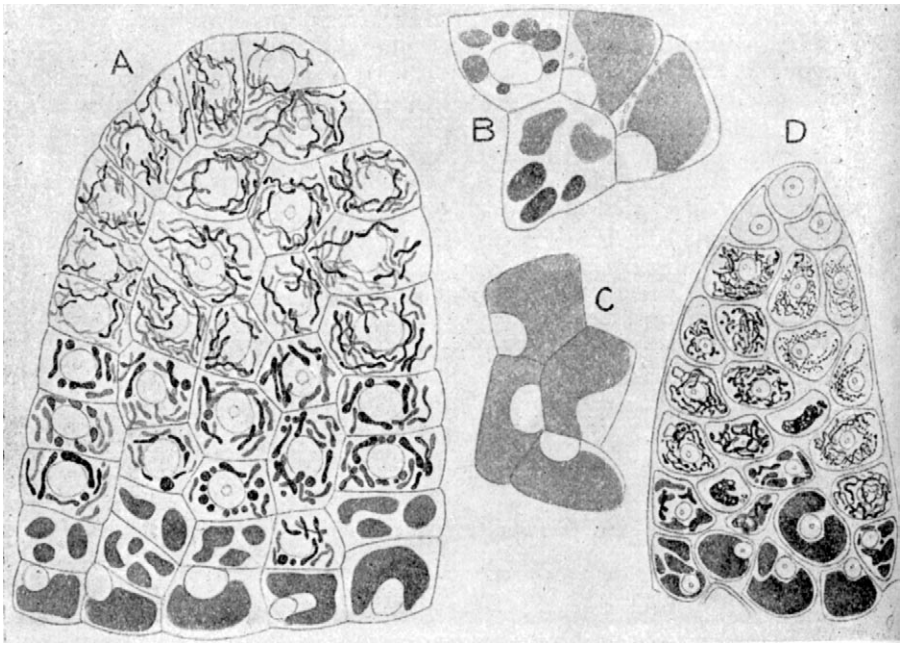


FIGURE 7.2 The anthocyanin-containing vacuolar system in the cells of the teeth of young, living rose leaflets. (a and d) Cells at tip; (b and c) older cells. (*Source:* From Guilliermond, 1941.)

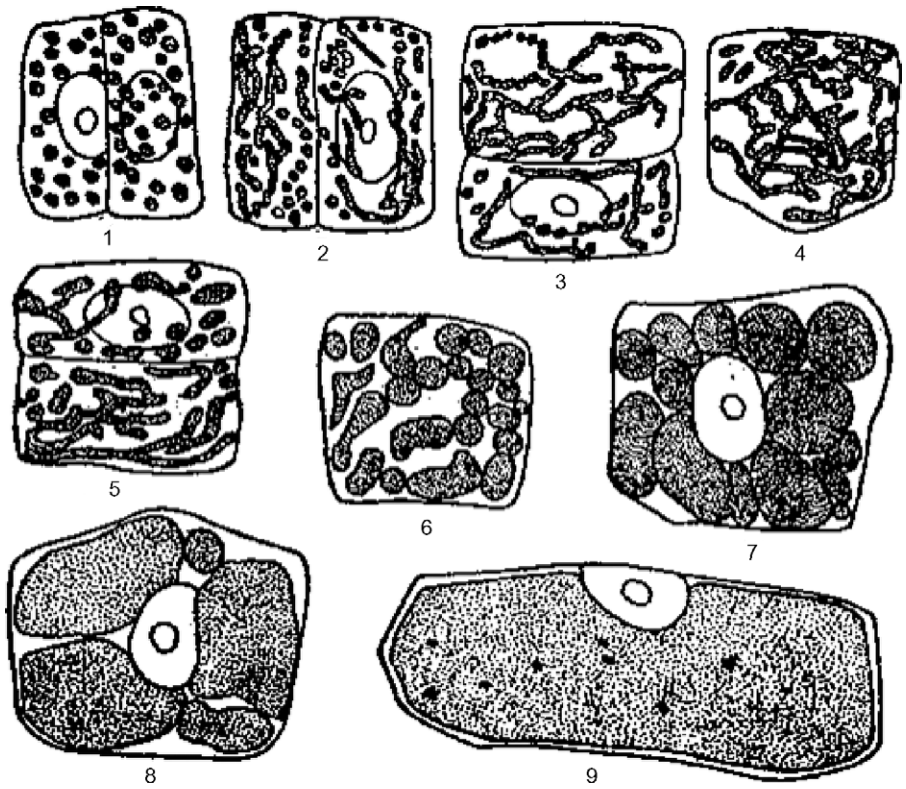


FIGURE 7.3 The vacuolar system in cells of a barley root vitally stained with neutral red. 1–5 are meristem cells, 6–8 are in the region of differentiation, and 9 is a mature cortical parenchyma cell. (*Source:* From Guilliermond, 1941.)

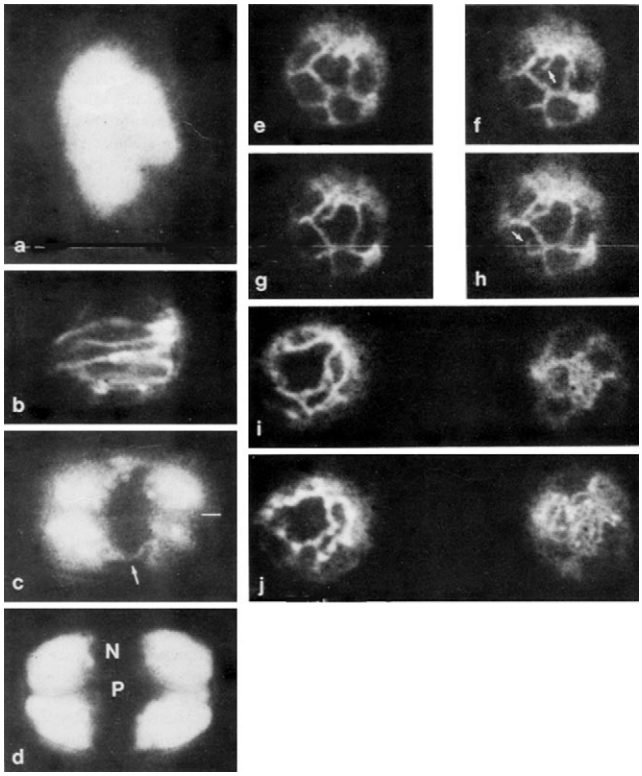


FIGURE 7.4 Autofluorescence images of various stages in vacuole development during stomatal differentiation in onion seedlings. (*Source:* From Palevitz and O'Kane, 1981.)

misidentified the vacuole as protoplasm.) The cells of the tentacles contain a single central anthocyanin-filled vacuole. At the moment of stimulation, the vacuole fragments into filamentous vacuoles. Immediately after stimulation, the filamentous vacuoles fuse to form a large central vacuole and the cell returns to its initial state (de Vries, 1886; Guilliermond, 1941; Lloyd, 1942; Juniper et al., 1989).

The vacuole can be defined operationally as a swollen terminally differentiated intracellular membrane-bound compartment of the secretory pathway (Marty, 1979). The minimal requirement for the formation of vacuoles is the synthesis of a vacuolar membrane that contains the transporters necessary to increase the osmotic pressure of the lumen. The increase in the osmotic pressure will allow the newly formed vacuoles to swell until the water potential of the vacuole is in equilibrium with the water potential of the cytosol. Moreover, continued membrane synthesis and/or delivery must occur if the vacuolar membrane thickness is to remain constant. The biogenesis of vacuoles does not occur by a single pathway (Robinson and Hinz, 1997; Marty, 1999), and, at the electron microscopic level, we can see that vacuoles can form in a variety of ways (Marinos, 1963; Ueda, 1966; Matile and Moor, 1968).

Using electron microscopy, Francis Marty (1978, 1997, 1999) studied vacuole formation in meristematic cells of roots. The cells adjacent to the quiescent cells of the root are the most undifferentiated and do not have any vacuoles.

In slightly older cells, primordial vacuole precursors, or provacuoles, arise from the trans-Golgi network (Figures 7.5–7.7). The provacuoles eventually form an anastomosing network of tubules, which then wrap themselves around portions of the cytoplasm like bars of a birdcage (Figure 7.8). Subsequently, the tubules fuse, thus entrapping the enclosed cytoplasm in a double membrane. At this point, various hydrolases are probably released from the E-space between the two vacuolar membranes. This leads to autophagy of the enclosed cytoplasm and the inner membrane of the provacuole becomes totally degraded (Figure 7.9). Eventually the newly formed vacuoles fuse to form larger vacuoles (Figure 7.10).

Vacuoles can also form directly from the endoplasmic reticulum (ER; see Figure 7.11; Herman, 2008; Sabelli and Larkins, 2009). Electron microscopy of rice endosperm cells shows that there is a population of ER, known as protein-body ER, which gives rise directly to protein storage vacuoles that store prolamins. Prolamins are proteins that are insoluble in water, but soluble in 50–95 percent aqueous ethanol. By contrast, protein storage vacuoles that store other storage proteins known as *glutelins* (which are soluble in dilute acid or base) form from clathrin-coated vesicles that bud from the trans-Golgi network and mature into protein bodies after the coats are shed (Nishimura and Beevers, 1978, 1979; Parker and Hawes, 1982; Nieden et al., 1984; Herman and Shannon, 1984a,b, 1985; Greenwood and Chrispeels, 1985; Harris, 1986; Faye et al., 1988; Robinson et al., 1989; Hoh et al., 1991; Levanony et al., 1992; Li et al., 1993a). Specifics of the biogenesis of protein bodies vary in a species-specific manner and during different stages of seed development within a given species (Marty, 1997). Further evidence that the ER is involved in vacuole formation comes from studies in *Arabidopsis* that show that at least some of the anthocyanin that is ultimately found in the vacuole is transported initially into the ER lumen (Poustka et al., 2007).

The protein bodies contain the hydrolytic enzymes necessary for the breakdown of the resident food storage proteins (Van der Wilden et al., 1980; Herman et al., 1981). There is evidence that mature vacuoles can form from the merger of vacuoles produced by two independent pathways; one that produces the storage proteins and one that produces the hydrolases (Paris et al., 1996). After fusion of the two types of provacuoles, the membranes of the provacuoles

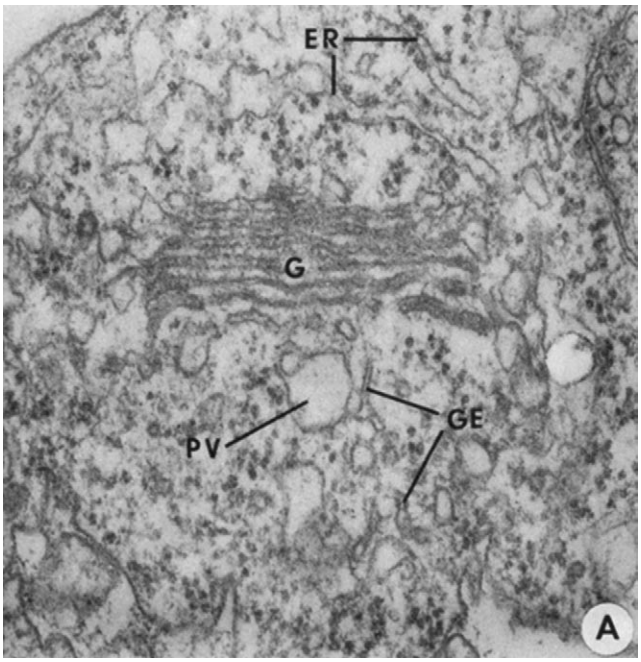


FIGURE 7.5 Electron micrograph of a meristematic root-tip cell of *Euphorbia* showing the relationship between the Golgi stack (G), the Golgi-associated endoplasmic reticulum from which lysosomes apparently form (GE), and a provacuole (PV). ×57,600. (*Source:* From Marty, 1978.)

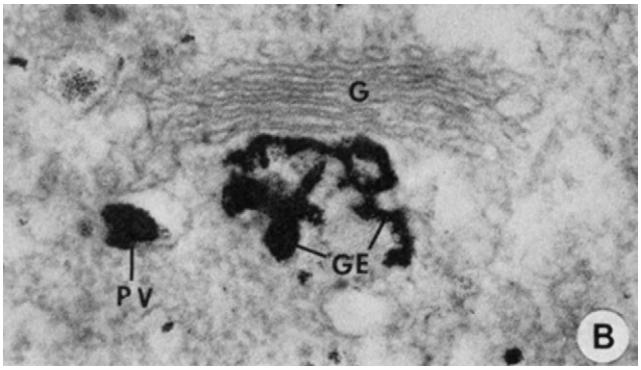


FIGURE 7.6 Electron micrograph of a meristematic root-tip cell of *Euphorbia* stained with zinc iodide and osmium tetroxide showing the relationship between the Golgi stack (G), the Golgi-associated endoplasmic reticulum from which lysosomes apparently form (GE), and a provacuole (PV). ×64,000. (*Source:* From Marty, 1978.)

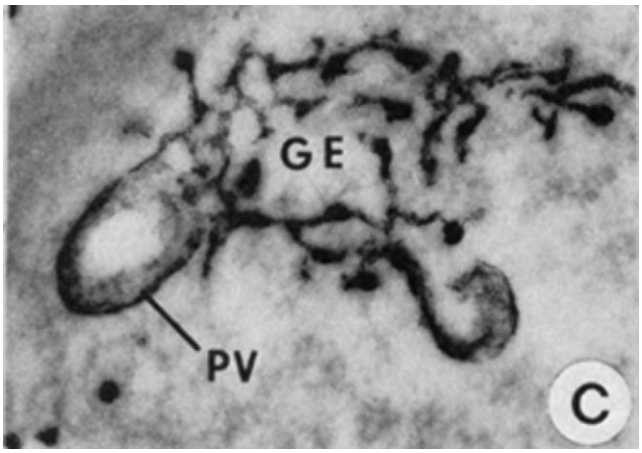


FIGURE 7.7 Electron micrograph of a meristematic root-tip cell of *Euphorbia* stained with zinc iodide and osmium tetroxide showing the relationship between the Golgi-associated endoplasmic reticulum from which lysosomes apparently form (GE) and a provacuole (PV). ×51,500. (*Source:* From Marty, 1978.)

containing the hydrolases remain intact within the vacuoles that contain the storage proteins (Jiang et al., 2000, 2001). Thus, topologically speaking, protein storage vacuoles contain hydrolase-containing vacuoles and the storage function is separated from the lytic function. Although the hydrolases are separated from the storage proteins during seed maturation, the hydrolases must be released from their inner sanctum in order to hydrolyze the storage proteins during germination.

When it comes to the interpretation of electron microscopic images, there is some contention concerning how vacuoles arise within the endomembrane system. In part, the disagreement comes from the lack of temporal resolution that is necessary to observe the dynamic three-dimensional behavior of the vacuolar compartment that is apparent in the light microscope. These dynamics are lost in static thin sections that have high two-dimensional spatial resolution, but low temporal and three-dimensional spatial resolution (Manton, 1962). Moreover, chemical fixation causes the transformation of a reticulate motile vacuolar system into spherical vesicles, causing the connections between various compartments to become obscured (Wilson et al., 1990). Thus, we must be cautious of interpretations of structure based on chemically fixed specimens and single sections.

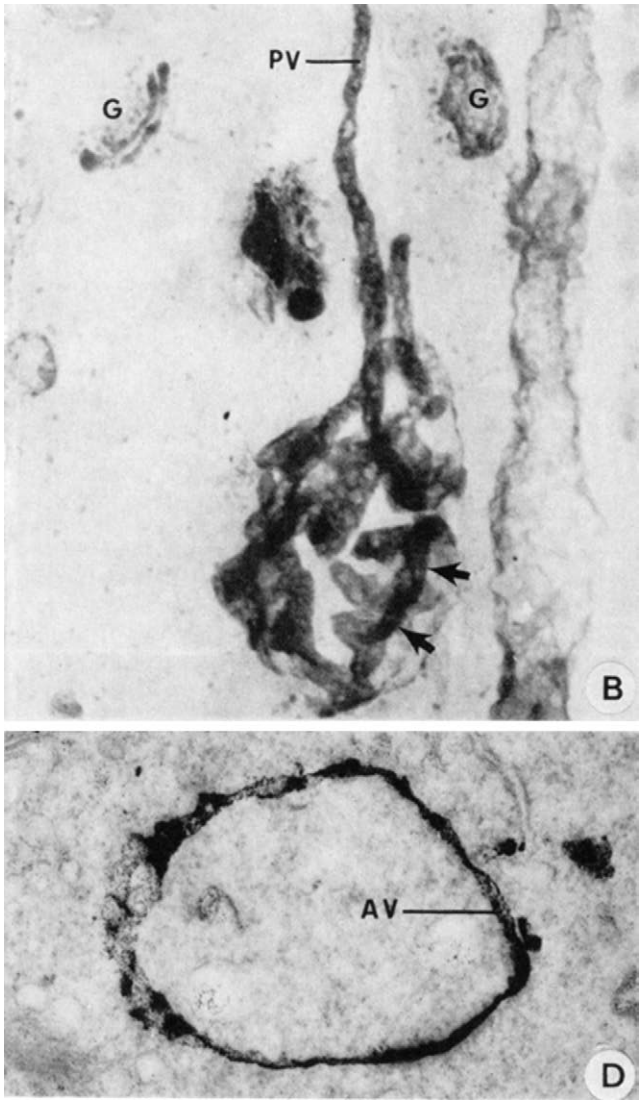


FIGURE 7.8 Electron micrographs of a meristematic root-tip cell of *Euphorbia* stained with zinc iodide and osmium tetroxide showing the sequestration cage structure of a provacuole (PV) (*top*) and a transverse section of the same structure showing the typical ringlike structure of an autophagic vacuole (AV) (*bottom*). (*Source:* From Marty, 1978.)

Building on a strong biochemical, biophysical, morphological, and physiological background, Yasuhiro Anraku and his colleagues have taken a genetic approach to understand vacuole biogenesis in yeast (Nishikawa et al., 1990; Wada et al., 1990, 1992). They have made a series of mutants that block vacuole biogenesis at a variety of points and even inhibit vacuole formation altogether. Yoshihisa and Anraku (1990) have found that while most proteins enter the vacuole through the ER-Golgi pathway, and that the majority of the vacuole forms from the budding of membranes from the trans-Golgi network, α-mannosidase, a marker enzyme for the vacuolar membrane, does not have a signal peptide, is not glycosylated at its N-X-S/T site, and contains no complex carbohydrates, indicating that it enters the vacuole in a manner that does not involve the ER-Golgi pathway. Other proteins also enter the vacuole directly from the cytosol (Klionsky and Ohsumi, 1999). Thus, even a single vacuole may result from the work of many subcellular units.

7.3 ISOLATION OF VACUOLES

The vacuoles of animal cells were first convincingly separated as a contaminant of the "mitochondrial pellet" by Christian de Duve in 1955 at the same time that he isolated peroxisomes (de Duve et al., 1955; de Duve, 1975). He called the vacuoles lysosomes because they had a number of nonspecific hydrolytic enzymes, which can cause the lysis (dissolution) of the soma (body). This is why lysosomes are sometimes called *suicide sacs*. He defined the lysosome as an organelle surrounded by a single membrane that contains a number of nonspecific hydrolases, including acid phosphatase, an enzyme that can be easily localized cytohistochemically. The nonspecific hydrolases exhibit latency. That is, they do not show any activity when assayed with exogenous substrates. However, once the lysosomal membrane is permeabilized with a detergent such as Triton X-100, the enzymes are very active toward their substrates.

A large quantity of vacuoles can be isolated rapidly by mechanically slicing fresh or plasmolyzed tissue with a razor blade in a medium containing an osmoticum. The homogenate is filtered and centrifuged at 1300–3500 g to recover the vacuoles. The pellet is resuspended in 15 percent Metrizamide. This suspension is overlayered with a layer of 10 percent Metrizamide and an uppermost layer

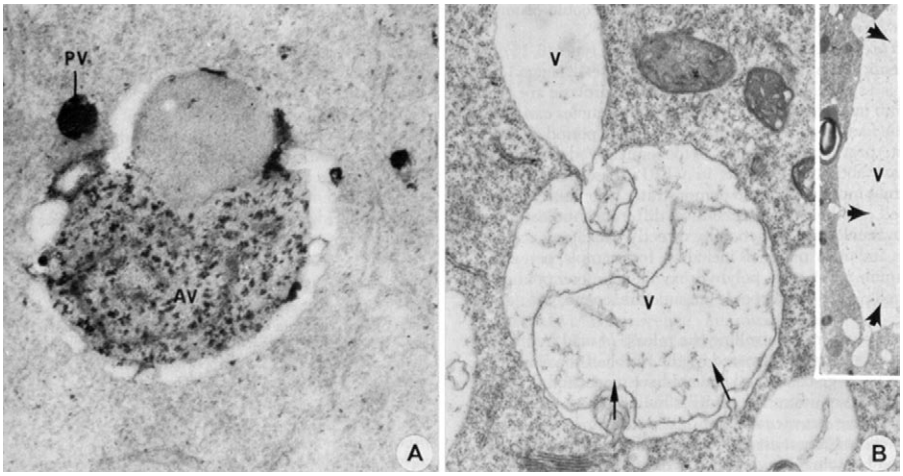


FIGURE 7.9 Electron micrograph of a meristematic root-tip cell of *Euphorbia* showing the sequestered cytoplasm within an autophagic vacuole (AV) (*left*) and a vacuole where the sequestered cytoplasm is almost degraded (*right*). The inset shows provacuoles merging with the large vacuole. Left, ×42,100; right, ×20,600; inset, ×8000. (*Source:* From Marty, 1978.)

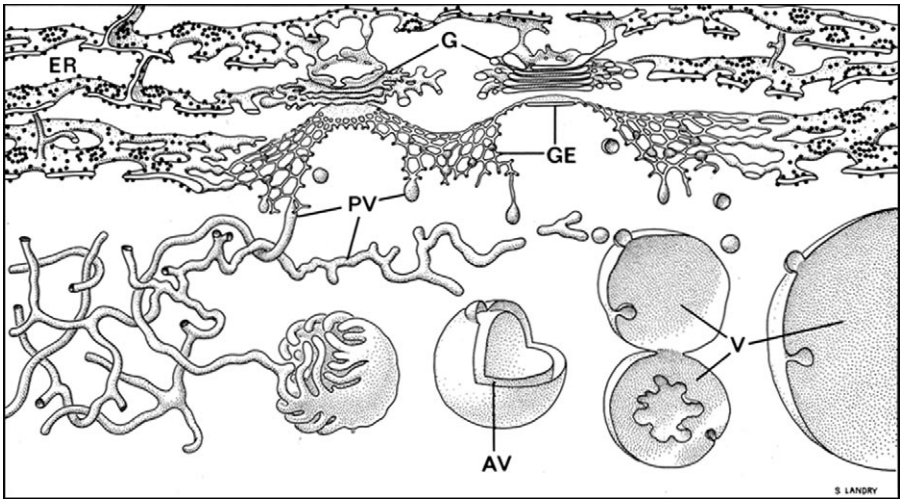


FIGURE 7.10 Various stages in the formation of vacuoles from the Golgi apparatus. (*Source:* From Marty, 1978.)

of 0 percent Metrizamide. During centrifugation (500 g, 10 minutes), the vacuoles float and collect at the 10–0 percent interface. The yield is low, but a large quantity of tissue can be processed in this manner (Wagner and Siegelman, 1975; Kringstad et al., 1980; Wagner, 1983).

Isolated vacuoles can be further purified to obtain only tightly sealed vacuolar membrane vesicles. This can be done by centrifuging the membrane fraction in a density gradient made with a high–molecular mass polymer that is unable to penetrate intact vesicles. The density of intact vesicles will depend on the density of the cell sap (ca. 1.01 g/mL; Kamiya and Kuroda, 1957), while the density of leaky vesicles will depend on the density of the membrane (ca. 1.1–1.2 g/mL; Sze, 1985). Thus, the leaky vesicles will move down further into the gradient away from the intact vacuoles. Highly purified vacuolar membranes have also been isolated by using aqueous two-phase partitioning followed by using free-flow electrophoresis (Scherer et al., 1992).

7.4 COMPOSITION OF VACUOLES

There are over 100 proteins in the vacuole according to a biochemical analysis (Kenyon and Black, 1986), or between 34 and 650 proteins according to proteomic analyses (Carter et al., 2004; Sazuka et al., 2004, Shimaoka et al., 2004; Szponarski et al., 2004; Endler et al., 2006; Jaquinod et al., 2007; Schmidt et al., 2007). The cell sap contains a number of nonspecific hydrolytic enzymes that typically have acidic pH optima. These include proteases that split

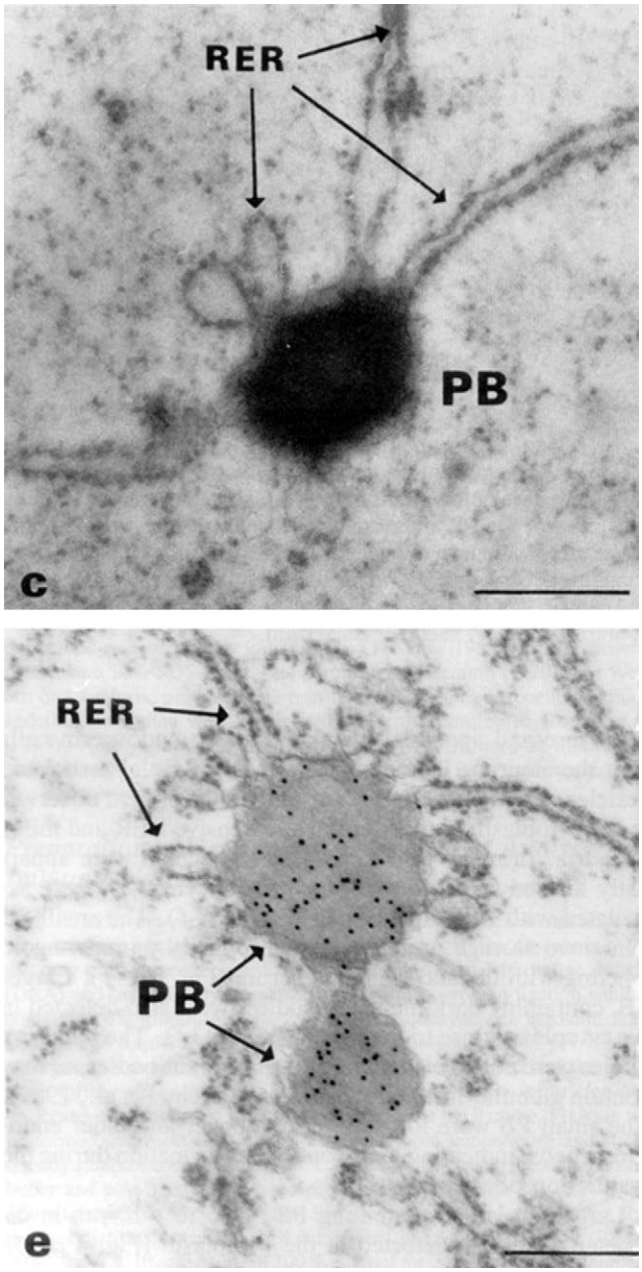


FIGURE 7.11 A protein body (PB) forming directly from the rough endoplasmic reticulum (RER) in endosperm cells of wheat. In the bottom figure, the protein body is immunolabeled with gold particles that are indirectly attached to an antibody directed against prolamins. Bars, 500 nm. (*Source:* From Levanony et al., 1992.)

polypeptides into fragments by digesting internal peptide bonds (endopeptidases), and proteases that digest the terminal amino acids (exopeptidases) from the amino-terminus (aminopeptidases) or the carboxy-terminus (carboxypeptidases). The cell sap can also contain esterases (e.g., acid phosphatase), phosphodiesterases (e.g., RNase and DNase), and acyl-esterases (e.g., lipases). The cell is doubly protected from these nonspecific hydrolases because they are sequestered in the acidic vacuole, and if they were to be released, they would not function in the neutral cytosol due to their acidic pH optima (Matile, 1975).

α-Mannosidase is usually used as a cell sap marker in plant cells, although it is a vacuolar membrane marker in animal and yeast cells. In plant cells, the nitrate-sensitive, vanadate-insensitive H^+-ATPase is often used as a vacuolar membrane marker (Sze, 1985), although it also occurs on the membranes that give rise to the vacuole (Herman et al., 1994).

The lipids of the vacuolar membrane have been characterized, and they are similar to but not identical with the other membranes (see Table 7.1; Yoshida and Uemura, 1986). The similarities may be due to their similar function as a barrier and their differences may have a functional basis. For example, the activity of the vacuolar membrane H^+-ATPase is affected by its lipid environment (Yamanishi and Kasamo, 1993, 1994).

Using free-flow electrophoresis, Leborgne et al. (1992) isolated vacuolar membranes from cell cultures of *Eucalyptus*. They tested two lines of cells, one that was frost-sensitive and one that was frost-tolerant. They used fluorescence redistribution after photobleaching (FRAP) with a fluorescent phosphatidylcholine to determine the diffusion coefficient of lipids in the vacuolar membranes of both cell types. They find that the diffusion coefficients of the frost-tolerant type are greater than those of the frost-sensitive type, indicating that the vacuolar membrane of the frost-tolerant type is more fluid or less viscous than that of the sensitive type (Table 7.2). Given that the radius of a lipid molecule is approximately 0.4 nm (see Chapter 1), the viscosities of the vacuolar membrane can be obtained from the Stokes-Einstein equation:

$$D = kT/(6\pi r_H \eta) \tag{7.1}$$

The data of Leborgne et al. (1992) show that the viscosity of the membrane varies between 1.7 and 3.3 Pa s, which is thousands of times greater than the viscosity of water (0.001 Pa s).

In order to confirm the difference in the viscosity of the vacuolar membranes of the two cell types, the rotational diffusion coefficients were determined by measuring the fluorescence polarization of 1,6-diphenylhexatriene. With this technique, the rate at which the probe spins around in the membrane is determined by measuring the degree of polarization of the fluorescent light emitted from the probe (Bull, 1964). If the fluorescent probe were fixed in a highly viscous membrane, the degree of polarization would be maximal. By contrast, if the fluorescent probe were rapidly spinning, the degree of polarization would be minimal. The degree of polarization is a measure of the rotational diffusion coefficient, which relates the kinetic energy of the probe (kT) to the viscosity of the membrane and the size and shape of the probe. The rotational diffusion coefficient is greater in frost-tolerant than in frost-sensitive vacuolar

TABLE 7.1 Lipid composition of vacuolar membrane of mung beans

Lipid Phospholipids	**mol %**						
Phosphatidylcholine (PC)	23.7						
Phosphatidylethanolamine (PE)	6.0						
Phosphatidylinositol (PI)	5.7						
Phosphatidylglycerol (PG)	2.3						
Phosphatidylserine (PS)	2.2						
Phosphatidic acid (PA)	1.1						
Subtotal	51.0						
Sterols							
Free sterols	18.2						
Acylated sterylglycoside	7.4						
Sterylglycoside	2.3						
Subtotal	27.9						
Other							
Ceramide monohexoside	16.6						
Monogalactosyldiglyceride	1.0						
Digalactosyldiglyceride	3.4						
Total	**99.9**						
Hydrocarbon Tails	**PI**	**PS**	**PC**	**PE**	**PG**	**PA**	**Total**
16:0	50.6	24.0	31.5	43.3	79.1	34.0	39.4
18:0	4.8	6.6	8.5	4.3	3.6	5.0	6.2
18:1	6.6	8.0	11.9	7.7	3.3	8.5	9.1
18:2	15.0	22.1	24.3	23.4	5.8	16.5	22.2
18:3	20.5	27.7	21.3	18.2	6.1	15.4	19.8
20:1	0.6	9.2	0.9	1.1	0.6	5.0	1.5
20:2	1.7	3.0	0.7	0.7	1.7	11.2	1.2
20:3		0.1	0.1	1.8			0.8
22:1		6.2	2.2	2.6			2.1
Unsaturated/saturated	0.81	2.27	1.50	1.10	0.21	1.56	1.19

Source: From Yoshida and Uemura (1986).

membranes, confirming that the vacuolar membrane of frost-tolerant cells is less viscous. These data indicate that through their effect on membrane viscosity, variations in lipid composition may be responsible for differences in frost tolerance or sensitivity.

7.5 TRANSPORT ACROSS THE VACUOLAR MEMBRANE

The lumen of the vacuole is an E-space and is topologically equivalent to the external space that surrounds the cell.

TABLE 7.2 Diffusion coefficients for phosphatidylcholine in vacuolar membrane

	D (in m^2/s)	
Cell Line	**280 K**	**296 K**
Frost-tolerant	2.14×10^{-13}	3.22×10^{-13}
Frost-sensitive	1.65×10^{-13}	2.37×10^{-13}

Source: *From Leborgne et al. (1992).*

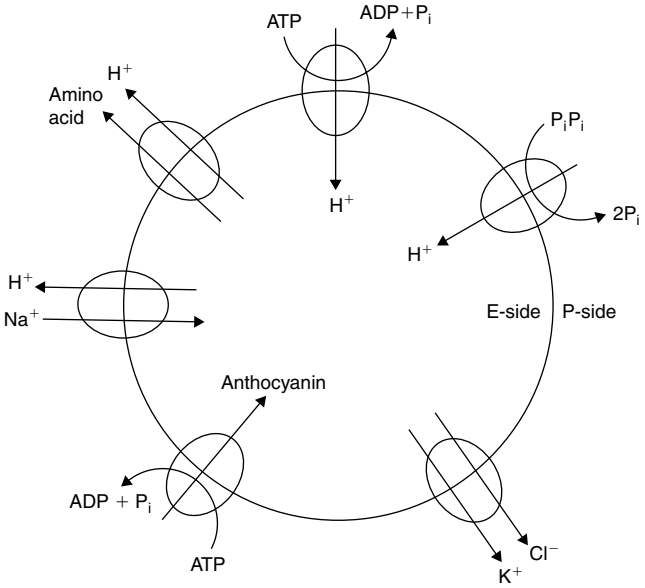


FIGURE 7.12 Diagram of the vacuolar membrane with a variety of transport proteins.

The vacuolar membrane complements the plasma membrane and ER in its ability to transport many molecules and help maintain a cellular homeostasis (Figure 7.12). The vacuolar membrane is also capable of generating an action potential (Kikuyama and Shimmen, 1997). Various vacuolar membrane channels, carriers, and pumps have been characterized (Bennett and Spanswick, 1983, 1984a,b; O'Neill et al., 1983; Kaiser and Heber, 1984; Bennett et al., 1985; Blumwald and Poole, 1985, 1986; Lew et al., 1985; Rea and Poole, 1986; Bush and Sze, 1986; Hedrich et al., 1986; Blumwald et al., 1987; Hedrich and Kurkdjian, 1988; Hedrich et al., 1989; Johannes and Felle, 1989; Blackford et al., 1990; Schumaker and Sze, 1990; Maathuis and Sanders, 1992; Müller et al., 1996, 1997, 1999; Hirschi, 1999, 2001; Yamaguchi et al., 2001, 2003, 2005; Cheng et al., 2002; Müller and Taiz, 2002; Pittman et al., 2002; Gaxiola et al., 2002; Sottosanto et al., 2004; Epimashko et al., 2006; Pottosin and Schönknecht, 2007; Schmidt et al., 2007; Shiratake and Martinoia, 2007; Schneider et al., 2008). Some of the carriers and pumps are involved in Ca^{2+} homeostasis. At least two classes of primary proton pumps are involved in building up a proton difference across the vacuolar membrane that is utilized by secondary transporters to facilitate the transport of ions, sugars, amino acids, and other small molecules (Blumwald and Gelli, 1999). Other carriers, which are known as *ATP-binding cassette* (ABC) *transporters* or *traffic ATPases*, transport relatively large organic solutes (Rea et al., 1998; Klein et al., 1996, 1998, 2000, 2001). Work on the receptors that recognize cytosolically synthesized proteins and translocate them through the membrane is lagging behind the studies aimed at elucidating how proteins from within the secretory pathway enter the vacuole (Ahmed et al., 1997, 2000; Sanderfoot et al., 1998).

7.5.1 Proton-Translocating Pumps

Traditionally, studies on the vacuolar proton-translocating pumps have begun with biochemical studies. The vacuolar H^+-pumping ATPase is known as the V-type ATPase and has been purified from isolated vacuolar membranes (see Figure 7.12). It accounts for 6.5–35 percent of the total vacuolar membrane protein and has a density of 970–3380 molecules per μm^2 (Ratajczak, 2000). The V-type ATPase is a 500-kDa protein complex (Rea et al., 1987a,b; Bowman et al., 1989; Nelson, 1989; Taiz et al., 1990; Ward et al., 1992; Ward and Sze, 1992b) that consists of two multipolypeptide components. One component (V_1) is a peripheral membrane complex, which contains the catalytic ATP hydrolyzing site. The other component (V_0) is an integral membrane complex that makes the proton channel. The ATPase can be dissociated from isolated vacuolar membranes by solubilizing it with Triton X-100. The solubilized protein is then separated from many of the other proteins by gel-filtration chromatography followed by anion-exchange chromatography. The canonical V-type H^+-ATPase is inhibited by NO_3, bafilomycin, and (dicyclohexylcarbodimide) DCCD, but not by (VO_4) or azide.

The purified V-type ATPase is composed of 10–13 polypeptides. Gene sequence information has revealed that there are several isoforms of these polypeptides. A V-type ATPase has been reconstituted into proteoliposomes in order to determine its transport characteristics (Kasamo et al., 1991; Ward and Sze, 1992a). The ability of the reconstituted protein to form an ATP-dependent pH difference across the membrane is inhibited by gramicidin. Since gramicidin (Dubos, 1939) is an antibiotic that forms monovalent cation–conducting pores in membranes, the pH difference must be due to proton pumping as opposed to the transport of organic acids.

Moreover, H^+ pumping is stimulated by valinomycin, an antibiotic that functions as a K^+-selective ionophore. In the presence of valinomycin, one K^+ leaves the vesicle for every H^+ that is pumped in, and consequently, an electrical potential does not build up across the membrane in response to proton pumping. If the ATPase were electroneutral, that

is, if it transported an anion in the same direction as the proton, or transported a cation in the opposite direction, then valinomycin would have no effect on proton pumping. Since valinomycin stimulates proton pumping, the proton pump must be electrogenic. That is, the V-type ATPase generates an electrical potential across the vacuolar membrane. In the cell, the greater the electrical potential difference that the vacuolar H^+-pumping ATPase generates across the membrane, the more energy it will take to transport a proton from the more negative side to the more positive side.

The K_m for the Mg^{2+}-ATP complex ranges between 0.2 and 0.81 mol/m^3. The ATPase hydrolyzes about 30–50 ATP/s and pumps between 60 and 90 H^+/s. The H^+/ATP stoichiometry varies between 1.75 and 3.28 (Ratajczak, 2000). Müller et al. (1996) discovered an unusual vacuolar ATPase from the fruits of lemons that differs from the canonical V-type ATPase, which is found in lemon epicotyls. Unlike the canonical V-type ATPase, the vacuolar ATPase in citrus fruits that are hyperacidic is sensitive to vanadate and insensitive to nitrate and bafilomycin. Interestingly, the unusual type of V-ATPase is found in the vacuolar membranes of acidic limes, but in not sweet limes, indicating that this protein is genetically adapted for vacuolar hyperacidity (Brune et al., 2002). By promiscuously exchanging DNA sequences, domains in a polypeptide can be swapped between the canonical ATPase polypeptides to form chimerical polypeptides with differing function, binding characteristics, localization, etc. This blurs the distinctions between the categories of ATPases one creates to pigeonhole transport proteins into a convenient system of classification before the diversity of proteins is established. So again, we learn that nature mocks human categories, and the vacuolar ATPases have evolved to ensure that lemons and acid limes are sour.

The vacuolar H^+-ATPase is a large protein and its structure can be seen in the electron microscope by negatively staining vesicles with phosphotungstic acid (Klink and Lüttge, 1991; Taiz and Taiz, 1991). The V_1 complex appears as an H- or V-shaped particle on a stalk with small projections emerging from the base. It is whimsical that the V-type ATPase looks like a V! Treatment with NO_3 inhibits the V-type ATPase because it causes the hydrophilic catalytic subunit to dissociate from the hydrophobic membrane channel complex (Adachi et al., 1990; Bowman et al., 1989). In electron micrographs of freeze-fractured preparations, the V-type ATPase appears as a particle 9.1 nm in diameter (Ratajczak, 2000). The vacuolar H^+-ATPase also occurs in other membranes in the cell and functions to acidify the compartments enclosed by these membranes (Maeshima et al., 1996). Grabe et al. (2000) suggest that the V-type ATPase is a mechanochemical enzyme and ATP hydrolysis by the V_1 complex causes a rotary torque on the V_0 complex that results in the translocation of a proton across the vacuolar membrane.

The vacuolar membrane contains another major H^+-translocating pump (Martinoia et al., 2007). The second one is composed of a single polypeptide and is fueled by the hydrolysis of pyrophosphate (P_iP_i) (see Figure 7.12; Rea and Sanders, 1987; Britten et al., 1992; Rea and Poole, 1993; Maeshima et al., 1996; Maeshima and Nakanishi, 2002). The H^+-$P_iP_i$ase is specifically inhibited by aminomethyldiphosphonate, a structural analog of pyrophosphate (Zhen et al., 1994).

H^+-pumping by both the V-type ATPase and the pyrophosphatase is stimulated by Cl^-. Cl^- stimulates the electrogenic H^+ pumps because it enters the vacuole though a Cl^- transporter and reduces, without eliminating, the net positive charge in the lumen. Thus, the vacuoles are able to generate a substantial pH difference. In essence, Cl^- permits the conversion of an electrical potential difference into a chemical difference. Thus, the lumen of the vacuole, like the stomach, is acidified by HCl (Wada and Anraku, 1994).

Why does the vacuolar membrane have two different H^+ translocators, an ATPase and a pyrophosphatase? Perhaps, at different times in a cell's life, the two substrates are more or less prevalent. For example, meristematic cells that are synthesizing DNA and RNA produce a lot of pyrophosphate. The pyrophosphatase can use the free energy of this "waste product" to build a proton motive force across the vacuolar membrane while helping to drive the reactions involving the synthesis of DNA and RNA. By contrast, in older cells, where biosynthetic reactions that generate pyrophosphate have slowed down or stopped, ATP is by far the most available substrate, so the ATPase should be more prevalent. This trend has been observed by Maeshima et al. (1996).

7.5.2 ABC (ATP-Binding Cassette) Transporters or Traffic ATPases

After intensive work on the vacuolar ATPase and pyrophosphatase, there was still room for the discovery of new carriers that are involved in the active transport of organic solutes. These carriers, known as ABC transporters or traffic ATPases, directly bind Mg-ATP and transport organic solutes, including alkaloids, endogenous toxins, xenobiotic toxins, and anthocyanins, into the vacuole (see Figure 7.12; Martinoia et al., 1993, 2000; Li et al., 1995; Goodman et al., 2004; Marinova et al., 2007). The ABC transporters are recognized by their requirement for Mg-ATP, insensitivity to the electrochemical potential of protons across the membrane, and inhibition by vanadate (Rea et al., 1998). There are large families of ABC transporters, each with a unique cellular or subcellular localization and function (Martinoia et al., 2002; Rea, 2007).

7.5.3 Slowly Activated Vacuolar Channels

Just as there are many channel types on the plasma membrane, there are also many channel types on the vacuolar membrane. Many of these kinds of channels may be related.

However, each class of channels differs in terms of its organ, tissue, and cellular and intracellular localization. Moreover, each type of channel has a certain conductance, ion selectivity, and type of regulation. Some kinds of channels are rapidly activated, others are slowly activated; some types of channels are voltage dependent or modified by pH or Ca^{2+}. It may be that there are sequences of amino acids that are capable of determining a given characteristic of a given class of channels. Combining such sequences over evolutionary time as a result of mixing and matching the gene sequences that encode the amino acid sequences may have created a single polypeptide or group of polypeptides that function as a channel for a specific ion with a certain type of regulation (Gilbert, 1978).

Using the patch-clamp technique invented by Bert Sakmann and Erwin Neher (1983), Rainer Hedrich and Erwin Neher discovered a channel in the vacuolar membrane of sugar beet vacuoles (Hedrich and Neher, 1987) that also exists in *Vicia faba* guard cells. This channel is neither a cation channel nor an anion channel but passes both K^+ and Cl^- with a $P_K/P_{Cl} \cong 3.5$. Since the permeability to K^+ is greater than the permeability to Cl^-, more positive charges pass through the channel than negative charges, and thus the net current is positive. Given the sign convention for patch clamping endomembranes, where the lumen of the vacuole is considered topologically equivalent to the external region surrounding a cell, a positive ionic current that passes from the vacuole to the cytosol is considered to be an inward current, and a positive current that passes from the cytosol to the vacuole is considered to be an outward current. The channel discovered by Hedrich and Neher (1987) is an inwardly rectifying channel, meaning that the net positive current, in the form of K^+ and Cl^-, passes from the lumen of the vacuole (E-space) to the cytosol (P-space). The single-channel conductance is approximately 280 pS, which is large for a single channel. The conductance is not constant, but varies with the KCl concentration. The channel is also activated by voltage when the potential on the E-side of the vacuolar membrane potential is approximately 0.06 V more positive than the potential on the P-side. Currently, it is not known whether the vacuolar membrane potential reaches this value in vivo. If it does, this class of channels, which is distributed on the vacuolar membrane with a density of $0.37/\mu m^2$, is extremely sensitive to the cytoplasmic concentrations of Ca^{2+} and H^+ and may function in the release of KCl from the vacuole during guard cell closure (Schulz-Lessdorf and Hedrich, 1995).

7.5.4 Water Channels

An abundant protein in the vacuolar membrane is called γ-TIP, which stands for *tonoplast intrinsic protein*. The vacuolar membrane intrinsic protein (γ-TIP) can act as a water channel (Maurel et al., 1993; Maurel, 1997; Niemietz and Tyerman, 1997; Tyerman et al., 1999, 2002; Baiges et al., 2002) and has been given the name *aquaporin*, suggesting that this is its function in vivo. Aquaporins are also found in the other organelle membranes in the cell (Katsuhara et al., 2008; Maurel et al., 2008). Given the fact that water can permeate the lipid bilayer (with its low specific hydraulic conductance but large area), as well as many proteins with aqueous channels (with their high specific hydraulic conductance but small area), I feel that it is unlikely that the selective advantage of aquaporins in plant cell membranes is to facilitate the permeation of water. It is possible that the physiological function of aquaporins is to pass small, nonionic molecules, including carbon dioxide, that are similar in chemical structure to water (Wayne and Tazawa, 1990; Wayne et al., 1994; Ishibashi et al., 1994; Nakhoul et al., 1998; Terashima and Ono, 2002; Uehlein et al., 2003, 2008; Hanba et al., 2004; Flexas et al., 2006; Kaldenhoff, 2006; Maurel et al., 2008; Warren, 2008).

7.6 FUNCTIONS OF THE VACUOLE

The five-kingdom classification system of Robert Whittaker (1969) separates organisms, in part, based on their mode of nutrition. The vacuolar compartment may have evolved in the various kingdoms to reflect these differences. Animals typically acquire food as either organisms or macromolecules and must digest them. This has led to the evolution of a vacuolar compartment that is primarily involved in digestion and is thus usually termed the *lysosomal compartment* (de Duve and Wattiaux, 1966). Plants, on the other hand, make their food out of small inorganic molecules like carbon dioxide, water, and nitrate, using the radiant energy of sunlight. In order to capture these molecules and energy, plants typically evolved an arborescent form and the vacuoles have evolved to take up space, which we will see allows the building of a structurally economical arborescent form. As a consequence of the multiplicity of functions of plant and fungal vacuoles, I will retain the name *vacuole* (Klionsky et al., 1990) to reflect its many functions (Marty, 1999; De, 2000; Robinson and Rogers, 2000). I will consider that one of the functions of the vacuole is to act as a lysosome.

7.6.1 Proteolysis and Recycling

In the plant, animal, and fungal kingdoms, cells must recycle their own protoplasm in times of starvation. They may recycle either within a cell or within the organism. During starvation conditions, the vacuolar compartment performs this function in a process known as autophagy (de Duve and Wattiaux, 1966; Dunn, 1990; Takeshige et al., 1992; Baba et al., 1994; Chen et al., 1994; Aubert et al., 1996; Moriyasu and Ohsumi, 1996; Niwa et al., 2004; Yano et al., 2004). Life, like Ouroboros, the self-sustaining, tail-eating

snake, is a balance between the synthesis and degradation of molecules (Bernard, 1865). Death is associated with a change in the balance, which leads to the destruction of biomolecules. Thus, within the cell, the basic unit of life, lies the very mechanism that can result in death. In fact, many diseases of humans result from malfunctions in the balance of synthesis and degradation, and even a decrease in degradation in the vacuole can be fatal (de Duve, 1981). Proteolysis, however, does not only occur in the vacuole, but in every cellular compartment (Vierstra, 1993).

When Rudolf Schoenheimer (1942) introduced stable isotope tracers into the study of metabolism, he was surprised to find that almost all the macromolecules in mature bodies undergo turnover, and thus, even the material of which living organisms are made is in constant flux, and we are not composed of the same atoms and molecules for our whole life. According to de Duve (1981), the average liver cell lives for many years, yet it destroys and rebuilds its protoplasm approximately every week. The potatoes we eat today become our brain tomorrow (Feynman, 1955). Some cells, like those in senescing leaves or those that will give rise to laticifers or to conducting elements of the phloem and xylem, undergo almost total proteolysis (Wodzicki and Brown, 1973; Matile, 1975). Such programmed cell deaths are known as *apoptosis* (Fukuda, 1996; Groover et al., 1997). Partial proteolysis may be important for dedifferentiation and redifferentiation. It is the vacuolar compartment that specializes in cellular recycling, and in the vacuole, organelles can be seen in the process of being degraded (Sievers, 1966; Villiers, 1967).

Many nonspecific hydrolytic enzymes with acidic pH optima occur in plant vacuoles (Matile, 1975; Nishimura and Beevers, 1978, 1979; Moriyasu, 1995; Muntz, 2007). Moriyasu and Tazawa (1988) tested the proteolytic capability of the vacuole by introducing an exogenous protein like bovine serum albumin (BSA) into the vacuole of giant algal cells. In this study, both ends of the cell were removed and about 10 μL of BSA were added to the vacuole, which contained approximately 50 μL of endogenous cell sap. The cell ends were then ligated and the cells were allowed to sit for various times. Then the proteins in the vacuole were collected, run on SDS polyacrylamide gels, transferred to nitrocellulose paper, and immunoblotted with antibodies directed against BSA. Indeed, the BSA was hydrolyzed, indicating that the vacuole is capable of proteolysis. Moriyasu et al. (1987) have also purified and characterized vacuolar proteases from *Chara*.

In the vacuole, the proteins are hydrolyzed into their constituent amino acids, and the amino acids are recycled back to the cytoplasm by way of an amino acid carrier on the vacuolar membrane. When the cell sap of *Chara* is replaced with artificial cell sap containing various amino acids, the amino acids leave the vacuole and enter the cytoplasm via an H^+/amino acid symporter (see Figure 7.12; Sakano and Tazawa, 1985; Amino and Tazawa, 1989).

Why don't the hydrolytic enzymes in the vacuole destroy the vacuolar membrane itself? According to Christian de Duve (1981), we could reply in the manner of the "medical student" in the last act of Molière's (1673) *Le Malade Imaginaire*. He answered the question, "Why does opium put you to sleep?" with the answer, "Opium puts you to sleep because it is a soporific." That is, we can say that the proteins in the vacuolar membrane have a conformation that makes them resistant to the vacuolar proteases. To paraphrase de Duve, as well as Bacon (1620), Locke (1824), and Hayakawa (1941), it would be just as well to say, "We do not know" than to worship the "Idols of the Marketplace."

7.6.2 Taking up Space

Unlike animals, which can gather food, plants are usually sessile and have a dendritic form that helps them acquire light and the necessary nutrients that are dilute in the environment. The vacuole is essential for plant survival in that it allows the plant to attain a large open dendritic structure with a minimum investment in energy-intensive compounds like cellulose or protein. Instead, the plant cell vacuole is filled with water, which is generally abundant and energetically cheap to obtain (Dixon and Joly, 1895; Dixon, 1938; Dainty, 1968; Wiebe, 1978; Taiz, 1992).

As a consequence of the large central vacuole, the cytoplasm is pushed to a parietal position, where the distance from the atmosphere to a chloroplast or mitochondrion is kept to a minimum. This can greatly enhance photosynthesis and respiration, since the diffusion rates of O_2 or CO_2 in air is approximately 10,000 times greater than they are in water (Table 7.3). The vacuole then ensures that resistance to diffusion of CO_2 and O_2 is kept to a minimum (Wiebe, 1978). According to Fick's Law, the flux of O_2 and CO_2 to the center of the cell will be proportional to the diffusion coefficient and inversely related to the distance it must travel:

$$J = -(DK/dx)dC \tag{7.2}$$

If CO_2 and O_2 are transported through the plasma membrane and utilized by the chloroplasts and mitochondria faster than they are transported through the cytosol, then photosynthesis and respiration, respectively, will be limited

TABLE 7.3 Diffusion coefficients for oxygen and carbon dioxide in air and water

		D (in m^2/s)	
Molecule	Temperature (K)	CO_2	O_2
Air	(273°K)	1.04×10^{-5}	1.89×10^{-5}
Water	(298°K)	1.94×10^{-9}	1.77×10^{-9}

Source: From Weast, R. C., ed. The Handbook of Chemistry and Physics. 54th Edition. Cleveland, OH: CRC Press, 1973–1974.

by diffusion through the cytosol. Although the permeability coefficient of the plasma membrane to CO_2 is between 2×10^{-6} and 3.5×10^{-3} m/s (Gutknecht et al., 1977; Gimmler et al., 1990; Wayne et al., 1994), with a CO_2 difference of approximately 0.05 mol/m^3 and a partition coefficient of 1, the flux across the plasma membrane would be at least 10^{-7} mol m^{-2} s^{-1}. The flux of CO_2 through the aqueous cytosol to the center of a 2×10^{-4} m in diameter mesophyll cell would be 9.7×10^{-8} mol m^{-2} s^{-1}. This flux may be limiting to photosynthesis and respiration. However, if the chloroplasts were pushed within 10^{-6} m of the plasma membrane, the flux would increase 100 times to 9.7×10^{-6} mol m^{-2} s^{-1}, and then photosynthesis and respiration would most likely be limited by their enzymes and not by the length of the diffusion pathway. Of course, the light intensity at the chloroplast is greater when the chloroplast is at the periphery of the cell compared to when it is in the center of the cell, and this too may enhance photosynthesis.

7.6.3 Storage and Homeostasis

Since vacuoles take up the better part of a cell, they contain the volume necessary to store levels of organic and inorganic molecules that would be toxic to the cytosol and the other organelles. In this way, vacuoles contribute to the protection of the cell and the maintenance of a cellular homeostasis in terms of ions, water, and amino acids (Matile, 1987; De, 2000). All vacuoles store water. This water is in equilibrium with the protoplasm and keeps the protoplasm hydrated so that enzymatic reactions can take place. This is a vital function in plants, which have undergone an evolutionary process from living in water to living on arid land. Water storage is particularly important to desert plants, which is one reason that they are so succulent (Walter and Stadelmann, 1968).

The acidic nature of most vacuoles has been known for a long time from looking at the color of natural or introduced dyes. Vacuoles are typically acidic (pH ~5) and thus act as a store of H^+. As a consequence of the large capacity of the vacuole to store H^+, it can function in pH regulation (Moriyasu et al., 1984; Takeshige et al., 1988; Takeshige and Tazawa, 1989b; Grabe and Oster, 2001). In fact, the vacuole is involved in the pH regulation necessary to protect plants from acid rain (Heber et al., 1994).

The pH of the vacuole of the brown alga *Desmerestia* is less than 1 (Wirth and Rigg, 1937; McClintock et al., 1982). The pH of the vacuoles of the juice cells of lemons and acid limes is also hyperacidic compared with most vacuoles, reaching values as low as 2–2.2 (Echeverria and Burns, 1989; Echeverria et al., 1992). Two factors may allow these hyperacidic vacuoles to store so many protons. First, the V-ATPase in these cells is atypical and may transport only one H^+ per ATP hydrolyzed (Figure 7.13), which would allow the vacuole to be more acidic, and second, the vacuolar membrane of the acidic juice cell vacuoles has less permeability to the passive movement of H^+ than vacuolar membranes in typical cells, so once H^+ are pumped into the vacuole, they will tend to stay there (Müller et al., 1996, 1997, 1999; Brune et al., 2002; Müller and Taiz, 2002).

The hydrolysis of a single molecule of ATP provides approximately 8×10^{-20} J of molecular free energy. According to the following equation, this is a sufficient quantity of energy to pump two protons from the cytosol to the vacuole when the pH of the cytoplasm ($-\log H^+_c$) is about 7, the pH of the vacuole ($-\log H^+_v$) is about 3, and the electrical potential (ψ_c) across the vacuolar membrane is about -0.02 V, but only enough to pump 1 H^+ from the cytosol to the vacuole when the pH of the vacuole is as low as 2.1. By initially equating the molecular free energy of ATP hydrolysis with the electrochemical energy necessary to transport an H^+ from the cytosol to the vacuole (Smith et al., 1982; Bennett and Spanswick, 1984b), we get:

$$E_{ATP} = n(ze\Psi_c + kT \ln(H^+_v/H^+_c))$$

where n is the number of H^+ transported per ATP molecule hydrolyzed and is known as the *coupling ratio* (Läuger, 1991; Schmidt and Briskin, 1993; Davies et al., 1994; Davies, 1999). Of course, at equilibrium, there is not any net H^+ transport since just as much ATP is hydrolyzed to pump protons across the vacuolar membrane as ATP is synthesized by the passive flow of H^+ through the pump. That means that to pump protons into the vacuole, $E_{ATP} > n\ [ze\psi_c + kT\ \ln(H^+_v/H^+_c]$. Remembering that death, not life, is characterized by the equilibrium state, we can nevertheless use equilibrium thermodynamics as a first approximation. However, once we are able to determine concentrations of metabolites and ions under nonequilibrium conditions, we can use irreversible or nonequilibrium

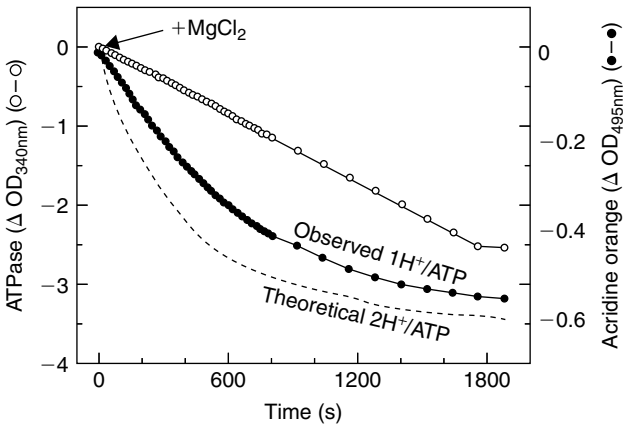


FIGURE 7.13 Simultaneous measurement of ATPase activity (open circles) and proton pumping (closed circles) from acid lime. (*Source:* From Brune et al., 2002.)

thermodynamics to provide a more realistic description of life. Solving for n under equilibrium conditions, we get:

$$n = E_{ATP}/(ze\Psi_c + kT \ln(H^+{}_v/H^+{}_c))$$

The electrochemical energy that is inherent in the extreme difference in the pH between the vacuole and the cytosol of the juice cells of lemons and acid limes provides the molecular free energy to transport high quantities of citric acid from the cytosol, where its concentration is less than 10 nM, to the vacuole, where its concentration is 325 mM (Brune et al., 1998; Ratajczak et al., 2003). The large pH gradient in juice cells is enhanced by the nonenzymatic hydrolysis of sucrose into organic acids in the vacuole (Echeverria and Burns, 1989; Echeverria et al., 1992).

While vacuoles are typically acidic, not all vacuoles are acidic, and the blue color of the epidermal cells of heavenly blue morning glories is due to the alkaline nature of the vacuole as a consequence of Na^+/H^+ exchange (Yoshida et al., 1995, 2005). Interestingly, the vacuole of the epidermal cells are basic when the flowers are ripe for pollination, but are acidic before the flowers are ripe for pollination and after the flowers have been pollinated. When the epidermal cell vacuoles are acidic, the anthocyanins are purple and the purple flowers do not compete for the attention of the pollinator bees.

Vacuoles also store nutrients, like PO_4 (Mimura et al., 1990) and other ions (Leigh, 1997), as well as sugars (Fisher and Outlaw, 1979; Kaiser et al., 1982; Gerhardt and Heldt, 1984; Keller and Matile, 1985; Matile, 1987; Keller, 1992; Martinoia and Ratajczak, 1999) and amino acids (Wagner, 1979; Wayne and Staves, 1991; Riens et al., 1991). Many plants that are salt tolerant store Na^+ in the vacuole, thanks to a Na^+/H^+ antiporter (Staal et al., 1991; Epimashko et al., 2004).

Vacuoles can act as an intracellular toxic waste site and store substances that would be harmful if kept in the cytoplasm. Some of these compounds, including nicotine (Saunders, 1979; Steppuhn et al., 2004; Howe and Jander, 2008), protect the plant from would-be predators. Many secondary substances, particularly alkaloids that are useful to cell biologists and other human beings, are stored in the vacuole (Hobhouse, 1986; Ziegler and Facchini, 2008). These include trypsin inhibitors, antifungal phytoalexins, vinblastine, vincristine, colchicine, rubber, morphine, serpentine, caffeine, etc. (Blom et al., 1991; Sottomayor et al., 1996; Costa et al., 2008; Hagel et al., 2008). Some of these substances, which are membrane permeant, are trapped in the vacuole and do not leak into the cytoplasm because they form complexes with other molecules, including polyphenols and tannins, which increases their apparent size and polarity (Mösli Waldhauser and Baumann, 1996). High concentrations of heavy metals, found in abandoned industrial and mining sites, would be toxic if they were in the cytosol. These are also sequestered in the vacuole.

The beautiful reds, blues, and purples of autumn leaves as well as fruits and flowers are a consequence of the anthocyanins that are stored in the vacuole (Thimann, 1950; Moskowitz and Hrazdina, 1981; Andersen and Markham, 2006). The term *anthocyanin* was coined by L. Marguartin in 1835, and over the next century, the anthocyanins were isolated and characterized and the structures were deduced and confirmed by synthesis due to a large extent to the work of chemists Richard Willstätter and Sir Robert and Lady Robinson (Onslow, 1916; Robinson, 1955; Willstätter, 1965).

Not only have chemists been instrumental in understanding anthocyanins, but anthocyanins have also been instrumental for the progress of chemists. Robert Boyle (1664) used the anthocyanins of violets as a pH indicator, and Jeremias Richter (1792–1794) used the pH-indicating ability of the anthocyanins of violets to determine the quantity of acid needed to neutralize a quantity of base, and in doing so, came to us with the concept of stoichiometry. The discovery of fixed stoichiometries formed the foundation necessary for the introduction of the mole concept in chemistry (Kieffer, 1963). The colors of the anthocyanins in the flower are determined by the pH of the vacuoles in the epidermal (Asen et al., 1975; Stewart et al., 1975; Kondo et al., 1992; Yoshida et al., 1995, 2005) or subepidermal (Yoshida et al., 2003) cells.

While studying the unstable inheritance of the mosaic pattern of blue, brown, and red spots that results from the differential production of vacuolar anthocyanins in the aleurone cells of a single maize kernel, Barbara McClintock (1950) discovered transposable elements. The transposable elements regulated the color of the kernels. When a transposable element moved into a gene-controlling anthocyanin synthesis (Grotewold, 2006; Lepiniec et al., 2006; Cone, 2007), anthocyanin synthesis was suppressed in the aleurone cells of the kernel, and when it moved out of a gene-controlling anthocyanin synthesis, the pigment was produced. The randomness of the color mosaicism in the kernel reflects the randomness of the spatial insertion of the transposable element in the genome of the aleurone cells. The size of the colored spot on the kernel depends on the randomness of the timing of the insertion of the transposable element into a gene that leads to anthocyanin synthesis. By studying the unstable inheritance of the distribution of vacuolar coloration, McClintock realized that functionally differentiated cells in a multicellular organism must be a result of the differential expression of an identical genome. Moreover, McClintock (1983) realized that the genome itself was not static, but could respond rapidly to challenges.

The dynamic nature of the genome as a result of "jumping genes" was not readily accepted by geneticists until they realized that the rapid evolution of antibiotic resistance in bacteria could be explained by a similar mechanism. McClintock's observations on the mosaic pattern of vacuolar anthocyanins in the aleurone cells of maize also lead to the understanding of how the movement of oncogenes from one position to another can result in cancer in humans.

Barbara McClintock was awarded the 1983 Nobel Prize in Physiology or Medicine. The presentation speech on behalf of the Nobel Assembly of the Karolinska Institute ended with the following words:

> *I have tried to summarize to this audience your work on mobile genetic elements in maize and to show how basic research in plant genetics can lead to new perspectives in medicine. Your work also demonstrates to scientists, politicians and university administrators how important it is that scientists are given the freedom to pursue promising lines of research without having to worry about their immediate practical applications. To young scientists, living at a time of economic recession and university cutbacks, your work is encouraging because it shows that great discoveries can still be made with simple tools.*

The readily visible anthocyanins in flower petals have also helped in the discovery that double-stranded RNAs are involved in gene expression (Fire, 2006; Mello, 2006). The discovery of gene silencing came unexpectedly from the observations of Napoli et al. (1990) and van der Krol (1990) who introduced a chalcone synthase gene into petunia plants with the hopes that the overexpression of this gene would increase anthocyanin synthesis and improve the color of the flowers. However, they found that the transformed plants lost the ability to produce anthocyanins in the vacuole and only produced white flowers. This led to a rethinking of what happens in the generation of transgenic plants and during normal gene expression (see Chapter 16; Dougherty and Parks, 1995).

Vacuoles also store flavones, and occasionally (e.g., in snapdragon flowers) the yellow color of petals are due to the presence of flavones, although typically, the yellow color of flowers comes from pigments in the plastids.

Desert plants as well as many other plants that have crassulacean acid metabolism (CAM) utilize the vacuole as a storage site for organic acids, including malic acid (Kenyon et al., 1978, 1985; Winter and Smith, 1996; Black and Osmond, 2003). Plants exhibiting crassulacean acid metabolism open their stomata at night in order to minimize transpirational water loss. These plants are able to use phosphoenolpyruvate (PEP) carboxylase to fix CO_2 at night and store the fixed CO_2 as malic acid (Pucher et al., 1947; Vickery, 1953; Bandurski and Greiner, 1953; Bandurski, 1955; Epimasko et al., 2004). During the day, the stomata close in order to conserve transpirational water loss. In the presence of light, electron transport occurs and adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) are formed by the light reactions of photosynthesis. Simultaneously, the CO_2 is released from the malic acid by the $NADP^+$-malic enzyme to become refixed by RuBP carboxylase (see Chapter 13).

In developing seeds, proteins are stored in the vacuole (Levanony et al., 1992; Li et al., 1993a,b; Jiang et al., 2000, 2001; Kumamaru et al., 2007). These protein-storing vacuoles are usually called *protein bodies*. During germination, the protein-storing vacuole acidifies (Swanson and Jones, 1996; Hwang et al., 2003). Subsequently, the proteins are hydrolyzed and the amino acids are mobilized to nourish the growing embryo (Filner and Varner, 1967; Graham and Gunning, 1970).

7.6.4 Role in Turgor Generation

The studies of osmotic and turgor pressure in plants done by Pfeffer (1877) and de Vries (1884) provided the experimental basis necessary for Jacobius van't Hoff (1888, 1901) to apply the gas laws to molecules in solution (Wald, 1986). Van't Hoff learned of Pfeffer's experimental results from his friend de Vries who asked van't Hoff to come up with a theoretical explanation of the results. Pfeffer had measured the osmotic pressure (P_π) at a given temperature of solutions made up of various concentrations of nonelectrolyes (C). Van't Hoff took Pfeffer's results and merely divided P_π by C and saw that this quotient was a constant at constant temperature (Figure 7.14a). Pfeffer also determined the effect of various temperatures on the osmotic pressure of a given solution, and again van't Hoff noticed that, for a given concentration of solute, P_π/T was a constant (see Figure 7.14b).

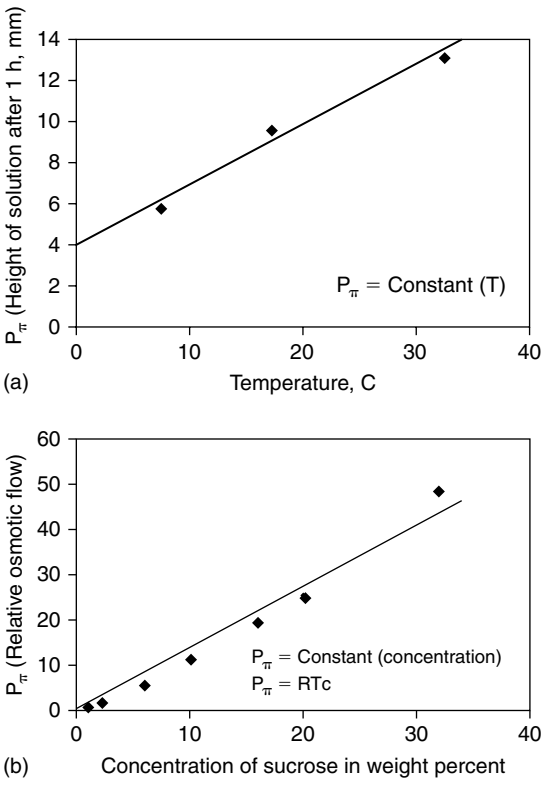


FIGURE 7.14 Two graphs of Pfeffer's (1877) tabular data. (a) The height of a sucrose solution in an osmometer held at various temperatures. (b) The osmotic flow of sucrose solutions of various concentrations at a given temperature.

By hypothesizing that liquids behave in an analogous manner to gases, he framed these two results in terms of Boyle's Law (PV = constant at constant temperature) and Gay Lussac's Law (P/T = constant at constant volume). Van't Hoff combined these two equations to deduce that

$$P_{\pi} = RTC \tag{7.3}$$

where R is a combination of the two constants mentioned above and is equal to the universal gas constant. Since concentration (C) = amount in moles (s) divided by volume (V), then

$$PV = sRT \tag{7.4}$$

which is a restatement of the gas law.

The introduction of the gas constant gave the equation immediate significance, since now the colligative properties of solutions could be examined from a thermodynamic perspective. Van't Hoff used his new equation, which was backed up by thermodynamic theory, to deduce the laws of many diverse phenomena, including the effects of solutes on the vapor pressure and freezing point of a solution, as well as Guldberg and Waage's law of chemical equilibrium. Here is a case where a little mathematics applied to empirical physiological observations helped to not only generalize the observations within the field of plant cell biology, but also to open up the field of physical chemistry.

One far-reaching effect of the van't Hoff equation is that it provided the theoretical and experimental basis for calculating the molecular mass of nonvolatile substances, an important procedure that was uncertain until this time (Ostwald, 1891; Pattison Muir, 1909). Since $P_{\pi} = RTC$ and C is equal to the number of moles of a substance (s) divided by the volume of the solution (V), then

$$P_{\pi} = (RT)(s/V) \tag{7.5}$$

Furthermore, the molecular mass of a given solute could be determined since s is equal to the number of grams added to the volume divided by the molecular mass of the substance (M_r):

$$M_r = (\text{grams added})(RT)/(VP_{\pi}) \tag{7.6}$$

That is, the molecular mass of a solute could be determined by measuring the osmotic pressure of a known mass in a given volume at a given temperature.

Equation 7.6 only holds for nonionized substances. However, van't Hoff also incorporated the observations of de Vries (1884, 1888a) on plasmolysis. De Vries noticed that it takes a lower concentration of KNO_3 compared with sucrose to plasmolyze various cells. De Vries determined the concentration of various compounds that were required to cause incipient plasmolysis. He then found the concentration of each chemical that was as effective as KNO_3 and ranked the effectiveness of all these compounds relative to KNO_3. He dubbed the ratio of the concentration of KNO_3 to the concentration of a given substance, the isotonic coefficient. Svante Arrhenius realized that the isotonic coefficient was an indication that salts ionized in solution, and van't Hoff included this interpretation in a latter form of his equation that applies to electrolytes:

$$P_{\pi} = iRTC \tag{7.7}$$

where the dimensionless ionization coefficient, i, represents the number of particles that each salt produces when it ionizes in solution. Peter Debye and Erich Hückel discovered that when the concentration of salts is high, the molecules do not dissociate completely, and corrections to the above formula must be made (Laidler, 1993).

It took a long time before chemists believed Svante Arrhenius' idea that when salts were dissolved in water they decomposed into their constituent charged atoms. The difficulty in believing this arose from the observations that pure metals like sodium reacted violently with water, and thus pure sodium could not be produced in the tranquil solution of NaCl. Likewise, chlorine was a green gas, yet a solution of NaCl did not turn green and bubble. The fact that the theoretically and experimentally robust van't Hoff equation would only apply to salts if they were considered to be ionized helped convince Arrhenius' contemporaries of the reality of ionization (Arrhenius, 1903, 1912).

Now back to plant cell biology! As a consequence of the presence of solutes in the cell, the differential permeability of the plasma membrane, and the rigidity of the extracellular matrix, water enters the cell and generates a turgor pressure of several 100,000 Pa. The potential energy of a volume of water in the cell is known as its *water potential* and it is given in J/m^3 or Pa. Since membranes that are not protected by the extracellular matrix typically lyse when the hydrostatic pressure difference across a membrane exceeds approximately 100 Pa (Wolfe et al., 1986), the water potential of every organelle must be the same as the cytosol or the organellar membrane will break. Therefore, the total concentration of solutes is essentially equal in all of the compartments of the cell and turgor pressure is only generated across the plasma membrane.

Plants growing in environments with high osmotic pressure, due particularly to the presence of salts, must produce sufficient osmotic pressure within their cells to allow the uptake of water necessary for life and growth. Under these conditions, the high concentrations of osmoticum in the cytosol and the matrix of each organelle must allow the normal enzymatic reactions to take place, and thus the osmoticum must not adversely affect the enzymes. Depending on the cell, glycerol, trehalose, betaines, and proline act as "compatible solutes" that can be used to increase the osmotic pressure of the cytosol or an organelle without adversely

affecting its enzymes. The compatible solutes often, but not always, mimic water by having many OH groups.

By contrast, the enzymes in the vacuole are not so particular and it appears that any osmoticum can be used to generate osmotic pressure. The osmotic pressure (P_π) of the vacuole and the cytoplasm must be equal. While the plasma membrane and not the vacuolar membrane may be the primary mediator of turgor regulation, the large size of the vacuole means that the number of moles of osmoticum is greater in the vacuole than in the rest of the cell. The components that usually contribute the most to the osmotic pressure of the vacuole are Na^+, K^+, and Cl^- (Bisson and Kirst, 1980; Okazaki, 1996). These are relatively "energetically cheap" turgor-generating substances. However, they are incompatible solutes and must be kept away from the enzymes localized in the cytosol.

Turgor pressure inside the cell (P_{ti}) results from the pressure that is exerted by the protoplast against the extracellular matrix when water moves down its water potential difference from outside the cell where $P_{to} = 0$ and $P_{\pi o}$ is small to inside the cell where $P_{\pi i}$ is large.

The water potential (P_w) is equal to the difference between the hydrostatic or turgor pressure (P_t) and the osmotic pressure (P_π). All quantities are given in Pascals.

$$P_w = P_t - P_\pi \tag{7.8}$$

Water passively moves from regions of high water potential (high energy/volume) to regions of low water potential (low energy/volume). Consequently, for passive flow, $P_{w\ \text{final}} - P_{w\ \text{initial}}$ is negative. Specifically, the water potential inside the cell (P_{wi}) and outside the cell (P_{wo}) are given by the following equations:

$$P_{wi} = P_{ti} - P_{\pi i} \tag{7.9}$$

$$P_{wo} = P_{to} - P_{\pi o} \tag{7.10}$$

At equilibrium, where there is no net water movement,

$$P_{wi} = P_{wo} \tag{7.11}$$

Therefore:

$$P_{to} - P_{\pi o} = P_{ti} - P_{\pi i} \tag{7.12}$$

Since $P_{to} = 0$ (by definition in most cases):

$$P_{ti} = P_{\pi i} - P_{\pi o} \tag{7.13}$$

A change in $P_{\pi i}$ will cause the turgor to increase or decrease. Since $P_{\pi i}$ depends mostly on Na^+, K^+, and Cl^-, an increase in these ions will result in an increase in turgor, while a decrease in these ions will result in a decrease in turgor (see Chapter 12). The turgor pressure of plant cells is typically between 10^5 and 10^6 Pa, although higher and lower values exist.

The turgor pressure that is generated by the cell is responsible for providing the motive force for continued cell expansion and shape generation (see Chapter 20; Harold, 1990). It also provides the motive force for leaflet movements, tendril curling, stomatal movements, and fungal invasions.

7.6.5 Other Functions

The small vacuoles in the tip of a *Chara* rhizoid are filled with barium sulfate crystals. These crystals act as statoliths and fall in a gravitational field. As they settle, they displace the Golgi-derived vesicles from the lowermost side. Growth that is dependent on the deposition of the Golgi-derived vesicles is restricted to the upper surface and the rhizoid bends toward the earth in a positive gravitropic manner (Schröter et al., 1975).

The low density of the cell sap compared to the rest of the cytoplasm is important for understanding buoyancy regulation (Raven, 1984; Walsby, 1975), dispersal mechanisms (Gregory, 1961), and gravity sensing (Wayne and Staves, 1991) in single cells.

7.7 BIOTECHNOLOGY

In a series of studies that range from biophysics to biotechnology, Eduardo Blumwald has been able to overexpress in tomato and canola plants a vacuolar Na^+/H^+ antiporter, which allows the plants to grow in 200 mM NaCl. The salt that enters the xylem is transported to the leaves and is sequestered in the vacuoles of the cells of the leaf. Since water is supplied to the fruits from the phloem and not the xylem, and since salt in general does not enter the phloem, the fruits from the plants growing in high salt are not salty (Epstein, 1983; Apse et al., 1999; Shi et al., 2000; Zhang and Blumwald, 2001; Zhang et al., 2001; Lv et al., 2008; Uddin et al., 2008).

7.8 SUMMARY

The vacuole is the most conspicuous organelle in plant cells. Because of its large volume, it is involved in storing many inorganic and organic molecules, and in so doing, functions in homeostasis. The vacuole is also important in storing the molecules necessary for plant defense and the osmoticum necessary for generating turgor pressure. The vacuole is also

the recycling center of the cell and has the mechanisms necessary for collecting organelles and macromolecules, degrading them, and returning their constituent parts to the cytosol.

In this chapter, I discussed the interplay between plant cell biology and physical chemistry and the importance of the vacuole and its contents in developing the foundations of physical chemistry.

7.9 QUESTIONS

7.1. What are the many functions of the vacuole?
7.2. Why is the vacuolar compartment so well developed in plants compared with animals?
7.3. How have the readily visible anthocyanins in the vacuole been important for making discoveries in genetics?

Chapter 8

Movement within the Endomembrane System

I get my best ideas while taking a bath. But with biology I have problems; I always have to jump out and look up a fact.

—Leo Szilard (quoted in George Feher, 2002)

8.1 DISCOVERY OF THE SECRETORY PATHWAY

We have seen that cells are composed of a multitude of membranous motifs, including the endoplasmic reticulum (ER), the Golgi apparatus, the vacuole, and the plasma membrane. In this chapter, I will discuss how the various membranes are generated, as well as the relationships between the various membrane systems (Claude, 1970; Griffiths, 1996; Robinson et al., 2007). The relationships between the various membranes were revealed to a large extent by studying the secretory process in pancreatic exocrine cells.

Henri Dutrochet (1824, quoted in Schwartz and Bishop, 1958) postulated that

> *it is within the cell that the secretion of the fluid peculiar to each organ is effected. … The cell is the secreting organ par excellence. It secretes, inside itself, substances which are, in some cases, destined to be transported to the outside of the body by way of the excretory ducts, and, in other cases, destined to remain within the cell which has produced them.*

In the 1870s and 1880s, Rudolf Heidenhain (1878) studied the exocrine cells of the pancreas of mammals. He noticed that shortly after an animal ate, microscopic granules disappeared from the apical part of their pancreatic cells, and reappeared a few hours later. He correlated the disappearance of the apical granules with the appearance of digestive enzymes in the pancreatic juices that he measured biochemically, and concluded that the granules, which he dubbed *zymogen granules*, contained the precursors of the digestive enzymes. The zymogen granules, he supposed, represented an available store of digestive enzymes that could be released upon eating.

Limited by technology, Heidenhain was unable to elucidate the intracellular pathways involved in the secretion of the chymotrypsinogen, trypsinogen, and α-amylase that are stored in the zymogen granules. However, impressed with Heidenhain's work that combined morphology with biochemistry, George Palade (1959) set out to understand the intracellular part of the secretory process using and, more importantly, integrating the newly developed techniques of electron microscopy and cell fractionation. Palade (1959) considered these studies to be "a collaboration over almost a century between Rudolf Heidenhain, Philip Siekevitz, and myself." The integrated studies by Palade and his colleagues have become a watershed in the study of the intracellular secretory pathway. These papers constitute a good pedagogical example of the interplay between theory and experiment, inductive and deductive reasoning, and technique and interpretation.

While all cells secrete one thing or another, Palade chose to study secretion in cells that specialized in secretion. Palade and his colleagues injected ^{3}H-leucine into guinea pigs to radiolabel the newly synthesized proteins and then rapidly isolated the pancreas to follow the intracellular movement of the nascent proteins. Siekevitz and Palade (1958a,b,c, 1959, 1960a,b), using subcellular fractionation techniques, and Caro and Palade (1964), using radioautography at the EM level, extended Heidenhain's conclusion by showing that the digestive enzymes, stored in the smooth membrane enclosed zymogen granules at the apical end of pancreatic exocrine cells, were synthesized on the rough ER at the basal region of the cell (Figures 8.1–8.3). However, their ability to resolve the pathway followed by the digestive enzymes as they moved from the rough membranes at the basal portion of the cell to the smooth membrane–enclosed vesicles at the apical region of the cell was compromised by the fact that it took too much time to label the newly synthesized proteins by intravenously supplying the pancreas with radioactive amino acids.

The lack of temporal resolution was overcome by Jamieson and Palade (1966, 1967a,b, 1968a,b, 1971a,b), who switched from whole guinea pigs to pancreatic tissue slices. This minimized the time it took for the tracer to travel to, and to diffuse into, the site of incorporation; since the

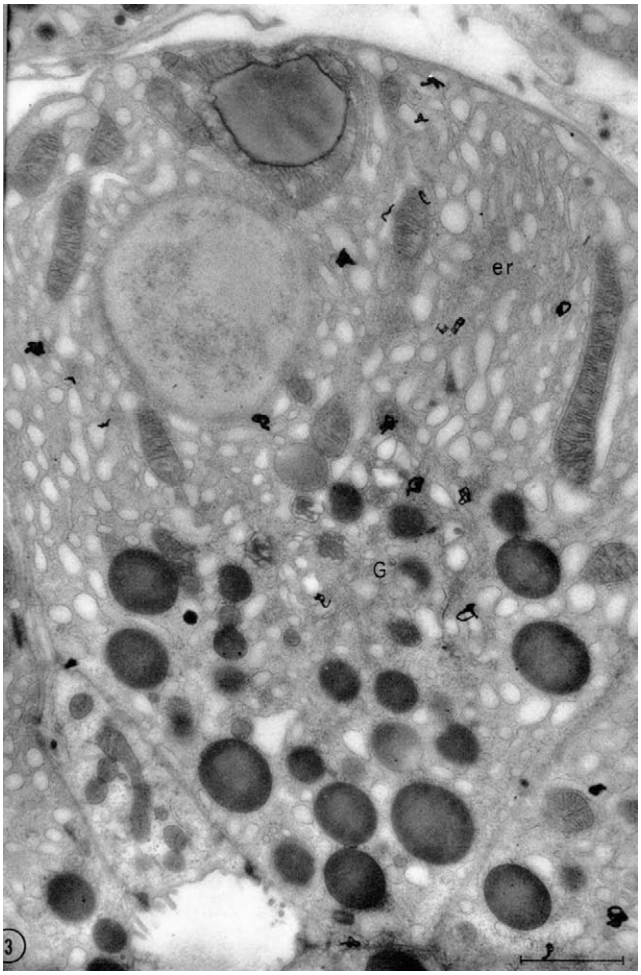


FIGURE 8.1 Electron microscopic autoradiograph of an exocrine cell 5 minutes after a pulse injection of ^{3}H-leucine into the guinea pig. Most of the grains are over the rough ER. ×21,000. (*Source:* From Caro and Palade, 1964.)

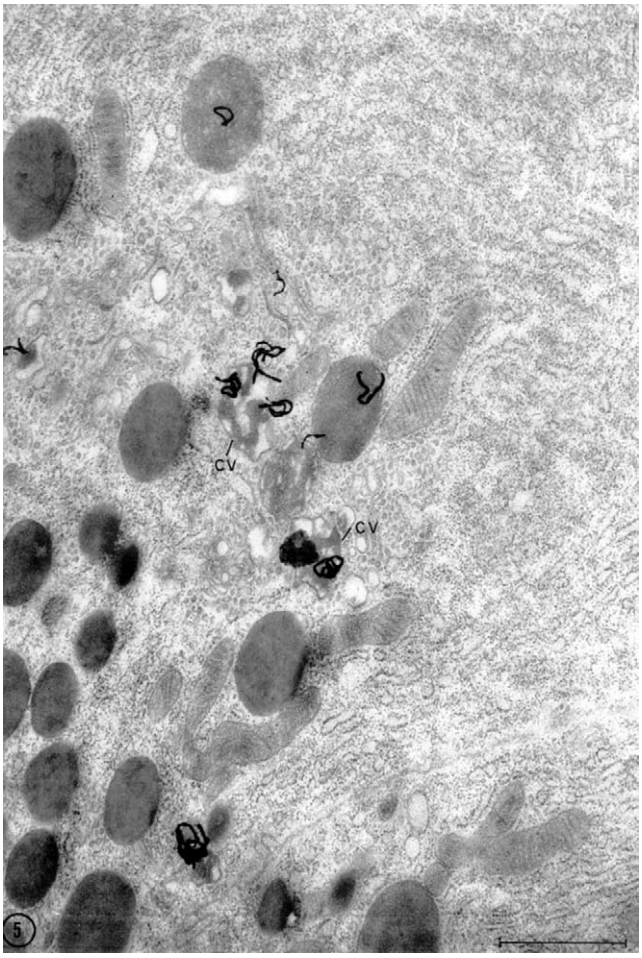


FIGURE 8.2 Electron microscopic autoradiograph of an exocrine cell 20 minutes after a pulse injection of ^{3}H-leucine into the guinea pig. The radioactivity has left the ER and most of the grains are over the Golgi stacks and condensing vacuoles (CVs). ×26,000. (*Source:* From Caro and Palade, 1964.)

time it takes to diffuse into the exocrine cells is minimized when the surface-to-volume ratio is maximized. Moreover, thin sections maximized the uniformity of labeling in each cell since the tracer enters all the cells at approximately the same time. Now Jamieson and Palade were able to see a precursor-product relationship between organelles just as biochemists had seen in chemical reactions. Tissue slices had previously been used successfully by many biochemists, including Otto Warburg, Albert Szent-Györgyi, and Hans Krebs, to maximize the temporal resolution necessary to determine the sequences in a pathway (see Chapter 14).

Jamieson and Palade labeled the cells for only 3 minutes with radioactive leucine and then replaced the radioactive leucine with an excess concentration of unlabeled leucine. They followed the movement of the label with both electron microscopic radioautography and by cell fractionation. They discovered that the label moved like a wave through the cell (Figure 8.4). It started at the ribosomes on the ER, which represent the site of protein synthesis. The protein then entered the lumen of the ER and was only released by treatments that break the membranes. After 7 minutes, the label appeared in the peripheral vesicles of the Golgi apparatus. The labeled protein probably took a membranous route from the ER to the Golgi apparatus since the label never increased in the cytosolic fraction. Moreover, the labeled protein traveled from lumen to lumen, since it could only be released from the Golgi membranes by high-pH treatments that destroyed membrane integrity. Thirty-seven minutes following the pulse label, the protein appeared in the condensing vacuoles at the trans-Golgi network. Approximately 2 hours after the pulse label, essentially all of the labeled protein was in the zymogen granules at the apical end of the cell where they are stored. Thus, the fact that they selected favorable material for studying secretion, combined with their biophysical insight in deciding to use tissue slices in order to obtain a uniform and rapid uptake of labeled amino acids, allowed Jamieson and Palade to see the movement of proteins from the ER to the Golgi apparatus.

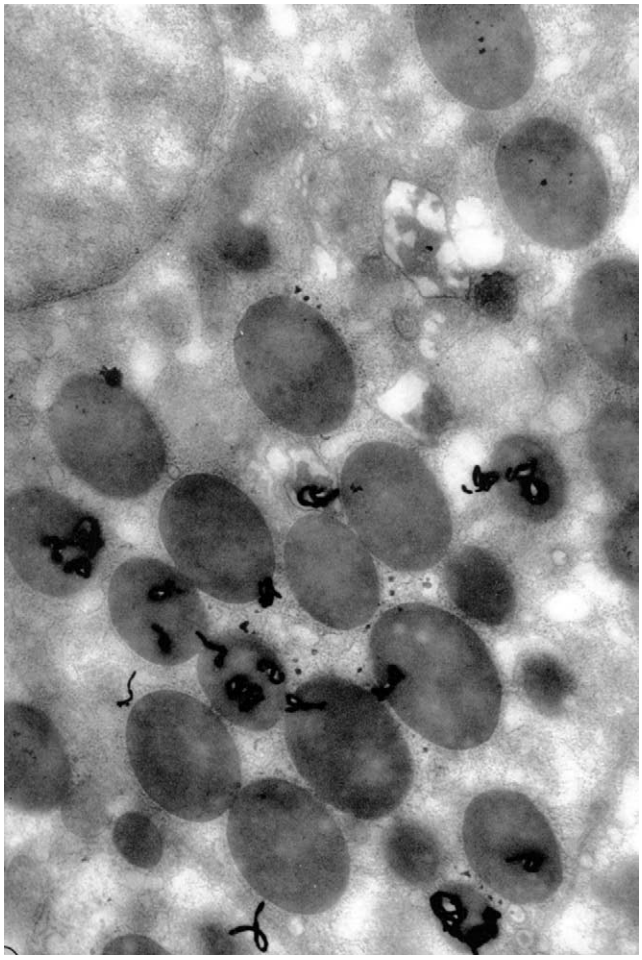


FIGURE 8.3 Electron microscopic autoradiograph of an exocrine cell 4 hours after a pulse injection of ^{3}H-leucine into the guinea pig. The label has left the ER and Golgi stacks and most of the grains are over the mature zymogen granules. ×24,000. (*Source:* From Caro and Palade, 1964.)

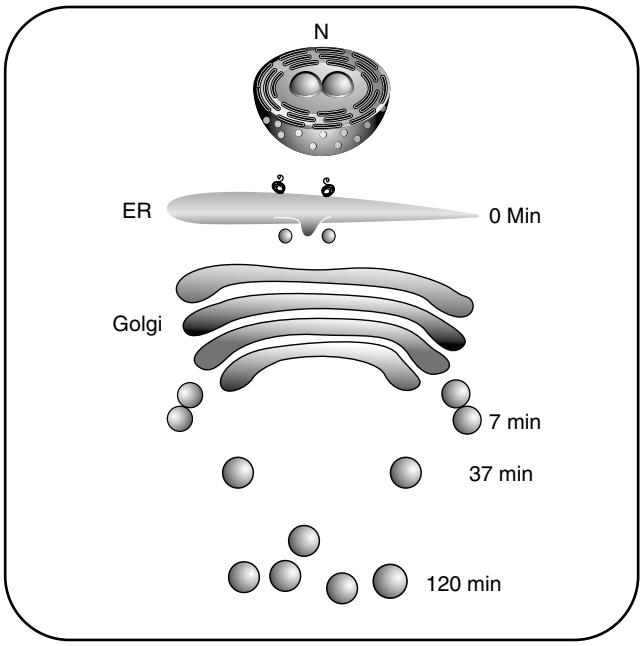


FIGURE 8.4 Spatiotemporal map of the secretory process discovered by Palade, Caro, Siekevitz, and Jamieson in pancreatic exocrine cells.

Interestingly, treating the tissue slices with chemicals that stimulate secretion caused an increase in secretory flow by increasing the amount of protein secreted, not by increasing the velocity of movement of a given protein through the secretory pathway. Thus, there must be an increase in the rate of protein synthesis and/or the area of the pathway. Indeed, the secretion-stimulating agents cause an elaboration of the Golgi apparatus!

While Jamieson and Palade followed intracellular transport through the secretory pathway with ^{14}C-leucine-labeled proteins, Northcote and Pickett-Heaps (1966) and Neutra and Leblond (1966) followed the movement of labeled glucose and discovered that the Golgi apparatus is a major site of glycosylation. Neutra and Leblond (1966) followed the movement of ^{3}H-glucose containing mucus glycoproteins through the secretory pathway of rat goblet cells with electron microscopic radioautography. They found that by 5 minutes after injecting the ^{3}H-glucose into a rat, the sugar was incorporated into glycoproteins in the Golgi apparatus. After 20 minutes of continuous labeling, the label is found in both the Golgi apparatus and the mucigen granules, and after 40 minutes, the label is in the mucigen granules. Unfortunately, these experiments were not done with tissue sections and with a short pulse, so the time resolution is marginal. However, at the same time, pulse-chase experiments with wheat root tips were done by Donald Northcote and Jeremy Pickett-Heaps although the extracellular matrix components they were interested in studying were not proteinaceous, but were exclusively composed of polysaccharides. They found that after a 5-minute pulse with radioactive glucose, the label showed up in the Golgi apparatus (Figure 8.5). Following a 10-minute pulse, the label was found in the Golgi apparatus and its associated vesicles. When the 10-minute labeling period was followed with a chase period (for 10–60 minutes), the label declined in the Golgi apparatus and its associated vesicles and appeared in the extracellular matrix (Figure 8.6). These data indicate that the Golgi apparatus in plant and animal cells serves as a site of protein glycosylation and polysaccharide synthesis in the intracellular secretory pathway.

Plant cells can have highly developed secretory systems, although the intracellular pathways have not been worked out with the high temporal resolution attained by Jamieson and Palade. While all cells in plants are involved in secretion to some extent, secretion often takes place in highly developed, taxonomically important, and truly gorgeous glandular trichomes. Many halophytes have glands that secrete salt in order to maintain a livable internal salt balance. Other plants secrete sweet nectar to attract pollinating insects, while insect-eating plants like *Drosera*

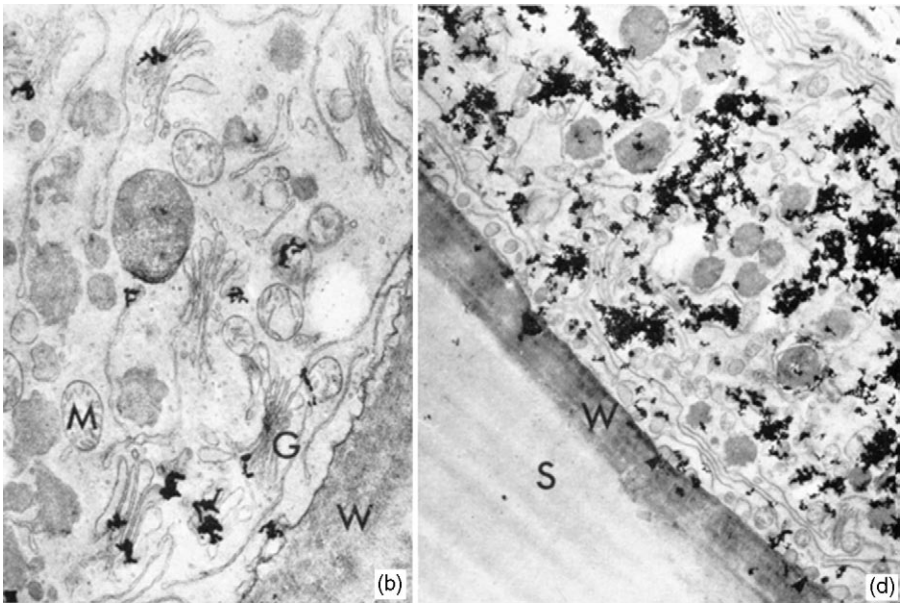


FIGURE 8.5 Root-cap cells of wheat exposed to D-[6-^{3}H] glucose for 5 minutes (left) and 10 minutes (right). The majority of the labeled compounds are located in the Golgi apparatus after 5 minutes. By 10 minutes, some of the label shows up in the extracellular matrix. Left, ×11,625; right, ×14,435. M, mitochondria; G, Golgi stack; W, extracellular matrix. (*Source:* From Northcote and Pickett-Heaps, 1966.)

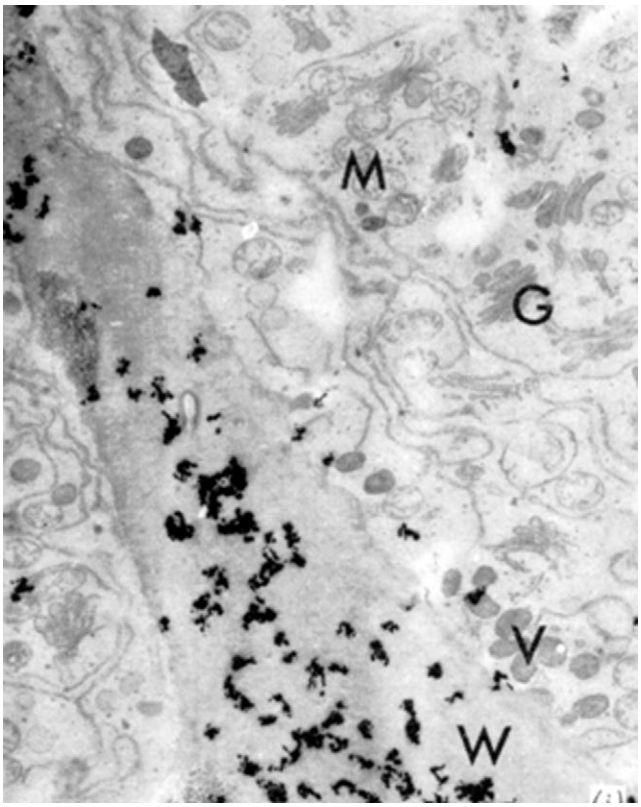


FIGURE 8.6 Root-cap cells of wheat exposed to D-[6-^{3}H] glucose for 10 minutes followed by a 60-minute chase period when the cells were treated with unlabeled glucose. The majority of the labeled compounds are now located in the extracellular matrix (W) and have left the Golgi apparatus. ×5625. (*Source:* From Northcote and Pickett-Heaps, 1966.)

secrete slime that includes coniine, an alkaloid that paralyzes insects. Insectivorous plants also secrete the enzymes necessary to digest their prey. Cells in the abscission zone secrete wall-digesting enzymes that cause the leaves to fall in the autumn. Stigmas also secrete proteins that are involved in determining whether or not a given pollen grain will germinate or have an incompatible reaction. Plant cells secrete essential oils that we associate with herbs, and resins that we associate with antimicrobial activity. Stinging trichomes secrete histamine, serotonin, and acetylcholine, molecules that we usually associate with nervous activity (Roshchina, 2001). Cereal grains have a layer of glandular cells known as the *aleurone layer* that surrounds the endosperm and secretes hydrolases that digest the stored food. The tapetal layer that surrounds the developing pollen grains secretes the proteins involved in sporophytic incompatibility that discourages self-pollination (Hesse et al., 1993). Many potentially fascinating aspects of the cell biology of these secretory pathways remain a mystery (Haberlandt, 1914; Fahn, 1979; Roshchina and Roshchina, 1993; Nicolson et al., 2007; Roshchina, 2008) as well as a source of new discovery and biotechnological innovation.

How does a protein end up in a given organelle? In the past, when cell biologists thought about the biogenesis of each organelle in the endomembrane system, it was assumed that each and every component of the organelle is made at the same time and in the same way. Now the focus is on how each individual component gets to its targeted organelle. This approach allows for the possibility of multiple pathways—and which pathways are used may be cell-type or species

specific and even developmentally or physiologically determined. Every protein has information encoded in its structure that determines where it will go, and, as I have already discussed, proteins take different pathways to enter the endoplasmic reticulum, the peroxisome, and the vacuole. The information encoded in the sequence of the protein is affectionately known as its *molecular zip code* (see Chapter 17).

When I discuss the intracellular pathway for the movement of a protein, I follow the lead of Adolf Fick (1855), a physiologist who, under the influence of Hermann von Helmholtz, pioneered the application of physical thought to biological transport processes. Fick decided not to look at diffusion in isolation, but within the context of transport of heat and electricity. I will treat protein transport like any transport phenomenon and consider the structure of the molecule being transported, including its signal sequences, the receptor for this protein, the affinity of the protein for the receptor, and the proportion of this protein compared with other proteins that may compete with it for the receptor. Any of these factors can vary from cell to cell and during the life of a cell and consequently can affect the transport of the protein at any stage in the pathway. Thus, it is conceivable that proteins with identical sequences may be targeted to one place in one cell, but another location in a different cell. Indeed, the uniformity found in targeting sequences in a single-celled organism like yeast may not exist in multicellular organisms. An indication of this comes from the observations that phytohemagglutinin, a seed protein that is normally targeted to the vacuole of beans, is secreted to the external solution in transgenic monkey COS cells, but transported to the vacuole in transgenic yeast (Voelker et al., 1986; Tague and Chrispeels, 1987). It is also possible that two proteins that only differ by their signal sequence may be targeted to the same class of organelles in neighboring cells due to differences in the receptor proteins or the concentration of proteins competing for the same receptor (Frigerio et al., 1998). Elucidating the cellular and molecular components of the secretory system has been driven, in part, by the hope of using plants as light-powered bioreactors that are capable of secreting or storing high concentrations of economically valuable proteins that can be used as pharmaceuticals (Vitale and Pedrazzini, 2005).

8.2 MOVEMENT TO THE PLASMA MEMBRANE AND THE EXTRACELLULAR MATRIX

According to the endomembrane concept, the pathway for creating new plasma membrane results from the synthesis of proteins and lipids on the ER. As a result of ER membrane growth, transition vesicles, covered with a COP II coat, bleb off from the transition elements of the ER and fuse with the *cis*-face of the Golgi apparatus or its associated membranes. The membrane coats are composed of proteins that allow the blebbing off and fusion of vesicles with specific membranes (Schekman, 1996). The new membrane and its luminal contents move through the Golgi stack where further processing of carbohydrates takes place. Once the membrane with its contents arrives at the trans-face of the Golgi apparatus, it becomes covered with another coat and moves to the plasma membrane. As it moves to the plasma membrane, it loses its coat. It then fuses with the plasma membrane in a process that involves bilayer adherence and bilayer joining. At this point, if the lumen of the vesicle contains something, that substance is secreted simultaneously in a process known as *exocytosis*. When the cell is no longer growing, plasma membrane replacement must be balanced by plasma membrane retrieval (Palade, 1959).

In plants, the secretion of α-amylase and the hydroxyproline-rich glycoprotein has been studied most extensively (Akazawa and Hara-Nishimura, 1985; Jones and Robinson, 1989; Jones and Jacobsen, 1991; Chrispeels, 1991). Paleg and Yomo independently discovered in 1960 that gibberellin, produced by the embryo of cereal grains, diffuses to a specialized secretory tissue known as the *aleurone layer* and stimulates the secretion of α-amylase (see Paleg, 1965; Chrispeels and Varner, 1967). Following secretion, the α-amylase diffuses through the extracellular matrix to the endosperm where it causes the breakdown of starch to maltose, which nourishes the growing embryo. Gubler et al. (1986) and Zingen-Sell et al. (1990) have labeled aleurone cells with a colloidal gold-tagged antibody directed against α-amylase and showed that α-amylase occurs in the ER, Golgi apparatus, and Golgi-derived vesicles (Figures 8.7 and 8.8). They suggest that α-amylase follows the same secretory pathway in aleurone cells that it does in the pancreatic exocrine cells. Unfortunately, the temporal sequence is not known since the labeling time used in the pulse-chase experiments was as long as its takes the protein to move through the entire secretory pathway (Jones and Jacobsen, 1982).

By contrast, the secretion of the hydroxyproline-rich glycoprotein of carrot phloem parenchyma into the extracellular matrix was studied with a pulse of only 2–3 minutes, and by 30 minutes, the radioactivity was found exclusively in the extracellular matrix, indicating a possible rapid movement through the intracellular secretory pathway from the site of synthesis to the extracellular space (Chrispeels, 1969). Unfortunately, the plant cell biologists involved in studying secretion found it "impossible to obtain even moderately pure particulate fractions from plant tissue homogenates," and thus did not fractionate the material into ER, Golgi, and vesicular fractions, but only into a membrane fraction and a supernatant fraction (Chrispeels, 1969).

The hydroxyproline-rich glycoprotein must move through the Golgi apparatus since it is a complex arabinose-containing glycoprotein, and the enzymes necessary for the addition of arabinose in these cells are found in the Golgi

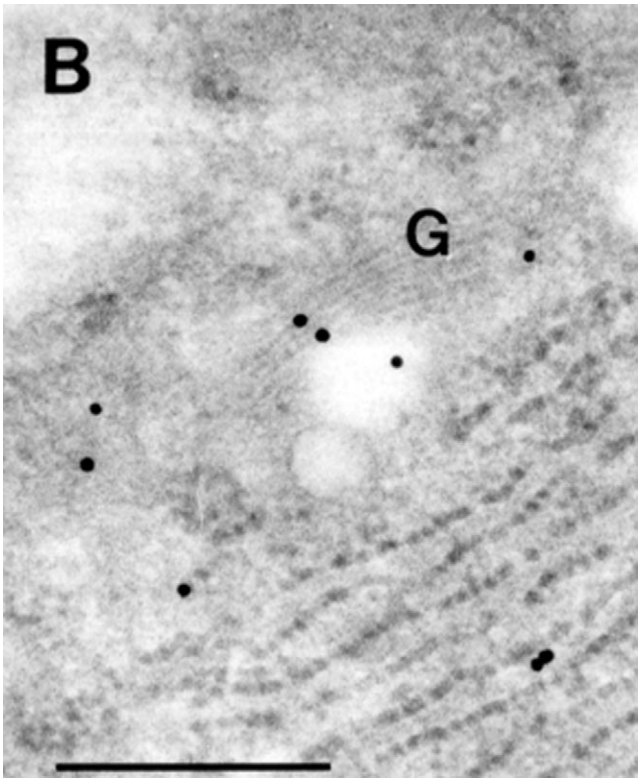


FIGURE 8.7 Immunolabeling of the ER and a Golgi stack in a cell of the aleurone of barley with an antibody to α-amylase. Bar, 300 nm. (*Source:* From Gubler et al., 1986.)

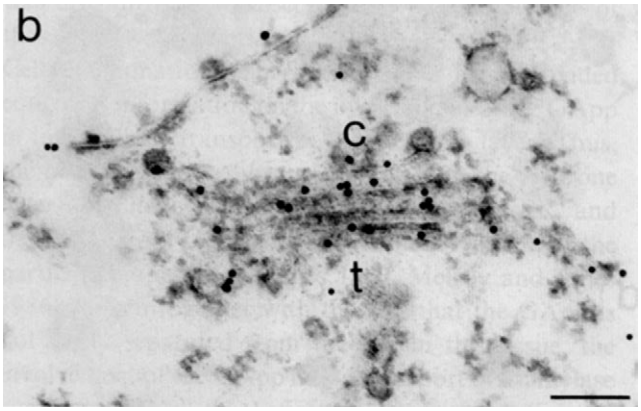


FIGURE 8.8 Immunolabeling of a Golgi stack in a cell of the aleurone of barley with an antibody to α-amylase. c, cis-face; t, trans-face. Bar, 500 nm. (*Source:* From Zingen-Sell et al., 1990.)

apparatus (Chrispeels, 1970; Gardiner and Chrispeels, 1975). By 1982, David Robinson and his colleagues overcame the problems associated with cell fractionation, and determined, with a 5-minute labeling period in a pulse-chase experiment, that, following the synthesis of the hydroxyproline-rich glycoprotein in the ER, the protein moves to the Golgi apparatus within 15 minutes and is secreted to the extracellular matrix within 30 minutes (Robinson and Glas, 1982; Wienecke et al., 1982).

The most common secretory products of all plant cells are hemicelluloses and pectins that will end up in the extracellular matrix. Jeremy Pickett-Heaps (1967b) has demonstrated in wheat root tips, with pulse-chase EM radioautography, that ^{3}H-glucose is incorporated into the Golgi apparatus within 10 minutes and transferred to the extracellular matrix after another 10 minutes (see Figure 8.5). By 30 minutes, the entire label is in the extracellular matrix (see Figure 8.6). Moore and Staehelin (1988) have shown that antibodies to the two polysaccharides are found localized in the Golgi apparatus and Golgi-derived vesicles but not in the ER, indicating that the Golgi apparatus is crucial for the secretion of the matrix of the cell wall.

Using the pulse-chase labeling method along with cell fractionation, Moreau et al. (1998) have shown that sterols are synthesized in the ER, transported through the Golgi apparatus, and transferred to the plasma membrane with a half-time of about 30 minutes. This movement is inhibited by brefeldin A and monensin.

8.2.1 Movement between the ER and the Golgi Apparatus

The newly synthesized proteins in the ER are folded by members of a class of proteins known as *chaperonins*. Incorrectly folded proteins, proteins with hydrophobic surfaces, free sulfhydryl groups, or incomplete glycosylation are usually captured in the lumen of the ER by special chaperonins like calreticulin and calrexin, which recognize incorrectly folded proteins. The chaperonins refold them to their correct configuration so they can leave the ER. This is an example of cellular quality control (Sonnichsen et al., 1994; Hammond and Helenius, 1995; Pelham, 1995; Opas et al., 1996).

There are two proposed mechanisms for the export of correctly folded proteins from the ER (Pimpl and Denecke, 2000). One proposal is that there is a constitutive, nonselective anterograde bulk flow of proteins into COP II–coated transition vesicles, which pinch off from the transition endoplasmic reticulum (TER) and move to the Golgi apparatus (Pelham, 1989; Phillipson et al., 2001). Thus, the membrane proteins of the ER involved in lipid synthesis, ribosome docking, protein translocation, etc., as well as the chaperonins in the lumen of the ER, leave the transition ER by bulk flow in or on transition vesicles that are destined to arrive at the cis-Golgi network. These proteins must be reclaimed and returned to the ER by a retrograde transport mechanism (Sabatini et al., 1991; Pelham, 1991). COP I–coated membranes may be responsible for recycling membrane proteins back to the ER (Pimpl et al., 2000). The alternative proposal is that correctly folded proteins are actively selected and enriched in or on the COP II–coated vesicles destined to arrive at the Golgi apparatus. This is supported by the observation that these vesicles are enriched in some ER proteins but lacking in others. There may be truth in both proposals.

Some of the resident proteins in the lumen of the ER contain the amino acid sequence K/H-D-E-L while the resident membrane-bound proteins have the sequence K-K or K-X-K on the cytoplasmically exposed carboxy-terminal region. These three sequences are recognized by receptor proteins in the cis-Golgi network. In search of such receptors, Vaux et al. (1990) made an antibody to a KDEL-containing protein and then made an antibody to that antibody. They assumed that the second antibody had a shape similar to that of the original protein and would thus bind to the receptor. In this way, they found a receptor protein that is localized on the cis-Golgi network. Somehow, binding to this receptor permits retrograde transport back to the ER. Thus, the vesicles or tubules seen in electron micrographs do not only move substances from the ER to the Golgi, but also in the other direction.

Saint-Jore et al. (2002) and Brandizzi et al. (2002b) have shown, using green fluorescent protein (GFP) fused to the HDEL receptor, that the movement of this membrane protein from the ER to the Golgi apparatus does occur. Moreover, the ER-to-Golgi movement of this protein requires adenosine triphosphate (ATP), is inhibited by brefeldin A, and is independent of the actin and microtubular cytoskeletons. They also determined, using fluorescence redistribution after photobleaching (FRAP), that the movement of this protein from the ER to the Golgi apparatus occurs within 5 minutes. There is currently a lot of work that is going on to understand the processes that take place in this "no organelles' land" between the ER and the Golgi apparatus, which is sometimes called the *ER-Golgi intermediate compartment* (ERGIC), the *cis-Golgi network* (CGN), or the *vesicular-tubular cluster* (VTC; Hammond and Helenius, 1995; Pelham, 1995; Robinson, 2003). Many of the genes and proteins involved in ER-to-Golgi transport are being identified (Bassham and Raikhel, 2000; Botoko et al., 2000).

The majority of proteins that are synthesized in the ER continue moving to the Golgi apparatus. These proteins bleb off from the transition ER as transition vesicles and fuse with the Golgi apparatus and/or its cis-associated membranes (Figure 8.9). Morré et al. (1989) have shown that (50–70 nm) transition vesicles from the transition ER are able to fuse with the Golgi apparatus by using a reconstituted cell-free system. They isolated transition elements, and labeled them with ^{125}I. They added ATP (with an ATP regenerating system) to the transition ER that caused vesicles to bleb off. Then they isolated the ^{125}I-labeled transition vesicles. Concurrently, the Golgi apparati were isolated and adsorbed to nitrocellulose strips.

When the labeled transition vesicles were mixed with the isolated Golgi apparati, the Golgi apparati became labeled with ^{125}I, indicating that the transition vesicles fuse with the Golgi apparatus. Interestingly, transition vesicles isolated from rat liver could fuse with the Golgi apparatus isolated from soybeans and vice versa, indicating that a common "receptor protein" may exist. However, the specificity of this fusion reaction is not known because other subcellular fractions like the plasma membrane, mitochondria, etc. were not tested as acceptors.

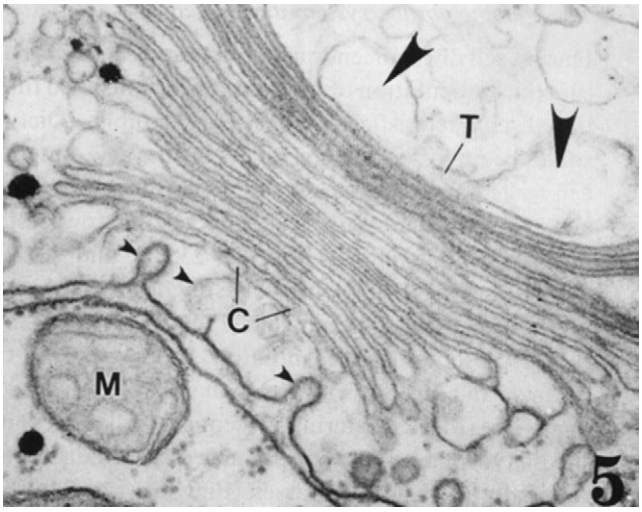


FIGURE 8.9 Small transition vesicles appear to bleb off from the ER that is associated with the cis-face (c) of the Golgi stack. t, trans-face; m, mitochondrion. ×78,200. (*Source:* Domozych, 1989a.)

Sturbois-Balcerzak et al. (1999) have shown that the transition vesicles that bleb off from the ER are enriched in phosphatidylserine compared to the ER membrane itself (Table 8.1). Thus, in the process of transition vesicle formation, sorting of phospholipids as well as proteins occurs.

There are some proteins that seem to never leave the Golgi apparatus. It is not known how these proteins stay in the Golgi apparatus without continuing through the endomembrane system. In the case of membrane proteins, perhaps the membrane-spanning regions of the Golgi-localized proteins are shorter than those of the plasma membrane–localized proteins, and as Bretscher and Munro (1993) suggest, the Golgi-localized membrane proteins get stuck when they reach the region of the Golgi stack that has a certain membrane thickness. Brandizzi et al. (2002c) have shown using genetically engineered GFP-containing fusion proteins with variable numbers of amino acids in the membrane-spanning region that proteins with membrane-spanning regions that contain 17, 20, and 23 amino acids end up in the endoplasmic reticulum, Golgi apparatus, and plasma membrane, respectively.

8.2.2 Movement from the Golgi Apparatus to the Plasma Membrane

The vesicular movement of polysaccharides from the Golgi apparatus to the extracellular matrix was discussed in Chapter 6. During such movements, the lipids that compose the membranes of the Golgi-derived vesicles must fuse with the plasma membrane. Wait et al. (1990) have investigated the transfer of sterols from isolated Golgi stacks to the plasma membrane

TABLE 8.1 Phospholipid composition of ER and ER-derived transition vesicles

	Phospholipid Composition (% of total)			
Membrane Fraction	PC	PS	PI	PE
ER	75.9 ± 5.8	1.7 ± 1.2	3.2 ± 1.3	19.2 ± 4
TV(−ATP)	67.1 ± 2.9	2.9 ± 0.5	3.7 ± 1.8	26.3 ± 4.5
TV(+ATP)	69.1 ± 3.6	6.9 ± 2.6	3.5 ± 1.9	20.5 ± 3.2

Note: PC, phosphatidylcholine; PS, phosphatidylserine; PI phosphatidylinositol; PE, phosphatidylethanolamine.The PS increase in the vesicles TV(+ATP) compared with the ER was significant ($P < 0.01$). Source*: Sturbois-Balcerzak et al., (1999).*

using a reconstituted cell-free system where the plasma membrane is adsorbed onto nitrocellulose strips and dipped in solutions containing radio labeled donor membranes. They found that the Golgi stacks were more effective as donors than other membrane fractions. The transfer required ATP. The cell-free transfer system is not yet completely efficient; that is, less than 1 percent of the label is transferred, yet it will be a very powerful system to understand the cellular components that regulate membrane trafficking.

The regulatory processes that determine whether a vesicle is secreted as soon as it is produced, stored in the cytoplasm until a stimulus induces its secretion, or stored in the cell indefinitely as a vacuole have yet to be elucidated. Currently, many of the genes and gene products involved in mediating vesicle flow in particular cells are being identified (Marsh and Goode, 1993; Denesvre and Malhotra, 1996; Seaman et al., 1996; Pimpl et al., 2000, 2003; Phillipson et al., 2001; Sohn et al., 2003; Happel et al., 2004; Pratelli et al., 2004; Surpin and Raikhel, 2004; Uemura et al., 2004; Sutter et al., 2006; Lipka et al., 2007; Matheson et al., 2007; Min et al., 2007; Robinson et al., 2007; Sanderfoot, 2007; Zhang et al., 2007; Groen et al., 2008; Nielsen et al., 2008; Rojo and Denecke, 2008).

8.3 MOVEMENT FROM THE ER TO THE GOLGI APPARATUS TO THE VACUOLE

The movement of proteins from the endoplasmic reticulum to the vacuole has been studied most extensively in seeds (Chrispeels, 1984, 1985). Protein bodies contain hydrolytic enzymes (and their inhibitors), indicating that they are part of the vacuolar compartment (see Chapter 7; Van der Wilden et al., 1980; Herman et al., 1981; Rasmussen et al., 1990). The best estimate of the temporal sequence of the intracellular secretory pathway to the vacuole comes from experiments done by Maarten Chrispeels (1983) who labeled bean cotyledons with ^{3}H-fucose and found, using cell fractionation, that the Golgi apparatus is labeled after approximately 45 minutes and the protein storage vacuoles are labeled after 60 minutes. The temporal resolution is poorer in all other vacuole-targeting studies since the labeling times used exceed the time it takes for the protein to move through the entire pathway (Chrispeels and Bollini, 1982; Vitale and Chrispeels, 1984; Lord, 1985).

Even though the temporal resolution of the pulse-chase experiments is not sufficient to determine the intracellular pathway, the Golgi apparatus must be involved in the trafficking of some storage proteins to the vacuole since some of the storage proteins contain complex carbohydrates (Vitale and Chrispeels, 1984), and immunocytohistochemistry at the EM level shows that storage proteins can be detected in the ER, Golgi apparatus, coated vesicles, and protein storage vacuoles of developing cotyledons (Nieden et al., 1982, 1984; Parker and Hawes, 1982; Herman and Shannon, 1984a,b, 1985; Greenwood and Chrispeels, 1985b; Boller and Wiemken, 1986; Harris, 1986; Faye et al., 1988; Kim et al., 1988; Robinson et al., 1989; Hoh et al., 1991).

8.4 MOVEMENT FROM THE ER TO THE VACUOLE

After studying their electron micrographs of corn endosperm, Khoo and Wolf (1970) and Larkins and Hurkman (1978) concluded that protein storage vacuoles containing water-insoluble prolamins form directly from the ER. The results of these and other studies that indicated that protein-containing bodies may arise directly from the ER (Bonnett and Newcomb, 1965) were ignored, in part, because it was believed by many plant cell biologists that there was only one pathway of vacuole formation—and that one pathway involved the Golgi apparatus. Thus, it was generally thought that any electron micrographs that showed vacuole formation directly from the ER must be riddled with artifacts. However, there has been a paradigm shift, and now it is believed that there are many pathways involved in vacuole formation, and that some protein storage vacuoles do in fact form directly from the ER (see Chapter 7; Galili et al., 1996; Robinson and Hinz, 1996; Robinson et al., 1996; Herman, 2008).

At least two of these pathways exist in rice endosperm cells, and in these cells, there are two different populations of ER known as *cisternal ER* and *protein-body ER*. The cisternal ER is enriched in glutelin mRNA as evidenced from in situ hybridization with the cDNA that codes for the glutelins, which are proteins that are soluble in dilute acids or bases. The glutelins that are translated on the ER probably go through the Golgi to form protein storage vacuoles. The prolamins in contrast are translated on the ER that is connected to protein bodies as evidenced by in situ hybridization with the cDNA that codes for prolamin (Levanony et al., 1992 ; Li et al., 1993). The water-insoluble prolamins that are retained in the ER lumen do not have the typical ER lumen-retention sequence KDEL or HDEL (Masumura et al., 1990). Perhaps the prolamins are retained in the ER as a consequence of their solubility properties. In rice endosperm cells, the prolamin-containing ER is sometimes engulfed by autophagosomes to form yet another kind of protein storage vacuole.

8.5 MOVEMENT FROM THE PLASMA MEMBRANE TO THE ENDOMEMBRANES

Christian de Duve (1963) coined the term *endocytosis* to name all the processes (e.g., phagocytosis, pinocytosis, micropinocytosis, etc.) whereby cells engulf small volumes of the external medium and *pari passu* internalize the plasma membrane. The movement of macromolecules into plant cells can occur by various mechanisms, including fluid-phase endocytosis and receptor-mediated endocytosis, both of which bring the macromolecules into the E-space. Many polypeptide toxins, including ricin and diphtheria, enter the cell through the endocytotic system (Lord and Roberts, 1998). Endocytosis occurs during plasmolysis, and it is has been suggested that the plasma membrane may be stored in the endocytotic vesicles readying the cell for deplasmolysis, although the endocytotic membranes have not yet been shown to be utilized during deplasmolysis (Oparka et al., 1990; Oparka, 1994; Lang-Pauluzzi, 2000). Another inwardly directed macromolecular transport system, sometimes called *piggyback endocytosis*, has been discovered that brings macromolecules into the P-space of the cell.

8.5.1 Fluid-Phase Endocytosis

When wall products or enzymes are rapidly secreted, new plasma membrane may be added at a rate that is greater than that necessary to keep up with growth. Therefore, a mechanism is needed to retrieve excess or old membrane from the plasma membrane and either recycle it by sending it back to the Golgi apparatus or degrade it by sending it to the vacuole. This is accomplished by a process known as *endocytosis* (de Duve, 1963). Endocytosis occurs incessantly in the wall-less alga *Dunaliella* (Ginzburg et al., 1999). Although initially deemed impossible in walled plant cells due to the presence of turgor pressure (Cram, 1980), endocytosis does occur in such cells, as evidenced by the uptake of the relatively impermeant La^{3+} ion into root cells (Samuels and Bisalputra, 1990); the uptake of Lucifer Yellow into the inner cortical cells of roots (Baluska et al., 2004); the uptake of large, polar FITC-dextrans into suspension culture cells (Cole et al., 1990) and pollen tubes (O'Driscoll et al., 1993); the uptake of fluorescently labeled plasma membrane in turgid guard cells (Meckel et al., 2004, 2005) and at the apex of growing pollen tubes (Zonia and Munnik, 2008); the uptake of CdSe/ZeS quantum dots into suspension culture cells (Etxeberria et al., 2006); and by using rapid-freezing techniques combined with electron microscopy (Ketelaar et al., 2008). Indeed, endocytosis may be important in recapturing molecules from the extracellular matrix that undergo turnover in the natural cycle of synthesis and degradation (Labavitch, 1981; Herman and Lamb, 1992; Baluska et al., 2005), for recycling the plasma membrane (Parton et al., 2001; Meckel et al., 2004), and in nutrient-starved cells undergoing autophagy (Yano et al., 2004).

As a first approximation, vesicle formation is a mechanical process, in which the free energy necessary to push a vesicle into a protoplast will be equal to the product of the volume of the vesicle (V) and the turgor pressure of the cell (P_t). Thus, for a given availability of free energy, cells with higher turgor pressures would have to have smaller endocytotic vesicles. The force needed to invaginate the membrane is supplied, in part, by a mechanochemical protein like dynamin that is energized by the hydrolysis of guanosine triphosphate (GTP) (Collings et al., 2008). The dynamin forms helical spirals around the neck of the forming vesicle. In vitro models of this mechanochemical process can be viewed under the microscope, and the change in the conductance of the membrane that occurs during the budding process can be followed with electrophysiology techniques (Bashkirov et al., 2008; Pucadyil and Schmid, 2008).

Let us study the parts of the endocytotic pathway individually. Plasma membrane vesicle formation begins where clathrin (see Chapter 6) binds to the plasma membrane. In the early stages of vesicle formation, the plasma membrane contains clathrin-coated pits that may have a surface density of about 0.1–4.5 μm^2 (Emons and Traas, 1986; Low and Chandra, 1994). The clathrin acts as a magnet for plasma membrane receptors, since coated pits in some animal cells contain 70 percent of certain plasma membrane receptors, while accounting for only 3 percent of the surface area (Pearse and Robinson, 1990; Robinson and Hillmer, 1990). As the membrane blebs in, the coated pits develop into clathrin-coated vesicles (Balusek et al., 1988; Robinson and Depta, 1988; Harley and Beevers, 1989; Coleman et al., 1987, 1991; Robinson et al., 1991; Beever, 1996; Blackbourn and Jackson, 1996). Clathrin forms a polygonal network around the vesicle, which gives

it a honeycomb-like appearance (Wiedenhoeft et al., 1988). Clathrin cages assemble into a honeycomb-like structure spontaneously in vitro when the proteins are above a critical concentration. Assembly does not require metabolic energy, but depends on $[Ca^{2+}]$, $[H^+]$, and ionic strength. The binding of clathrin to receptor proteins is mediated by adaptor proteins (Pearse and Robinson, 1990; Holstein et al., 1994; Drucker et al., 1995).

The coated vesicles originating from the plasma membrane lose their coats and then fuse with the partially coated reticulum (PCR) (see Chapter 6). The partially coated reticulum was first observed in plants by Tom Pesacreta and Bill Lucas (1984, 1985). The partially coated reticulum can be sparsely branched or extensively anastomosed. The membranes are coated along various regions throughout the reticulum. It seems to have a variable relationship with the Golgi apparatus (Hillmer et al., 1988; Mollenhauer et al., 1991). Some people believe that the partially coated reticulum is an independent structure (Pesacreta and Lucas, 1984, 1985); others, after viewing three-dimensional reconstructions of serial sections, believe that it is interconnected with the trans-face of the Golgi apparatus and is thus equivalent to the trans-Golgi network (TGN) (Sluiman and Lokhorst, 1988; Hillmer et al., 1988). The partially coated reticulum, at least in some soybean cells, may be synonymous with the early endosome in animal cells (Dettmer et al., 2006; Lam et al., 2007). The early endosome is defined as the first internal membranous body in the peripheral cytoplasm to which the endocytotic vesicles fuse (Brown et al., 1986; Mellman, 1996; Robinson et al., 2008).

As endocytosis proceeds, the endocytotic organelles change their appearance. As a result of maturation or of vesicle exchange, they become late endosomes (Tse et al., 2004; Ueda et al., 2004). The late endosomes in soybean cells look like multivesicular bodies (MVBs). At one time multivesicular bodies were considered to be fixation artifacts; however, Tanchak and Fowke (1987) demonstrated their importance in endocytosis. Multivesicular bodies are usually 250–500 nm in diameter and contain a number of smaller vesicles, usually 40–100 nm in diameter. The multivesicular bodies may be specialized lysosomes that help degrade plasma membrane proteins. Some multivesicular bodies have been seen attached to tubules extending from the PCR or TGN (Noguchi and Kakami, 1999). The MVBs leave the vicinity of the TGN and then fuse with the central vacuole, or vesicles may bleb off the multivesicular bodies and fuse with the central vacuole, where final degradation takes place and the degraded components can be recycled. I would like to stress that, while fluid phase endocytosis may be a common process in all cell types, the actual intracellular pathway followed by the endocytotic vesicles is cell-type specific. For example, in many mammalian cells, the early endosomes appear as multivesicular vesicles. The membranous organelles that participate in endocytosis may also form an endosomal reticulum (Hopkins et al., 1990; Mironov et al., 1997). The pleiomorphic nature of the early and late endosomes may reflect the particular balance of multidirectional transport processes that occur in these multifunctional organelles.

In order to determine the pathway and kinetics of endocytosis in soybean cells, Tanchak et al. (1984, 1988) treated protoplasts with cationized ferritin. Within 10 seconds the cationized ferritin is found evenly labeling the plasma membrane and coated pits (Figure 8.10). After 30 seconds, the cationized ferritin is found in coated vesicles in the vicinity of the Golgi apparatus and in smooth vesicles (Figure 8.11). After 30–120 seconds, the cationized ferritin is found in partially coated vesicles (Figure 8.12). After 12 minutes, the cationized ferritin is found in the partially coated reticulum and the Golgi stacks.

These results are consistent with those of Hübner et al. (1985), who looked at the uptake of heavy metals in intact root-cap cells. They find that lead is localized in coated pits, coated vesicles, and the membranes near the trans-face of Golgi apparatus, which these authors believe to be the partially coated reticulum. This could mean that vesicles can move from the plasma membrane to the partially coated reticulum to the Golgi apparatus.

A more complete pathway of endocytosis was found by Tanchak and Fowke (1987) and Record and Griffing (1988; see Table 8.2). Soybean protoplasts were exposed to cationized ferritin for 5, 30, or 180 minutes and then the cells were fixed to localize the cationized ferritin and stained with acid phosphatase in order to visualize the vacuolar compartment. After 5 minutes, the cationized ferritin is found in the coated pits, coated vesicles, smooth vesicles, and partially coated reticulum. After 30 minutes, the cationized ferritin is found in the Golgi complex and the multivesicular bodies (Figure 8.13). After 3 hours, the cationized ferritin is found in the large central vacuole. Acid phosphatase occurs in the smooth vesicles, Golgi apparatus, multivesicular bodies, and the vacuole (Fowke et al., 1991). A similar sequence is observed with the uptake of bovine serum albumin-gold, except that this probe, unlike the cationized ferritin, does not end up in the vacuole (Villanueva et al., 1993; Griffing et al., 1995). Recent work comparing the uptake of CdSe/ZeS quantum dots with soluble dextrans shows that the quantum dots also do not move all the way to the vacuole in suspension culture cells (Etxeberria et al., 2006). Thus, just as there are variations in the secretory pathway that depend on the substance secreted, there also seem to be variations in the endocytotic pathway that depend on the substance taken up (Onelli et al., 2008).

Many symbionts and pathogens enter the cell by endocytosis (Son et al., 2003). In some cases (e.g., *Listeria* and *Shigella*), the endocytotic membrane dissolves, and the pathogens reproduce in the cytoplasm. In other cases (e.g., *Chlamydia* and *Toxoplasma*), the endocytotic membrane is resistant to lysis and the pathogens reproduce within the endocytotic vesicle. In still others (e.g., *Coxiella*

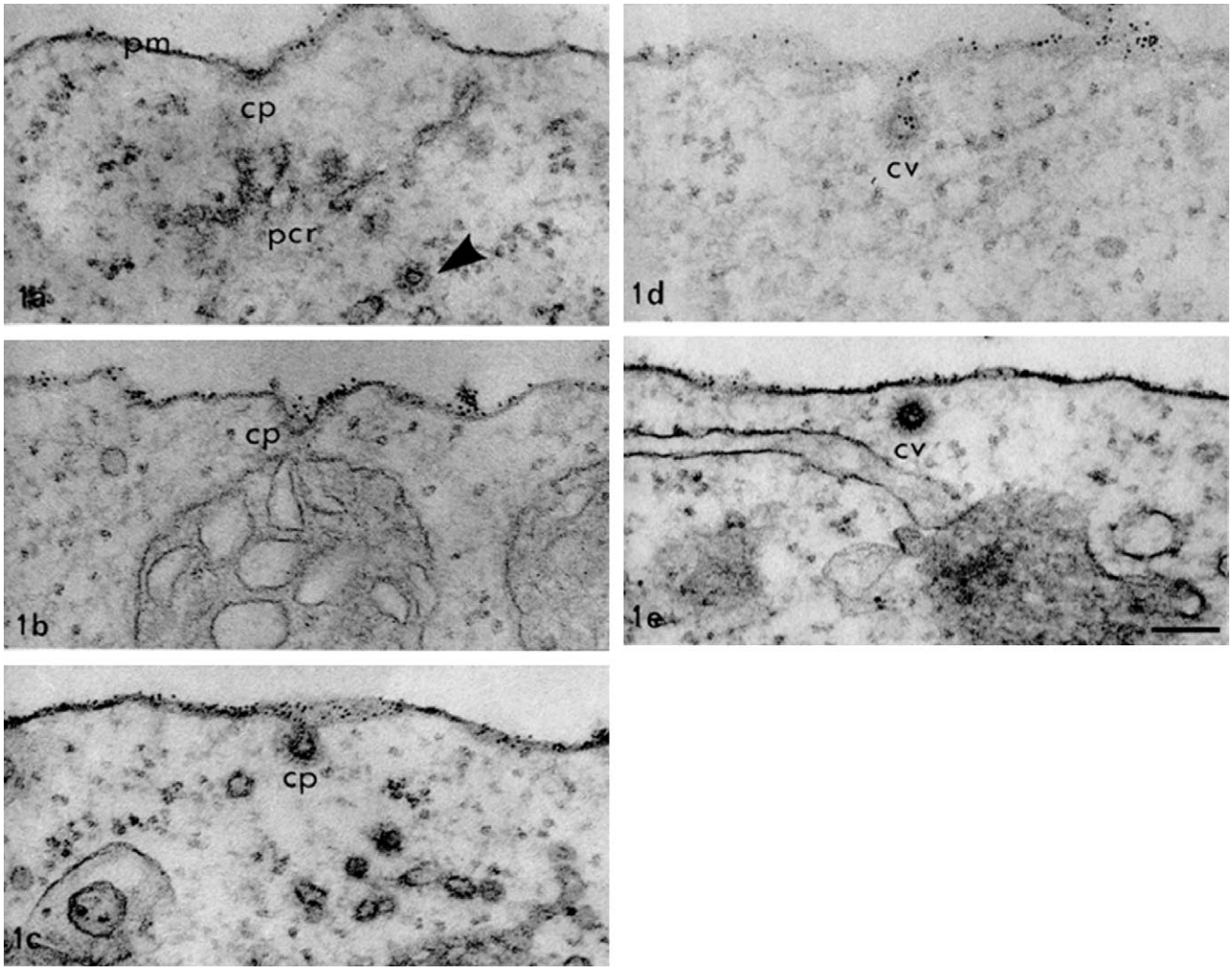


FIGURE 8.10 Endocytosis in plant cells. Cationized ferritin is seen as electron-dense dots in a coated pit (a). Cationized ferritin is seen as electron-dense dots in a deep, coated pit (b). Cationized ferritin is seen as electron-dense dots in a coated pit with a narrow neck (c). Cationized ferritin is seen as electron-dense dots in a coated vesicle (d). Cationized ferritin is seen as electron-dense dots in a coated vesicle (e). The soybean protoplast was treated with cationized ferritin for 10 seconds prior to fixation. cp, coated pit; pm, plasma membrane; pcr, unlabeled partially coated reticulum. Arrow points to an unlabeled coated vesicle. Bar, 100 nm. (*Source:* From Tanchak et al., 1984.)

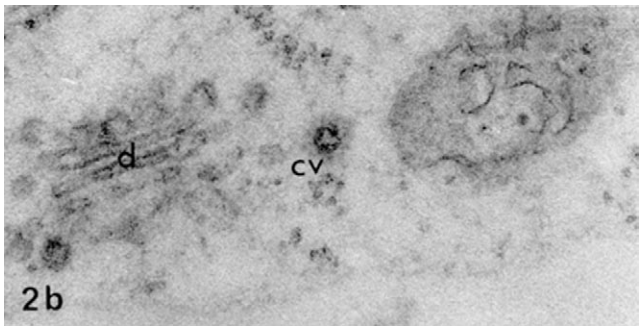


FIGURE 8.11 Endocytosis in plant cells. Cationized ferritin is seen as electron-dense dots in a coated vesicle. The soybean protoplast was treated with cationized ferritin for 30 seconds prior to fixation. (*Source:* From Tanchak et al., 1984.)

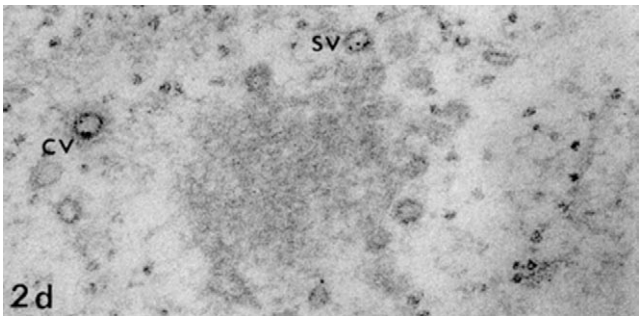


FIGURE 8.12 Endocytosis in plant cells. Cationized ferritin is seen as electron-dense dots in a smooth vesicle. The soybean protoplast was treated with cationized ferritin for 2 minutes prior to fixation. (*Source:* From Tanchak et al., 1984.)

TABLE 8.2 Time course of endocytosis in protoplasts

Time	Structure Labeled
10 seconds	Coated pits, coated vesicles
30 seconds	Coated vesicles near Golgi apparatus and smooth vesicles
30–120 seconds	Partially coated vesicles
12 minutes	PCR and Golgi stacks
30 minutes	Golgi and MVBs
3 hours	Large central vacuole

and *Leishmania*), the endocytotic vesicle fuses with the lysosome and the pathogen divides in the lysosomal compartment. In the most unusual case (e.g., *Legionella*), the endocytotic vesicle transforms into rough ER (Tilney, 2001). Is it possible for the endocytotic membranes in uninfected cells to transform into rough ER, providing yet another pathway for membrane traffic?

An additional kind of membrane coat called *caveolin* has been found in animal cells and may be involved in capturing glycosyl-phosphatidylinositol–anchored proteins and low–molecular weight substances in a vesiculating process termed *potocytosis* (Anderson, 1993). Potocytosis and caveolin have not yet been found in plants.

8.5.2 Receptor-Mediated Endocytosis

Following binding of extracellular ligands, including hormones, lectins, or antibodies to the plasma membrane of animal cells, the ligand and its receptor are typically taken up into the cell (Pastan and Willingham, 1985). This process is known as *receptor-mediated endocytosis*. The removal of receptors from the plasma membrane is one way to terminate a given response and there is evidence that a part of the signal transduction chain occurs in the endosomal compartment (Geldner and Robatzek, 2008). The receptor proteins that are internalized by endocytosis have specific amino acid sequences in their cytoplasmic domains (Trowbridge, 1991; Geldner and Robatzek, 2008).

Horn et al. (1989, 1992) have developed a fascinating system to study receptor-mediated endocytosis in plants. Soybean suspension culture cells are induced to make fungal defense molecules in response to fungal attack. When the fungus begins to degrade the plant extracellular matrix it releases large, polar oligosaccharide molecules ($M_r > 30{,}000$ Da) that are known as *elicitors*. Elicitors are too large and polar to passively diffuse through the membrane; however, they bind to the plasma membrane

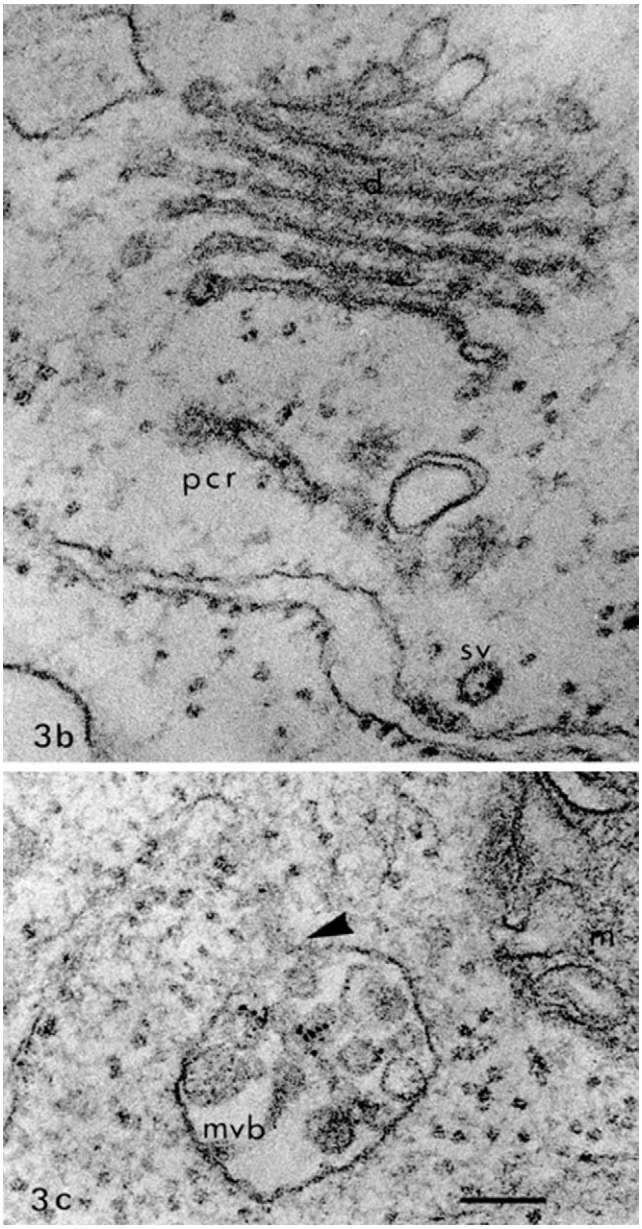


FIGURE 8.13 Endocytosis in plant cells. In a, cationized ferritin is seen as electron-dense dots in the trans-cisternae of a Golgi stack (d), the partially coated reticulum (pcr), and a smooth vesicle (sv). In b, cationized ferritin is seen as electron-dense dots in a multivesicular body (mvb). The arrow points to a tubular extension of the mvb. The soybean protoplast was treated with cationized ferritin for 12 minutes prior to fixation. Bar, 100 nm. (*Source:* From Tanchak et al., 1984.)

(Schmidt and Ebel, 1987) and cause the cell to produce antifungal defense molecules, including glyceolin, pisatin, phaseolin, and H_2O_2 (Low and Heinstein, 1986; Apostol et al., 1987, 1989).

When cells are challenged with polygalacturonic acid elicitors, which are fluorescently labeled, the elicitors are first observed to bind to the plasma membrane. They enter the

cytoplasm, and after approximately 2 hours, they end up in the large central vacuole, where the β-glucanases, which digest the elicitors, are concentrated (van den Bulcke et al., 1989).

In order to test whether the elicitor entered the cell through nonspecific fluid-phase endocytosis or by receptor-mediated endocytosis, Horn et al. (1989) labeled bovine serum albumin and inulin to see if these large molecules, which presumably do not have receptors on the plasma membrane, are taken up by the plant cell. Neither molecule is taken up into the plant cell, indicating that the elicitor is taken up specifically by receptor-mediated endocytosis. Horn et al. (1989) studied the uptake of ^{125}I-labeled elicitors and found that 10^6 molecules are taken up per cell per minute (which is approximately 4.6×10^{-11} mol m^{-2} s^{-1}). They also found that 1 mM KCN and low temperatures (4°C), two treatments that inhibit energy-dependent processes, inhibit elicitor uptake. Furthermore, they found that uptake of the elicitor does not change its molecular size, eliminating the possibility that only small molecules or breakdown products are taken up. Robatzek et al. (2006) have shown that receptor-mediated endocytosis is also involved in the defense response stimulated by a bacterial flagellin peptide (Robatzek, 2007).

Receptor-mediated endocytosis may also be important in understanding the mechanism of auxin action in plants (Geldner et al., 2001, 2003; Geldner and Jürgens, 2006; Dhonukshe et al., 2007), the cell-to-cell communication that occurs during pollination (Lind et al., 1996; Luu et al., 2000; Sanchez et al., 2004), as well as other developmental responses (Geldner and Robatzek, 2008).

8.5.3 Piggyback Endocytosis

Horn et al. (1990) reasoned that water-soluble vitamins like folate, vitamin B_{12}, and biotin, which are too large to passively diffuse across the plasma membrane, may have receptors in the plasma membrane of plant cell membranes like those in animal cells. They also hypothesized that when compounds like biotin are attached to other large molecules that are normally impermeant, it may facilitate the transport of the large molecule across the plasma membrane and into the cytoplasm in a "piggyback" manner. In this way, large molecules could be introduced into the P-space of the cell. Indeed, Horn et al. found that fluorescently labeled molecules, including hemoglobin, RNAse, and bovine serum albumin, which normally do not pass the plant plasma membrane, can permeate the plasma membrane of soybean suspension cells when the macromolecules are tagged with biotin. This may be a great way to introduce antisense RNA, toxins (e.g., phalloidin, cholera toxin, pertussis toxin), antibodies, and individual genes into cells, especially since biotinylation of macromolecules is easy and the reagents are commercially available.

8.6 DISRUPTION OF INTRACELLULAR SECRETORY AND ENDOCYTOTIC PATHWAYS

While mutations have easily led to the discovery of hundreds or thousands of genes and gene products involved in the secretory and endocytotic pathways of various cells in various organisms from various kingdoms, finding specific inhibitors of the proteins involved in specific stages of the secretory pathway has been difficult. It is difficult to find a specific inhibitor of a specific stage in the secretory process because the proteins involved in vesicle blebbing, fusion, and other aspects of transport through the endomembrane system have many similarities with each other, as well as differences. Moreover, the identification of homologous stages of the secretory process in two cell types that each has a very protean secretory system is difficult. Consequently, chemicals that influence the structure, function, or distribution of the membranes that comprise the endomembrane system have had only modest success in helping to elucidate the intracellular pathway taken by a given protein.

Brefeldin A is one such chemical that is used to test the importance of the Golgi apparatus in a given secretory pathway in plants (Klausner et al., 1992; Bauerfeind and Huttner, 1993; Driouich et al., 1993b, 1994; Satiat-Jeunemaitre et al., 1994, 1996; Kaneko et al., 1996). In animal cells, brefeldin A inhibits the ADP ribosylation factor (ARF) that results in the formation of anterograde membrane blebs without affecting retrograde membrane tubularization. This causes the Golgi stacks to be reabsorbed by the ER, and consequently, any trafficking that normally occurs through the Golgi apparatus is inhibited. In plant cells, brefeldin A does not always result in the reabsorption of the Golgi stacks (Langhans et al., 2007), but only induces a redistribution of the Golgi stacks (Satiat-Jeunemaitre and Hawes, 1994); thus, inhibition of secretion in plants by brefeldin A indicates either the Golgi stacks themselves or the arrangement of the Golgi stacks is important for a given secretory pathway.

Secretion can also be influenced by the monovalent cationophore, monensin, an antibiotic isolated from *Streptomyces cinnamonensis* that cause structural changes in the Golgi apparatus (Morré et al., 1983; Mollenhauer et al., 1983; Cornejo et al., 1988; Simon et al., 1990). Monensin has been shown to inhibit the secretion of α-amylase (Melroy and Jones, 1986). Monensin also inhibits the secretion of xyloglucans, but not pectins, in carrot and sycamore culture cells (Moore et al., 1991; Zhang et al., 1993), indicating that these two polysaccharides follow different secretory pathways in the cell. Interestingly, monensin redirects the movement of vicilin, legumin, and concanavalin A so that they are secreted extracellularly instead of being deposited in the protein bodies (Craig and Goodchild, 1984; Bowles et al., 1986).

Treating cells with 2-deoxyglucose to inhibit glycolysis inhibits ER to Golgi traffic, indicating that this transport process is not passive, but requires ATP (Brandizzi et al., 2002b). Other drugs that disrupt the secretory pathway include cyclopiazonic acid and tunicamycin. These agents inhibit the Ca^{2+}-ATPase in the ER and N-linked glycosylation in the ER, respectively (Höftberger et al., 1995).

Inhibitor studies have also indicated that the cytoskeleton is involved in the transfer of vesicles from the Golgi apparatus to the plasma membrane. In plant cells, movement may be mediated by actin microfilaments (see Chapter 10) and not microtubules (see Chapter 11), since microfilament antagonists, but not microtubule disrupting agents, inhibit vesicle migration away from the Golgi stacks and the subsequent secretion (Franke et al., 1972; Mollenhauer and Morré, 1976a).

Endocytosis is also inhibited by Wortmannin, Ikarugamycin, and tyrosine kinase inhibitors (Aniento and Robinson, 2005; Müller et al., 2007; Onelli et al., 2008; Robinson et al., 2008).

8.7 SUMMARY

Movement is the natural condition of living cells, and at the macromolecular and ultrastructural levels, we see that movement is incessantly occurring throughout the endomembrane system. Proteins and polysaccharides move throughout the endomembrane system of the cell in 30–60 minutes. It is still not known how each membrane maintains its unique mixture of proteins and lipids in the face of intense transfer between compartments (van Meer, 1993; Harryson et al., 1996).

We have also seen how the ER and the Golgi apparatus cooperate in the synthesis and secretion of substances that are destined to go to either the vacuolar compartment or the plasma membrane. We have also seen that the plasma membrane and the vacuolar compartment are also in communication with each other through the endocytotic pathway, which includes the endosomes (multivesicular bodies and partially coated reticulum) and the trans-Golgi network. It will be fascinating to determine how these organelles initiate, maintain, or change their spatial localization relative to each other. In Chapter 9, I will discuss the structure of the cytoplasm through which the vesicles, membrane tubules, and organelles move.

A deep and broad understanding of the secretory pathway was discovered in the "virtual century-long collaboration" between Dutrochet, Heidenhain, Palade, Blobel, Rothman, and Schekman. Since then, there have been an enormous number of papers published on the secretory pathways in plant, fungal, and animal cells, and many of these papers have provided deep insight into the importance of specific aspects of the secretory pathway in the life of a cell as it grows, develops, and responds in an adaptive manner to the environment. On the other hand, in elucidating the layers of a never-ending complexity, orders of magnitude of more papers have provided little more than impressive-sounding buzzwords and acronyms that include three letters and a number followed by more letters, which can be used in grant proposals, reviews, and at cocktail parties.

I think that the situation can be compared with the discovery of the structure of the atom. The virtual collaboration between Dutrochet, Heidenhain, Palade, Blobel, Rothman, and Schekman is one biological equivalent to the "virtual collaboration" in physics between J. J. Thomson, Ernest Rutherford, and James Chadwick, who discovered the electron, proton, and neutron, respectively, and elucidated the structure of the atom. However, in the years following these pivotal discoveries, physicists imitating these discoverers searched for more elementary particles and found so many that the collection of them became known as the "particle zoo." There were so many new "elementary particles" discovered that they began to look like epicycles upon epicycles in older versions of astronomy. Willis Lamb (1955) said in his Nobel Prize acceptance speech, "I have heard it said that 'the finder of a new elementary particle used to be rewarded by a Nobel Prize, but such a discovery now ought to be punished by a $10,000 fine.'"

Eventually, Murray Gell-Mann (1969) joined the "virtual collaboration" when he found a new way of looking at the growing list of new particles and developed a theory of elementary particles that simplified the "particle zoo" and provided a new foundation on which to build new physics. Likewise, while so many cell biologists work on discovering the growing list of genes and gene products involved in secretion, I hope that one of them will join the "virtual collaboration of secretory biologists" and find a new way of looking at the secretory pathways in order to find the fundamental laws that unify the "secretory gene zoo."

8.8 QUESTIONS

8.1. How may the flux of proteins influence the structure of the membranes in the endomembrane system of cells undergoing a development change or a physiological change in response to an environmental stimulus?

8.2. How can you explain the protean nature of the membranes of the endomembrane system?

8.3. How may the architecture of the membranes in the endomembrane system differ in growing cells, stationary-state cells, and senescing cells?

Chapter 9

Cytoplasmic Structure

What community of form, or structure, is there between animalicule and the whale, or between the fungus and the fig-tree? And a fortiori, between all four?

—Thomas H. Huxley (1890)

9.1 HISTORICAL SURVEY OF THE STUDY OF CYTOPLASMIC STRUCTURE

In the previous chapters, I discussed the evidence that proteins, vesicles, membranous tubules, and organelles move throughout the cytoplasm. In this chapter, I will discuss the structure of the cytoplasm through which they move. Remember that when the cell was discovered by Robert Hooke (1665), he could only imagine that there was a possibility of an internal structure within the walls composed of passages, valves, instruments, and contrivances, which would be discovered by "some diligent observer, if helped by better microscopes."

In the 17th and 18th centuries, the lenses in light microscopes had various spherical and chromatic aberrations that made it difficult to see minutely-detailed structures in nearly transparent objects (Wayne, 2009). By the 19th century, the optics of microscopes were improved thanks to the invention of the achromatic doublet by Chester Moor Hall, John Dolland, and/or James Ramsden, and its introduction of achromatic lenses into microscopes in the 1820s and 1830s by scientists and inventors, including Giovanni Battista Amici (1818) and Joseph Jackson Lister (1830), the father of the surgeon who pioneered the use of antiseptics. The newly developed lenses were corrected for spherical and chromatic aberrations, and allowed light microscopists such as Félix Dujardin (1835, 1841) to resolve objects that were less than 1 μm, about 100 times smaller than that resolvable by the naked eye (Claude, 1948; Bradbury, 1967). The new microscopes with achromatic lenses provided the means to explore the structure of living beings at the subcellular level.

Robert Brown (1828, 1829) could see that cells consisted of spherical particles and molecules about 1/20,000 of an inch in diameter, and moreover, it was easy to see that these particles and molecules moved independently and incessantly when squeezed out into water. Dujardin (1835, 1841) could see and study the nature of the transparent, water-insoluble, glutinous, contractile substance that held together the food vacuoles of ciliates, and gave it the name *sarcode*, from the Greek word for "flesh." In 1840, Jan Purkinji used the term *protoplasm*, a term long used in religious contexts to mean the first created thing (protoplast = Adam and protoplasmator = God), to designate the living substance of animal embryos. And in 1846, Hugo von Mohl independently applied the term *protoplasm* to the living substance of plant cells, since he believed that the protoplasm was capable of giving rise to all other parts of the cell. By 1848, Alexander Ecker suggested that the sarcode is a fundamental substance of all animal life, from the cells of *Hydra*, to those of muscles in higher animals, and Ferdinand Cohn (1853) further emphasized the ubiquity, constancy, and importance of protoplasm when he wrote:

> *All these properties, however, are possessed by that substance in the plant-cell, which must be regarded as the prime seat of almost all vital activity, but especially of all the motile phenomena in its interior—the protoplasm. Not only do its optical, chemical and physical relations coincide with those of the "Sarcode" or contractile substance, but it also possesses the faculty of forming "vacuoles" … From these considerations it would therefore appear … that the protoplasm of the Botanists, and the contractile substance and sarcode of the Zoologists, if not identical, are at all events in the highest degree analogous formations.*

Calling attention to the similarity of the living substance of all cells and giving it a common name—protoplasm—propelled the search to find a definite structure within the protoplasm that would prove to be the essence of life itself (Beale, 1872; Drysdale, 1874; Brücke, 1898). All the solutions to the problems of life were to be found in the identification of this one structure. The search ensued and dualistic theories, which distinguished between the living part of the cell and the lifeless part, were all the rage. Realizing that animal cells lacked the thick extracellular matrix typically found on the exterior of plant cells, Max

Schultze (1863) decided that the extracellular matrix could be eliminated as a possible candidate. This left the naked protoplasm as the part of the cell that was endowed with all the attributes of life.

The 19th-century biologists were interested in dissecting the protoplasm down to the ultimate constituent of life. Schleiden was enamored with the idea that the nucleus, or the *cytoblast* as he called it, was the elementary particle of life. This was because he could see that cells that had a nucleus were able to reproduce, while those without one could not. Since the cytoblast was not always easy to see, Schleiden later believed that the cytoblast was an elaboration of the invisible cytoblastema, the true elementary substance (Schleiden, 1853). Later work showed that the nucleus existed in all living cells, divided prior to cell division, and as a consequence of its continuity, must house the living substance (von Mohl, 1852; Wilson, 1925; Goebel, 1926). Rudolf von Kölliker coined the term *cytoplasm* in 1862 to distinguish the nucleus from everything else in the protoplasm. The nucleus, like the protoplasm, showed substructure, and, of course, one part was thought to be more vital than its counterpart was. For example, the idiochromatin was considered to be the portion of the nucleus that contained the hereditary material, and was thus more vital than the trophochromatin, which served merely to nourish the idiochromatin (Wilson, 1925).

While one school believed the nucleus or some of its contents were the true living substance, others felt that the surrounding elements in the cytoplasm were more vital. Thus, the cytoplasm was differentiated into various parts to distinguish the most vital part. For example, the cytoplasm was divided into the inner region of granular matter known as the *endoplasm* (Pringsheim, 1854; Hofmeister, 1867) and the outer border of a clearer substance called the *ectoplasm*. Johannes von Hanstein (1868) distinguished the protoplasm from the metaplasm, where the metaplasm performed certain duties necessary for life, but the protoplasm was the true living substance and retained all the properties of life. The metaplasm, which later became known as the ergastic substances, included the cell sap, starch grains, crystals, and the extracellular matrix.

Under the bright-field microscope, the cytoplasm appears as a fine dispersion of particles of different sizes (1–10 μm), freely suspended in a liquid medium. This led Hanstein to propose that the granules form the fundamental nature of cytoplasm (Figure 9.1), that is, the fundamental nature of life. Hanstein (1882) named the granules *microsomes*—a term later used by Albert Claude to designate a membrane fraction isolated from rat liver cells (see Chapter 4). After Hanstein christened these granules, which were previously known as small bodies (i.e., *kleinkörperchen*), with the name *microsomes*, which comes from Greek for "small bodies," Otto Bütschli (1892, 1894) sarcastically wrote that microsomes had now "obtained the right of entry among the privileged and recognized units of cytoplasmic structure, for anything that is called by a Greek name at once seems to many people to be much better known, and as something which must be definitely reckoned with." Richard Altmann suggested that the granules, which he called *bioblasts*, were equivalent to living bacteria, and the cell was really a colony of minute organisms, each of which was the true vital agent in the cell (Altmann, 1890; see Chapters 14 and 15).

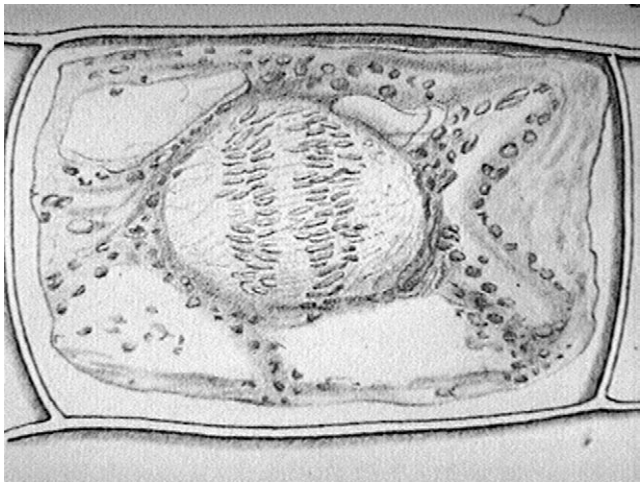


FIGURE 9.1 A dividing cell of *Equisetum* showing the granular nature of cytoplasm. (*Source:* From Hanstein, 1882.)

Others turned their attention to the elements that surrounded the granules. They felt that undue attention was being given to the motley collection of granules, which included vacuoles, crystals, oil droplets, etc., for surely not all the granules were important in understanding the vital nature of cytoplasm; some must only serve as food or contain wastes. The framework that surrounded the granules was studied by two groups of biologists, the histologists and the physical chemists, who did not see eye to eye.

Between 1870 and 1890, the techniques involved in the cytological staining of cells were being developed, and fixed and stained sections revealed an apparently three-dimensional meshwork or entanglement of fibers (see Figure 9.2; Flemming, 1882; Wilson, 1895; Strasburger, 1897; Lee, 1893; Heidenhain, 1907, 1911). The fibers were associated with the parts of the cytoplasm that moved, and Eduard Strasburger called the active, moving parts of the cytoplasm, which appeared fibrous in stained material, the *kinoplasm*. He gave the name *trophoplasm* to the substance that surrounded and supposedly nourished the kinoplasm (Strasburger et al., 1912). The kinoplasm included the plasma membrane, spindle fibers, centrosome, and cilia. Walther Flemming felt that the fibrils were the "seat of the energies on which life depends," while others felt that the hyaloplasm or substance that bathed the fibrillar framework was the real living substance and not just there to feed the kinoplasm (Seifriz, 1936).

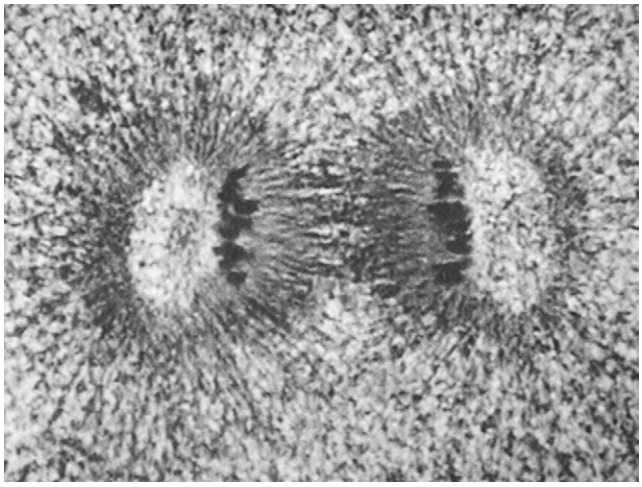


FIGURE 9.2 The fibrous nature of the cytoplasm can be seen in this zygote of *Toxopneustes* photographed in late anaphase. (*Source:* From Wilson, 1895.)

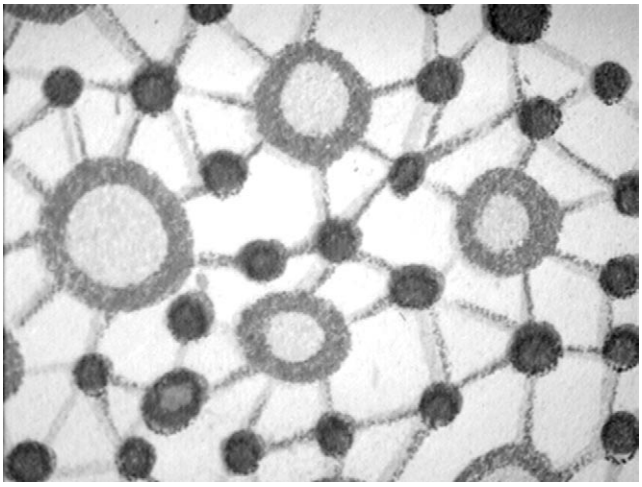


FIGURE 9.3 The aveolar protoplasm in the ovum of *Hydatina senta*. (*Source:* From Bütschli, 1894.)

While cytologists were discovering the unexpected and astonishing behavior of chromosomes and bringing to light new aspects of cell structure, as they observed the spindle fibers and cilia, they worked under the assumption that their techniques disclosed real, preexisting structures that were not visible in the optically transparent living cells. However, the physico-chemically oriented cell biologists, influenced by the tenets of colloidal chemistry, argued bitterly that cytological techniques involving killing, fixing, staining, dehydrating, embedding, and sectioning material caused an artifactual phase separation of the hydrophilic and the hydrophobic substances, which resulted in the production of structures that do not exist in the living cell (Fischer, 1899; Hardy, 1899; Ostwald, 1922). W. B. Hardy (1899) wrote,

> *It is, I think, one of the most remarkable facts in the history of biological science that the urgency and priority of this question should have appealed to so few minds. … It is notorious that the various fixing reagents are coagulants of organic colloids, and that they produce precipitates which have a certain figure or structure. It can also readily be shown … that the figure varies … according to the reagent used. It is therefore cause for suspicion when one finds that particular structures which are indubitably present in preparations are only found in cells fixed with certain reagents.*

Bütschli (1894) further admonished that many of the fibrous elements were probably diffraction artifacts since they could be seen best when using the poorest microscope illumination (Hacking, 1981).

Bertold (1886) and Bütschli (1894) among others looked at living cells and treated the cytoplasm as a semi-solid/semi-liquid or gel/sol colloidal system. Colloids, as we know them now, are macromolecules that are permanently dispersed in solution (Graham, 1842, 1843, 1850–1857; Zsigmondy, 1909, 1926; Zsigmondy and Spear, 1917; Staudinger, 1961). Colloids are approximately 1–1000 nm—larger than low–molecular mass molecules but smaller than bacteria. Wolfgang Ostwald (1922) called this the domain of neglected dimensions (Frey-Wyssling, 1957). Colloids remain suspended because the electromagnetic force that results from their surface properties dominates over the gravitational force that results from their density.

In order to understand the structure of cytoplasm, the physico-chemically oriented biologists followed the teachings of Lord Kelvin and made physical models that looked like protoplasm and imitated some of its properties, including movement (Seifriz, 1936). Bütschli (1894) considered the cytoplasm to be an emulsion of alveolae or vesicles that contained cell sap dispersed in a continuous phase that consisted of the vital element (Figure 9.3). Ahead of his time, feeling like an alchemist and unappreciated (Goldschmidt, 1956), using simple ingredients and techniques originally developed by Quincke (1888) like shaking up salt, water, and oil to create a foam, Bütschli created artificial physico-chemical models of amoeboid motion in order to understand protoplasmic structure and its role in living processes.

Others considered the cytoplasm to be a complex emulsion containing a mixture of oil in water and one of water in oil (Figure 9.4). The dispersed phase was given the name *phaneroplasm* and the invisible, continuous phase was called the *cryptoplasm*. The cryptoplasm was considered to be vitally more important than the phaneroplasm.

As soon as any visible component of the cytoplasm seemed to lack the fundamental properties of life, the next most elusive and smaller component was considered to be the vital part of the cell. In fact, Gwendolen Andrews (1897) wrote, "Nature might be well liked to a great spider, spinning and spinning the living stuff and weaving it into tapestries; and still hiding herself and the ever-lengthening thread of vital phenomena behind the web already spun."

Throughout history, monks, scientists, and philosophers have searched for essences and elixirs that they hoped

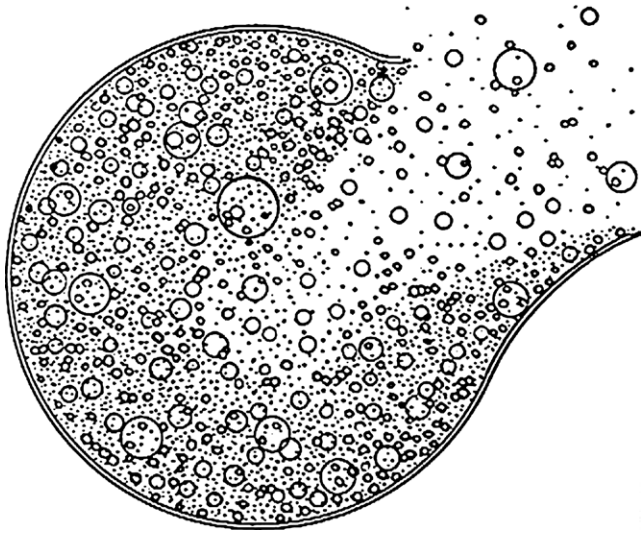


FIGURE 9.4 The protoplasm of a torn *Fucus* egg looks like an emulsion. (*Source:* From Seifriz, 1938b.)

would be the most fundamental unit of life (Berthelot, 1885; Taylor, 1953; Forbes, 1970). For example, Thales believed that water was the essence of all matter, and Jean Baptiste van Helmont provided evidence for this theory by showing that a tree grew and flourished when apparently all he provided it with was water (see Chapter 13). More recently, the putative vital elixir, or essence of life, has been given many names, including biogen, physiological units, bioblasts, micelles, plastidules, plasomes, ideoblasts, biophores, gemmules, pangenes, genes, genomes, transcriptomes, proteomes, metabolomes, ionomes, signalomes, etc. (Harper, 1919; Wilson, 1925; Seifriz, 1936; Conklin, 1940). More recently (in Lewontin, 2001), Sidney Brenner has said, "if he had the complete sequence of DNA of an organism and a large enough computer then he could compute the organism." With a like mind, Walter Gilbert has claimed, "that when we have the complete sequence of the human genome we will know what it is to be human."

The search for the essence of life is based on the assumption that the characteristics of the whole can be found in the constitutive parts. So far the search to find a single structure or compound, smaller than that of a whole cell, that has all the properties of life, including the ability to take up molecules, generate electricity, grow, reproduce, and respond to external stimuli, have failed. According to Frederick Gowland Hopkins (1913),

> *it is clear that the living cell … is … a highly differentiated system; … a system of coexisting phases of different constitutions. Corresponding to the differences in their constitution, different chemical events may go on contemporaneously in the different phases, though every change in any phase affects the chemical and physico-chemical equilibrium of the whole system. … It is important to remember that change in any one of these constituent phases … must affect the equilibrium of the whole cell system, and because of this necessary equilibrium-relation it is difficult to say that any one of the constituents phases … is less essential than any other to the "life" of the cell.*

The reductionist approach has led to the discovery of much cellular structure and function, though ironically in the quest to find the singular secret of life, these discoveries have only emphasized the intricate organization that is necessary for life, and indeed, that the whole is greater than the sum of its parts. None of the isolated microscopic parts exhibits all the properties of life, including assimilation, growth, reproduction, ability to respond to external stimuli, and adaptability (Blackman, 1906). To maintain the living condition for an extended period of time, all the parts of the protoplasm are necessary and together form the basis of life. "Life is not found in atoms or molecules or genes as such, but in organization" (Conklin, 1940).

In several notable articles, all entitled, "The Physical Basis of Life," T. H. Huxley (1890), W. B. Hardy (1906), E. B. Wilson (1923), and J. D. Bernal (1951) came to the conclusion that protoplasm is "the physical basis of life" rather than its essence. I will discuss the structure of cytoplasm, with a view to understanding the physico-chemical milieu in which organelles move and function, vesicles and membranous tubules move, and chemical reactions necessary for life take place.

9.2 CHEMICAL COMPOSITION OF PROTOPLASM

Protoplasm is not a chemical, but an elaborate organization of some of the most complex chemical substances known. Moreover, the chemical composition differs in every species and in every cell of the same organism. As studies on the transcriptome, posttranscriptome, proteome, lipidome, metabolome, signalome, ionome, and all other "-omes" intimate, the chemical composition also varies during the lifetime of a single cell. As a first approximation, however, protoplasm contains proteins, lipids, carbohydrates, nucleic acids, and their constituents (Table 9.1).

Henry Lardy (1965) defined the cytosol as the portion of the cell that is found in the supernatant fraction after centrifugation at 105,000 g for 1 hour. At that time, it referred specifically to the cytoplasm minus the mitochondria and the endoplasmic reticulum (ER). In the cytosol of *E. coli*, the protein concentration is about 200–320 mg/mL, the RNA concentration is about 75–120 mg/mL, and the DNA concentration is about 11–18 mg/mL (see Elowitz et al., 1999; Zimmerman and Trach, 1991).

The substances that make up the cytosol are dissolved in an aqueous salt solution that contains about 75 percent water and 100 mol/m^3 K^+, tens of mol/m^3 Cl^-, 1 mol/m^3 Mg^{2+}, 10^{-4} mol/m^3 each of H^+ and Ca^{2+}, as well as trace quantities of other ions. Most of the water may be free, forming an aqueous phase through which ions can freely diffuse; however,

TABLE 9.1 Composition of dehydrated protoplasm of the slime mold *Reticularia*

Substance	Percent Dry Weight
Protein	28
Nucleic acids	4
Other nitrogen-containing compounds	12
Fat	18
Lecithin	5
Cholesterin	1
Carbohydrates	23
Unknown	9

Source: *From Kiesel (1930).*

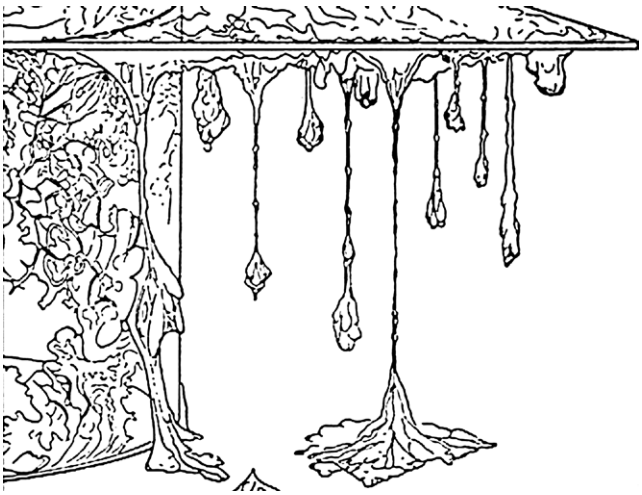


FIGURE 9.5 The behavior of the hanging strands of *Physarum polycephalum* indicates that the protoplasm is highly viscous. (*Source:* From Seifriz, 1938b.)

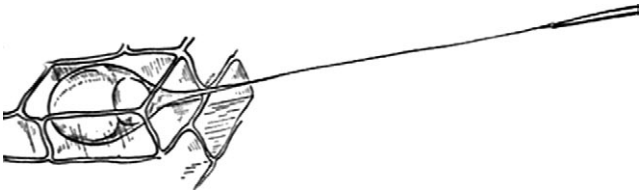


FIGURE 9.6 Stretching the protoplasm with the aid of a microneedle. (*Source:* From Seifriz, 1936.)

a portion of the water is bound to proteins, forming a glass-like phase (Garlid, 2000). The concentrations of ions, as measured with fluorescent dyes, vary spatially and temporally throughout the protoplasm (Rathore et al., 1991; Pierson et al., 1994, 1996; Kropf et al., 1995). The redox potential (ca. −0.309 to −325 V, depending on cell type) and pH of the cytosol (6.5–7.6, depending on cell type and gravistimulation) have been observed in transformed cells using redox- and pH-dependent forms of green fluorescent protein (GFP; Fasano et al., 2001; Moseyko and Feldman, 2001; Jiang et al., 2006). The concentration of various small organic molecules, including adenosine triphosphate (ATP), amino acids, and sugars, usually falls between 0.1 and 10 mol/m^3 (Mimura et al., 1990b; Scott et al., 1995; Haritatos et al., 1996). The various ions and molecules are not necessarily uniformly distributed throughout the cell (Aw, 2000).

Knowing the chemical composition at this level gives us little knowledge of the structure of the cytoplasm. While we might be tempted to conclude that the cytoplasm could behave like a viscous protein solution, we will find that it does not (Luby-Phelps and Weisiger, 1996).

9.3 PHYSICAL PROPERTIES OF CYTOPLASM

Because of the primacy of manual labor in doing work, the ancient Greeks realized the importance of quantifying resistance to movement in order to optimize the number of people necessary for moving a given object (Cohen and Drabkin, 1958; Franklin, 1976). Since the internal resistance or viscosity of solutions influence the mobility of particles contained in it as well as the movement of the solution itself, the viscosity must also be accounted for if we wish to understand the relationship between the motive force and the velocity of movement (Maxwell, 1873, 1878; Garber et al., 1986). Accounting for the resistance quantitatively and from first principles is very difficult and tedious (Tait and Steel, 1878; Synge and Griffith, 1949), and consequently, resistance is often ignored in mechanics. However, resistance is taken into consideration in the fields of rheology and biorheology—fields that study the flow of matter (see Appendix 2). In general, resistance of gases and liquids results in viscous flow (Figure 9.5), while resistance in solids results in the reversible (elastic) and irreversible (plastic) deformation of matter (see Figure 9.6; Blair and Spanner, 1974).

The resistance of the cytoplasm affects all aspects of cellular motion, including the transit of water on or off of ions, the translational diffusion of substrates to enzymes, the rotational diffusion of substrates so that they can properly bind to an enzyme, as well as the movement of membrane vesicles, tubules, and organelles (Fulton, 1982; Goodsell, 1991; Welch and Easterby, 1994; Luby-Phelps, 2000; Zhu et al., 2000; Weihs et al., 2006: Guigas et al., 2007; Jonas et al., 2008). The resistance to movement in the cytoplasm is quantified as the cytoplasmic viscosity (Figure 9.7), which is the primary physical factor that influences the flow of material through the cell (Heilbrunn, 1958; Bereiter-Hahn, 1987; Hiramoto, 1987; Hiramoto and Kamitsubo, 1995).

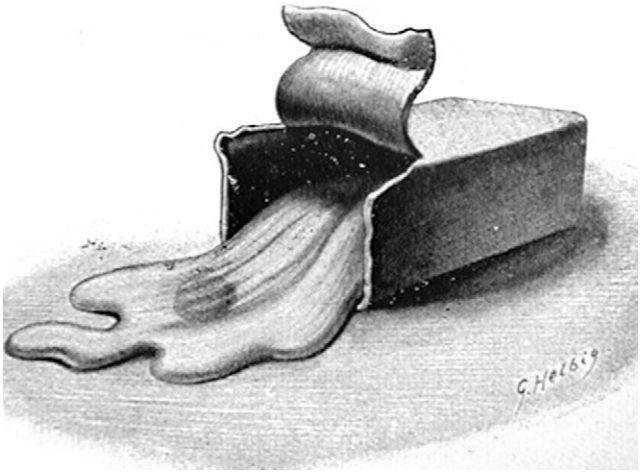


FIGURE 9.7 The viscous cytoplasm of a plant cell. (*Source:* From Kahn, 1919.)

I will present viscosity explicitly as a physically real frictional and dissipative component that resists the movement of something with a constant mass in response to a constant applied force. If one did not account for the viscosity, it would appear that the motive force was weaker or the mass that is moving was greater. Studies on the viscosity and elasticity of cytoplasm have contributed greatly to our understanding of the structure of this living milieu. Rheological studies of the cell wall have also contributed to our understanding of cell growth (see Chapter 20).

9.3.1 Viscosity of the Cytoplasm

Cytoplasm is a viscous fluid, and thus resists flow. Viscosity is a measure of the resistance to flow and is given in units of Pa s, N m^{-2} s, or kg m^{-2} s^{-1}. In order to get a feel for how the viscosity of bulk fluids is measured, imagine placing a fluid between two glass plates that are 1 m apart, and each plate has an area of 1 m^2 (Figure 9.8). Imagine pushing the upper plate to the right with a force of 1 N. (The force exerted by the falling of a 100-g apple is approximately 1 N.) The more viscous the fluid, the longer it will take for the top plate to slide completely past the stationary lower plate. If the fluid (e.g., water) has a viscosity of 0.001 Pa s, it will take 0.001 s for the top plate to slide past the bottom plate. By contrast, if the fluid has a viscosity of about 0.1 Pa s (e.g., olive oil), about 1 Pa s (e.g., glycerol), or about 10 Pa s (e.g., honey), it will take 0.1, 1, or 10 s, respectively, for the top plate to slide past the bottom plate. In the examples given, the top plate travels at a velocity of 1000, 10, or 1 m/s, respectively, relative to the stationary plate.

Newtonian Fluids

A Newtonian fluid is one that obeys the law of fluid flow found in Isaac Newton's *Principia* (1729; Chandrasekhar, 1995). The law states that the velocity of flow (v) of a liquid is proportional to its fluidity (f, in Pa^{-1} s^{-1}) and the shearing stress (σ, in Pa) on either of two plates separated by a distance x (in m). The fluidity of a Newtonian fluid is independent of its velocity. The ratio of the velocity to the distance (v/x) is known as either the velocity gradient or the rate of shear (γ, in s^{-1}). The relationship between velocity and fluidity is given by the following formulae:

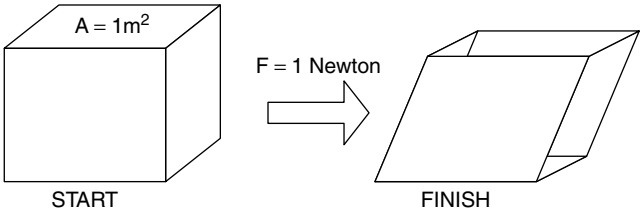


FIGURE 9.8 The deformation of a viscous liquid when it is exposed to a shearing stress.

$$v = f\sigma x \tag{9.1}$$

and

$$v/x = f\sigma \tag{9.2}$$

Newton did not define *viscosity*, the term we use today. This was done by James Clerk Maxwell (1891), who stated that "the viscosity of a substance is measured by the tangential force on the unit area of either of two horizontal planes at the unit of distance apart, one of which is fixed, while the other moves with the unit of velocity, the space being filled with the viscous substance." The viscosity (η) is the reciprocal of fluidity, and it is given by the following formula:

$$\eta = 1/f = (\sigma x)/v = \sigma/(v/x) \tag{9.3}$$

Maxwell (1891) defined a shearing stress as one that moves tangentially along a fluid, and induces movement within the fluid. The shearing stress exerted on a plane causes the adjacent substance to move with a rate of shear that depends on the viscosity of the fluid. The following formula gives the relationship between shearing stress, the rate of shear, and the viscosity:

$$\sigma = \eta(v/x) \tag{9.4}$$

In order to get a feel for the rate of shear, imagine the spinning of a compact disc or DVD. The center point does not move and the edge moves the fastest. Thus, there is a velocity gradient from outside to inside. The difference in the maximal and minimal velocity divided by the distance between them gives the rate of shear. The velocity (v_i) at any distance (x_i) from the nonmoving plate is given by:

$$v_i = (\sigma x_i)/\eta \tag{9.5}$$

and at $x_i = 0$, $v_i = 0$.

Newton's law of fluid flow was finally tested in the 1840s by Jean Poiseuille who came up with a formula that related the velocity of flow of a liquid in a capillary to the pressure difference across the capillary, the radius and length of the capillary, and the viscosity of the solution (Poiseuille, 1940). Poiseuille was a physiologist who was interested in studying the flow of blood in vertebrates. Specifically, he wanted to know why some organs receive more blood than others do. To this end, he studied how fluids moved though glass tubes the size of capillaries. He varied the pressure difference across the tube, the length of the tube, and the diameter of the tube, and measured the time that it took a given volume of liquid to move through the tube.

When Poiseuille varied the pressure difference (dP), he found that the volume flow (Q, in m^3/s) was proportional to the pressure difference. However, as I will discuss below, the coefficient of proportionality (K) also depended on the geometry of the tube as well as the consistency of the fluid:

$$Q = K\ dP \tag{9.6}$$

When he varied the length of the tube (x), he found that the volume flow was inversely proportional to the length. However, the proportionality coefficient (K') still depended on the tube and the fluid:

$$Q = K'(dP/x) \tag{9.7}$$

When he varied the diameter ($2r$), he found that the volume flow was proportional to the fourth power of the diameter. Now all the variation due to the tube was accounted for and the coefficient of proportionality (K'') was only a function of the fluid:

$$Q = K''(2r)^4(dP/x) \tag{9.8}$$

Poiseuille noticed that his law did not hold when either the diameter or the pressure difference was too large or the length was too short. The law no longer holds under these conditions because the flow is no longer laminar but becomes turbulent.

Poiseuille also noticed that K'' was dependent on temperature, and increased as the temperature increased. Thus, he reasonably assumed that K'' was inversely related to the density of the solution. He thus made mixtures of alcohol and water to make solutions of different densities. However, he found that as he increased the density of the solution by increasing the water content, the flow increased and then decreased at even higher densities. This indicated to him that K'' does not depend exclusively on the density, but is inversely proportional to another property of the solution. In fact, the flow can be different in two solutions with the same density, and the same in two solutions with different densities. We now know that the property of a solution that influences flow through pipes is not density, but viscosity.

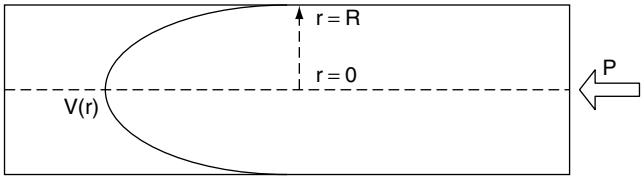


FIGURE 9.9 Parabolic flow of a Newtonian fluid through a tube.

Viscosity was defined by Maxwell years after Poiseuille's experiments.

Maxwell integrated Poiseuille's equation and showed its equivalence with Newton's equation that relates the rate of shear to the shear stress. Integrating Poiseuille's equation allows us to determine the shape of the velocity profile, the maximal velocity, the half-maximal velocity, and the average velocity.

In order to integrate the equation we must define our parameters and assumptions. Let us assume that a fluid is moving in a tube at a constant velocity, which according to Newton, indicates that there is no acceleration, and thus no net force. Therefore, the sum of all the forces acting on the liquid must be zero. We will assume that there are two forces: one due to the pressure difference that pushes the liquid through the tube, and one due to the friction that resists movement through the tube. Given the following tube, let us consider a section of length x and diameter R. r_r is any distance starting from the center of the tube, and parallel to a radius, and varies from 0 to R (Figure 9.9).

The pressure difference along the tube is dP (in Pa or N/m^2) and it induces an inertial force (F_i, in N) that is equal to the product of the pressure difference and the area of a cylinder on which the pressure is exerted (πr_r^2). The inertial force is the force that tends to cause liquids to accelerate:

$$F_i = dP\pi r_r^2 \tag{9.9}$$

The acceleration of a liquid is resisted by the viscous force (F_v). The flow is resisted because each molecule in a liquid is attracted to its neighbors. This attraction, which is due to electrostatic interactions between molecules, causes friction between each layer of the fluid. The electrostatic interactions that cause friction are quantified by measuring the viscosity of the fluid.

The viscosity of a fluid in a capillary can be measured by determining the relationship between the shear stress on the fluid and the rate of shear, just as it was done using two plates.

In a set of planes, $F_v = \eta(dv/dx)A$, where x is the distance between the planes and A is the product of the length and width of the plane. In a cylinder, $F_v = \eta(dv/dr)A$, where r is the radius of the cylinder and A is the area of the tube ($2\pi rx$). That is, the viscous force is equal to the product of the viscosity, the rate of shear, and the area of the outside skin of the cylinder fluid:

$$F_v = \eta(dv/dr)(2\pi rx) \tag{9.10}$$

Consider a fluid that is flowing at a constant velocity; that is, it is exhibiting laminar flow and is not accelerating. In this case, the inertial force is balanced by the viscous force, so that the net force on the liquid is zero:

$$F_i + F_v = 0 \tag{9.11}$$

Thus, $F_i = -F_v$ and

$$dP\pi r^2 = -\eta(dv/dr)(2\pi rx) \tag{9.12}$$

Cancel like terms and solve for dv/dr:

$$dv/dr = -dPr/(2x\eta) \tag{9.13}$$

According to the rules of calculus, we can find the maximum or minimum velocity by finding where $dv/dr = 0$.

In order to determine the velocity at any distance r from the center of the tube, we must integrate Eq. 9.13. First, multiply both sides by dr:

$$dv = -(dP/(2x\eta))r\,dr \tag{9.14}$$

Now take the integral of both sides, leaving the constant $dP/(2x\eta)$ outside the integral:

$$\int dv = -(dP/(2x\eta))\int r\,dr \tag{9.15}$$

After taking the antiderivative of each side, we get:

$$v(r) + A = -(dP/(2x\eta))(r^2/2) + B \tag{9.16}$$

where A and B are constants of integration and can be combined into C, as follows:

$$v(r) = -(dP/(2x\eta))(r^2/2) + C \tag{9.17}$$

In order to solve for C we use the "no slip" condition—that is, the velocity at the boundary of the cylinder and the wall of the tube is zero. So $v = 0$ when $r = R$:

$$v(R) = 0 = -(dP/(2x\eta))(R^2/2) + C \tag{9.18}$$

and

$$C = (dP/(2x\eta))(R^2/2) \tag{9.19}$$

Thus:

$$v(r) = -(dP/(2x\eta))(r^2/2) + (dP/(2x\eta))(R^2/2) \tag{9.20}$$

which can be simplified to:

$$v(r) = (R^2 - r^2)(dP/(4x\eta)) \tag{9.21}$$

Solving this equation for various values of r shows that when a pressure is applied across a tube containing a solution with a constant viscosity, the velocity is maximal in the center of the tube, and declines with the square of the distance as we move toward the edge of the cylinder where $v = 0$. Thus, there is parabolic flow. In 1860, Jacob Eduard Hagenbach named this equation after Poiseuille. Gotthilf Hagen, an engineer, independently discovered the law of parabolic flow, and in 1925, Wilhelm Ostwald, who invented an instrument to measure viscosity and who also defined happiness in terms of one's ability to overcome resistance (Boltzmann, 1904; Gadre, 2003), renamed the law of parabolic flow the Hagen-Poiseuille Law. This law has been useful for describing the flow of water through the xylem and the flow of sugar through the phloem (Zimmermann and Brown, 1971).

It is common, although not entirely correct, to refer to the half-maximal velocity as the average velocity. The average and half-maximal velocity are only equal when the rate of shear is linear. The half-maximal velocity is given by:

$$v_{0.5\max} = dPr^2/(8x\eta) \tag{9.22}$$

The average velocity of parabolic flow, which I will not derive, is given by:

$$v_{\text{ave}} = dPr^2/(6x\eta) \tag{9.23}$$

The viscosity of a number of fluids is given in Table 9.2. The viscosity of a fluid can be measured in the manner described previously, or, as I will discuss later, it can also be calculated by measuring the velocity of a falling ball through the liquid (Stokes, 1922). Viscosity is not only important at the cellular level, but affects many organismal behaviors. The morphologies of plants and animals have

TABLE 9.2 Viscosity of various newtonian fluids

Substance (°C)	Viscosity (Pa s)
Glycerin (25)	0.954
Castor oil (20)	0.986
Heavy machine oil (15.6)	0.6606
Light machine oil (15.6)	0.113
Olive oil (20)	0.084
Water (20, 25, 37)	0.001, 0.0009, 0.0007
Air (18)	0.0000002

Source: *From Weast, R. C. ed. The Handbook of Physics and Chemistry. 54th Edition. Cleveland, OH: CRC Press, 1973–1974.*

evolved in part due to the influences of viscosity (Vogel, 1981; Niklas, 1992; Denny, 1993).

Non-Newtonian Fluids

Many solutions do not show parabolic flow and thus do not obey Poiseuille's Law. These are called *non-Newtonian fluids* (Seifriz, 1920, 1921, 1929, 1931, 1935; Kamiya, 1956; Allen and Roslansky, 1959). Newtonian solutions obey Poiseuille's Law because they have a single viscosity. However, non-Newtonian solutions possess an infinite number of viscosity values, where the viscosity of the solution depends on the rate of shear, otherwise known as the *velocity gradient*. Noburô Kamiya (1950) used Poiseuille's Law to study the flow of the endoplasm of *Physarum*, using the ectoplasm as the tube. He noticed that the velocity of the flowing endoplasm did not show a parabolic profile, where the rate of shear would be proportional to the shearing stress. Kamiya found that most of the particles in the endoplasm travel at the same speed (Figure 9.10). More recent measurements using laser Doppler velocimetry show that there is more variation in the velocities than Kamiya observed, but still less than would be predicted if the cytoplasm moved by parabolic flow (Mustacich and Ware, 1977b; Earnshaw and Steer, 1979). Kamiya concluded that the nonparabolic flow indicated that the viscosity of the endoplasm depended on the rate of shear. That is, close to the ectoplasm, where the rate of shear was highest, the viscosity of the endoplasm was low, while in the center of the cell, where the rate of shear was lowest, the viscosity was highest, and the endoplasm there moved as a block.

The dependence of the viscosity on the rate of shear depends on the molecular structure of the fluid. When the viscosity of the solution is independent of the rate of shear, the solution is probably composed of noninteracting spherical molecules. In non-Newtonian fluids, the electrostatic attraction between the molecules is not symmetrical, and depends on the position of the molecules relative to each other. The relative position depends on the flow, and thus, the non-Newtonian properties depend on the relationship between the electrostatic energy between the molecules and the mechanical energy that can change their position.

If we know the viscous properties of a solution, we can make estimates of its molecular structure. For example, if the viscosity decreases as the rate of shear increases, we can infer that the solution is composed of asymmetrical molecules that may have the appearance of linear fibers. Solutions that show this property are called *thixotropic* (from the Greek words for "change by touch") solutions. When the viscosity increases as the rate of shear increases, the solution is called dilatent (Reynolds, 1885, 1886). We can surmise that such a solution is compressible, and in response to a force, the particles come together to form a "tighter" solution. A dilatent solution may also be composed of highly branched, knotted, or hooked molecules that get entangled when exposed to a force. Figure 9.11 shows the relationship between the shearing stress and rate of shear, and Figure 9.12 shows the relationship between the rate of shear and viscosity for Newtonian, thixotropic, and dilatent solutions. In thixotropic solutions, the viscosity decreases as the rate of shear increases, and in dilatent solutions, the viscosity increases as the rate of shear increases. Catsup, an extract of plant cells, is thixotropic (Vonnegut, 1999). Cornstarch, another plant extract, is dilatent (Seifriz, 1936; Heilbrunn, 1958).

Because the viscosity of thixotropic solutions is highest when they are not disturbed or nothing is moving through them, and decrease as something moves through them, they possess what is called a *yield value*. The yield value is the minimum shearing stress required to produce a flow. In the case of cytoplasm, the yield value is a measure of the minimum force per unit area necessary to move a vesicle, chromosome, or organelle through the cytoplasm. The cytoplasmic motors I will discuss in Chapters 10 and 11 are capable of providing the force per unit area necessary to overcome the yield value.

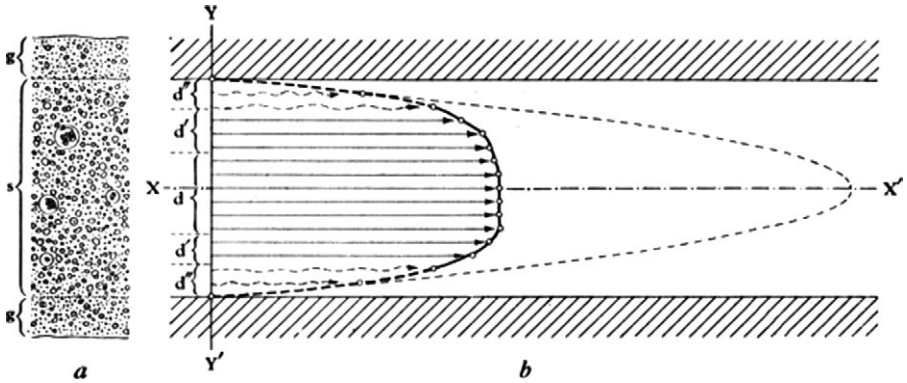


FIGURE 9.10 (a) A drawing of the sol-like endoplasm (s) and gel-like ectoplasm (g) of a plasmodial strand of *Physarum*. (b) The velocity distribution of endoplasmic flow. The small circles represent experimental values, and the broken line represents what the velocity distribution would look like if the endoplasm were Newtonian and the flow were parabolic. (*Source:* From Kamiya, 1950.)

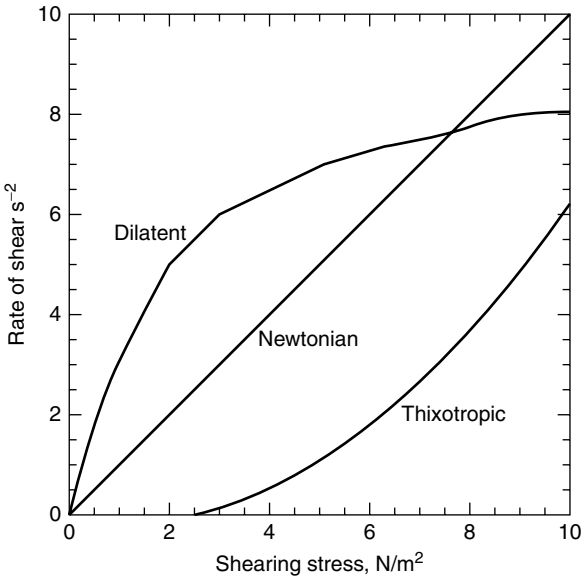


FIGURE 9.11 Graph of curves relating the rate of shear to the shearing stress. The viscosity of the solution is obtained from the reciprocal of the slope (or the tangent to the line at any point). The relationship between shearing stress and rate of shearing is linear for Newtonian fluids and nonlinear for non-Newtonian fluids. The non-Newtonian fluids are either dilatent or thixotropic. The viscosity of dilatant fluids increases as the shearing stress increases. Consequently, the rate of shear does not rise as fast at higher shearing stresses. The viscosity of thixotropic fluids decreases as the shearing stress increases. Consequently, the rate of shear rises faster at higher shearing stresses.

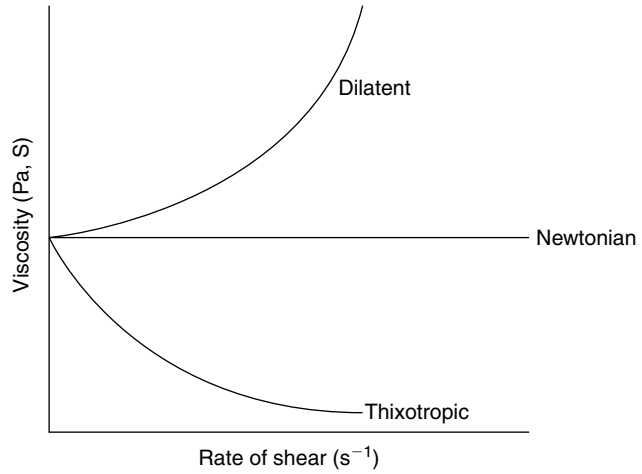


FIGURE 9.12 Newtonian and non-Newtonian (dilatent and thixotropic) viscosity. The viscosity of a dilatent solution can be proportional to the rate of shear, the rate of shear squared, or by a more complicated function of the rate of shear (see Appendix 2). The viscosity of a thixotropic solution can be inversely proportional to the rate of shear, the rate of shear squared, or by a more complicated function of the rate of shear.

Experimental Approaches to Measuring Cytoplasmic Viscosity

Cell biologists have come up with a variety of ingenious methods for measuring the viscosity of cytoplasm. For example, cytoplasmic viscosity can be studied with a centrifuge microscope, which is essentially a microscope with a rapidly rotating stage (Hiramoto and Kamitsubo, 1995). Kamitsubo et al. (1988) determined the viscosity of cytoplasm by observing the velocity of lipid droplets moving through the cytoplasm when the cell was exposed to a centrifugal force, which put a shearing stress on the lipid droplets. Eiji Kamitsubo and his colleagues used Stokes' Law to calculate the viscosity from the applied shearing stress and the observed rate of shear.

Stokes' Law was derived from empirical observations that determined the relationship between the forces that resist the movement of a sphere of a given radius through a viscous medium of a certain viscosity, with the velocity of the sphere. Stokes' Law is:

$$F_v = 6\pi r_H \eta v \tag{9.24}$$

We can determine the velocity of a sphere, if it falls under the influence of gravity, because the inertial force exerted on the sphere due to gravity is given by Newton's Second Law:

$$F_i = mg \tag{9.25}$$

where m is the mass of the sphere and g is the acceleration due to gravity (9.8 m/s^2). In the absence of friction, the sphere will continue to travel due to its inertia, which is a function of its mass. In the absence of friction, the velocity will also increase over time as a result of the acceleration due to gravity.

Since the mass of a sphere acted upon by gravity depends on the density difference ($\rho_s - \rho_m$) between the sphere and the medium it passes through, the mass must be calculated from the following formula:

$$m = (\rho_s - \rho_m)(4/3)\pi r^3 \tag{9.26}$$

Thus, Newton's Second Law can be rewritten as:

$$F_i = g(\rho_s - \rho_m)(4/3)\pi r^3 \tag{9.27}$$

Newton's First Law states that a body remains at rest or in uniform motion unless a force acts upon it. According to this law, if a sphere falls at a constant velocity, there must be no net force exerted on it. In other words, the inertial force is opposed by the force resisting the travel; that is, the inertial force is opposed by a frictional force. Thus, the velocity of a sphere falling in a viscous fluid due to the force of gravity can be found by setting $F_i + F_v = 0$, where there is no acceleration, or simply, $F_i = -F_v$. Thus:

$$-6\pi r_H \eta v = g(\rho_s - \rho_m)(4/3)\pi r^3 \tag{9.28}$$

Assuming that the gravitational force is constant, we can solve for v. After rearranging terms, canceling, and simplifying, we get:

$$v = \frac{2g(\rho_m - \rho_s)r^2}{9\eta} \tag{9.29}$$

Thus, the velocity of a falling sphere is directly proportional to the acceleration due to gravity and the difference in density between the medium and the sphere. The velocity is proportional to the square of the radius of the sphere, and inversely proportional to the viscosity of the medium. Moreover, in a viscous solution, the velocity is constant with respect to time. (On the other hand, in cases where the viscosity is nil, the change in velocity with respect to time will be constant. That is, $F = mg = mdv/dt$. Therefore, $g(dt) = dv$, $g\int dt = \int dv$, and $v(t) = gt$). It is important to realize that this case is only an idealization presented by Galileo and Newton and can never be realized because if η were equal to zero, the terminal velocity of any particle or projectile, which is the velocity at $t = \infty$, would be infinite.

In order to calculate the viscosity of the cytoplasm, we have to know the density of the lipid droplets and the cytoplasm through which they move, the radius of the lipid droplets, and their velocity.

The density of the endoplasm of characean cells was determined by cutting the cell and allowing the endoplasm to fall in a density gradient of dextran dissolved in artificial cell sap, which had an osmotic pressure equal to that of the cell. Due to its high molecular mass, the dextran exerts a negligible osmotic pressure so that the vesicles maintain their original density without swelling or shrinking. The endoplasmic drops are then left to fall into the gradient. Eventually, $v = 0$ and the drop stops when it reaches its own density, that is, when $\rho_m = \rho_s$. The average density found by this method is $1.015 \times 10^3\,kg/m^3$ (Kamiya and Kuroda, 1957). Have you noticed that I just explained the principle behind density gradient centrifugation?

It is also possible to measure the time it takes for a drop of endoplasm to fall a certain distance in a medium of known density and viscosity. Using this method, Kamiya and Kuroda (1957) found that the density of the endoplasm is $1.0145 \times 10^3\,kg/m^3$. These values are very close to those found recently using optical methods (Wayne and Staves, 1991).

Once the density of the endoplasm is known, it is possible to determine the viscosity with a centrifuge microscope using Stokes' Law. Prior to the measurement of cytoplasmic viscosity, the endoplasm of a characean cell is centrifuged down to one end of the cell. This takes about 5–10 minutes at 1000 g. Then the viscosity of the endoplasm is determined by measuring the movement of oil droplets, which have a density of approximately $960\,kg/m^3$, through the endoplasm under various amounts of centrifugal acceleration (Kamitsubo et al., 1989).

TABLE 9.3 Effect of centrifugal acceleration on shearing stress

Acceleration (g)	σ (N/m^2)
100	0.178
200	0.356
300	0.534
400	0.712
500	0.890

Source: ***From Kamitsubo et al. (1988).***

If the cytoplasm were a Newtonian fluid, the viscosity would be constant at all rates of shear. Furthermore, in a Newtonian fluid, the rate of shear would be linearly related to the shear stress and the relationship, plotted on a graph, would go through the origin. In order to test whether or not the cytoplasm is a Newtonian or non-Newtonian fluid, Kamitsubo and his colleagues carried this experiment out at different shearing stresses and different rates of shear. At various amounts of centrifugal acceleration (100–500 g), the interface between the oil droplet and the endoplasm experiences various shearing stresses and rates of shear (Kamitsubo et al., 1988).

The shearing stress on a spherical particle, which is the amount of force per unit area (F/A) that is experienced by the sphere in a centrifugal field is given by the following equation:

$$\sigma = F/A = (V/A)\alpha g(\rho_m - \rho_s) \tag{9.30}$$

This is just a restatement of Newton's Second Law ($F = ma$) where both sides are divided by area to convert force into stress. The shearing stress (σ) is proportional to the volume of the sphere (V), the centrifugal acceleration (αg), and the density difference ($\rho_m - \rho_s$). The shearing stress is inversely proportional to the surface area of the sphere (A). Since for a sphere, $V/A = r/3$, Eq. 9.30 becomes:

$$\sigma = (r/3)\alpha g(\rho_m - \rho_s) \tag{9.31}$$

The relationship between centrifugal acceleration and the shearing stress on a lipid droplet, with a radius of 10^{-5} m and a density of $960\,kg/m^3$, moving through a cytoplasm with a density of $1014.5\,kg/m^3$ is given in Table 9.3.

The rate of shear is the velocity gradient across a solution in which a body may or may not be immersed. The rate of shear is zero when the solution and/or object are at rest. The viscosity of a solution measured by the falling

ball method, in which a spherical body falls, is given by Eq. 9.32, which I already derived:

$$\eta = \sigma/\gamma = \frac{2\alpha g(\rho_m - \rho_s)r^2}{9v} = ((r/3)\alpha g(\rho_m - \rho_s))/(3v/(2r)) \quad (9.32)$$

Thus, we can use Eq. 9.32 combined with the definition of the shearing stress on a sphere to determine the mathematical definition of the rate of shear of a sphere. The rate of shear of a sphere is given by the following formula:

$$\gamma = v(3/(2r)) \quad (9.33)$$

The rate of shear (γ) of a sphere is proportional to the velocity of the sphere, and a geometric factor that is given by ($3/2r$).

Kamitsubo et al. (1988) measured the velocities of various lipid bodies moving in a centripetal direction under a variety of centrifugal forces and calculated the rate of shear of each particle and the shearing stress that caused that rate of shear. These data are presented in Table 9.4.

Plotting the rate of shear versus the shearing stress (Figure 9.13), Kamitsubo et al. (1988) got a straight line that intercepts the *x*-axis at about 0.5 Pa. This means that at shearing stresses greater than 0.5 Pa, the lipid droplets move at a velocity that is proportional to the shearing stress. However, at shearing stresses less than 0.5 Pa, the cytoplasm has a very high resistance and the oil droplets are unable to move in the cytoplasm. The point where the relationship of rate of shear versus shearing stress crosses the *x*-axis is called the yield value. The possession of a *yield value* is characteristic of thixotropic, non-Newtonian fluids.

We can obtain the cytoplasmic viscosity by dividing the shear stress by the rate of shear (Table 9.4):

$$\eta = \sigma/\gamma = \frac{2\alpha g(\rho_m - \rho_s)r^2}{9v} \quad (9.34)$$

Now we can now plot the viscosity against the rate of shear (Figure 9.14). We can see that the viscosity is very high at low rates of shear, and very low at high rates of shear. That is, the cytoplasm is thixotropic.

These measurements come from characean cells that have very active streaming. Since the endoplasm travels with a velocity of about 100 μm/s at the outside of the endoplasm and about 90 μm/s approximately 10 μm toward the center, the endoplasm travels with a rate of shear of (10 μm/s)/(10 μm) or about 1 s^{-1}. With this rate of shear, the viscosity of the streaming endoplasm must be very

TABLE 9.4 Relationship between rate of shear, shearing stress, and cytoplasmic viscosity

Lipid Droplet No.	γ (s^{-1})	σ (Pa)	η (Pa s)
1	0.69	0.545	0.790
2	0.79	0.673	0.852
3	1.88	0.609	0.324
4	2.56	0.660	0.258
5	3.85	0.644	0.167
6	5.18	0.805	0.155
7	8.09	0.877	0.108
8	11.00	1.45	0.132
9	11.90	0.887	0.075

Source: *From Kamitsubo et al. (1988).*

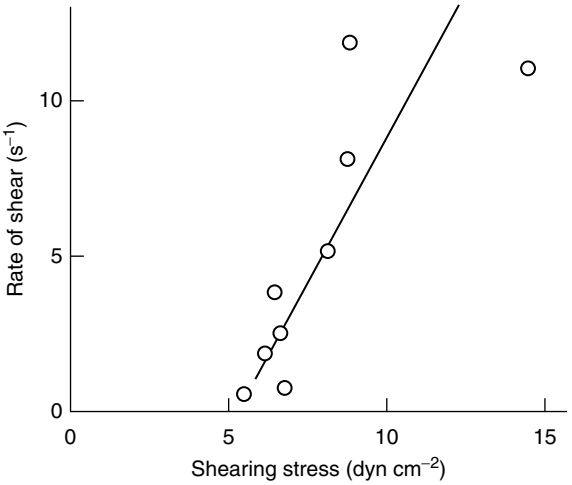


FIGURE 9.13 The relationship between shearing stress and rate of shear for the endoplasm of *Nitella axilliformis* determined with a centrifuge microscope. (*Source:* From Kamitsubo et al., 1988.)

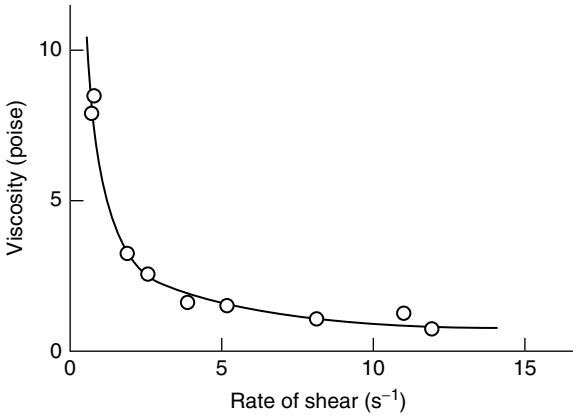


FIGURE 9.14 The relationship between rate of shear and viscosity for the endoplasm of *Nitella axilliformis* determined with a centrifuge microscope. (*Source:* From Kamitsubo et al., 1988.)

high (0.8 Pa s), about 800 times the viscosity of water. The narrow range of velocities in the streaming cytoplasm observed with laser Doppler velocimetry is consistent with the non-Newtonian nature of the cytoplasm of characean cells (Mustacich and Ware, 1974, 1976, 1977; Langley et al., 1976; Sattelle and Buchan, 1976).

The viscosity of neutrophils is approximately 0.131 Pa s, as measured by pulling the cells into a pipette and measuring the deformation. The viscosity decreases as the rate of shear increases, indicating that the cytoplasm of neutrophils is also thixotropic (Tsai et al., 1993, 1994). As a rule, we can consider the bulk viscosity of cytoplasm to be between 0.1 and 0.8 Pa s, which is 100–800 times the viscosity of water, and between the viscosity of olive oil and glycerol.

According to Newton's Second Law, the acceleration is proportional to the force. By contrast, according to Stokes' Law, the velocity is proportional to the force. Which law is a better predictor of what happens in the cytoplasm? Newton's Second Law is not valid when friction is not negligible and Stokes' Law typically applies when the movement of the sphere is slow and its size is small (Rayleigh, 1893). But how slow is slow, and how small is small? Quantitatively, this is measured with the Reynolds number (Reynolds, 1901). The Reynolds number (*Re*, dimensionless) is the ratio of the inertial force to the viscous force. While the Reynolds number for moving solids is an approximation, which cannot be rigorously derived, it is valuable for approximations (Dodge and Thompson, 1937).

When the Reynolds number is greater than 1, friction is negligible, inertial forces dominate, and a body in motion will tend to stay in motion. Newton's Second Law best describes the motion of a spherical particle in an inertial system:

$$F_i = a(\rho_s - \rho_m)(4/3)\pi r^3 \tag{9.35}$$

For any shaped particle with a "characteristic length" of x, Newton's Second Law is:

$$Fi \cong a(\rho_s - \rho_m)x^3 \tag{9.36}$$

When the Reynolds number is less than 1, viscous forces dominate and Stokes' Law best describes the motion of a spherical particle:

$$F_v = 6\pi r_H \eta v \tag{9.37}$$

For any shaped particle with a "characteristic length" of x, Stokes' Law is:

$$F_v \cong x\eta v \tag{9.38}$$

When viscous forces predominate, there is no inertia and a body needs a constant force to stay in motion; otherwise it will stop instantly (Purcell, 1977). The mechanics of Aristotle apply to situations where the Reynolds numbers are low (Franklin, 1976).

Since $Re = \left|F_i/F_v\right|$, for any shaped particle:

$$Re \cong \left|[a(\rho_s - \rho_m)x^3]/(x\eta v)\right| \tag{9.39}$$

$$Re \cong \left|[a(\rho_s - \rho_m)x^2]/(\eta v)\right| \tag{9.40}$$

Since $a = v/t$, we can simplify:

$$\begin{aligned} Re &\cong \left|[(v/t)(\rho_s - \rho_m)(x^2)]/(\eta v)\right| \\ &= \left|[(\rho_s - \rho_m)x^2]/(\eta t)\right| \end{aligned} \tag{9.41}$$

Since dimensionally, $t = x/v$, we can simplify again:

$$Re \cong \left|[v(\rho_s - \rho_m)x]/\eta\right| \tag{9.42}$$

Thus, the Reynolds number is proportional to the velocity, the density difference, and the characteristic length. It is inversely proportional to the viscosity.

In the cytoplasm, where the viscosity is typically between 0.1 and 0.8 Pa s, the density differences between a moving body and the cytoplasm are less than 250 kg/m^3, the velocity of movement is between 0.1 and 100 μm/s, the characteristic length of a moving body is between 1 and 10 μm, the Reynolds number is typically much less than 1, and viscous forces predominate over inertial forces by approximately one million times. Consequently, in the cytoplasm, Newton's Second Law is not valid. When viscous forces predominate, the inertial forces are negligible, and movement stops the instant the applied force is removed.

Kikuyama and Tazawa (1972) measured the viscosity of the cytoplasm of characean cells using a really clever method. They introduced *Tetrahymena* into the vacuolar space of *Nitella* by means of vacuolar perfusion. After closing both ends by cellular ligation, the *Tetrahymena* was forced into the endoplasm by centrifugal force. After centrifugation, the cell was ligated near the centrifugal end to obtain an endoplasm-rich cell fragment containing *Tetrahymena*. The viscosity of the cytoplasm was estimated by measuring the swimming speed of the *Tetrahymena* in the endoplasm. This was compared to a standard curve where the swimming speed of *Tetrahymena* was measured in media of various viscosities. Using this method, they estimated that the bulk viscosity was between 0.04 and 1 Pa s. We do not know whether the variation is due to the non-Newtonian properties of the cytoplasm, where the faster the *Tetrahymena* swim, the more they reduce the viscosity.

As Heilbronn did in 1914, Staves et al. (1997) observed the falling of amyloplasts through the cytoplasm of columella cells, and used Stokes' Law to estimate the bulk viscosity

of the cytoplasm. They estimated the cytoplasmic viscosity of columella cells to be approximately 0.268 Pa s. From an analysis of the amyloplast sedimentation data of Yoder et al. (2001), one can conclude that the cytoplasmic viscosity of the columella cells is anisotropic.

The viscoelastic properties of the cytoplasm can be measured with laser tweezers. Arthur Ashkin discovered that lasers are able to move organelles with the force provided by photons (Ashkin and Dziedzic, 1987; Ashkin et al., 1987; Leitz et al., 1995; Schindler, 1995; Berns and Greulich, 2007). The force per unit area provided by the laser is calculated from the following equation:

$$F/A = EFR/(nc) \tag{9.43}$$

where *EFR* is the energy fluency rate of the laser (in J m^{-2} s^{-1}), n is the refractive index of the cytoplasm (dimensionless), and c = speed of light (3×10^8 m/s). Since the energy fluency rate of the laser is approximately 10^{11} J m^{-2} s^{-1}, it is capable of producing a shearing stress of hundreds of N/m^2 (Ashkin et al., 1987).

Using laser tweezers to move particles through the cytoplasm, Ashkin and Dziedzic (1989) find that cytoplasm has a yield value and is thus a non-Newtonian fluid. They find that the yield value of the moving endoplasm of scallion epidermal cells is approximately 0.1 N/m^2, and the yield value of the stationary ectoplasm is between 10 and 1000 N/m^2. The yield value for the endoplasm is similar to those found previously in other cells. The yield value of the endoplasm is 0.5 Pa in *Nitella*, and in *Physarum* it is between 0.06 Pa and 0.11 Pa, depending on the direction of movement (Sato et al., 1989). The presence of a yield value means that the cytoplasm in these cells is also non-Newtonian. Cytoplasmic viscosity has also been measured by injecting magnetic particles into cells or allowing them to be taken up by phagocytosis and then determining the ability of magnetic tweezers to move the particles (Bausch et al., 1999; Scherp and Hasenstein, 2007).

As I have already stated, the viscosity of the cytoplasm is, in part, the relationship between the velocity a particle moves and the force to which it is subjected. However, in a non-Newtonian cytoplasm, the viscosity that is measured also depends on the size of the particle. Thus, at a given shearing stress, the cytoplasm may have differing viscosities that depend on the size of the moving particle. The viscosity of the cytoplasm toward large particles like organelles (>100 nm) is known as the bulk viscosity. The viscosity experienced by metabolite-sized molecules (<1 nm) is known as the *microviscosity*, and the viscosity experienced by macromolecules (~1–100 nm) is called the *intermediate viscosity*. The studies mentioned above measured the bulk viscosity of cytoplasm using organelles as moving bodies.

Kate Luby-Phelps et al. (1985, 1986, 1988) have estimated the intermediate and microviscosity of the cytoplasm by measuring the diffusion coefficient of a number of different molecules in the cytoplasm with the aid of the fluorescence redistribution after photobleaching (FRAP) technique (see Chapter 2). As I stated in Chapter 2, the diffusion coefficient of a spherical particle depends on the viscosity, according to the Stokes-Einstein equation: $D = kT/(6\pi r_H \eta)$.

Luby-Phelps et al. fluorescently labeled dextrans of various sizes and microinjected them into the cytoplasm. Then they measured the diffusion coefficient of the dextran in the cytoplasm by bleaching a region of the cytoplasm and watching the recovery of fluorescence over time. They also measured the diffusion coefficient of the dextran in water by bleaching a region of the water and watching the recovery of fluorescence over time. Then they plotted the ratio of the two diffusion coefficients, which is an estimate of the relative viscosity of the cytoplasm ($D_c/D_w = \eta_w/\eta_c$) versus the hydrodynamic radii of the molecules tested (Figure 9.15). They estimate, by extrapolation, that the viscosity of the cytoplasm for infinitesimally small molecules is about four times the viscosity of water. They also find that the viscosity of the cytoplasm is not a constant but is proportional to the radius of the molecule for molecules with hydrodynamic radii from 2–15 nm. Thus, the larger the molecule, the greater the viscosity it experiences in the cytoplasm. This means that diffusion in the cytoplasm is hindered by some kind of network. For molecules with radii between 15 and 60 nm, the viscosity remains constant at a high value, approximately 12.5 times the viscosity of water. The constant viscosity for molecules with different hydrodynamic radii indicates that the elongated molecules may reptate through the netlike cytoplasm like worms. If we extrapolate the slope to the x-axis, where the diffusion

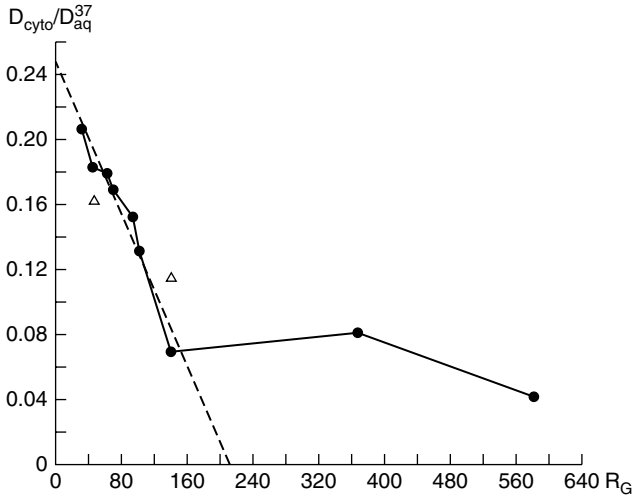


FIGURE 9.15 The effective viscosity experienced by dextrans in the cytoplasm depends on the size of the dextran (R_G, which is the radius of gyration). D_{cyto}/D_{aq} is the ratio of the diffusion coefficient of the dye in the cytoplasm relative to the diffusion coefficient of the dye in water. D_{cyto}/D_{aq} is related to the reciprocal of the effective viscosity. (*Source:* From Luby-Phelps et al., 1986.)

in the cytoplasm becomes zero, we find that the radius is 20.7 nm. This may indicate that the diameter of the holes in the mesh of the cytoplasm is about 41.4 nm (Provance et al., 1993). Fluorescently labeled proteins diffuse throughout *E. coli* cells with a diffusion coefficient that indicates that, for proteins, the cytoplasm is about 11 times more viscous than water. The apparent viscosity depends not only on the size of the protein, but also on its charge (Elowitz et al., 1999).

In order to visualize the microviscosity of the cytoplasm, Luby-Phelps et al. (1993) have used a very small fluorescent probe (Cy 3.18), the quantum yield of which varies with viscosity, and (Cy 5.18), another small fluorescent probe, the quantum yield of which is independent of viscosity. They then visualized the viscosity of the fluid phase of the cytoplasm by ratio imaging the two dyes. They find that the viscosity of the fluid phase is not significantly different from that of water (Luby-Phelps, 1994). Bicknese et al. (1993) and Kao et al. (1993) also measured the microviscosity of the cytoplasm next to the plasma membrane to be approximately 0.0011 Pa s.

By contrast, Keith and Snipes (1974) studied the microviscosity of the aqueous portion of the cytoplasm using spin labels. They found that the viscosity of this aqueous space is about 100 times greater than the viscosity of water. These experiments were done with human cells, bean cells, and *Chlamydomonas*.

We can conclude that the cytoplasm is a non-Newtonian viscous fluid where the viscosity depends on the rate of shear and the size of the moving object. It also changes throughout the life of the cell. Since viscosity can affect all transport processes, it must always be taken into consideration. According to R. J. P. Williams (1961), "It may well be that the achievement of a separation of activated reagents in space plus restricted diffusion provides the fundamental distinction between biological chemistry and test-tube chemistry."

The Effect of Environmental Stimuli on Cytoplasmic Viscosity

Virgin (1954) observed that the chloroplasts in the leaf cells of *Elodea* were more easily displaced by centrifugation when they were treated with blue light. Similarly, Seitz (1967, 1979) observed the same phenomenon in the leaf cells of *Vallisneria spiralis*. Takagi et al. (1989, 1991, 1992) have shown that blue light has the same effect in *Vallisneria gigantea*, whereas red light has the opposite effect. These results and others indicate that the viscosity of the cytoplasm is not constant but can be influenced by environmental factors, and perhaps these factors influence some cellular processes, in part through their effect on viscosity (Stafelt, 1955; Seitz, 1987; Virgin, 1987; Mansour et al., 1993). Thus, the translational diffusion of substrates to enzymes, the rotational diffusion of substrates so that they can properly bind to an enzyme, as well as the movement of membrane vesicles, tubules, and organelles may be affected by environmental factors.

9.3.2 Elasticity of the Cytoplasm

The conclusion that the cytoplasm is constructed of fibrous elements is supported by studies on the elasticity of cytoplasm. Elasticity is the property of a body to resist deformation and to reversibly recover from deformation produced by force. The elasticity of a material is defined by Young's modulus of elasticity, which can be determined by stretching the body in question. In the case of a wire, the elastic modulus, M (in Pa or N/m^2), is given by determining the elongation (dx) of a wire of length x and radius r that is produced by a given force (mg). The relation between stress ($mg/\pi r^2$) and strain (dx/x) is given by the following formula:

$$M = \text{stress/strain} = ((mg)/(\pi r^2))/(dx/x) \qquad (9.44)$$

The elastic modulus, also known as Young's modulus, is equal to the stress ($mg/\pi r^2$) needed to produce a doubling of the length (when $dx = x$), which is a unit strain (dx/x). Young's modulus is usually determined by extrapolation because many substances break before they double in length.

In William Seifriz's time (1924), elasticity was the best indicator of the structure of living cytoplasm. Since elasticity depends on the presence of linear molecules, he assumed that the cytoplasm was composed of a network of linear molecules. This is also consistent with the thixotropic behavior of cytoplasm. He demonstrated that protoplasm is elastic by stretching it between microneedles. He found that live protoplasm is very elastic, while dead protoplasm is not elastic. Seifriz looked at plasmolyzed cells of the onion epidermis. After plasmolysis, the tissue is cut across to expose some cells without touching the protoplasm within. The cells were entered with a microneedle, and the naked protoplasm touched. The protoplasm stuck to the needle and could be drawn out to great lengths. When the thread snapped, its elastic limit had been passed. Once the elastic limit was passed, the protoplasmic thread snapped back, becoming reincorporated into the protoplasm, usually without even disturbing the continuous cytoplasmic streaming (Figure 9.6).

Crick and Hughes (1950) and Crick (1950) estimated that the elastic modulus of cytoplasm is about 10 N/m^2 using a magnetic particle technique. With this technique, magnetic particles were introduced into the cytoplasm of chick fibroblasts by phagocytosis and then "pulled" with a magnetic field to determine the relationship between the stress applied in the form of a magnetic field and the strain observed. When the magnetic field was shut off the particles recoiled, providing further evidence for the elastic

nature of cytoplasm. As a comparison, rubber has an elastic modulus of 5×10^8 N/m^2. Francis Crick and Arthur Hughes (1950) described the cytoplasm like so: "If we were compelled to suggest a model we would propose Mother's Work Basket—a jumble of beads and buttons of all shapes and sizes, with pins and threads for good measure, all jostling about and held together by 'colloidal forces.'"

The elastic modulus can also be determined by coating magnetic beads with a peptide containing RGD (Arg-Gly-Asp), and subjecting the bead to a magnetic field. The RGD binds to the integrins in the plasma membrane and the magnetic field causes the bead to rotate. The degree of bead displacement is a function of the elastic modulus of the cell (Fabry et al., 2001).

9.4 MICROTRABECULAR LATTICE

Can we see the linear structures in the cytoplasm that lead to its thixotropic behavior and elastic properties? Keith Porter and others have observed a microtrabecular lattice in animal (see Figure 9.16; Wolosewick and Porter, 1979; Porter and Tucker, 1981; Porter and Anderson, 1982; Porter, 1984) and plant cells (see Figure 9.17; Hawes et al., 1983; Wardrop, 1983) using the million-volt electron microscope that allows one to observe thick sections. While there are arguments whether the microtrabecular lattice is fact or artifact, it may represent the fibrous network of the cytoplasm as envisioned by Rudolph Peters (1929, 1937) and Joseph Needham (1936).

Albert Szent-Györgyi (1941) proposed that enzymes may form a solid-state structure in the cell, and such a structure may be necessary for their action in vivo. Perhaps metabolite channeling has resulted in the evolution of the microtrabecular lattice, which is actually composed of all the proteins in the cell that form a quintinary structure, including those enzymes of the glycolytic pathway (see Chapter 14; Clarke and Masters, 1975; Knull et al., 1980; McConkey, 1982; Srere, 1985; Svivastava and Bernhard, 1986; Schliwa et al., 1987; Pagliaro, 1993, 2000; Ovádi and Srere, 2000).

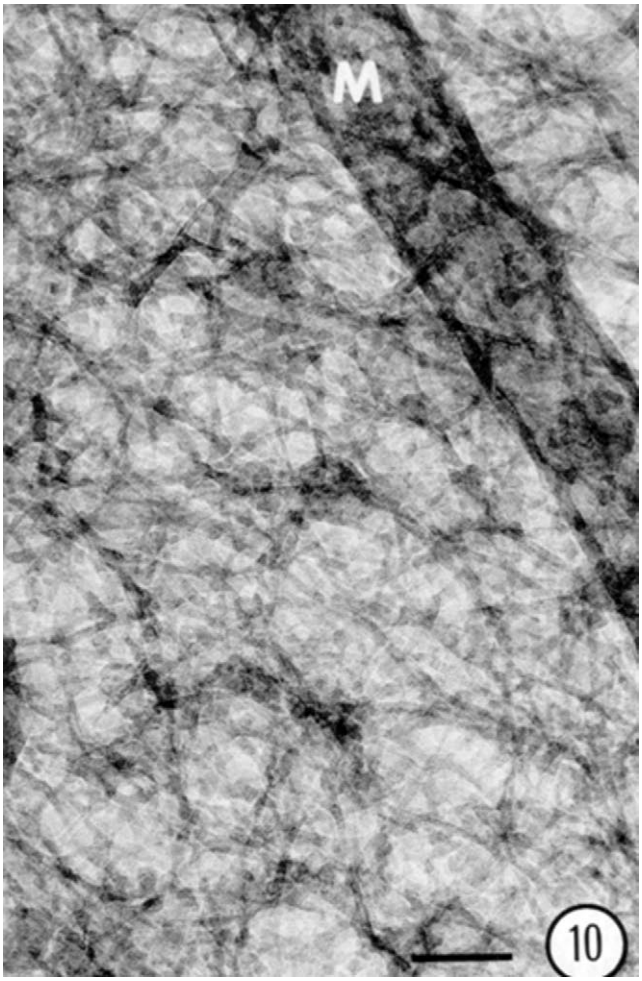


FIGURE 9.16 The microtrabecular lattice of a WI-38 cultured cell prepared by the freeze-substitution method. M, mitochondrion. ×100,000. (*Source:* From Wolosewick and Porter, 1979.)

9.4.1 Function of the Microtrabecular Lattice in Polarity

The microtrabecular lattice may influence differentiation of cell types. Cell differentiation may be brought about by the differential localization of various determinants in embryos (Wilson, 1925; Davison, 1976). These cytoplasmic determinants appear to be, in part, maternal messenger RNA molecules. The cytoplasmic determinants are localized in various regions of the egg cell, and are selectively distributed to particular embryonic cell lineages where they may initiate specific developmental programs (Jeffrey, 1982, 1984a,b, 1985; Jeffrey and Wilson, 1983; Jeffrey et al., 1983; Jeffrey and Meier, 1984; Swalla et al., 1985). The eggs have three distinct regions with specific morphogenetic fates: the ectoplasm, endoplasm, and myoplasm. I will only discuss the myoplasm, which gives rise to muscle cells. When radiolabeled poly-U is used as an in situ hybridization probe for poly-A RNA, a general feature of all messenger RNAs (see Chapter 16), it is found that 45 percent of the poly-A RNA is in the ectoplasm, 50 percent is in the endoplasm, and 5 percent is in the myoplasm. However, when a radiolabeled actin cDNA is used, it is found that 40 percent of the actin mRNA is present in the ectoplasm, 15 percent is in the endoplasm, and 45 percent is in the myoplasm. Thus, the myoplasm, which is destined to be muscle, is specifically enriched in actin mRNA.

When the eggs are extensively extracted with Triton X-100 so that almost everything in the cell is washed away, a detergent-resistant lattice remains. This lattice includes actin, tubulin, and intermediate filaments. When this lattice is probed with radiolabeled cDNA using in situ hybridization,

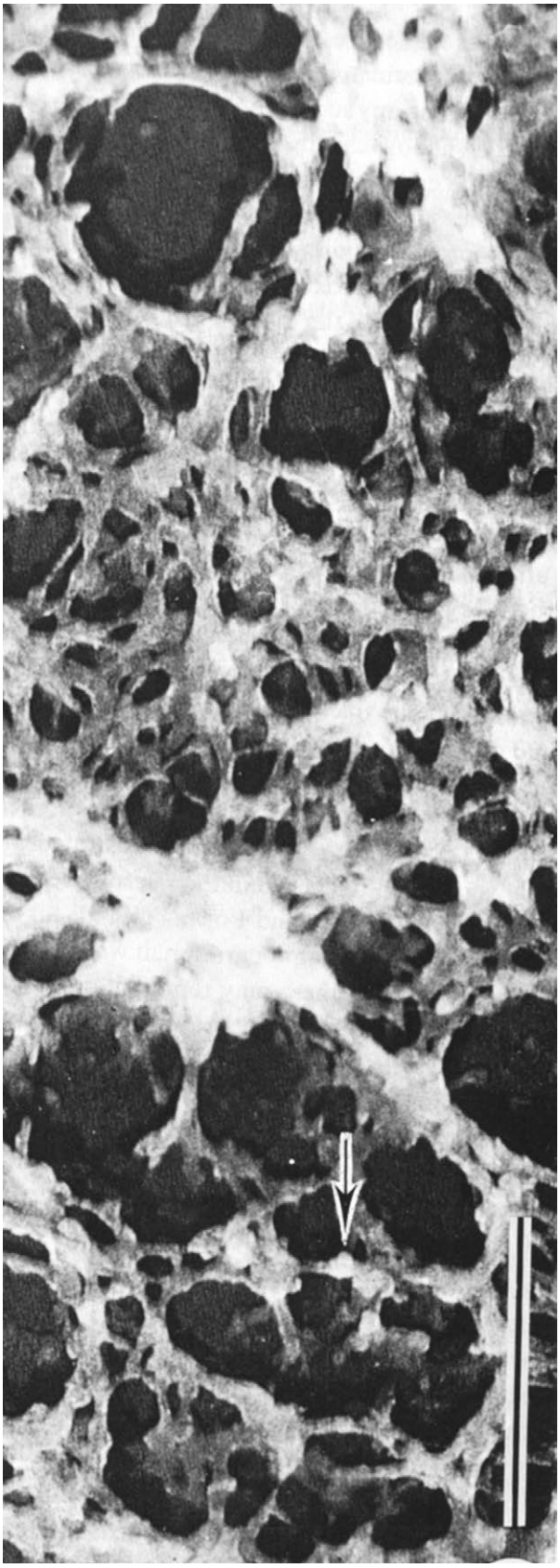


FIGURE 9.17 The microtrabecular lattice in a parenchyma cell of *Zea mays*. Bar, 500 nm. (*Source:* From Wardrop, 1983.)

it is found that the actin mRNA is bound to the lattice and remains in the same position it was in before detergent extraction. It appears that the cytoplasmic lattice can help maintain a polarity in cells so that the daughter cells of a division get unequal components, which may determine their developmental fate.

9.5 SUMMARY

We have seen from cytological evidence provided by Walther Flemming and Eduard Strasburger; biophysical evidence provided by William Siefriz, Noburô Kamiya, Eiji Kamitsubo, Kate Luby-Phelps, Arthur Hughes, and Francis Crick; as well as electron microscopic evidence provided by Keith Porter and Alan Wardrop, that the cytoplasm consists of a three-dimensional network of fibrous elements of unknown composition. This anatomizing reticulum may form a structure for the enzymes of various biochemical pathways, and *pari passu* provides a resistance to the movement of intracellular macromolecules, vesicles, membranous tubules, and organelles. We see that the cytoplasm behaves as a non-Newtonian fluid. When the rate of shear is low, it behaves as a gel (gelatin) and has a high viscosity. When the rate of shear is high, it behaves as a sol (solution) and has a low viscosity. We see that the cytoplasm has a yield value and at shearing stresses below the yield value, the cytoplasm provides such a high resistance to movement that increasing the shearing stress up to the yield value does not induce movement. Movement only occurs when the shearing stress is greater than the yield value. The low Reynolds number tells us that inertial forces are essentially nonexistent and viscous forces predominate. Thus, there is no inertia in the cell. Movement requires the application of a constant force and movement will stop instantly upon the removal of the force.

So in order to induce a vesicle to move in the cytoplasm, we have to apply a force per unit area on the vesicle that is greater than the yield value of the cytoplasm ($\sim$0.5 Pa) and we have to continue to apply this force for as long as we want the vesicle to move. Thus, we need intracellular motors to move the vesicles. We have at least two classes of motors available: one that uses microfilaments as a track and one that uses microtubules as a track. These motors are discussed in Chapters 10 and 11.

9.6 QUESTIONS

9.1. Would it be productive today to try to discover the part of the protoplasm that is essential for life? Why or why not?

9.2. How would cellular processes differ if the cytoplasm were not viscous?

9.3. How would cellular processes differ if the cytoplasm were not thixotropic?

Chapter 2

Plasma Membrane

By fate, not option, frugal Nature gave …
It was her stern necessity: all things
Are of one pattern made …
Deceive us, seeming to be many things,
And are but one …
To know one element, explore another,
And in the second reappears the first …

—Ralph Waldo Emerson, "Xenophanes"

2.1 THE CELL BOUNDARY

The plasma membrane provides a barrier that separates the living cellular protoplasm from the external environment, and as such, resists the attainment of equilibrium prophesied by the kinetic theory of molecules and the Second Law of Thermodynamics (Clausius, 1879; Bünning, 1989). For this reason, the plasma membrane is a *sine qua non* for life (Just, 1939). However, the boundary must not be absolutely impassable but must allow the entrance of nutrients into and the excretion of waste products out of the cell. Moreover, the plasma membrane, by virtue of its position at the frontier of the protoplasmic substance, is particularly suited to sense changes in the external environment so that the cell can act appropriately. Of course, the external environment for a cell in a multicellular plant also includes all the other cells in the same plant that are connected through chemical signals or physical forces! As the interface of the cell, the plasma membrane is involved in every cellular process that depends on the cell's ability to respond to external stimuli, including light, gravity, hormones, salinity changes, pollination, and pathogen attack.

The honeycomb-like appearance of the thick walls found in wood and cork inspired Hooke (1665) to name the compartments *cells*. However, confusion concerning the definition of a cell ensued when it was discovered that animal cells lack such a wall even though they contain a nucleus and protoplasm (Baker, 1988). As a result of this discovery, the cell was redefined by Franz von Leydig (1857).

Leydig (1857) defined the cell as a soft substance containing a nucleus and surrounded by a plasma membrane. Johannes von Hanstein (1880) used the term *protoplast* for the soft substance containing a nucleus and surrounded by a plasma membrane, in order to avoid the confusion that could be caused by having two definitions of a cell. However, the term never caught on (see Sachs, 1882). The great plant physiologist Julius Sachs (1892) rejected the use of the word *cell* for a wall-less protoplast, and said sarcastically, that, if it were correct to call the protoplast a cell, then a bee should be called a cell and the honeycomb should be called the capsule of the cell! Unbelievably, this argument over terminology was resurrected 100 years later (Robinson, 1991; Sack, 1991; Staehelin, 1991; Stafford, 1991; Connolly and Berlyn, 1996)! In this book, I will use the words *protoplast* and *cell* interchangeably to emphasize that the plasma membrane and not the wall provides the major functional division between the living matter and the external environment.

2.2 TOPOLOGY OF THE CELL

The plasma membrane divides the volumes inside and outside the cell topologically into two compartments: the external space or E-space, which is the volume external to the plasma membrane; and the protoplasmic space or P-space, which is the volume immediately inside the plasma membrane (Figure 2.1). That is, the plasma membrane separates the "living" space from the "lifeless" space. In most bacteria, which typically have only one membrane, these are the only compartments, although this too is a generalization since there can be more than one aqueous phase in a single membrane-enclosed compartment (Tehei et al., 2007). At first glance, the situation appears unreasonably complicated in eukaryotic cells, which contain many membranous compartments. However, we will see that keeping track of the topology of each compartment in the cell will help us to develop some generalizations about the cell. For example, the P-space will generally have a lower Ca^{2+} concentration than the E-space.

The E-space is not totally lifeless or abiotic. For example, surrounding the epidermal cells in many roots, symbiotic

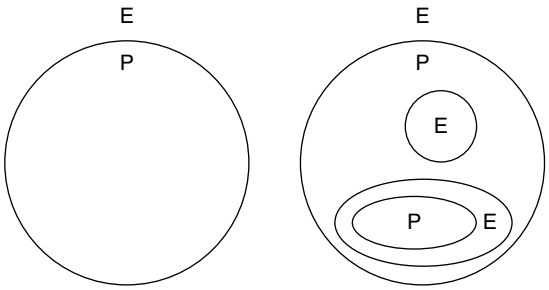


FIGURE 2.1 The topology of the cell. The external space (E-space) and protoplasmic space (P-space) are separated by a membrane. The figure on the right shows the topology a cell with many compartments.

mycorrhizal fungi live, which secrete a protein known as glomalin that allows better root development by structuring the soil so that it is more permeable to air and water (Wright and Upadhyaya, 1996, 1998, 1999).

Gilbert Ling (1984, 2001) proposed an alternative to the membrane theory of separation between the E- and P-spaces. He proposed that the colloidal nature of the proteins in the P-space differentially bind ions and cause a matrix or Donnan potential similar in magnitude to the observed "membrane" potential. If Ling were correct, the high electrical resistance of the plasma membrane (discussed in the following section) would be superfluous because the proteins in the cytosol would bind the ions so tightly that the proteins would resist the movement of ions more than the membrane would. While there may be some truth in the membrane and matrix theories, evidence including the high electrical resistance of the plasma membrane (Walker, 1960) and the rapid diffusion of dyes in the cytoplasm but not across the membrane (Chambers, 1922; Plowe, 1931) suggest that the membrane theory is the best first approximation of reality.

2.3 EVIDENCE FOR THE EXISTENCE OF A PLASMA MEMBRANE

In 1844, Carl von Nägeli noticed that the protoplasts of various algae, including *Nitella* and *Bryopsis*, pull away from the wall when the cells are exposed to various concentrated solutions, and that they return to their normal size when the concentrated solutions are replaced by dilute solutions (Figure 2.2). Realizing that protoplasts exhibited the same osmotic properties that Jean-Antoine Nollet and Henri Dutrochet described for animal bladders, Nägeli as well as Nathaniel Pringsheim (1854) concluded that there must be a differentially permeable membrane around the protoplast. After investigating the plasmolysis of many plant cells that could be easily visualized as a consequence of their anthocyanin content, Nägeli and Cramer (1855) concluded that a cell membrane was a typical characteristic of plant cells.

In 1867, Wilhelm Hofmeister found that the protoplasts that make up beet roots shrink in concentrated NaCl solutions. However, Hofmeister turned his attention to the shrinking of the easily visible red-colored vacuole and concluded that the entire protoplast, and not an invisible surface layer, is responsible for the osmotic properties of the cell. Hofmeister proposed that the osmotic movement of water into and out of the protoplast is primarily responsible for plant movements, including the touch-sensitive movements of the leaves of *Mimosa*, the temperature-induced opening and closing of tulip flowers, and the light- and/or gravity-induced bending of plant organs (Goebel, 1926).

Hugo de Vries (1885, 1888a), like Hofmeister, performed similar plasmolytic experiments on the violet epidermal cells of *Tradescantia discolor*. He also noticed that the protoplast detached from the cell wall and it was de Vries who named this phenomenon *plasmolysis*. However, due to the obvious shrinkage of the violet vacuole, he believed that the vacuolar membrane, which he termed the *tonoplast* because of its putative role in tonicity or turgor, and the protoplasm surrounding the tonoplast were responsible for the osmotic properties of the cell. While in reality, these botanists demonstrated the differential permeability of the plasma membrane, they did not think that the surface layer was an important regulator of the osmotic properties of the cell (Briggs and Robertson, 1957). Many influential botanists held on to this view up until the 1960s, thus impeding the advancement of plant membrane biology (Dainty, 1962; Hope and Walker, 1975; Wayne, 1994).

Wilhelm Pfeffer, a botanist influenced by the physicochemical philosophy of Hermann von Helmholtz, turned to the study of cellular mechanisms in order to satisfy his curiosity to understand the thigmonastic or touch-induced leaf movements of the sensitive plant, *Mimosa pudica* and the rapid movements of the stamen of the knapweed, *Centaurea jacea* (Bünning, 1989). In order to understand osmosis,[1] the basis of the pushing force that causes the leaf movement, Wilhelm Pfeffer (1877) turned to the model membranes that Justus Liebig's student, Moritz Traube, designed out of copper ferrocyanide.[2] Traube (1867) designed these membranes in order to create "artificial cells" so he could study the processes of living cells, including growth and osmosis (Figure 2.3). The artificial cells could expand and bud like living cells; however, the artificial membranes were not strong enough to withstand the osmotic pressure that developed within them, and consequently broke easily. To overcome this problem, Pfeffer

[1] From *osmos*, the Greek word meaning "to push."

[2] To make artificial cells, fill a beaker three-quarters full with a 5 percent copper sulfate solution ($CuSO_4$). Use forceps to drop a small crystal of ferrocyanide ($K_4Fe(CN)_6$) into the solution. Do not disturb. Observe the formation of a precipitation membrane of copper ferrocyanide. Note the growth of the "cell" and the different colored solutions that are outside and inside the precipitation membrane. For other work on artificial membranes, see Collander (1924, 1925) and Michaelis (1926a).

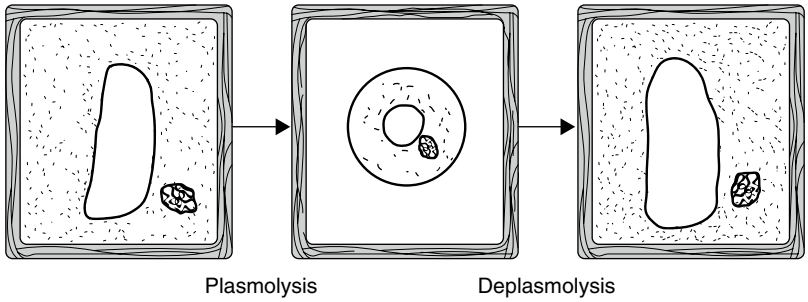


FIGURE 2.2 Plasmolysis and deplasmolysis of a plant cell.

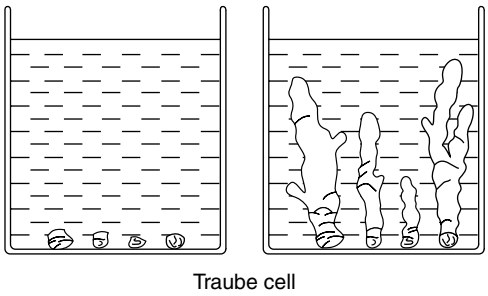


FIGURE 2.3 A Traube cell. The diagram on the left shows the initial situation and the diagram on the right shows "cell" growth after approximately 15 minutes.

deposited the copper ferrocyanide membranes on a porous clay pot, imagining that the pot would protect the artificial membrane from lysing, much like a plant cell wall prevents the rupturing of the protoplast. The membrane-covered porous pot was connected to a thin capillary tube (Figure 2.4). When he added solutions of sucrose to the inside of the copper ferrocyanide membrane, the water moved from the outside of the membrane into the sucrose solution and caused the sucrose solution to rise in the capillary. He defined osmotic pressure as the pressure that must be added to the top of the capillary to prevent the water from entering the sucrose solution surrounded by the differentially permeable membrane. Pfeffer's membranes were strong enough to perform repeated measurements, and he was able to get quantitative results (see Chapter 7). Noticing that thin artificial membranes exhibited the same osmotic phenomena as protoplasts, Pfeffer postulated that a thin plasma membrane surrounded the entire protoplast and regulated the osmotic properties of the cell (Bünning, 1988). Indeed, he also mentioned that the analogous behavior of cells and model membranes indicates that a vital force is not responsible for the permeability properties of cells.

Pfeffer also used classical anatomical techniques to understand the nature of the plasma membrane. He noticed that the plasma membrane was stained by iodine or mercury, and thus concluded that it was composed, at least in part, of proteins. Pfeffer (1877) also believed that physiological

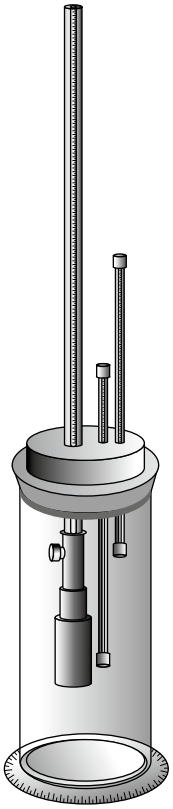


FIGURE 2.4 Wilhelm Pfeffer's osmometer.

experiments would provide a method for understanding the structure of the plasma membrane. He said:

> *Though our understanding of the experimental results does not necessarily compel us to presuppose a very definite conception of the molecular structure of precipitation membranes, we feel even more an intellectual need for deeper insight because only on the basis of such insight can our thoughts follow the path of a solute through the membrane.*

Pfeffer coined the term *plasma membrane* to emphasize its differentially permeable nature. Others, who did not believe that there was a functional differentially permeable boundary at the surface of the cell, called this region the *plasmalemma*, which just means the surface of the protoplast (Mast, 1924).

In 1899, Ernest Overton, a distant cousin of Charles Darwin, examined the permeability of a medley of living plant and animal cells, including root hairs, algal filaments, muscle cells, and red blood cells, to about 500 compounds and came up with a series of generalizations—sometimes called Overton's Rules. Overton measured permeability by observing the ability of a substance to induce cell shrinkage (that is, to cause osmotic water flow out of the cell). He postulated that less permeable substances would cause plasmolysis while the more permeable substances, which moved into the cell as fast as the water could move out, would not cause plasmolysis. He also postulated that substances of intermediate permeability would cause an initial plasmolysis, resulting from the initial flow of water out of the cell, followed by deplasmolysis, resulting from the permeation of the solute and an equilibration of the osmotic pressure on both sides of the membrane. From his plasmolysis studies, he found that sugars, amino acids, neutral salts of organic acids, and glycerol barely enter living cells, while alcohols, aldehydes, ketones, and hydrocarbons permeated rapidly. He concluded that the possession of a charged or polar group (COO^-, OH, NH_2) in a chemical substance decreased its ability to permeate living cells.

The polarity of a functional group can be estimated from the polar nature of the bonds that make up the functional group. The greater the difference between the electronegativities of the two atoms that make up a bond, the greater the ionic or electrical dipole nature of the bond. A functional group composed of primarily ionic bonds will be polar. On the other hand, the smaller the difference is between the electronegativities of the two atoms that make up a bond, the more covalent the bond is. Because a pure covalent bond has a vanishingly small electrical dipole, functional groups composed primarily of covalent bonds are nonpolar. Linus Pauling (1954a) created the electronegativity scale to characterize the electrical nature of bonds. From Figure 2.5, one sees why functional groups composed primarily of OH bonds are more polar than functional groups composed of CH bonds.

Consistent with the electrical interpretation of the chemical bonds just described, Overton (1900) also found that the ability of chemicals to permeate living cells increased as the length of their hydrocarbon chains increased. His observations indicated a positive correlation between lipid solubility or lipophilicity and permeability. He postulated that the plasma membrane, which regulated the permeability of the cell, must be composed of lipid. Overton went on to show that dyes that were soluble in lipid permeated the cell faster than those that were not.

Considerably, more support for Overton's theory came from the work of Collander and Bärlund (1933), who analyzed chemically the amount of a given substance that appeared in the cell sap of characean cells a certain time after the cells were placed in a given concentration of the substance. Runar Collander (1937, 1959) found that, in general, the more lipophilic a substance is, the greater its ability to permeate the membrane (Figure 2.6). However, some small hydrophilic molecules, like water, also permeate quickly. Collander concluded that while the membrane

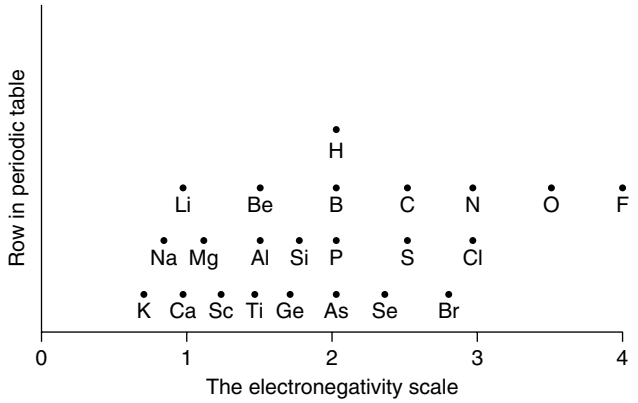


FIGURE 2.5 Linus Pauling's electronegativity scale. The greater the difference between the electronegativities of the two atoms that make up a bond, the greater the polarity of the bond and the greater the polarity of the functional group (e.g., OH) that is composed of these bonded atoms. The smaller the difference between the electronegativities of the two atoms that make up a bond, the more covalent the bond and the less polar the functional group (e.g., CH) that is composed of these bonded atoms. (*Source:* Data from Pauling, 1954a.)

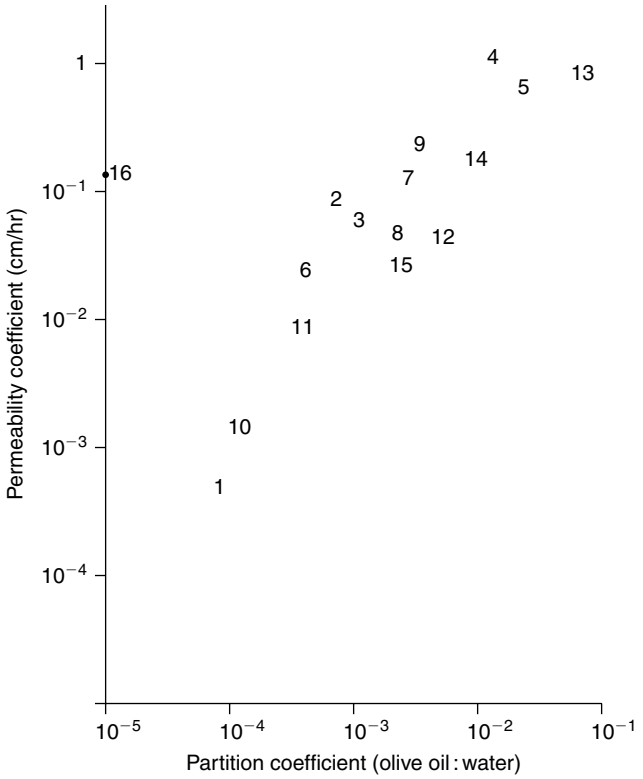


FIGURE 2.6 The relationship between the permeability coefficient and olive oil:water partition coefficient. 1, glycerol; 2, formamide; 3, acetamide; 4, methanol; 5, ethanol; 6, ethylene glycol; 7, propylene glycol; 8, succinamide; 9, cyanamide; 10, urea; 11, methylurea; 12, dimethylurea; 13, urethane; 14, n-butyramide; 15, dimethyl urea; 16, DHO. (*Source:* Data from Collander, 1937, 1959.)

is made primarily of lipid, it is really a mosaic, and must contain aqueous pores to account for the high permeability of some small polar molecules (Höber, 1945; Ling, 1984).

In order to determine the arrangement of the lipids in the plasma membrane, Gorter and Grendel (1925) isolated the lipids of chromocytes (i.e., red blood cells) by dissolving them in acetone. Mammalian red blood cells are a favorite material of plasma membrane biologists since, unlike most eukaryotic cells, the plasma membrane in mammalian red blood cells is the only membrane in the cell (Bretscher and Raff, 1975). Gorter and Grendel floated the isolated lipids on the surface of a Langmuir trough (Adam, 1941; Taylor et al., 1942; Becher, 1965) and decreased the surface area of the trough until the lipids formed a monolayer (Figure 2.7). They measured the surface area of the monolayer and calculated the surface area of the original red blood cells from measurements of the cell radius (r). The area of the monolayer was twice as large as the area of the red blood cells, and so they concluded that the plasma membrane is composed of a lipid bilayer, with the hydrocarbon tails oriented inward and the polar head groups facing the outside.

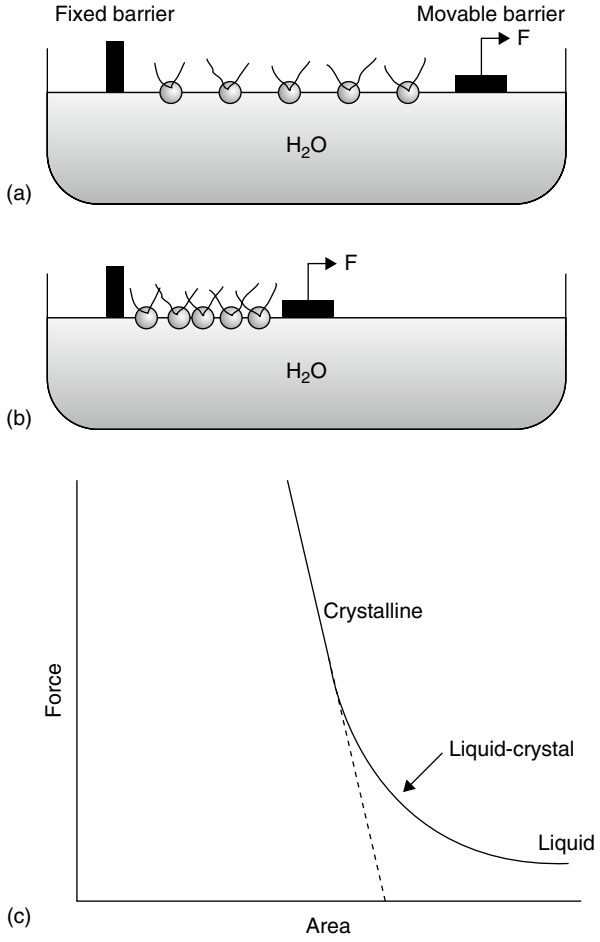


FIGURE 2.7 Using a Langmuir trough to determine the area taken up by a known amount of lipids. The lipids are placed on the surface of the water (a) of the trough and a movable barrier attached to a force transducer is moved toward the lipids (b). As the lipids are compressed, the force increases as the lipids are transformed from a liquid state to a liquid crystalline state to a crystalline state (c). The area of the lipids in the liquid crystalline state is determined by extrapolating the force-area curve to zero force.

Actually, Gorter and Grendel's conclusion was not justified because the acetone did not extract all the lipids from the membrane. Luckily, they also made a mistake in calculating the surface area of the cells, which they underestimated to be equal to $8r^2$, so they could conclude, serendipitously, that the lipids form a bilayer around the cell. Newer experiments show that there are only enough lipids to cover approximately 1.5 cell surface areas, not two (Bar et al., 1966). It is possible that an overlapping of the hydrocarbon tails comprising each leaflet of the membrane could increase the surface covered by the lipids somewhat, but we now know that much of the surface of the membrane is taken up by proteins.

Hugh Davson and James Danielli (1943, 1952), two physical chemists, put together the known physico-chemical data on plasma membranes in order to deduce the structure of the plasma membrane. They included Overton's observations on permeability; the determination by Parpart and Dziemian (1940) that the plasma membrane is composed of lipids and proteins with a ratio (w/w) of 1:1.7 (Jain, 1972); Hugo Fricke's (1925) and McClendon's (1927) measurements on the electrical impedance of red blood cells, which showed that the membrane had a high resistance and a capacitance reminiscent of a lipid bilayer (see Section 2.8); the measurements of the tension at the surface of the plasma membrane that indicated that the low value for the surface tension may be due to a coating of protein (Danielli and Harvey, 1935); the observations by Schmitt et al. (1936, 1938) that plasma membranes have radially positive intrinsic birefringence and negative-form birefringence when viewed with a polarization microscope (Wayne, 2009); and the observations by Waugh and Schmitt (1940) using interference microscopy that showed that the plasma membrane is approximately 20 nm thick and that roughly half of the thickness is due to lipids. They also considered the then newly acquired X-ray diffraction data of Schmitt and Palmer (1940) that indicated that the membrane may be composed of one or two bilayers.

From these data, Davson and Danielli proposed a structure for membranes that came to be known as the *paucimolecular membrane model* (Figure 2.8). According to this model, the plasma membrane is composed of one or two bimolecular leaflets of lipid and a single layer of protein that covers each exposed polar surface of lipid. Danielli (1975) believed that he had solved the problem of membrane structure using physico-chemical theory. By contrast, Robertson (1987), as we will see in Section 2.4, believed as an electron microscopist that visible structures have a

reality far greater than structures implied from physiological experiments. Science is never complete and a synthetic theory of a given structure or process requires the combination of many kinds of data gained with many different techniques—that is, the synthetic picture requires the synthesis of the thesis with the antithesis. Because theory and experiment are always limited, the synthesis, which is never a final solution, often involves conflict and compromise. In this case, the difference in opinion caused great arguments between the two camps.

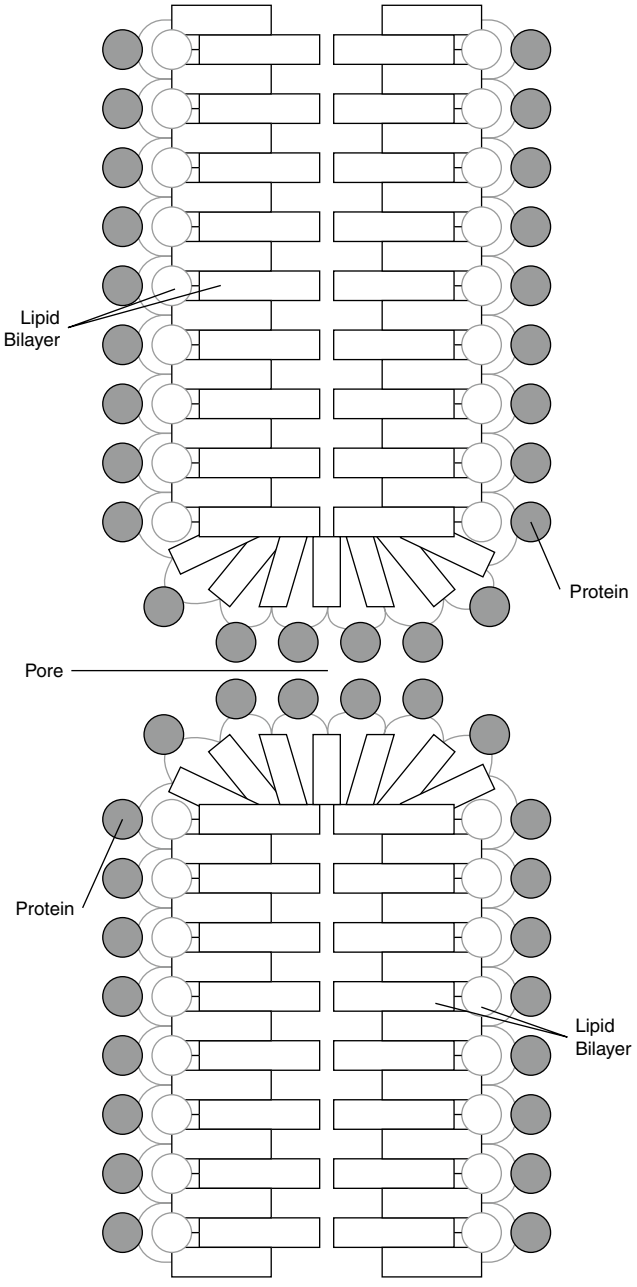


FIGURE 2.8 Danielli and Davson's paucimolecular model of a membrane.

2.4 STRUCTURE OF THE PLASMA MEMBRANE

The introduction of $KMnO_4$ and glutaraldehyde fixatives as well as plastic embedding media allowed J. David Robertson (1959, 1964) to visualize the architecture of the plasma membrane of cells with the transmission electron microscope (Figure 2.9). Imagine Robertson's delight when he saw the trilamellar structure proposed by Danielli and Davson. Since it was only 7.5 to 10 nm thick, he determined that there was only one 3.5-nm lipid bilayer, coated on both sides with approximately 2-nm-thick protein layers. Moreover, due to the differential fixation and staining properties of the two protein layers, Robertson concluded that the membrane was asymmetric. Robertson also stressed that the membrane had no pores since he could not visualize them at the level of resolution attainable at the time. He proposed that all membranes have the same structure and proclaimed the concept of the "unit membrane." This model became unfashionable, not because it is so wrong, but because it emphasized the static, constant properties of membranes at a time when biochemistry was showing

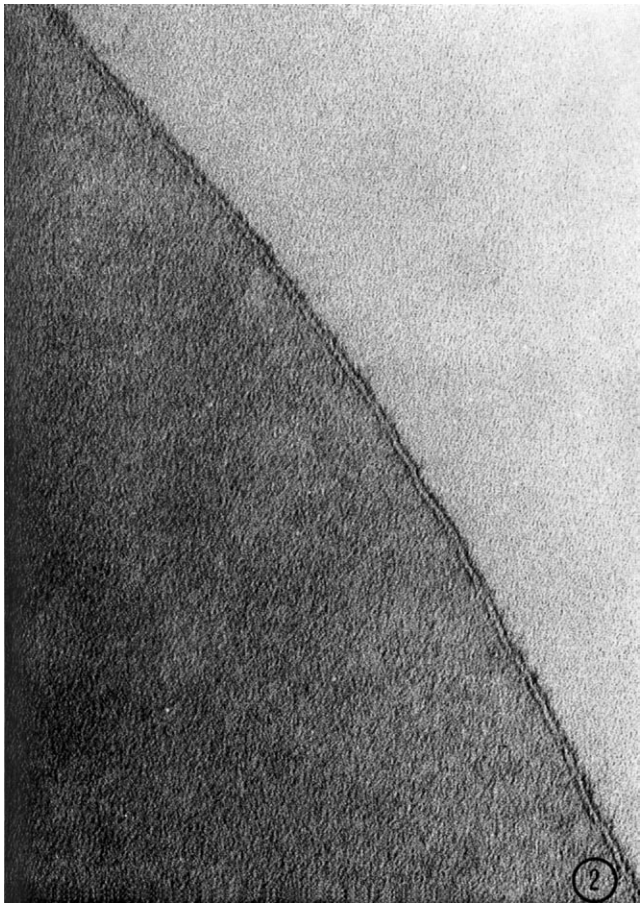


FIGURE 2.9 Electron micrograph of the plasma membrane of a red blood cell showing the structure of a unit membrane. (*Source:* From Robertson, 1964.)

that each membrane was chemically distinct, enzymatically unique, and dynamically active (Korn, 1966; Stoeckenius and Engelman, 1969; Robertson, 1987).

Moor et al. (1961) and Moor and Mühlethaler (1963) introduced a new method called *freeze-fracture* or *freeze-etching* for visualizing cells that prepared the way for a dynamic view of the plasma membrane. With this technique, shown in Figure 2.10, frozen cells are nicked with a razor blade in such a manner that the membrane splits between the two leaflets of the lipid bilayer, disclosing face views of the membrane that show the distribution of particles that are approximately 8–10 nm in diameter (Branton, 1966; see Figure 2.11). Every membrane has characteristic particles, which are not uniformly distributed over the membrane, but are restricted to either the external surface (ES), the protoplasmic surface (PS), or the fracture face of either the leaflet on the external side (EF) or the leaflet on the protoplasmic side (PF).

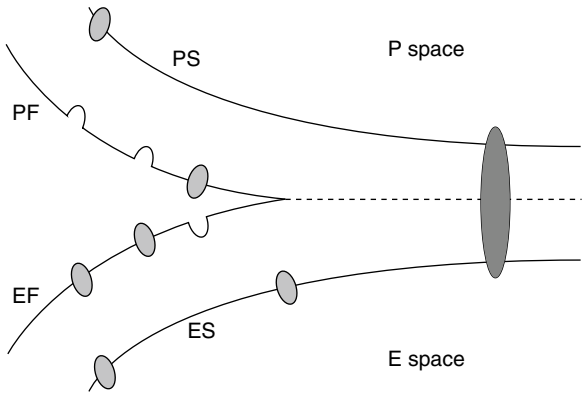


FIGURE 2.10 Diagram of a freeze-fractured membrane showing the PS, PF, EF, and ES.

The present model of the architecture of the plasma membrane, known as the *fluid mosaic model,* explains elegantly the structure of the membrane observed with freeze-fracture electron microscopy (Singer and Nicolson, 1972). In this model, shown in Figure 2.12, the particles are considered to be proteins. The fluid mosaic model arose from the curiosity of Jonathan Singer (1975, 1992), who as a protein physical chemist, wondered why some proteins are soluble in the cytoplasm while others are associated with membranes. He suggested that an accurate model of membrane structure must be able to provide an explanation for the specific association of proteins with membranes. Singer (1990) grouped membrane proteins into two classes: peripheral proteins, which are primarily hydrophilic and

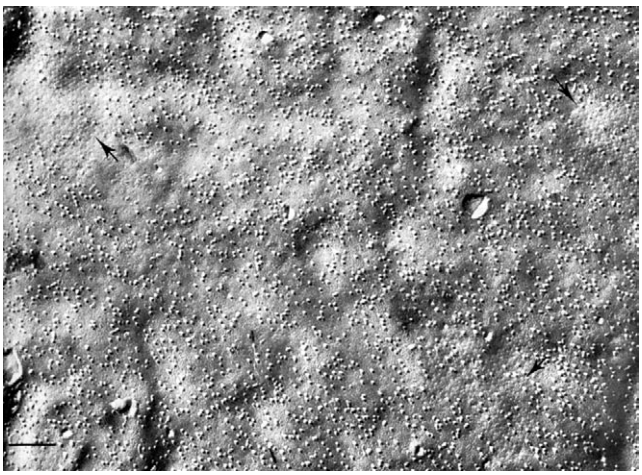


FIGURE 2.11 Freeze-fracture micrograph of the plasma membrane of a mesophyll cell. The arrows point to hexagonally arranged depressions from which particles have been pulled away during the freeze-fracturing process. Bar, 100 nm. (*Source:* From Schnabl et al., 1980.)

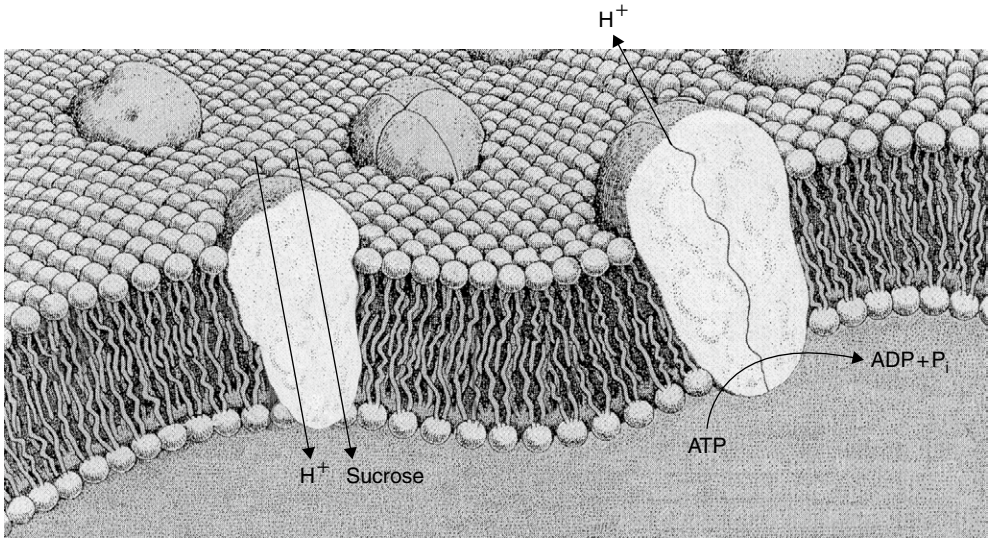


FIGURE 2.12 Singer and Nicholson's fluid mosaic model of a membrane.

can be removed by mild treatments like raising or lowering the ionic strength, changing the pH, or using bivalent ion chelators like EDTA or EGTA; and intrinsic proteins, which are primarily hydrophobic and can be removed only by detergents or organic solvents. Singer realized that the Davson-Danielli-Robertson model of the membrane was thermodynamically unsound because it would require hydrophobic membrane proteins to be in contact with aqueous solutions. One can get a feel for the high amounts of free energy required to mix hydrophobic and hydrophilic molecules by trying to mix an oil and vinegar–type salad dressing using various amounts of shaking.

Singer visualized that the intrinsic membrane proteins were globular and amphipathic; that is, they had both hydrophilic and hydrophobic ends. He proposed that the hydrophobic portions of proteins would be embedded in the hydrocarbons derived from fatty acids in the lipid bilayer and either one or two hydrophilic portions would extend into the polar head groups and out into the aqueous media. He visualized the proteins as icebergs floating in a sea of lipid, and imagined that the structure of the membrane is determined primarily by the various hydrophobic and hydrophilic interactions between the proteins and lipids. Some of the proteins, he guessed, traversed the entire thickness of the membrane. This was confirmed by Mark Bretscher's (1971) study in which he labeled a protein (later named Band 3, or the anion transporter) in the intact erythrocyte membrane with a radioactive probe. Presumably, the protein would be labeled only if it was exposed to the E-surface of the plasma membrane. When Bretscher previously treated the intact erythrocytes with pronase, an impermeable enzyme that digests the parts of the proteins on the outside of the membrane, the Band 3 protein in the intact cell could not be labeled.

Subsequently, Bretscher made membrane ghosts, which are either inside out or right side out. This allowed the probe to label the inside and the outside. Since a peptide fragment of the same protein becomes labeled in ghosts made from cells that were previously treated with pronase, Bretscher concluded that the Band 3 protein spanned the entire width of the membrane.

If the membrane proteins coated the lipid bilayer, as suggested by the Davson-Danielli-Robertson model, it would be unlikely that the membrane proteins would be mobile. However, Singer's thermodynamic calculations showed that membrane molecules should be able to move in the plane of the membrane. Thus, according to the fluid mosaic model, lateral movements of proteins and lipids in the plane of the membrane are possible, and in fact do occur. Movements of membrane molecules can be observed with a technique known as *fluorescence redistribution after photobleaching* (FRAP; Axelrod et al., 1976). With this technique, shown in Figure 2.13, membrane molecules are selectively labeled with fluorescent probes. The distribution of the probe in the membrane is then observed with a fluorescence microscope.

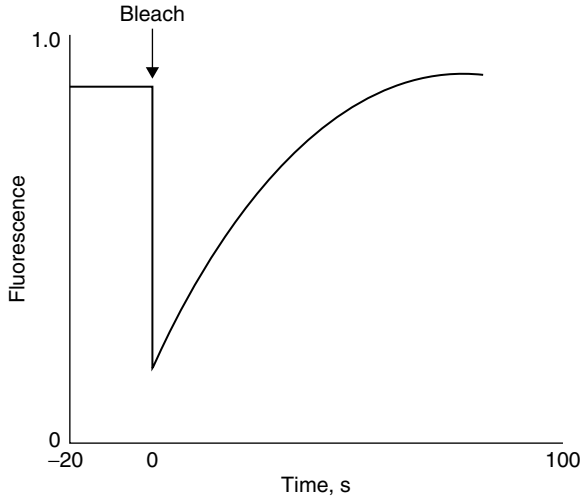


FIGURE 2.13 Results from a fluorescence redistribution after photobleaching (FRAP) experiment. The sample is initially fluorescent. Then the fluorescence is bleached at time zero. The fluorescence recovers over time and the diffusion coefficient is calculated from the slope of the recovery curve.

Initially, the fluorescence is uniform. Then the fluorescent molecules in an area of the membrane are destroyed with a laser and the fluorescence decreases. The fluorescence begins to recover over time as fluorescent molecules diffuse back into the bleached area.

The rate of fluorescence recovery is an indication of the rate of diffusion of the molecules in the plane of the membrane. The diffusion coefficients for proteins in plant plasma membranes fall between 5×10^{-15} and 3×10^{-14} m^2/s (Metcalf et al., 1986a; Dugas et al., 1989). While the proteins are able to diffuse within the plane of the membrane, they are not free to diffuse anywhere. In fact, the values obtained for the diffusion coefficients of proteins vary in part because peripheral proteins known as the *membrane skeleton* may cause a compartmentalization of the plasma membrane into domains that are approximately $0.1–1\,\mu m^2$. The membrane skeletal proteins "corral" the intrinsic proteins, and consequently, the smaller the domain tested with FRAP, the larger the diffusion coefficient appears to be (Kusumi and Sako, 1996).

The mobility of individual plasma membrane proteins can also be observed using single particle–tracking techniques that depend on computer-enhanced microscopy (Saxton and Jacobson, 1997; Tomishige et al., 1998; Smith et al., 1999; Tomishige and Kusumi, 1999; Mirchev and Golan, 2001; Douglass and Vale, 2005). The diffusion-restricted domains in the plasma membrane can be evanescent or stable, and they may represent regions of the plasma membrane with specialized functions (Edidin, 1992, 2001). As we will see later, the diffusion coefficients for protein in water are between 10^{-11} and 10^{-10} m^2/s, indicating that the lipid bilayer is really a very viscous solution through which the proteins diffuse.

The lipids also diffuse through the leaflet of the bilayer, although due to their small size, they diffuse 10–100 times faster than the proteins. Their diffusion coefficients fall

between 3×10^{-14} and 10^{-12} m^2/s (Metcalf et al., 1986b; Walko and Nothnagel, 1989). As we will see, the ability of molecules to move translationally in the membrane does not mean that they necessarily become randomly arranged. Indeed, like the proteins, the lipids can form microdomains, known as *lipid rafts* (Simons and Ikonen, 1997; Simons and Toomre, 2000; Mongrand et al., 2004; Bhat and Panstruga, 2005; Borner et al., 2005; Martin et al., 2005; Lefebvre et al., 2007).

In contrast to the array of movements that take place in the plane of the membrane, it is thermodynamically unlikely for a lipid or protein to flip-flop from one leaflet of the bilayer to the other as a consequence of the high energies that are required to make contact between hydrophilic and hydrophobic molecules. This kind of movement can be monitored by labeling membrane molecules with a molecule that contains an unpaired electron (i.e., a spin label), which acts like a magnet that can be aligned in a magnetic field (Compton, 1921a,b; Uhlenbeck and Goudsmit, 1926; McMillan, 1968; Goudsmit, 1971). Some spins are aligned parallel to the magnetic field and others are aligned antiparallel to the magnetic field. The greater the number of spin labels in the system, the greater the amount of microwave energy that can be absorbed by the system to flip the spin label from a lower-energy spin parallel to the field to the higher-energy spin antiparallel to the field.

The spin label is inserted into one side of the membrane and observed with an electron spin resonance spectrometer. If the labeled lipids flip-flop to the other side of the membrane, the signal will not be able to be quenched with ascorbic acid, a quencher of free radicals. The percentage of lipid molecules that flip across the membrane during a given time can be estimated by determining the proportion of the signal that is protected from being quenched by ascorbic acid. These measurements indicate that 10^{-6}–10^{-4} lipids flip across the membrane every second, or putting it another way, it takes 10^4–10^6 seconds (i.e., several days) for a single lipid to flip-flop across the membrane. While the rate of lipid flip-flop is slow, the rate of protein flip-flop is even slower and has not yet been observed. The high energies required for lipids and proteins to flip-flop between the two sides of the membrane assures that if the two leaflets of the membrane are synthesized asymmetrically, they will maintain their asymmetry.

All the data obtained to date are consistent with the fluid mosaic model. Moreover, this model of a dynamic membrane helps us to conceptualize many cellular processes, including energy generation, nutrient uptake, and those processes involved in responding to the external environment.

2.5 ISOLATION OF THE PLASMA MEMBRANE

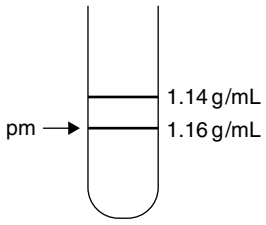
In most cells, the plasma membrane accounts for less than 5 percent of the cellular membranes. Thus, if we want to be sure that the plasma membrane itself is involved in a given process, we must isolate it from all the other membranes in the cell. However, one of the rules of cell biology is to analyze the properties of the plasma membrane in vitro in light of the known properties in vivo, and once information about the properties of this membrane is gathered in vitro, apply it right away to understand the function of the plasma membrane in the living cell. That is, we must always keep in mind the relationship of the parts to the whole, and in the words of Lester Sharp (1934), "a true conception of the organism can be approached only when analysis into physico-chemical components is followed by resynthesis into a biological whole."

Christian de Duve (1975) described his journey through the cell with the aid of a centrifuge, and in the process described the history, theory, and practice of cell fractionation. Anyone interested in isolating organelles should read his Nobel lecture. In each chapter of this book, I describe a general procedure for isolating a given organelle, although it must be realized that the procedure usually has to be modified for each tissue and species. Furthermore, it is likely that techniques will be developed that further maximize the yield, minimize the contamination due to other cellular components, as well as minimize the loss of molecules from the organelle that are present in vivo. With these and other (Hillman, 2001) caveats in mind, I describe the isolation of the plasma membrane.

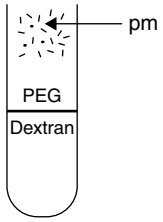
In order to isolate the plasma membrane, the tissue must first be homogenized. The homogenate is then passed through a filter to remove the walls and whole cells. The filtrate is then centrifuged at 10,000 g for 15 minutes to get a supernatant free of nuclei, plastids, and mitochondria. This differential centrifugation separates organelles solely on the basis of their differential rates of sedimentation. In general, since the density of particles is similar, the larger the particle, the faster it sediments. The supernatant from the differential centrifugation step contains small particles. This supernatant is centrifuged at 100,000 g for 30 minutes to separate the membranes from the majority of cytosolic proteins. The supernatant is discarded and the pellet is then resuspended. At this stage, the plasma membrane can be isolated from the other cellular membranes in one of three ways. One way of isolating the plasma membrane is based on equilibrium density-gradient sedimentation, where the membranes are separated on the basis of their densities. Before the membranes are applied to the centrifuge tube, the tube is filled with sucrose or a polymer and centrifuged to establish a gradient of densities. The gradient is maintained indefinitely due to the opposing actions of diffusion and sedimentation (Meselson et al., 1957). Once the gradient is established, the membranes are loaded on the top of the gradient and centrifuged at approximately 100,000 g for a few hours.

Each type of membrane forms a band at its particular density (Figure 2.14a). In general, the greater the proportion of protein ($\rho \approx 1.33$ g/mL) to lipid ($\rho \approx 0.9$ g/mL), the greater the density of the membrane. The density of the

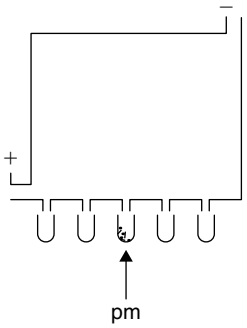
plasma membrane is typically about 1.16 g/mL. The identity of the plasma membrane is confirmed by assaying whether or not it has high vanadate-sensitive, K^+-stimulated H^+-ATPase activity for the amount of protein in the sample. Each membrane has a specific enzyme that can be used to identify it. These enzymes are called *marker enzymes* (Quail, 1979). The plasma membrane fraction should also be assayed for the marker enzymes of the other membranes in order to determine the amount of contamination in the fraction.



(a) Equilibrium density gradient centrifugation



(b) Aqueous two-phase partitioning



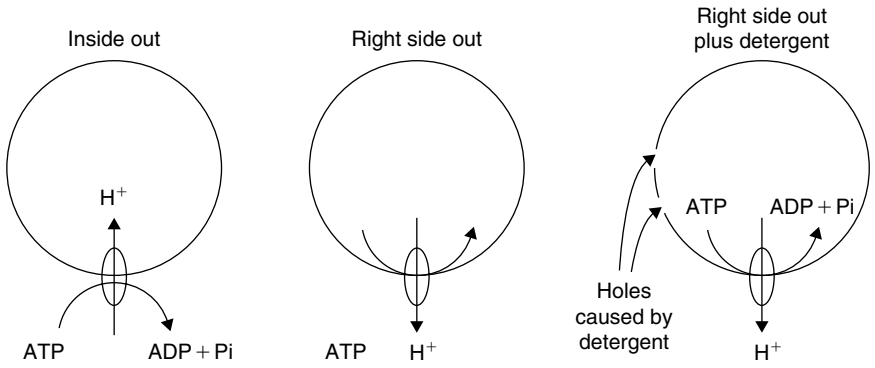
(c) Free-flow electrophoresis

FIGURE 2.14 Techniques used to isolate plasma membranes: (a) equilibrium density-gradient centrifugation, (b) aqueous two-phase partitioning, and (c) free-flow electrophoresis.

The plasma membrane can also be isolated from the other membranes on the basis of its surface properties, including charge and hydrophobicity using an aqueous two-phase partitioning technique (see Figure 2.14b; Kjellbom and Larsson, 1984). To perform this technique, the membranes are mixed with a solution of 6.4 percent dextran T500 and 6.4 percent polyethylene glycol 3400. The solutions are then centrifuged so that the polyethylene glycol forms a layer above and the dextran forms a layer below. Right-side-out plasma membrane vesicles end up in the upper phase.

Plasma membranes can also be purified on the basis of their charge densities with a technique known as free-flow electrophoresis (see Figure 2.14c; Sandelius et al., 1986). With this technique, a mixture of membranes is introduced into a separation buffer flowing perpendicular to an electric field. Membranes bearing different electrical charge densities will migrate different distances along the separation chamber. Each type of membrane flows into a different collecting tube, which is then centrifuged at 110,000 g for 30 minutes to concentrate the membranes. This technique can resolve vesicles of different sidedness.

How can we determine the sidedness of the membranes? There is a good trick (see Figure 2.15; Canut et al., 1987). Do you remember when we talked about the E- and P-spaces? Adenosine triphosphate (ATP) is almost always in the P-space. Therefore, the portions of membrane proteins that bind ATP must be on the P-side of the membrane. Thus, if all the membranes are tightly sealed and right side out, and we add ATP in order to assay the VO_4-sensitive, K^+-stimulated, H^+-ATPase activity, we should see no activity because the membrane prevents a large hydrophilic molecule like ATP from getting to the P-space. If we then add a



(a) High activity (b) Low activity (c) High activity

FIGURE 2.15 Determination of the orientation of a membrane vesicle. (a) intact inside-out vesicle; (b) intact right-side-out vesicle; (c) detergent-permeabilized right-side-out vesicle.

detergent like Triton X-100 to permeabilize the membrane so that ATP can enter the P-space and bind to the enzyme on the P-side of the plasma membrane, we should see an enhancement of the ATPase activity. If we see a detergent enhancement, which is known as *latency,* we say that the membrane vesicles are tightly sealed and are right side out. If the ATPase exhibits high activity both with and without the detergent, the membranes are either inside out or leaky.

Once we have isolated right-side-out plasma membrane vesicles we can use them to characterize the function of the lipids and proteins that determine the permeability of the membrane. We can also use the isolated plasma membranes to determine the chemical profile of the lipids and proteins that reside in the membrane.

2.6 CHEMICAL COMPOSITION OF THE PLASMA MEMBRANE

Since most nutrients and metabolites are polar, the nonpolar lipids in the membrane bilayer act as a barrier to their passage. On the other hand, once a given molecule gets to the right place in or out of the cell, the lipid bilayer will help it stay there. As we will see in Section 2.8, the lipid bilayer also forms a nonconducting layer that gives the plasma membrane the capacity to store charge (i.e., act as a capacitor), and the stored charge can be used to do work. The lipids also provide the fluid environment where the integral membrane proteins involved in transporting polar molecules reside.

In order to characterize the lipid fraction, the plasma membranes must be extracted with nonpolar organic solvents (e.g., chloroform, methanol, HCl). The lipids in the extract are then separated by polarity on silica Sep Pak cartridges and the fractions are identified by thin-layer chromatography, gas chromatography, and mass spectrometry (Schneiter et al., 1999). Lipids have been characterized in the plasma membrane of a variety of plants (Sheffer et al., 1986; Lynch and Steponkus, 1987; Sandstrom and Cleland, 1989; Brown and DuPont, 1989; Navari-Izzo et al., 1989; Peeler et al., 1989; Cahoon and Lynch, 1991; Bohn et al., 2001; Kerkeb et al., 2001; Quartacci et al., 2002; Lin et al., 2003; Welte and Wang, 2004). Omic approaches are now being applied to the study of lipids in plants (Welti and Wang, 2004; Welti et al., 2007).

The membrane lipids make up about 30 percent of the weight of the plasma membrane. The membrane lipids of the plasma membrane are mainly represented by phospholipids, glycolipids, and sterols. Sterols are neutral and nonpolar lipids. In plants, unlike animals where cholesterol is the only sterol, the sterols form a complex mixture, which may include sitosterol, stigmasterol, cholesterol and brassicasterol (Nes, 1977; Hartmann and Benveniste, 1987; Gachotte et al., 1995; Hartmann, 1998). The glycolipids are polar lipids that contain one or more sugar groups. The phospholipids are polar lipids that contain a glycerol molecule with a phosphate group attached through an ester bond to one carbon, and two hydrocarbon tails, which result from the esterification of fatty acids to hydroxyl groups attached to the remaining two carbons of the glycerol molecule (Figure 2.16). A polar group, like choline, serine, inositol, glycerol, or ethanolamine, is attached to the phosphate group through an ester bond. The combination of the glycerol phosphate and the additional group is known as the *polar head group*. Careful workers determine the true molecular species of each lipid, which involves identifying the polar head group and the fatty acids that are derived from the hydrocarbon tails. They also determine the position of each hydrocarbon tail in each lipid. Less analytically precise workers analyze the polar head groups and hydrocarbons separately. The polar head group composition of a variety of plasma membranes are given in Table 2.1.

Notice that there is a basic similarity between different plasma membranes. On the average, the noted membranes are composed of (in mole percent): 40.7 percent phospholipids, 27.3 percent glycolipids, and 25.4 free sterols—values very similar to those found in the plasma membranes of animal cells.

By contrast, the hydrocarbon compositions of plant and animal plasma membranes are somewhat different. Animal membranes are enriched in hydrocarbons derived from stearic acid (18:0) and oleic acid (18:1), while plant plasma membranes are enriched in hydrocarbons derived from palmitic (16:0), linoleic (18:2), and linolenic (18:3) acids. This is particularly intriguing since the hydrocarbon composition may have a large effect on the fluidity of the membrane. If we consider enzymes as little machines that must be well oiled in order to change their conformation so that they can mechanically split a molecule apart, fuse two together, or push one through a membrane (Johnson et al., 1974), then we can visualize the possible importance of membrane fluidity and the diversity in membrane lipids.

You can easily see for yourself the effect of hydrocarbon composition in fluidity by observing a variety of oils and butter at room temperature and on ice. Butter fat and vegetable oils are the readily available source of lipids used to nourish the next generation of animals and plants. Butter, an animal product, is primarily a mixture of hydrocarbons derived from palmitic and oleic acids and is solid both at room temperature and on ice. Palm oil, which contains hydrocarbons derived from palmitic acid, is solid at room temperature. Olive oil, which is high in hydrocarbons derived from oleic acid, is fluid at room temperature and gelled after being put on ice. Corn oil, sunflower oil, and soybean oil, which are rich in a hydrocarbon derived from linoleic acid, remain fluid at both temperatures. Likewise, linseed (flax) oil, which is rich in the hydrocarbon derived from linolenic acid (omega-3 fatty acid), remains liquid at both temperatures.

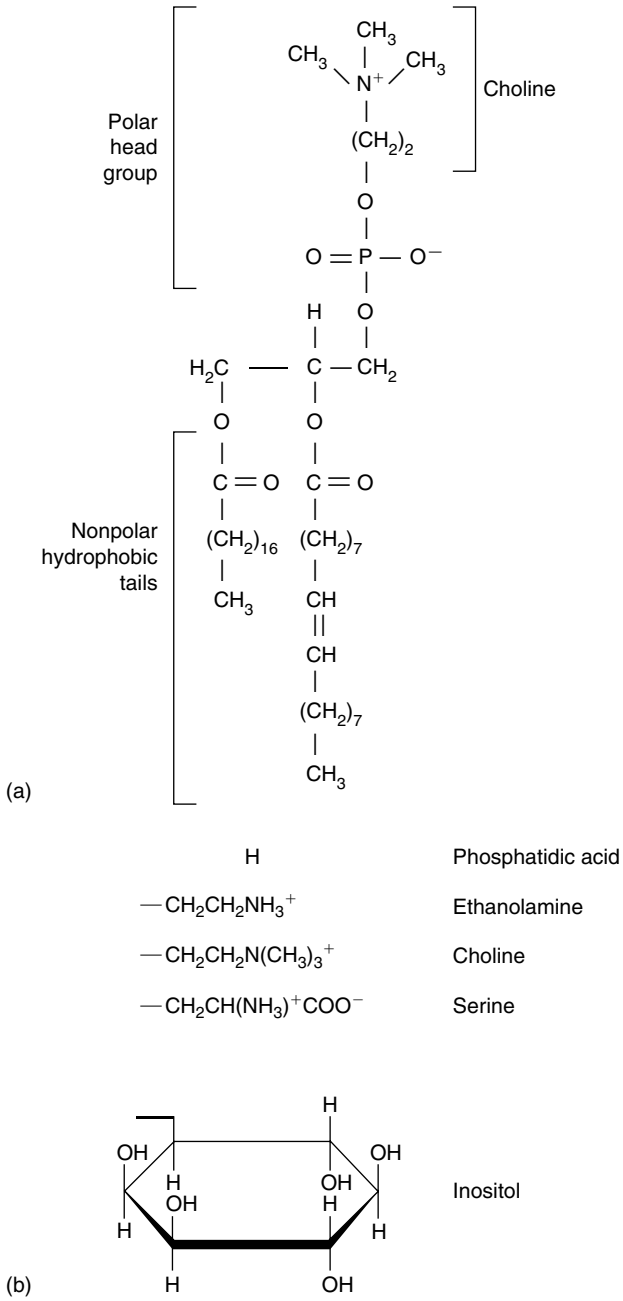


FIGURE 2.16 (a) Chemical structure of phosphatidylcholine composed of a stearic acyl group and an oleic acyl group. (b) chemical structures of the common polar head groups found in phospholipids.

The diversity in chemical composition of lipids is probably adaptive, since butter comes from warm-blooded animals, palms live in the tropics, olive trees live in warm Mediterranean climates, the crops rich in the hydrocarbons derived from linoleic acid grow in temperate regions, and crops rich in hydrocarbons derived from linolenic acid come from frost-resistant flax grown in the colder regions of the temperate zone. Cold-water fish also contain large amounts of linolenic acid.

The membrane fluidity is affected by the chain length of the hydrocarbons. As the chain length increases, the hydrocarbons interact with each other to a greater extent and form a more gel-like membrane. This causes the melting point to increase. The melting point decreases as the number of double bonds in the hydrocarbons increase. This is because the double bonds cause a kink in the hydrocarbons, which prevents them from interacting with each other. This decreases their gel-like properties and thus decreases the melting

TABLE 2.1 Lipid composition of plasma membranes (polar head groups)

	Oat Coleoptile	Oat Root	*Dunaliella*	Barley Root	Corn Shoot	Rye Leaves
Total phospholipid	**41.7**	**50.1**	**29.2**	**43.3**	**47.2**	**31.7/41.9**
LPC	1.0	1.7				
PI	2.3	1.5				0.7/ < 0.5
PS	3.2	4.2				1.5/1.0
PC	9.6	14.3	13.2			14.8/19.5
PG	1.8	1.3	5.3			1.8/2.1
PA	14.8	11.8				1.7/2.3
PE	9.0	15.3	10.7			10.9/15.7
Total Glycolipid	**38.9**	**25.8**	**7.7**	**25.5**	**44.2**	**35.6/13.3**
ASG	5.5	4.5				4.3/1.1
SG	7.3	10.6				15.1/5.7
GC	26.1	10.1				16.2/6.8
Total Free Sterol	**19.4**	**24.6**	**26.4**	**25.5**	**4.6**	**32.7/44.4**
Cholesterol	3.1	0.1				0.5/0.4
Brassicasterol	2.0	n.d.				
Campesterol	1.9	2.0			3.5/1.3	
Stigmasterol	1.6	12.1			0.6/ < 0.1	
ß-Sitosterol	9.2	5.1				20.8/32.6
Unknown	1.5	5.2				

Note: *LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine; ASG, acylated sterol glycoside; SG, steryl glycoside; GC, glycocerebroside.*

point. Therefore, the relative proportion of each hydrocarbon determines the fluidity of the membrane at a given temperature (Table 2.2). Indeed, the hydrocarbon composition of some phospholipids changes after cold acclimation or osmotic stress, indicating that there is a relationship between the lipid composition of a membrane and its function (Lynch and Steponkis, 1987; Peeler et al., 1989).

Why are there approximately 100 different kinds of lipids that coexist in the plasma membrane? We really do not know. It is possible that a variety of lipids is required in order to maintain the membrane fluidity in the correct range throughout the day and throughout the seasons. Interestingly enough, just the presence of many types of lipids will maintain the fluidity of the bilayer since each one acts like an impurity for the others and prevents crystallization. Each lipid has a different conformational shape, and we may find that different lipids are necessary to maintain a tight barrier against the free diffusion of polar molecules or ions across the membrane in curved areas and in flat areas of the membrane—or in regions of the membrane with various intermediate curvatures. We may find that many lipids are necessary because each molecular species of lipid performs a specific function. For example, some proteins, including the proton ATPase on the plasma membrane, require certain lipids for activation (Kasamo and Sakakibara, 1995; Kasamo, 2003). Some lipids, including phosphatidylinositol and sphingolipids, participate in cell signaling.

The plasma membrane is also composed of proteins, which make up approximately two-thirds of the weight of the membrane. Proteins are composed of one or more polypeptide chains of amino acids. Amino acids are bifunctional molecules with the following structure: H_2N-CRH-COOH (Figure 2.17). The properties of the proteins are due, in part,

TABLE 2.2 Names and melting points of various fatty acids

Symbol[1]	Common Name	Melting Point (°C)
14:0	myristic	53.9
16:0	palmitic	63.1
18:0	stearic	69.6
20:0	arachidic	76.5
$16{:}1^{9}$	palmitoleic	−0.5
$18{:}1^{9}$	oleic	13.4
$18{:}2^{9,12}$	linoleic	−5
$18{:}3^{9,12,15}$	linolenic	−11
$20{:}4^{5,8,1,14}$	arachidonic	−49.5

[1]The numbers in the superscript represent the positions of the double bond(s) counting from the carbon that occurs in the carboxylic acid group. For example, $18{:}2^{9,12}$ indicates that there are 18 carbons in the fatty acyl chain with two double bonds. One double bond is in the 9th position and the other is in the 12th position from the carboxylic end.

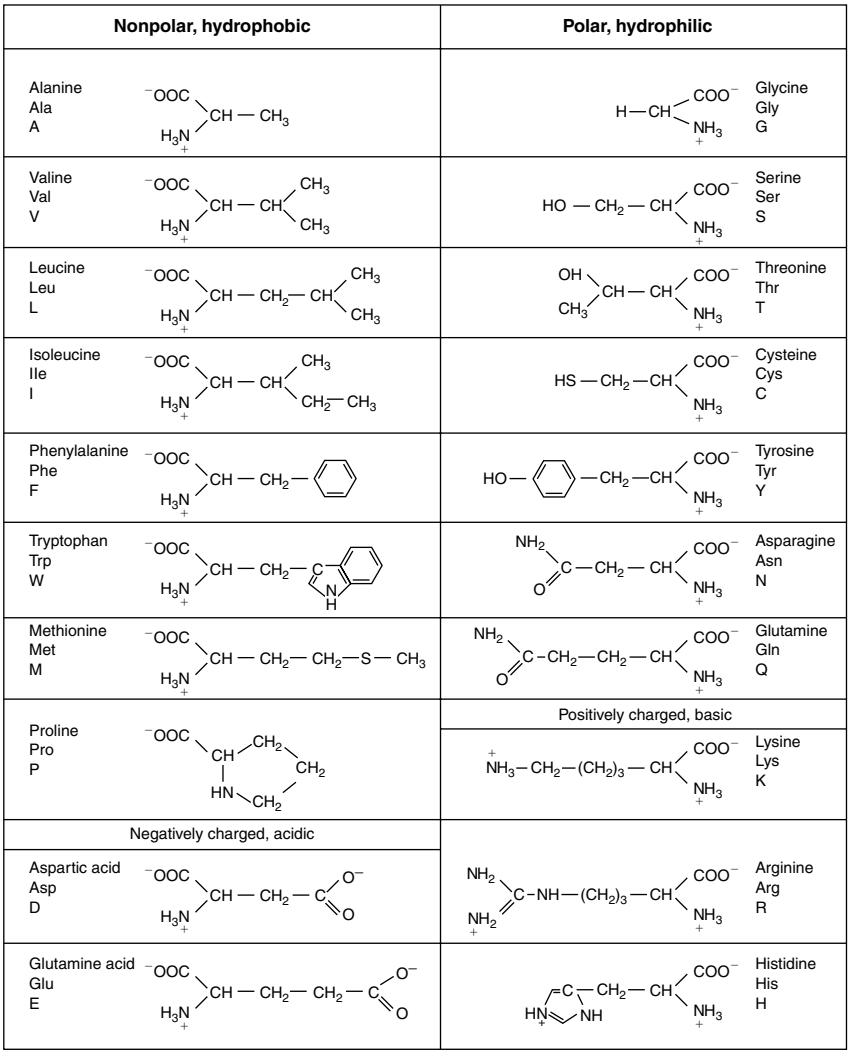


FIGURE 2.17 Structure of amino acids with their three-letter and one-letter abbreviations.

to the properties of their amino acids, the properties of the amino acids are due to the various radical or R groups, and the properties of the R groups depend on the properties of the atoms that constitute them. The salient property of the atoms is their electronegativity, which is a semiquantitative value that can be given in Joules (J) and represents their affinities for electrons (Pauling, 1932, 1940, 1954a, 1970). Higher electronegativities represent greater affinities for electrons. In general, carbon (2.5) and hydrogen (2.1) atoms have equal electronegativities. Consequently, R groups that contain many hydrocarbon (CH) bonds are typically nonpolar. By contrast, the oxygen (3.5) atom has a greater electronegativity than the hydrogen atom, and thus the OH bond has a partial ionic character and acts like an electrical dipole, which makes R groups with an OH group polar.

R groups with more than one polar group may be charged as a result of the combined action of the polar groups on a given atom. In complex R groups, nitrogen becomes positively charged and oxygen becomes negatively charged. As a result of the different electronegativities of the C, H, O, N, and S that make up the R groups, the amino acids that make up the proteins can be nonpolar, polar, or charged. The charged and polar amino acids will be soluble in water and are thus called *hydrophilic*. The relative hydrophilicities of the 20 amino acids that make up proteins are given in Table 2.3. The amino acids with the lowest hydrophilicities are the most likely to be in contact with the hydrocarbons derived from fatty acids in the lipid bilayer.

The various polypeptides that reside in the plasma membrane can be observed using polyacrylamide gel electrophoresis (PAGE) after dissolving the isolated plasma membrane in an ionic detergent like sodium dodecyl sulfate (SDS). The SDS unfolds and coats each integral and peripheral protein so that the number of SDS molecules attached is proportional to the mass of the protein. The SDS molecules are negatively charged and this overwhelmingly

TABLE 2.3 Amino acids and their average hydrophilicity-hydrophobicity

Amino Acid	Three-Letter Symbol	One-Letter Symbol	Hydrophilicity*
Tryptophan	Trp	W	2.57
Phenylalanine	Phe	F	2.64
Leucine	Leu	L	3.29
Isoleucine	Ile	I	3.64
Tyrosine	Tyr	Y	4.57
Methionine	Met	M	6.57
Valine	Val	V	7.50
Proline	Pro	P	7.57
Cysteine	Cys	C	8.29
Alanine	Ala	A	12.07
Histidine	His	H	12.79
Threonine	Thr	T	13.64
Glutamine	Gln	Q	14.36
Glutamic acid	Glu	E	14.64
Glycine	Gly	G	14.79
Serine	Ser	S	14.93
Arginine	Arg	R	15.93
Asparagine	Asn	N	16.14
Lysine	Lys	K	16.21
Aspartic acid	Asp	D	16.29

**The greater the hydrophilicity number, the more hydrophilic the amino acid.*

negative charge masks the intrinsic charge of the protein so that each protein travels through the gel with essentially the same charge-to-mass ratio.

The SDS-protein complexes are put on top of an acrylamide gel that has a given pore size. When an electric field is placed across the gel, the proteins are separated by mass since the smaller proteins migrate through the gel faster than the larger ones as they move toward the positive pole (anode). There are approximately 100 polypeptides in the plasma membrane as determined by SDS polyacrylamide gel electrophoresis (see Figure 2.18) and over 200 by proteomic analysis using mass spectrometry (Alexandersson et al., 2004; Lefevre et al., 2007; Marmagne et al., 2004, 2007; Tang et al., 2008; Zhang et al., 2008). Out of these, only a few have been well characterized.

While the lipid bilayer prevents water-soluble solutes from entering or leaving the cell, membranes contain special transport mechanisms made of proteins that contain an aqueous pathway that facilitates the movement of hydrophilic solutes that permeate the membrane (Bethe, 1930). Depending on the tortuosity or openness of the pathway, the proteins are classified into two groups: carrier proteins and channel proteins. As with any system of classification, the division is convenient, but artificial, and intermediates between carriers and channels exist (Eisenberg, 1990; Läuger, 1991).

Carrier proteins act like enzymes in that their ability to transport a solute increases as the solute concentration increases, but eventually saturates. Carrier proteins can also be characterized by their maximal velocity and their affinity for the transported solute (Epstein et al., 1963; Welch and Epstein, 1968; Weiss, 1996). Since carrier proteins undergo a conformational change to move solutes through a relatively tortuous pathway, they are relatively slow and can transport only 10^2–10^4 solutes/s. A carrier protein can act as a uniporter, which transports one type of ion in one direction (e.g., H^+-ATPase), or it can be involved in coupled transport, where it transports two types of solutes in the same direction (e.g., H^+/amino acid symporter; Borstlap and Schuurmans, 2001) or in opposite directions (e.g., Ca^{2+}/H^+ antiporter). When carrier proteins facilitate the movement of solutes down an electrochemical difference, in a process known as *facilitated diffusion*, the process is passive. That is, it does not require any additional inputs of free energy.

Carrier protein transport can also be coupled to ATP hydrolysis. When this occurs, the carriers can facilitate the movement of a solute against its electrochemical difference. This process is known as *active transport* because a free-energy input in the form of ATP is required. When a carrier protein requires the hydrolysis of a phosphoanhydride bond (e.g., ATP or pyrophosphate), thereby converting the free energy of chemical bonds into the free energy of an electrochemical difference, the carrier is considered to be a primary transporter or pump. In plant cells, primary pumps typically transport protons. By contrast, secondary transporters transport ions and organic molecules by using the free energy inherent in the electrochemical difference across the membrane formed by a primary transporter. In plant cells, secondary transport dissipates the electrochemical difference of protons that is established by the primary transporter. In secondary transport, the free energy made available by the movement of protons down their electrochemical difference is used to move another type of ion or uncharged organic molecule against its electrochemical or concentration difference, respectively. While most primary pumps transport ions using the free energy made available from ATP hydrolysis, some primary pumps transport protons and other ions at the expense of free energy made available by electron transport chains (Conway, 1953; Møller and Lin, 1986; Rubinstein and Luster, 1993; Trost, 2003).

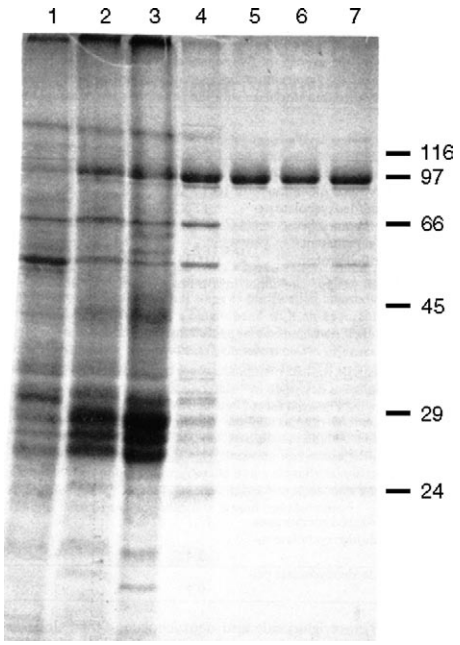


FIGURE 2.18 SDS polyacrylamide gel of (1) crude membranes, (2) membranes that have been washed with Triton/KBr, (3) membranes that have been extracted with octylglucoside plus deoxycholate, (4) the lysolethicin-solubilized supernatant. (5), (6), and (7) are three different glycerol-gradient purified protein preparations. (*Source:* From Anthon and Spanswick, 1986.)

Channel proteins contain relatively large aqueous pores that have a diameter of about 0.6 nm (Hille, 1992). As a reference, ions have a diameter of about 0.2 nm. Because the pores are so large, they can pass solutes at a rate of 10^7–10^8 particles/s. Eisenberg (1990) suggests that channel proteins should also be considered as enzymes that catalyze the flow of current. However, for a channel protein, the maximal velocity is higher and the affinity for a solute is lower than it would be for a carrier protein that binds a solute and transports it through a tortuous path. However, unlike carrier proteins,

channel proteins cannot be coupled to use the free energy of ATP hydrolysis, and thus can only transport solutes down an electrochemical gradient and thus dissipate it. Some channels pass only a given solute, while others are rather nonselective. In fact, the selectivity for a particular channel can be changed by mutating a single amino acid (Yang et al., 1993; Tang et al., 1993; Catterall, 1994, 1995; Jan and Jan, 1994; Uozumi et al., 1995; Mäser et al., 2001, 2002). Information on ion channels can be found at *http://www.ionchannels.org/*.

If the pores of channel proteins were always open, the movement of solutes would continue until the free energies inherent in the electrochemical gradients of various solutes were dissipated. Thus, the pores must be opened and closed in a regulated manner. This is called *gating*. Channel gates can be opened and closed by the mechanical energy of compression, tension and stretch, the electromagnetic energy of light, chemically by the binding of a ligand (e.g., hormone or nucleotide), or electrically by the change in the electrical potential across the membrane. These channels are referred to as *mechanically gated* (Falke et al., 1988; Cosgrove and Hedrich, 1991; Dutta and Robinson, 2004; Qi et al., 2004; Haswell, 2007; Haswell et al., 2008; Martinac et al., 2008), light-gated (Li et al., 2005; Zhang et al., 2006; Hegemann and Tsunoda, 2007; Zhang and Oertner, 2007; Hegemann, 2008), ligand-gated, and voltage-gated channels, respectively. The gates regulate the communication between the living protoplasm and the lifeless environment—the P- and E-spaces.

Many of the transport proteins may have related domains, which have joined together in various ways to make related yet unique proteins (Doolittle, 1995). By comparing the sequence of genes that code for the diverse transport proteins, one can make predictions about the function of a given domain. Currently, the structures of various plasma membrane proteins are studied in silico, using software that predicts the structure and function of the protein from its amino acid sequence inferred from the nucleotide sequence of the gene that encodes the protein. The transmembrane domains can be predicted from the nucleotide sequence by using TMHMM2 (*http://www.cbs.dtu.dk/services/TMHMM/*), the beta-barrel regions can be predicted by using Pred-TMBB (*http://biophysics.biol.uoa.gr/PRED-TMBB/*), the presence of a signal peptide can be predicted by using SignalP (*http://www.cbs.dtu.dk/services/SignalP-2.0/*), the presence of a myristoylation site can be predicted by using the MYR Predictor (*http://mendel.imp.ac.at/sat/myristate/SUPLpredictor.htm*), the presence of a GPI anchor can be predicted by using big-PI Plant Predictor (*http://mendel.imp.ac.at/sat/gpi/plant_server.html*), and the subcellular localization of the protein can be predicted by using Psort (*http://psort.nibb.ac.jp/form.html*) or by using the SUBA database (*http://www.plantenergy.uwa.edu.au/applications/suba*). Other functional domains of the protein can be predicted using Prodom (*http://prodes.toulouse.inra.fr/prodom/current/html/home.php*).

Now that I have discussed the general nature of membrane transport, I will discuss two proteins that function in transporting solutes across the lipid bilayer, but first let us become familiar with the ways to quantify transport.

2.7 TRANSPORT PHYSIOLOGY

Plasmolysis studies show that the plasma membrane is readily permeable to water, but not to the salts and sugars. Yet these solutes, which are so necessary for life, must be taken up and the plasma membrane has the mechanisms necessary to take up and eliminate each solute in a regulated manner. Differential membrane permeability and the mechanisms that cause it can be observed in certain cells as well as organs, including the roots of plants and the intestines of animals that specialize in nutrient uptake. Likewise, differential membrane permeability and the mechanisms that cause it can be observed in certain cells as the organs, including the salt glands of plants and the kidneys of animals that specialize in the elimination of wastes. The differential ionic permeability and the mechanisms that cause it can be observed in the guard cells of the stomatal complexes of plants, which are important in regulating the balance between carbon dioxide uptake and water loss. Differential membrane permeability and the mechanisms that regulate it can be observed in pollen tubes and root hairs in plants and myoblasts and neurons in animals, cells that grow in a polarized manner (Lund, 1947; Jaffe and Poo, 1979; Hinkle et al., 1981; Patel and Poo, 1984; Robinson, 1985). The differential membrane permeability and the mechanisms that control it can be observed in the neurons of animals and in some plant cells responsible for the coordination between parts of large organisms (Cole and Curtis, 1938, 1939; Eccles, 1963; Hodgkin, 1963; Huxley 1963; Erickson and Nuccitelli, 1982; Nuccitelli and Erickson, 1983; Cooper and Keller, 1984; Cooper and Schliwa, 1985). Differential membrane permeability and the mechanisms that cause it can also be observed in pathogens that use electric fields to target roots (Morris and Gow, 1993; Robinson and Messerli, 2002; van West et al., 2002). Differential membrane permeability and the mechanisms that cause it are not static, but change during cell development and aging (Laties, 1964). It is interesting to think about all the plasma membranes that a nutrient crosses as it is transported through the food chain from the soil, to the plant, to the herbivore, carnivore, or omnivore, and back to the soil (Weiss, 1996).

As important as membrane permeability to particular solute is, it cannot be measured directly (Jost, 1907). Rather, we must postulate a relationship between the permeability of the membrane for a given solute and the measurable quantities, including the amount of the solute transported, time, area, and driving force. Consequently, according to Jack Dainty (1962), “no permeability coefficient should ever

be quoted without stating the theory according to which it was calculated." Since no theory of membrane permeability was available to Adolf Fick, a physiologist who was searching for a physical description of how kidneys work, he had to come up with his own theory. Using the concept of analogy, Fick (1855) considered that a

> *law for the diffusion of a salt in its solvent must be identical with that … [which describes] the diffusion of heat … and … the diffusion of electricity. According to this law, the transfer of salt, and water occurring in a unit of time, between two elements of space filled with differently concentrated solutions of the same salt, must be cœteris paribus, directly proportional to the difference in the concentration, and inversely proportional to the distance of the elements from one another.*

When nonelectrolytes are the permeators, the driving force is expressed as the concentration difference divided by the distance between the high and low concentrations, according to Fick's First Law:

$$(ds/dt)/A = J = -D(dC/dx) \tag{2.1}$$

where ds is the amount of solute (in mol) passing through the membrane in a given time (dt, in s); A is the area of the membrane (in m^2); and $[(ds/dt)(1/A)]$ is defined as the flux in mol m^{-2} s^{-1} and is often denoted by J. dC is the concentration difference (in mol/m^3) over distance dx (in m), and it is defined as the low concentration minus the high concentration (i.e., dC/dx is the concentration gradient, which is defined as the negative of the concentration drop). D is the diffusion coefficient (in m^2/s) that relates the flux to the concentration difference. When the concentration differences of two different substances are identical but the fluxes are different, the substance with the greater flux will have a greater diffusion coefficient. The diffusion coefficient is related to velocity. Alternative definitions of the diffusion coefficient are given in equations 2.5 and 2.8.

Fick (1855) investigated the diffusion of salt in water or across aqueous porous membranes and did not have to take into consideration the presence of a membrane barrier that could hinder the passage of the solute on the basis of the lack of solubility of the substance in the membrane. However, when we apply Fick's Law to biological membranes we have to account for the fact that each solute must enter and leave the membrane, and the solubility of the solute in the membrane material will be a factor that will also determine its flow. We use the dimensionless partition coefficient, K, introduced by the chemist Marcellin Pierre Berthelot, as an estimate of how soluble a given solute is in the membrane relative to its solubility in an aqueous solution:

$$J = -D \times K(dC/dx) \tag{2.2}$$

Since we do not know the actual partition coefficient of the solute in the membrane compared to water, we estimate it by measuring the relative distribution of that solute in olive oil, or any other solvent (e.g., octanol) that mimics the hydrophobic properties of the membrane, and water (Stein, 1986).

In the case of plasma membranes, dx is usually not a measured or measurable quantity and consequently it is almost impossible to determine the diffusion coefficient. Thus, we use Runnström's (1911) modification of Fick's Law to relate the flux of nonelectrolytes to the magnitude of measurable quantities:

$$J = -P(dC) \tag{2.3}$$

where P is equal to $(D)(K)/dx$, and is called the *permeability coefficient*. It is given in units of m/s. Consequently, the permeability coefficient gives us an idea of the velocity with which a given substance will permeate a given membrane.

Let us do an example. Assume that the concentration of sucrose outside the cell is 100 mol/m^3, the concentration inside is 1 mol/m^3, and the cell is a cube the sides of which have a length (10^{-5} m). Using a radioactive tracer, we measure how many moles of sucrose are in the cell after a given time and we find that the flow (ds/dt) is 1.2×10^{-15} mol/s. Now we will calculate the permeability coefficient:

$$\begin{aligned}
P &= -(ds/dt)/(A)(dC) \\
A &= 6(10 \times 10^{-6}\,\text{m})^2 = 6 \times 10^{-10}\,\text{m}^2 \\
dC &= 1\ \text{mol m}^{-3} - 100\ \text{mol m}^{-3} = -99\ \text{mol m}^{-3} \\
P &= -(1.2 \times 10^{-15}\ \text{mol s}^{-1})\,(6 \times 10^{-10}\ \text{m}^2)^{-1} \\
&\quad (-99\ \text{mol m}^{-3})^{-1} \\
P &= 2 \times 10^{-8}\ \text{m s}^{-1}
\end{aligned}$$

Remember that the use of these equations depends on the validity of the assumptions. First, we assume that the concentration difference does not change during the experiment; therefore, we must use short transport times. We also assume that the concentration of the bulk solution is identical to the concentration at the membrane and therefore there are no unstirred layers (Dainty, 1990). Lastly, it is the absolute activity of the solute, and not the concentration, that is important. The absolute activity is less than the concentration because some of the molecules in question may be bound to each other or to other molecules. The absolute activity is equal to the concentration times the activity coefficient, which is the proportion of the free to the total solute. Thus, we are assuming that the activity coefficients on both sides of the membrane are equal to 1.

The equations can only be used for calculating the permeability coefficients of nonelectrolytes, since they assume that the only driving force is the concentration difference. While Fick studied the movement of electrolytes, he used membranes that had little or no capacitance (see Section 2.8) and thus could not develop an electrical

potential across them. Without an electrical potential difference, there can be no electrical driving force. The flux of electrolytes is not only affected by the concentration difference but also by the electrical properties of the membrane (Michaelis, 1925; Höber, 1930), including the electrical driving force, which can develop when ions diffuse across a differentially permeable membrane that has capacitance. Thus, the equation used to determine the permeability coefficient of an electrolyte must be an expanded form of Fick's Law. Such an expansion was done by Walther Nernst (1888). The Nernst equation will be used for many applications, which will allow us to determine the contribution of a given ion to the resting membrane potential; to determine if transport is active or passive; to determine the driving force for ion movements; and to determine the specificity of the ionic channels that are observed in patch clamp studies.

Like any robust equation, the Nernst equation can be derived from many starting points. I will derive the Nernst equation by considering two basic principles behind the movement of ions: Fick's Law and Ohm's Law.

As I already discussed, Fick's Law describes the movement of uncharged solutes. In order to get a better understanding of Fick's Law, consider two groups of solutes in communication with each other. As a consequence of the thermal motion of the particles, there will be a tendency for the two groups to mix and a net flux (J_{dif}) of particles will occur from the dense to the sparse group. The magnitude of the flux will depend on the concentration gradient and the diffusion coefficient in the following manner:

$$J_{dif} = -D \times (dC/dx) \tag{2.4}$$

The average flux is also proportional to temperature; thus the diffusion coefficient is proportional to absolute temperature (T, in K). This seems reasonable since diffusion is a consequence of the thermal motion of the solutes (Figure 2.19).

Boltzmann's constant ($k = 1.38 \times 10^{-23}$ J/K) relates the free energy of the solute to the temperature. The higher the temperature, the more free energy the solute has and the faster it moves. The coefficient of proportionality that relates the diffusion coefficient to kT is called the mobility (u, in $m^2\ J^{-1}\ s^{-1}$). The relation is given by the Nernst-Einstein equation (Einstein, 1956):

$$D = ukT \tag{2.5}$$

where u (in m^2/J s) relates the diffusion coefficient to the thermal energy. In a more graphic sense, since Joules/meter = Newton, u (in (m/s)/N) is a measure of the velocity (v, in m/s) a given solute travels when subjected to a given force. Therefore, Fick's Law can be rewritten as:

$$J_{dif} = -ukT \times (dC/dx) \tag{2.6}$$

Equation 2.6 describes the movement of solutes as a result of diffusion. The concentration difference produces the force needed for movement and u is a coefficient that relates the velocity of movement to the applied force. That is, $u = v/F$. Note that this equation assumes that velocity and not acceleration, as is found in Newton's Second Law, is proportional to force. That is, a molecule does not accelerate in response to a force because it constantly collides with other molecules. In the microscopic world, molecular resistance is so great that Newton's Second Law does not apply.

Since the mobility is a coefficient that relates the velocity of a particle to the force that causes it to move, we can consider it in terms of Stokes' Law, which states that a force causes a spherical particle to move with a given velocity. However, as a consequence of friction, the velocity of the particle is inversely proportional to the hydrodynamic radius of the particle (r_H, in m) and the viscosity of the medium through which it moves (η, in Pa s). While this relationship was worked out by George Stokes (1922) for the macroscopic movement of pendulums, it is also applicable to the microscopic movement of atoms and

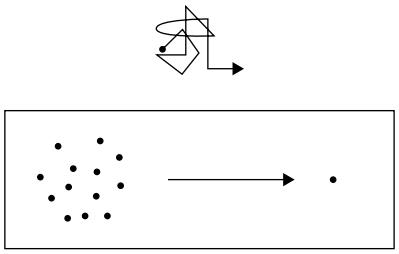


FIGURE 2.19 Thermal motion of a single particle. Due to the uneven initial distribution of particles (i.e. chemical potential), a particle will typically diffuse along an axis from regions where the initial concentration is high to regions where the initial concentration is low. The speed of the particles will remain constant in an isolated system. However, the average velocity vector of the particles, which will initially be finite and pointing from high concentration to low concentration, will vanish as the distribution equalizes. The vanishing of the velocity vector makes diffusion irreversible, and contrary to Poincare's recurrence theorem, the initial condition can only be achieved with an input of energy.

molecules, although one can still introduce higherorder corrections (Millikan, 1935). Stokes' Law is:

$$v = F/(6\pi r_H \eta) \tag{2.7}$$

and since $v/F = u$, then $u = 1/(6\pi r_H \eta)$ and

$$D = kT/(6\pi r_H \eta) \tag{2.8}$$

Equation 2.8 is known as the Einstein-Stokes (or Stokes-Einstein) equation (Weiss, 1996). It describes the diffusion coefficient as the ratio of the amount of free energy in a spherical particle at a given temperature (kT) to the friction experienced by that particle (with a hydrodynamic radius of r_H) moving through a solution with a viscosity, η. Larger solutes experience more friction than smaller solutes. Thus, Ca^{2+} with a radius of 99 picometers will have a smaller diffusion coefficient than Mg^{2+} with a radius of 65 picometers. Likewise, a protein with a hydrodynamic radius of 2.5 nm will have a smaller diffusion coefficient than a glucose molecule that has a hydrodynamic radius of 350 picometers *ceteris paribus*. In general, the diffusion coefficient for "typical" small molecules like glucose ($r_H = 0.35$ nm) in water ($\eta = 0.001$ Pa s) at room temperature (298 K) is about 6×10^{-10} m^2/s.

In order to model the electrical effects on solute movement we will use the relation discovered by Georg Ohm that describes the flow of current in wires. Ohm's Law (1827) describes the current (in A) that results when a potential, ψ (in V), is put across a given resistance, R (in Ω):

$$I = -\psi/R \tag{2.9}$$

where 1 A = 1 C/s, 1 V = 1 J/C, and 1Ω = 1 Vs/C = 1 Js/C^2. The negative sign indicates that a positive current moves away from a positive voltage source. The negative sign is not used in most forms of Ohm's Law, but is used here because it is consistent with the use of negative signs in all other flux equations.

The transport of electricity can take place in two different ways: with or without the simultaneous transport of atomic nuclei (Nernst, 1923). In wires (i.e., metallic conductors), electricity is transported without the simultaneous transport of atomic nuclei. In fact, in wires, electricity is carried by the movement of electrons from one potential to a more positive potential. Unfortunately, Ben Franklin defined the movement of electricity as the movement of positive charge from one potential to a more negative potential. In electrolyte solutions, electricity is transported by atomic nuclei and thus can be described by fluxes. In order to convert Ohm's Law into a flux equation, I will write Ohm's Law in a form that describes the simultaneous transport of electrons and matter (be it atomic nuclei and electrons or electrons alone).

Ohm's Law can also be used to describe the net motion of charged solutes in an electric field ($d\psi/dx$) where $d\psi$ is the electrical potential difference (in V) across the distance x (in m).

The flux of monovalent cations in an electric field, J_{el} (in mol m^{-2} s^{-1}) is related to the current (in A or C s^{-1}) by the following relation:

$$J_{el} = I/(FA) \tag{2.10}$$

where F is the Faraday (9.65×10^4 C/mol) and A is the area perpendicular to the electric field through which the solutes move (in m^2).

In order to derive the Nernst equation, I will start with Ohm's Law. However, in order to determine the movement of electrolytes in solution instead of electrons in a wire, both sides of the equation must be divided by FA. This converts the current (I) into a flux (J_{el}).

$$J_{el} = I/(FA) = -\psi/(RFA) \tag{2.11}$$

Equation 2.11 is only true for the flux of monovalent cations, yet the flux of ions for a given current density (in A/m^2) depends on the valence of the ion (z). Thus, for a given current density, the flux of a bivalent ion is one-half of the flux of a monovalent ion, and the general equation is:

$$J_{el} = I/(zFA) = -\psi/(RzFA) \tag{2.12}$$

Equation 2.12 is actually a typical flux equation since $I/(zFA)$ is equal to J_{el} and $\psi/RzFA$ is equal to $uzeC(d\psi/dx)$. In order to show this, I will use dimensional analysis; that is, I will write out the units of each term.

$$\frac{\text{C/s}}{(\text{C/mol})(\text{m}^2)} = \frac{-(V)}{(\text{Js/C}^2)(\text{C/mol})(\text{m}^2)}$$

After canceling C on both sides, we get:

$$\frac{(\text{s}^{-1})}{(\text{mol}^{-1})(\text{m}^2)} = \frac{-\text{V}}{(\text{Js/C})(\text{mol}^{-1})(\text{m}^2)}$$

We can see that the left side is in units of flux, and thus is equal to $J_{el,}$ which is the flux of ions in response to an electric field.

$$J_{el} = \frac{-\text{V}}{(\text{Js/C})(\text{mol}^{-1})(\text{m}^2)}$$

Now we have to use a mathematical trick. The first time you use it, it is a trick. The second time you use it, it seems clever. By the third time you use it, it will seem self-evident and will become one of your mathematical skills. Now for the trick: We are going to multiply the right side by 1. We know that multiplying anything by 1 (or adding 0) does not change its value, but it will help us to put the equation in a simple form. This trick takes advantage of the identity

property of multiplication (or addition). Now, there are many 1s—for example, 1, 2/2, 100/100, m/m, and m^2/m^2 all equal to 1—so we have to make the right choice. We will multiply the right side by $1 = m^2/m^2$ in order to end up with the equation in a convenient form.

$$J_{el} = \frac{-V(m^2)}{(JsC^{-1})(mol^{-1})(m^2)(m^2)}$$

After rearranging terms and converting C/J into V^{-1}, we get:

$$J_{el} = \frac{-V}{m}\frac{m^2}{(Vs)}\frac{mol}{m^3}$$

Replacing the units with symbols, we get:

$$J_{el} = -(d\psi/dx)(u')(C)$$

where $d\psi$ is the electrical potential difference (in V) across a membrane of thickness x (in m) and u' is the electrical mobility (in $m^2\ V^{-1}\ s^{-1}$). Again, even though an electric field accelerates a charged particle, the particle collides with other particles. Upon collision, the acceleration stops and must start anew after each collision. Consequently, the velocity, and not the acceleration, is proportional to the electrical force. According to Robinson and Stokes (1959), u' is equal to uze by definition. Thus,

$$J_{el} = -uzeC(d\psi/dx) \tag{2.13}$$

and we see that the electrical flux equation is really nothing more than Ohm's Law. The total flux due to diffusion and electric forces is

$$J_{tot} = J_{dif} + J_{el} \tag{2.14}$$

and the total flux of a given solute in response to the driving forces, (dC/dx) and $(d\psi/dx)$, will be a function of the mobility of the solute.

$$J_{tot} = -ukT(dC/dx) - zeCu(d\psi/dx) \tag{2.15}$$

Equation 2.15 also assumes that the solubility of the solute in the membrane and the solutions on either side of the membrane are the same. This is not a valid assumption for biological membranes, so we must account for this by including the partition coefficient:

$$J_{tot} = -KukT(dC/dx) - KzeCu(d\psi/dx) \tag{2.16}$$

Now consider two solutions of monovalent ions (e.g., KCl) separated by a membrane. Imagine that the membrane passes only the positively charged cation (K^+) but not the negatively charged anion (Cl^-), which is a good assumption for a plasma membrane. On each side of the membrane, the concentrations of ions are different, although according to the rule of electroneutrality, on the macroscopic level there must be almost the same number of cations and anions. Let us consider a situation where there is no net flux (i.e., $J_{tot} = 0$).

$$J_{tot} = -KukT(dC/dx) - KzeCu(d\psi/dx) = 0 \tag{2.17}$$

This is an equilibrium situation, so any term that involves a rate should cancel out. First, let us rearrange the terms:

$$KuzeC(d\psi/dx) = -KukT(dC/dx) \tag{2.18}$$

Now let us solve for $d\psi/dx$:

$$d\psi/dx = -KukT(dC/dx)(KzeCu) - 1 \tag{2.19}$$

After canceling u and K, we get:

$$d\psi/dx = -(kT/ze)(dC/C)/dx \tag{2.20}$$

In order to eliminate the derivatives and get a simple, easy-to-use, powerful equation, we must integrate the equation. I will integrate between the two limits of the membrane thickness from outside to inside and assume k, T, z, and e are constant to get a simple yet powerful equation (Lakshminarayanaiah, 1965, 1969).

We take the constants out of the integral and then integrate. To integrate, we must remember that $\int_o^i dC/C\,dx = (\ln C_i - \ln C_o)$. I set up this and every equation in this book to reflect a change from the initial state (outside the cell) to the final state (inside the cell). According to the fundamental law of calculus, we must subtract the final state (inside the membrane) from the initial state (outside the membrane). Thus, upon integration, we get:

$$\psi_i - \psi_o = -kT/ze\ (\ln C_i - \ln C_o) \tag{2.21}$$

where C_o and C_i are the concentration outside and inside the membrane, respectively, and ψ_i and ψ_o are the electrical potentials inside and outside the membrane, respectively. Remember that $\ln C_i - \ln C_o = \ln C_i/C_o$, and $-\ln C_i/C_o = \ln C_o/C_i$; thus

$$\psi_i - \psi_o = -kT/ze\ (\ln C_o/C_i) \tag{2.22}$$

Boltzmann's constant (k) and the elementary charge (e) are related to the universal gas constant (R) and the Faraday (F), respectively, through Avogadro's number (Perrin, 1926). Since $R = kN_A$ and $F = eN_A$, kT/e is also equal to RT/F. Thus,

$$\psi_i - \psi_o = RT/zF\ \ln(C_o/C_i) \tag{2.23}$$

And since, by convention $\psi_o = 0$, which means that practically, we zero the potential measuring electrode outside the cell:

$$\psi_i = (RT/zF)\ln(C_o/C_i) = (kT/ze)\ln(C_o/C_i) \qquad (2.24)$$

which are the familiar forms of the Nernst equation.

Let us use the Nernst equation right away to calculate the electrical potential across a membrane that has a concentration of 100 mol/m^3 K^+ inside and 1 mol/m^3 K^+ outside (Figure 2.20).

$$\psi_i = (RT/zF)\ln(C_o/C_i)$$
$$\psi_i = [(8.31\ \text{J mol}^{-1}\ \text{K}^{-1})(298\ \text{K})]/[(1)9.65 \times 10^4\ \text{C/mol}]\ln(1/100)$$
$$\psi_i = [(8.31\ \text{J mol}^{-1}\ \text{K}^{-1})(298\ \text{K})]/[9.65 \times 10^4\ \text{C/mol}](-4.6)$$
$$\psi_i = -0.118\ \text{V or } -118\ \text{mV}$$

The result from the Nernst equation tells us that leakage of K^+ down its concentration difference creates an electrical difference across the membrane, such that the inside of the membrane becomes more and more negatively charged until the electrical potential difference that develops exactly balances the concentration difference. At equilibrium, the resulting voltage difference is −0.118 V.

In order to maintain an electrical potential across the plasma membrane, the membrane must have a property known as capacitance, which is the ability to store charge or resist changes in voltages. A capacitor results when two conductors are separated by a nonconductor (Figure 2.21). The plasma membrane is a capacitor since the lipid bilayer serves as a nonconductor that separates the aqueous conducting fluids on both sides of the membrane. The specific capacitance (C_{sp}, in F/m^2, where F = C/V) is the proportionality coefficient that relates the charge per unit area (q/A in C/m^2) that is produced on either side of a nonconductor to a given electrical potential difference (ψ, in V). The capacitance (C) is defined as q/ψ and the specific capacitance (C_{sp}) is defined as $q/(A\psi)$.

The capacitance of the membrane determines how many K^+ ions have to move across the membrane in order to obtain the membrane potential predicted by the Nernst equation. How many K^+ ions have to move across the membrane in order to obtain a membrane potential of −0.118 V? Assume the cell is a cube where the length of each edge is 10^{-5} m and the specific capacitance of the membrane is 10^{-2} F/m^2.

$$q/A = C_{sp}\psi_i \qquad (2.25)$$

After plugging in the above values for the specific capacitance and the membrane potential, we get:

$$q/A = 10^{-2}\ \text{C V}^{-1}\ \text{m}^{-2}\ (-0.118\ \text{V})$$
$$= -1.18 \times 10^{-3}\ \text{C m}^{-2}$$

The number of K^+ per unit area needed to charge the membrane is obtained by dividing the charge per unit area (on either side of the membrane) by the elementary charge (e):

$$(-1.18 \times 10^{-3}\ \text{C m}^{-2})/(1.6 \times 10^{-19}\ \text{C/K}^+)$$
$$= -7.36 \times 10^{15}\ \text{K}^+\ \text{m}^{-2}$$

Since the surface area = 6 $(10^{-5}\text{m})^2 = 6 \times 10^{-10}$ m^2, then $(-7.36 \times 10^{15}\ \text{K}^+\ \text{m}^{-2})(6 \times 10^{-10}\text{m}^2) = -4.4 \times 10^6\ \text{K}^+$ must cross the membrane to charge it to a voltage of −0.118 V. Since the sign of the membrane potential was determined by the fact that we integrated from outside the

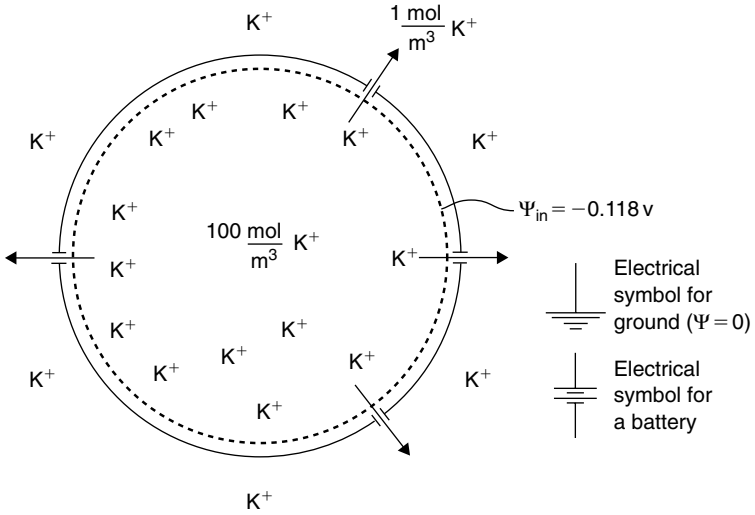


FIGURE 2.20 A membrane potential (e.g. battery) develops as a result of the unequal distribution of ions across the membrane and transport proteins that allow the ions to permeate across the membrane.

membrane to inside, the negative sign for the number of K^+ that must cross the membrane in order to charge it to $-0.118\,V$ means that the ions must move from inside the cell to outside the cell, which as we know is down their concentration difference.

When approximately four million ions leave the cell, what is left? Or, put another way, how does this change the initial conditions in a cell with a volume of $10^{-15}\,m^3$?

Since the initial concentration of K^+ in the cell was $10^2\,mol/m^3$ and the volume of the cell is $10^{-15}\,m^3$, then the number of K^+ initially in the cell was:

$$(10^2\ \text{mol/m}^3)(10^{-15}\ \text{m}^3)(6.02 \times 10^{23}\ \text{K}^+/\text{mol}) = 6.02 \times 10^{10}\ \text{K}^+$$

Thus, only $(4.4 \times 10^6/6.02 \times 10^{10})100\%$, or 0.007 percent, of the K^+ leaves the cell due to its concentration difference. After this trivial loss, the membrane potential is charged to $-0.118\,V$, which prevents any additional net loss of K^+. Therefore, K^+ diffuses out of the cell until the membrane potential becomes negative enough to balance the driving force due to diffusion. If the membrane capacitance were zero, it would be impossible to develop an electrical potential across it. It would run down over time. Since the capacitance is due to the lipid bilayer, the membrane potential is, in part, due to the lipids, as well as the transport proteins and the concentration differences of the various ions.

The creation of a membrane potential due to the passive movement of ions across a membrane was just described. However, the membrane potential created by the diffusion of ions depends on the ability of that ion to dissolve in and diffuse across the membrane. Therefore, if a membrane is completely impermeable to an ion, the ion will not be able to diffuse and leave behind the opposite charge and establish a membrane potential. In reality, membranes are usually much more permeable to K^+ than to any other abundant ion due to the large proportion and high conductance of K^+ channels. For this reason, K^+ is the ion that contributes the most to the resting diffusion potential (Figure 2.22).

Nernst determined the relationship between the diffusion of a single type of ion to the electrical potential at equilibrium where the net flux equals zero ($J_{tot} = 0$). Max Planck integrated the flux equation for situations where $J_{tot} \neq 0$. Planck's form of the equation can be used to determine the permeability coefficients of a membrane for ions from flux experiments. Later, David Goldman, Alan Hodgkin, and Bernard Katz derived an equation to account for the diffusion of multiple ions at equilibrium. We can calculate the resting membrane potential due to passive diffusion of all the abundant monovalent ions using the Nernst potential for these ions and the relative permeabilities of each ion. These are all combined into the Goldman-Hodgkin-Katz equation:

$$\psi_i = (RT/F)\ln\frac{(P_K C^o{}_K + P_{Na} C^o{}_{Na} + P_{Cl} C^i{}_{Cl})}{(P_K C^i{}_K + P_{Na} C^i{}_{Na} + P_{Cl} C^o{}_{Cl})} \qquad (2.26)$$

of which the complete derivation is given in Wayne (1994). Only the monovalent ions are considered in this equation since they are the most abundant and their mobilities are greater than the mobilities of the abundant bivalent cations due to their low charge density (Nernst, 1923).

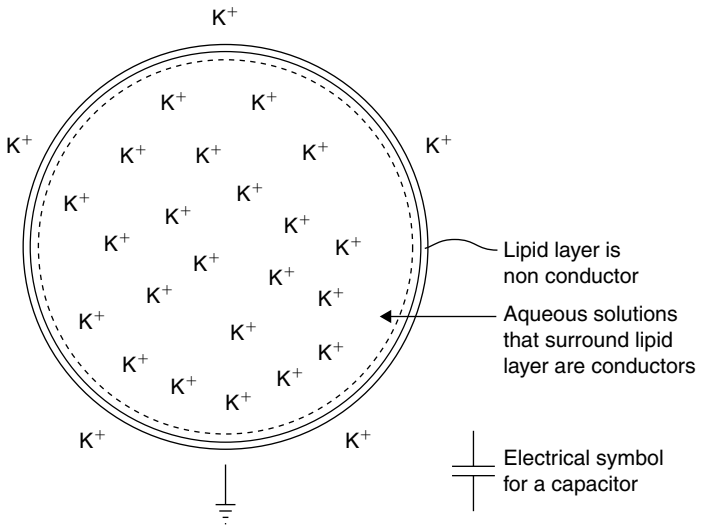


FIGURE 2.21 The membrane potential developed by the permeation of ions across the membrane would dissipate if the membrane did not have the electrical capacity (capacitance) to store electrical charge. The lipid bilayer functions as the nonconducting or dielectric layer between the two conducting aqueous layers. The three layers form a capacitor.

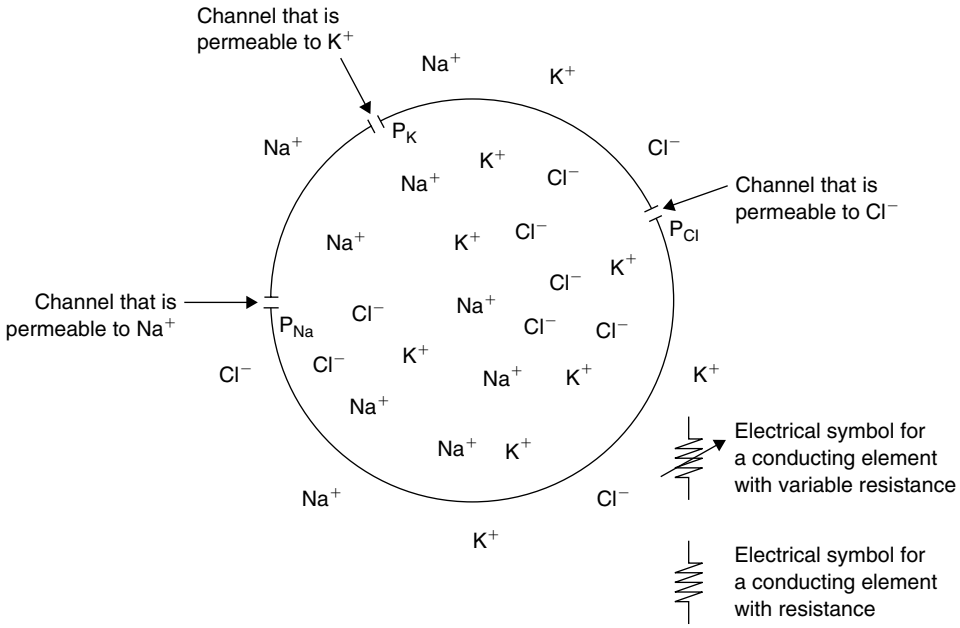


FIGURE 2.22 The magnitude of the membrane potential depends, in part, on the relative permeability of the membrane to various ions. The permeability of a channel to a given ion is a variable, not a constant.

Once the permeabilities are known, we can simplify the equation by using relative permeabilities:

$$\psi_i = (RT/F)\ln\frac{(C^o_{\mathrm{K}} + \alpha C^o_{\mathrm{Na}} + \beta C^i_{\mathrm{Cl}})}{(C^i_{\mathrm{K}} + \alpha C^i_{\mathrm{Na}} + \beta C^o_{\mathrm{Cl}})} \qquad (2.27)$$

where $\alpha = P_{\mathrm{Na}}/P_{\mathrm{K}}$ and $\beta = P_{\mathrm{Cl}}/P_{\mathrm{K}}$. When solving Eq. 2.27, remember the order of operations in arithmetic. Add the concentration terms in the parentheses before dividing the two sums.

When $\alpha = 0$ and $\beta = 0$, the Goldman-Hodgkin-Katz equation reduces to the Nernst equation. The permeability coefficients vary from about 10^{-4} m/s for water to about 10^{-11} m/s for Cl^-. Moreover, the permeability coefficients are not constant, but depend on such factors as the age of the cell, the light quality, pH, and Ca^{2+}. Indeed, fluctuations in the permeability coefficients of ions lead to dramatic changes in cell physiology and development (Jaffe, 1980, 1981, 2006, 2007; Harold, 1986, 1990; Raschke et al., 1988).

Typically, the membrane potential of plant cells is far more negative (hyperpolarized) than would be predicted by the Goldman-Hodgkin-Katz equation. When the membrane potential is greater than that accounted for by the passive diffusion of ions, then active transport must be taking place. Active transport can be diagnosed by treating the cells with metabolic inhibitors and seeing whether the membrane potential rapidly and reversibly depolarizes.

In animal cells, the membrane potential is only slightly greater than the diffusion potential and a Na^+/K^+-ATPase is the most common electrogenic pump (Ussing and Zerahn, 1951; Kerkut and York, 1971). While there are also Na^+-ATPases in plant cells, fungi, and bacteria, in these organisms, the H^+-ATPase is the most common electrogenic pump (Spanswick, 2006).

We can already determine the Nernst potential for K^+, Na^+, and Cl^-. Now we will determine whether or not the uptake or effluxes of these ions are active or passive. Assume that the membrane potential is -0.25 V, $C^o_{\mathrm{K}} = 0.1$ mol/m^3, $C^o_{\mathrm{Na}} = 0.1$ mol/m^3, $C^i_{\mathrm{Cl}} = 20$ mol/m^3, $C^i_{\mathrm{K}} = 100$ mol/m^3, $C^i_{\mathrm{Na}} = 10$ mol/m^3, $C^o_{\mathrm{Cl}} = 0.2$ mol/m^3, and $T = 298$ K. We will determine if an ion is at equilibrium by determining if the Nernst potential for that ion is equal to the membrane potential. We will determine whether the movement is active or passive by calculating the driving force for an ion and multiplying that value by ze. The free energy (in J) needed to move an ion is given by the following formula:

$$\Delta E = (\psi_m - \psi_{\mathrm{ion}})ze \qquad (2.28)$$

where ψ_m is the membrane potential (in V = J/C), ψ_{ion} is the Nernst potential for the ion (in V = J/C), z is the valence of the ion (dimensionless), and e is the elementary charge (in C). Since we integrated the Nernst equation from outside to inside, that set all the signs. If the free energy obtained is negative (exergonic), the flux into the cell is spontaneous, or passive. If the free energy is positive (endergonic), the flux into the cell is active.

The difference between the membrane potential and the Nernst potential for an ion provides the driving force[3] for the uptake of that ion. In terms of K^+, $(\psi_m - \psi_{ion})$ is given by $-0.250\,V - (-0.177\,V) = -0.073\,V$. The free energy involved in moving a K^+ from outside the cell to the inside is $(-0.073\,V)ze = -1.2 \times 10^{-20}$ J. Since the free energy is negative, the inward movement is passive. The inward movement of positive charge results in an inward current.

In terms of Na^+, $(\psi_m - \psi_{ion})$ is given by $-0.250\,V - (-0.118\,V) = -0.132\,V$, and the free energy of transport is given by $(-0.132\,V)ze = -2.1 \times 10^{-20}$ J. Since the free energy is negative, there is a passive inward movement of positive charge.

In terms of Cl^-, $(\psi_m - \psi_{ion})$ is given by $-0.250\,V - (0.118\,V) = -0.368\,V$, and the free energy of transport is given by $(-0.368\,V)ze = +5.9 \times 10^{-20}$ J. Since the free energy is positive, the inward movement of ions requires active transport. However, the outward movement of Cl^- is passive. The outward movement of negative charges is also called an inward current because it behaves as if positive charges move into the cell.

In conclusion, if the product of $(\psi_m - \psi_{ion})$ and ze is negative, it means that ions will move into the cell passively. If the product of $(\psi_m - \psi_{ion})$ and ze is positive, it means that ions will move out of the cell passively. If the product of $(\psi_m - \psi_{ion})$ and ze is zero, there will be no net movement of anions or cations and the ion is at equilibrium. If the net movement in one direction is spontaneous (i.e., passive), it must be active in the other direction. When we measure the distribution of all ions on both sides of the membrane, we see that none of the ions is in equilibrium. That is, maintaining the normal distribution of ions requires a constant input of energy.

A change in the membrane potential (ψ_m) can determine whether uptake is active or passive. An environmental stimulus (e.g., touch, light, or hormones) can often cause a membrane depolarization and thus a change in influx and efflux. Given the above situations, if the membrane potential depolarized to $-0.1\,V$, would the flux change from passive to active or from active to passive for any of the ions?

2.8 ELECTRICAL PROPERTIES OF THE PLASMA MEMBRANE

As we have already seen, the electrical properties of the plasma membrane influence the transport of electrolytes across the membrane. The membrane electrical potential has a great influence, and it can be readily measured. Our study of permeability and transport physiology is bringing us into the field of electrophysiology. Although this field is unfamiliar to many botanists, historically the study of electricity began with plants. Thales of Miletus discovered that amber (e.g., fossilized pine sap from which succinic acid was first extracted), when rubbed with fur, attracts little pieces of pith and cork. William Gilbert (1600), named this attraction electricity, after *electron*, the Greek work for "amber" (Laidler, 1993).

It turns out that there are two kinds of electricity with opposite properties. Rubbed amber contains resinous electricity (i.e., an excess of electrons) and is negatively charged, while rubbed glass repels the things that amber attracts and contains vitreous electricity (i.e., a dearth of electrons) and is positively charged. Electricity is dynamic and current flows from positively charged substances to negatively charged substances. In the late 18th century, animal biologists played a role in the development of galvanic electricity from static electricity when Luigi Galvani noticed that when two different metals touched a frog's leg, electricity was generated (Galvani, 1953a,b). This work was followed up by Alessandro Volta, who found that he could still generate electricity without the frog as long as the two different metals were placed in a solution more conductive than water. These observations led Volta to invent the battery (Conant, 1947). In the early 18th century, the voltaic pile was used to separate chemical compounds into their constituent elements (Davy, 1821; Nicolson and Carlisle, 1800; Arrhenius, 1902; Nernst, 1923; Ostwald, 1980). At this time, it was believed in some circles that electricity could also be used to animate matter and to create life (Aldini, 1803; Shelley, 1818; Ure, 1819). The importance of electricity in living organisms was further established when Emil DuBois Reymond (1848) showed that electricity was the "nervous principle" transmitted by nerves, and Guillaume Duchenne (1862, 1871, 1949) showed that electrical stimulation was involved in the contraction of all muscles—including the ones that give rise to smiles. The electrical nature of the nervous system of animals was further characterized by Sherrington (1906), Lucas (1917), Langley (1921), Creed et al. (1932), and Eccles (1964). There is currently a call for a resurgence in studies of the electrical nature of plant communication (Staves et al., 2008). The techniques involved in electrophysiology are described briefly next. A fuller discussion on the techniques can be found in Bures et al. (1967), Hille (1992), Weiss (1997), and Volkov (2006).

In order to measure the membrane potential, two electrodes are connected to an electrometer (see Figure 2.23; Walker, 1955). Then the two electrodes are placed in the solution bathing the cell and the electrometer is zeroed. This is why the external electrical potential is considered zero. Then the glass microcapillary electrode, filled with 3M

[3] The driving force, like any force, is properly given in Newtons. Thus, the proper driving force is equal to the negative spatial derivative (or gradient, in vector calculus notation) of the electrochemical potential. That is, the proper driving force is the difference between the high electrochemical potential and the low electrochemical potential divided by the distance between the high and low electrochemical potentials.

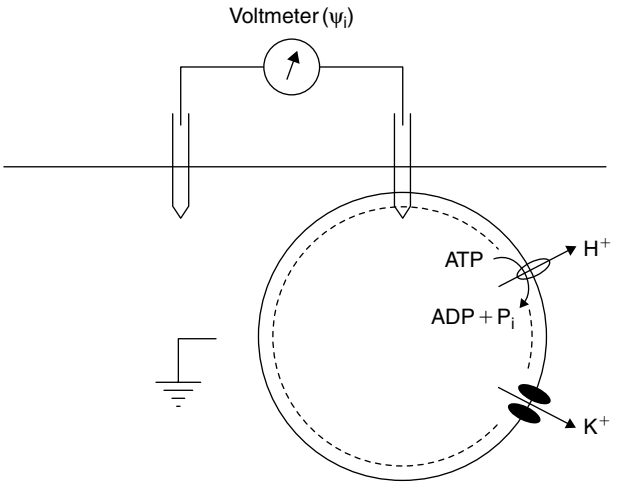


FIGURE 2.23 Measuring the membrane potential with two microcapillary electrodes connected to a voltmeter in the current clamp mode. By convention, the electrical potential difference between the two electrodes is set to zero before the microcapillary electrode is inserted into the cell.

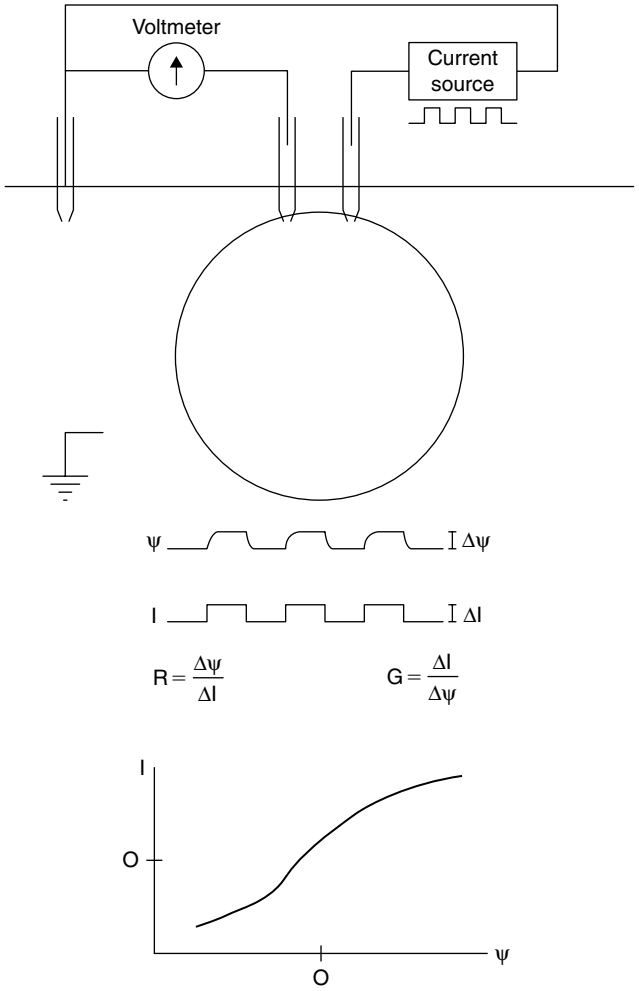


FIGURE 2.24 A voltage clamp measures the amount of current that passes through a membrane when an electrical potential difference is established artificially across that membrane. The current is plotted against the membrane potential. The conductance of the membrane at a given voltage is calculated from the slope of the *I*-ψ curve.

KCl, is inserted into the cell with the help of a micromanipulator and ψ_i is measured (in V). The membrane potentials of plant cells typically range from -0.12 to -0.25 V (Etherton and Higginbotham, 1960; Etherton, 1963). Using their wits, Wright and Fisher (1981) measured the membrane potential of narrow and difficult-to-access sieve tubes by using aphid stylets as microcapillaries. Membrane potentials found in animal cells range from -0.06 to -0.1 V.

The resistance of the membrane (in Ω) is the property of the membrane that determines the relationship between the current and the electrical potential. The greater the resistance, the smaller the change in current for a given change in voltage. Alternatively, the greater the resistance, the greater the change in voltage across a resistor for a given change in current. In order to measure the membrane resistance, a tiny current is intermittently passed through the membrane while the membrane potential is being measured (see Figure 2.24; Blinks, 1930, 1939; Walker, 1960). The membrane potential changes when the current flows. The membrane resistance is then obtained from Ohm's Law, where the membrane resistance is calculated by dividing the change in membrane potential by the change in membrane current. Since the resistance is a function of cell size, we usually use the specific resistance (in Ω m^2) to characterize the membrane. The specific resistance is obtained by multiplying the membrane resistance by the surface area of the cell. The reciprocal of the specific resistance is the specific conductance (in S/m^2). The specific conductance of the membrane is a measure of its permeability to all ions and is determined by the quantity and type of transport proteins embedded in the lipid bilayer.

Capacitance is the property of a membrane that resists changes in voltage when a current is applied. When a current is applied to the cell, the voltage does not instantaneously attain the value predicted by the resistance, but rises logarithmically to that value. Once the resistance is known, the capacitance is measured by determining the time it takes for the membrane potential to reach 63 percent of the maximal value it reaches at infinite time (Figure 2.25). The time it takes to reach this value is equal to the product of the resistance and the capacitance. The time needed to reach 63 percent of the maximal value is known as the *time constant,* and in general is around 10 ms. Capacitance, like resistance, also depends on the surface area of the cell, and thus we usually talk about the specific capacitance (in F/m^2), which is given by the capacitance divided by the surface area.

Hugo Fricke (1925) measured the specific capacitance of the plasma membrane of red blood cells to be 0.01 F/m^2. The specific membrane capacitance depends on an electrical property of the membrane known as the dielectric

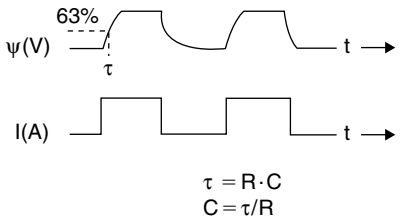


FIGURE 2.25 When a current is applied to a membrane in a square wave, the membrane potential follows a sawtooth pattern. The shape of the sawtooth depends on the membrane resistance and the membrane capacitance. The voltage change reaches 63 percent of the maximum in a time (τ) equal to the product of the resistance (R) and the capacitance (C). The membrane capacitance is equal to τ/R.

constant or the *relative permittivity* (ε, dimensionless) and the thickness of the nonconducting layer (dx). Fricke assumed that the nonconducting layer was made of lipids and guessed that the relative permittivity (or dielectric constant) of the membrane was the same as it is for lipids ($\varepsilon = 3$). By assuming that the relative permittivity of the plasma membrane was 3, the thickness of the lipid layer can be calculated from the specific capacitance of the membrane. Plugging these values into the following equation, which is used to calculate the specific capacitance of a parallel plate capacitor, Fricke estimated that the thickness of the lipid layer was approximately 3 nm.

$$C_{\text{sp}} = \varepsilon_o \varepsilon / dx \quad \text{or} \quad dx = \varepsilon_o \varepsilon / C_{\text{sp}} \tag{2.29}$$

where ε_o, the permittivity of a vacuum, is 8.85×10^{-12} F/m.

2.9 CHARACTERIZATION OF TWO TRANSPORT PROTEINS OF THE PLASMA MEMBRANE

The plasma membrane of plants contains a diverse array of transport proteins including a H^+-ATPase, a Ca^{2+}-ATPase, a Na^+-ATPase (Benito and Rodriguez-Navarro, 2003), a Cl^--ATPase (Gradmann and Klempke, 1974; Mummert et al., 1981), an ATP-binding cassette-type transporter (Jasinski et al., 2001; Sanchez-Fernandez et al., 2001; Kobae et al., 2006), an amino acid symporter (Etherton and Rubinstein, 1978; Kinraide and Etherton, 1980), a Ca^{2+}/H^+ antiporter, a sucrose/H^+ antiporter, as well as Cl^-, K^+, and Ca^{2+} channels (Mäser et al., 2001; Axelsen and Palmgren, 2001; Hedrich and Marten, 2006). There are also channel proteins that pass small polar and nonpolar molecules, including H_2O and CO_2 (Wayne and Tazawa, 1990; Wayne et al., 1994; Tyerman et al., 2002; Terashima and Ono, 2002). The approaches used to characterize two of the major and ubiquitous transport proteins—the H^+-pumping ATPase and the K^+ channel—are discussed next.

2.9.1 Proton-Pumping ATPase

The H^+-ATPase is one of the best characterized proteins in the plasma membrane of plants. Its presence in the plasma membrane was first inferred by H. Kitasato in 1968 when he noticed that, contrary to the predictions of the Goldman-Hodgkin-Katz equation, the plasma membranes of characean cells were relatively insensitive to changes in the external K^+ at concentrations below 1 mol/m^3. He did observe, however, that the membrane potential was sensitive to changes in the external H^+ concentration (Kitasato, 2003).

Using the Nernst equation, Kitasato calculated that if the protons were distributed passively, the internal pH should be <3 given the external pH and the observed membrane potential. Since the internal pH is approximately 7, Kitasato proposed that H^+ were actively pumped out of the cell. He found that dinitrophenol (DNP), a protonophore, reduced the membrane potential. Later, Roger Spanswick (1972, 1974a) and Keifer and Spanswick (1978, 1979) provided evidence that H^+ was the ion pumped since dicyclohexylcarbodiimide (DCCD), an inhibitor of H^+ transport in mitochondria and chloroplasts, decreased the membrane potential to the value predicted by the Goldman-Hodgkin-Katz equation. DCCD also increased the membrane resistance, indicating that there is a conductance in the plasma membrane for H^+. Teruo Shimmen and Masashi Tazawa (1977) perfused the inside of characean internodal cells with ATP and demonstrated that the membrane potential and the efflux of H^+ were dependent of the intracellular ATP concentration. Takeshige et al. (1986) have shown that the extrusion of H^+ can be quantitatively accounted for by the action of the proton pump by showing the equivalence between the proton efflux (J^{H+} in mol m^{-2} s^{-1}) and the pump current density (I/A in A/m^2). They used the following equation:

$$J^{\text{H}+} = I/(zFA) \tag{2.30}$$

Not every segment of the plasma membrane is identical. This can be visualized easily and elegantly in the large internodal cells of *Chara* (Shimmen and Wakabayashi, 2008). The plasma membrane is differentiated into regions that have a net proton efflux and regions that have net proton influx. These regions are known as the acid and alkaline bands, respectively. The bands can be beautifully visualized by placing cells on nutrient agar containing phenol red. The phenol red will turn yellow where the pH is acidic and red where the pH is basic (Spear et al., 1969).

At the same time that work with whole cells or cell models was advancing, investigations at the biochemical level were also making progress. Knowing that ion transport is often dependent on respiration (Briggs and Petrie, 1931; Steward, 1933, 1941; Lundegarth, 1955; Laties, 1959), Tom Hodges and his colleagues (Fisher and Hodges, 1969; Hodges et al., 1972) began searching for the molecular mechanism that converts respiratory energy into the work of

ion transport. They purified plasma membranes from roots and discovered that purified plasma membranes had the ability to hydrolyze ATP, a product of respiration. In fact, if the plasma membrane ATPase ran continuously, it would consume 25–50 percent of the cellular ATP (Felle, 1982).

The H^+-ATPase has been purified from many plants and accounts for approximately 1 percent of the plasma membrane protein and approximately 0.01 percent of the total cellular protein (Sussmann and Harper, 1989). Anthon and Spanswick (1986) purified the H^+-ATPase from the plasma membranes of tomato. They washed a crude membrane fraction with high salt and 0.1 percent Triton to remove the peripheral and loosely held integral membrane proteins. The membranes were further extracted with octylglucoside/deoxycholate, a detergent that removes other integral proteins, but not the H^+-ATPase. The ATPase was finally solubilized with lysolecithin and released into the supernatant fraction. The supernatant was centrifuged through a glycerol gradient, and the H^+-ATPase was collected in the 37 percent glycerol fraction. It is possible to follow the purification of the H^+-ATPase since the specific activity increases as the protein is purified.

Purity can also be estimated from SDS page (see Figure 2.18) and the activity of the purified protein can be measured with functional assays of its ATPase activity and its ability to pump protons. As the specific activity increases, a single band becomes more and more prominent and this is assumed to be the H^+-ATPase polypeptide.

The H^+-ATPase can be characterized based on the nature of the compounds that inhibit it (Figure 2.26). For example, the plasma membrane proton ATPase activity is inhibited by vanadate, which inhibits all ATPases that form an inorganic phosphate (P_i)-enzyme intermediate. By contrast, it is not inhibited by nitrate, which is an inhibitor of the vacuolar membrane proton ATPase. The plasma membrane ATPase is also inhibited by DCCD, a compound that depolarizes the membrane potential.

The purified ATPase is able to pump H^+ after it is inserted into proteoliposomes filled with a fluorescent dye, quinacrine, the fluorescence of which depends on the pH of its environment, and the fluorescence of quinacrine decreases upon the accumulation of H^+. H^+ pumping requires ATP and is inhibited by DCCD and vanadate (Figure 2.27). Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a proton ionophore, increases the fluorescence by releasing protons, providing evidence that the fluorescence decrease is due to H^+ pumping and that the ATPase is an H^+ pump.

The functions of the various segments of the H^+-ATPase are becoming clear (Portillo, 2000; Bukrinsky et al., 2001; Kühlbrandt et al., 2002; Wurtele et al., 2003). For example, when inside-out vesicles are challenged with ATP, they pump protons at the expense of the ATP. If the vesicles are treated with trypsin, so that a 7-kDa polypeptide

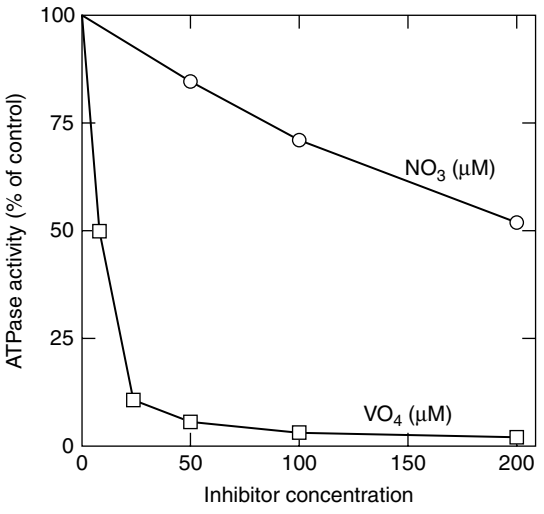


FIGURE 2.26 Inhibition of plasma membrane ATPase activity by vanadate but not by nitrate. (*Source:* From Anthon and Spanswick, 1986.)

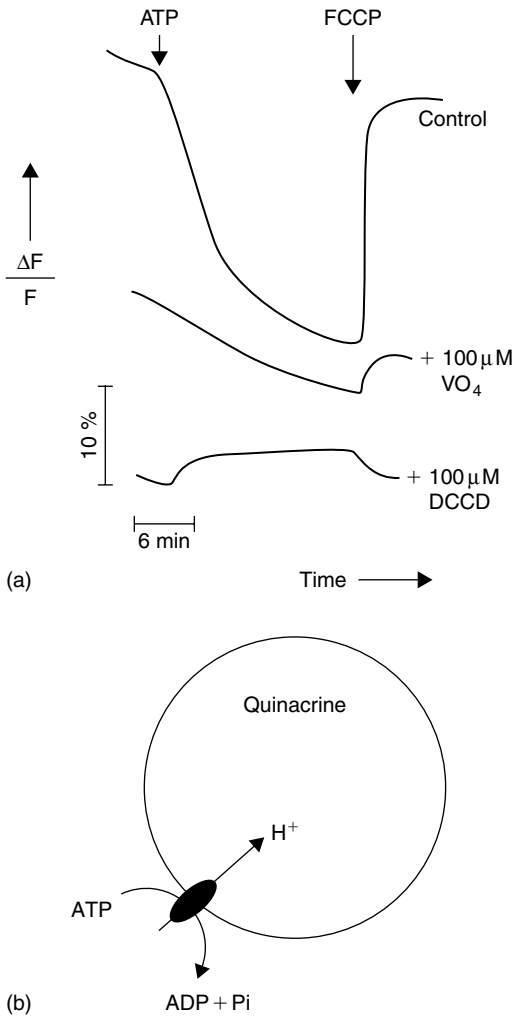


FIGURE 2.27 (a) Inhibition of plasma membrane ATP-dependent proton-pumping activity in proteoliposomes by vanadate and DCCD. (b) Only the activities of the proton pumps in the "inside out" orientation are measured. From Anthon and Spanswick (1986).

is removed from the carboxy-terminus of the H^+-ATPase, both the ATP hydrolyzing and proton-pumping activities are stimulated. This stimulation can again be inhibited by the addition of the 7-kDa fragment, indicating that the carboxy-terminal end of the ATPase regulates the activity of the rest of the protein (Palmgren et al., 1991).

The H^+-ATPase is regulated by its phosphorylation state. The carboxy-terminal end has a threonine residue that can be phosphorylated. Upon phosphorylation, a regulatory protein known as 14-3-3 binds to the carboxy-terminal end and activates the H^+-ATPase (Jahn et al., 1997; Svennelid et al., 1999). The carboxy-terminal end also has a serine residue, which upon phosphorylation, inhibits the binding of the 14-3-3 protein and thus inactivates the H^+-ATPase (Fulsang et al., 2007). The activated protein complex consists of six phosphorylated proton ATPase molecules and six 14-3-3 molecules assembled in a hexameric structure (Kanczewska et al., 2005).

The mechanism of how the H^+-ATPase pumps protons across the membrane has been postulated by looking for analogies with the better known Na,K-ATPase and Ca-ATPase of animal cells (Jørgensen and Pedersen, 2001; Toyoshima and Nomura, 2002; Buch-Pedersen and Palmgren, 2003). The phosphorylation of the enzyme, which results from the incorporation of the phosphate from the ATP used to power the enzyme, probably induces a conformational change in the protein that moves the H^+-binding site of the protein from the protoplasmic side of the plasma membrane to the extracellular side of the plasma membrane. Concurrently, the affinity of the H^+-binding site for H^+ decreases. These changes result in the release of the H^+ to the external space and the net transport of protons across the membrane. Dephosphorylation of the H^+-transporting ATPase returns it to its initial conformation where it can again bind an H^+ on the protoplasmic side of the membrane.

The powers of electrophysiological techniques and biochemical techniques have been combined to study the proton ATPase by reconstituting the proton ATPase into a planer lipid bilayer. In this way, the electrical and chemical environments on both sides of the proton ATPase can be regulated at the same time (Briskin et al., 1995).

In 1989, the gene for the H^+-ATPase was cloned and sequenced (Serrano, 1989). Using the hydrophilic and hydrophobic properties of the amino acids that are encoded by the sequence, a first approximation of the structure and topography of this H^+-ATPase was made and the structure suggested that this canonical H^+-ATPase is a multipass integral membrane protein.

Molecular genetics has taught us that there are typically multiple and distinguishable copies of genes that encode transport proteins like the plasma membrane H^+-ATPase, and that the first transport protein characterized will most likely turn out to be just one example of a class of transport proteins that may vary from cell to cell or during the life of a cell in a single organism. Thus, we must not be too dogmatic and we must be sure to remember that the canonical protein with its characteristics may just be one example of the range of possible transport proteins that may differ in their kinetics, their sensitivity to inhibitors, their regulation, and their transport selectivity, as a result of being encoded by genes of which the domains have been joined together in various ways though evolutionary time to code for related yet unique proteins (Doolittle, 1995).

Genetic analysis shows that the proton ATPase is a member of a class of ion-translocating ATPases called the *P-type ion-translocating ATPases*. P-type ATPases are characterized by the formation of a phosphorylated intermediate in its reaction cycle and thus are inhibited by vanadate. In plants, the plasma membrane–bound and endoplasmic reticulum (ER)–bound Ca^{2+}-ATPases are also P-type ATPases and are related to the Na^+/K^+-ATPase and the Ca^{2+}-ATPase of animal cells, the H^+-ATPase of fungal cells, and the K^+-ATPase of bacterial cells (Wimmers et al., 1990). Information about P-type ATPases can be found at *http://www.patbase.kvl.dk/*.

The H^+ pump has been characterized, purified, and cloned because it is so important to the life of the cell (Felle, 2002; Tazawa, 2003). The H^+-ATPase creates an electrochemical difference of protons. As long as the plasma membrane is not freely permeable to the protons being pumped out, the free energy stored in the electrochemical difference of protons set up by the proton pump can be used to drive a number of secondary transport processes, including sugar and amino acid transport, and the passive transport of K^+ (Vreugdenhil and Spanswick, 1987; Raschke et al., 1988; Sanders, 1990). The proton ATPase is abundant in cells specialized for the transport of nutrients, including root epidermal cells, phloem companion cells, and transfer cells (Jahn and Palmgren, 2002).

The proton pump uses the energy of ATP to pump protons out of the cell in an electrogenic manner. The membrane potential, which becomes negative inside as a result of the activity of the pump, drives the inward flux of K^+. The increased osmotic pressure due to the increased K^+ concentration within the cell causes water to move into the cell, which in turn causes an increase in the turgor pressure. This turgor pressure, which follows indirectly from the activity of the electrogenic proton pump, is necessary for cell and plant growth as well as movements, including tropisms, leaflet movement, and stomatal opening/closure. In the cells of many tissues, the proton ATPases is not uniformly distributed throughout the plasma membrane, but is differentially and/or asymmetrically localized (Bouchè-Pillon et al., 1994; Jahn et al., 1998; Jahn and Palmgren, 2002; Certal et al., 2008).

The H^+ pump is involved in another aspect of growth. It acidifies the wall, thus activating the wall-loosening enzymes, which are necessary so that the wall yields to the pressure due to turgor (Hager et al., 1971; Cleland and Rayle, 1978; Rayle and Cleland, 1977; Cleland, 2002). The H^+-ATPase is regulated by both development and environment (Michelet

et al., 1994)—nature and nurture. The proton ATPase is regulated by auxin (Gabathaier and Cleland, 1985; Frias et al., 1996), light (Spanswick, 1974a), salt stress (Perez-Prats et al., 1994), and internal pH (Vesper and Evans, 1979), indicating that it may participate in all aspects of signal transduction (Felle, 1989b). The proton ATPase is also regulated by various toxins and fungal elicitors, including fusicoccin (Rasi-Caldogno et al., 1986; Hagendoorn et al., 1991).

It is now possible to utilize a few techniques to visualize this important protein in living cells. For example, recombinant DNA techniques allow the insertion of a sequence into a gene that encodes for a protein (green fluorescent protein, GFP) that will give off green fluorescent light (Chalfie et al., 1994; Hadjantonakis and Nagy, 2001; Hanson and Kohler, 2001; van Roessel and Brand, 2002; Luby-Phelps et al., 2003). This allows one to visualize the distribution of the proton ATPase in various cell types, the change in distribution of the protein in response to developmental and external stimuli, and its targeting to and removal from the plasma membrane (Certal et al., 2008). Soon, the proton ATPase will be studied with microscopic techniques that allow one to visualize the interactions between different domains of a single-proton ATPase molecule or the interaction between a single-proton ATPase and the proteins that interact with it in a living cell (Gadella et al., 1999; Uhlén, 2006).

2.9.2 The K^+ Channel

K^+ is an essential macronutrient that accounts for 1–10 percent of the dry mass of a plant and, as the major ionic contributor to cell turgor, plays a role in cell growth and other turgor-dependent cell movements (Epstein, 1972; Epstein and Bloom, 2005; Moran, 2007; Britto and Kronzucker, 2008). Although water-selective channels known as aquaporins exist, by virtue of their aqueous pore, K^+ channels also serve as water channels (Wayne and Tazawa, 1990; Tazawa et al., 2001). The K^+ channels of the plasma membrane of plants, particularly those found in guard cells, are becoming well understood as a consequence of the introduction of the patch-clamp technique (Schroeder, 1988, 1989; Schroeder et al., 1987, 1994; Cao et al., 1995; Schachtman et al., 1992).

Patch clamping is an electrophysiological technique. However, unlike classical electrophysiological methods, where a microcapillary electrode is inserted into the cell, with patch clamping, a microcapillary electrode is pressed against a clean membrane surface, and suction is applied to make a tight seal (Hedrich, 1995). The high-resistance seal that is formed between the pipette and the membrane allows the recording of tiny currents, including those that pass through single channels.

There are several configurations used in the patch-clamp technique (Hamill et al., 1981), one of which is the whole-cell configuration, in which the current through the whole membrane is studied.

Using the whole-cell configuration, Julian Schroeder and his colleagues (Schroeder et al., 1987; Schroeder, 1988) discovered that the activity of K^+ channels is controlled by membrane potential (Figure 2.28). To perform these experiments, the electrical potential across the membrane is varied and the steady-state current that flows at each potential is measured. The steady-state current is then plotted with respect to the membrane potential used to elicit those currents. Such a plot is referred to as an *I*-ψ curve or, more commonly, an *I*-V curve where *I* represents current (a variable) and V stands for voltage (a unit of measurement). We can determine if K^+ is the ion that flows through the channel by calculating the equilibrium potential for K^+ using the Nernst equation and the concentrations of K^+ on

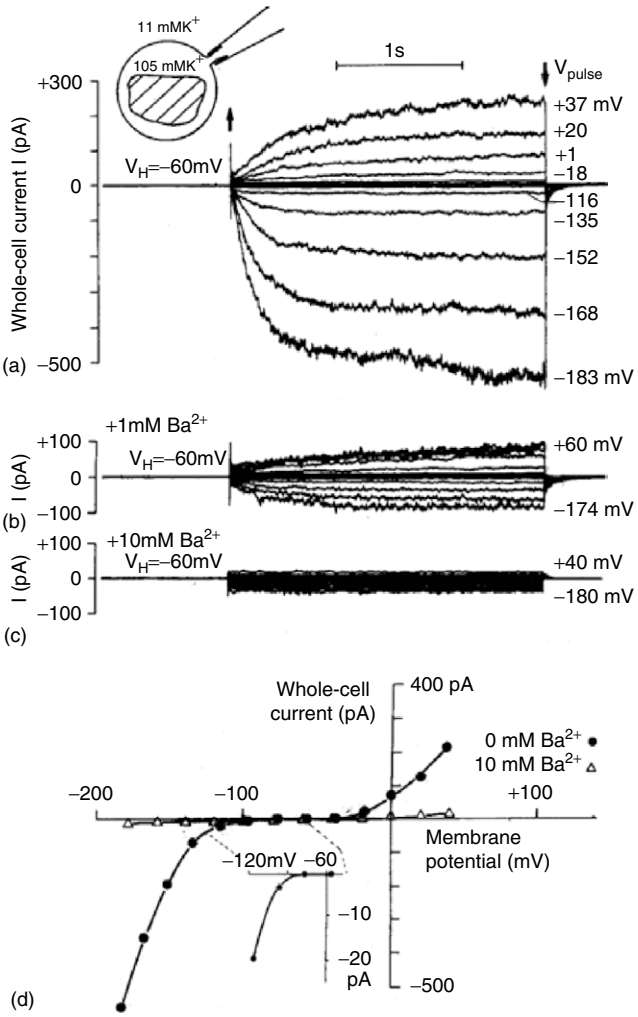


FIGURE 2.28 Recordings of K^+ channel currents in guard-cell protoplasts of *Vicia faba* using the whole-cell configuration of recording. (a) Current versus time curves at the indicated pulsed voltages (right) (b and c) Current versus time curves at pulsed voltages when the cells are treated with Ba^{2+}. (d) Current voltage or *I*-ψ curve that represents the data shown in (a) and (c). (*Source:* From Schroeder et al., 1987.)

both sides of the membrane. Since the external and internal concentrations of K^+ are 11 and 105 mol/m^3, respectively, the equilibrium potential is approximately -0.058 V. If K^+ is the ion moving through the channels, there should be no current at the applied potential that is equal to the equilibrium potential for K^+. We see in the figure that the curve in Figure 2.28d intercepts the *x*-axis at approximately the voltage equal to the equilibrium potential of K^+ and there is no current flow. Moreover, the direction and magnitude of the currents passing through the channels depend on the deviation of the electrical potential from the equilibrium potential for K^+, and are consistent with the movement of positive charge. The currents can be interpreted as an uptake of K^+ at potentials more negative than the equilibrium potential and a release at more positive potentials. If the electrical potential only influenced the K^+ currents by changing the displacement of the membrane potential from the equilibrium potential for K^+, then the I-ψ curve would be linear with an *x*-intercept at the K^+ equilibrium potential. However, since the curve is nonlinear, the electrical potential must be activating voltage-gated channels, which are virtually closed when the membrane potential lies between -0.05 and $-0.08\,\psi$. The channels are activated at both hyperpolarized and depolarized potentials and the conductances of the membrane at hyperpolarized or depolarized potentials can be calculated from the slope of the curve.

We can determine the selectivity of the channels for K^+ by doing the following experiment (Figure 2.29). First, the channel in question is activated by applying either a hyperpolarizing or a depolarizing pulse. Then the voltage is rapidly changed to various values to see where there is neither inward nor outward "tail" current flow. The potential that causes neither an inward nor an outward current represents the reversal potential (ψ_{rev}). If there are only two permeant ions used at a time and one is on one side of the membrane and one is on the other, the relative permeabilities can be calculated from the reversal potential obtained using an exponentiated form of a simplified version of the Goldman equation, where everything cancels except the following terms:

$$\psi_{rev} = (kT/e)\ln(P_{Na}/P_K) \text{ or } P_{Na}/P_K = e^{(e\psi^{rev}/kT)} \qquad (2.31)$$

These experiments show that the permeability sequence for the inward-rectifying channel is $K^+ > Rb^+ > Na^+ >$

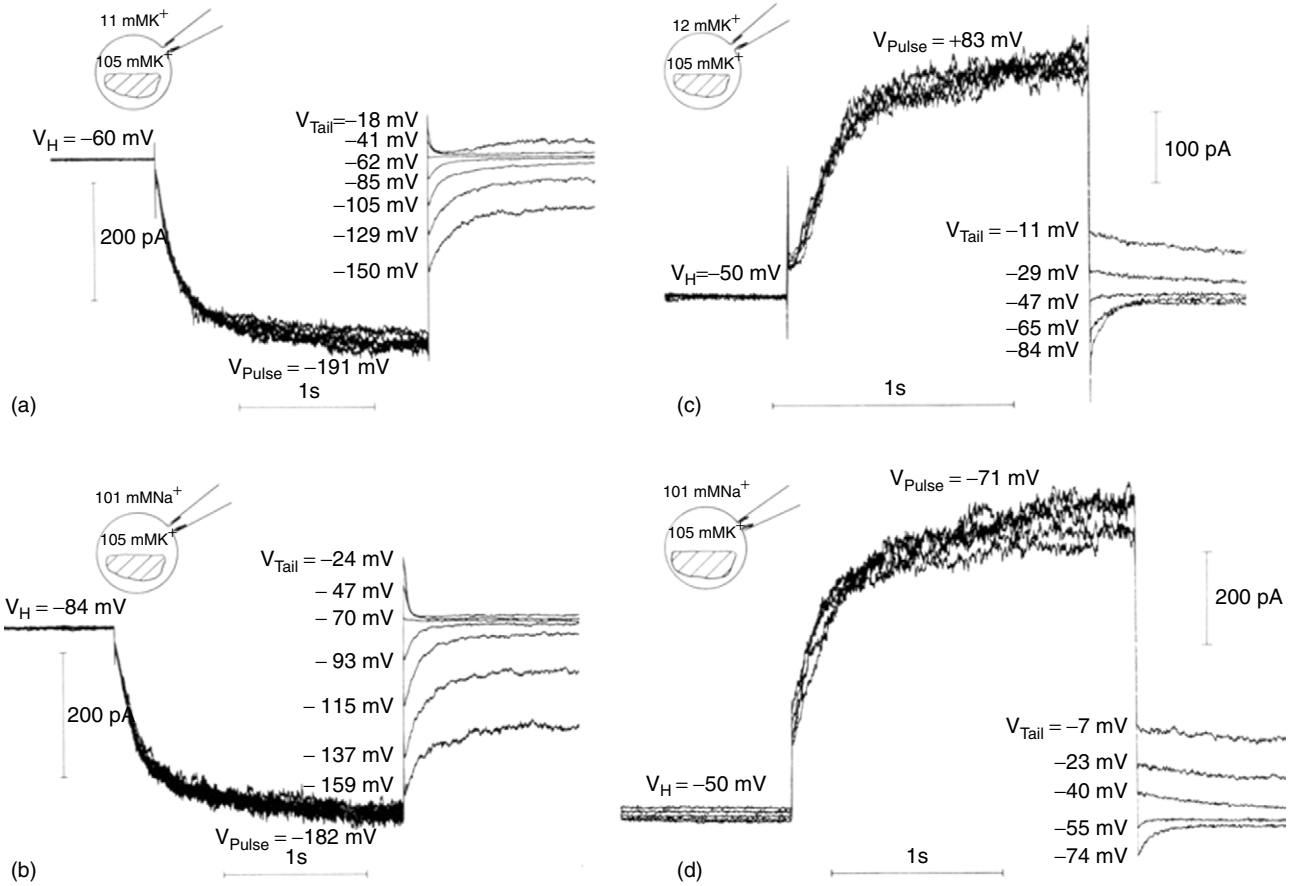


FIGURE 2.29 Recordings of K^+ currents in guard-cell protoplasts of *Vicia faba* using the whole-cell configuration of recording in order to determine the specificity of the inward (a and b) and outward (c and d) currents for K^+. Notice that the concentrations of Na^+ and K^+ have been varied in each experiment. (*Source:* From Schroeder et al., 1987.)

$Li^+ >> Cs^+$. P_{Na}/P_K for the inward-rectifying channel is 0.06. It is 0.132 for the outward-rectifying channel.

Ba^{2+} blocks the inward and outward current (Figure 2.28) while Al^{3+} only blocks the inward current but not the outward current. This provides evidence that there are two distinct classes of channels, one that allows K^+ to move into the cell (e.g., inward rectifying) and one that facilitates the movement of K^+ out of the cell (e.g., outward rectifying).

Currents that pass through a single channel can be visualized with the patch-clamp technique even though they may be less than 1 pA in magnitude (Figure 2.30). In order to accomplish this, a single patch of membrane approximately 1 μm in diameter must be removed from the cell. This is done by pulling the tightly attached patch-clamp pipette away from the cell. The single-channel currents consist of rectangular pulses of random duration. The upward and downward spikes represent small conformational changes in the channel-gating polypeptide. Each upward step represents the closing and each downward step represents the opening of a single inward-rectifying cation (e.g., K^+) channel. By convention, inward current, which is defined as the movement of positive charge from an E-space to a P-space, is presented as a downward deflection from zero, and outward current is presented as an upward deflection. The height of the opening is a measure of the current that passes through the channel. As long as the channel is open, ions pass through it driven by their electrochemical difference. The current amplitude indicates how many ions pass through the channel in a given time, since the number of ions/s passing through a channel times *ze* equals the current.

$$\begin{aligned}\text{Single-channel flow} &= \text{ions/s}\\ &= (\text{single-channel current})/ze\\ &= 2 \times 10^{-12}\ \text{A}/1.6 \times 10^{-19}\ \text{C/K}^+\\ &= 1.25 \times 10^7\ \text{K}^+/\text{s}\end{aligned}$$

We can estimate the conductance of the K^+ channels from the current (I) and electrical potential (ψ), or I-ψ curves, for the membrane patch since the slope of the curve is equal to the conductance ($-I/\psi$). At hyperpolarizing potentials, the single-channel conductance is 10 pS, and at depolarizing potentials, the single-channel conductance is 25 pS, where 1 S = 1 A/V. The difference in conductance supports the contention that there are two distinct types of channels on the plasma membrane of guard cells, one inwardly rectifying and the other outwardly rectifying. Notice that the observed conductance depends on the concentration of K^+ on each side of the membrane.

How many inward-rectifying K^+ channels are there on the plasma membrane? If the whole-cell current is approximately 300 pA and each channel passes 2 pA, then there are 150 channels/cell. If the area of the cell is $6 \times 10^{-10}\text{m}^2$, then there are 2.5×10^{11} channels/m^2 or 0.25 channels/μm^2. That is, about one channel can be found for every four patches made. The inward-rectifying K^+ can be observed in the plasma membrane using GFP fusion proteins (Hurst et al., 2003).

The genes for K^+ channels have been cloned by transforming yeast that is unable to grow on low K^+ with cDNA from *Arabidopsis* (Anderson et al., 1992; Sentenac et al., 1992). If the transformed yeast can grow on low K^+ with a given DNA, then that DNA used to transform it is likely to be some kind of K^+ transporter. Subsequently, many families of genes that encode K^+ channels have been discovered using other cloning strategies (Maser et al., 2001; Véry and Sentenac, 2003; Hosy et al., 2003; Gierth and Mäser, 2007; Grapov, 2007; Ward et al., 2009). There are many different kinds of K^+ channels that are expressed throughout the plant, consistent with the idea that DNA is promiscuous, and thus regions that code for properties such as K^+ selectivity, K^+ affinity, certain gating characteristics, channel

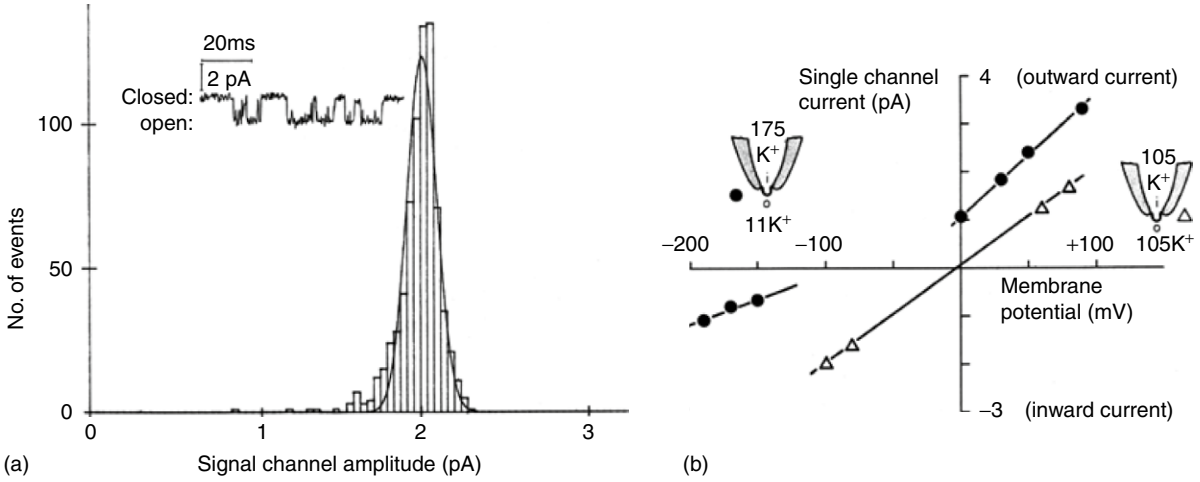


FIGURE 2.30 Recordings of K^+ currents through single channels in guard-cell protoplasts of *Vicia faba* using the patch-clamp configuration of recording. (a) Shows the current recording of an individual channel and a histogram showing the distribution of amplitudes through a channel. (b) Shows current-voltage curves for channels in outside-out patches. (*Source:* From Schroeder et al., 1987.)

regulation, and kinetics, as well as placement in the membrane with respect to space and time, can be mixed and matched through evolutionary time in a number of ways to result in a great variety of adaptive channels. The various polypeptides that confer the ability to transport K^+ come together as subunits to form dimers or heterotetrameric K^+ channels with diverse properties that depend on the relative composition of the subunits (Hoth et al., 2001; Véry and Sentenac, 2003; Xixluna et al., 2007). Molecular genetics has taught us that a single transporter is often made up of the polypeptides encoded by several members of a gene family (Hedrich and Marten, 2006).

Using recombinant DNA technology, Julian Schroeder and his coworkers (Cao et al., 1995; Rubio et al., 1995; Uozumi et al., 1995; Mäser et al., 2002) have identified the amino acids or peptide regions of various K^+ channels that confer ion selectivity and the ability to act as a rectifier, and this work has been extended by others (Marten and Hoshi, 1998; Hoth et al., 2001). The properties of the K^+ channels can be regulated through the action of many regulatory proteins, including protein kinases, protein phosphatases, and 14-3-3 proteins (Véry and Sentenac, 2003).

2.10 PLASMA MEMBRANE–LOCALIZED PHYSIOLOGICAL RESPONSES

2.10.1 Guard Cells

In the 19th century, plant physiologists coated leaves in which the stomates are restricted to a given surface with Vaseline and found that CO_2 uptake and water loss occur through stomates (Darwin and Acton, 1894). The stomates are composed of guard cells, which surround a pore in the epidermis known as the stoma. When the guard cells swell, the pore opens and CO_2 can readily diffuse through the epidermis to be used for photosynthesis; however, water from transpiration is lost at the same time. If too much water escapes, the plant may wilt, making it essential that the plant be able to regulate the size of its stoma. The stoma closes when the guard cells shrink, and this closure not only prevents the loss of water, but also prevents the influx of CO_2 necessary for photosynthesis. The swelling and shrinkage of the guard cells are consequences of their water uptake or loss, respectively. The guard cells act as osmometers; water moves in and out of them passively depending to a large extent on the difference in the osmotic pressure on both sides of the plasma membrane and to a smaller extent on the elasticity of the guard cell wall (Roelfsema and Hedrich, 2002). In the main, the osmotic pressure in the guard cells is due to K^+ and Cl^-. Thus, the channels involved in K^+ transport across the plasma membrane provide the molecular mechanism for regulating many aspects of whole-plant physiology, including photosynthesis, transpiration, thermoregulation, and the ascent of sap due to transpiration (Dixon and Joly, 1895; Larmor, 1905; Ewart, 1906; Dixon, 1938; Nobel, 1983, 1991, 2005). The properties of the K^+ channels that I discussed above can account for the known properties of guard cells that were obtained from physiological studies. That is, the properties of these channels can account for guard cell swelling, which requires an increase in the $[K^+]$ of about 400 mol/m^3.

Assume that at rest, the membrane potential of guard cells is equal to the Nernst potential of K^+ (-0.058 V), so that there is no net movement of K^+ into or out of the cell (Saftner and Raschke, 1981). The opening and closing of the stomatal pore are regulated by a myriad of environmental signals, which are perceived and integrated by the guard cells themselves (Schroeder et al., 2001). Blue light, which signals the beginning of the day, is absorbed by phototropins, and causes guard cells to swell as a result of the blue light–induced activation of the H^+-ATPase in the plasma membrane. The activation of the H^+-ATPase results in the hyperpolarization of the membrane potential to approximately -0.16 V (Shimazaki et al., 1992; Kinoshita and Shimazaki, 1999, 2001, 2002; Kinoshita et al., 2001, 2003; Inoue et al., 2008). The activation of the H^+-ATPase occurs through the phosphorylation of its C-terminus followed by the binding of the 14-3-3 protein to the phosphorylated H^+-ATPase. The blue light–induced, H^+-ATPase—mediated hyperpolarization is facilitated by a blue light–induced inhibition of an anion channel on the plasma membrane, which, if active, would have short-circuited the proton-mediated hyperpolarization (Marten et al., 2007).

The hyperpolarization of the plasma membrane by the H^+-ATPase activates the voltage-dependent, inward-rectifying K^+ channels and causes a whole cell current of approximately 300 pA. Given that 300 pA is equivalent to 300×10^{-12} C/s and that there are 1.6×10^{-19} C/K^+, the flow of K^+ into the cell would be about 1.9×10^9 K^+/s. Given that there are approximately 150 inward-rectifying channels per cell, then approximately 1.25×10^7 K^+ must pass through each channel every second. If the volume of a guard cell is approximately 10^{-14} m^3 and the $[K^+]$ in the guard cell must increase by 400 mol/m^3, then the channels must pass 400×10^{-14} mol of K^+, which, using Avogadro's number as a conversion factor, is equivalent to 2.4×10^{12} K^+. Thus, if all the channels were activated it would take $(2.4 \times 10^{12}\ K^+)/(1.9 \times 10^9\ K^+/s)$ or approximately 1260 seconds (21 minutes) for the guard cell to swell. The opening of some stoma, like those from corn, takes only a few minutes, while those from the broad bean take hours (Blatt, 1991; Tallman, 1992; Assmann, 1993). Variation in the kinetics of opening depend not only in variations in the properties and abundance of potassium channels and guard cell size, but also in part on the fact that opening can result from the accumulation of solutes besides potassium, including chloride, malate, and sucrose (Schroeder et al., 2001). There are many environmental factors, hormones, nucleotides, enzymes, and ions that regulate stomatal opening

and closure in the treasure house of plant species (Kim et al., 1995; Eun and Lee, 1997, 2000; Li et al., 1998, 2000; Li and Assmann, 2000; Mori et al., 2000; Eun et al., 2001; Hwang and Lee, 2001; Schroeder et al., 2001; Jung et al., 2002; Taiz and Zeiger, 2006).

2.10.2 Motor Organs

Many legumes, including *Mimosa*, the sensitive plant, *Neptunia*, *Albizzia*, and *Samanea*, show leaflet movements. The leaflet movements result from changes in turgor. The changes in turgor result from water movement that is controlled by ion movements across the plasma membrane of specialized cells in organs known as pulvini (Moran et al., 1988; Satter et al., 1988; Suh et al., 2000; Moshelion and Moran, 2000; Yu et al., 2001; Moshelion et al., 2002a,b; Okazaki, 2002).

2.10.3 Action Potentials

The necessity of cells to osmoregulate rapidly resulted in the evolution of ion channels. Once these channels evolved, they could be used to communicate electrical signals within a cell or from cell to cell in the form of action potentials (Di Palma et al., 1961; Sibaoka, 1962, 1966; Cole, 1968, 1979; Kishimoto, 1968; Huxley, 1992, 1994; Wayne, 1994; Shimmen, 2001, 2003, 2008; Johnson et al., 2002; Baudenbacher et al., 2005; Iwabuchi et al., 2005, 2008; Kaneko et al., 2005). An action potential is a transient depolarization of the plasma membrane that is propagated along the length of the cell. In characean cells, a mechanical or electrical stimulus transiently activates a mechanosensitive calcium channel. The resulting influx of Ca^{2+} causes an increase in cytosolic Ca^{2+} that activates Cl^- channels on the plasma membrane. The efflux of Cl^- along its electrochemical gradient through the channels depolarizes the adjacent membrane, which opens more Ca^{2+} channels, and the cycle repeats as the depolarization propagates along the cell. In the case of characean cells, the action potential results in an electrically or mechanically induced cessation of cytoplasmic streaming (see Chapter 12).

2.10.4 Cell Polarization

Many cells exhibit a polarized distribution of ionic currents, which most likely result from the unequal distribution of pumps and channels in the plasma membrane. These currents are involved in many aspects of cell polarization that occur during development (Jaffe, 1979, 1981; Harold, 1990; Feijó et al., 1995, 2001; Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997; Franklin-Tong, 1999; Messerli et al., 1999; Hepler et al., 2001; Griessner and Obermeyer, 2003). Indeed, cells can also generate electric fields that may participate in localizing the proteins of the plasma membrane in a polar manner by electrophoresis in the plane of the membrane (Jaffe, 1977; Poo and Robinson, 1977; Poo, 1981; see also Chapter 19).

2.11 STRUCTURAL SPECIALIZATIONS OF THE PLASMA MEMBRANE

Invaginations of the plasma membrane, analogous to brush borders in intestines, increase the surface area in a variety of plant cells. *Dunaliella*, an alga that lives in the Dead Sea, increases the surface area of its plasma membrane through a rapid and continuous process of endocytosis and exocytosis (Ginzburg et al., 1999). Other organisms increase the area of the plasma membrane by forming apparently less dynamic invaginations known as lomasomes, charasomes, or plasmalemmasomes (Moore and McAlean, 1961; Chau et al., 1994). In some cells, which occur at bottlenecks in solute transport pathways, invaginations of the extracellular matrix occur that increase the surface area of the plasma membrane. Such cells are known as *transfer cells* (Pate and Gunning, 1972; Gunning and Pate, 1974; Offler et al., 2003; Royo et al., 2007). Invaginations of the plasma membrane are observed in gland cells of carnivorous plants that secrete digestive enzymes (Robins and Juniper, 1983; Scala et al., 1968; Schwab et al., 1969), in the cells of flowering plants bordering mycorrhizal fungi (Allaway et al., 1985; Ashford and Allaway, 1985), in cells at the interface of two generations (Offler et al., 2003), and in the salt glands of *Limonium* (Faraday and Thomson, 1986a,b,c; see Figure 2.31). In fact, when limnologist Robert Lauterborn declared that the self-purification of fresh water necessary to prevent eutrophication is directly proportional to the surface area of the flora, he essentially realized the relationship between the area of the plasma membrane and its ability to take up nutrients.

The facilitated transport of solutes across the plasma membrane does not depend solely on transport proteins, including pumps, channels, and carriers, but can also occur as a result of exocytosis and endocytosis, which are discussed in Chapter 8. Ed Etxeberria and his colleagues (Etxeberria et al., 2005a,b,c, 2007; Baroja-Fernandez et al., 2006; Pozueta-Romero et al., 2008) have demonstrated that the uptake of apoplastic sugars into plant cells depend on two parallel pathways—one consisting of a carrier protein and the other consisting of endocytotic vesicles. Transport due to the carrier protein has rectangular hyperbolic uptake kinetics and is inhibited by phloridzin (Bush, 1993), while transport due to the endocytotic mechanism is linear and inhibited by wortmannin-A (Emans et al., 2002) and latrunculin-B (Baluska et al., 2004).

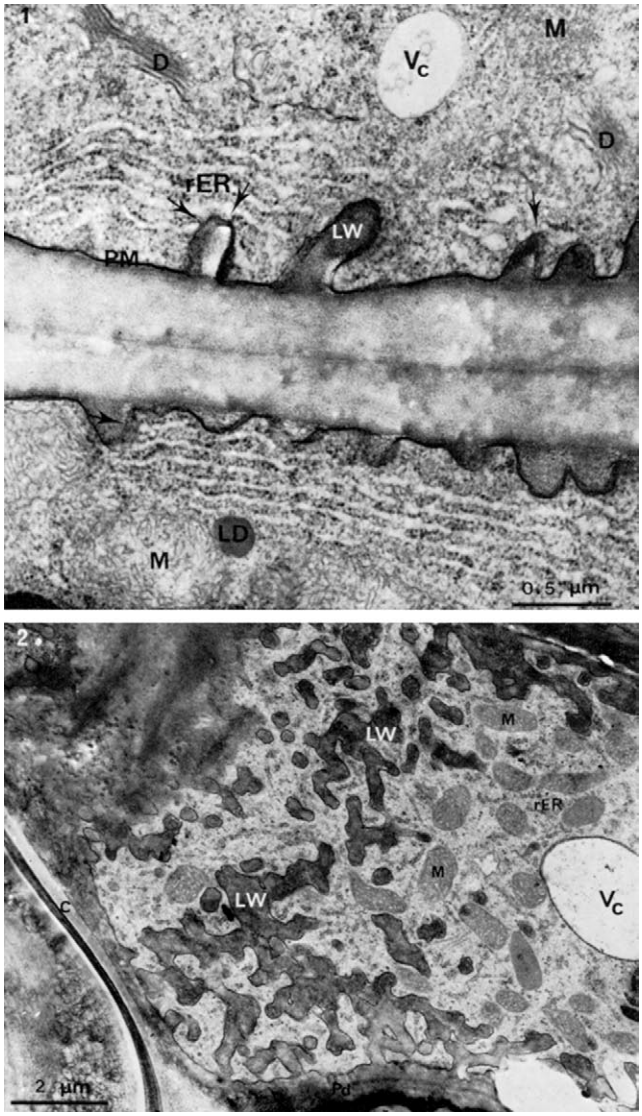


FIGURE 2.31 The surface area of the plasma membrane of the glandular cells of *Dionea muscipula* is increased due to the labyrinthine invaginations in the extracellular matrix (LW). (1) Cross-section, (2) tangential section. V_c, vacuole; M, mitochondrion; PM, plasma membrane; rER, rough endoplasmic reticulum; D, Golgi stack; c, cuticle. (*Source:* From Robins and Juniper, 1980.)

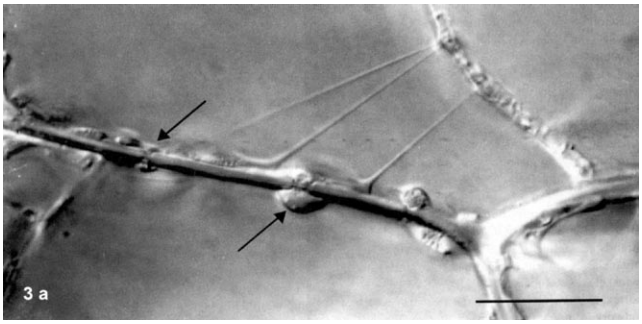


FIGURE 2.32 Hechtian strands in onion epidermal cells that demonstrate the connections between the protoplast and the extracellular matrix. Bar, 20 µm. (*Source:* From Lang-Pauluzzi and Gunning, 2000.)

2.12 THE CYTOSKELETON–PLASMA MEMBRANE–EXTRACELLULAR MATRIX CONTINUUM

While investigating plasmolysis in a number of plants, Bower (1883) noticed that the protoplasm does not detach uniformly from the cell wall as was often shown in studies of plasmolysis, but does so in a nonuniform manner, as if the protoplasm adhered to the cell wall in a number of places. The thin strands of protoplasm that adhere to the cell wall are now know as Hechtian strands, named after Hecht (1912), who observed them while studying plasmolysis in onion cells (Figure 2.32; Küster, 1929; Oparka, 1994; Lang-Pauluzzi, 2000; Lang-Pauluzzi and Gunning, 2000). In both plant and animal cells, the plasma membrane does not exist in isolation, but is intimately attached to the extracellular matrix on the outside and the cytoskeleton on the inside. The plasma membrane proteins that connect the extracellular matrix proteins to the cytoskeleton are usually called *integrins* (McDonald, 1988; Ruoslahti, 1988; Burridge et al., 1988; Pennell et al., 1989; Schindler et al., 1989; Roberts, 1990; Humphries, 1990; Kaminsky and Heath, 1995; Canut et al., 1998; Laval et al., 1999; Nagpal and Quatrano, 1999; Swatzell et al., 1999; Sun et al., 2000; Sonobe et al., 2001; Sakurai et al., 2004). The cytoskeleton and extracellular matrix are discussed in Chapters 10, 11, and 20.

The attachment of the plasma membrane to the extracellular matrix can be seen easily by plasmolyzing the cells (see Figure 2.32; Cholodny and Sankewitsch, 1933; Lang-Pauluzzi and Gunning, 2000). Interestingly, the attachments are not uniform, but may show a distinct polarity within the cell (Strugger, 1935; Stebbins and Jain, 1960). The plasma membrane is attached structurally and functionally to an underlying skeleton known as the membrane skeleton (Bennett and Gilligan, 1993). The membrane skeleton is composed of proteins, including spectrin, ankryn, etc., and ankryn may bind directly to some of the transport proteins. The membrane skeleton may also attach directly to the cytoskeleton. The integrin-like proteins that connect the extracellular matrix with the cytoskeleton appear to be involved in the ability of cells to sense gravity (Wayne et al., 1990, 1992; Hemmersbach and Braun, 2006) and touch-induced responses (Haberlandt, 1914; Junker, 1977; Bünning, 1989; Jaffe et al., 2002). The cytoskeleton–plasma membrane–extracellular matrix continuum also appears to be the way pathogenic fungi sense the epidermal cell pattern on leaves that facilitates the directional growth of the mycelium and the formation of an appressorium (Johnson, 1934; Hoch et al., 1987; Correa et al., 1996).

2.13 SUMMARY

The plasma membrane is at the frontier of the plant cell, and not only separates the living protoplasm from the external medium, but also coordinates the relationships between the protoplasm and the external world. In general, the lipids in the plasma membrane provide a barrier to mixing while the membrane proteins facilitate the transport of polar substances across the membrane. In this chapter, I have discussed how to quantify phenomena that are not directly measurable by postulating relationships between the desired quantities and measurable quantities. Remember that these relationships are postulates and must be changed to accommodate newly discovered relationships and interactions. I have also discussed the techniques used to characterize ion fluxes and visualize the movement of ions through a single channel. Imagine how delighted Wilhelm Pfeffer would be to know that we now have the theoretical and technical tools to understand leaflet movement in *Mimosa*, the phenomenon that started Pfeffer in his investigations of the plasma membrane.

2.14 QUESTIONS

2.1. What is the evidence that the plasma membrane provides a barrier between the living and nonliving world of a cell?

2.2. What are the mechanisms by which the plasma membrane and its components regulate transport between the inside and outside of the cell?

2.3. What are the limitations of thinking about the plasma membrane as a barrier or as the sole barrier?

Chapter 10

Actin and Microfilament-Mediated Processes

You got to move
You got to move
You got to move, child
You got to move
But when the Lord
Gets ready
You got to move.

—Mississippi Fred McDowell

10.1 DISCOVERY OF ACTOMYOSIN AND THE MECHANISM OF MUSCLE MOVEMENT

Movement is one of the most easily distinguished characteristics of life. Theodor Engelmann (1879) noticed all kinds of motion in plants and protozoa, including amoeboid movement and cytoplasmic streaming (Figure 10.1). He suggested that these activities might be a primitive version of the specialized movements that occur in muscle, and indeed, the same molecular mechanisms may be involved in them all. Seventy years later, Albert Szent-Györgyi (1949b) put it this way:

> *All living organisms are but leaves on the same tree of life. The various functions of plants and animals and their specialized organs are manifestations of the same living matter. This adapts itself to different jobs and circumstances, but operates on the same basic principles. Muscle contraction is only one of these adaptations.*

If all life shows motion, which cell, tissue, organ, or organism shall we choose to study in order to unravel the mysteries that underlie the vital process of movement in living organisms, and to give us the clearest and most profound answers? Szent-Györgyi (1948) suggests that we use the cells that are most specialized for movement: skeletal muscle. The excitement of some of the pioneers in muscle research has been captured in their published lectures and monographs (Szent-Györgyi, 1947, 1948, 1953; Mommaerts, 1950b; Weber, 1958; Huxley, 1966, 1969, 1996; Huxley, 1980; Straub, 1981; Engelhardt, 1982).

While most biochemists in the 1930s were studying water-soluble enzymes, the husband-and-wife team of Vladimir Engelhardt and Militza Ljubimowa violated one of the canons of biochemistry, and studied the "residue instead of the extract" (Engelhardt, 1982). In those days, following the acceptance of Sumner's (1926) work, the residue was thought to be composed of mundane structural proteins and not exciting enzymes. However, while studying muscle, Engelhardt and Ljubimowa (1939) found that myosin, a "structural" protein that had previously been isolated from muscle by Wilhelm Kühne (1864), was also an enzyme capable of hydrolyzing adenosine triphosphate (ATP).

Szent-Györgyi became interested in muscle after he read about the ATPase activity of myosin. He thought that myosin might be the mechanochemical transducer that coupled the chemical energy of ATP to the mechanical energy of contraction, and he set out to test his hypothesis. Realizing that he was standing on the shoulders of giants, Szent-Györgyi repeated the work of the "old masters" and isolated myosin using the method of Engelhardt and Ljubimowa (Szent-Györgyi and Banga, 1941). He extracted the muscle for an hour with an alkaline 0.6M KCl solution to get the typical syrupy myosin preparation. He

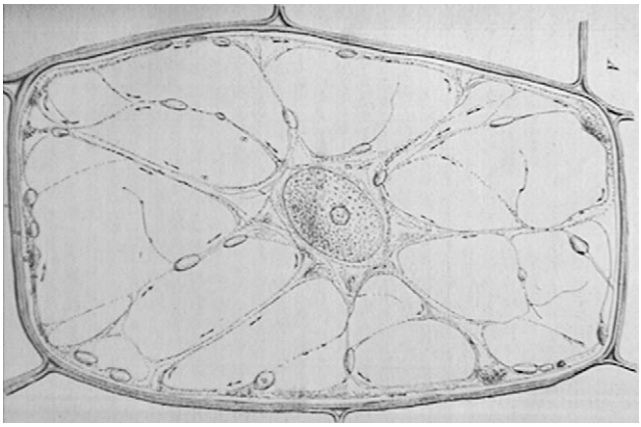


FIGURE 10.1 Cytoplasmic streaming in a parenchyma cell. (*Source:* From Hanstein, 1880.)

then prepared threads of myosin and put them on a slide and watched them under a microscope. Then he added ATP to the slide and, mirabile dictu, they contracted! It was as if he had seen life itself!

Ilona Banga continued to isolate myosin in Szent-Györgyi's laboratory, but had to go home early one day and left the minced muscle in KCl all night. The next morning they realized that the extract was thicker than the usual extract and it also contracted more vigorously upon the addition of ATP. They called the original extract myosin A and the thick extract myosin B. It turned out that the difference between the two extracts was that myosin A was extracted while the muscle still contained ATP, and myosin B was isolated after all the ATP had been hydrolyzed.

Szent-Györgyi suggested that Ferenc Brunó Straub investigate the difference between the weakly contracting myosin A and the forceful myosin B (Straub, 1981). Straub postulated that myosin B was enriched in a protein that was a contaminant in myosin A. Unbeknownst to Szent-Györgyi and Straub, the protein contaminant had been isolated by Halliburton in 1887 under the name *myosin-ferment* (see Finck, 1968). Straub extracted an ATP-containing muscle with 0.6M KCl, and then washed and dried the remaining muscle with acetone. The acetone powder was then extracted with water and a protein went into solution. This protein solution, when added to myosin A in the presence of ATP, caused the myosin to contract. Straub named this protein *actin*, because it caused myosin to go into action (Moss, 1988), and then he and Szent-Györgyi renamed myosin B *actomyosin*. Actin had the ability to activate the ATPase activity of myosin by about ten-fold, in addition to being able to cause the actomyosin mixture to contract.

Szent-Györgyi resurrected an earlier proposal by Karl Lohmann (Meyerhof, 1944), the discoverer of ATP, that the chemical energy of ATP provided the energy for muscle contraction, and moreover, that muscle contraction was essentially due to the interaction of actomyosin and ATP. However, this conclusion was not widely accepted for a number of reasons, one of which was that the magnitude of the free energy released by the measured amount of ATP hydrolyzed was insufficient to account for the work performed by the contracting muscle (Mommaerts and Seraidarian, 1947; Perry et al., 1948; Hill, 1949; Mommaerts, 1950a; Szent-Györgyi, 1963; Gergely, 1964).

Szent-Györgyi (1949a) decided to demonstrate beyond a shadow of a doubt that ATP provides the chemical energy for contraction. He and Varga (1950) developed a glycerinated muscle preparation. They extracted the muscle with 50 percent glycerol at low temperatures to make a permeabilized cell model (Arronet, 1973). Then, upon addition of ATP, the model contracted and developed the same tension as if it were an intact muscle. Contraction is thus due to the conversion of the chemical energy of ATP to the mechanical energy of muscle contraction. The inability to detect the relationship between free energy release from ATP and work was due to the fact that the magnitude of ATP hydrolyzed by a contracting muscle was underestimated, since, in muscle, ATP is constantly being regenerated through a creatine phosphate system.

From the first observation of the contraction of actomyosin threads under the microscope, Szent-Györgyi (1948) believed that the proteins themselves contracted. However, structural data, which included X-ray diffraction images, as well as polarization, interference, and electron microscopic images obtained by Jean Hanson, Hugh Huxley, and Andrew Huxley (unrelated), indicated that the contractile proteins were not contractile at all, but slide past each other when they effected the shortening of a muscle (Hanson and Huxley, 1953, 1955; Huxley and Hanson, 1954, 1957; Huxley and Niedergerke, 1954).

Hugh Huxley was one of the nuclear physicists who left physics and entered biology in the late 1940s after the mass killing of Japanese by the atomic bomb. After all, the bomb was, and still is, the most visible by-product of nuclear physics. Will a similar migration occur from biology into fields concerned with human understanding if we allow genetically engineered diseases to be released accidentally or in an act of war? At present there is a paucity of discussion on the ethical concerns of basic biological research (Bush, 1967; Chargaff, 1976), even though scientists should continually examine and reexamine the fruits of their labors and take responsibility for them (Williams, 1993a). Anyhow, Hugh Huxley decided to enter biology and figure out how muscles worked by combining the power of X-ray diffraction, a technique he believed provided true data in an enigmatic form, with the power of electron microscopy, a technique that provided tangible images even though, at that time, the images were laden with artifacts. Huxley decided to take a multidisciplinary approach, where he himself became well versed in many aspects of science. He already knew X-ray diffraction, and he went to the Massachusetts Institute of Technology (MIT) to learn electron microscopy from Frank Schmitt. With his multidisciplinary approach, where he himself understood and combined many techniques, as opposed to an interdisciplinary approach, where each member of a team is an expert in a given technique, Hugh Huxley contributed to the understanding we now have concerning the mechanism of muscle contraction.

Although structural studies on muscle began in the 19th century when histologists observed that muscle cells contained repeating units called *sarcomeres*, they were forgotten or unknown to the muscle biochemists (Huxley, 1980). The observations by Theodor Engelmann that the sarcomeres, which were separated by the "in between bands" or Z-bands (zwichen-bands), were birefringent under a polarizing microscope were repeated by the structural biologists in the 1950s (Figure 10.2). The birefringent area was given the English name *A-band* (meaning anisotropic) and the two nonbirefringent areas between the Z-bands and the

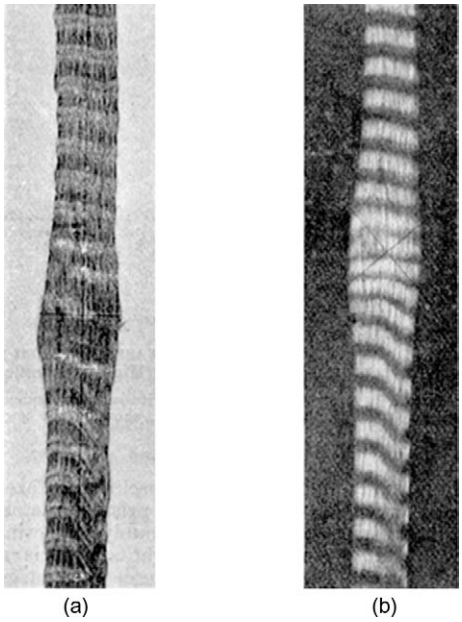


FIGURE 10.2 Photomicrographs taken by Professor Engelmann of a leg-muscle fiber from *Chrysomela coerulea* observed with a polarized light microscope with (a) parallel and (b) crossed polars. (*Source:* From Schäfer, 1902.)

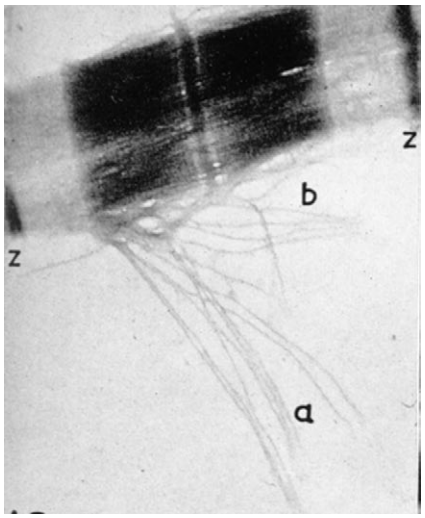


FIGURE 10.3 A myofibril from a toad muscle showing one sacromere. This sacromere has frayed, showing (a) the filamentous nature of its components. The z-band, z; the A-band (b). ×28,000. (*Source:* From Hodge, 1956.)

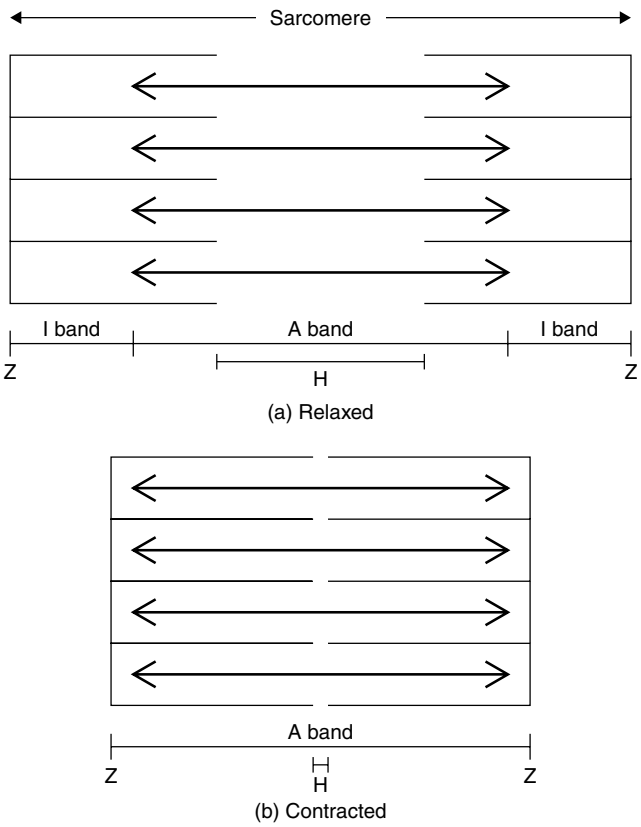


FIGURE 10.4 Diagram of the relative movement of actin and myosin as described by the sliding filament hypothesis: (a) relaxed and (b) contracted.

A-band were given the name *I-bands* (meaning isotropic). X-ray diffraction data confirmed that the A-band had a repeating structure and provided data on the size and distribution of the repeating units.

Electron microscopy showed that the A-band was composed of thick filaments approximately 16 nm in diameter and 1.6 μ in length, and the I-bands were composed of thin filaments that were 5–6 nm in diameter and about 1 μm in length (Figure 10.3). The thick filaments also contained globular regions, some of which made cross-bridges with the thin filaments. Studies utilizing phase and interference microscopy showed that treating the muscle fibers with high salt, which caused the extraction of myosin, simultaneously resulted in the disappearance of the A-bands! Longer extractions, which resulted in the subsequent loss of actin, caused the I-bands to disappear. These results indicated that the thick filaments were made out of myosin, and the thin filaments were composed of actin. These results were later confirmed in situ using immunolocalization techniques.

Phase and interference microscopy showed that the length of the A-bands as well as the distance between the Z-band and the edge of the H-band (an area of variable width in the middle of the A-band where the actin filaments do not reach) stayed constant during contraction, while the I-bands decreased in length (Figure 10.4). These data were interpreted by Jean Hanson and the two Huxleys to mean that the contractile proteins remain constant in length, but contraction occurs when the thin filaments slide past the thick filaments. This idea, however, was not supported by electron microscopic data, which showed that the filaments decreased in size. However, it turned out that the proteins were depolymerizing during fixation, and later, good fixation procedures revealed that the filaments do not change size during contraction.

The filamentous nature of purified actin and myosin can be observed in the electron microscope using negatively stained preparations. Under high-salt conditions, actin forms filaments known as *F-actin*, and under low-salt conditions, the filaments depolymerize into globular subunits known as *G-actin*. An individual myosin molecule is a polar filamentous structure with two globular heads and a long tail. Under physiological conditions, the myosin molecules join together to form a bipolar thick filament where the head groups are at the ends of the filament.

Further support for the sliding filament model came from experiments that showed that the actin filaments have a polarity, and moreover, the actin filaments on each side of the sarcomere are antiparallel. This was discovered as a result of Andrew Szent-Györgyi's (1953) research on the proteolytic cleavage products of purified myosin. He found that the treatment of myosin with trypsin yields a rodlike segment known as *light meromyosin* and a head region known as *heavy meromyosin*. Huxley (1963) treated isolated Z-bands with the heavy meromyosin fragment and observed them in the electron microscope. He noticed that the heavy meromyosin bound to the actin filaments, and decorated them with an arrowhead-like arrangement where the arrowheads pointed away from the Z-bands. This meant that the actin filaments had a polarity. During contraction, the myosin moves along the actin filament from the end farther away from the Z-band (the minus end) toward the end nearer the Z-band (the plus end).

Following the work of the Huxleys and their coworkers, the sliding filament model became universally accepted. Subsequently, biophysicists and biochemists have worked to understand how the chemical energy of ATP is converted into the mechanical energy of actomyosin by studying actomyosin kinetically (Lymm and Taylor, 1971) and structurally (Rayment et al., 1993). In order for the myosin molecule to generate movement along an actin microfilament, it must bind ATP. This causes the myosin to break its tight binding to the actin filament. Subsequently, myosin hydrolyzes the ATP and undergoes a conformational change so that the head is adjacent to the next actin monomer. Then the myosin releases the terminal phosphate of the ATP and binds actin tightly. This tight binding initiates a ratcheting of the myosin molecule that results in the power stroke and the release of adenosine diphosphate (ADP). The myosin head continues to bind tightly to the actin filament until it binds to another molecule of ATP, and the rowing motion continues as the myosin moves from the minus end of an actin filament to the plus end. When a cell dies and no longer produces ATP, the myosin head can no longer dissociate from the actin filament and the cell becomes nonelastic, a state known as *rigor mortis*. Andrew Huxley (1980) considers myosin to be a step-down transformer that converts the very strong chemical forces (involved in the hydrolysis of ATP) that act over a short distance (0.1 nm) into a much weaker mechanical force that acts over a greater distance (5 nm).

10.2 ACTIN IN NONMUSCLE CELLS

Actin is not only found in muscle cells; it also occurs in all eukaryotic cells. Actin is one of the most abundant proteins in the world, second only to RuBP carboxylase (see Chapter 13). Actin has been purified from a number of cells, including pollen, root cells, protozoa, and slime molds, and it can make up as much as 5 percent of the cellular protein (Loewy, 1954; Ts'o et al., 1957; Nakajima, 1960; Hatano and Tazawa, 1968; Adelman and Taylor, 1969; Vahey and Scordilis, 1980; Vahey et al., 1982; Ma and Yen, 1989; Liu and Yen, 1992; Andersland and Parthasarathy, 1992, 1993; Andersland et al., 1992; Igarashi et al., 1999).

Actin filaments, or *microfilaments* as they are called, can be observed in nonmuscle cells at the electron microscopic level (Wohlfarth-Bottermann, 1962; Porter et al., 1965; Rhea, 1966; O'Brien and Thimann, 1966; Nagai and Rebhun, 1966; Parthasarathy and Mühlethaler, 1972; Lancelle et al., 1986, 1987, 1989; Ding et al., 1991a,b). The actin filaments in nonmuscle cells, like their muscle counterparts, have the ability to bind heavy meromyosin (Ishikawa et al., 1969; Nachmias et al., 1970; Condeelis, 1974) and the S-1 subfragment of myosin (Igarashi et al., 1999). The arrowhead decorations indicate that nonmuscle actin filaments are also polar.

10.2.1 Temporal and Spatial Localization of Actin in Plant Cells

Actin filaments in nonmuscle cells form the actin cytoskeleton. However, the actin cytoskeleton in nonmuscle cells is a dynamic structure. The actin filaments polymerize and depolymerize, and consequently appear in various places around the cell in a cell cycle–dependent manner. The three-dimensional architecture of the microfilament-based cytoskeleton can be visualized by fixing plant cells and treating them with fluorescently labeled phalloidin, a fungal toxin from *Amanita* that specifically binds to filamentous actin (see Figure 10.5; Barak et al., 1980; Nothnagel et al., 1981, 1982; Parthasarathy, 1985; Parthasarathy et al., 1985). The introduction of this technique into plant biology resulted in an explosion of papers where the architecture of the actin cytoskeleton has been demonstrated in hundreds of cell types (Lloyd, 1987, 1988, 1989). Peter Hepler and his colleagues then developed the technology to microinject fluorescently labeled phalloidin so that the dynamic aspects of actin filaments can be observed in living cells (Zhang et al., 1992, 1993; Meindl et al., 1994; Wasteneys et al., 1996). Actin can also be visualized in cells that have been transformed with genes coding for fusion proteins composed of actin-binding proteins and green fluorescent protein (GFP; Benedikt et al., 1998).

While the actin cytoskeleton has a unique arrangement in each cell type, there are some basic similarities in all cells. For example, in the interphase cells of higher plants, the actin microfilaments typically appear either transverse

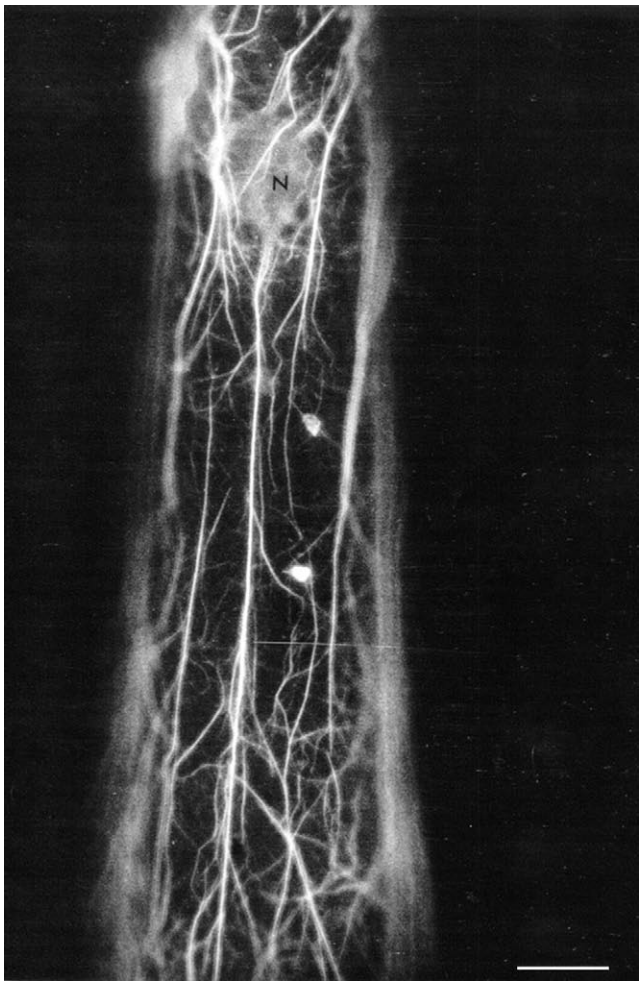


FIGURE 10.5 F-actin in a stem hair cell of a tomato. F-actin is stained with rhodamine-phalloidin and viewed with a fluorescence microscope. Bar, 20 μm. (*Source:* From Parthasarathy et al., 1985.)

to the long axis of the cell or as a random mesh in the cortical cytoplasm. Before the cell enters prophase, the actin forms a band that predicts the future site of cell division. During metaphase, there are actin filaments near the spindle poles and parallel to the spindle fibers. Some actin filaments are in the spindle, particularly associated with kinetochore fibers. During anaphase, the actin filaments become more and more aligned with the spindle fibers. In telophase, the actin filaments become part of the phragmoplast. These actin filaments are parallel to the spindle and connected to the reforming cortical actin network. Double labeling suggests that the actin in the phragmoplast is newly assembled during cell plate formation and does not come from the spindle-associated microfilaments (Gunning and Wick, 1985; Kakimoto and Shibaoka, 1987, 1988; Seagull et al., 1987; Traas et al., 1987; Clayton and Lloyd, 1985; Palevitz, 1988a; Schmidt and Lambert, 1990; Zhang et al., 1992). As cells elongate and stop dividing, the actin filaments are typically longitudinally oriented throughout the ectoplasm and traverse the transvacuolar strands (Thimann et al., 1992; Shimmen et al., 1995). There are reports that actin also occurs in mitochondria (Lo et al., 2003) and nuclei (Paves and Truve, 2004).

The arrangement of the actin cytoskeleton in epidermal cells is correlated with the ability of the cells to elongate. In cells that elongate slowly, the actin microfilaments are organized in dense bundles. Upon activation of elongation by continuous far-red light, which is mediated by phytochrome, the bundles split into fine strands (Waller and Nick, 1997). A similar response occurs after the addition of the elongation-inducing hormone auxin (Wang and Nick, 1998).

10.2.2 Biochemistry of Actin

Structural studies with fluorescently labeled actin filaments indicate that the actin cytoskeleton is an extremely dynamic structure. The biochemistry of actin and its associated proteins provide a molecular mechanism for the dynamic behavior of actin filaments. The behaviors of the replicate actin solutions that Straub (1981) isolated were erratic. Sometimes the solution was highly viscous and sometimes it was not. In order to increase the reproducibility of the extraction procedure, Straub varied the salt concentration and noticed that he obtained a highly viscous extract when the salt concentration was high (0.1 M NaCl or KCl) and a less viscous extract when the salt concentration was lower. The highly viscous extract, but not the fluid one, had fibers that were visible in the electron microscope. The two extracts could be interconverted by adding or dialyzing away the salt. Later Straub discovered that concentrations of NaCl and KCl greater than 0.1 M caused a decrease in the viscosity of the extract and that the salts had an optimal concentration in which they promoted polymerization. The optimal concentration was around the point at which the salts were isoosmotic with intact muscle cells. Straub and Szent-Györgyi postulated that the actin was a fibrous polymer made out of globular subunits, and named them *F-actin* and *G-actin*, respectively.

Actin can be purified by allowing it to go through several polymerization-depolymerization cycles using 0.1 M and 0.6 M KCl, respectively and centrifuging the filamentous actin away from the other cellular proteins. The physico-chemical properties of purified actin have been studied (Janmey et al., 1990; Janmey, 1991). G-actin has now been studied by X-ray diffraction and it is a bilobed, pear-shaped, 42-kDa globular protein (Kabsch et al., 1990; Holmes et al., 1990; Otterbein et al., 2001; De La Cruz and Pollard, 2001). There are probably many isoforms of actin and actin-related proteins since there are many more or less related genes in a single organism (Meagher, 1991; Frankel and Mooseker, 1996).

Each actin monomer is associated with one molecule of ATP. The polymerization of actin is accompanied by the hydrolysis of the terminal phosphate of the bound ATP. However, the energy released by the hydrolysis of ATP is

not required for polymerization since either ADP or adenylimidodiphosphate (AMP-PNP), a nonhydrolyzable analog of ATP, can substitute for ATP in the polymerization reaction.

Actin microfilaments can form and grow in vitro. When the ionic strength of the actin-ATP solution is increased, there is a lag phase that reflects the initial step in polymerization. The slow step involves the formation of a nucleating site. Once nucleation occurs, polymerization takes place rapidly by the addition of monomers. The assembly reaction is reversible and eventually the monomer concentration decreases until disassembly proceeds at the same rate as assembly. This monomer concentration is known as the critical concentration (Korn et al., 1987).

The rate of polymerization of actin depends on the concentration of monomers ([*C*], in M) and the on-rate constant (k_{on}, in $M^{-1}\ s^{-1}$) according to the following equation (Figure 10.6):

$$\text{rate of polymerization} = k_{on}\ [C] \qquad (10.1)$$

The on-rate constant is a measure of the rate of diffusion of the monomers to the site of polymerization. The rate that actin monomers dissociate from the filament is determined by the off-rate constant (k_{off}, in s^{-1}), which is independent of concentration according to the following equation:

$$\text{rate of depolymerization} = k_{off} \qquad (10.2)$$

There is a critical concentration (C_c) where the rate of polymerization equals the rate of depolymerization:

$$k_{on}\ [C_c] = k_{off} \quad \text{and} \quad [Cc] = k_{off}/k_{on} \qquad (10.3)$$

When the monomer concentration is greater than the critical concentration, polymerization continues. When the monomer concentration is less than the critical concentration, depolymerization occurs. At the critical concentration, there is no net filament growth. While polymerization does not require the hydrolysis of ATP, when hydrolyzable forms of ATP are present, as they are in the cell, a new property of actin known as *treadmilling* is exposed. In the presence of ATP, growth takes place at one end, while shrinkage occurs at exactly the same rate at the other end. Thus, even though the filament maintains a constant length, the individual actin monomers are constantly being transferred from one end to the other. This can be demonstrated by decorating short filaments of actin with heavy meromyosin, which will mark their polarity. The decorated actin filaments are then put back into the polymerizing solution. The end marked by the barb of the heavy meromyosin arrows grows 5–10 times faster than the end marked by the arrowheads (Bonder et al., 1983; Estes et al., 1992). The fast-growing end is known as the *plus end* and the slow-growing end is known as the *minus end*.

Cytochalasins, a family of fungal metabolites that bind to the plus end of actin filaments, prevent their further growth (Cooper, 1987). Mycalolide B or latrunculin, a toxin produced by a sponge, has a similar effect (Shimmen et al., 1995; Saito and Karaki, 1996). Phalloidin, a toxin produced by *Amanita*, on the other hand, stabilizes actin filaments so they cannot depolymerize. Treating motile processes with these pharmacological agents is a good way to test whether or not actin is involved in a given process.

The dynamic behavior of the actin filaments is an intrinsic property of the actin filament itself. However, the behavior of the actin filaments can be further modified by other cellular proteins (Hussey et al., 2002; Staiger and Hussey, 2004). For example, there are a number of proteins in plant and animal cells, including profilin, which interact with G-actin and prevent it from polymerizing (Giehl et al., 1994;

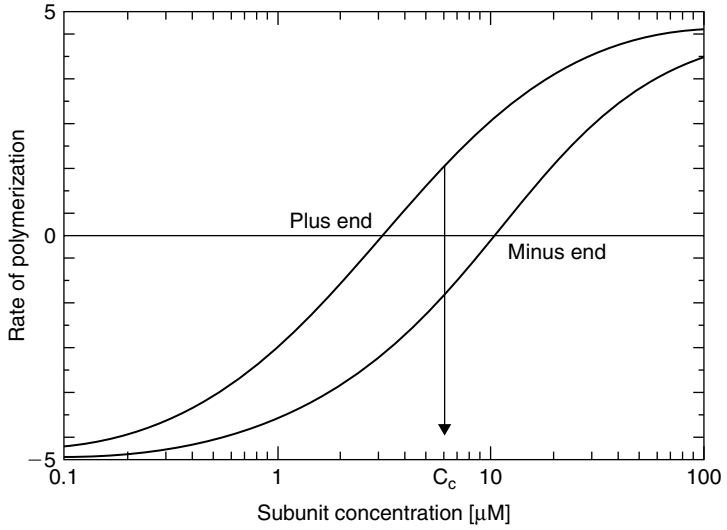


FIGURE 10.6 Graph of rate of polymerization versus concentration of actin subunits.

Darnowski et al., 1996). Other proteins, including severin, fragmin, and gelsolin, bind to actin filaments and either cap the plus end and prevent polymerization or bind to the middle of the filament and cut it (Weeds and Maciver, 1993). A third class of proteins interacts with actin filaments and induces gel formation. This class of cross-linking proteins includes spectrin (de Ruijter and Emons, 1993; Faraday and Spanswick, 1993). A fourth class of actin-binding proteins, including villin, causes bundling (Yokota and Shimmen, 1999; Vidali et al., 1999; Yokota et al., 2000a,b, 2003; Tominaga et al., 2000b). Other proteins are involved in the interaction between actin microfilaments and microtubules (Igarashi et al., 2000).

10.2.3 Biochemistry of Myosins

Myosins are actin-binding mechanochemical transducer proteins, which are capable of generating force along actin filaments as a result of their ability to hydrolyze ATP. There are at least 24 classes of myosin and probably one or more types occur in all plant and animal cells (Kato and Tonomura, 1977; Ohsuka and Inoue, 1979; Vahey and Scordilis, 1980; Vahey et al., 1982; Parker et al., 1986; Qiao et al., 1989; Kohno et al., 1991; Higashi-Fujime, 1991; Yokota and Shimmen, 1994; Yokota et al., 1995, 1999a,b; Miller et al., 1995; Plazinski et al., 1997; Kashiyama et al., 2000; Shimmen et al., 2000; Li and Nebenführ, 2007). The multiple forms of myosin may be due to repeated duplication of the myosin gene combined with variation introduced into the repeated gene through mutation. As an alternative to the "repeat and vary" theme, the multiple forms of myosin may be due to the promiscuity of DNA (Doolittle, 1995), which results in the DNA sequences encoding the actin binding domain, the ATPase activity, the length of the lever arm and the cargo binding regions being mixed and matched to produce each type of myosin (Knight and Kendrick-Jones, 1993; Kinkema and Schiefelbein, 1994; Kinkema et al., 1994; Hasson and Mooseker, 1995; Yamamoto et al., 1995; Yokota et al., 1999a,b; Foth et al., 2006; Reisen and Hanson, 2007; Yamamoto, 2007; Avisar et al., 2008b; Golomb et al., 2008; Hashimoto et al., 2008; Sparkes et al., 2008; Yokota et al., 2008) that is specialized to produce or maintain tension and elasticity in the cell or to pull a specific cargo at a given rate and a given direction. The activity of myosins can be regulated by calcium (Szent-Györgi, 1996; Szent-Györgi et al., 1999) and through phosphorylation (Karcher et al., 2001).

Minimyosins with molecular masses close to 100 kDa, have only one ATP hydrolyzing head and a tail that is capable of binding with a high affinity to membranes and liposomes, which indicates that they are involved in vesicle transport (Adams and Pollard, 1989; Titus et al., 1989; Miyata et al., 1989; Haydon et al., 1990; Schroer, 1991; Zot et al., 1992). Indeed, Golgi-derived vesicles contain minimyosin as a peripheral membrane protein on the cytosolic leaflet (Fath and Burgess, 1994). The tail of a minimyosin isolated from *Chara* binds to vesicles made from phosphatidlyserine or phosphatidylinositol with dissociation constants of 273 nM and 157 nM, respectively (Nunokawa et al., 2007). Minimyosin can be inactivated by calcium (Coluccio and Bretscher, 1987; Collins et al., 1990; see Chapter 12).

Myosin II is the myosin found in skeletal muscle. Unlike minimyosin, it is specialized to form bipolar filaments, which are necessary for skeletal muscle contraction. Microscopic assays have been developed to image the hydrolysis of an individual ATP molecule by a single myosin II molecule (Funatsu et al., 1995). In contrast to minimyosin, myosin II is twice the size, forms bipolar filaments, has two ATP hydrolyzing heads, and is activated by calcium.

Just as it became conventional wisdom that all myosin motors were plus end–directed motors, Wells et al. (1999) discovered a myosin, known as myosin VI, which is a minus end–directed motor (Schliwa, 1999; Cramer, 2000; Vale and Milligan, 2000). This was discovered by Wells et al. (1999) in an in vitro motility assay in which they labeled the barbed (+) end of actin microfilaments with rhodamine phalloidin and labeled the remainder of the microfilaments with FITC-phalloidin, and noticed that when these filaments were placed on a slide containing myosin V, a "typical myosin," the pointed (−) end moved first that is, the myosin walked toward the plus end. However, when they placed the actin microfilaments on a slide coated with myosin VI, the barbed (+) end moved first, indicating that the myosin was walking toward the minus end.

10.3 FORCE-GENERATING REACTIONS INVOLVING ACTIN

10.3.1 Actomyosin

I have discussed the force-generating reactions that take place in muscle. Could actomyosin also be involved in moving vesicles through the cytoplasm? Vesicle movement is often inhibited by cytochalasin and latrunculin, as well as the sulfhydryl-binding agent N-ethylmaleimide. These agents are inhibitors of actin and myosin, respectively. Let us see if the interaction of actin and myosin provide enough force to overcome the yield value of the cytoplasm (0.5 N/m^2 or 0.5 Pa; see Chapter 9). Imagine a typical vesicle, with a diameter of 10^{-6} m and a surface area ($4\pi r^2$) of approximately 3.14×10^{-12} m^2, moving through the cytoplasm. It would need a force of $(0.5\,\text{N m}^{-2})(3.14 \times 10^{-12}\,\text{m}^2) = 1.6 \times 10^{-12}$ N or 1.6 pN to overcome the viscous resistance of the cytoplasm (yield value) and move through it. Is this the ballpark for the forces exerted by myosin? How much force can each myosin molecule exert? It is possible to measure the force exerted by a single myosin molecule in a variety of ways.

Studies aimed at measuring the force of a single myosin molecule began when Shimmen and Tazawa (1982a,b)

discovered that they could reconstitute cytoplasmic streaming in *Nitella* internodal cells using endoplasm from another source. Then Sheetz and Spudich (1983a,b), Sheetz et al. (1984), and Shimmen and Yano (1984) found that the characean actin bundles would support active streaming of myosin-coated latex beads. Thus, myosin molecules are capable of exerting force when they move along actin bundles in plant cells. Unfortunately, the researchers did not know how many myosin molecules on each bead were in contact with the actin bundles and thus could not determine the force exerted by a single myosin molecule. Chaen et al. (1988) opened up a characean cell to expose the actin cables. Then they placed a myosin-coated microneedle against the actin bundles and the needle began to bend. The glass needle had an elastic coefficient of 40 pN/μm. If the researchers had known the number of myosin molecules attached to the glass, it would have been possible to calculate the force due to one myosin molecule after measuring how far the needle bent.

It is also possible to cover a glass slide with myosin so that the myosin molecules become attached to the glass slide, and then put fluorescently labeled actin filaments on top of them (Yanagida et al., 1984; Kron and Spudich, 1986). Upon the addition of ATP, the actin filaments move over the myosin. We can attach a small glass rod to the actin filament and measure how much it bends. Using the elastic coefficient of the glass and the number of myosin molecules touching the actin filament, it is possible to calculate the force due to one myosin molecule. According to Kishino and Yanagida (1988) and Ishijima et al. (1991, 1996), the minimum force that one myosin exerts is 0.2 pN. The movement of actin bundles over immobilized myosin molecules is a good functional assay that can be used for the purification of actin.

Finer et al. (1994) have used laser tweezers to stop actin filaments from moving across a slide sparsely coated with myosin so that only one myosin molecule will attach to an actin filament at a time. In this way, they determined that a single myosin molecule can exert a force of 3–4 pN. Optical trapping measurements of the force exerted by myosin XI show that the maximal force of this myosin is approximately 0.5 pN (Tominaga et al., 2003). Given that the average measured force of a single myosin molecule is 1.8 pN, and the yield value of the cytoplasm is approximately 0.5 Pa, a single myosin molecule would be capable of moving a typical 1 μm in diameter vesicle through the cytoplasm. Because of the low Reynolds numbers, which indicate that the viscous forces in the cytoplasm are greater than the inertial force exerted by myosin, myosin molecules must continually exert a force or the vesicles will stop.

Based on the results of in vitro motility assays, Leibler and Huse (1991, 1993) have come up with a theory of motor proteins that provides an understanding of the kinetics of the mechanochemical cycle that goes beyond that deduced by Lymm and Taylor (1971) for myosin in solution. Leibler and Huse conclude that when a motor protein, such as a single molecule of minimyosin, pulls a vesicle or organelle through the cytoplasm, it must remain attached to the actin microfilament for the majority of the mechanochemical cycle or the load will diffuse away. By contrast, a motor such as a myosin II molecule, which in skeletal muscle works in concert, yet asynchronously, with other myosin II molecules, must detach from the actin filament for a considerable portion of its mechanochemical cycle in order to not increase the friction against which the other myosin molecules must work. The details of the mechanochemical cycle of *Chara* myosin, which is approximately 20 times faster than skeletal muscle myosin, are still unknown (Higashi-Fujime et al., 1995; Uyeda, 1996; Kashiyama et al., 2000).

Szent-Györgyi (1947) wrote: "Like most children, the biochemist, when he finds a toy, usually pulls it to pieces, and he can seldom keep his promise to put it together again." However, we can see that very definite progress is being made when it comes to reconstituting actin-based motility systems!

10.3.2 Polymerization of Actin Filaments

The mere polymerization of actin can provide a force (Mahadevan and Matsudaira, 2000). This is well documented in the acrosomal reaction of some invertebrate sperm (Tilney, 1976). The acrosomal region of the sperm of *Thyone* is packed with monomeric actin. This actin stays as a monomer because it is bound to profilin, a protein that prevents the polymerization of actin. When the sperm touches the egg, the pH of the sperm cytoplasm rises, and the actin dissociates from the profilin and rapidly polymerizes at a rate of approximately 9 μm/s into a long thin acrosomal process "which punctures the egg coat like a harpoon." This allows the membranes of the egg and sperm to fuse.

The polymerization of actin in the host cell is responsible for providing the motive force for the movement of *Listeria*, a bacterial pathogen (Tilney and Portnoy, 1989; Tilney et al., 1990a; Theriot et al., 1992). The bacterium is taken up into a macrophage by phagocytosis. Subsequently, the phagosomal membrane dissolves and the bacterium causes the nucleation of actin filaments. The polymerization of actin provides the force necessary to propel the bacterium into an extended region of the cell. A neighboring macrophage then takes up the cell extension that contains the bacterium by phagocytosis, and the cycle continues. Other bacteria also harness the power of actin polymerization to propel them from cell to cell (Laine et al., 1997). It appears that viruses may also take advantage of the cytoskeleton to move around a cell (McLean et al., 1995).

10.4 ACTIN-BASED MOTILITY

Many motile processes in plant cells depend on the actin cytoskeleton (Grolig, 2004). Actin can be definitively

considered to be involved in a given process in a cell based on the following criteria:

- Descriptive analysis of the location and pattern of microfilaments
- Demonstration that actin antagonists inhibit the observed response
- Test the characteristics of the system in a cell model
- Isolate the proteins involved in the process
- Reconstitute of a functional system

Given these criteria, cytoplasmic streaming is the best-characterized actin-based motile process in plants (Shimmen and Yokota, 2004; see Figure 10.7).

10.4.1 Cytoplasmic Streaming

Cytoplasmic streaming is one of the most unforgettable processes that can be seen under the microscope, and a sensational and must-read account of it has been written by T. H. Huxley (1890). Cytoplasmic streaming, which occurs in almost all plant cells, facilitates the transport and mixing of substances in large cells by causing convection, which is much faster than diffusion (Darwin and Acton, 1894; Pickard, 1974; Hochachka, 1999; Goldstein et al., 2008). Even the smell of smoke or perfume would take hours to cross a room if its movement depended on diffusion alone (Clausius, 1858, 1860, 1879; Maxwell, 1873, 1878; Garber et al., 1986). Cytoplasmic streaming also occurs in the large embryonic cells of animals, where diffusion would be rate limiting (Hird and White, 1993; Cramer et al., 1994).

The time it takes for a substance to diffuse a given distance can be calculated by Einstein's (1906) random-walk equation:

$$t = x^2/(2D) \tag{10.4}$$

Given that $D = kT/(6\pi r_H \eta)$, T is usually close to 300 K, the microviscosity of the cytoplasm is approximately 0.004 Pa s, and the radius of typical atoms and molecules falls between 10^{-10} and 10^{-9} m, the diffusion coefficient of low–molecular mass molecules in the cell is typically between 0.5 and 5×10^{-10} m^2/s. If $D = 10^{-10}$ m^2/s, it would take 0.5 s to diffuse across a 10-μm-long cell and 5×10^7 s (≈1.5 years) to diffuse from one end to the other in a 10-cm-long characean cell.

It can be seen that the time increases with the square of the distance, so movement of a given substance across a cell will be 100 times slower for a 10-μm cell than for a 1-μm cell and 100 times slower for a 100-μm cell than for a 10-μm cell. It will be really slow in a 100,000-μm-long characean cell. Thus, it is understandable that the rate and organization of cytoplasmic streaming, as I will discuss in the following, are related to cell size.

The fascinating movements of the cytoplasm were first observed by Bonaventura Corti in 1774, and later by Giovanni Amici (1818) after he invented the achromatic lens. There are many manifestations of cytoplasmic streaming from the slow saltatory movement found in the small cells of *Spirogyra* to the rapid rotational streaming found in the giant *Chara* cells (Hofmeister, 1867; Berthold, 1886; Hörmann, 1898; Kamiya, 1959; Kuroda, 1990).

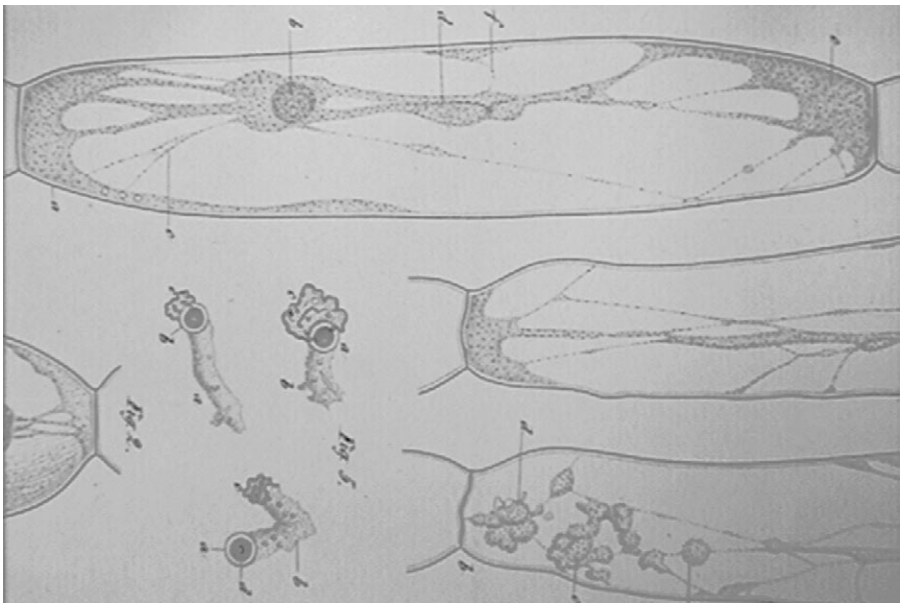


FIGURE 10.7 Cytoplasmic streaming in *Tradescantia virginica*: (a) plasma membrane, (b) nucleus, (c) protoplasm, (d) contracted area of protoplasm, (e, f) areas of flowing protoplasm. (*Source:* From Kühne, 1864.)

The slowest cytoplasmic movements, which occur in small cells like those of *Spirogyra*, are called *agitation* or *saltatory motion*. In this class of streaming, the motion of vesicles is erratic and haphazard, but statistically speaking, not devoid of directional movement. More organized and faster movements, known as circulation streaming, are characteristic of moderate-sized cells having transvacuolar strands like the hair cells of *Tradescantia*. In these cells, vesicles of various sizes differ in speed and direction as they pass through the cytoplasm, indicating that there is a very complicated network of roads. The long root-hair cells of *Limnobium* and the long pollen tubes of *Plantago major* have a type of streaming known as *fountain streaming*, which is highly organized and fast. Fountain streaming results when the stream of cytoplasm flows up the middle of the cell toward the tip and then flows back along the sides, looking much like a fountain in a town square. Reverse fountain streaming, where the flow reaches the apex from the sides, takes place in the long pollen tubes of *Camellia japonica*.

An extremely fast type of streaming, found in the giant internodal cells of characean algae, is called *rotational streaming*, since the protoplasm is limited to the periphery of the cell and it streams like a rotating conveyor belt. The fastest type of streaming is found in the slime mold *Physarum*. In the plasmodial stage of this giant single-celled organism, the cytoplasm moves back and forth in a rhythmic fashion with a velocity as great as 2 mm/s. The stream is reminiscent of a shuttle used in weaving, and is thus called *shuttle streaming* (Kamiya 1940, 1942, 1950a,b,c; Kishimoto, 1958; Mustacich and Ware, 1977b; Newton et al., 1977). In addition and unrelated to shuttle streaming, a suspended plasmodium will rotate alternately clockwise and counterclockwise in a period of about 2 minutes (Kamiya and Seifriz, 1954).

Cytoplasmic streaming is affected by plant hormones (Sweeney and Thimann, 1942; Sweeney, 1944; Kelso and Turner, 1955; Ayling et al., 1990; Ayling and Butler, 1993), light (Nagai, 1993), electricity (Tazawa and Kishimoto, 1968), and gravity (Wayne et al., 1990; Staves et al., 1992), and can thus be used as an indicator of how cells respond to these stimuli (see Chapter 12). Moreover, cytoplasmic streaming is an excellent indicator of cell viability and can be used to determine whether or not a given treatment is lethal to a cell.

The velocity of cytoplasmic streaming depends on the magnitude of the inertial motive force and the viscous force that provides the resistance to flow. The motive force results from the conversion of chemical energy into mechanical energy by actomyosin, and the resistance to flow depends on the viscosity of the cytoplasm.

In order to determine the site where the motive force for streaming is generated, Kamiya and Kuroda (1956) mea sured the velocity gradient in a single characean internodal cell. They found that the velocity of the ectoplasm is zero, and increases from 0 μm/s to about 100 μm/s, depending on temperature, about 1 μm into the interior of the endoplasm. Thus, in this region there is a large velocity gradient and a large rate of shear. Since the cytoplasm of characean cells is non-Newtonian (see Chapter 9), the viscosity depends on the rate of shear. If the velocity gradient is 100 μm/s per μm, then the rate of shear at the ectoplasmic/endoplasmic interface is 100 s^{-1}, and consequently, the viscosity at the interface is approximately 0.01 Pa s. The velocity of endoplasm itself decreases from 100 μm/s to 90 μm/s over 10 μm. Thus, the rate of shear is only about 1 s^{-1}, and its viscosity is high at about 0.8 Pa s. Thus, the internal friction of the endoplasm resists the flow induced by the shearing stress.

The flowing endoplasm ruffles the vacuolar membrane and this transmits a force into the vacuole that causes streaming in the cell sap (Staves et al., 1995). The velocities of the cell sap particles are as fast next to the vacuolar membrane as they are in the endoplasm. The velocities of the cell sap inclusions decrease to zero near the middle of the vacuole and then they slowly increase in a symmetrical way, albeit in the opposite direction. A similar velocity gradient can be seen in cytoplasm-rich cells, which have had their vacuole removed by centrifugation, indicating that neither the vacuole nor the vacuolar membrane is a *sine qua non* for cytoplasmic streaming.

From the velocity profiles, Kamiya and Kuroda hypothesized that the ectoplasm/endoplasm interface is the site of the generation of the shear stress. Can you imagine the excitement when Eiji Kamitsubo (1966), using a phase-contrast microscope, first saw linear fibrillar structures at this interface, or when Reiko Nagai and Lionel Rehbun (1966), using electron microscopy, first observed the bundles of 5-nm-diameter microfilaments, which were oriented parallel to the direction of flow, at this interface (Kamitsubo, 1972a,b,c, 1980)?

Barry Palevitz and Peter Hepler (1975) decorated the bundles at the ectoplasmic/endoplasmic interface with heavy meromyosin and confirmed that they were actin. Yolanda Kersey et al. (1976) then showed that the pointed ends (the minus ends) are directed away from the direction of streaming. So, characean myosin must move from the pointed (minus) end toward the barbed (plus) end, just as it does in skeletal muscle. Therefore, the microfilaments have the right polarity to act in concert with a plus end–directed myosin to provide the motive force for cytoplasmic streaming. The fact that these bundles bind fluorescently labeled phallotoxins (Barak et al., 1980; Nothnagel et al., 1981, 1982) and anti-actin antibodies (Grolig et al., 1988; Williamson et al., 1986, 1987) provides further evidence that these microfilaments are composed of actin.

Myosin is not part of the large actin-containing bundles, but it seems to be attached to the organelles in the flowing endoplasm and may form a network in the endoplasm (Nagai and Hayama, 1979; Grolig et al., 1988). A network of some kind of filament seems to be important in coupling the motive force to the endoplasmic flow (Nothnagel and Webb, 1982). Katcher and Reese (1988) present beautiful

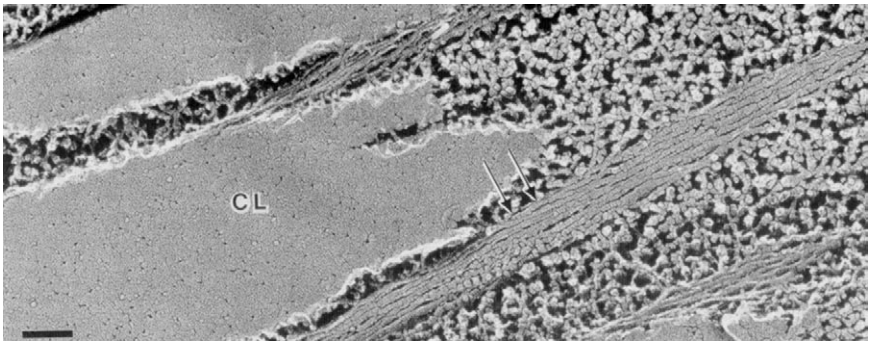


FIGURE 10.8 Freeze-etch micrograph showing the actin bundles (arrows). The flat region is part of the chloroplast envelope. Bar, 120 nm. (*Source:* From Katcher and Reese, 1988.)

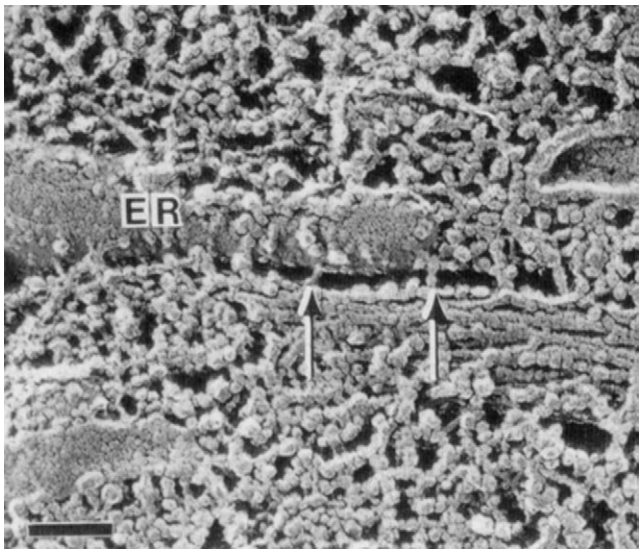


FIGURE 10.9 Freeze-etch micrograph showing the actin bundles and areas where they seem to be attached to the ER (arrows). Bar, 100 nm. (*Source:* From Katcher and Reese, 1988.)

pictures showing that the endoplasmic reticulum (ER) may be responsible for coupling the moving endoplasm so that it moves as a whole along the actin bundle (Figures 10.8 and 10.9). However, I find that cytochalasin causes the separation of water and solids (syneresis) in the endoplasm, indicating that actin filaments may also provide the framework that gives the flowing cytoplasm a high viscosity and couples the bulk of the endoplasm to the moving vesicles.

The evidence that actin and myosin provide the motive force for streaming comes from experiments that show that treatments with cytochalasins, or cytochalasin given with latrunculin, which inhibit actin function, or N-ethylmaleimide, 2,3-butanedione monoxime (BDM), and heat, which inhibit myosin function (Chen and Kamiya, 1979, 1981; Kamitsubo, 1981; Kuroda, 1983; Tominaga et al., 2000a; Seki et al., 2003; Funaki et al., 2004; Foissner and Wasteneys, 2007), inhibit streaming. Evidence that a specific myosin is involved in the movement of a particular organelle comes from studies in which a given myosin gene was knocked out, overexpressed, or modified using genetic techniques (Peremyslov et al., 2008), including RNA interference (Avisar et al., 2008b).

The velocity distribution indicates that the endoplasmic layer moves passively as a unit within the ectoplasm. It also indicates that the motive force responsible for this streaming is provided by a shearing stress, generated at the boundary between the cortical gel and the streaming endoplasm (Kamiya and Kuroda, 1956, 1965; Tazawa, 1968; Donaldson, 1972; Pickard, 1972). The inertial motive force (F_m) exerted by the shearing stress (σ, in N/m^2) is equal to σA, where A is the area of the endoplasm acted upon by the shearing stress on one side of the internodal cell.

How can we measure the magnitude of the inertial motive force per unit area responsible for cytoplasmic streaming? We can put the characean cell in a centrifuge microscope, and determine the inertial force per unit area due to centrifugal acceleration (αg, in m/s^2) that is required to stop the cytoplasmic streaming in the centripetal direction. The acceleration needed to stop cytoplasmic streaming is also known as the *balance acceleration*. In a centrifugal field, the net shearing stress is given by the following equation:

$$\sigma = F_w/A - F_i/A \tag{10.5}$$

The inertial force (F_i, in N) supplied by the centrifugal force and applied to the streaming endoplasm is given by Newton's Second Law:

$$F_i = ma = \alpha g(\rho_e - \rho_v)Ax \tag{10.6}$$

where ($\rho_e - \rho_v$) is the density difference between the endoplasm and the vacuolar sap, A is the area against which the shearing stress exerts itself, and x is the thickness of the endoplasm under centrifugal accelerations. The volume of the endoplasm flowing in the centripetal direction is thus Ax. The motive force per unit area that powers cytoplasmic streaming can be determined at the balance acceleration,

where the velocity gradient = 0. According to Eq. 9.4 (in Chapter 9):

$$\eta(v/z) = \sigma \tag{10.7}$$

Thus, at the balance acceleration, the shearing stress also vanishes as shown in the following equation:

$$\eta(v/z) = \sigma = 0 \tag{10.8}$$

Substituting Eq. 10.5 into Eq. 10.8, we get:

$$\eta(v/z) = (F_m/A - F_i/A) = 0 \tag{10.9}$$

Since, according to Eq. 10.6, $F_i = \alpha g(\rho_e - \rho_v)Ax$, then:

$$(F_m/A - (\alpha g(\rho_e - \rho_v)Ax)/A) = 0 \tag{10.10}$$

Thus:

$$(F_m/A) = (\alpha g(\rho_e - \rho_v)Ax)/A \tag{10.11}$$

and after canceling like terms:

$$F_m/A = \alpha g x(\rho_e - \rho_v) \tag{10.12}$$

Thus, the force per unit area that powers cytoplasmic streaming can be calculated from Eq. 10.12 as long as αg, x, and $(\rho_e - \rho_v)$ are known. Assuming that g, x, and $(\rho_e - \rho_v)$ were constants, Kamiya and Kuroda (1958) calculated the shearing stress to be about 0.16 N/m^2. Kamitsubo and Kikuyama (1994) calculated it to be higher. However, since the thickness of the endoplasm decreases as the centrifugal acceleration increases, due to pooling of the endoplasm at the centrifugal end of the cell, the actual centrifugal force applied to the cell decreases over time. Thus, Staves et al. (1995) propose that the shearing stress is overestimated by the balance acceleration, and have determined it to be about 0.1 N/m^2 by extrapolation from the linear portion of a streaming velocity versus centrifugal force graph. If the motive force generated by a single myosin molecule is about 10^{-12} N, and the shearing stress powering cytoplasmic streaming is about 0.1 N/m^2, then there should be approximately 10^{11} myosin molecules per meter squared at the interface of the endoplasm/ectoplasm or 0.1 myosin molecule/μm^2. The concentration of myosin found in *Chara* cells is approximately 200 nM, which is equal to about 10 myosin molecules/μm^2—more than enough to account for the observed motive force. If the total population of myosin was attached to the actin cables, this myosin concentration, with its actin-activated ATPase activity, would hydrolyze ATP faster than respiration could produce it. Consequently, Yamamoto et al. (2006) suggest that the majority of myosin must not be attached to the actin cables at the same time.

Cell models have been important in the study of cytoplasmic streaming. Permeabilized and vacuolar membrane-free cell models have been used to show that ATP provides the energy for cytoplasmic streaming (Williamson, 1975; Shimmen, 1988b; Shimmen and Tazawa, 1982a,b, 1983) and that streaming is regulated through phosphorylation reactions (Tominaga et al., 1987; Awata et al., 2001, 2003; see Chapter 12).

Characean actin bundles can be used as a tool for studying various aspects of actomyosin-based motility (Shimmen, 1988a; Shimmen and Tazawa, 1982a; Shimmen and Yano, 1984; Sheetz and Spudich, 1983, 1983a; Sheetz et al., 1984; Kohno and Shimmen, 1988; Katcher, 1985; Kohno et al., 1990; Rogers et al., 1999). For example, the actin bundles from characean internodal cells have been used in situ as a "common garden" to test the ability of various myosins to move just as yeast cells are currently being used as a common garden to test the function of a given DNA sequence. Interestingly, and perhaps unexpectedly, it turns out that myosin from plants exerts approximately 20 times more force than does skeletal muscle myosin (Shimmen, 1988a). Characean myosin is being studied to understand its interesting properties as the "fastest motor protein in the world" (Yamamoto et al., 1994, 1995; Kashiyama et al., 2000; Morimatsu et al., 2000; Awata et al., 2001, 2003; Kashiyama and Yamamoto, 2001; Ito et al., 2003, 2007; Kimura et al., 2003). Characean myosin hydrolyzes ATP faster, binds tightly to actin longer, and has a longer lever arm than other myosins (Ito et al., 2007). By genetically engineering the length of the lever arm of myosin, Schott et al. (2002) have been able to show that the transport velocity of exocytotic vesicles in living yeast cells is linearly related to the length of the lever arm.

10.4.2 Chloroplast Movements

There are many beautiful and fascinating light-stimulated, actomyosin-mediated motile responses in plant cells, including the light-induced chloroplast turning response in *Mougeotia* (see Figure 10.10; Haupt, 1965, 1983; Wagner et al., 1972; Wagner, 1979; Klein et al., 1980; Wagner and Klein, 1981); the light-stimulated chloroplast aggregation response in *Vaucheria* (Blatt and Briggs, 1980; Blatt et al., 1981; Blatt, 1983, 1987); the light-stimulated aggregation and dispersal response of chloroplasts in the protonema of the fern *Adiantum* (Yatsuhashi et al., 1985, 1987a,b; Wada and Kadota, 1989); and the light-stimulated chloroplast orientation and induction of cytoplasmic streaming response in *Vallisneria* (see Figures 10.11 and 10.12; Ishigami and Nagai, 1980; Yamaguchi and Nagai, 1981; Takagi and Nagai, 1985, 1986; Dong et al., 1995, 1996, 1998; Ryu et al., 1995, 1997; Takagi, 1997, 2003; Takagi et al., 2003). Presumably, all these light-stimulated, actomyosin-mediated chloroplast movements optimize photosynthesis, and they are discussed further in Chapter 13.

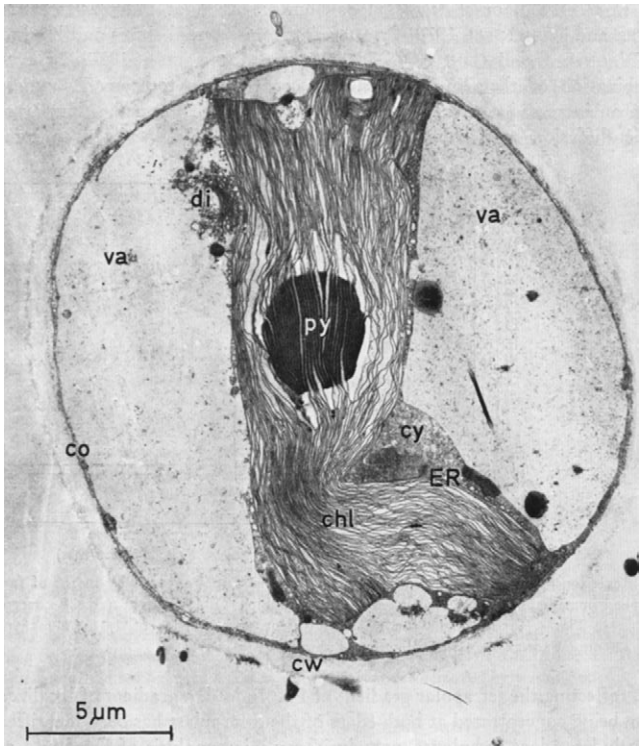


FIGURE 10.10 A cross-section of a *Mougeotia* cell showing the shape and orientation of the chloroplast. py, pyrenoid; va, vacuole; cy, cytoplasm; chl, chloroplast; di, Golgi stack; ER, endoplasmic reticulum. (*Source:* From Wagner and Klein, 1981.)

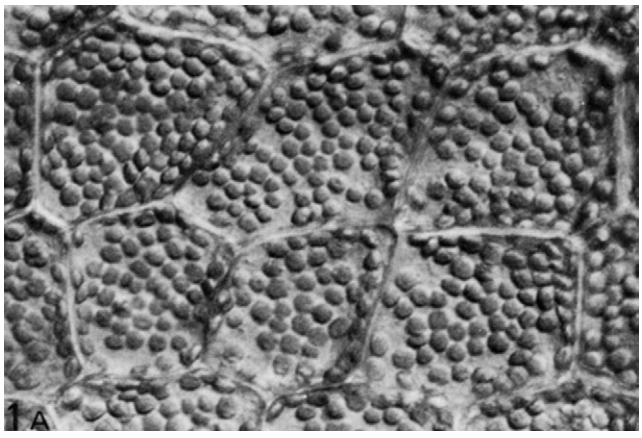


FIGURE 10.11 Epidermal cells of *Vallisneria gigantea* kept under low-intensity light. The chloroplasts are along the periclinal walls. (*Source:* From Yamaguchi and Nagai, 1981.)

10.4.3 Cell Plate Reorientation in *Allium*

At the end of guard cell differentiation in *Allium*, the spindle in the guard mother cell ultimately lies along the longitudinal axis of the cotyledon, in contrast to the spindles of the epidermal cells proper. Initially, the spindle in the guard mother cell is oriented transversely to the long

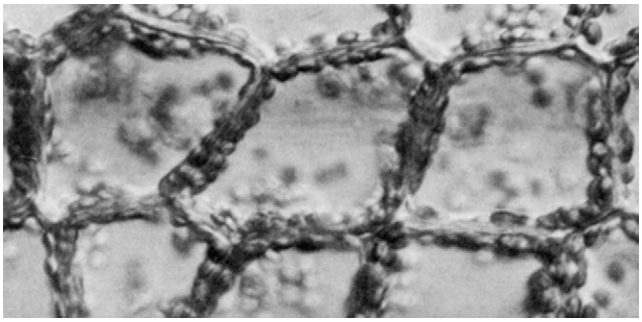


FIGURE 10.12 Epidermal cells of *Vallisneria gigantea* in which the chloroplasts have been induced to move to the anticlinal walls. (*Source:* From Yamaguchi and Nagai, 1981.)

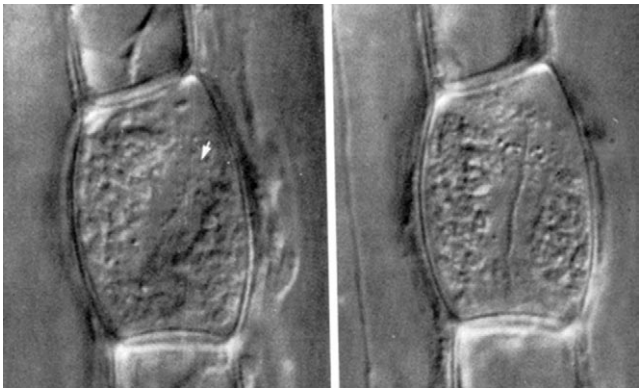


FIGURE 10.13 Nomarski differential interference contrast micrographs of an *Allium* guard mother cell in which the cell plate was caught in the process of reorienting. (*Source:* From Palevitz and Hepler, 1974a.)

axis. However, during anaphase and telophase, the spindle reorients until it is aligned with the long axis of the cotyledon (Figure 10.13). This process is inhibited by cytochalasin, indicating that actin is involved in the reorientation mechanism (Palevitz and Hepler, 1974a,b). Actin is involved in a number of other movement and morphogenetic processes (Mineyuki and Palevitz, 1990; Menzel, 1996; Kennard and Cleary, 1997).

10.4.4 Secretion of Vesicles Involved in Tip Growth and Auxin-Induced Growth

Many plant and fungal cells as well as neuronal cells grow predominantly at the tip. In all these cases of tip growth, actin filaments are involved. In pollen tubes and other tip-growing cells, actomyosin is involved in the delivery of Golgi-derived vesicles to the growing point of tip-growing cells (Picton and Steer, 1982; Kohno and Shimmen, 1988; Kohno et al., 1991, 1992; Lancelle and Hepler, 1988; Steer and Steer, 1989; Heath, 1990; Braun and Sievers, 1994; Yokota and Shimmen, 1994; Yokota et al., 1995a). Waller et al. (2002) suggest that an auxin-induced reconfiguration of the actin cytoskeleton induces growth in non-tip-growing

cells by transporting vesicles containing cell wall components in the lumen and auxin efflux carriers in the membrane to the preferred region of the cells.

10.4.5 Contractile Vacuoles

Contractile vacuoles were the first organelle seen in cells and they are just as exciting to see today (Allen and Naitoh, 2002; Allen et al., 2009) as they were over 200 years ago when Lazzaro Spallanzani (1776) observed the "stars he thought were respiratory organs." The contraction of these osmoregulatory organelles, which expel water and allow wall-less protozoan and algal cells to live in dilute solutions without bursting, is powered by actin and myosin (Zhu and Clarke, 1992; Domozych and Nimmons, 1992; Dobberstein et al., 1993; Domozych and Dairman, 1993; Ishida et al., 1993; Nolta et al., 1993; Heuser et al., 1993; Nolta and Steck, 1994). Interestingly, the osmotic pressure of the cytosol of cells can be determined by increasing the osmotic pressure of the medium to the point where the contractile vacuoles disappear.

10.5 ROLE OF ACTIN IN MEMBRANE TRANSPORT

In animal cells, the actin cytoskeleton is intimately connected to the plasma membrane through such proteins as talin, vinculin, α-actinin, spectrin, and ankyrin (Luna, 1991; Hitt and Luna, 1992, 1994; Ervasti and Campbell, 1993; Paller, 1994; Calderwood et al., 2000; Kawakatsu et al., 2000). Actin and spectrin are also associated with the plasma membrane of plant cells (de Ruijter and Emons, 1993; Faraday and Spanswick, 1993; Sonesson and Widell, 1993; Kobayashi, 1996). There is evidence in plant cells that the actin cytoskeleton may influence membrane permeability (Wayne and Tazawa, 1988; Tazawa and Wayne, 1989; Hwang et al., 1997; Khurana, 2000), as well as the position of Ca^{2+} channels (Brawley and Robinson, 1985; Saunders, 1986a,b). Class VIII myosins are required to target proteins to the plasmodesmata for intercellular transport (Avisar et al., 2008a).

10.6 SUMMARY

Movement is one of the basic characteristics of life. In this chapter, I have provided evidence for the basic unity of nature that Theodor Engelmann and Thomas Huxley believed existed when they compared cytoplasmic streaming in the hair of a stinging nettle with muscle movements that allow a human mouth to recite poetry. However, among this unity we also found that there is diversity: Some actin-mediated processes are driven by actomyosin, while others are driven by actin polymerization. We have also learned that there are many kinds of myosins. In Chapter 11, we will see that there is even greater diversity among the various motile systems, and that many motile processes do not depend on actin at all.

10.7 QUESTIONS

10.1. How does actin participate in motile processes?
10.2. Why are there so many types of myosin?
10.3. What is the function of cytoplasmic streaming, and what is the relationship between the pattern and velocity of cytoplasmic streaming and cell size?

Chapter 11

Tubulin and Microtubule-Mediated Processes

According to a valley girl in the 1980s, "This organelle is totally tubular."

11.1 DISCOVERY OF MICROTUBULES IN CILIA AND FLAGELLA AND THE MECHANISM OF MOVEMENT

Imagine the excitement Antony van Leeuwenhoek (1677, 1678) felt when he first looked at a drop of water through the microscope he made with his own hands, and saw a whole new world of little playful swimming creatures. Leeuwenhoek (1677) saw that "when these animalcula or living atoms did move, they put forth two little horns, continually moving themselves" and he noticed that others were "furnished with diverse incredibly thin feet, which moved very nimbly." Two hundred years later, with the advantage of better microscopes, cytologists could see that the flagella and cilia that powered the little protozoa were composed of fibers (see Figure 11.1; Ballowitz, 1888), and Prénant (1913) suggested that these little fibers were contractile. The fibrous nature of the filaments within a cilium was confirmed using an electron microscope (see Figure 11.2; Jakus and Hall, 1946; Grigg and Hodge, 1949). With the resolution attainable at the time, the filaments seemed similar to those found in muscle (Hall et al., 1946; Draper and Hodge, 1949). However, the introduction of the ultramicrotome allowed Fawcett and Porter (1954) to section cilia transversely, and thin enough to reveal a structure different from that of muscle, a structure that has come to be known as the *9 + 2 arrangement* of tubules with which we are familiar today (see Figure 11.3; Satir, 1974; Berger et al., 1975).

I will use the terms *cilia* and *flagella* interchangeably to describe the whiplike structures of eukaryotic cells. At one time, A. P. Shmagina suggested that the appendages be called *undulipodia*, but that term never caught on (Margulis, 1980; Corliss, 1980). Others have suggested that *flagella* be used to describe the whiplike appendages of prokaryotes, and *cilia* be used to describe those of eukaryotes. This suggestion, which also did not catch on, was based on the facts that prokaryotic and eukaryotic appendages are composed of different proteins and have different structures. Thus, in eukaryotes, we are stuck with two terms for organelles with identical internal structure and composition. Many people consider flagella to be longer than cilia, more sparsely arranged on a cell, and to have a symmetrical beating motion, compared with the asymmetrical beat of cilia.

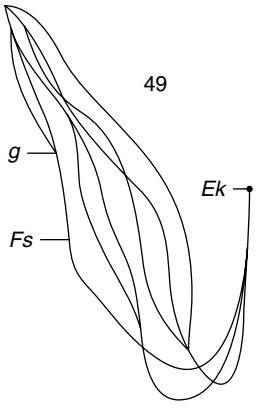


FIGURE 11.1 The sperm of *Passer domesticus* in which filamentous components are visible in the splayed flagella. (*Source:* From Ballowitz, 1888.)

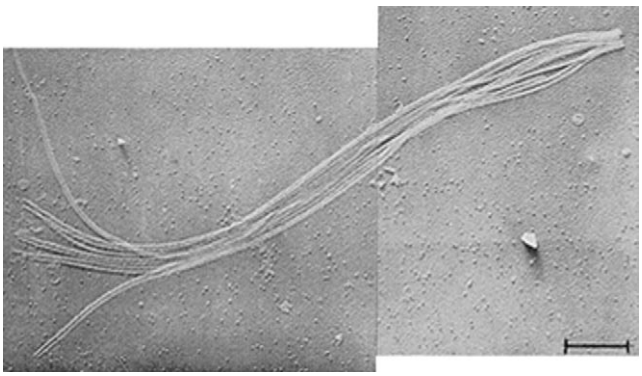


FIGURE 11.2 Electron micrograph of a splayed filaments of a cilium from *Paramecium* that have been shadow-cast with chromium. ×11,000. (*Source:* From Jakus and Hall, 1946.)

However, as I will discuss in the following, the flagellar and ciliary beat can occur at different times in the same structure. Because of this, combined with the fact that there are intermediates in all the characteristics and that there is no universally accepted distinction, I will use the terms interchangeably.

While ciliary motion is widespread in unicellular plants, fungi, and animals, it also occurs in multicellular organisms, although it is restricted to specialized cells (Gray, 1928; Sleigh, 1962, 1974). In the plant kingdom, the sperm of some embryophytic taxa, including mosses, fern allies, ferns (Figure 11.4), cycads and *Ginkgo*, are ciliated (Manton, 1950, 1959; Hepler, 1976; Wolniak and Cande, 1980; Paolillo, 1981; Li et al., 1989). In animals, sperm are powered by flagella and cilia line the respiratory tract where they sweep mucus, dead cells, and dust up toward the mouth; and the oviduct, where they move the oocyte, egg, zygotes and blastocyst toward the uterus. The structure of cilia and flagella in all the above examples are extremely similar, which led Peter Satir (1961) to state that "cellular structure, down to its minute details, remains constant as long as function is constant." The exceptions often prove the rule and some cilia, including the rods and cones in our retina, as well as our olfactory and auditory cells, have highly modified structures, and consequently have lost their motile abilities in exchange for sensory functions (Porter, 1957; Pazour and Witman, 2003).

Cilia, like muscle, require adenosine triphosphate (ATP) for movement, as was shown by Hartmut Hoffmann-Berling

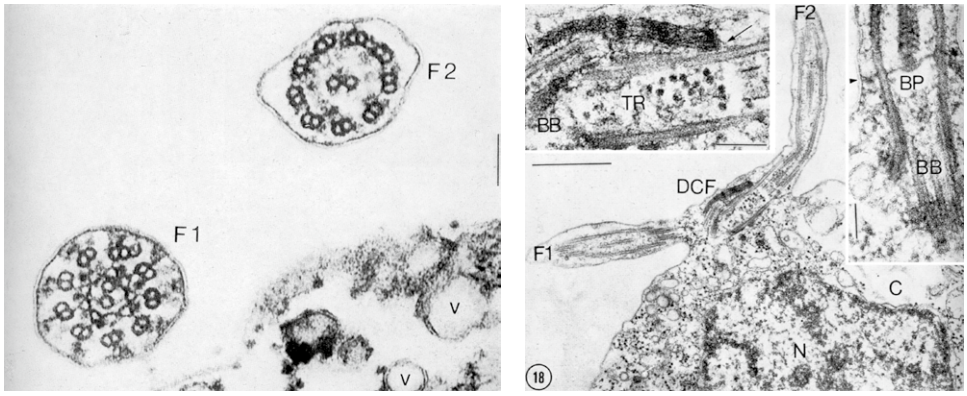


FIGURE 11.3 Electron micrograph of the cilia of the motile gametes of *Acetabularia:* (a) transverse view, bar, 100 nm, and (b) longitudinal view, bar, 500 nm. Inset bar, 100 nm. (*Source:* From Berger et al., 1975.)

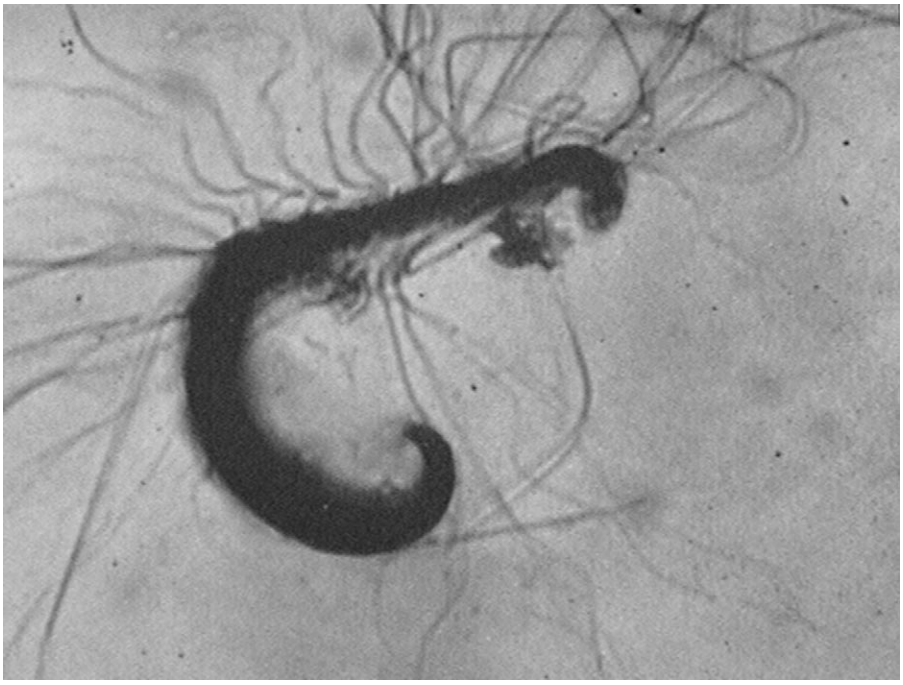


FIGURE 11.4 The sperm of *Dryopteris villarsii* taken with an ultraviolet microscope. The long and numerous cilia are clearly visible. (*Source:* From Manton, 1950.)

(1955), who used glycerinated cell models. Isolated cilia contain everything they need to beat except Mg^{2+}-ATP. When isolated cilia are given Mg^{2+}-ATP, they beat by themselves exactly as if they were intact and attached to a cell. In order to understand how cilia convert the chemical energy of ATP into the mechanical energy that causes the cilia to beat, Tibbs (1957) and Child (1959) took a biochemical approach and discovered that a protein isolated from the cilia of algae and protozoa has ATPase activity.

Given the success of the structural approach in elucidating the mechanism of muscle contraction, Gibbons and Grimstone (1960) and Satir (1961) used electron microscopy to understand ciliary motion. Cilia are structurally complex, membrane-enclosed organelles approximately 0.2 μm in diameter and 10 μm long. Cilia can be as short as 5 μm and as long as 150 μm. The internal structure is known as the *axoneme* and is mainly composed of nine doublets of tubules that surround a central pair of tubules. The central pair is composed of two complete tubules, while each doublet is composed of one complete and one partial tubule called the *A tubule* and the *B tubule*, respectively (Pease, 1963; Andre and Thiery, 1963). Each tubule in the axoneme is approximately 24 nm in diameter and as long as the cilium. The cilia are asymmetric in every way, and thus the individuality of each doublet can be unambiguously recognized.

With the introduction of better fixation procedures, new structures appeared in the electron micrographs that hinted at how the cilia and flagella may produce force in order to generate movement. For example, Björn Afzelius (1959) discovered radial spokes that extended from the A tubule toward a central sheath. He also found arms along the length of the A tubule that form cross-bridges with the adjacent B tubule. Afzelius suggested that these arms, like the heads of myosin, might generate force by inducing sliding between adjacent doublets in a mechanism analogous to that found in muscle cells (see Chapter 10).

Gibbons and Grimstone (1960) suggested that the current microscopic data could not distinguish between the possibilities that the bending movement was due to a localized shortening of longitudinal contractile elements or to sliding of the tubules in a manner similar to that described by the sliding filament model of muscle contraction. By looking at cilia at different stages in their beat cycle with an electron microscope, Peter Satir (1965) concluded that there was no change in the tubule length during ciliary motion, as would be predicted if the tubules were contractile proteins. He also noticed that some tubules, which by this time were universally called *microtubules*, extended further at the tip of the cilia than other microtubules did. The time of their extension correlated perfectly with the position of the cilium during the beat cycle when it was fixed. That is, the microtubules on the convex side of the cilium were extended into the tip. These data supported the sliding filament model for ciliary beating.

Biochemical, genetic, and proteomic data indicate that there are over 200 polypeptides in axonemes (Warner et al., 1989; Dutcher, 1995; Li et al., 2004; Pazour et al., 2005; Stolc et al., 2005). Presumably, most of the proteins are involved in the production of force and the regulation of motility. Gibbons (1963) isolated one protein from axonemes that had ATPase activity. He called the ATPase *dynein*, from the Greek for "force protein," and suggested it may be important for many aspects of cell motility, including ciliary motion (Gibbons and Rowe, 1965). Ian Gibbons localized the dynein in the axoneme by extracting the ATPase activity from the axonemes and then seeing which structure disappeared. When the dynein was extracted, the arms disappeared. The arms could be reconstituted by adding back the purified dynein. The purified dynein was observed with the electron microscope. It had a head and tail structure similar to that of myosin. Both ciliary motion and dynein ATPase activity are inhibited by 1 mM N-ethylmaleimide and 25 μM vanadate (Vale and Toyoshima, 1988, 1989). Thus, dynein has the structure, localization, enzymatic activity, and pharmacological sensitivity that is consistent with its being the mechanochemical transducer involved in microtubule sliding.

Summers and Gibbons (1971), using dark-field microscopy, and Sale and Satir (1977), using electron microscopy, showed that the doublets in the axoneme are capable of sliding past each other. They observed axonemes that had been treated with trypsin, a protease that disrupts the radial spokes, and a protein known as *nexin* that links the outer doublets, but leaves the dynein and microtubules intact. When the trypsin-treated axonemes were treated with ATP, the axoneme elongated up to five times its original length before the cilia disintegrated, indicating that the microtubule doublets are capable of sliding. This makes it likely that the cilia beat as a result of the dynein arms walking along the adjacent doublets. The sliding of microtubules on one side of a cilium at a time results in the generation of a shearing stress that bends the cilium and propels the cell. While it seems certain that the sliding filament model explains how force is generated in cilia and flagella, we still do not know the role of most of the over 200 axonemal proteins. Some of them may provide elastic or rigid structures that help in the generation of shearing stresses. Others may be involved in regulating the activity of the motor and structural proteins in order to generate the three-dimensional beat that propels a cell through a viscous medium, or a viscous medium over stationary cilia (Brokaw, 1972; Satir, 1974, 1975).

Cilia originate from a structure known as the *basal body*, which is a small organelle at the base of the cilium that is about 0.2 μm wide and 0.4 μm long. It is composed of nine groups of three tubules (Figure 11.5). The basal bodies are in turn connected to filamentous structures known as *roots* that permeate the inner surface of the cell body (Pickett-Heaps, 1975; Lee, 1995). The outer tubules of the axoneme are continuous with the inner two tubules of each triplet of the basal body, while the central pair grows from an amorphous area at the distal end of the basal body. Basal bodies are formed either from self-duplicating centrioles or from

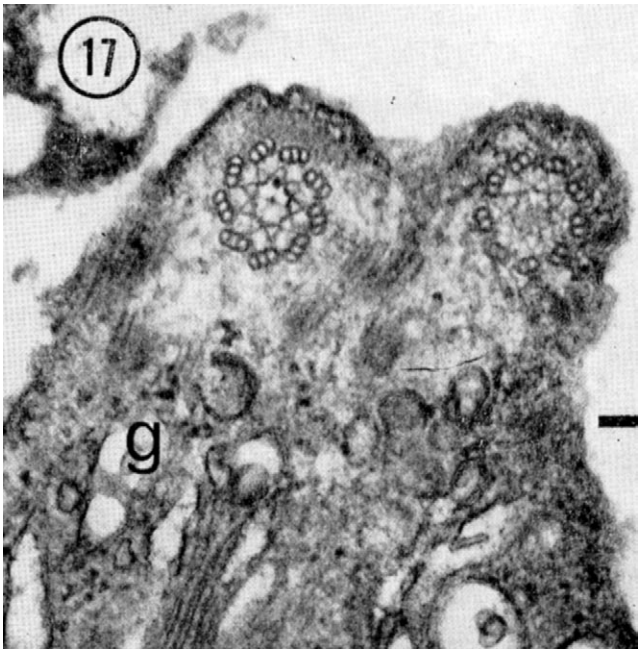


FIGURE 11.5 An electron micrograph of basal bodies in *Acetabularia*. Bar, 100 nm. (*Source:* From Woodcock and Muller, 1973.)

blepharoplasts, which are essentially centrioles that form in the cell *de novo* (Sharp, 1914; Mizutani and Gall, 1966; Hepler, 1976; Duckett and Renzaglia, 1986; Kalnins, 1992; Keller et al., 2005; Nick, 2008a; Vaughn and Bowling, 2008). The cilia can be cut off from the basal bodies, and if they are, they regrow from the basal bodies synchronously in a couple of hours (Rosenbaum and Carlson, 1969). Thus, the assembly of all the proteins of the axoneme can be studied with extreme precision (Lefebvre and Rosenbaum, 1986).

While I am using the terms *cilia* and *flagella* to mean the same structure, I will use the term *flagellar motion* to mean a symmetrical snakelike motion that pushes the organism through a medium, and the term *ciliary motion* to characterize an asymmetrical whiplike motion that is reminiscent of the breaststroke. Ciliary motion either pulls the organism through a medium, or if it is anchored to a stable structure, pulls medium over it.

The same axoneme is capable of both ciliary and flagellar motion. Indeed, an increase in the ciliary Ca^{2+} concentration from 0.1 to 1 μM causes a change from a ciliary beat to a flagellar beat. This can be demonstrated by observing the shape of the ciliary beat in isolated axonemes placed in solutions containing different concentrations of Ca^{2+} (Hyams and Borisy, 1976). Environmental cues can stimulate the increase in intraciliary Ca^{2+}. For example, when cells of *Chlamydomonas* are subjected to an increase in light intensity they undergo a change from a ciliary beat shape to a flagellar beat shape. This is called the step-up photophobic response. Channelrhodopsin, a protein related to the photoreceptor for human vision, is the photoreceptor for this response (Foster, 2001; Foster and Smyth, 1980; Foster et al., 1984, 1988, 1989a,b, 1991; Symth et al., 1988; Saranak and Foster, 1994, 1997, 2000; Sineshchekov et al., 2002; Govorunova et al., 2004; Berthold et al., 2008; Hegemann, 2008). In intact cells, the light-stimulated reversal requires at least 1-μM extracellular Ca^{2+} and is inhibited by Ca^{2+} channel blockers (Schmidt and Eckert, 1976). Mechanical stimulation causes a similar reversal in *Paramecium* (Eckert, 1988). The contribution of Ca^{2+} in the coupling of a stimulus to a response is discussed in Chapter 12.

Due to the similarity between the cilia of *Paramecium* and the cilia of the respiratory organs, which are subjected to the nicotine from tobacco smoke, toxicity tests on *Paramecium* are routinely run by cigarette companies. One test, referred to as the "hanging drop *Paramecium* test," exposes a hanging culture of *Paramecium* to puffs of cigarette smoke to determine the number of puffs required to stop all ciliary movement. Another test exposes *Paramecium* overnight to a homogenate of smoke collected in water to determine the concentration of smoke required to kill a standard volume of *Paramecium*.

11.2 MICROTUBULES IN NONFLAGELLATED OR NONCILIATED CELLS AND THE DISCOVERY OF TUBULIN

In the 19th century, Walther Flemming and Eduard Strasburger saw filamentous structures in the cytoplasm that were associated with movement. As I discussed in Chapter 9, many physico-chemically oriented cytologists believed that the fibers, filaments, or kinoplasm were artifacts of the fixation process and could only be seen in fixed cells, or as diffraction artifacts in living cells when the optics were poorly adjusted. However, with the introduction of polarizing microscopes, it became apparent these fibers did exist in living cells, and were very dynamic (Inoué, 1951, 1952, 1959). Many of the filamentous structures or kinoplasm seen in the 19th century (Ranvier, 1875; Flemming, 1881; Strasburger, 1897, 1898; Strasburger and Hillhouse, 1911) turned out to be tubules when they were visualized with the electron microscope (see Figures 11.6 and 11.7; Bernhard and de Haven, 1956; Roth and Daniels, 1962; Ledbetter and Porter, 1963, 1964; Slautterback, 1963; Hepler and Newcomb, 1964; Esau and Grill, 1965).

Tubules were seen by electron microscopists in the cytoplasm and spindle of a variety of nonciliated cells. The diameters of the various tubules varied and the tubules were often misidentified as tubules of the endoplasmic reticulum (ER). Nevertheless, David Slautterback (1963) reasoned that the variation may be artifactual and may result from the shrinkage and swelling that can occur during fixation, dehydration, and staining. Slautterback (1963) and Ledbetter and Porter (1963) independently proposed to call all the tubules in the cell body and cilia that were approximately

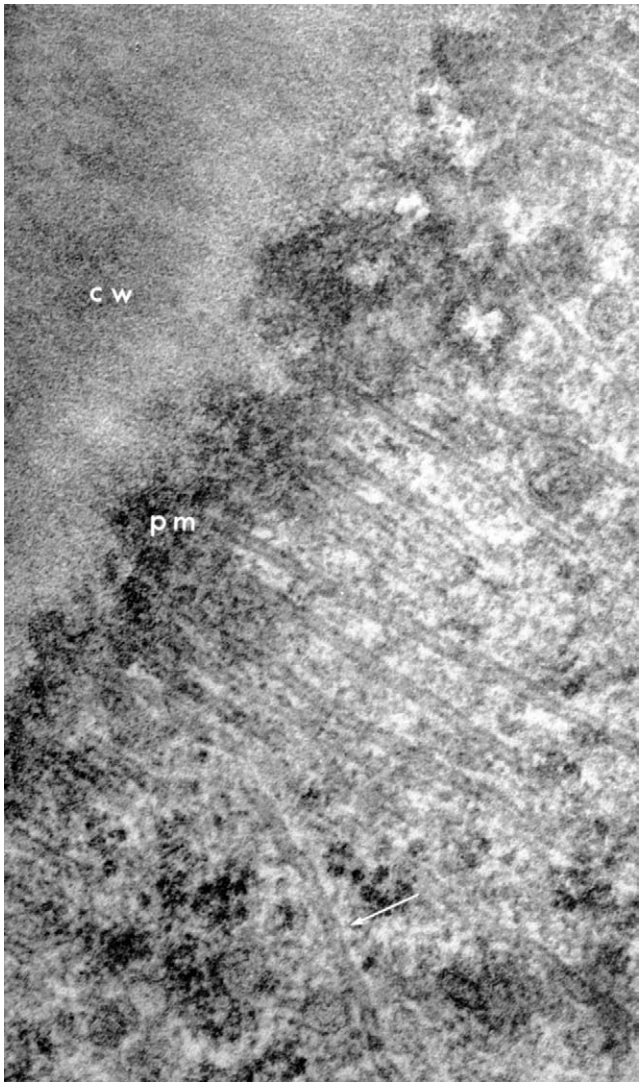


FIGURE 11.6 A grazing section of the extracellular matrix (CW), plasma membrane (pm), and cortex of a *Phleum* root cell showing microtubules that are coparallel and oriented circumferentially around the cell. (*Source:* From Ledbetter and Porter, 1963.)

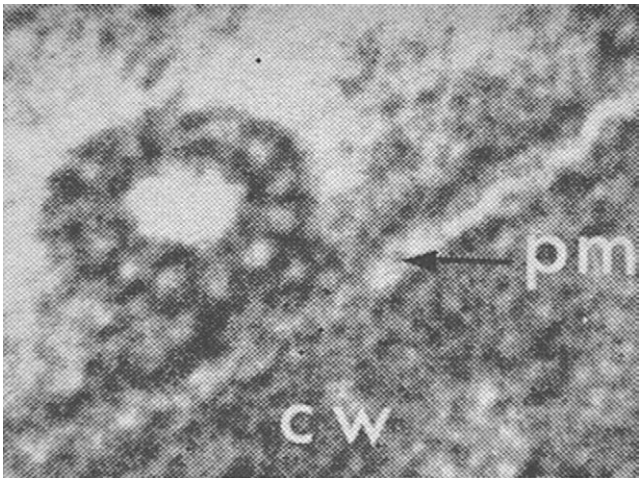


FIGURE 11.7 Transverse section of a microtubule from the cortex of the tannin-rich cells of *Juniperus chinensis*. The microtubule is composed of 13 protofilaments. The natural tannins in *Juniper* provide a natural contrasting agent for microtubules. ×740,000. (*Source:* From Ledbetter and Porter, 1964.)

24 nm in diameter *microtubules*. Slautterback thought the interior of the microtubules might function in the transport of water, ions, and small molecules, while Ledbetter and Porter considered that the microtubules might be involved in regulating cell shape. Later, Ledbetter and Porter (1964) and Porter and Tilney (1965) proposed that microtubules were involved in intracellular motility based on their intracellular distribution, their relationship to the kinoplasm, and the effects of colchicine on morphogenesis.

Colchicine is a fascinating drug that was used by the Egyptians around 1550 BCE for treating gout and rheumatism (Eigsti and Dustin, 1955). It is extracted from the autumn crocus (*Colchicum autumnali*), which is a native plant of Colchis, a region on the Black Sea. Colchis is where Jason and the Argonauts went to capture the Golden Fleece. There, Medea helped Jason capture the Golden Fleece by giving him various life-saving brews. Perhaps one of the brews contained colchicine!

Colchicine inhibits mitosis (Pernice 1889; Eigsti et al., 1949). However, Pernice initially believed that colchicine stimulated mitosis, since he found that after a dog had been treated with colchicine, there would be an inordinate number of mitotic figures in the cells of its stomach and intestine. However, by the 1930s, it was established that colchicine did not stimulate mitosis, but prolonged mitosis, thus increasing the chance of catching dividing cells in a given section (Wellensiek, 1939). Dustin (1947) hypothesized that colchicine directly caused the spindle fibers to break down. However, he could not make a very good argument, since at that time many people believed that the spindle was only an artifact of fixation. Later, electron micrographs taken by Seder and Wilson (1951) showed that colchicine broke down the spindle fibers; however, the quality of the fixation was too poor to put much stock in their interpretation.

Acceptance of the reality of spindle fibers and the direct effect of colchicine on them arose from the polarized light microscopy studies of Shinya Inoué (1951, 1952), who showed that colchicine caused a decrease in the birefringence of the spindle fibers. Later, Harris and Bajer (1965) combined polarization microscopy with electron microscopy and showed that spindle fibers were indeed microtubules. Pickett-Heaps (1967a) and Shelanski and Taylor (1967) showed that colchicine caused microtubules to disappear from plant cells and isolated mitotic apparati, respectively. Taylor and his colleagues, using ^{3}H-colchicine, purified the colchicine-binding protein (Borisy and Taylor, 1967; Shelanski and Taylor, 1967; Weisenberg et al., 1968), which they later named *tubulin* (Adelman et al., 1968). Plant tubulin differs from animal tubulin in terms of its drug sensitivity. For example, animal tubulin is depolymerized by

5×10^{-8} M colchicine and is insensitive to the herbicide oryzalin, while plant tubulin is depolymerized by millimolar concentrations of colchicine and is very sensitive to oryzalin (Morejohn and Fosket, 1984a,b, 1992).

Tubulin is a 110-kDa heterodimer that is composed of two globular subunits—α-tubulin and β-tubulin (Dustin, 1978)—encoded by two gene families (Breviario, 2008). Each subunit has a mass of approximately 55 kDa, and is associated with one molecule of guanosine triphosphate (GTP). Initially, it was difficult to get tubulin to polymerize in vitro or to isolate microtubules. Then Richard Weisenberg (1972) noticed that the ability of tubulin to polymerize depended on which pH buffer he used. He discovered serendipitously that the ability of microtubules to polymerize in vitro was correlated with the Ca^{2+}-binding ability of the buffer. Subsequently, he routinely added EGTA, a Ca^{2+} chelator, to the extraction buffer and polymerization solutions to obtain microtubules.

Ceteris paribus (all other factors being the same), isolated tubulin polymerizes into microtubules in vitro as long as the concentration of tubulin is greater than the critical concentration. Like actin filament growth, microtubule growth shows a lag phase, indicative of the need for nucleation. Nucleation in vivo may require a third form of tubulin known as *γ-tubulin* (Oakley and Oakley, 1989; Oakley et al., 1990; Oakley, 1992; Ledueña et al., 1992; Liu et al., 1993; McDonald et al., 1993; Joshi, 1994; Hoffman et al., 1994), which may also initiate microtubule branches along existing microtubules (Murata et al., 2005; Wasteneys and Collings, 2007).

The tubulin dimers are arranged in a specific orientation in microtubules, and consequently, microtubules, like microfilaments, are polar structures, and the two ends of microtubules are different. If purified tubulin is allowed to polymerize on fragments of a ciliary axoneme and the products are observed in the electron microscope, it can be seen that the microtubules elongate three times faster on one end than the other end. Growth at each end occurs when the polymerization reactions take place faster than the depolymerization reactions. As is the case with microfilaments, the rapidly polymerizing end is called the *plus end* and the slowly polymerizing end is called the *minus end*.

While GTP is necessary for polymerization, the hydrolysis of GTP is not, since nonhydrolyzable analogs of GTP support polymerization (Kirschner, 1978). Microtubules in the presence of GTP exhibit treadmilling just like actin filaments do in the presence of ATP. At the critical concentration, tubulin polymerization occurs at the plus end at the same rate that depolymerization occurs at the minus end, and, while the tubulin dimers are translocated along the microtubule from the plus end to the minus end, there is no net change in microtubule length (Kirschner, 1980; Bergen and Borisy, 1980; see Figure 10.6 in Chapter 10). While treadmilling could presumably cause the movement of a vesicle bound to a tubulin dimer from one end of a microtubule to the other, it is probably not involved in intracellular motility since the flux of subunits occurs at an excruciatingly slow rate of about 0.5 μm/h, far slower than the slowest known microtubule-mediated motile process (~1 μm/min).

In the dark-field microscope, microtubules reveal another fascinating behavior known as *dynamic instability* (Bayley, 1990). When microtubules assemble from pure tubulin, we can see the microtubules shrink and grow rapidly, alternating between the two states in a seemingly random manner. The growing end, or plus end, appears to switch between a slowly growing to a rapidly shrinking state. When the hydrolysis of GTP is slower than the rate of GTP-tubulin addition, GTP-tubulin dimers accumulate at the plus end and form a GTP-tubulin cap. GTP-tubulin dissociates 100 times less readily than guanosine diphosphate (GDP)-tubulin, and thus, when there is a GTP-tubulin cap, the microtubule is stable. However, when the rate of hydrolysis of GTP is greater than the rate of polymerization, the microtubule becomes capped with GDP-tubulin, and the microtubules rapidly depolymerize. Once the rapid depolymerization begins, the GTP-tubulin cap is hard to regain and the shrinking microtubule usually completely depolymerizes (Carlier, 1991). Shaw et al. (2003) characterized the dynamic behavior of cortical microtubules in living epidermal cells, and they found that the plus end grows and shrinks at a rate of 3.69 and 5.80 μm/min, respectively, while the minus end grows and shrinks at a rate of 1.98 and 2.78 μm/min, respectively.

Microtubule polymerization and depolymerization can be affected by many natural products. These include vinblastine and vincristine (isolated from *Catharanthus roseus*), podophyllotoxin (isolated from *Podophyllum peltatum*), taxol (isolated from *Taxus brevifolia*) and griseofulvin (isolated from *Penicillium griseofulvum*). Furthermore, many herbicides, including IPC (isopropyl-N-phenylcarbamate), CIPC (N-[3-chlorophenyl]carbamate) and trifluralin (trifluoro-2,6-dinitro-N,N-dipropyl-*p*-toluidine), APM (aminoprophos methyl), and oryzalin affect microtubule polymerization or organization.

11.2.1 Temporal and Spatial Localization of Microtubules in Animal and Plant Cells

In vivo, microtubules always originate from regions of the cell known as *microtubule organizing centers* (MTOCs; Figure 11.8). In many mammalian cells, the microtubules typically radiate from a single region known as the *cell center* or *centrosome* (Mazia, 1987), while in plant cells, microtubules originate from many spatially separated MTOCs. While basal bodies and centrioles are MTOCs with an easily discernable structure, most MTOCs and the pericentriolar regions of the centrosome are amorphous areas. Studies in which the readily visible structures in the MTOCs have been removed genetically or mechanically

FIGURE 11.8 Immunofluorescence micrograph showing microtubules in an internodal cell of *Nitella tasmanica* regrowing from MTOCs following the removal of oryzalin, a microtubule-depolymerizing agent. Bar, 20 μm. (*Source:* From Wasteneys and Williamson, 1989.)

show that the readily visible structures are more efficient in generating microtubule arrays than the amorphous regions alone (Ambrose and Cyr, 2007). MTOCs can be identified by depolymerizing microtubules with various microtubule-depolymerizing agents, and after removing the agent, watching where the microtubules reform (Falconer et al., 1988; Wacker et al., 1988; Cleary and Hardham, 1988; Wasteneys and Williamson, 1989). The MTOCs themselves are dynamic and move throughout the cell (Chan et al., 2003).

We can detect the polarity of microtubules in the cell by adding free tubulin molecules to the existing microtubules. Under special conditions, the tubulin does not add to the ends, but forms curved protofilament sheets on the sides. In cross-section, the sheets resemble hooks, and depending on the polarity of the microtubule, the hooks appear either clockwise or counterclockwise. When we look at a microtubule from the plus end, the hooks are oriented in the clockwise direction (Euteneuer and McIntosh, 1981; Schliwa, 1984). Using this method, we can see that typically the plus ends are distal to the MTOCs and the minus ends are embedded in the MTOCs. The polarity of microtubules can also be determined by decorating them with dynein (Telzer and Haimo, 1981).

In some, but not all animal cells in interphase, the microtubules radiate from the centrosome in the center of the cell. In most cases, the end of the microtubules attached to the centrosome is the minus end, and the distal end is the plus end. This is known as the *plus-end distal arrangement*. As we will see in the following, the organization of the microtubules is important for moving organelles to the correct location. Roger Penrose (1994) has suggested that the microtubule cytoskeleton in brain cells has an even bigger role. He thinks that it is the material basis of the mind!

In higher plant cells, during interphase, microtubules occur in the cortical cytoplasm, and have a transverse orientation relative to the long axis of the cell (see Figure 11.9; Vesk et al., 1996: Kumagai et al., 2001). The cortical microtubules in the end wall are oriented randomly. As the cell elongates, the cortical microtubules along the cell flanks become oriented longitudinally (see Figure 11.10; Lloyd,

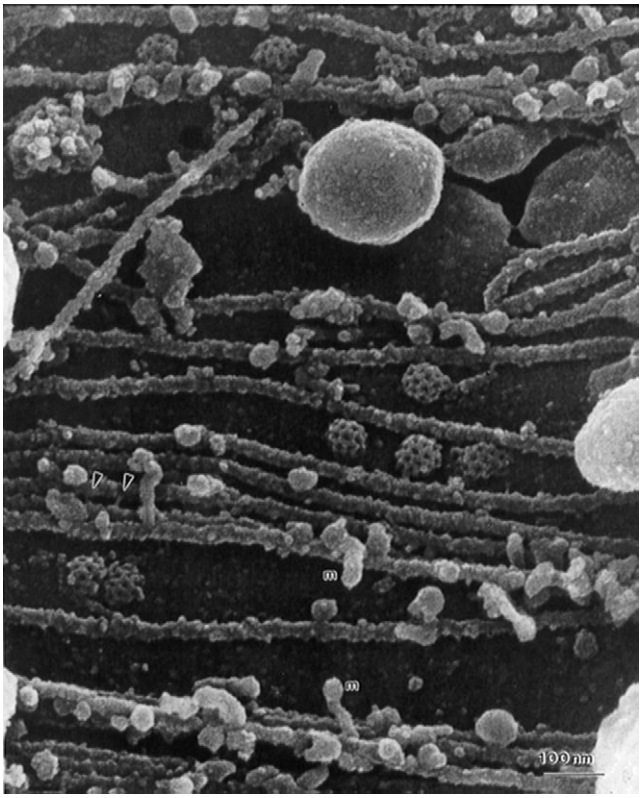


FIGURE 11.9 Cortical microtubules in an onion root cell that have been freeze-fixed, freeze-fractured, dried, and observed with a field-emission scanning electron microscope. There are many cross-bridges (arrowheads) and appendages (m) on the microtubules. The plasma membrane is in the background and coated vesicles can be seen. Bar, 100 nm. (*Source:* From Vesk et al., 1996.)

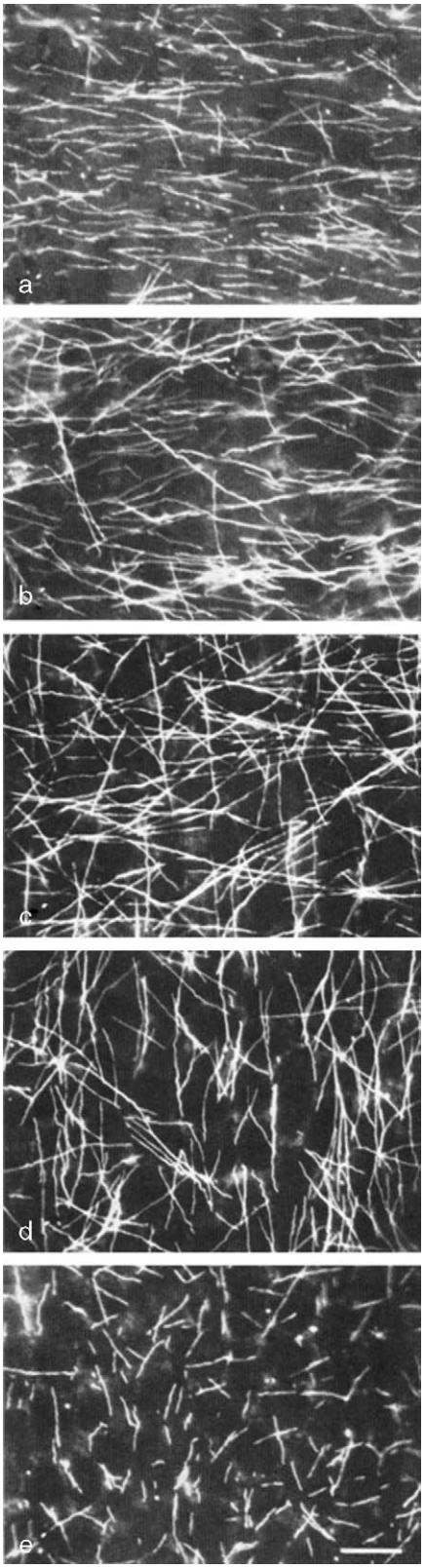


FIGURE 11.10 Immunofluorescence micrograph showing the transverse orientation of microtubules in internodal cells of *Nitella tasmanica*: (a) taken from the youngest internodal cell, and (b–e) taken from cells that are successively older. Bar, 10 μm. (*Source:* From Wasteneys and Williamson, 1987.)

1987). In naturally wall-less plant cells, the microtubules typically are oriented parallel to the long axis of the cell (Pickett-Heaps, 1975). Tobacco BY-2 cells are particularly good material in which to study microtubules in vitro and in vivo (Sonobe et al., 2001; Nagata et al., 2004; Dhonukshe et al., 2005).

Just before prophase, a unique arrangement of microtubules known as the *preprophase band* forms in the cortex of most plant cells (see Figures 11.11 and 11.12; Northcote, 1967). Pickett-Heaps and Northcote (1966b) found the preprophase band while they were looking for a cytoplasmic structure that may be related to the plane of cell division:

> *It seemed most likely that spindle organization, and in particular microtubule synthesis, might be observable in what was going to be the future polar zone of these cells, but long and careful scrutiny of these regions failed to reveal any changes or structures that could be implicated in mitosis. However, a band consisting of a large number of microtubules was found near the wall of the cell, far removed from the polar zone.*

The preprophase band is formed from the gradual rearrangement of the randomly or transversely oriented cortical microtubules into a tightly packed transverse band (Mineyuki et al., 1989). The nucleus may control the position of the preprophase band, since when the nucleus of an *Adiantum* protonema is displaced by centrifugation, the preprophase band appears at the new nuclear position (Murata and Wada, 1991; Wada, 1992). The nucleus may also be responsible for the disintegration of this transient structure since the preprophase band does not break down at metaphase if the nucleus is centrifuged away from it. In all cases, the position of the preprophase band predicts the site of cell plate formation (Pickett-Heaps, 1969b; Cleary and Smith, 1998; Mineyuki, 1999).

During prophase, the nuclear envelope serves as an MTOC, and microtubules polymerize around the nucleus, forming the prophase spindle (Mizuno, 1993). The nuclear envelope breaks down at prometaphase and the microtubules permeate the nucleus and form the spindle fibers (Zhang et al., 1990b). The shape of the spindle is determined by the compactness of the MTOCs, and consequently, animal spindles are more pointed at the poles than plant spindles are. Basically, there are two groups of microtubules in the spindle, ones that terminate at the kinetochores of chromosomes and ones that do not, although the two groups are interconnected (Euteneuer and McIntosh, 1980; McIntosh and Euteneuer, 1984; Bajer and Mole-Bajer, 1986; Kubiak et al., 1986; Schibler and Pickett-Heaps, 1987; Palevitz, 1988b; Fuge and Falke, 1991). As a rule, the microtubules in each half spindle are oriented so that their minus ends are embedded in the poles, and their plus ends are near the chromosomes (Euteneuer and McIntosh, 1981a,b).

Following nuclear division, a group of microtubules known as the *phragmoplast* organizes the developing cell plate (Nemec, 1899; Inoué, 1964; Esau and Grill, 1965;

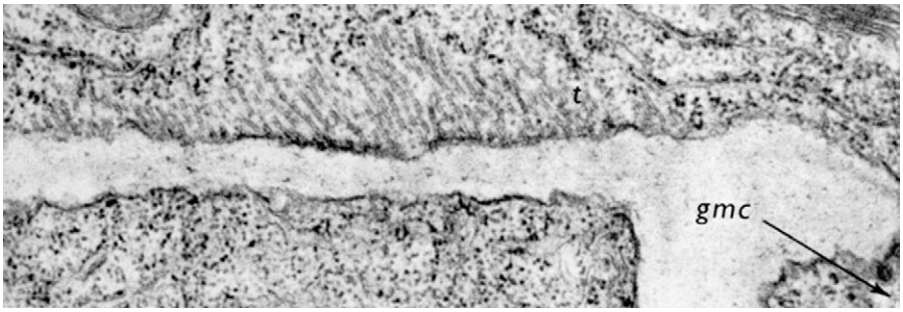


FIGURE 11.11 Electron micrograph of preprophase band in an epidermal cell of wheat that will be cut off a subsidiary cell. gmc, guard mother cell. ×36,000. (*Source:* From Pickett-Heaps and Northcote, 1966a.)

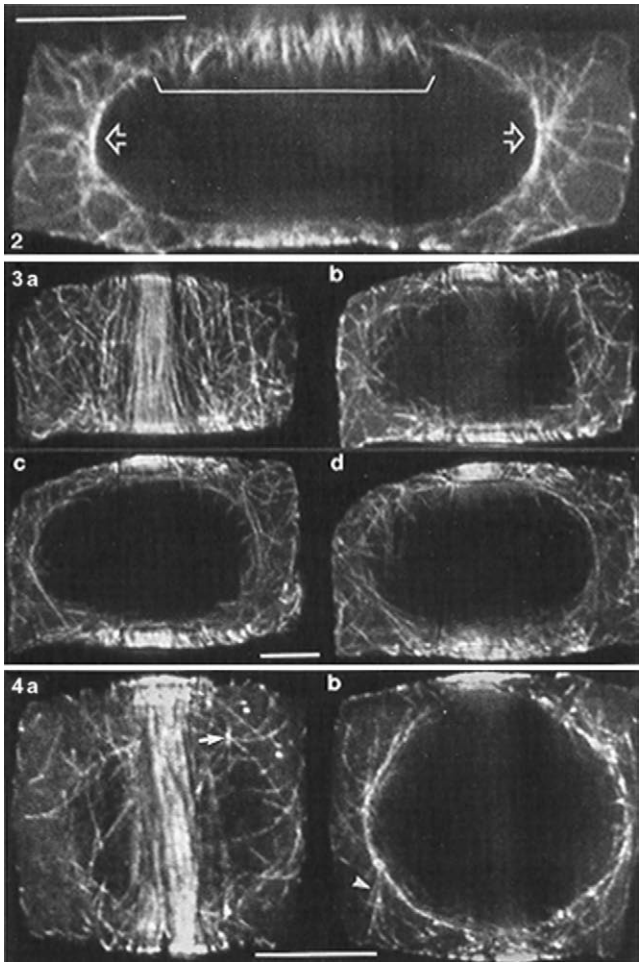


FIGURE 11.12 Immunofluorescence confocal micrographs showing the development of a preprophase band in the root-tip cells of wheat. Bar, 10 μm. (*Source:* From Gunning, 1992.)

Hepler and Newcomb, 1967; Hepler and Jackson, 1968, 1969; Palevitz, 1987a,b; Shibaoka, 1992). The phragmoplast begins to form at the center of the future cell plate and moves in a centrifugal manner in most cases. However, in *Haemanthus* endosperm cells, the phragmoplast begins as a ring at some distance from the center, and then moves both centrifugally and centripetally. The phragmoplast moves slightly ahead of the developing cell plate. The plus ends of the microtubules are embedded near the forming cell plate, and the minus ends stick out (Enteneuer and McIntosh, 1980). Microtubules can also be visualized in transgenic plant cells transformed with the gene for either tubulin or microtubule-associated proteins fused to the green fluorescent protein (GFP; Marc et al., 1998; Ueda et al., 1999; Granger and Cyr, 2000; Dixit and Cyr, 2002; Chan et al., 2003; Shaw et al., 2003; Dhonukshe et al., 2005).

When microtubule organization is observed with fluorescence microscopy, we must remember that fluorescence-microscopic images may be misleading since microtubules are only 24 nm in diameter, but appear to be about 240 nm in diameter in the light microscope due to diffraction (Williamson, 1990, 1991; Wayne, 2009). Therefore, microtubules near each other, but not touching, may appear to be connected to each other or grouped in bundles. Thus, while light microscopy gives a good impression of the three-dimensional architecture of the microtubule cytoskeleton, electron microscopy of serial sections is essential for visualizing the true spatial relationship of microtubules.

The dynamic nature of the microtubule arrangements that occur throughout the cell cycle may result from a change in the distribution of MTOCs or of capping proteins that stabilize the plus ends of microtubules so that they do not depolymerize as a result of dynamic instability. The polymerization and depolymerization of microtubules as well as their organization can be modified by microtubule-associated proteins (Hotani and Horio, 1988; Cyr and Palevitz, 1989; Cyr, 1991a,b; Yasuhara et al., 1992; Chang Jie and Sonobe, 1993; Chan et al., 2003; Shaw et al., 2003).

11.2.2 Characterization of Microtubule-Associated Motor Proteins

When Melanie Pratt (1980) discovered dynein in nonciliated cells, the exciting implication was that the molecule could potentially participate in intracellular motility (Asai and Wilson, 1985; Vallee et al., 1988). Cytoplasmic dynein, like ciliary dynein, is a high–molecular mass protein with a

similar head and tail structure. When cytoplasmic dynein is immobilized on a glass cover slip, it is capable of translocating a microtubule in the direction of its plus end. That is, if the microtubule were immobilized, the dynein would walk to the minus end of the microtubule (Paschal et al., 1987; Lye et al., 1989). Dynein induces the movement of vesicles from the plus end to the minus end of microtubules at a rate of 1.25 μm/s. Both motility and the dynein ATPase activity are inhibited by 1 mM N-ethylmaleimide, 25 μM vanadate, and the adenine derivative erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA).

Since vesicles can move both directions on a single microtubule, it seemed likely that another motor existed in cells that can walk to the plus end of microtubules. Kinesin is just such a motor (Sheetz, 1989). Like dynein, it is a mechanochemical enzyme that converts the chemical energy of ATP into mechanical energy. Kinesin was first found in squid axons, but probably occurs in most cells (Sheetz, 1989; Mitsui et al., 1993; Hoyt, 1994). It was discovered by squeezing out the axoplasm from the axon of a squid and watching organelles move along single microtubules with the aid of video-enhanced light microscopy. Adenylyl-imidodiphosphate (AMP-PNP), a nonhydrolyzable analog of ATP, stopped the movement of organelles, and they become tightly bound to the microtubules. This property helped in the identification and purification of the motor protein. Vale et al. (1985a,b,c) identified kinesin as a protein that bound to microtubules in the presence of AMP-PNP, but was released upon the addition of ATP. Like dynein and myosin, kinesin is a large elongated protein that contains two heads and a tail (Vale, 1987; Sheetz, 1989). Kinesin transports vesicles from the minus end to the plus end at a rate of approximately 0.5 μm/s. In contrast to dynein, kinesin is relatively insensitive to 1 mM N-ethylmaleimide (NEM) and 25 μM vanadate.

There is a family of kinesin-related proteins, all with similar gene sequences and with similar pharmacological properties (Richardson et al., 2006; Ambrose and Cyr, 2007; Lee and Liu, 2007). However, one of them, known as *Ncd*, is a minus end–directed motor and not a plus end–directed motor as is the original kinesin (Sharp et al., 1997; Sablin et al., 1998; Liu and Lee, 2001). A single amino acid substitution from asparagine to lysine in the neck region of the protein is sufficient to transform Ncd from a minus end–directed motor to a plus end–directed motor (Endow and Higuchi, 2000).

Another translocator protein has been isolated from *Reticulomyxa* (Euteneuer et al., 1988). It has a high molecular mass and binds to microtubules in the absence of ATP, and is released in the presence of ATP. This protein causes bidirectional movement at a rate of 3.6 μm/s. Phosphorylation of this protein by cyclic AMP-dependent protein kinase converts this bidirectional motor to a unidirectional motor.

Molecular biology has taught us that there are many genes that encode dyneinlike proteins, kinesinlike proteins, and proteins that have some properties of each. In general, each motor protein has a similar structure, even when the amino acid sequence differs greatly (Kull et al., 1996; Sablin et al., 1996). It is likely that nature is just as promiscuous when it comes to motor proteins as it is with ion channels, and sequences of DNA that code for functional domains in proteins were mixed and matched to form chimeric motor proteins that will transport a given cargo in the desired direction along a microtubule track (Gilbert, 1978; Doolittle, 1995).

11.3 FORCE-GENERATING REACTIONS INVOLVING TUBULIN

11.3.1 Sliding

The movement of dynein and kinesin along a microtubule is thought to occur in a similar manner to the way myosin slides along an actin microfilament (Warner et al., 1989). Kamimura and Takahashi (1981) and Oiwa and Takahashi (1988) have measured the force generated by the microtubule/dynein interaction using the glass microneedle method (see Chapter 10). They held a single demembranated sea urchin sperm flagellum between two microneedles, and measured the amount the needle bent when the flagella was reactivated with Mg^{2+}-ATP. They found that a single microtubule/dynein association could produce a force of about 3 pN (assuming that there are 83 dynein molecules per μm flagellum). Using laser tweezers, Ashkin et al. (1990) estimate that a single dynein molecule exerts a force of approximately 2.6 pN. This is in the same ballpark as the force resulting from an actin/myosin association, and consequently, a single dynein molecule can move a 1-μm-diameter vesicle through a non-Newtonian cytoplasm that has a yield value of 0.5 Pa.

The force exerted by a single kinesin molecule has also been measured with laser tweezers. Block et al. (1990) estimated the force exerted by a single kinesin molecule to be between 0.5 and 5 pN. Moreover, a special interferometric version of the laser tweezers suggests that the kinesin molecule moves 8 nm with every stroke (Svoboda et al., 1993; Svoboda and Block, 1994). Thus, a single kinesin molecule, like dynein and myosin, is capable of moving a vesicle that is 1 μm in diameter through a non-Newtonian cytoplasm that has a yield value of 0.5 Pa.By contrast, it appears that more than one kinesin molecule is required to move a vesicle though the cytoplasm of some cells, including kidney epithelial cells, since the measured maximum force, steplike movement, and rate of ATP hydrolysis for a single kinesin molecule is not great enough (Holzwarth et al., 2002).

11.3.2 Polymerization/Depolymerization

The polymerization/depolymerization of microtubules can provide a motive force for cytoplasmic movement. It is possible that the depolymerization of microtubules plays a role in the movement of chromosomes in mitosis (see Chapter 19).

11.4 TUBULIN-BASED MOTILITY

Microtubules are involved in moving organelles around the cell. In Chapter 19, I will discuss the role of microtubules in moving chromosomes during mitosis. The involvement of microtubules in positioning the Golgi apparatus has been studied in Chinese hamster ovary (CHO) cells. The microtubule in these interphase cells have a plus-end distal arrangement typically found in centrosome-containing cells, and the minus ends are embedded in the centrosome adjacent to the central nucleus. When isolated Golgi stacks are added to semi-intact, permeabilized CHO cells, they are captured and transported to the nuclear periphery. Golgi capture and translocation is inhibited by nocodazole, a microtubule inhibitor, indicating that microtubules are necessary for the capture and translocation of the Golgi apparati. Capture and translocation also requires ATP. The capture and translocation do not occur in CHO cells that have been immunodepleted of dynein. Moreover, adding back dynein to the dynein-depleted CHO cell models yields a functional system. Thus, dynein is the translocator that moves the Golgi apparatus from the plus ends of the microtubules to the minus ends so that the Golgi apparatus can go to its typical position in the nuclear periphery of nonmitotic mammalian cells (Corthesy-Theulaz et al., 1992).

Nuclear migration is a prerequisite for asymmetric cell division, and in many plant cells, microtubules are associated with the migrating nucleus. Microtubule depolymerizing agents prevent nuclear migration, indicating that microtubules are involved in providing the tracks for nuclear migration (Kiermayer and Hepler, 1970; Kiermayer, 1972; Schnepf et al., 1982; Mineyuki and Furuya, 1985).

While the majority of movement in pollen tubes can be attributed to actin and myosin, the movement of some organelles is driven by dyneinlike and kinesinlike motors, which are differentially localized along microtubules in various regions of the cell (Tiezzi et al., 1992; Cai et al., 1993, 2001, 2000; Moscatelli et al., 1995, 1998; Romagnoli et al., 2003).

Actin and myosin are typically responsible for cytoplasmic streaming. However, microtubules occasionally play a role too. Microtubules are involved in organizing the actin microfilaments, which provide the tracks for cytoplasmic streaming in *Hydrocharis* (Tominaga et al., 1997). Moreover, microtubules are directly involved in powering cytoplasmic streaming in the oocytes of *Drosophila* (Theurkauf, 1994) and the rhizoids of the alga *Caulerpa* (Manabe and Kuroda, 1984; Kuroda and Manabe, 1983). In *Caulerpa*, the cytoplasm streams at a rate of about 3 μm/s, and is inhibited in this cell by colchicine, but not by cytochalasin. Microtubules also provide the tracks for cytoplasmic streaming in a *Chlorella*-containing autotrophic species of *Paramecium* (Sikora and Wasik, 1978; Wasik and Sikora, 1980; Cohen et al., 1944; Nishihara et al., 1999). Microtubules are also involved in the intracellular transport of viral movement proteins (Laporte et al., 2003; Heinlein, 2008).

11.5 MICROTUBULES AND CELL SHAPE

It has been known since the 1930s that colchicine causes plant organs and cells to lose their cylindrical form and swell isodiametrically. These early studies showed that colchicine has an effect on the deposition of the extracellular matrix (Eigsti and Dustin, 1955). These results were extended in 1962, when Paul Green showed that colchicine caused the cylindrical cells of *Nitella* to become isodiametric, and proposed that a spindle fiberlike element may be responsible for ordering the wall microfibrils. Soon after, Hepler and Newcomb (1964) and Ledbetter and Porter (1963) independently visualized microtubules approximately 0.0175–1 μm from the plasma membrane—a position from where the microtubules may be able to affect cellulose microfibril orientation (VandenBosch et al., 1996). The cortical microtubules were parallel to each other, and as Ledbetter and Porter (1963) wrote, "They are … like hundreds of hoops around the cell."

The orientation of cellulose microfibrils is thought to regulate the direction of cell growth (see Chapter 20; Green, 1969, 1988; Laskowski, 1990; Harold, 1990; Williamson, 1990, 1991; Kropf et al., 1997; Nick, 2000, 2008; Inada and Shimmen, 2001; Inada et al., 2002). Randomly arranged microfibrils will give rise to a roughly spherical cell, like a cortical parenchyma cell. Cells with transversely oriented microfibrils will elongate in a direction perpendicular to the long axis of the microfibrils, and will give rise to cylindrical cells like those of the procambium. Microtubules probably direct the orientation of cellulose microfibril deposition. This hypothesis is supported by the following observations:

1. Microtubules are parallel to cellulose microfibrils and predict the orientation of cellulose microfibrils.
2. Agents that inhibit microtubule polymerization or organization affect cellulose microfibril orientation.
3. Extracellular stimuli (e.g., light, gravity, hormones) that affect cellulose microfibril orientation also affect microtubule orientation.

While many studies support the relationship between microtubule orientation and growth, it must be remembered that growth requires the coordination of many cellular processes, and consequently, the transverse orientation of microtubules is not sufficient in itself for elongation (Kropf et al., 1997).

11.5.1 Apical Meristems

Let us look at the organization of microtubules and microfibrils in the cells of the apical meristem (Hanstein, 1870). Schmidt (1924) proposed that the apical meristem of angiosperms was organized into two main layers. The surface layer or layers, which he called the *tunica*, divided anticlinally and gave rise to the epidermis. The internal layer, which he called the *corpus*, gave rise to the rest of the plant

body. This theory was later incorporated into the cytohistological zonation theory (Foster, 1938), which describes many types of meristems, and is based on differences in cell staining. According to the cytohistological theory, the apical meristem is divided into the distal axial zone, the proximal axial zone, and the peripheral zone. The leaf primordia and procambium arise from the peripheral zone. The proximal axial zone becomes a rib meristem and gives rise to the pith.

Sakaguchi et al. (1988a,b, 1990) were interested in determining what causes the specific division and expansion patterns seen in the apex. They found that there is a relationship between the orientation of the microtubules in the tunica, corpus, and rib meristem cells as determined with immunofluorescence microscopy, and the orientation of the cellulose microfibrils in these cells as determined by polarization microscopy. In the meristem region, the microtubules and microfibrils are coparallel. Briefly, the orientations of the microtubules and microfibrils in the tunica cells are anticlinal (perpendicular to the surface); in the corpus cells, the orientations are random; and in the rib meristem, the orientations are transverse to the axis of the plant (Figure 11.13).

These data support the hypothesis that cortical microtubules determine the alignment of adjacent cellulose microfibrils. The reinforcement exerted by the cellulose microfibrils causes the cell to expand at right angles to the long axis of the cellulose microfibrils, and the orientation of the cortical microtubules determine the structure of the stem apex.

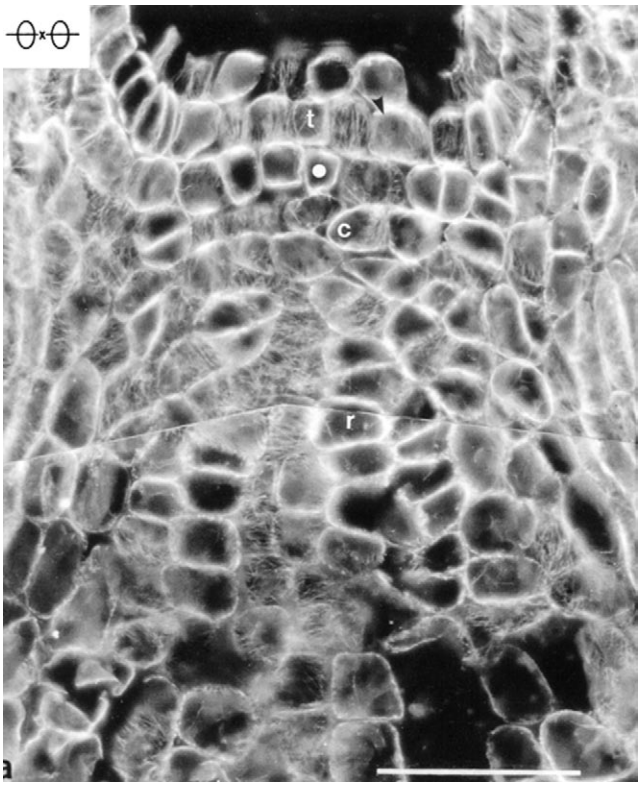


FIGURE 11.13 Immunofluorescence image of microtubules in the apical meristem of *Vinca major* showing the orientation of microtubules in the tunica (t), corpus (c), and rib meristem (r). Bar, 50μm. (*Source:* From Sakaguchi et al., 1988a.)

11.5.2 Tracheary Elements

Tracheary elements are the cells that comprise the water-conducting system of the plant, which is what Brisseau-Mirbel (1808) referred to as *tubes and spirals*. Tracheary elements are dead at maturity, but of course, they are alive during their development. They have very elaborate and taxonomically distinctive cell wall patterns (Bierhorst, 1971). These include annular, spiral, scalariform, and reticulate thickenings (Figure 11.14). Much elegant work has been done on determining the contribution of microtubules to cellulose microfibril orientation in these cell types.

In the root apex of *Azolla*, the fate of every cell is known (Gunning et al., 1978a,b,c). Using this material, Hardham and Gunning (1979, 1980) determined the orientation of microtubules in the cells that would give rise to the tracheary elements. In this case, the microtubules were coparallel with the microfibrils in the developing tracheids, and moreover, they predicted the site and orientation of microfibril deposition. Colchicine also prevented the normal-ordered deposition of microfibrils, and following colchicine treatment, the normal annular rings were not formed and the secondary wall material was deposited in irregular masses. This is good evidence that microtubules determine the orientation of cellulose microfibrils in the tracheids of *Azolla*.

Microtubules are oriented parallel to the orientation of cellulose microfibrils in the tracheary elements of many species (Figure 11.15), and microtubule depolymerizing or disorganizing agents disrupt normal wall deposition in these cells (see Figure 11.16; Pickett-Heaps, 1967a; Roberts and Baba, 1968; Hepler and Fosket, 1971; Robinson and Quader, 1982; Kobayashi et al., 1988; Falconer and Seagull, 1988). The concentric orientation

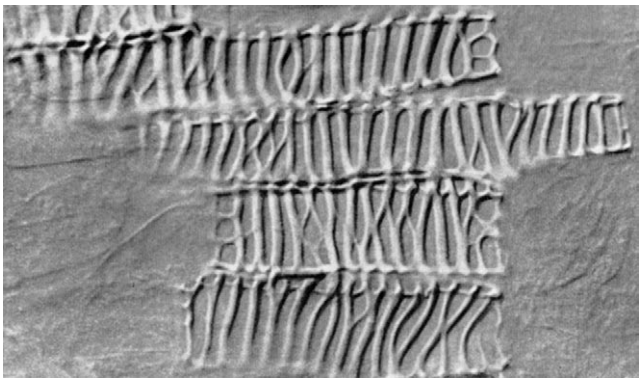


FIGURE 11.14 Nomarski differential interference contrast micrograph of a wound xylem element of *Coleus* showing the secondary wall structure. ×1400. (*Source:* From Hepler and Foskett, 1971.)

of the cellulose microfibrils in bordered pits of conifer tracheids is correlated with the concentric orientation of the microtubules beneath (Uehara and Hogetsu, 1993).

Interestingly, the cell wall deposition patterns and the formation of pits in one tracheary element are coordinated with those in the adjacent cells, indicating the possibility of transcellular or tissue level communication (Sinnott and Bloch, 1944, 1945; Witztum, 1978).

11.5.3 Guard Cells

Guard cells are another specialized cell type that have an unusual but very characteristic cell wall morphology. The cellulose microfibrils are arranged radially around the cell, and this arrangement, known as *radial micellation*, is important for proper stomatal function. Using polarization light microscopy combined with electron microscopy, Palevitz and Hepler (1976) showed that microtubules are coparallel with cellulose microfibrils and they are both arranged radially (Figure 11.17). Furthermore, treatment of the cells with microtubule antagonists prevents the normal development of radial micellation and induces a random arrangement of microfibrils (Figure 11.18). Interestingly, γ-tubulin, which is associated with sites of microtubule initiation, is present at

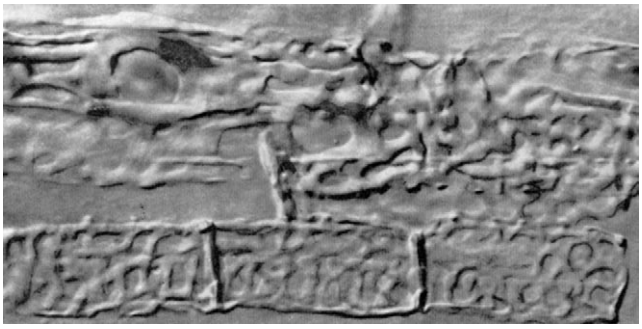


FIGURE 11.16 Nomarski differential interference contrast micrograph of a wound tracheary element of *Coleus* treated with colchicine. The secondary wall structure is unorganized. ×1400. (*Source:* From Hepler and Foskett, 1971.)

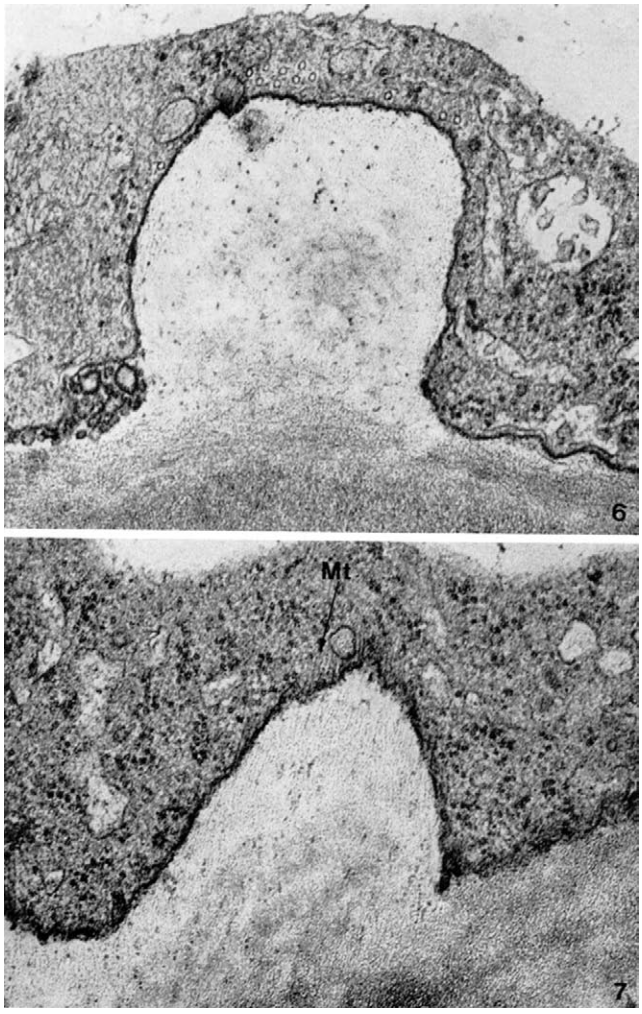


FIGURE 11.15 Transverse (top) and oblique (bottom) sections through a secondary-wall thickening of a wound tracheary element of *Coleus*. In the transverse section, transverse sections of microtubules are evident. In the oblique section, coparallel microtubules and cellulose microfibrils are evident. ×60,000. (*Source:* From Hepler and Foskett, 1971.)

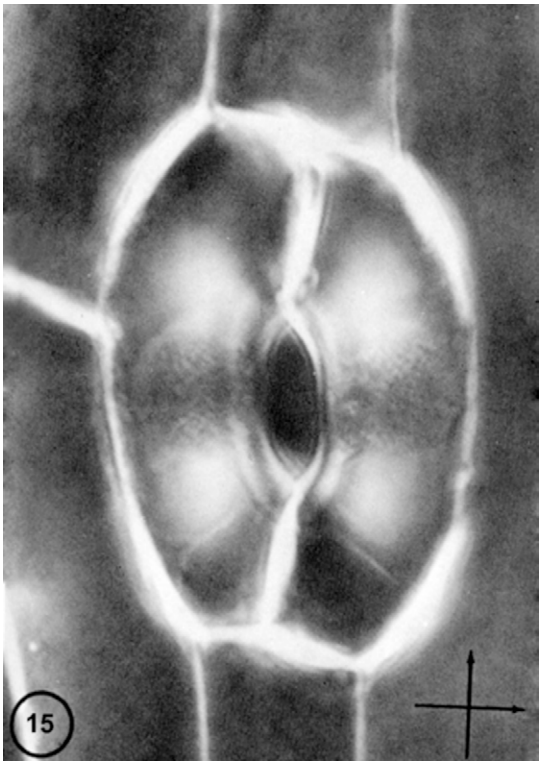


FIGURE 11.17 The guard cells of *Allium* viewed with polarization microscopy. The pattern of birefringence indicates the microfibrils in the extracellular matrix are arranged radially. ×1260. (*Source:* From Palevitz and Hepler, 1976.)

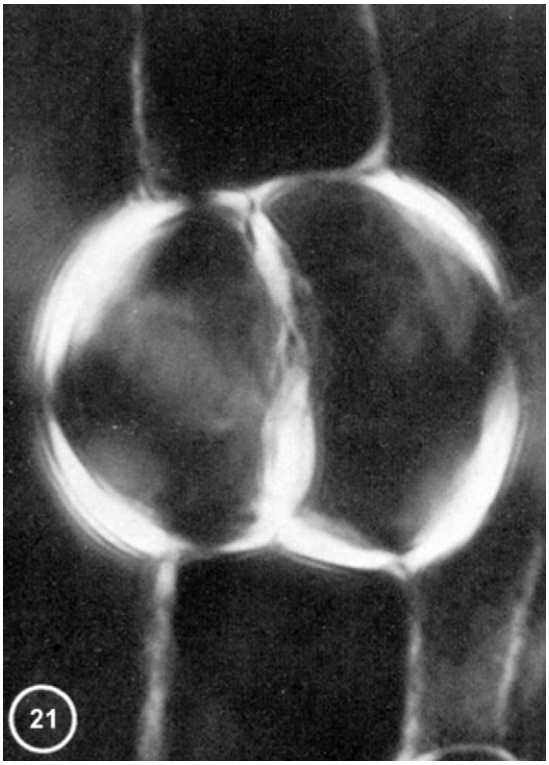


FIGURE 11.18 The guard cells of *Allium* that had been treated with colchicine during development. The pattern of birefringence indicates the microfibrils in the extracellular matrix are arranged randomly. ×1503. (*Source:* From Palevitz and Hepler, 1976.)

the ventral side of the cell. Thus, the ventral side is the site from which the microtubules radiate (McDonald et al., 1993).

Microtubules probably influence the shape of all nonspherical plant cells. For example, the morphogenesis of the highly branched and lobed mesophyll cells also depends on microtubules. The microtubules are coparallel with the microfibrils that surround and "reinforce" the lobes (Jung and Wernike, 1990; Panteris et al., 1993, Wernicke et al., 1993).

11.5.4 Extracellular Matrix of *Oocystis*

Perhaps the most dramatic example of a microtubule–microfibril relationship occurs in the alga *Oocystis* (Sachs et al., 1976; Quader et al., 1978). In this alga, the extracellular matrix is polylaminate and layers of microfibrils regularly change their orientation by 90 degrees (Figure 11.19). This is correlated with a 90-degree change in microtubule orientation. Colchicine inhibits the 90-degree change in microfibril orientation. However, the newly formed microfibrils are not random, but are deposited in the same alignment that they were before colchicine treatment (see Figure 11.20; Robinson et al., 1976b; Grimm et al., 1976). Many other microtubule depolymerizing or disorganizing agents have similar effects (Robinson and Herzog, 1977; Quader, 1986). Perhaps we can conclude that microtubules are responsible for initiating microfibril orientation or changing a given pattern, but are not required for the maintenance of a given orientation.

11.5.5 Mechanism of Microtubule-Mediated Cellulose Orientation

How do microtubules influence the orientation of cellulose microfibrils? Cellulose-synthesizing complexes can be readily visualized as rosettes on the P-leaflet of freeze-fractured plasma membranes (Brown, 1985). What is the spatial relationship between these rosettes and the cytoplasmic microtubules? Do the rosettes ride on the microtubules, or do the microtubules form membrane channels or riverbeds through which the rosettes ride? There is currently no simple answer to these questions and the final answers may depend on the cell type and whether the cell is making a primary or secondary wall. Support for the direct involvement of microtubules comes from fluorescence studies in which the cellulose synthesizing complexes seem to ride on top of the microtubules (Lloyd, 2006; Paredez et al., 2006), although the limit of resolution of the light microscope does not allow one to strongly make this case. The direct involvement of microtubules is also supported by the fact that a microtubule-associated protein is one target of an inhibitor of cellulose synthesis (Rajangam et al., 2008). On the other hand, Giddings and Staehelin (1988), using rapid freeze-fixation combined with freeze-fracture and freeze-etching, show that two to seven rosettes, spaced at a constant interval of 30 nm, appear in a row in *Closterium*. Then they subjected the plasma membrane to prolonged etching, which caused the plasma membrane to collapse—except in the areas supported by microtubules. They find that the rosettes are always found adjacent to or between microtubules, but never directly over them. Occasionally they have observed filaments extending between the microtubule and the plasma membrane. They interpret these data to mean that the microtubules make canals or domains in the membrane through which cellulose-synthesizing centers move.

Once the cellulose begins polymerization, it continues in the same direction, thus becoming independent of the microtubule orientation. This may be why microtubules seem to be responsible for the initiation or reorientation of microfibrils, but not the maintenance of a specific orientation. Once initiated, the rigid microfibrils keep growing in the same direction, and the polymerization of cellulose provides the motive force for the movement of the cellulose-synthesizing centers through the canals (Diotallevi and Mulder, 2007).

11.5.6 Tip-Growing Cells

The correlation between microtubules and cellulose microfibrils is not clear in the tip-growing root hairs of *Equisetum hyemale*. Perhaps in this and other tip-growing

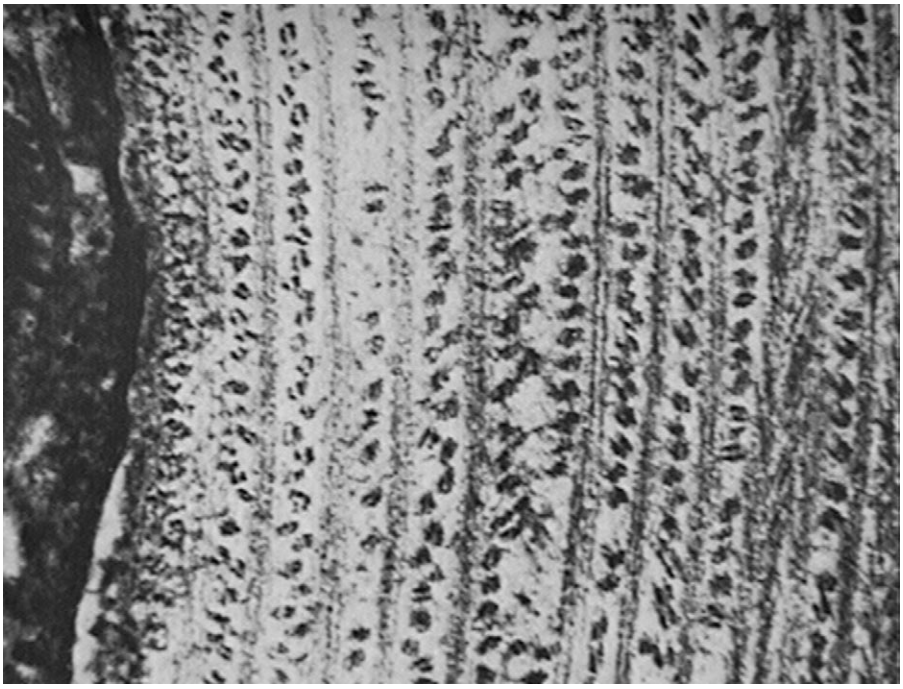


FIGURE 11.19 The polylaminate extracellular matrix of *Oocystis solitaria* showing the alternating perpendicular layers of cellulose microfibrils. Bar, 500 nm. (*Source:* From Sachs et al., 1976.)

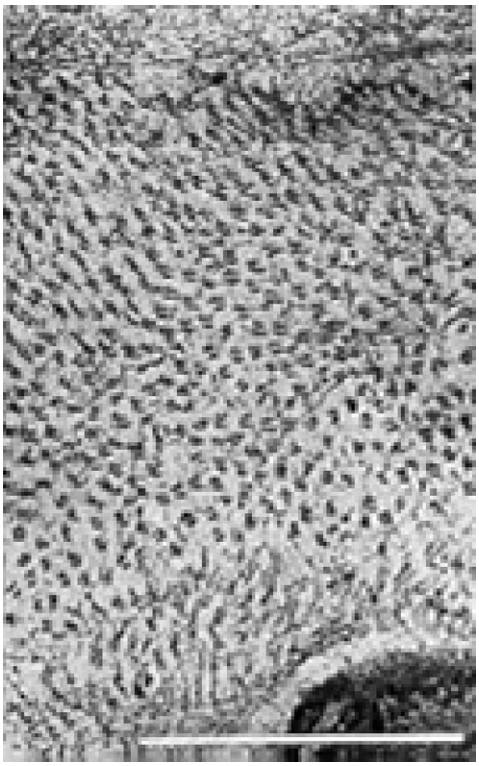


FIGURE 11.20 The extracellular matrix of *Oocystis solitaria* following the application of colchicine. The microfibrils are all coparallel and the extracellular matrix is no longer polylaminate. Bar, 500 nm. (*Source:* From Grimm et al., 1976.)

cells, other mechanisms may influence the orientation of cellulose microfibrils (Emons and Wolters-Art, 1983; Traas et al., 1985; Emons, 1989). The causal relationship between the orientation of microtubules and the orientation of cellulose microfibrils in cortical cells is being questioned (Baskin, 2001). Himmelspach et al. (2003) have shown that the cellulose microfibrils orient transversely in cortical cells in which the cell wall had been disrupted previously with a cellulose synthesis inhibitor and in which the microtubules were disrupted as a result of a temperature-sensitive mutation. These data indicate that there are still yet to be discovered mechanisms that regulate the orientation of cellulose microfibrils, which results in the shapes of plant cells and the forms of plants (Wasteneys and Collings, 2006).

11.6 VARIOUS STIMULI AFFECT MICROTUBULE ORIENTATION

Hormones, light, gravity, fungi, and other stimuli influence the orientation of microtubules in plant cells (Fischer and Schopfer, 1997; Genre and Bonfante, 1997). Hiroh Shibaoka has pioneered the study of the effect of hormones on microtubule orientation. He and his colleagues have shown that cells treated with hormones that cause elongation (e.g., auxin and gibberellic acid [GA]) have transverse microtubules, while cells treated with hormones

that cause isodiametric growth (e.g., ethylene and cytokinins) have randomly arranged microtubules (Shibaoka, 1972, 1974; Shibaoka and Hogetsu, 1977; Takeda and Shibaoka, 1981a,b; Mita and Shibaoka, 1984a,b; Mita and Katsumi, 1986; Akashi and Shibaoka, 1987; Ishida and Katsumi, 1991; Hamada et al., 1994). These data support the hypothesis that microtubules control the orientation of microfibrils.

Light also affects the orientation of microtubules (Wada et al., 1981, 1983, 1990; Kadota et al., 1982, 1985; Murata et al., 1987; Murata and Wada, 1989a,b; Iino et al., 1990). The protonema of the fern *Adiantum* are phototropic toward red light. If the protonema are irradiated on one side of the cell with a microbeam, they bend toward the light. Preceding the bend, the microtubular band remains on the shaded side but disappears on the lighted side, perhaps allowing a randomization of the microfibrils on the lighted side and the formation of a new growing tip (Wada et al., 1990).

Irradiation of the cells with polarized red light oriented 45 degrees relative to the long axis of the cell causes bending. This bending, which is known as *polarotropism*, is preceded by a shift in the angle of the microtubule band so it predicts and surrounds the new growing tip (Wada et al., 1990). Likewise, the microfibrils change their orientation in parallel with the microtubules (Wada et al., 1990).

Hush and Overall (1991) find that both electrical and mechanical fields are capable of orienting cortical microtubules in the cells of pea roots. Electric fields of only 0.36 V/cm (≈0.001 V/cell), oriented perpendicular to the long axis of the root, cause a change in the microtubule arrangement from transverse (relative to the axis of the root) to longitudinal (which is perpendicular to the applied electric field). Similar results were found by White et al. (1990) in *Mougeotia* protoplasts. Since the resistance of the plasma membrane is so much greater than the resistance of the cytoplasm, the voltage drop is almost exclusively across the plasma membrane. Therefore, it is possible that the electric field causes a reorientation of a dipole in a plasma membrane protein, which produces a conformational change in the rest of the protein that affects its ability to bind and/or orient microtubules or microtubule-associated proteins (Hush and Overall, 1991).

Hush and Overall (1991) have also applied mechanical forces of 0.12 N perpendicular to the longitudinal axis of the roots and observed that the microtubules reorient from transverse to longitudinal—that is, perpendicular to the direction of applied force. Williamson (1990) proposes that the mechanical stress applied to the extracellular matrix causes a strain in the cellulose microfibrils. This strain in turn causes a conformational change in transmembrane proteins that bind the cellulose microfibrils in the E-space and cortical microtubules in the P-space. The conformational change in these proteins can then cause a change in the orientation of cortical microtubules. Thus, microtubules can cause the orientation of cellulose microfibrils, and the microfibril orientation may also influence the orientation of microtubules in a feedback loop (Williamson, 1990, 1991). As I will discuss in Chapter 20, microtubules may be part of a structural continuum that includes the cytoskeleton, the plasma membrane, and the extracellular matrix (Akashi et al., 1990; Laporte et al., 1993; Joos et al., 1994; Gardiner et al., 2001).

11.7 MICROTUBULES AND CYTOPLASMIC STRUCTURE

Isolated microtubules behave as a non-Newtonian fluid consistent with the postulate that microtubules are one of the protein polymers that are responsible for the viscoelastic, thixotropic, and non-Newtonian properties of the cytoplasm (Sato et al., 1988). Similar to cytoplasm, the elastic modulus of microtubules is $\approx 4\,N/m^2$ and the viscosity varies from 0.01 to 10 Pa s, as the rate of shear changes from 10^2 to $10^{-2}\,s^{-1}$ (Buxbaum et al., 1987; Sato et al., 1988).

11.8 INTERMEDIATE FILAMENTS

A third major cytoskeletal system exists that is composed of intermediate filaments (Figure 11.21). These filaments are approximately 10 nm in diameter and therefore intermediate between actin microfilaments (5 nm) and microtubules (24 nm). There are a variety of proteins that make up the various types of intermediate filaments (Goodbody et al., 1989; Hargreaves et al., 1989a,b; Ross et al., 1991; Staiger and Lloyd, 1991; Mizuno, 1995). Yang et al. (1993) suggest that the intermediate filament protein keratin may form the microtrabecular lattice of plant cells. Beven et al. (1991) suggest that the intermediate filament protein lamin may form the network of 10-nm filaments that are found just inside the inner membrane of the nuclear envelope.

11.9 CENTRIN-BASED MOTILITY

Some motile proteins are actually contractile elements themselves (Stebbings and Hyams, 1979). These include the contractile protein in the spasmoneme of *Vorticella* and centrin, which was discovered in *Chlamydomonas* (Salisbury et al., 1984, 1988; Wright et al., 1985; McFadden et al., 1987; Melkonian, 1989). Centrin is a component of the MTOCs or pericentriolar material in algae, protozoa, mammals, and higher plants (Baron and Salisbury, 1988; Wick and Cho, 1988; Hiraoka et al., 1989; Katsaros et al., 1991). Centrin is also found in the multilayered structure in motile plant sperm cells (Vaughn et al., 1993; Hoffman et al., 1994).

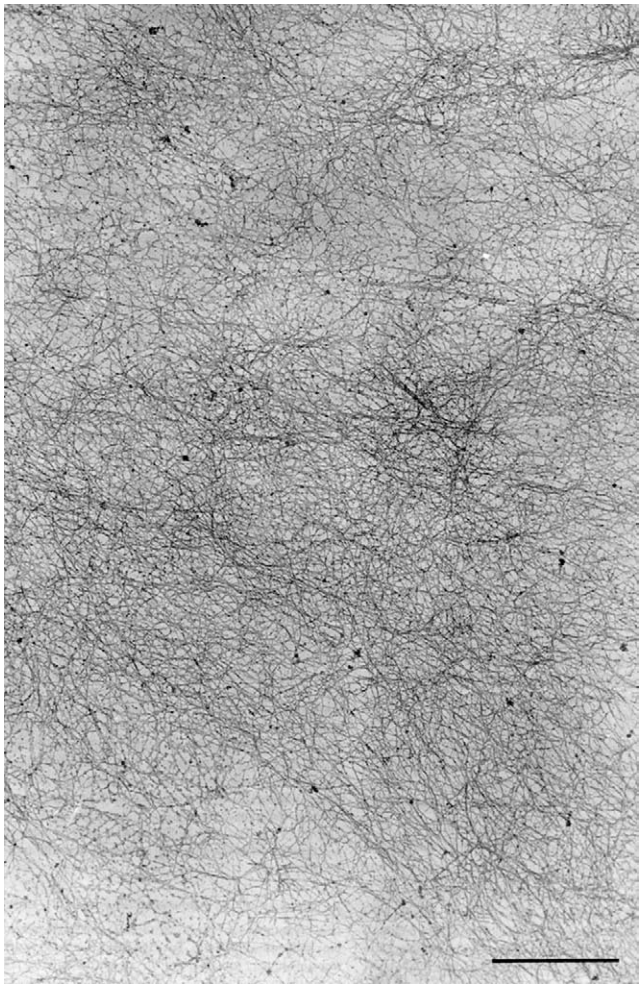


FIGURE 11.21 Intermediate filaments in a whole mount of a carrot suspension culture cell that has been extracted with detergent. Bar, 1 μm. (*Source:* From Yang et al., 1992.)

11.10 TENSEGRITY IN CELLS

As a rule, the human-made structures around us are built "brick upon brick" and are thus under compression. An exception to this rule is a suspension bridge, which is under tension. It turns out that nature, unlike humans, builds structures, like spider webs, that depend on tension to maintain their integrity. Buckminster Fuller coined the term *tensegrity* to describe these structures, the integrity of which is tension based, and Don Ingber (1993) has applied this concept to cellular structures. There are many proteins in the cell that are capable of making filamentous structures that are either elastic or rigid. There are also motor proteins that are able to produce shearing forces that can cause either tension or compression. Laser microbeam experiments have shown that the cytoplasm is under tension, since irradiation causes a rapid retraction of the cytoplasm (Goodbody et al., 1991; Schmid, 1996).

The architectural entity formed from the tensile elements is capable of transmitting mechanical information throughout the cell. Ingber and Jamieson (1985) and Ingber and Folkman (1989a,b) suggest that the intermediate filaments, actin filaments, microtubules, and their associated motor proteins form a tensionlike scaffold in the cell, and have shown that the potential energy stored in this tension is capable of doing work and directing cell differentiation. A dynamic, information-bearing, and transmitting cellular structure was envisioned by Rudolph Peters as early as 1929, and was called the *cytoskeleton* by Joseph Needham in 1936.

11.11 SUMMARY

In Chapter 10, I discussed the involvement of actin in cell motility, and in this chapter, I have discussed another motility-generating system based on microtubules. Ciliary motion is the best characterized microtubule-based system. The shearing stress that powers the cilia is generated by the interaction of dynein with microtubules. We have learned that dynein is also present in nonciliated cells where it acts as a minus end–directed motor that walks down a microtubule to move vesicles through the cytoplasm. We also learned about kinesin, which is a mechanochemical transducer that moves vesicles along a microtubule or can move microtubules relative to a place where kinesin is anchored. The canonical kinesin is a plus end–directed motor while some kinesin-related proteins are minus end–directed motors. A single amino acid substitution can change a minus end–directed motor to a plus end–directed motor. This indicates to me that assigning a function to a protein that has 99 percent homology with another protein of known function may give misleading results in cases where the identity of a single amino acid confers on the protein a specific and crucial function.

We have also learned that microtubules are involved in orienting cellulose microfibrils and thus determine the shape of the cell. In general then, microtubules are involved in cell motility and cell shape.

11.12 QUESTIONS

11.1. Why do you think there are two classes of motile systems in cells: one based on actin and one based on tubulin?

11.2. Why do you think there is more than one class of mechanochemical ATPases that move along microtubules?

Chapter 12

Cell Signaling

Between stimulus and response there is a space. In that space is our power to choose our response. In our response lies our growth and our freedom.

—Viktor E. Frankl, 1985

12.1 THE SCOPE OF CELL REGULATION

Life, according to Herbert Spencer (1864), is "a continuous adjustment of internal relations to external relations." Morley Roberts (1938) wrote, "The irritability, or excitability, of the cell, or of the minutest possible portion of protoplasm, is a *sine qua non* of its existence and powers of reaction, and the sole source of feeling and life in all animals." What happens in the cell when it is faced with a stochastic or planned change in its environment? The cell undergoes adjustments that result in a variety of changes that range from the maintenance of homeostasis to a change in the developmental program. The regulatory mechanisms discussed in this chapter are relatively rapid processes that take place in the time scale of tens of milliseconds to several minutes. How much does the quality and quantity of our own life as well as the lives of others around us depend on the biophysical and biochemical events involved in cell signaling?

The vitality of plants is often underappreciated (Hallé, 2002; Baluska et al., 2006). However, if we were to walk quietly and observantly through a garden, it would become increasingly clear that it is a normal and ubiquitous property of plants to sense and respond to their environment (Pfeffer, 1875; Darwin, 1881, 1897; Bose, 1906, 1913, 1926, 1985; Haberlandt, 1906, 1914; Goebel, 1920; Bünning, 1953, 1989; Jaffe and Galston, 1968; Sibaoka, 1969; Jaffe, 1980; Bradbeer, 1988; Simons, 1992; Sopory et al., 2001; Darnowski, 2002; Trewavas, 2006; Ueda and Nakamura, 2007; Volkov et al., 2008). The sensing behavior of plants becomes most obvious when we watch their movements. The leaves of *Albizzia*, for example, open during the day and show sleep movements at night; the leaves of *Mimosa* fall rapidly in response to the touch of an animal; the flowers of the daylily open at dawn and close at dusk. Even the seeds in the ground beneath our feet are able to exquisitely sense the temperature and light conditions, and then break dormancy so that the radical emerges in the appropriate season. If we were lucky enough to walk around a bog, we might even see the leaves of the Venus flytrap or the hairs of a sundew capture its meaty meal! We do not even have to leave our houses to see plants in action—houseplants in the window bend toward the light, and in every pot, the roots grow down and the shoots grow up in response to gravity.

Alexander Pope (1871) wrote, "Know then thyself, presume not God to scan. The proper study of Mankind is Man." Perhaps we should ask: Could the study of plant behavior help us to understand man and the evolution of consciousness? Raoul Francé (1905) wrote, "What grander lesson could the speechless plants give than that which they have taught us: *that their sense life is a primitive form, the beginning of the human mind* … it tells us that after all the living world is but mankind in the making, and that we are but a part of all." According to Lynn Margulis (2001), consciousness, as defined as an awareness of the external environment, began with life itself.

Herbert Jennings (1906) concluded that there is a biological basis for the distinctions between right and wrong that is based on the processes of cell signaling. Jennings (1933) wrote:

> *To determine what is to be done, what not to be done; in other words, to determine right and wrong, is an insistent problem for all organisms. … The daily, the hourly, occupation of most organisms—high or low—is the seeking of conditions that are favorable for life and the avoiding of conditions that are unfavorable. … With all organisms, life is a continuous process of selecting one line of action and rejecting another, of determining whether certain actions are right or wrong. The life of the single-celled organism is such a continual process of trial … it has its dramatic crises as has the life of higher creatures.*

Are the adaptive responses of an organism to the environment deterministic or subject to physical processes that are fundamentally probabilistic (Lillie, 1927)? According to Arthur Compton (1931), "the actions of the organism depend

upon events on so small a scale that they are appreciably subject to Heisenberg uncertainty. This implies that the actions of a living organism cannot be predicted definitively on the basis of its physical condition." Indeed, Pascual Jordan (1938, 1939, 1942a,b, 1948; Jordan and Kronig, 1927) began a program to eliminate any mechanistic and deterministic basis for biology and tried to start "quantum biology" (Heilbron, 1986; Beyler, 1996, 2007; Popp and Beloussov, 2003).

Is a highly individualistic and courageous response determined by an individual's cellular balance of stochastic and deterministic material elements, or are one's actions dependent on nonmaterial elements, including free will? What causes a man like Martin Niemöller (1941) to shun cowardly, sheeplike, faddist behavior and stand up to an authority like Adolf Hitler (1943)? Niemöller initially supported Hitler, but by 1937, he was arrested by the Gestapo for his open opposition to Hitler and incarcerated in the Sachsenhausen and Dachau concentration camps. Nevertheless, he still berated himself for not doing more to fight the tyranny, and he was paraphrased in the *Congressional Record* (October 14, 1968, page 31,636) as having said:

> *When Hitler attacked the Jews I was not a Jew, therefore I was not concerned. And when Hitler attacked the Catholics, I was not a Catholic, and therefore, I was not concerned. And when Hitler attacked the unions and the industrialists, I was not a member of the unions and I was not concerned. Then Hitler attacked me and the Protestant church—and there was nobody left to be concerned.*[1]

Does this kind of behavior depend on quantum uncertainty and statistical variation? Is free will a type of usable energy that, according to John Eccles (1979), is capable of inducing physico-chemical reactions such as ion channel gating or the secretion of neurotransmitters (Popper and Eccles, 1977)?

12.2 WHAT IS STIMULUS-RESPONSE COUPLING?

Until recently, stimulus-response coupling was studied using the "black-box approach." The black-box approach is described by Ashby (1958):

> *The Problem of the Black Box arose in electrical engineering. The engineer is given a sealed box that has terminals for input, to which he may bring any voltages, shocks, or other disturbances he pleases, and terminals for output, from which he may observe what he can. He is to deduce what he can of its contents.*

With the help of biophysical, biochemical, genetic, and various "-omic" tools, cell biologists are now cracking open the black box and understanding each step in the signal transduction chain.

We will consider a stimulus to be any environmental, physiological, or biological signal that induces a change in a biophysical, biochemical, physiological, morphological, or developmental process in the cell. Common stimuli include light (Bünning and Tazawa, 1957), hormones (Leopold, 1964; Leopold and Kriedermann, 1975; Thimann, 1977; Strader and Bartel, 2008), florigen (Ayers and Turgeon, 2004), neurotransmitters (Roshchina, 2001), touch (Jaffe and Galston, 1968; Jaffe, 1980; Jaffe et al., 2002; McCormack et al., 2006), time (Cole, 1957; Hastings and Sweeney, 1957; Sweeney and Hastings, 1958; Sweeney and Haxo, 1961; Broda and Schweiger, 1981; Sweeney, 1987; Berger et al., 1992; Chandrashekaran, 1998; Suzuki and Johnson, 2001; Mittag et al., 2005), gravity (Nemec, 1899; Wayne and Staves, 1996a; Sack, 1997), and biological interactions (e.g., pollen and stigma or fungus and plant).

A stimulus must be able to impart a certain amount of free energy to the cell that is greater than the energy of thermal noise ($E \approx kT$) or a receptor will not be able to perceive the stimulus (Bialek, 1987; Block, 1992). A stimulus will have no effect on a cell unless that cell has the appropriate receptor, and if the cell has an appropriate receptor, the stimulus will provide the cell with information. The presence or absence of a certain constellation of receptor proteins will provide a certain degree of selectivity in terms of which cells respond to a stimulus. This competence of a cell to perceive a stimulus is predetermined by the genetic system. Substantial progress is going on in identifying hormone (auxin, gibberellin, cytokinin, ethylene) and other chemical (salicylic acid and nitric oxide) receptors, light receptors (phytochrome, cryptochrome, and phototropin), and gravity receptors (Jones and Venis, 1989; Hooley et al., 1991; Wayne et al., 1992; Furuya, 1993, 2005; Ahmad and Cashmore, 1996; Lin et al., 1996a,b; Cashmore, 1997, 1998; Christie et al., 1998, 1999; Trewavas, 2000; Salomon et al., 2000; Briggs et al., 2001a,b; Christie and Briggs, 2001; Briggs and Christie, 2002; Briggs, 2005; Quail, 2005; Gilliham et al., 2006; Hagemann, 2008).

We will consider a response to be any biophysical, biochemical, physiological, morphological, or developmental process that changes after the cell receives the stimulus. Common responses include germination, flowering, osmoregulation, turgor regulation, chloroplast movement, phototaxis, leaf movements, gravitropism, senescence, abscission, and the processes involved in plant defense. The presence or absence

[1] Others quote him to have said, "In Germany they came first for the Communists and I didn't speak up because I wasn't a Communist. Then they came for the Jews and I didn't speak up because I wasn't a Jew. Then they came for the trade unionists and I didn't speak up because I wasn't a trade unionist. Then they came for the Catholics and I didn't speak up because I was a Protestant. Then they came for me—and by that time no one was left to speak up." Sibylle Sarah Niemöller von Sell, in response to a student's question, "How could it happen?," quoted her husband, saying: "First they came for the Communists, but I was not a Communist so I did not speak out. Then they came for the Socialists and the Trade Unionists, but I was neither, so I did not speak out. Then they came for the Jews, but I was not a Jew, so I did not speak out. And when they came for me, there was no one left to speak out for me."

of a constellation of response elements that are required for each of these responses will also provide a certain degree of selectivity. The competence of a cell to respond to a stimulus in a given manner is predetermined by the genetic system.

A signal transduction chain comprises all the biophysical and/or biochemical steps that occur between the perception of the stimulus and the response. In the simplest case, the signal transduction chain acts like a switch. It initiates the response when the cell is presented with a stimulus. As an analogy, think of the electric system in your house. All the switches are very similar, yet when you turn on the television set you see a television show; when you turn on the toaster, your bread gets brown; when you turn on your radio, you hear music. Your appliances are preprogrammed to respond to stimuli in a special way, so when you turn on a switch on the television, you see a show and your bread does not get brown. When a fern spore is given red light, it germinates and it does not make a nitrogen-fixing nodule; or when a characean cell gets an electrical stimulus, it stops streaming and it does not flower. In the simplest cases, the cell is preprogrammed to respond to one stimulus with one given response. A part of the response may be to reprogram the cell with new receptors and/or response elements so that it continues its developmental fate.

Considering the signal transduction chain as a switch has widespread appeal because of its elegant simplicity. Yet there is a growing awareness that the mechanisms that underlie cellular signaling are more complex and intricate. We already know that more than one switch exists: one switch is turned on by Ca^{2+} and others by cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP). Moreover, any two switches can be redundant, antagonistic, hierarchical, or sequential (Rasmussen, 1981; Barritt, 1992). The common intracellular switches like Ca^{2+} and cyclic nucleotides are known as *second messengers*. A second messenger is the first relatively stable intracellular chemical that increases its concentration in response to the stimulus and can influence the response elements in the cell. Cyclic AMP at first was proposed to be the second messenger for all hormone responses, and Ca^{2+} the second messenger in muscle contraction, secretion, and egg activation. However, through continued experimentation, it became apparent that the participation of both of these signaling systems was more widespread than had been thought originally. The totality of the components involved in cellular signaling has been dubbed the *signalome* (Reddy, 2001).

It is becoming clear that interacting and independent pathways are involved in coupling stimuli with responses when a number of responses can be identified in a single plant cell (Bowler et al., 1994; Wu et al., 1996; Neuhaus et al., 1997; Iseki et al., 2002). For example, *Dunaliella* cells have three independent responses to light: the phototactic response, the step-up photophobic response, and the step-down photophobic response. All these responses involve a rapid and subtle regulation of the ciliary beat (Wayne et al., 1991). The phototactic response involves a rapid turn toward blue light; the step-up photophobic response involves a change from a forward-swimming ciliary beat to a backward-swimming flagellar beat when green light is turned on; and the step-down photophobic response involves a turn of 90 degrees or more immediately after the green light is shut off. Therefore, different signal transduction chains must exist in the single *Dunaliella* cell or else all three responses would be turned on simultaneously. The three different chains are not completely independent, but have common components. For example, they all require external Ca^{2+} (Noe and Wayne, 1990).

It is important to understand at the outset that we are discussing the effect of a stimulus on the response of single cells, whether they be individual organisms or part of a multicellular organism. This is important since neighboring cells in a multicellular organism can have quite different responses to the same stimulus. Hans Mohr (1972) has illustrated this elegantly in mustard seedlings. Red light causes the epidermal cells of the hypocotyl to differentiate hairs and the hypodermal cells to synthesize anthocyanins. Furthermore, the competence of the hypodermal cells to produce anthocyanins depends on the duration of time the seedling spent in the dark before it was irradiated. Thus, Mohr has shown that competence is a dynamic spatiotemporal phenomenon that depends on time as well as the position of the cell in the organism. Consequently, when investigating stimulus-response coupling, it is essential to isolate a given cell type at a given time in order to characterize how it responds to a stimulus, if we want to use established cellular paradigms to describe the signal transduction chain. These paradigms explain what happens in a single cell—not a whole cow or a whole plant.

Plant physiological experiments are often interpreted as if the plant were a single cell, or as if all the cells in the plant body were identical. If in fact each cell in a multicellular organism has a different constellation of receptors and response elements as well as differences in the kinetics or types of signal transduction chains, then it would be ludicrous to provide a stimulus to the whole seedling and then grind it all up to find a change in the concentration of a second messenger. First of all, the temporal resolution would be low, and second, the response of all the cells would be averaged. Thus, if only the epidermal or hypocotyl cells responded to a stimulus, but the cortical or pith cells did not, a real change in the concentration of a second messenger in the responding cells could go undetected.

Of course, higher levels of regulation exist in plants where communication mediated by plasmodesmata, as well as chemical, electrical, and mechanical gradients, between cells in different tissues or tissue systems occurs (Chapter 3; Sachs, 1887; Osterhout, 1906; Leopold and Kriedermann, 1975; Thimann, 1977; Sachs, 2006; Taiz and Zeiger, 2006). There is also integration at the organ level where two or more cells in an organ may communicate (e.g., abscission), or at

the whole plant level where one or more cells in an organ may compare their states with one or more cells in another organ (e.g., apical dominance). An understanding of these higher-level processes requires experimental designs that take into consideration both the cellular and organismal levels of organization.

A stimulus contains energy, and a signal transduction chain involves the conversion of the energy of the primary stimulus, which may be light, gravity, chemical, heat, or electrical energy, to the energy of an intracellular molecule that can be coupled to the biochemical machinery in the cell. Indeed, as James Clerk Maxwell (1877) wrote, "The transactions of the material universe appear to be conducted, as it were, on a system of credit (except perhaps that credit can be artificially increased, or inflated). Each transaction consists of the transfer of so much credit or energy from one body to another." The energy of the stimulus is transferred to a receptor. The receptor often takes advantage of the potential energy already present, typically in the form of Ca^{2+} difference across the plasma membrane, to activate the cell.

12.3 RECEPTORS

There are a variety of types of receptors in cells. The receptor proteins that act on ion channels are called *channel-linked receptors*, and the acetylcholine receptor is the canonical example of this class. Channel-linked receptors in plants (Demidchik, 2006) include channelrhodopsin, which mediates phototaxis and photophobic responses in *Chlamydomonas*. Channelrhodopsin is an example of a channel-linked receptor that passes cations, including H^+, K^+, Na^+, and Ca^{2+}, in response to light. The activation of this channel results in a depolarization of the plasma membrane (Sineshchekov et al., 2002; Govorunova et al., 2004; Berthold et al., 2008; Hegemann, 2008). By transforming cells with the gene for channelrhodopsin, the channelrhodopsin can be used as a nanoswitch to rapidly and noninvasively activate action potentials with light in normally light-insensitive neurons (Li et al., 2005; Nagel et al., 2005; Miller, 2006; Zhang et al., 2006; Hegemann and Tsunoda, 2007; Zhang and Oertner, 2007).

Other receptors operate directly as enzymes after binding a ligand or after being activated by a physical stimulus. The insulin receptor is the canonical example of the catalytic receptor class. The insulin receptor is an integral, transmembrane protein with a cytoplasmic domain that functions as a protein kinase. In plants, the receptor involved with pollen-stigma incompatibility reactions (Stein and Nasrallah, 1993; Stein et al., 1996) and a blue-light photoreceptor, phototropin, which is a plasma membrane–localized photoreceptor involved in phototropism, stomatal opening, chloroplast movement, leaf expansion, and hypocotyl growth inhibition (Huala et al., 1997; Christie et al., 1998; Kagawa et al., 2001; Kinoshita et al., 2001; Sakai et al., 2001; Sakamoto and Briggs, 2002; Briggs, 2005), are integral membrane proteins with protein kinase activity. The blue-light photoreceptor, cryptochrome (Cashmore, 2005), and the red light photoreceptor, phytochrome (Yeh and Lagarias, 1998; Suetsuga et al., 2005; Rockwell et al., 2006), both show protein kinase activity. However, the contribution of this activity to signal transduction is still being investigated and alternative hypotheses are being advanced (Quail, 2005). Indeed, just as related members of the rhodopsin family can initiate signal transduction chains through differing mechanisms (Hagemann, 2008), allied members of the phytochrome family may also initiate responses through different mechanisms. Interestingly, plants have made use of the promiscuity of DNA to create chimeric phytochrome-like photoreceptors from the canonical red-light and blue-light photoreceptors. For example, neochromes found in the alga *Mougeotia* and the fern *Adiantum* are red- and blue-light photoreceptors, the genes of which are composed of phytochrome and phototropin nucleotide sequences (Nozue et al., 1998; Suetsuga et al., 2005; Suetsuga and Wada, 2007).

Other types of catalytic receptors have been discovered in plants. These include the blue-light receptor for the step-up photophobic response in *Euglena* and the blue-light receptor for the branching response in golden algae. The photoreceptor in *Euglena*, which is not an integral membrane protein, is a flavoprotein that is found in the quasi-crystalline paraflagellar body in *Euglena* and along the whole length of the flagellum in related genera. The photoreceptor protein catalyzes the conversion of ATP to cAMP in a light-dependent manner (Iseki et al., 2002; Häder et al., 2005). By transforming cells with the gene for this receptor, Schröder-Lang et al. (2007) have used this photoactivated adenylate cyclase (PAC) as a tool to use light to increase the concentration of cAMP in transformed cells that are normally light insensitive.

A third class of receptors is known as G-protein–linked receptors (Ma et al., 1990, 1991; Coughlin, 1994; Ma, 1994, 2001; Mu et al., 1997; Assmann, 2002; Perfus-Barbeoch et al., 2004; Assmann, 2005; Pandey et al., 2006; Grill and Christmann, 2007; Liu et al., 2007a; Hegemann, 2008; Martinac et al., 2008). These receptors are mainly integral plasma membrane proteins with seven transmembrane domains that indirectly activate or inactivate plasma membrane–bound enzymes or ion channels through the activation of a G-protein. Vertebrate rhodopsin and the norepinephrine receptor are examples of G-protein–linked receptors and similar G-protein–linked receptors can be found in plants. The best characterized G-protein–linked receptor in higher plants is the abscisic acid receptor (Liu et al., 2007a), although there is some controversy surrounding this research (Johnston et al., 2007; Liu et al., 2007b). In almost all cases, activated G-protein–linked receptors bring about an increase in the concentration of Ca^{2+} or cyclic nucleotides.

G-proteins involved in cell signaling are composed of three subunits (α, β, and γ) and consequently are known as *heterotrimeric* G-proteins. The α subunit hydrolyzes guanosine triphosphate (GTP), and the β and γ subunits form a dimer that anchors the G-protein to the cytoplasmic side of the plasma membrane. In the inactive form, the G-protein exists as a trimer with guanosine diphosphate (GDP) bound to the α subunit. When a G-protein becomes activated, the α subunit binds a molecule of GTP in exchange for its bound GDP, and then dissociates from the βγ dimer and diffuses in the plane of the membrane until it encounters a protein to which it can bind. The α subunit then binds tightly to the protein and either activates or inactivates it. The α subunit is active for only as long as the GTP molecule remains intact, which is typically 10–15 seconds. Once the GTP is hydrolyzed, the α subunit becomes inactive and dissociates from the protein to which it had been bound, and that protein is no longer activated or inactivated.

The activation of G-proteins provides an amplification step in cell signaling because the α subunit can remain activated for 10–15 seconds; long after the primary stimulus has dissociated. G-proteins can remain artificially activated for a long time by introducing GTP-γ-S into the cell. This molecule cannot be hydrolyzed, and thus the G-protein remains activated for a long time. This is an experimentally good way to determine whether G-proteins are involved in cell signaling. Stimulatory G-proteins can also be permanently activated by cholera toxin while inhibitory G-proteins can be inhibited by pertussis toxin.

A fourth class of receptor is a zinc-containing transcription factor that acts directly on gene expression (see Chapter 16). The steroid hormone receptor is the canonical example. The hormone binds to receptors in the cytosol and the receptor-ligand complexes dimerize and move into the nucleus, where the dimer binds to a specific DNA sequence. Like the steroid hormone receptor aureochrome, the blue-light photoreceptor that is involved in stimulating branching and initiating sex organs in gold-colored stramenopile algae, including *Vaucheria* and *Fucus*, is also a transcription factor (Takahashi et al., 2001, 2007). Hirnao Kataoka hopes to support science in fields other than plant photobiology by promoting aureochrome as a light-activated transcription factor that can be used to activate specific genes in the nucleus of cells that typically do not respond to light.

12.4 CARDIAC MUSCLE AS A PARADIGM FOR UNDERSTANDING THE BASICS OF STIMULUS-RESPONSE COUPLING

I will first discuss the role of Ca^{2+} in the contraction of cardiac muscle, since cardiac muscle exhibits many ways in which Ca^{2+} acts as a second messenger (Figure 12.1). Normally, our hearts beat with a rhythm set by the pacemaker cells. The pacemaker cells send a periodic electrical stimulus to the cardiac muscle cells that depolarizes the plasma membrane or sarcolemma of the cardiac cells (Hoffman and Cranefield, 1960). Consequently, a voltage-dependent Ca^{2+} channel in the plasma membrane opens and allows Ca^{2+} to enter the cell in the direction of its electrochemical difference. The increase of Ca^{2+} in the cytosolic P-space is augmented because the Ca^{2+} that enters the cell through the plasma membrane binds to the Ca^{2+}-release channel in the endoplasmic reticulum (sarcoplasmic reticulum) and causes a large release of Ca^{2+} as a result of a Ca^{2+}-induced Ca^{2+} release.

As a result of this cascade effect, the Ca^{2+} concentration in the cytosol rises from 0.1 μM to 1–10 μM. At this elevated concentration, Ca^{2+} binds to one of the subunits of an intracellular Ca^{2+}-binding protein called *troponin*. Each troponin molecule binds four Ca^{2+} ions. Once troponin binds Ca^{2+}, the complex displaces a filamentous protein known as *tropomyosin* from actin so that myosin can interact with actin and cause contraction. Other proteins that bind Ca^{2+} include the Ca^{2+}-ATPases of the sarcolemma and the sarcoplasmic reticulum. The increase in cytosolic calcium is transient, in part, because these proteins pump Ca^{2+} from the cytosol (P-space) into the extracellular space (E-space) and the lumen of the sarcoplasmic reticulum (E-space). In cardiac muscle cells, the increase in the concentration of intracellular-free Ca^{2+} lasts about 30 milliseconds. The activation of the actin-activated myosin ATPase depends on the increase in the concentration of intracellular Ca^{2+}, and is thus an example of amplitude modulation.

When we are excited, our hearts beat faster. This is due to norepinephrine (=noradrenaline), an adrenaline-like neurotransmitter that is released by the sympathetic nervous system. Norepinephrine initiates a rise in cAMP. Norepinephrine does this by binding to a membrane receptor (β-adrenergic receptor). Subsequently, the receptor activates trimeric G-proteins by causing them to bind GTP in exchange for GDP. The GTP-binding proteins then activate adenylate cyclase molecules. Each adenylate cyclase molecule converts many ATP molecules to cAMP. The many cAMP molecules then bind to many cAMP-dependent protein kinases. The cAMP increase is only transient, and soon after the cAMP increases, it is converted to the inactive 5′-AMP by phosphodiesterase. Thus, like the Ca^{2+} signal, the cAMP signal is also transient.

One of the substrates activated by the cAMP-dependent protein kinase is phosphorylase kinase. Phosphorylase kinase is a regulatory protein that activates glycogen phosphorylase, the enzyme responsible for the breakdown of glycogen. Phosphorylase kinase is a calcium-binding protein. However, phosphorylase kinase does not bind calcium directly, but binds a calcium-binding protein known as *calmodulin*. Calmodulin is a ubiquitous protein, and is related to the Ca^{2+}-binding subunit of troponin.

The phosphorylation of phosphorylase kinase increases the affinity of phosphorylase kinase-calmodulin for Ca^{2+}

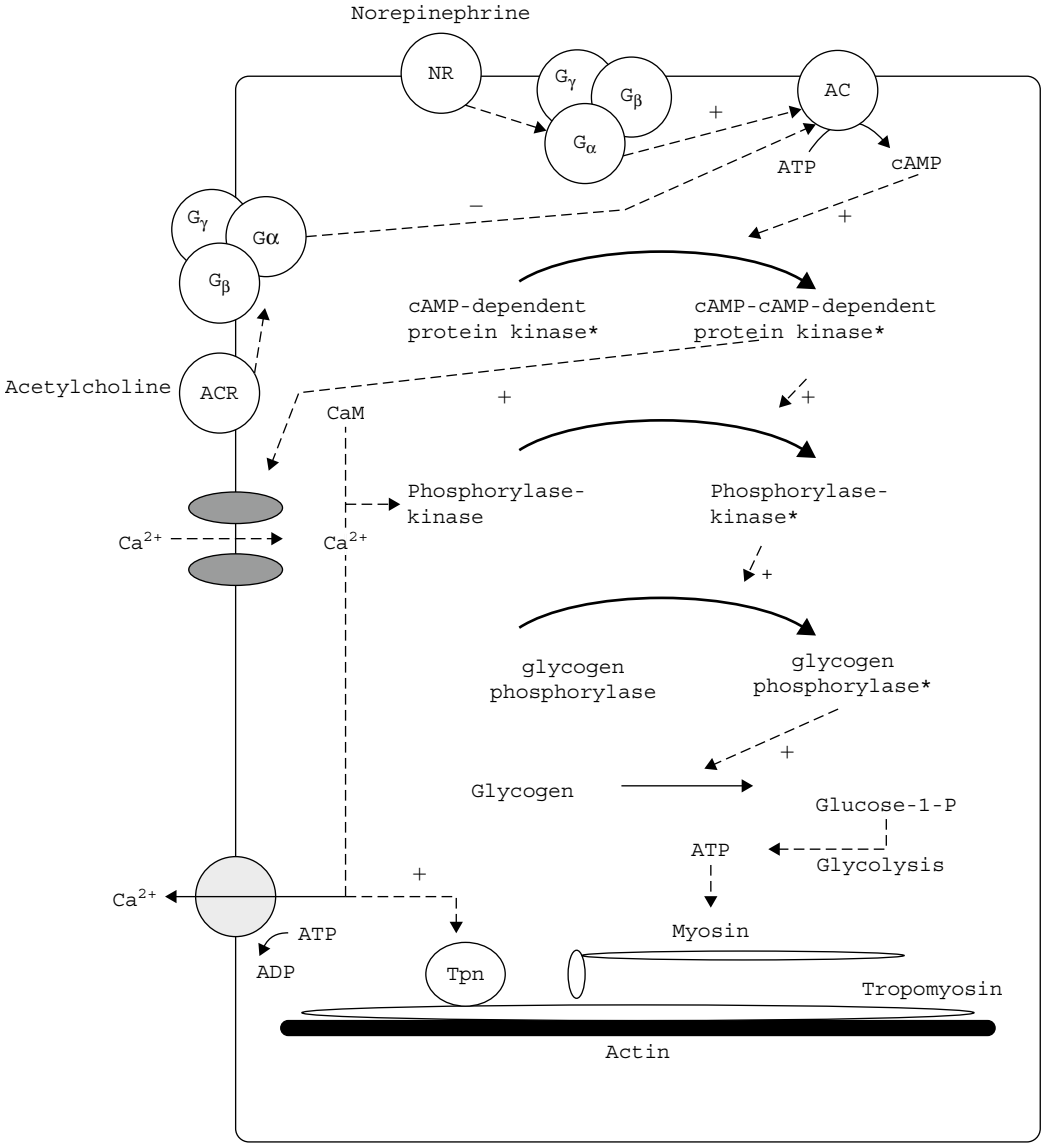


FIGURE 12.1 Scheme of a signal transduction network in a generalized cardiac muscle cell. Contraction is initiated when an electrical stimulus causes the plasma membrane of the muscle cell to depolarize. The depolarization activates the plasma membrane localized, membrane potential–dependent Ca^{2+} channels. The activated muscle cell then contracts due to the interaction of actin and myosin. Simultaneous activation of the receptors for norepinephrine results in the breakdown of glycogen and the formation of ATP necessary for contraction. Alternatively, activation of the acetylcholine receptor results in a diminished formation of ATP. An asterisk indicates the active form of an enzyme. NR, norepinephrine receptor; ACR, acetylcholine receptor, AC, adenylate cyclase, Tpn, troponin; CaM, calmodulin.

and lowers the concentration required for half-maximal activation from 3 μM to 0.3 μM Ca^{2+}. The activated phosphorylase kinase then phosphorylates many glycogen phosphorylase molecules. The phosphorylase catalyzes the breakdown of glycogen to glucose-1-phosphate. The sugar phosphate then goes through glycolysis and respiration to provide the chemical energy, in the form of ATP (see Chapter 14), needed for muscle contraction. Since the activation of phosphorylase kinase depends on the change in its sensitivity to Ca^{2+}, not on an increase in the intracellular Ca^{2+} concentration, this type of regulation is known as *sensitivity modulation*.

Our heartbeat can also slow down when we are relaxed. This occurs as a consequence of the release of acetylcholine by the parasympathetic nervous system (Dale, 1936; Loewi, 1936; Valenstein, 2005). In cardiac muscle, acetylcholine binds to the inhibitory G-protein–linked muscarinic acetylcholine receptors. The inhibitory G-protein that is activated by this receptor inhibits the activity of adenylate cyclase and reduces the concentration of cAMP. This causes a slowdown of the heartbeat by reducing the production of ATP. At the same time, the inhibitory G-protein activates a K^+ channel in the sarcolemma. This leads to a slowdown of the heartbeat by causing a hyperpolarization

of the plasma membrane that desensitizes the cell to the depolarization induced by the pacemaker cells.

In a cardiac muscle cell, a variety of stimuli act on a single cell in a coordinate manner to regulate muscle contraction. Cardiac muscle cells serve as a paradigm of the variety of signaling phenomena. There are examples of amplification, where a single molecule can activate many other molecules; amplitude modulation, where a change in the concentration of a chemical leads to a response; sensitivity modulation, where an increase in the affinity of a molecule for a second messenger leads to a response; covalent modifications, where the formation of an ester bond upon the addition of a phosphate group to a protein initiates a response; ionic regulation, where the electrostatic binding of an ion leads to a response; negative feedback, where the induction of a response leads to its termination; positive feedback, where the induction of a response leads to its amplification; and crosstalk or interactions between two signal transduction chains (Rasmussen, 1981). Actually, the interactions are even more complex than I have already described. For example, the cAMP-dependent protein kinase phosphorylates Ca^{2+} channels in the plasma membrane and increases their opening probability. When the Ca^{2+} current increases, so does the force of contraction. The Ca^{2+} signaling system integrates many different regulatory elements in a cell, and consequently, a variety of pharmacological agents can have a similar effect. For example, the Ca^{2+} current is increased by isoproterenol, a β-adrenergic receptor activator; cholera toxin, an activator of G proteins; forskolin, an activator of adenylate cyclase; methylxanthines, inhibitors of phosphodiesterase; and okadaic acid, an inhibitor of protein phosphatases (Hille, 1992). Elucidating any physiological process depends on being able to reconstruct the relationship between the parts and the whole.

Why is Ca^{2+} such a good second messenger? Perhaps its fitness as a second messenger comes from the fact that it is abundant in the environment and thus there is always a reliable source for it to act as a regulatory chemical (Jaiswal, 2001). On the other hand, Ca^{2+} is a cytotoxin and at elevated cytoplasmic levels, it will bind to inorganic phosphate and form an insoluble precipitate known as *hydroxyapatite* (Weber, 1976). Thus, phosphate-based energy metabolism would be severely inhibited if the intracellular Ca^{2+} concentration approached the millimolar quantities found outside the cell. Rather than change energy metabolism, cells seemed to deal with this crisis by evolving an efficient method for removing Ca^{2+} from the cytosol, lowering its concentration to approximately $0.1\,\mu M$, at which point the reaction between Ca^{2+} and inorganic phosphate is insignificant (Kretsinger, 1977). The concentration of free Ca^{2+} in the cell is therefore 10,000 times lower than the concentration in the environment.

Since the Ca^{2+} concentration is typically low on the P-sides of membranes and high on the E-sides of membranes, the entropy of the cell in terms of Ca^{2+} is low. According to Leo Szilard (1964), the information content of a system is proportional to the negative of the entropy. Thus, if a stimulus were to open Ca^{2+} channels in the membranes and the cytosolic concentration of Ca^{2+} were to rise as the concentration outside and inside equalized *pari passu*, the entropy would increase and information could be imparted to the cell. The increase of entropy (ΔS) results in a release of molecular free energy (ΔE) that can be harnessed to perform work, given that $\Delta E = [(\Delta H - T\Delta S)/N_A]$. An ion is thus able to do work on an intracellular receptor. The magnitude of the work depends on the magnitude of the change in entropy and the magnitude of heat loss accompanying the change in entropy. The ability of an ion to transmit information to the receptor depends, in part, on a change in the concentration of the ion, not on the absolute concentration. Let's look at enzyme kinetics to get a better feel for this.

12.5 A KINETIC DESCRIPTION OF REGULATION

In order for a cell to undergo a physiological or developmental change in response to its environment, the biophysical changes in the Ca^{2+} concentration must be converted into biochemical changes that involve proteins. Thus, we must investigate the affinity between Ca^{2+} ions and the proteins they bind. Either of the binding partners in a reaction can be called a *ligand*. The binding of Ca^{2+} to a ligand puts a process in motion. Kinetics comes from the Greek word *kinetikes*, which means "putting in motion." *Kinetos* is the verbal adjective of *kinein*, which means "to move." I will begin the discussion of kinetics from a historical point of view.

12.5.1 Early History of Kinetic Studies

The study of affinity began with Empedocles (≈450 BCE), who thought that chemicals had the qualities of love and hate. To him, chemical combination and decomposition was analogous to marriage and divorce, respectively. Hippocrates generalized this idea somewhat and concluded that only chemicals that shared a kinship with each other combined to form compounds. This thinking has been captured in terms like *hydrophilic* and *hydrophobic*! By contrast, Heraclitus argued that chemicals with opposite properties attract and thus form compounds. While Hippocrates was correct for the interactions between polar and nonpolar molecules, Heraclitus was right when it came to the interactions between charged chemicals. Neither theory was all-encompassing (Clark, 1952; Kaufmann, 1961).

Throughout the 18th century, chemists, including Torbern Bergman and Georg Stahl, set up affinity tables for various chemicals. They concluded that the order of affinity is $A > B > C$, if A displaced B and B displaced C from a given chemical. It was as if A bound with more force than

B, and B bound with more force than C. At the end of the 18th century, Karl Wenzel applied Newtonian mechanics to the study of affinity. He proposed that chemical affinity is a force, and since a force causes a change in the velocity of a particle, an increase in the chemical force should cause an increase in the velocity of a chemical reaction. Thus, he studied the rate of decomposition of metals in various acids and concluded that the rate of the reactions depended on both the affinity of the acid for the metal and the quantity of the acid. Unfortunately, Wenzel's work was unappreciated and forgotten (Ostwald, 1900, 1906).

The fact that the nature and quantity of a chemical are important in predicting the reactions in which it will participate came to light again in 1799 when Claude Berthollet, one of the scientists who accompanied Napoleon Bonaparte to Egypt, came up with a theory that explained how enormous quantities of sodium carbonate appeared on the shores of the salt lakes in Egypt. While it was already known that calcium chloride combined with sodium carbonate to make sodium chloride and the insoluble calcium carbonate according to the following reaction:

$$CaCl_2 + Na_2CO_3 \Leftrightarrow CaCO_3 + 2NaCl$$

the reverse reaction was not known. Berthollet postulated that the reverse reaction occurred when the amount of NaCl was very high, like it was in the salt lakes. He concluded that sodium carbonate was formed instead of calcium carbonate, even though calcium had a greater affinity than sodium for carbonate, because the chemical force depended on both the concentration and the affinity (Mellor, 1914). Berthollet's work was ignored by fellow scientists because they were afraid that it insinuated that chemicals could combine in any proportion and this inference might undermine the atomic theory. In the 1860s, Cato Guldberg and Peter Waage built a theory that has been called the Law of Mass Action that incorporated all the known observations at the time and allowed the transformation of chemical reactions into mathematical equations (Guldberg and Waage, 1899; Bastiansen, 1964).

The Law of Mass Action may be limited when applied at the cell or organismal level. In biological systems composed of many enzymes, pathways, compartments, cells, tissues, and organs, a single chemical (e.g., drug, hormone, toxin, etc.) may have opposing effects at low and high concentrations. The slight stimulation caused by low concentrations of inhibitors, known as *hormesis*, may cause the organisms to "prepare" for larger doses by turning on pathways necessary to deal with higher concentrations of the chemical in question (Southam and Erhlich, 1943; Davis and Svendsgaard, 1990; Calabrese and Baldwin, 2000a,b; Kaiser, 2003; Calabrese, 2003, 2004). So, we must be careful in applying single-enzyme models to whole cells and organisms. Even so, the single-enzyme models have been very productive in understanding how cells respond to stimuli. I will now describe the importance of concentration and affinity in understanding enzyme reactions.

12.5.2 Kinetics of Enzyme Reactions

Now let us consider a generalized enzyme reaction that can be described by Michaelis-Menten kinetics (Michaelis and Menten, 1913; Briggs and Haldane, 1925; Haldane, 1930). In this case, the substrate (S) binds to an enzyme (E) to make an enzyme-substrate complex (ES) that decomposes to form a product (P) and the regenerated enzyme (E).

$$S + E \underset{k_2}{\overset{k_1}{\Leftrightarrow}} ES \underset{k_4}{\overset{k_3}{\Leftrightarrow}} P + E$$

k_1 and k_4 (in $M^{-1}\ s^{-1}$) represent the rate constants that describe the formation of the ES complex, while k_2 and k_3 (in s^{-1}) represent the rate constants that describe the decomposition of the ES complex.

In analogy with the Law of Mass Action formulated by Guldberg and Waage, this equation tells us that the rate of formation of ES equals $k_1[E][S] + k_4[P][E]$ and the rate of decomposition of ES equals $k_2[ES] + k_3[ES]$. At steady state, the rate of formation of ES equals its rate of decomposition, and the concentration of ES does not change. Thus:

$$k_1[E][S] + k_4[P][E] = k_2[ES] + k_3[ES] \qquad (12.1)$$

In spite of the current ubiquity of string theorists in physics, who call unknowns *free parameters*, there has been a long tradition among mathematically minded scientists to create equations where there are no more unknowns than can be measured. Among these traditional mathematically minded scientists, there is a saying, "1,2,3, infinity." That is, any equation that has more than three unknown variables is as useless as an equation with an infinite number of variables. Equation 12.1 has too many unknown quantities. Thus, Leonor Michaelis and Maud Menten used a little algebra to combine all the unknown quantities into a single measurable quantity, known as the *Michaelis-Menten constant*, that turns out to be easy to determine experimentally, and very useful for understanding regulatory proteins and/or enzymes. I will derive the Michaelis-Menten equation by first rearranging the terms in Eq. 12.1:

$$[E]\,(k_1[S] + k_4[P]) = [ES]\,(k_2 + k_3) \qquad (12.2)$$

and solve for [ES]/[E]:

$$[ES]/[E] = \frac{k_1[S] + k_4[P]}{(k_2 + k_3)} = \frac{k_1[S]}{(k_2 + k_3)} + \frac{k_4[P]}{(k_2 + k_3)} \qquad (12.3)$$

Since the concentration of the product [P] in the initial stages of the reaction is very small and does not influence

the initial velocity, we can simplify the model by studying the initial reaction where [P] = 0. Thus:

$$[ES]/[E] = \frac{k_1[S]}{(k_2 + k_3)} \text{ and } [E]/[ES] = \frac{k_2 + k_3}{k_1[S]} \quad (12.4)$$

Since the total enzyme concentration $[E]_T$ is equal to the concentration of free enzyme [E] plus the concentration of bound enzyme [ES], then $[E] = [E]_T - [ES]$. Thus:

$$\begin{aligned}[E]/[ES] &= \frac{[E]_T - [ES]}{[ES]} \\ &= ([E]_T/[ES]) - ([ES]/[ES]) \\ &= ([E]_T/[ES]) - 1 \end{aligned} \quad (12.5)$$

and

$$([E]_T/[ES]) - 1 = \frac{k_2 + k_3}{k_1[S]} \quad (12.6)$$

If we define the Michaelis-Menten constant K_m as $(k_2 + k_3)/k_1$, then:

$$[E]_T/[ES] = (K_m/[S]) + 1 \quad (12.7)$$

Unfortunately, $[E]_T$ and [ES] cannot be readily determined. However, they can be expressed in terms of the initial velocities of the reaction (v) at any given substrate concentration [S], and the maximum initial velocity (v_{max}) at saturating concentrations of [S].

The initial velocity (v) at any given substrate concentration is proportional to the concentration of the enzyme-substrate complex [ES]. Thus, v is proportional to [ES], and consequently v is an estimate of [ES]. Likewise, the maximal initial velocity (v_{max}) is proportional to the total enzyme present when $[ES] = [E]_T$—That is, when all the enzyme is in the ES complex. Thus, v_{max} is proportional to $[E]_T$, and consequently, v_{max} is an estimate of $[E]_T$. Assuming that the proportionality constants are equal, $[E]_T/[ES] = v_{max}/v$, and Eq. 12.7 becomes:

$$v_{max}/v = (K_m/[S]) + 1 \quad (12.8)$$

After solving for v, we get the typical form of the Michaelis-Menten equation:

$$v = \frac{v_{max}}{((K_m/[S]) + 1)} \quad (12.9)$$

From Eq. 12.9, we see that the relative velocity of a reaction depends on the substrate concentration. When $[S] = K_m$, $v = v_{max}/2$. Thus, K_m is also defined as the concentration of S that supports a reaction that proceeds at the velocity of $v_{max}/2$. When $[S] = 10\ K_m$, $v = v_{max}/1.1$ and the reaction proceeds at approximately 90 percent of its maximal velocity and we say the enzyme is activated. When $[S] = 0.1\ K_m$, $v_{max}/11$ and the reaction proceeds at approximately 10 percent of its maximal velocity and we say the enzyme is inactive. A reaction that obeys Michaelis-Menten kinetics is activated from 0.1 v_{max} to 0.9 v_{max} by an 81-fold change in the substrate concentration. When an increase in the substrate concentration causes an increase in the velocity of the reaction, the regulation is known as *amplitude modulation*.

The Michaelis-Menten constant is a steady-state constant attained under initial conditions, and not an equilibrium constant, so it cannot be analyzed with equilibrium thermodynamics. However, K_m $(=(k_2 + k_3)/k_1)$ is equivalent to the dissociation constant K_d $(=k_2/k_1)$ when $k_2 >> k_3$. The dissociation constant (in M) is a measure of the affinity of two chemicals for each other. The dissociation constant is an equilibrium constant, and consequently can be treated thermodynamically. The dissociation constant of E for S in the reaction shown below is equal to k_2/k_1 where k_1 is called the *on-rate constant* (in $M^{-1}\ s^{-1}$) and k_2 is called the *off-rate constant* (in s^{-1}):

$$S + E \underset{k_2}{\overset{k_1}{\Leftrightarrow}} ES$$

Let us consider the equilibrium state where the rate of formation of the active complex [ES] equals the rate of dissociation of the active complex [ES]. Thus:

$$k_1[S][E] = k_2[ES] \quad (12.10)$$

Solving for [E]/[ES] and defining K_d as k_2/k_1 we get:

$$[E]/[ES] = (k_2/k_1)/[S] = K_d/[S] \quad (12.11)$$

Remember that $[E]/[ES] = ([E]_T/[ES]) - 1$. Thus:

$$([E]_T/[ES]) - 1 = K_d/[S] \quad (12.12)$$

Since $[E]_T/[ES] = v_{max}/v$, then

$$v_{max}/v = \{(K_d/[S]) + 1\} \quad (12.13)$$

and

$$v = v_{max}/\{(K_d/[S]) + 1\} \quad (12.14)$$

Thus, like a reaction that follows Michaelis-Menten kinetics, the velocity of a reaction activated by the ES complex depends on the concentration of the activator relative to the dissociation constant. The true dissociation constant is determined by incubating a known amount of enzyme in various concentrations of substrate and measuring how much substrate is bound at equilibrium (time = ∞). The results are plotted, and the K_d is equal to the substrate concentration at the inflection point. The association constant (K_a, in M^{-1}) is the reciprocal of K_d.

Most enzymes involved in cell regulation do not show typical Michaelis-Menten kinetics, where a plot of the reaction velocity versus the substrate concentration is a rectangular hyperbola. In the case of regulatory enzymes, a plot of velocity versus substrate concentration is typically sigmoidal. A sigmoidal response is indicative of multiple or cooperative binding sites for the substrate on the enzyme. If the binding of the substrate shows positive cooperativity, then the binding of the first substrate causes a conformational change in the enzyme so that the affinity of the next binding site is increased, etc. Thus, the substrate also acts as an activator. Multiple, positively cooperative binding sites allow more sensitive control of a reaction by a substrate/activator. Let us look at the following reaction where an enzyme binds four substrate/activator molecules.

$$[\mathrm{E}] + [\mathrm{S}] + [\mathrm{S}] + [\mathrm{S}] + [\mathrm{S}] \underset{k'_2}{\overset{k'_1}{\Leftrightarrow}} [\mathrm{ES}_4]$$

This equation is really a short hand version of four equations, including [E] + [S] ⇔ [ES], [ES] + [S] ⇔ [ES_2], [ES_2] + [S] ⇔ [ES_3], and [ES_3] + [S] ⇔ [ES_4], and thus k'_1 is the product of four on-rate constants and is given in units of $M^{-n}\,s^{-n}$ where n is the number of substrate molecules that bind to the receptor. k'_2 is the product of four off-rate constants and is given in units of s^{-n}. Thus:

$$k'_1[\mathrm{E}][\mathrm{S}][\mathrm{S}][\mathrm{S}][\mathrm{S}] = k'_2[\mathrm{ES}_4] \tag{12.15}$$

and if we define K' (in M^n) as k'_2/k'_1, we get

$$[\mathrm{E}]/[\mathrm{ES}_4] = (k'_2/k'_1)/[\mathrm{S}]^4 = K'/[\mathrm{S}]^4 \tag{12.16}$$

For simplicity, we assume that ES_4 is the only active form of the enzyme. Then we can use the following equation: $[E]/[ES_4] = ([E]_T/[ES_4]) - 1$. If we again assume that $v_{max}/v = ([E]_T/[ES_4])$, then

$$v = v_{max}/\{(K'/[\mathrm{S}]^4) + 1\} \tag{12.17}$$

Equation 12.17 can be written in a more generalized form:

$$v = v_{max}/\{(K'/[\mathrm{S}]^n) + 1\} = v_{max}/(K''/\mathrm{S})^n + 1 \tag{12.18}$$

where K'' is the nth root of K', and K' is the concentration of substrate to the nth power that activates the enzyme to a level of $v_{max}/2$. This equation, which was originally produced to describe the binding of oxygen to hemoglobin, is known as the *Hill equation*; n is called the *Hill coefficient*, named after Archibald V. Hill (1962), and it represents the number of binding sites on the enzyme for the substrate (Hill, 1965). When $n = 1$, the Hill equation is identical to the equation that relates the velocity to the dissociation constant.

By solving the Hill equation (12.18), we see how the velocity of a reaction depends on the substrate concentration. For example, when $n = 4$ and $[S] = K''$, $v = v_{max}/2$, and the reaction will proceed at its half-maximal velocity. When $n = 4$ and $[S] = 0.2K''$, $v = v_{max}/1.06$, and the reaction will proceed at approximately its maximal velocity. When $n = 4$ and $[S] = 0.2K''$, $v = v_{max}/626$, and the reaction will proceed at an infinitesimally slow velocity, and we say that the enzyme is inactive. In general, the change in the concentration of a substrate required to activate an enzyme with n binding sites from 10 percent to 90 percent of its maximal activity is equal to $\sqrt[n]{81}$ (Segal, 1968). We can see this easily by solving Eq. 12.18 for $[S]^n$. I will solve for $[S]^n$ using a few algebraic steps:

$$v\{(K'/[\mathrm{S}]^n) + 1\} = v_{max} \tag{12.19}$$

$$vK'/[\mathrm{S}]^n + v = v_{max} \tag{12.20}$$

$$vK'/[\mathrm{S}]^n = v_{max} - v \tag{12.21}$$

$$vK'/(v_{max} - v) = [\mathrm{S}]^n \tag{12.22}$$

Now I will select two concentrations of $[S]^n$, which will result in two velocities, and set up Eq. 12.22 to solve for the ratio of $[S_1]^n/[S_2]^n$ that leads to a desired ratio of velocities.

$$[\mathrm{S}_1]^n/[\mathrm{S}_2]^n = \{v_1K'(v_{max} - v_2)\}/\{v_2K'(v_{max} - v_1)\} \tag{12.23}$$

Cancel like terms:

$$[\mathrm{S}_1]^n/[\mathrm{S}_2]^n = \{v_1(v_{max} - v_2)\}/\{v_2(v_{max} - v_1)\} \tag{12.24}$$

Let's find the ratio of $[S_1]^n/[S_2]^n$ that will lead to a reaction where $v_1 = 0.9\ v_{max}$ and $v_2 = 0.1\ v_{max}$:

$$[\mathrm{S}_1]^n/[\mathrm{S}_2]^n = \{0.9\ v_{max}(v_{max} - 0.1\ v_{max})\}/\{0.1\ v_{max}(v_{max} - 0.9\ v_{max})\} \tag{12.25}$$

$$[\mathrm{S}_1]^n/[\mathrm{S}_2]^n = \{0.9\ v_{max}(0.9\ v_{max})\}/\{0.1\ v_{max}(0.1\ v_{max})\} \tag{12.26}$$

$$[\mathrm{S}_1]^n/[\mathrm{S}_2]^n = \{0.81\ v_{max}{}^2\}/\{0.01\ v_{max}{}^2\} = 81 \tag{12.27}$$

$$\sqrt[n]{([\mathrm{S}_1]^n/[\mathrm{S}_2]^n)} = \sqrt[n]{81} \tag{12.28}$$

When an enzyme has a Hill coefficient of 1, large changes in the concentration of the substrate cause small changes in the activity. Thus, the enzyme activity is stable

with respect to that substrate. When the Hill coefficient is 4, small changes in substrate concentration around the K' lead to large changes in enzyme activity.

Thus, an enzyme that shows positive cooperativity is able to recognize small changes in the concentration (i.e., amplitude) of a substrate. Consequently, such an enzyme is exquisitely designed to act as a sensitive switch in a signal transduction chain (Figure 12.2).

I will now discuss the fundamental significance of the relationship between the dissociation constant of a regulatory protein and the cellular concentration of an activator (Figure 12.3). Consider a receptor that has a $K_d = 10^{-3}$M ($= 1000\,\mu$M) and a substrate of which the intracellular concentration varies from 3×10^{-7}M to 3×10^{-5}M. This receptor does not have a high enough affinity for the substrate, so it will never bind it. If the substrate is needed to activate the receptor, this receptor will never be activated.

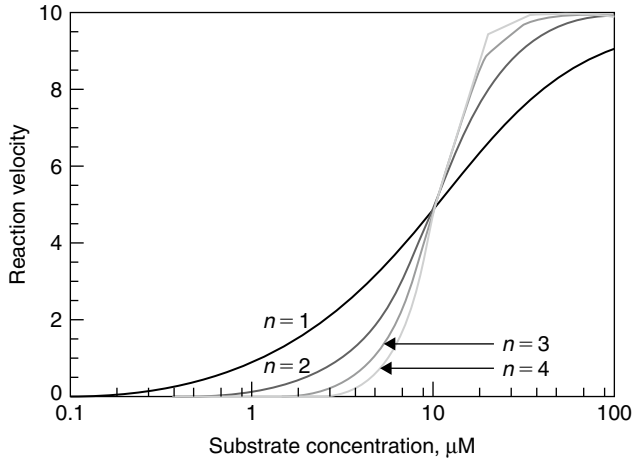


FIGURE 12.2 The relationship between reaction velocity and substrate concentration for enzymes with various Hill coefficients (n).

Consider a receptor that has a $K_d = 10^{-7}$M ($= 0.1\,\mu$M) and a substrate of which the intracellular concentration varies from 3×10^{-7}M to 3×10^{-5}M. This receptor has too high an affinity for the substrate, and even when the substrate is at its lowest concentration, the receptor will bind it. If the substrate is needed to activate the receptor, this receptor will always be activated.

Consider a receptor that has a $K_d = 10^{-6}$M ($= 1\,\mu$M) and a substrate of which the intracellular concentration varies from 10^{-7}M to 10^{-5}M. This receptor has too low of an affinity to bind the substrate when the substrate is at its lowest concentration (3×10^{-7}M) and will not be activated, but when the substrate reaches its highest concentration (3×10^{-5}M) the receptor will be activated. The substrate can act as a switch because the dissociation constant of the receptor is matched with the intracellular concentrations of the substrate in the resting and activated states. That is, the K_d is between the resting level and the activated level of the substrate. For example, when Ca^{2+} is the substrate, the K_d of the receptor should be about 10^{-6}M because the resting concentration is 10^{-7}M and the activated concentration is about 10^{-5}M. Of course, a cell may have a variety of intracellular Ca^{2+} receptors with K_d near 10^{-6}M, but with different Hill coefficients. Thus, at a given rate of increase in the concentration of intracellular Ca^{2+}, each one binds Ca^{2+} at a different rate, and thus they become activated at different times. Differential activation can also occur because the intracellular Ca^{2+} receptors may be closer or farther from the site of Ca^{2+} entry. Let us look at the chemistry of Ca^{2+} and ask why Ca^{2+} is a ubiquitous second messenger while Mg^{2+}, a similar bivalent ion, is not.

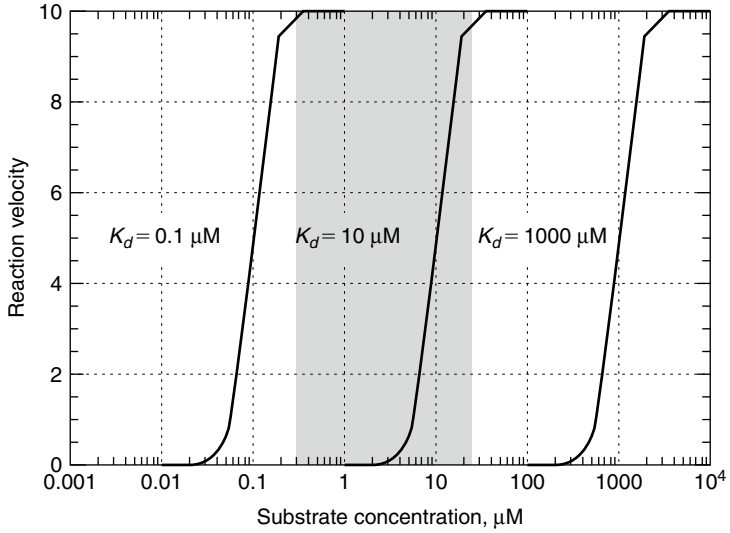


FIGURE 12.3 The relationship between reaction velocity and substrate concentration for proteins that have various affinities for the substrate. The smaller the dissociation constant (K_d), the greater the affinity. The gray region indicates the range in which the concentration of the substrate varies.

12.5.3 Kinetics of Diffusion and Dehydration

Calcium ions must diffuse through the cell to a receptor before they are able to activate the receptor. In solution, water molecules are bound to ions as a consequence of the polar or electrical dipolar nature of water and the electrical charge of the ions. Thus, ions structure the water in the cytoplasm to some degree and the water of hydration surrounding the ions hinders the ions from binding to other ligands. The water that immediately surrounds an ion may thus have a different viscosity than the bulk water in a cell. According to R. J. P. Williams (1974, 1976, 1980), Ca^{2+} is more fit than Mg^{2+} to act as a second messenger because Ca^{2+} sheds its water of hydration more quickly than Mg^{2+}. But is the dehydration step the limiting step in ionic reactions in cells? I will discuss the factors that limit the overall rate of a reaction.

In order for Ca^{2+} and Mg^{2+} to enter a reaction, they must be dehydrated. The dehydration reactions are as follows:

$$Ca^{2+} + H_2O \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} Ca^{2+}H_2O$$

$$Mg^{2+} + H_2O \underset{k_{off}}{\overset{k_{on}}{\Leftrightarrow}} Mg^{2+}H_2O$$

In water, the concentration of free cations is less than the concentration of total cations because a majority of the ions is bound to molecules of water. These water molecules make concentric shells of more and more loosely bound water around the cation. The binding energies between water and a given ion are calculated using electrostatic models (Williams and Williams, 1965, 1966). Because Mg^{2+} and Ca^{2+} have the same charge, but Ca^{2+} has a larger radius than Mg^{2+}, Ca^{2+} does not hold on to the negatively charged oxygen atoms in water as tightly as Mg^{2+} does. Due to its smaller charge density, Ca^{2+} sheds its water of hydration more than 1000 times more quickly than Mg^{2+}, and the off-rate constants (k_{off}) are $5 \times 10^8\ s^{-1}$ for Ca^{2+} and $10^5\ s^{-1}$ for Mg^{2+} (Eigen and Kruse, 1962). Because of this, dehydration limits the rate in which magnesium can bind to a ligand.

In order to convert these off-rate constants of dehydration into potential on-rate constants for the ion to bind with an intracellular ligand, the off-rate constants must be divided by the intracellular concentration of the ion that is able to activate the receptor (e.g., the K_d; see Table 12.1A). Likely physiological on-rate constants are $12.5 \times 10^8 M^{-1}\ s^{-1}$ and $10^8 M^{-1}\ s^{-1}$ for Ca^{2+} and Mg^{2+}, respectively.

While the speed in which an ion sheds its water of hydration will influence its ability to bind a ligand, the dehydration step is not always the step that limits the rate of a reaction. The rate of a reaction can depend on the speed in which an ion diffuses to the ligand. While the rate of dehydration for similarly charged ions is proportional to the radius (r_H) of the ion, the rate of diffusion is inversely proportional to the radius of the ion. In addition, the rate of binding of an ion to a stationary ligand is proportional to the radius of the ligand (r_b). The on-rate constant for a nonelectrolyte to enter a diffusion-limited reaction is given by the equation derived by Marian von Smolokowski (1917):

$$\begin{aligned} k_{on} &= [4\pi r_b RT/(6\pi r_H \eta)](10^3\ L/m^3) \\ &= 4\pi r_b D N_A (10^3\, L/m^3) \end{aligned} \qquad (12.29)$$

where $4\pi r_b$ represents the size of the receptor, $RT/(6\pi r_H \eta)$ represents the molar diffusion coefficient of the ion (in $m^2/[s\ mol]$), and ($10^3 L/m^3$) is the factor that converts cubic meters into liters. The on-rate constant for ions must take into consideration the charge of the ion and the strength of the electric field. Peter Debye (1942) has derived more sophisticated equations for describing the diffusion of ions to a receptor, and Glasstone et al. (1941) have formulated the Smolokowski equation in quantum mechanical terms. For simplicity, we will estimate the on-rate constant for ions using Smolochowski's classic equation. In general, the on-rate constant of diffusion-limited reactions can be considered to be approximately $10^8 - 10^9 M^{-1}\ s^{-1}$ given the viscosities, temperatures, and hydrodynamic radii frequently encountered in a cell. Consistent with the inverse relationship between on-rate constants and hydrodynamic radii of the diffusing ions, the diffusion-limited on-rate constants for Ca^{2+} are approximately 30 percent smaller than those for Mg^{2+} (Table 12.1B). Typically, Ca^{2+}-mediated reactions are limited by diffusion, while Mg^{2+}-mediated reactions are limited by dehydration.

As I discussed in the previous section, kinetics dictates that the K_d of a ligand for an ion must be close to the intracellular concentration of that ion if that ligand is to act as a signaling

TABLE 12.1A On-rate constants (k_{on}) for Ca^{2+} and Mg^{2+} when dehydration is limiting

K_d	Ca^{2+}	Mg^{2+}
	k_{on} ($M^{-1}\ s^{-1}$)	k_{on} ($M^{-1}\ s^{-1}$)
10^{-7}	5×10^{15}	10^{12}
10^{-6}	5×10^{14}	10^{11}
10^{-5}	5×10^{13}	10^{10}
10^{-4}	5×10^{12}	10^{9}
10^{-3}	5×10^{11}	10^{8}
10^{-2}	5×10^{10}	10^{7}

Note: *Values are calculated by dividing the off-rate constants for dehydration by the K_d of the receptor ligand.*

TABLE 12.1B On-rate constants (k_{on}) for Ca^{2+} and Mg^{2+} when diffusion is limiting

	Ca^{2+}	Mg^{2+}
Radius of Binding Site	k_{on} (M^{-1} s^{-1})	k_{on} (M^{-1} s^{-1})
1×10^{-10} m	4.2×10^8	6.3×10^8
2×10^{-10} m	8.3×10^8	12.7×10^8
3×10^{-10} m	12.5×10^8	19.0×10^8

Note: *Values are calculated from Smolochowski's equation assuming $\eta = 0.004$ Pa s, $T = 298$ K, and the radii of Ca^{2+} and Mg^{2+} ions are 99 and 65 pm, respectively.*

$$k_{on} = [4\pi r_b RT/(6\pi r_H \eta)](10^3 \text{ L/m}^3)$$

In order to convert on-rate constants into time, we must combine Einstein's random-walk equation ($t = x^2/(2D)$ with Smolochowski's equation ($k_{on} = [4\pi r_b RT/(6\pi r_H \eta)](10^3 \text{ L/m}^3) = 4\pi r_b D N_A (10^3 \text{ L/m}^3)$). Since $D = k_{on}/(4\pi r_b N_A (10^3 \text{ L/m}^3))$, then

$$t = x^2(4\pi r_b N_A(10^3 \text{ L/m}^3))/(2k_{on})$$

or

$$t = x^2(2\pi r_b N_A(10^3 \text{ L/m}^3))/(k_{on})$$

or

$$t \propto 1/(k_{on})$$

molecule. Thus, at a Ca^{2+} concentration of 0.1–10 μM and a Mg^{2+} concentration of 1–10 mM, the K_d for a Ca^{2+}-binding or an Mg^{2+}-binding ligand must be between 0.1–10 μM and 1–10 mM, respectively. Given these K_ds, and the calculated maximum on-rate constants, for a given concentration of the ion, the off-rate constants of Ca^{2+}-activated and Mg^{2+}-activated reactions would be:

- For Ca^{2+}: (10^{-5}M) $(12.5 \times 10^8 \text{M}^{-1} \text{s}^{-1})$ $= 1.25 \times 10^4 \text{ s}^{-1}$
- For Ca^{2+}: (10^{-6}M) $(12.5 \times 10^8 \text{M}^{-1} \text{s}^{-1})$ $= 1.25 \times 10^3 \text{ s}^{-1}$
- For Ca^{2+}: (10^{-7}M) $(12.5 \times 10^8 \text{M}^{-1} \text{s}^{-1})$ $= 1.25 \times 10^2 \text{ s}^{-1}$
- For Mg^{2+}: (10^{-3}M) $(10^8 \text{M}^{-1} \text{s}^{-1}) = 10^5 \text{ s}^{-1}$
- For Mg^{2+}: (10^{-2}M) $(10^7 \text{M}^{-1} \text{s}^{-1}) = 10^5 \text{ s}^{-1}$

Wilhelm Ostwald defined the half-time of a reaction to be $(\ln 2)/k_{off}$. The half-time of the reaction indicates how long an ion and a ligand will stay together before they dissociate. The shorter the half-time, the more rapidly the molecules involved can pass through the cycle of movements necessary to complete an elementary reaction and be ready for the next one. Regulatory proteins with short half-times can regulate rapid reactions with sharp and keen precision. Regulatory proteins with long half-times can regulate reactions with lower temporal resolution. If we consider diffusion to be limiting, the half-times for Ca^{2+}-mediated reactions will fall between 55 μs and 5.5 ms. If we consider diffusion to be limiting for Mg^{2+}-mediated reactions, the half-times are approximately 6.9 μs. As I discussed previously, when dehydration is limiting, the half-times for Ca^{2+}-activated and Mg^{2+}-activated reactions are 1.39 ns and 6.93 μs, respectively. Thus, Ca^{2+}-mediated reactions are typically limited by diffusion, while Mg^{2+}-mediated reactions are limited by dehydration. Thus, by considering diffusion, affinity, and dehydration, we find that, under typical cellular conditions, Mg^{2+} could actually be a faster signaling agent than Ca^{2+}.

Thus, it is incorrect to assume that Mg^{2+} is an inferior ion when it comes to cell signaling compared with Ca^{2+} because it is small and slow, while "Ca^{2+} is fat and fast." I do not know why Ca^{2+} is a ubiquitous second messenger while Mg^{2+} is not. Perhaps one reason is the fact that fewer Ca^{2+} ions have to enter the cell to raise the concentration 3- to 81-fold compared with Mg^{2+}. A 10-fold increase in the intracellular Mg^{2+} concentration may be an ionic and osmotic burden. Moreover, since the Mg^{2+} concentration outside the cell is likely to be equal to or less than the intracellular concentration, the electrochemical difference will be smaller for Mg^{2+} than it would be for Ca^{2+}, and an influx of Mg^{2+} may actually be the rate-limiting step.

It is possible that Ca^{2+} is a better signaling ion than Mg^{2+} because it is more flexible in forming coordinate bonds with a ligand. Mg^{2+} is a rigid ion that forms exactly six coordinate bonds with lengths that vary a little from 200–212 pm, while Ca^{2+} can form six to eight coordinate bonds where the lengths of the various bonds are between 206 and 282 nm (Martell and Calvin, 1952). Perhaps the flexible Ca^{2+} ion can resonate and harmonize with conformational changes that occur in the protein to which it binds, whereas Mg^{2+} would be stiff and better suited to act as a bridging ion that can bring together a protein with ATP. It is also possible that there are Mg^{2+}-regulated reactions that have yet to be discovered.

We can use thermodynamics to help us understand the relationship between affinity and the change in free energy of a receptor (Lewis and Randall, 1923). Given the likely on-rate constants, the off-rate constant varies inversely with the dissociation constant. The molecular free energy released upon binding is related to K_d and K_a. The relationship is shown in Eq. 12.30:

$$E^{eq} - E^{Real} = kT \ln\{(K_a)/(K)\} \tag{12.30}$$

$$\begin{aligned} E^{eq} - E^{Real} &= kT \ln\{1/(KK_d)\} \\ &= -kT \ln\{KK_d\} \end{aligned} \tag{12.31}$$

where K is the ratio of products to reactants under real conditions, K_a represents the ratio of products to reactants at equilibrium, and $K_d = 1/K_a$. The product of K and K_d is always dimensionless no matter what the order of the reaction. Since E^{eq} is defined to be zero, Eq. 12.31 becomes

$$E^{\text{Real}} = kT \ln\{KK_d\} \quad (12.32)$$

The smaller the K_d, the greater the decrease in the molecular free energy, and consequently, the more stable the binding. Therefore, the high-affinity receptors regulate reactions with a low temporal precision compared with low-affinity receptors. Thus, for a given ion, a low-affinity receptor that becomes activated by an increase in the ion concentration (amplitude modulation) will always be able to regulate faster processes than the same receptor that increases its affinity for the ion (sensitivity modulation).

Calcium ions and H^+ are found in the cell at concentrations around 10^{-7} M, whereas other cations are either much more abundant (K^+, Na^+, Mg^{2+}, Mn^{2+}) or much less abundant (Cu^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+}). Thus, the binding of Ca^{2+} or H^+ to a ligand will be intermediate between a stable binding and a loose binding. Perhaps this is important in its fitness as the universal second messenger.

12.5.4 A Thermodynamic Analysis of the Signal-to-Noise Problem

In order for a primary or secondary stimulus to be perceived, the energy of the signal must be greater than the ambient energy or noise. Thus, we must be concerned with the signal-to-noise ratio of reactions. The energy of a stimulus has to be greater than $\approx kT$ because each and every molecule in the cell has a certain amount of energy that results from the thermal energy of the cell. The thermal energy of a molecule at any temperature is approximately equal to kT. Thus, the minimum energy needed to activate a receptor is $\approx kT$, and using the tenets of quantum mechanics, the receptor will become active when it absorbs an amount of energy, in the form of gravitational energy, radiant energy, chemical energy, etc., that is equal to the difference in energy between that of the active and inactive states. I will use enzyme kinetics and thermodynamics in order to show the relationship between the energy input and the probability (K_s) that a receptor will become activated.

Let us assume that we have a receptor protein that becomes activated according to the following reaction:

$$\text{inactive} \Leftrightarrow \text{active}$$

Let us assume that in order to trigger a response, there has to be a probability of 100:1 that the receptor will become activated by the stimulus. Put another way, following stimulation, there must be a ratio of 100 active receptors to 1 inactive one. The probability of reaching this activation level depends on the energy of the stimulus. This is equal to the energy difference between the active and inactive states:

$$E - E^\circ = kT \ln(K_s) \quad (12.33)$$

where E and E° are the molecular free energies of the receptor in the active state and the inactive state, respectively, relative to a standard energy. In this case, we will take the standard energy to be kT, the approximate energy of thermal noise. K_s is the ratio of active to inactive receptors and is equal to [active]/[inactive]. Thus:

$$[\text{active}]/[\text{inactive}] = K_s = e^{((E-E^\circ)/kT)} \quad (12.34)$$

The energy input necessary to induce a ratio of 100 active receptors to 1 inactive receptor is obtained by putting Eq. 12.34 in the following form:

$$\ln([\text{active}]/[\text{inactive}]) = \ln(100) = 4.6 = (E - E^\circ)/kT \quad (12.35)$$

which simplifies to:

$$(E - E^\circ) = 4.6\,kT = 1.89 \times 10^{-20}\ \text{J} \quad (12.36)$$

Thus, the difference between E and E° necessary to create a ratio of active to inactive receptors of 100:1 is equal to $4.6kT$. Thus, if $E^\circ = 1kT$, the energy of the activated receptor must be $5.6kT$. We can also look at this energy as the amount of energy needed to give a probability of 100:1 that a single receptor will become activated after an energy input of $4.6kT$. The difference in energy has to be $2.3kT$, $1.6kT$, and $0.69kT$ to create a 10:1 probability, a 5:1 probability, and a 2:1 probability, respectively. In these cases, the energies of the activated receptors will be $3.3kT$, $2.6kT$, and $1.69kT$, respectively. The ratio of active to inactive receptors is a function of the energy difference between the inactive and active receptor. From a thermodynamic point of view, I cannot imagine cellular receptors that can be activated by energies that are less than that of thermal noise. Now that I have discussed the theoretical aspects of cell signaling, I will discuss the components of the cell that participate in cell signaling.

12.6 Ca^{2+} SIGNALING SYSTEM

Since Ca^{2+} is a cytotoxin, cells have evolved an efficient method for lowering the Ca^{2+} concentration in the cytosolic P-space to 0.1 μM, approximately 10,000 times lower than the concentration in the environment. The low Ca^{2+} concentration in the cell is maintained by a plasma membrane–bound Ca^{2+}-ATPase, an ER-bound Ca^{2+}-ATPase, a vacuolar membrane–bound Ca^{2+}/H^+ antiport system (Schumaker and Sze, 1985, 1986, 1987) and Ca^{2+}-ATPase (Berkelman and Lagarius, 1990), and a mitochondrial uptake system (Dieter and Marme, 1980).

The challenge the cells faced to lower their intracellular-free Ca^{2+} concentration provided an opportunity for the cells to use this 10,000-fold gradient as a cellular switch to couple an extracellular stimulus with a cellular response. All that is needed is a control mechanism in the cell to induce a transient 10- to 100-fold increase in the Ca^{2+} concentration, which is still below the toxic level. This is accomplished by the opening of Ca^{2+} channels.

A Ca^{2+} channel is a protein made up of one or more polypeptides that form a hydrophilic pore in a membrane. The channel allows Ca^{2+} ions to pass relatively unimpeded at a rate of about 10^6 s^{-1} or more. Ca^{2+}-permeable channels may be either nonselective or highly selective for Ca^{2+}. The selectivity depends on the pore size and the charge density of the binding sites at the mouth of the pore. These sites directly affect the ability of Ca^{2+} to shed its outer shells of water so that it can pass as a dehydrated ion ($\approx$0.2 nm in diameter).

All Ca^{2+} channels mediate the transfer of Ca^{2+} from E-spaces to P-spaces. Ca^{2+} channels occur in the plasma membrane, as well as in the internal membranes. There can be many types of Ca^{2+} channels on the same membrane. The structure of channels is often deduced from molecular studies where a computer program calculates a hydropathy plot of the encoded amino acids (Kyte and Doolittle, 1982). The program is used to assess where the membrane-spanning regions of a protein may be, by equating a series of hydrophobic amino acids with a membrane-spanning region. The structure of channels can also be deduced from electrophysiological studies and biochemical studies. In the biochemical studies, radiolabeled channel blockers are used to follow the purification of the channel (Graziana et al., 1988; Harvey et al., 1989a,b; Thuleau et al., 1990).

The polypeptides of a Ca^{2+} channel complex isolated from muscle cells have been resolved by SDS polyacrylamide gel electrophoresis (Catterall et al., 1989; Catterall, 1995). The channel consists of five polypeptides. Their designations and relative molecular masses are α_1 (175 kDa), α_2 (143 kDa), β (54 kDa), γ (30 kDa), and δ (20 kDa). The α_1 and α_2 polypeptides run together in the absence of dithiothreitol (DTT), an agent that cleaves disulfide bonds, indicating that they are attached through disulfide bonds. The α_1 polypeptide probably forms the pore since it is the polypeptide that binds the Ca^{2+} channel blocker ^{3}H-azidopine.

The ability to determine which polypeptides have transmembrane-spanning segments comes from experiments where the polypeptides are challenged with the photoaffinity-labeling hydrophobic probe [^{125}I]3-(trifluoromethyl)-3-(m-iodo-phenyl)diazirine (TID). The α_1 and γ polypeptides are predominantly labeled, and the α_2 and δ polypeptides are slightly labeled. Thus, these polypeptides may have membrane-spanning regions. The α_1 polypeptide is the most abundantly labeled, indicating that it has the most membrane-spanning domains. The β polypeptide is not labeled at all. Therefore, it must be a peripheral protein. The α_2, γ, and δ polypeptides bind ^{125}I-wheat germ agglutinin, a sugar-binding agent, indicating that they are glycoproteins, and thus have domains on the E-side of the membrane.

Ca^{2+} channels are regulated through phosphorylation (Shiina et al., 1988) and it appears that the Ca^{2+} channel itself may be the substrate since the receptor complex becomes phosphorylated in the presence of the catalytic subunit of cAMP-dependent protein kinase and [^{32}P]ATP. Only the α_1 and β subunits are phosphorylated, indicating that they may contain the sites that are regulated by phosphorylation in vivo, and thus have domains on the P-side of the membrane.

A Ca^{2+} channel has been reconstituted in vitro, and its transport activity has been tested in a functional assay by inserting it into liposomes and determining their ability to take up $^{45}Ca^{2+}$, or by inserting it in a lipid bilayer and assaying its Ca^{2+}-transport activity with the patch-clamp technique (Pelzer et al., 1989).

Once Ca^{2+} enters the cell, and its concentration rises from the resting level of 30–200 nM to the activated level of 0.3–40 μM (Bush, 1993), it binds to specialized proteins, like calmodulin, which influence many aspects of cell metabolism (Kawasaki and Kretsinger, 1995). Activated calmodulin activates many enzymes, including protein kinases. A number of Ca^{2+} or Ca^{2+}-calmodulin-activated protein kinases have been found in plant cells. These include soluble, membrane-bound, and cytoskeleton-associated proteins (Polya et al., 1983, 1990; Harmon et al., 1987; Roberts, 1989). One of the actions of calmodulin is to activate the plasma membrane–bound Ca^{2+}-ATPase that pumps Ca^{2+} out of the cell and subsequently restores the cell to its resting state.

While the concentration of free Ca^{2+} is in the nanomolar range in the cytosol of resting cells, the total concentration of calcium is in the millimolar range (Wayne and Hepler, 1985b; Tazawa et al., 2001). This is because most of the calcium is sequestered in the E-space of the endoplasmic reticulum (ER), which contains various high-capacity, low-affinity calcium-binding proteins, including calsequestrin and calreticulin, with millimolar dissociation constants (see Chapter 4).

12.7 MECHANICS OF DOING EXPERIMENTS TO TEST THE IMPORTANCE OF Ca^{2+} AS A SECOND MESSENGER

In order to establish whether Ca^{2+} is involved in a given response, it is helpful to keep three rules, which have been named Jaffe's Rules in honor of Lionel Jaffe, in mind (Hepler and Wayne, 1985):

1. The response should be preceded or accompanied by an increase in intracellular Ca^{2+} concentration.
2. Blockage of the natural increase in the intracellular Ca^{2+} concentration should inhibit the response.
3. The experimental generation of an increase in the intracellular Ca^{2+} concentration should mimic the stimulus and stimulate the response.

In order to determine whether or not a response is preceded or accompanied by an increase in the intracellular [Ca^{2+}], the intracellular [Ca^{2+}] must be measured. This can be done by microinjecting a luminescent protein from the jellyfish *Aequoria aequoria* into the cytoplasm (Shimomura,

2008). This protein, called *aequorin*, luminesces in the presence of Ca^{2+}. The amount of luminescence is a function of the Ca^{2+} concentration; however, the relationship is not linear, which makes the calibration somewhat difficult (Blinks et al., 1982). This protein is large enough (≈20,000 Da) that once it is injected into the cytosol it stays there and reports on cytosolic Ca^{2+} concentrations, and not that of the ER, Golgi apparatus, etc. that could be measured by a small permeant Ca^{2+} indicator. Knight et al. (1991, 1992) have transformed plants with the aequorin gene so that the luminescence of the protein will indicate the cytosolic Ca^{2+} concentrations without the difficulty of microinjection.

Ca^{2+} concentrations can be measured with fluorescent dyes, including Fura-2 and calcium green. These dyes can be loaded into the cells by diffusion if the cell is put in an acid environment. Acetoxymethyl ester derivatives of the dye readily penetrate the plasma membrane, and once inside, the intracellular esterases cleave off the hydrophobic ester making the dye hydrophilic so it stays in the cytosol. Unfortunately, over time the low–molecular mass dye diffuses into the membranous compartments, thus giving a misleading estimate of the cytosolic Ca^{2+} concentration (Bush and Jones, 1990). However, these dyes can be conjugated to high–molecular mass dextrans so that they will stay in the cytosol (Miller et al., 1992; Gilroy and Jones, 1992).

Roger Tsien (2008) has designed a genetically engineered calcium-sensitive fluorescent protein by combining the gene sequences for the green fluorescent protein (GFP; Chalfie, 2008; Shimomura, 2008) and calmodulin. Intracellular calcium concentrations can be measured in cells that have been transformed with the gene that encodes this protein (Palmer et al., 2004).

Ca^{2+} concentrations can also be measured with Ca^{2+}-selective microelectrodes. These are glass microcapillary electrodes that have a synthetic resin membrane in them that has a high permeability to Ca^{2+} but not to other ions (Felle, 1989a). The difference in the concentration of Ca^{2+} in the cytosol relative to the inside of the Ca^{2+}-selective electrode generates an electrical potential. The Ca^{2+} concentration in the cytosol is determined using the Nernst equation. Each technique described previously has advantages and limitations and they really should be used in combination.

In order to test if a blockage of the stimulus-induced increase in the intracellular Ca^{2+} concentration inhibits the response, we must either lower the external Ca^{2+} concentration with Ca^{2+} chelators (e.g., EGTA) or lower the intracellular Ca^{2+} concentration with the chelator BAPTA. Ca^{2+} entry can be prevented with inorganic blockers, including Nd^{3+}, La^{3+}, and Gd^{3+}, and organic blockers, including nifedipine and verapamil. Since each class of channels is only affected by some of the inhibitors, the identity of the channels involved in a given response can be deduced from their inhibitor specificity.

Lastly, it should be possible to generate an increase in the intracellular Ca^{2+} concentration in the absence of a stimulus and consequently induce the response. This can be done by microinjecting Ca^{2+} into the cell; microinjecting into the cell-caged Ca^{2+}, which can be released on demand by irradiating the cell with light (Gilroy et al., 1990); or by treating cells with Ca^{2+}-selective ionophores, including A23187 or ionomycin. An ionophore is an artificial carrier that diffuses through the membrane-carrying Ca^{2+} and thus collapses the Ca^{2+} difference, thereby increasing the intracellular Ca^{2+} concentration.

12.8 SPECIFIC SIGNALING SYSTEMS IN PLANTS INVOLVING Ca^{2+}

12.8.1 Ca^{2+}-Induced Secretion in Barley Aleurone Cells

The secretion of α-amylase by aleurone cells is perhaps the best studied secretory system in plants and has also been used to study stimulus-response coupling (Bethke et al., 2006; Sreenivasulu et al., 2008). Secretion of α-amylase is stimulated by gibberellic acid (GA), and the GA-induced stimulation is inhibited by abscisic acid (AbA; Chrispeels and Varner, 1967). The secretion of α-amylase is dependent on external Ca^{2+} (Moll and Jones, 1982; Jones and Jacobsen, 1983; Jones and Carbonell, 1984). GA-induced secretion of α-amylase is blocked by the Ca^{2+} channel blocker La^{3+} and by calmodulin inhibitors. Moreover, the release of α-amylase is stimulated in the absence of GA by the Ca^{2+} ionophore A23187 (Mitsui et al., 1984). These data suggest that Ca^{2+} acts as a second messenger in GA-induced α-amylase secretion.

Gilroy and Jones (1992) have shown that GA induces an increase in the intracellular Ca^{2+} concentration after 4 hours, which is 2 hours before it induces α-amylase secretion. The cytosolic Ca^{2+} concentration increases from 50 nM to 200 nM and is dependent on external Ca^{2+}. AbA reverses the GA-induced increase. Confocal microscopy shows that the GA-induced increase in intracellular Ca^{2+} is localized just inside the plasma membrane.

Bush et al. (1993) show that much of the GA-induced Ca^{2+} uptake is taken up into the ER by a Ca^{2+}-ATPase. They suggest that elevated levels of Ca^{2+} may be necessary in part for the correct folding of α-amylase in the ER. α-amylase is a Ca^{2+} requiring metalloprotein and its correct folding may depend on the Ca^{2+} binding chaperonin BiP (Jones and Bush, 1991). Heterotrimeric G-proteins are also involved in the GA-induced stimulation of α-amylase secretion (Ueguchi-Tanaka et al., 2000).

12.8.2 Excitation-Cessation of Streaming Coupling in Characean Internodal Cells

While secretion of α-amylase is a relatively slow response and has a protracted signal transduction chain, the cessation

of cytoplasmic streaming is rapid and dramatic. Once the mechanism of cytoplasmic streaming was elucidated, Masashi Tazawa, Richard Williamson, and their colleagues turned their attention to how it is regulated and influenced by external stimuli (Kikuyama et al., 1996; Kikuyama, 2001). The cessation of streaming can be induced either electrically or mechanically (Kishimoto, 1968; Staves and Wayne, 1993; Wayne, 1994; Shimmen, 1996, 1997, 2001, 2003, 2008; Iwabuchi et al., 2005; Kaneko et al., 2005). These stimuli transiently eliminate the motive force that drives cytoplasmic streaming. Cytoplasmic streaming stops instantly in response to these stimuli as would be expected in a cell in which viscous forces predominate over inertial forces (Tazawa and Kishimoto, 1968). While the cessation of streaming is rapid and occurs in less than a second, the recovery is slow and takes a few minutes. The slow recovery may be due, in part, to restarting movement in a thixotropic solution where the viscosity at the motor region increases 100-fold upon stopping. It may also be a result, in part, from the slowness of the biochemical reactions needed to reactivate streaming.

Williamson and Ashley (1982) injected aequorin into cells to monitor the free calcium concentration, and found that an action potential caused a transient increase in the cytoplasmic Ca^{2+} concentration from approximately 0.1 μM to tens of micromolar. Williamson (1975) removed the vacuolar membrane from cells so that he could artificially increase the Ca^{2+} concentration in the cytosol. He found that when the Ca^{2+} concentration was greater than 10 μM, streaming stopped. It follows that an action potential may induce an increase in the intracellular Ca^{2+} concentration to 10 μM and consequently stop streaming.

At that time, vacuolar membrane-free cells did not show much sensitivity to Ca^{2+} (Tominaga and Tazawa, 1981). This was probably due to a washing out, during the preparation of vacuolar membrane-free cells, of a Ca^{2+}-binding protein or other factor needed for Ca^{2+} sensitivity. This led to the development of a plasma membrane permeabilized cell model. The permeabilized cell models are sensitive to Ca^{2+}, and increasing the cytoplasmic concentration above 1 μM causes the cessation of streaming (Tominaga et al., 1983). Streaming can be inhibited in intact cells by injecting the cells with Ca^{2+} (Kikuyama and Tazawa, 1982), indicating that artificially increasing the intracellular Ca^{2+} concentration mimics the stimulus. Restricting the influx of Ca^{2+} with the lanthanides or by placing the cells in media with submicromolar concentrations of Ca^{2+} prevents the stimulus-induced cessation of streaming (Staves and Wayne, 1994).

What does the Ca^{2+} bind to once it enters the cell? It is not currently known, but perhaps the Ca^{2+} activates a Ca^{2+}-dependent protein kinase, which can covalently modify one or more of the response elements crucial for streaming. This is likely since the ATP analog ATP-γ-S, which acts as a substrate of protein kinases but cannot be removed by protein phosphatases, prevents the recovery of streaming that occurs following the Ca^{2+}-induced cessation of streaming. Thus, the phosphorylation of a protein may cause the cessation of streaming, and the dephosphorylation of this protein may be required for the recovery of streaming. This suggestion is supported by the fact that protein phosphatase, isolated from rabbit skeletal muscle and introduced into internodal cells, prevents or reverses the inhibition of streaming caused by Ca^{2+} (Tominaga et al., 1987). It is likely that this phosphatase also exists in characean cells since a very specific protein inhibitor of this phosphatase, also isolated from rabbit skeletal muscle, brings streaming to a standstill in the absence of Ca^{2+}. The phosphorylated protein may be myosin itself.

12.8.3 Regulation of Turgor in Cells

Before I discuss turgor regulation in characean cells, I will discuss the thermodynamic basis of the van't Hoff equation, which is fundamental for understanding turgor regulation. The van't Hoff equation relates the osmotic pressure of a solution to the concentration of solutes in it (see Chapter 7).

Thermodynamic Basis of the van't Hoff Equation

Depending on the field, the tendency of water to move is described in many ways. It is discussed in terms of energy, pressure, potential, etc. To put water movement on par with every other transport process I have discussed, I will begin by describing its movement in terms of free energy. The molecular free energy of water relative to pure water at atmospheric pressure is described, in part, by Eq. 12.37:

$$\Delta E = (\overline{V}_w/N_A)\Delta P + kT \ln a_w \qquad (12.37)$$

where ΔE is the free energy (in J) of a single water molecule in the solution in question relative to a single molecule of pure water, $\overline{V}_w$ is the partial molar volume of water (in m^3/mol), N_A is Avogadro's number, k is Boltzmann's constant, T is the absolute temperature, ΔP is the hydrostatic pressure exerted on the water molecule relative to atmospheric pressure, and a_w is the activity of water in the solution in question compared to pure water (i.e., the relative activity). There are other terms that can be added to Eq. 12.37 to account for such things as the effect of gravity and electro-osmosis (House, 1974; Spanner, 1979). However, for most cellular phenomena, Eq. 12.37 is sufficient.

Water will move from one place to another if there is a difference in the molecular free energy of water in the two regions. For example, water will spontaneously move into a cell if the molecular free energy of a water molecule inside the cell is smaller than the molecular free energy of a water molecule outside the cell—that is, if $\Delta E_i - \Delta E_o$ is negative.

Thus, the movement of water into the cell depends on the following relationship:

$$\Delta E_i - \Delta E_o = \{(\overline{V}_w/N_A)\Delta P + kT \ln a_w\}_i - \{(\overline{V}_w/N_A)\Delta P + kT \ln\ a_w\}_o \quad (12.38)$$

Equation 12.38 is not an easy equation to work with, since the activity of water is not a readily measurable quantity. Therefore, I will describe how we can replace this term with one that describes directly measurable quantities.

The relative activity of water is defined as the product of the mole fraction of water (N_w) and its activity coefficient (γ_w) relative to pure water, where $a_w = \gamma_w N_w = 1$. The mole fraction of water is the ratio of moles of water to the total number of moles in the solution. That is,

$$N_w = n_w/(n_w + \Sigma n_j) \quad (12.39)$$

where n_w is the number of moles of water and n_j is the number of moles of solute j. In order to simplify our final equation, we will use the mathematical trick discussed in Chapter 2 and add zero to the numerator in the form of $(\Sigma n_j - \Sigma n_j)$.

$$\begin{aligned} N_w &= (n_w + \Sigma n_j - \Sigma n_j)/(n_w + \Sigma n_j) \\ &= 1 - \{\Sigma n_j/(n_w + \Sigma n_j)\} \end{aligned} \quad (12.40)$$

Thus:

$$\ln a_w = \ln\{\gamma_w(1 - (\Sigma n_j/(n_w + \Sigma n_j)))\} \quad (12.41)$$

Remember that the concentration of pure water is 55.5 M. If we assume that we have an ideal solution, the activity coefficient of water is 1, and all the water is unbound or "free." A dilute solution where $n_w >> \Sigma n_j$ can be considered to be an ideal solution. In an ideal solution, where $\gamma_w = 1$, $a_w = N_w$, and

$$\ln a_w = \ln(1 - (\Sigma n_j/(n_w + \Sigma n_j))) \quad (12.42)$$

We can use the series $\{-x - x^2/2 - x^3/3 - \ldots - x^m/m\}$ to approximate $\ln(1 - x)$. When x is not small, the higher-order terms, known as the *virials*, must be used. When x is small, $\ln(1 - x) = -x$. Thus:

$$\begin{aligned} \ln\ a_w &= \ln[1 - [\Sigma n_j/(n_w + \Sigma n_j)]] \\ &\cong -[\Sigma n_j/(n_w + \Sigma n_j)] \end{aligned} \quad (12.43)$$

Since for a dilute solution, $n_w >> \Sigma n_j$, then

$$\ln\ a_w \cong -[\Sigma n_j/n_w] \quad (12.44)$$

Divide both sides by $\overline{V}_w$:

$$(\ln\ a_w)/\overline{V}_w \cong -[\Sigma n_j/n_w]/\overline{V}_w \quad (12.45)$$

Since $(\Sigma n_j/n_w)/\overline{V}_w$ is equal to the concentration of solute j, then

$$\ln\ a_w \cong -C\overline{V}_w \quad (12.46)$$

where C is the concentration of solute j (in mol/m^3).

As a first approximation, the osmotic pressure is a measure of the decrease in the concentration of water due to the displacement of water from a solution by a solute (Nobel, 1991). We can rewrite Eq. 12.38 like so:

$$\Delta E_i - \Delta E_o = \{(\overline{V}_w/N_A)\Delta P - kTC\overline{V}_w\}_i - \{(\overline{V}_w/N_A)\Delta P - kTC\,\overline{V}_w\}_o \quad (12.47)$$

Water movement is typically discussed by plant biologists in terms of water potential (P_w), which is given in units of pressure (Pa) and not energy. $\Delta E_i - \Delta E_o$, in units of Joules, can be converted into water potential, in units of Pascals, according to Eq. 12.48:

$$(P_w)_i - (P_w)_o = (\Delta E_i - \Delta E_o)(N_A/\overline{V}_w) \quad (12.48)$$

Thus, Eq. 12.47 becomes

$$\begin{aligned} &(P_w)_i - (P_w)_o = (N_A/\overline{V}_w) \\ &[\{(\overline{V}_w/N_A)\Delta P - kTC\,\overline{V}_w\}_i - \\ &\{(\overline{V}_w/N_A)\Delta P - kTC\,\overline{V}_w\}_o] \end{aligned} \quad (12.49)$$

After canceling like terms, and substituting R for $N_A k$, Eq. 12.49 becomes

$$(P_w)_i - (P_w)_o = \{\Delta P - RTC\}_i - \{\Delta P - RTC\}_o \quad (12.50)$$

Van't Hoff (1898–1899) defined the osmotic pressure as RTC. I will denote it by P_π. Thus, Eq. 12.50 becomes

$$(P_w)_i - (P_w)_o = \{\Delta P - P_\pi\}_i - \{\Delta P - P_\pi\}_o \quad (12.51)$$

Assuming that the hydrostatic pressure outside the cell is equal to the atmospheric pressure, then $\Delta P_o = 0$ (by convention), and Eq. 12.51 becomes

$$(P_w)_i - (P_w)_o = \Delta P_i + P_{\pi o} - P_{\pi i} \quad (12.52)$$

Water will passively move into a cell when $(P_w)_i - (P_w)_o$ is negative. Decreasing ΔP_i and $P_{\pi o}$, or increasing $P_{\pi i}$, will favor the movement of water into a cell. Likewise, increasing ΔP_i and $P_{\pi o}$, or decreasing $P_{\pi i}$, will favor the movement of water out of the cell.

At equilibrium, when $(P_w)_i - (P_w)_o = 0$, there is no net movement of water into or out of the cell, and the hydrostatic pressure in the cell, which is referred to as the *turgor pressure*, is equal to the osmotic pressure difference:

$$P_{ti} = P_{\pi i} - P_{\pi o} \quad (12.53)$$

While the turgor pressure is a measure of the hydrostatic pressure experienced by the water molecules in the protoplast,

turgor pressure typically considered to be the force per unit area that the protoplast exerts against the wall. However, consistent with its parity with hydrostatic pressure, as defined by Pfeffer (1877), it is equal to the pressure or the force per unit area the wall exerts as it pushes back against the protoplast and forces water to leave the cell. A turgor pressure develops because the osmotic pressure is typically greater inside the cell than outside the cell, and thus water moves into the cell along its activity difference. However, the protoplast cannot expand indefinitely because of the presence of an extracellular matrix with a high tensile strength. Thus, at equilibrium, the wall exerts a pressure that is equal in magnitude, but opposite in sign to the osmotic pressure difference. By contrast, animal cells and wall-less plant cells cannot develop a significant hydrostatic pressure due to the absence of an extracellular matrix with high tensile strength. These wall-less cells must live in isotonic conditions or possess a contractile vacuole to pump out the excess water. Turgor pressure is an important property of plant cells and may affect a number of responses, including sugar uptake (Diettrich and Keller, 1991; Keller, 1991), gravity sensing (Staves et al., 1992), wall extensibility and yield threshold (Pritchard et al., 1991), plasmodesmata conductance (Ding and Tazawa, 1989), abscisic acid (ABA) accumulation (Creelman and Mullet, 1991), and wall deposition (Proseus and Boyer, 2006c). Consequently, many methods have been developed to measure turgor pressure (Tazawa, 1957; Boyer, 1995; Tomos and Leigh, 1999; Lintilhac et al., 2000; Wei et al., 2001; Geitman, 2006).

Inside the cell, at equilibrium, the water potential of all the compartments must be equal or the various organelles will shrink or swell. The hydrostatic pressure in all the organelles must be similar since the tensile strength of a typical membrane is so low that the maximum tensile stress the membrane can withstand before breaking is less than 0.1 MPa. The relations between the tensile stress a membrane experiences (σ) and the difference in the hydrostatic pressure across that membrane (dP) is given by the following formula:

$$\sigma = rdP/(2w) \tag{12.54}$$

where r is the radius of a spherical membrane-enclosed cell or organelle, and w is the thickness of the membrane (Nobel, 1983). Given that $\sigma = 0.1$ MPa, $w = 7 \times 10^{-9}$ m, and $r = 8 \times 10^{-6}$ m, the pressure difference across the membrane will be 175 Pa. Since this value is small compared to the osmotic pressure of each compartment ($\approx$700,000 Pa), we can assume that the hydrostatic pressure is essentially equal in all compartments. Since at equilibrium the water potential is equal in all compartments, the osmotic pressure must also be equal in all compartments.

Turgor Pressure in Characean Cells

Lamprothamnium is a characean alga that lives in brackish water (Okazaki, 1996; Beilby et al., 1999; Shepherd and Beilby, 1999; Shepherd et al., 1999). As in other characean cells, the vacuole takes up 90–95 percent of the cell volume, and thus accounts for most of the solutes that make up the osmotic pressure of the cell. For example, the volume of a 2×10^{-2} m long (x) $\times$ 0.5×10^{-3} m diameter ($2r$) cylindrical cell is $x\pi r^2 = 3.9 \times 10^{-9}$ m^3. If the vacuole takes up 95 percent of the cell volume, the volume of the vacuole is 3.7×10^{-9} m^3 and the volume of the cytoplasm is 0.2×10^{-9} m^3. If the osmotic pressure of the vacuole is 1.77 MPa, then it contains 715 mol/m^3 of osmotically active solutes. In the cytoplasm, the osmotic pressure must also be 1.77 MPa, and there must also be 715 mol/m^3 of osmotically active solutes in it. We can calculate how many solutes are in each compartment. In the vacuole, there are (715 mol/m^3) (3.7×10^{-9} m^3) = 2.6×10^{-6} moles of solute. In the cytoplasm, there are (715 mol/m^3) (3.7×10^{-9} m^3) = 1.43×10^{-7} moles of osmotically active solute. Therefore, the osmotic pressure of the cell sap usually gives us a good estimate of the osmotic pressure of the whole cell. *Lamprothamnium* internodal cells normally have an osmotic pressure of 1.77 MPa when they grow in brackish water with an osmotic pressure of 0.89 MPa. Thus, at equilibrium, their turgor pressure is 0.88 MPa.

Turgor Regulation in Characean Cells

Cells may regulate either their turgor pressure or their osmotic pressure (Bisson and Kirst, 1980). The internodal cell of *Lamprothamnium* is a particularly good example of a cell that regulates its turgor pressure (Okazaki, 1996). When the cells are transferred from their normal medium, which has an osmotic pressure of 0.89 MPa, to a hypotonic medium, which has an osmotic pressure of 0.51 MPa, the turgor pressure of the cells increases to 1.26 MPa. The cells then must lose solutes in order to decrease their internal osmotic pressure and to bring their turgor pressure back to 0.88 MPa (Figure 12.4).

How much must the osmotic pressure of the vacuole decrease in order to regain a turgor pressure of 0.88 MPa? It must decrease by 0.38 MPa. This represents a decrease in the concentration of osmotically active solutes of 153.4 mol/m^3. If the volume of the vacuole is 3.7×10^{-9} m^3, then 5.6×10^{-7} mol of osmotically active solutes must be lost. This is equivalent to 3.4×10^{17} solutes. Most of the solutes lost are ions, and thus in order to maintain electroneutrality, one-half of the solutes must be negatively charged while the other half must be positively charged. Assuming that K^+ and Cl^- make up the greatest part of the osmoticum lost, 1.67×10^{17} Cl^- ions and 1.67×10^{17} K^+ ions must leave the cell.

Yoshiji Okazaki and his colleagues (Okazaki and Tazawa, 1987b; Okazaki et al., 1984a,b; Okazaki and Iwasaki, 1991) have shown that Ca^{2+} acts as a second messenger in the turgor regulation response in *Lamprothamnium*. External Ca^{2+} is required for the transient increase in membrane conductance (Figure 12.5) and the efflux of osmoticum from the cells (Figure 12.6). The threshold concentration of external

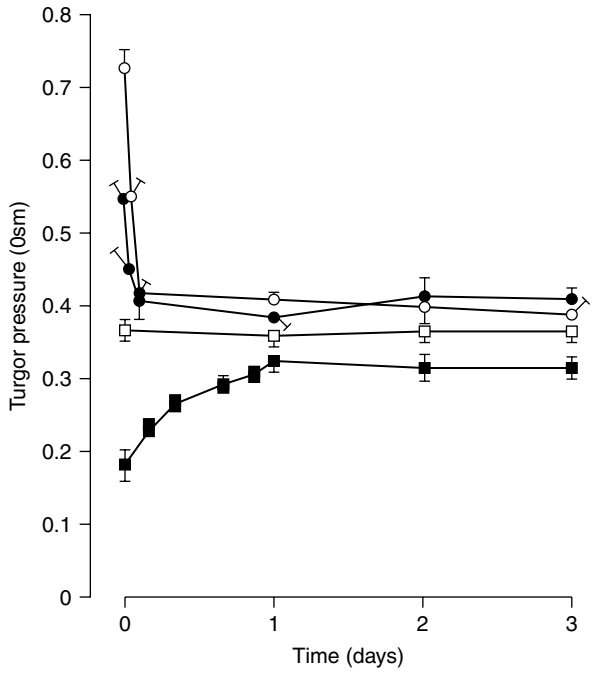


FIGURE 12.4 Time course of turgor regulation in *Lamprothamnium*. Cells were preconditioned in one-third–strength seawater and then placed in zero-strength seawater (open circles), one-sixth–strength seawater (filled circles), one-third–strength seawater (open squares), and one-half–strength seawater (filled squares). In all cases, the turgor pressure returned approximately to the control pressure. (*Source:* From Okazaki et al., 1984a.)

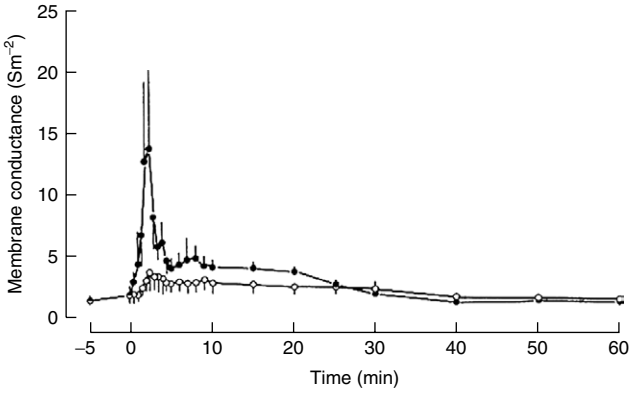


FIGURE 12.5 Time course of the change in membrane conductance and its dependence on external Ca^{2+}. Open and closed circles represent 0.01 and 3.9 mol/m^3 external Ca^{2+}, respectively. (*Source:* From Okazaki and Tazawa, 1986c.)

Ca^{2+} is 10 μM, and the optimum is approximately 1 mM (Okazaki and Tazawa, 1986a,b,c). The hypotonic medium–induced change in cellular osmotic pressure is inhibited by nifedipine, a Ca^{2+} channel blocker (Figure 12.7).

The plasma membrane has a memory. If the hypotonic treatment is given in the absence of Ca^{2+}, the cell does not undergo an increase in the electrical conductance; however, if the Ca^{2+} concentration is increased about 30 minutes

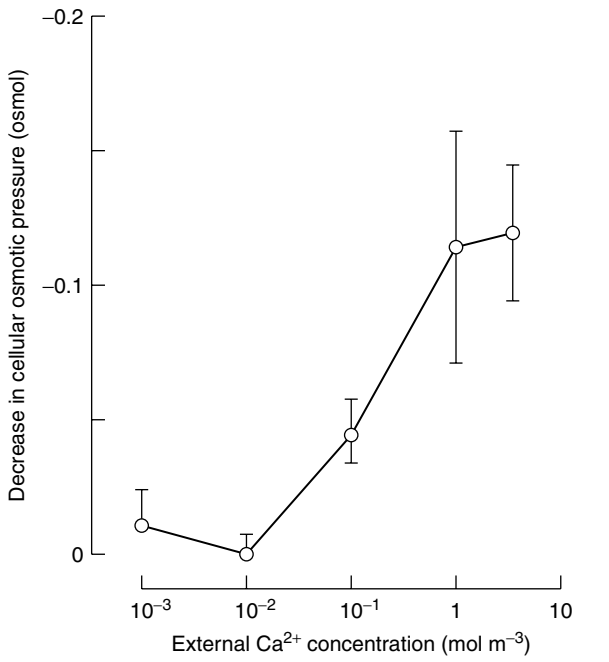


FIGURE 12.6 The dependence of the change in osmotic pressure on the external Ca^{2+} concentration. (*Source:* From Okazaki and Tazawa, 1986c.)

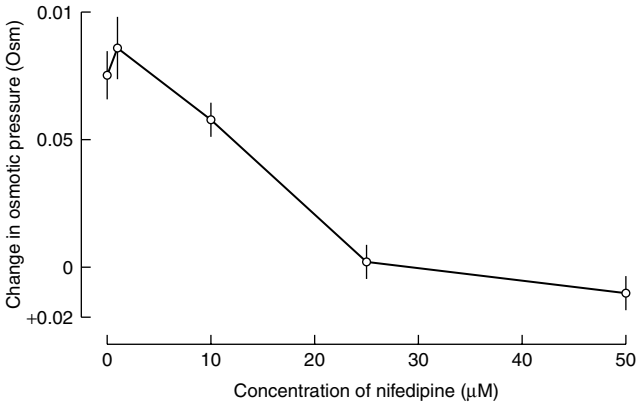


FIGURE 12.7 The effect of nifedipine, a Ca^{2+} channel blocker, on turgor regulation. (*Source:* From Okazaki and Tazawa, 1986.)

later, the membrane conductance rapidly increases following the addition of Ca^{2+} (Figure 12.8).

The rate of cytoplasmic streaming in *Lamprothamnium* stops instantly and transiently upon hypotonic treatment, but only if Ca^{2+} is in the external medium (see Figure 12.9; Okazaki and Tazawa, 1986b). This is an indication that hypotonic treatment causes an increase in the intracellular Ca^{2+} concentration. An increase in the intracellular Ca^{2+} concentration can be measured with an atomic absorption spectrophotometer. Measurements with atomic absorption spectrophotometry (AAS) show that the total cytoplasmic calcium concentration rises from 2 to 3.7 mM (Okazaki and Tazawa, 1987a). This means that there is an increase in the total cytoplasmic

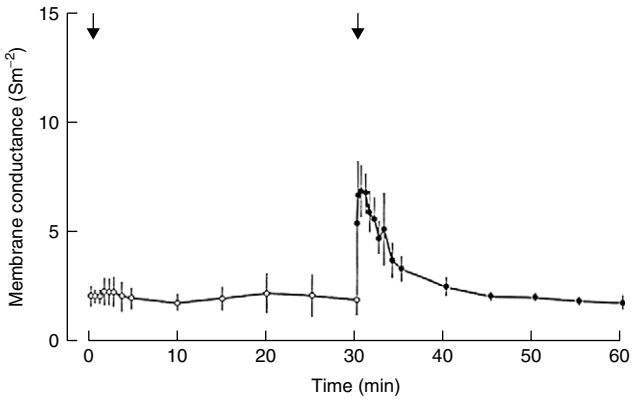


FIGURE 12.8 The membrane conductance does not increase when the cells are transferred into a hypotonic medium in the presence of 0.01 mol/m^3 external Ca^{2+} (first arrow). However, when 3.9 mol/m^3 external Ca^{2+} is added to the medium (second arrow), the membrane conductance rapidly increases, indicating that the membrane remains poised to respond to the turgor change, but the change in conductance itself requires external Ca^{2+}. (*Source:* From Okazaki and Tazawa, 1986c.)

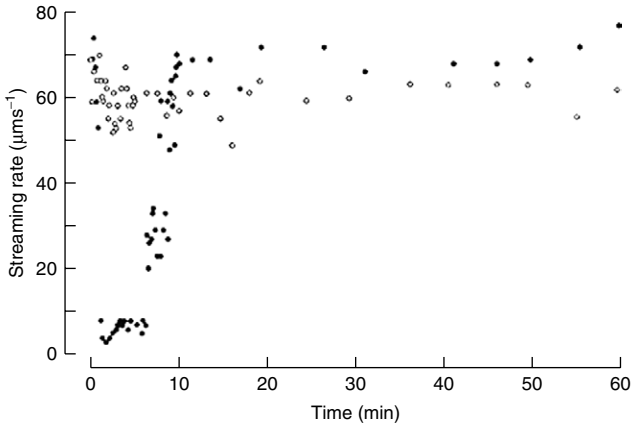


FIGURE 12.9 The change in the velocity of cytoplasmic streaming following transfer of the cells into a hypotonic medium containing 0.01 mol/m^3 external Ca^{2+} (open circles) or 3.9 mol/m^3 external Ca^{2+} (filled circles). (*Source:* From Okazaki and Tazawa, 1986b.)

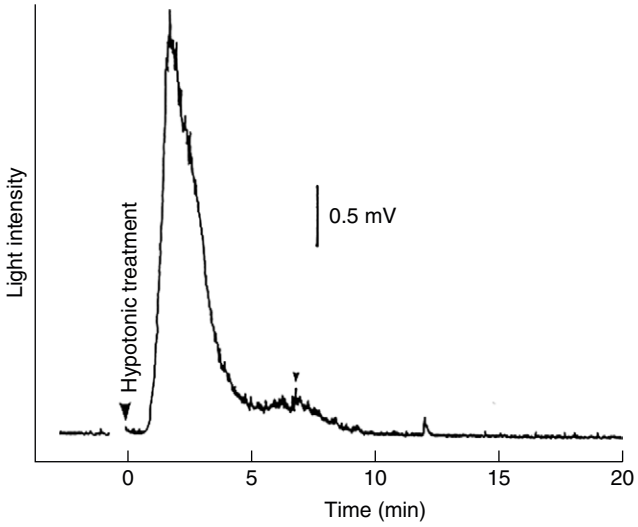


FIGURE 12.10 Time course of light emission of microinjected aequorin following hypotonic treatment. The light intensity is related to the intracellular-free [Ca^{2+}]. (*Source:* From Okazaki et al., 1987.)

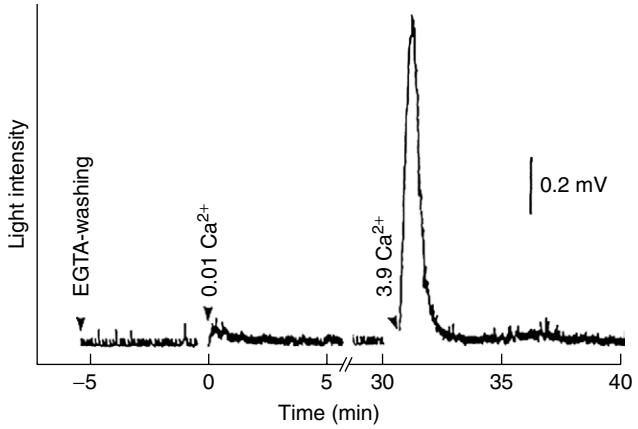


FIGURE 12.11 Time course of light emission of microinjected aequorin following hypotonic treatment in the presence of varying concentrations of extracellular Ca^{2+}. (*Source:* From Okazaki et al., 1987.)

concentration of calcium of 1.7 mM. Using aequorin that had been microinjected into the cell, Okazaki et al. (1987) showed that hypotonic treatment causes an immediate transient increase in the intracellular-free Ca^{2+} concentration (Figure 12.10). This increase requires external Ca^{2+} (Figure 12.11). The intracellular-free calcium concentration rises to an estimated 10–50 μM, which is approximately 30–100 times smaller than the rise in total cytoplasmic calcium. This means that there is a buffering capacity in the cytoplasm for Ca^{2+}. Some of the buffering may be due to intracellular Ca^{2+}-binding proteins and active sequestering by organelles.

According to Yoshiji Okazaki and Masashi Tazawa (1990), hypotonic stress causes an increase in the tension of the plasma membrane. This tension is sensed by a mechanoreceptor (Morris, 1990), which causes a membrane depolarization. The ion(s) that carry the initial depolarizing current are unknown, although K^+, Cl^-, and Ca^{2+} have been eliminated as candidates (Beilby and Shepherd, 1996). The depolarization then activates a voltage-dependent Ca^{2+} channel. The increased influx of Ca^{2+} results in an increase in the cytosolic Ca^{2+} concentration. At the elevated concentration, Ca^{2+} in turn causes the opening of K^+ and Cl^- channels. Patch-clamp studies show that a Ca^{2+}-dependent K^+ channel exists in the vacuolar membrane of *Lamprothamnium* (see Figure 12.12; Katsuhara et al., 1989). Water follows the K^+ and Cl^- out of the cell and the turgor pressure returns to its normal value.

Calcium may activate the monovalent ion channels involved in turgor regulation through the mediation of a protein kinase. Support for this idea comes from the facts that a Ca^{2+}-dependent protein kinase has been isolated from

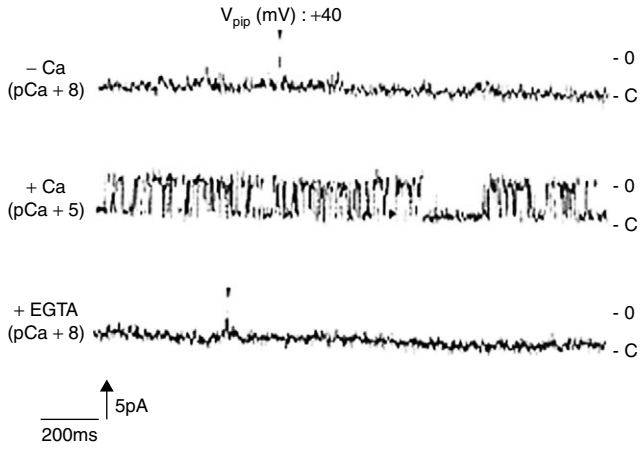


FIGURE 12.12 Recordings of K^+ channels from a cytoplasmic-side-out patch from the vacuolar membrane of *Lamprothamnium* at two different Ca^{2+} concentrations. The arrows indicate occasional channel openings at 10 nM Ca^{2+} (pCa 8). (*Source:* From Katsuhara et al., 1989.)

Lamprothamnium; microinjection of antibodies directed against a Ca^{2+}-dependent protein kinase inhibits turgor regulation; and last, K-252a, an inhibitor of protein kinases, inhibits turgor regulation in *Lamprothamnium* cells (Yuasa et al., 1997). Older cells respond more slowly than young cells (Belby et al., 1999a,b).

Turgor is an important indicator of the status of plant cells. Wounding results in the loss of turgor pressure in a cell. In *Chara*, the change in pressure is converted into electrical signals that pass the "death message" from the wounded cell to the neighboring cells (Shimmen, 2001a, 2002, 2003).

12.9 PHOSPHATIDYLINOSITOL SIGNALING SYSTEM

The Ca^{2+} signaling system interacts with other signaling systems in plant cells, including one that is based on phosphatidylinositol (Boss and Massel, 1985; Berridge, 1987; Coté et al., 1987; Morse et al., 1987b, 1989; Pfaffman et al., 1987; Sandelius and Morré, 1987; Wheeler and Boss, 1987; Drøbak et al., 1988; Ettlinger and Lehle, 1988; Sommarin and Sandelius, 1988; Murthy et al., 1989; Peeler et al., 1989; Einspahr and Thompson, 1990; Drøbak, 1991; Coté and Crain, 1993; Yang et al., 1993). The phosphatidylinositol pathway is involved in stomatal movements, the regulation of mitosis (Chen and Wolniak, 1987; Wolniak, 1987; Larsen et al., 1991), plasmodesmata conductance (Tucker, 1988; Tucker and Boss, 1996), leaflet movements (Morse et al., 1987a, 1989, 1990; Kim et al., 1996), osmotic adaptation in *Dunaliella* (Einspahr et al., 1988, 1989), the deflagellation response of *Chlamydomonas* (Yueh and Crain, 1993), pollen tube growth (Franklin-Tong et al., 1996), and α-amylase secretion in rice.

12.9.1 Components of the System

Phosphatidylinositol (PI) is a lipid that is formed in the ER by the CDP-dependent PI exchange protein that catalyzes the attachment of inositol phosphate onto diacylglycerol (see Chapter 4; Sandelius and Morré, 1987). Inositol lipids are enriched in the cytosolic leaflets of membranes. The PI gets to the plasma membrane through the mechanisms involved in membrane flow (Wheeler and Boss, 1987; Peeler et al., 1989; see Chapter 8). Phosphatidylinositol-4-phosphate (PIP) is formed from the PI by PI kinase, and phosphatidylinositol-4,5-bisphosphate (PIP_2) is formed from phosphatidylinositol-4-phosphate by PIP kinase according to the following reaction:

$$\text{PI} \xrightarrow{\text{PI kinase}} \text{PI-4-P} \xrightarrow{\text{PIP kinase}} \text{PI-4,5-P}$$

Activation of the PI signaling pathway begins with the activation of a PIP_2-specific phospholipase C. This enzyme splits PIP_2 into diacylglycerol and the water-soluble inositol trisphosphate. There are many classes of phospholipase C; some are activated by G-proteins, others by Ca^{2+}, and some by both (Dillenschneider et al., 1986; Melin et al., 1987; Einspahr et al., 1989; Tate et al., 1989; Buffen and Hanke, 1990).

The water-soluble inositol phosphate IP_3 causes the release of Ca^{2+} from microsomal and vacuolar membrane fractions (Drøbak and Ferguson, 1985; Rincon and Boss, 1987; Schumaker and Sze, 1987; Ranjeva et al., 1988; Reddy et al., 1988; Alexandre et al., 1990; Brosnan and Sanders, 1990; Taylor and Marshall, 1992). This is one way the components of the PI pathway interact with Ca^{2+}-mediated signal transduction chains.

The IP_3-stimulated Ca^{2+} release is transient because the IP_3 is rapidly dephosphorylated by a specific phosphatase to IP_2. Eventually the IP_2 is dephosphorylated to inositol where it will be a substrate of PI. IP_3 can also undergo phosphorylation. More and more isomers of inositol phosphates are being found and some of these may play a role in signaling (Berridge and Irvine, 1990).

Phospholipase C also causes the release of diacylglycerol (DAG) from PIP_2 (Morse et al., 1989; Morré et al., 1989b). DAG is capable of stimulating the activity of a Ca^{2+} phospholipid-dependent protein kinase, called *protein kinase C* (Elliot and Kokke, 1987a,b). Diacylglycerol increases the affinity of protein kinase C for Ca^{2+}. The activation by diacylglycerol is also transient because diacylglycerol is either cleaved to form arachidonic acid or phosphorylated to form phosphatidic acid within seconds.

12.9.2 Phosphatidylinositol Signaling in Guard Cell Movement

Water stress induces the formation of abscisic acid (AbA), which induces the closing of stomatal pores to prevent

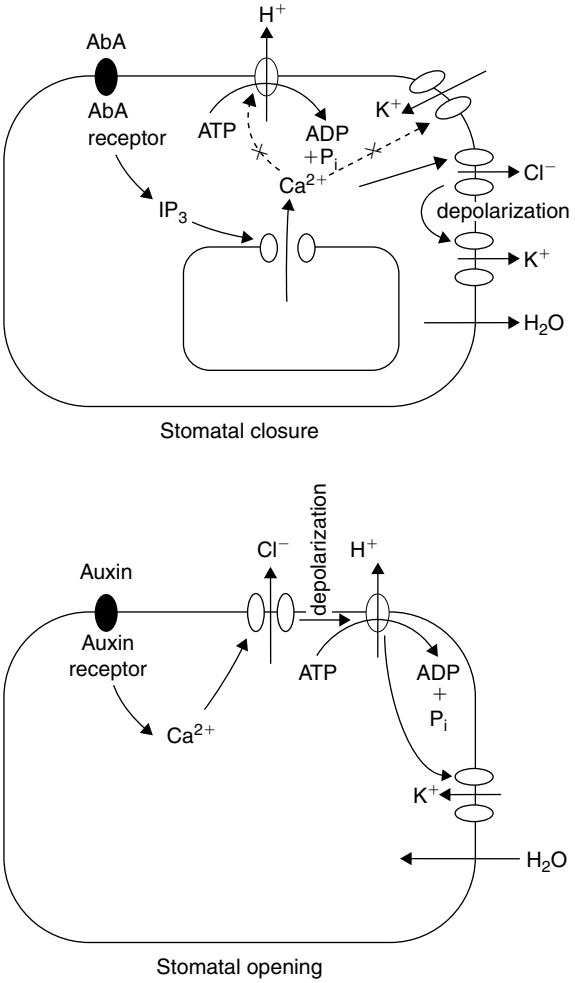


FIGURE 12.13

further water loss (Yamazaki et al., 2003). The closing of stomatal pores is due to the shrinkage of guard cells. The shrinkage results from the loss of K^+ through the outwardly rectifying K^+ channels (Figure 12.13a; see Chapter 2; Schroeder, 1988; Assmann and Shimazaki, 1999). The efflux of K^+ is followed by the passive efflux of water.

AbA can cause an increase in the concentration of IP_3 (Lee et al., 1996), perhaps mediated by a heterotrimeric G-protein (Liu et al., 2007a). Such an increase in IP_3 may result in the increase in the cytoplasmic-free $[Ca^{2+}]$ that occurs in response to AbA (McAinsh et al., 1990, 1992; Schroeder and Hagiwara, 1990; Gilroy et al., 1990, 1991; Schroeder and Thuyleau, 1991). The increase in the cytoplasmic-free calcium concentration is sufficient to inhibit the plasma membrane H^+-ATPase (Kinoshita et al., 1995) and activate a Ca^{2+}-activated anion channel in the plasma membrane (Keller et al., 1989). The efflux of Cl^- through this channel causes both a decrease in turgor and a membrane depolarization. The membrane depolarization activates the outwardly rectifying K^+ channels, which results in a further decrease in turgor and a consequent closing of the stoma. Elevated levels of cytoplasmic Ca^{2+} inhibit the inwardly rectifying K^+ channels, which further enhances the loss of turgor (Pei et al., 1998a). AbA may also regulate the anion channel through phosphorylation (Pei et al., 1997) and farnesylation (Pei et al., 1998b).

The inwardly rectifying K^+ channel can also be inhibited experimentally by releasing caged IP_3 into the guard cell (Blatt et al., 1990). In fact, releasing either caged IP_3 or caged Ca^{2+} into the cytosol induces stomatal closure even in the presence of 1 mM La^{3+}. This indicates that AbA initiates stomatal closure by stimulating the formation of IP_3, which releases Ca^{2+} from internal stores (Mansfield et al., 1990; Gilroy et al., 1991). This Ca^{2+} release channel is also regulated through phosphorylation (Mori et al., 2000).

Auxin can cause stomatal opening (Figure 12.13b; Marten et al., 1991). It does so by activating an anion channel, which causes a transient membrane depolarization. Subsequently, the H^+-pumping ATPase is activated and the membrane hyperpolarizes. This activates the inwardly rectifying K^+ channel. Thus, K^+ accumulates in the guard cell, water enters the guard cell, and the stoma opens.

Irving et al. (1992) show that an increase in the intracellular Ca^{2+} concentration precedes both stomatal opening and closure. Thus, while Ca^{2+} may be necessary for the individual signal transduction chains, the specificity in each chain comes from other elements and/or differences in the spatial and/or temporal change in Ca^{2+} (Leckie et al., 1998; Blatt, 2000; McAinsh et al., 2000; Assmann, 2002; Luan, 2002; MacRobbie, 1997, 2000, 2002). One of the unknown elements may be H^+ since an initial acidification of the cytosol is correlated with stomatal opening, whereas an initial alkalinization of the cytoplasm is correlated with stomatal closure (Blatt, 1992; Blatt and Armstrong, 1993; Grabov and Blatt, 1997). Other regulatory elements include protein kinases and phosphatases (Theil and Blatt, 1994; Armstrong et al., 1995; Li and Assman, 1996; Pei et al., 1997; Mori and Muto, 1997; Kinoshita and Shimozaki, 1999; Allen at al., 1999; Li et al., 2000, 2002; Hwang and Lee, 2001; Eun et al., 2001). Last, the channels involved in guard cell swelling and shrinking may be directly affected by turgor (Cosgrove and Hedrich, 1991).

Guard cell swelling may be accompanied by new membrane arriving at the plasma membrane through the endomembrane system (Duque et al., 2004). Likewise, shrinking may be accompanied by endocytosis. Vesicle movement may be mediated through the actin and/or the microtubular cytoskeleton. Presumably, these processes would also be regulated by the various signal transduction chains.

12.10 THE ROLE OF IONS IN CELLS

Calcium has been widely known to be a necessary element for plant growth ever since Benjamin Franklin wrote in large letters, formed by using ground plaster of Paris ($CaSO_4$),

"This has been plastered" in a clover field along the road to Washington, DC, and every passerby noticed that the clover grew lushly in the plastered portion (Chaptal, 1836). I have discussed the function of Ca^{2+} in cell signaling. Ever since the early chemist's work on the inorganic constituents of plants, we have known that other ions, including K^{+}, Fe^{2+}, Cu^{2+}, Mo^{2+}, Mn^{2+}, Mg^{2+}, Co^{2+}, and Zn^{2+}, are important macro- and micronutrients for the normal functioning of cells (Davy, 1821, 1827; Chaptal, 1836; Liebig, 1841; Hoagland, 1948; Epstein, 1972; Clarkson and Hanson, 1980; Ochiai, 1987; Fraústo da Silva and Williams, 1991; Williams and Fraústo da Silva, 1996; Gluska et al., 1999). Each ion has particular properties that make each one unique for fulfilling its function.

Many ions, however, can be toxic, particularly those of heavy metals. Plant and animal cells contain polypeptides that bind toxic heavy metals, including Pb^{2+}, Cd^{2+}, Ni^{2+}, and Hg^{2+}, as well as micronutrients like Cu^{2+} and Zn^{2+}, which become toxic at high concentrations (Murasugi et al., 1980). These heavy metal–binding polypeptides called *phytochelatins* and *metallothioneins* bind heavy metals with high-association constants so that the free concentrations of these ions in the cytosol are reduced and the cells are detoxified (Rauser, 1990; Steffens, 1990; Evans et al., 1990; De Miranda et al., 1990; Kawashima et al., 1991). Cysteine is a sulfur-containing amino acid that provides the necessary high-affinity binding sites for heavy metals. The heavy metal–binding polypeptides are synthesized in response to exposure by heavy metals (Ahner et al., 1994, 1995). Unlike metallothioneins, which are primary gene products, phytochelatins are polypeptides that are not primary gene products formed in ribosomes, but are formed without a template by the enzyme phytochelatin synthase. In general, phytochelatin synthase is activated by heavy metals and is completely inactive in their absence (Grill et al., 1985, 1989).

12.11 SUMMARY

We have discussed the various adaptive and developmental responses that plants undergo in response to their environment. We have studied the signal transduction chains that are involved in coupling the stimulus to the response in individual cells. We have learned that the competence to respond to a given stimulus is dependent on the receptors in the cell, and the response that the cell undergoes depends on its response elements. We have seen that Ca^{2+}, inositol, and phosphorylation are essential elements in signal transduction chains, and moreover that the specificity of a given response results from the fact that there are independent and interacting elements in the signal transduction chains that truly form a signal transduction network. We have also spent a good deal of time discussing kinetics in order to truly understand the regulatory aspects of cell signaling.

12.12 QUESTIONS

12.1. Why do various cell types have very similar signal transduction chains to couple the stimulus to the response?

12.2. Why do various cell types have very different signal transduction chains to couple a stimulus to a response?

12.3. Why is there more than one kind of signal transduction chain within a cell?

Chapter 13

Chloroplasts

The MOUSE'S PETITION, Found in the TRAP where he had been confin'd all Night.*

Parcere subjectis, & debellare superbos. —VIRGIL.

OH! hear a pensive prisoner's prayer,
For liberty that sighs;
And never let thine heart be shut
Against the wretch's cries.

For here forlorn and sad I sit,
Within the wiry grate;
And tremble at th' approaching morn,
Which brings impending fate.

If e'er thy breast with freedom glow'd,
And spurn'd a tyrant's chain,
Let not thy strong oppressive force
A free-born mouse detain.

Oh! do not stain with guiltless blood
Thy hospitable hearth;
Nor triumph that thy wiles betray'd
A prize so little worth.

The scatter'd gleanings of a feast
My frugal meals supply;
But if thine unrelenting heart
That slender boon deny,

The cheerful light, the vital air,
Are blessings widely given;
Let nature's commoners enjoy
The common gifts of heaven.

The well-taught philosophic mind
To all compassion gives;
Casts round the world an equal eye,
And feels for all that lives.

If mind, as ancient sages taught,
A never dying flame,
Still shifts through matter's varying forms,
In every form the same,

Beware, lest in the worm you crush
A brother's soul you find;
And tremble lest thy luckless hand
Dislodge a kindred mind.

Or, if this transient gleam of day
Be all of life we share,
Let pity plead within thy breast
That little all to spare.

So may thy hospitable board
With health and peace be crown'd;
And every charm of heartfelt ease
Beneath thy roof be found.

So, when destruction lurks unseen,
Which men like mice may share,
May some kind angel clear thy path,
And break the hidden snare.

**To Doctor PRIESTLEY.*

The Author is concerned to find, that what was intended as the petition of mercy against justice, has been construed as the plea of humanity against cruelty. She is certain that cruelty could never be apprehended from the Gentleman to whom this is addressed; and the poor animal would have suffered more as the victim of domestic economy, than of philosophical curiosity.

—From Anna Lætitia Aikin. *Poems*. London: Printed for Joseph Johnson, in St. Paul's Churchyard, 1773, pp. 37–40

13.1 DISCOVERY OF CHLOROPLASTS AND PHOTOSYNTHESIS

Up until now, I have been discussing cells that are surrounded by a plasma membrane, contain endoplasmic reticulum (ER), Golgi stacks and their associated membranes, coated and naked vesicles, vacuoles, and peroxisomes. Such cells could have been from either a plant or an animal. In this chapter, I will discuss an organelle that is found exclusively in plant cells: the chloroplast. The chloroplast is involved in photosynthesis and consequently cells that contain chloroplasts are autotrophic—that is, able to make their own food from inorganic molecules using the radiant energy of sunlight (Blankenship, 2002). It must be remembered that most plants contain both photosynthetic and colorless cells. In most plants, the majority of cells,

including the cells in the root, the pith of the stem, and the epidermis of the leaves, do not perform photosynthesis, and thus, they must be considered to be heterotrophic. Even so, each year, all the chloroplasts in the world fix about 10–100 billion tons of carbon dioxide, which is approximately equal to the mass of metropolitan New York City (Kamen, 1963) and significant in mitigating the effect of this greenhouse gas on global warming (Arrhenius, 1896; Callendar, 1938, 1949; Gore, 2006).

13.1.1 Discovery of Photosynthesis

Experimental studies of plant assimilation began when the alchemist Jean Baptiste van Helmont (1683) used his balance, one of the only tools available to scientists at the time, to study plant growth. He believed that all matter was built up from the single essence of water, an idea that can be traced back to Thales. Van Helmont used the growth of plants to test this thesis. According to van Helmont:

> *I have learned from the following clear experiments that all plants make up their matter completely from the element of water. I conclude this because, I have taken an earthen-ware container and placed in it 200 pounds of earth, that I have placed in a baking oven and allowed to dry. I moistened this earth with rain-water and placed in it a willow stem which weighed five pounds. To ensure that the dust from the air didn't add to the weight, I threw a screen over the soil. I watered it when necessary with only rainwater or distilled water. The tree grew and set into the ground. After five years, the willow became a tree that weighed 169 pounds and about 3 ounces and I did not even take into consideration the weight of the leaves, which fell off every autumn for four years. Eventually I took the earth out of the container and found that it weighed only two ounces less than the original two hundred pounds. Thus the 164 pounds of wood, bark and knots had grown alone from the water.*

In performing experiments in other realms of alchemy, van Helmont found that when he burned 62 pounds of coal he was left with only 1 pound of ashes. He called the material that made up the escaping 61 pounds the *spiritis sylvestres,* wild spirit or "gas," and although van Helmont was the discoverer of gases, he did not realize that the tree that grew in the pot was created from gas as well as from water. The idea that the leaves of plants assimilated air was originally proposed by Empedocles (Lambridis, 1976) but dismissed by Aristotle (Barnes, 1984) and his disciple Theophrastus (1916), who believed that all the nourishment came in through the roots. The proposal that plants assimilated air was not taken seriously until 1727. At this time, Stephen Hales (1727) burned plants and quantified the amount of gas given off and the amount of ash that remained. From the results of these experiments, Hales suggested that plants might assimilate air, just as animals do (Boyle, 1662; see Chapter 14). Experiments on the role of gases in plant growth were renewed at the end of the 18th century when Joseph Priestley decided to take up where Stephan Hales left off (Birch and Lee, 2007).

Like a burning candle, living beings require "clean air." A test for the presence of clean (or dephlogistonated) air is to see if a mouse can live, or a candle can burn, when placed in a container of the sample air (Faraday, 1860). Using such a test, Joseph Priestley (1774) accidently found that a sprig of mint could purify the air that had been previously fouled by the breathing of an animal or the burning of a candle. Perhaps Priestley was lucky to find the oxygenic (or dephlogistonating) property of plants, and just chose mint to purify the air because of its refreshing smell. However, he also found that groundsel, a bad-smelling weed, and spinach also had the ability to purify the air, and this ability was a general property of plants.

Priestley later found his experiments to be irreproducible, perhaps because he did not control the light conditions in his laboratory. However, it was difficult for the politically incorrect Priestley to continue his experiments since his home and laboratory were burned down as a result of his antiauthoritarian views, including his support of the French Revolution and the Unitarian church (Priestley, 1809). John Ingen-Housz (1796) continued experiments on the purification of air by plants, and he solved the problem of irreproducibility by showing that oxygen evolution required light. Moreover, Ingen-Housz showed that only the green parts of the plant evolved oxygen.

Joseph Priestley had discovered how to make soda water by impregnating water with fixed air derived from acidified sodium carbonate. By varying the amount of carbonic acid in the soda water surrounding immersed leaves, Jean Senebier (1788) was then able to show that oxygen evolution was dependent on CO_2. Théodore de Saussure (1804) quantified the relationship between CO_2 fixation and O_2 evolution and established that the amount of O_2 given off was equivalent to the amount of O_2 taken up in the form of CO_2. However, the mass gained by the plant was greater than the mass contributed by the C and O in CO_2. Thus, he concluded that water must also be taken up and the C combined with H_2O.

While Johann Wolfgang von Goethe (1952), Alphonse de Candolle, and Henri Dutrochet accepted the results of Ingen-Housz, Senebier, and de Saussure, it seemed ludicrous to many plant physiologists, including Gottfried Treviranus and Franz Meyen (1837–1839), to think that plants assimilated carbon from the tiny amounts found in air when they were surrounded by vast quantities in the form of humus or humic acid. They thought that it was more likely that plants absorbed their carbon from humus or humic acid, which surrounded the plants as was proposed by Aristotle (Sachs, 1906).

Justus von Liebig (1840) railed against his fellow German botanists, insisting that

> *… in botany the talent and labour of inquirers has been wholly spent in the examination of form and structure: chemistry and physics have not been allowed to sit in council upon*

the explanation of the most simple processes; their experience and their laws have not been employed, though the most powerful means of help in the acquirement of true knowledge.

Liebig went on to say that "the art of experimenting is not known in physiology." The whole matter as to the origin of carbon in plant nutrition was finally settled in favor of the atmospheric source when Jean Boussingault succeeded in growing plants in a totally inorganic soil and proved once and for all that plants do not obtain their carbon from humus or humic acid, but from the carbon dioxide in the air (Dumas and Boussingault, 1844; Sachs, 1906).

In *The Vegetable Cell*, Hugo von Mohl (1852) offered two possible reaction mechanisms for the first step in carbon assimilation. First, one proposed by Humphry Davy (1827) that the C combined with H_2O to make $C(H_2O)$ or carbohydrate as the first product. Second, one proposed by Justus von Liebig that an organic acid was the first product since "it was far more probable that it was not the difficultly decomposable carbonic acid [bond energy = 13.2×10^{-19} J], but the readily decomposable water [bond energy = 7.65×10^{-19} J] which was separated into its elements." Liebig's idea, although correct, was not so simple and was forgotten for almost 100 years and independently rediscovered by van Niel (1941) using the perspective of comparative physiology.

The biotic carbon cycle was understood by chemists in the mid-19th century. J. B. Dumas (1844, quoted in Fruton, 1972) wrote,

… green plants constitute the great laboratory of organic chemistry. It is they which, with carbon, hydrogen, nitrogen, and ammonium oxide, slowly build the most complex organic materials. They received from the solar rays, in the form of heat or chemical radiation, the power needed for this work.

Animals assimilate or absorb the organic materials made by plants. They change them bit by bit. … They therefore decompose bit by bit these organic materials created by plants; they bring them back bit by bit toward the state of carbonic acid, of water, of nitrogen, of ammonia, the state that permits them to be restored in the air.

Thomas Huxley (1893) described the cycle by means of a sociological metaphor: "Thus the plant is the ideal *prolétaire* of the living world, the worker who produces; the animal, the ideal aristocrat, who mostly occupies himself in consuming."

While the identity of the first product of carbon assimilation would not be known for almost 100 years and would have to wait for the introduction of ^{14}C and paper chromatography, Julius von Sachs (1887) stained leaves with iodine and established that the starch grains contained in the chlorophyll granules were the end result of carbon assimilation. The starch grains only appeared when the plant was exposed to light, and disappeared after the plant was put into darkness. Hans Molisch (1916) capitalized on this fact to use leaves as photographic paper. He put a negative image over a dark-adapted, starch-depleted leaf and exposed the leaf to bright sunlight. The light passed through the transparent parts of the negative, and initiated starch formation. Molisch then stained the leaf with iodide and the image developed right on the leaf, just as it would have on photographic paper (Figure 13.1)!

At the end of the 19th century, plant physiologists were realizing that the term *assimilation*, a term that had long been used by animal physiologists to designate the appropriation of digested food and its subsequent conversion into other substances, did not describe the unique aspects of the light-mediated synthesis of carbohydrates that occurred in plants. Plant physiologists began to look for a new term. In a published version of an address given before the American Association for the Advancement of Science, Charles Barnes (1893) wrote,

FIGURE 13.1 A leaf of *Tropaeolum majus* upon which a photographic negative of a man's face had been placed. Then the leaf was placed in the light and allowed to photosynthesize. Starch was consequently formed under the clear areas of the negative. Subsequently, the leaf was stained with iodide and photographed. (*Source:* From Molisch, 1916. See Hangarter and Gest (2004) for more fascinating pictorial examples of photosynthesis. Instructions for this fascinating process can be found in a book by David Walker (1992) that covers photosynthesis from the quantum processes to its impact on ethical decisions.)

> *For the process of formation of complex carbon compounds out of simple ones under the influence of light, I propose that the term* photosyntax *be used. … I have carefully considered the etymology and adaptation, as well as the expressiveness, of the word proposed, and consider it preferable to* photosynthesis *which naturally occurs as a substitute.*

The term *photosynthesis*, which was coined by Conway MacMillan, in a discussion following the presentation of Barnes's paper, gained universal acceptance (Oels, 1894; Barnes, 1898; Gest, 2002).

Isaac Newton (Qu.30, 1730) had suggested that light and matter may be convertible, and Stephen Hales (1727) added that this convertibility might play a role in plant nutrition. Eventually chemists began searching for the pigment that aided in the conversion of sunlight into carbohydrate. The green pigment, which was soluble in an alcohol extract of leaves, was dubbed *chlorophyle*, from the Greek for "green leaf," by Joseph Pelletier and Joseph Caventou in 1818. Work by Jöns Jacobius Berzelius, David Brewster, George Gabriel Stokes, Richard Willstätter (1920), Hans Fischer (1930), Harold Strain, James Conant, and others led to the elucidation of the structure of chlorophyll (Willstätter and Stoll, 1928; Strain, 1958; Willstätter, 1965). Paul Castelfranco and Sam Beale (1983), among others, elucidated how chlorophyll is synthesized in plants. In 1960, it was synthesized for the first time by humans at Harvard University (Woodward et al., 1960; Woodward, 1965). Interestingly, unlike the rhodopsin in our eyes, chlorophyll is terribly inefficient, since it does not absorb green light, which is the most prominent color of the sunlight that passes through the clouds to Earth (Barbour et al., 1980).

In order for a pigment to do photochemistry, it must absorb photons. Einstein's photochemical equivalence law states that each molecule in a linear photochemical process absorbs one photon of radiation, and the law predicts that the pigment involved in a photochemical process must have an absorption spectrum that closely follows the action spectrum for the process (Gerlach, 1921)—that is, in a linear photoreaction, the response at each wavelength will be proportional to the number of photons absorbed at that wavelength. Indeed, the action spectrum of carbon assimilation, originally obtained by Theodor Engelmann (1881, 1882) by observing the movement of oxytactic bacteria to various portions of photosynthetic cells that were irradiated with different-colored light, almost exactly mimics the absorption spectrum of chlorophyll—almost.

Robert Emerson and Charlton Lewis (1943) noticed that while red light between 700 and 730 nm was absorbed by chlorophyll, it was not very effective in stimulating photosynthesis. This was known as the *red-drop effect*. However, when the ineffective wavelengths were supplemented with short-wavelength light, the photosynthetic rate was greater than the rate induced by the sum of the two wavelengths alone (Emerson et al., 1957). In fact, the two wavelengths could still enhance photosynthesis when they were given sequentially (Myers and French, 1960). This enhancement, which is known as the *Emerson effect*, gave the first indication that there may be two sequential photosystems involved in photosynthesis: one that absorbs 700-nm light and one that does not (Govindjee and Rabinowitch, 1960; Rabinowitch and Govindjee, 1969). Hill and Bendall (1960) proposed that these results could be explained if there were two photosystems, and Duysens et al. (1961) named them *Photosystem I* and *Photosystem II*.

The reactions involved in the conversion of light energy into carbohydrate have become crystal clear over the past 270 years, in part due to the introduction of favorable material by Otto Warburg and Cornelius van Niel, including single-celled algae and bacteria for the study of the rapid reactions of photosynthesis (Kluyver and van Niel, 1956; Myers, 1974; Wayne and Staves, 1996b; Feher, 2002). The success obtained in photosynthetic research is perhaps also a result of the excellence of the individual scientists involved, who as a rule, have acted on the admonishments of Liebig, and combined an interest in biology with chemistry and physics (Rabinowitch, 1945, 1955; Franck and Loomis, 1949; Hill and Whittingham, 1955; Bassham and Calvin, 1957; Rabinowitch and Govindjee, 1969; Clayton, 1980; Duysens, 1989, 1996; Calvin, 1992; Walker, 1997; Feher, 1998, 2002; Jagendorf, 1998; Fuller, 1999; Krogmann, 2000; Govindjee and Krogmann, 2004; Govindjee et al., 2004; Rosenberg, 2004).

The individual reactions of photosynthesis span times from femtoseconds to hours, which provide work for scientists that range from physicists to ecologists. In order to help us comprehend the vast range in times, as well as making it easier to speak and write the various times, Martin Kamen (1963) introduced the symbol pt_s, which stands for "the minus log of the time in seconds." The symbol p was first employed by Sørensen (1909) when he used pH to stand for the minus log of the $[H^+]$.

The reactions of photosynthesis are divided into two major groups: the reactions that require light directly (i.e., the light reactions), and the reactions that do not require light directly (i.e., the dark reactions). The light reactions are involved in the conversion of light energy to chemical energy in the form of ATP and NADPH and take place on the time scale of pt_s15–pt_s3. The dark reactions, which take place on a time scale of pt_s4–pt_s0 or longer, are involved in the conversion of chemical energy in the form of ATP and NADPH to a more stable form of energy in the form of carbohydrate ($C[H_2O]$).

13.1.2 Discovery and Structure of Chloroplasts

Nehemiah Grew (1682) developed an interest in the theory of colors along with his contemporaries, Boyle, Descartes, Hooke, and Newton. Combining this interest with his interest in the anatomy of plants, he investigated the colors of

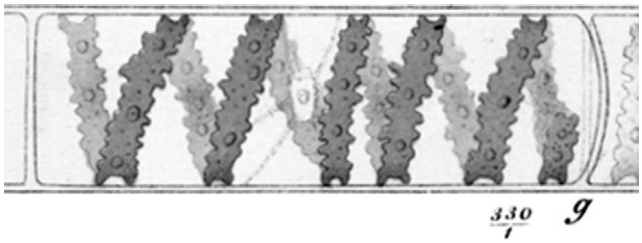


FIGURE 13.2 Chloroplast of *Spirogyra quinina*. ×330. (*Source:* From Pringsheim, 1879–1881.)

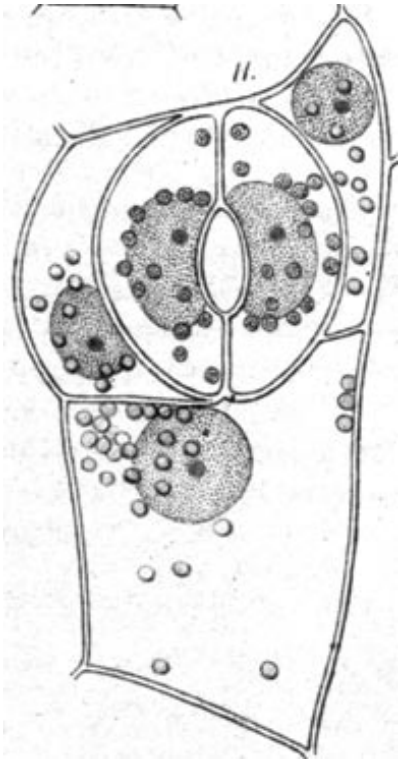


FIGURE 13.3 Chloroplasts in the guard cells of *Tradescantia subaspera*. (*Source:* From Schimper, 1883.)

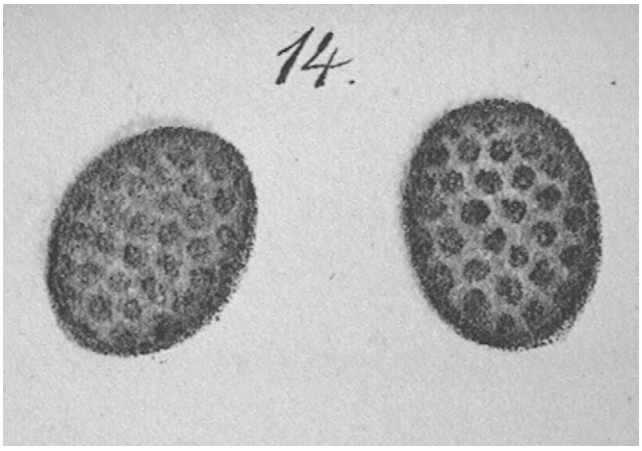


FIGURE 13.4 Chloroplasts with visible grana from the mesophyll of *Acanthephippium*. (*Source:* From Meyer, 1883.)

plants and noticed green precipitates in leaves. With the introduction of achromatic lenses in the first part of the 19th century, it became apparent to plant cell biologists that the green precipitates, which came to be known as the *chlorophyll granules*, had fascinating shapes, including stars, plates, and spirals (Figure 13.2), which were diagnostic of a given taxon, especially in the green algal order Zygnematales (Mohl, 1852). In the majority of plants, however, the chlorophyll granules are just ellipsoidal and about 2.5-μm long (Figure 13.3). Other granules, approximately the same size, were also observed. These included the orange-yellow carotenoid-containing granules and clear granules, which due to the differences in color and shape were given various names. Schimper (1883, 1885), fascinated by the fact that these organelles could metamorphose into the other, and were thus different facets of the same organelle, named the whole group *plastids* to emphasize their plasticity. He called the green plastids *chloroplasts*, the orange-yellow plastids *chromoplasts*, and the clear plastids *leucoplasts*.

Arthur Meyer (1883) noticed that the chlorophyll granules contained spherical grains that he called *grana* (Figure 13.4). However, as the application of colloid chemistry to biological problems became popular, the grana were interpreted as an artifact of fixation and the internal organization of the chloroplast in the living cell was considered to be a homogeneous emulsion of a very fine chlorophyll-containing lipoidal phase distributed throughout a hydrophilic phase (Guilliermond, 1941). Frederick Czapek (1911) believed that the fine emulsion separated during fixation and artifactually formed grana. This argument was considerably strengthened by the observations that grana in fixed material showed varied structures that depend on the fixative employed. Heitz (1937) clearly resolved grana in the living cells of 180 different species of plants using a high–numerical aperture apochromatic objective and blue-light illumination. He described the 40–60 grana that were in each chloroplast as discs that varied in size from 0.3 to 2 μm, depending on the species. Heitz also observed the grana with polarized light microscopy, and concluded that the chloroplast was not homogeneous. Even so, the reality of the grana continued to be debated until the 1960s (Küster, 1935; Weier, 1938, 1961; Strugger, 1951).

The newly invented technique of electron microscopy was applied to understand the nature of the grana in disrupted chloroplasts. The ultramicrotome had not yet been invented, so the only way to determine whether or not the grana existed was to shadow-cast the material (Algera et al., 1947; Granick and Porter, 1947; Frey-Wyssling and Mühlethaler, 1949). However, these images of grana were not of the quality we are used to today and were not very convincing to the nonbelievers (Figure 13.5). Following the invention of the ultramicrotome, however, Steinmann and Sjöstrand (1955) were able to obtain excellent images of

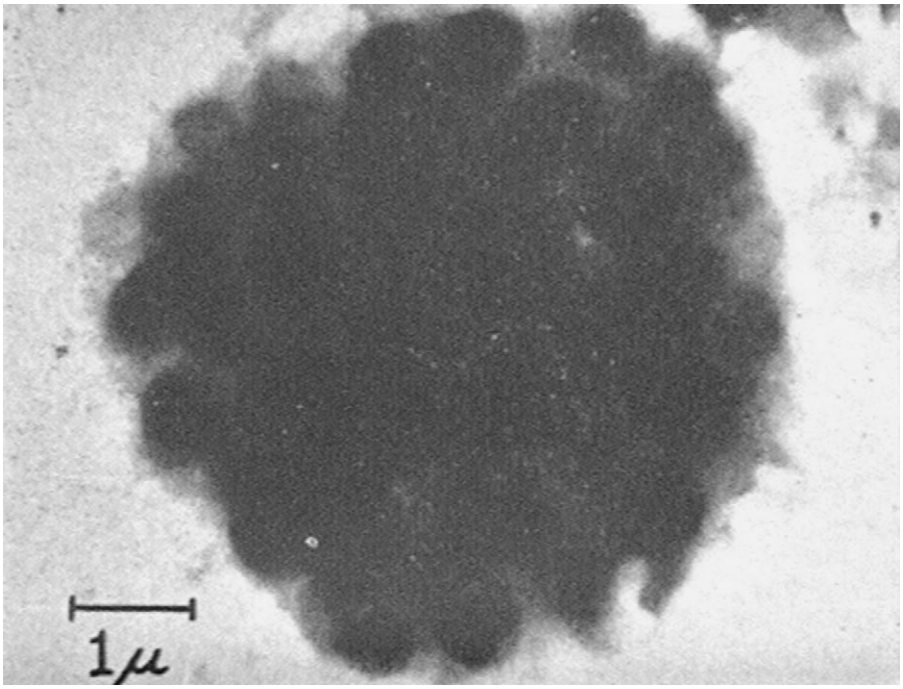


FIGURE 13.5 Electron micrograph of an isolated spinach chloroplast showing dense grana embedded in a paler matrix. The chloroplast preparation was dried directly on a wire mesh and observed with the electron microscope. (*Source:* From Granick and Porter, 1947.)

the grana that revealed that they were composed of 7- to 9-nm-thick membranes (Figure 13.6), which were later given the name *thylakoids* by Menke (1962). Thylakoids comes from a Greek word meaning "sacklike." The membranous structure of the plastid membranes of many species of plants was confirmed (Heslop-Harrison, 1962; Paolillo, 1962; Weier and Thomson, 1962; Paolillo and Falk, 1966; Paolillo and Reighard, 1967; Paolillo et al., 1969; Shimoni et al., 2005; Brumfeld et al., 2008; Garab and Mannella, 2008; Mustárdy et al., 2008).

The chloroplasts are delineated by a double membrane called the *envelope*. The two 6- to 8-nm-thick membranes are separated by a distance of approximately 10–20 nm. The membranes inside the chloroplast envelope are known as the thylakoids. A granum is formed when approximately 10–20 thylakoids, separated from each other by 3–4 nm, form a cylindrical stack, 300–600 nm in diameter and 200–600 nm in height (Shimoni et al., 2005). There are typically many grana per chloroplast (Figure 13.7). The space between the thylakoid membrane and the inner membrane of the chloroplast is called the *stroma*. Topologically, the space within the two membranes of the envelope, as well as the lumen of the thylakoids, is the E-space; the stroma is a P-space.

Dominick Paolillo (1970) proposed a model for grana structure based on serial sections of known orientation (Figure 13.8). He described the grana as cylinders that are surrounded by stromal lamellae that are arranged in right-handed helical frets (Figure 13.9). The stromal lamellae interconnect the lumen of the thylakoids below and above each other within a grana stack and they also interconnect the grana within the chloroplast. The stromal lamellae rise progressively at an angle to the grana stack like a spiral

FIGURE 13.6 Electron micrograph of a thin section of the chloroplast of *Aspidistra elatior* showing grana. (*Source:* From Steinmann and Sjöstrand, 1955.)

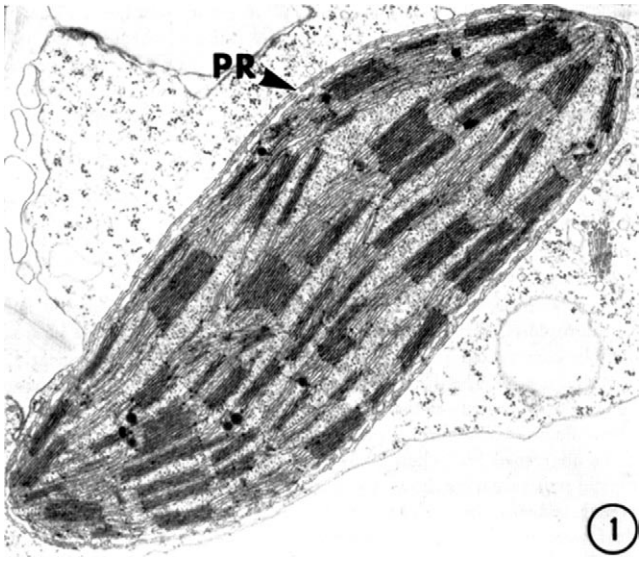


FIGURE 13.7 An electron micrograph of a chloroplast of *Zea mays* showing grana and a peripheral reticulum (PR) of membranes. (*Source:* From Chollet and Paolillo, 1972.)

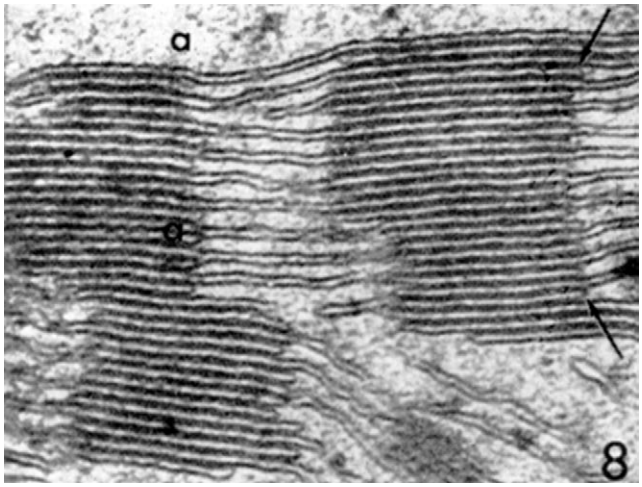


FIGURE 13.8 Thin-section electron micrograph of the grana and frets that connect the compartments in a chloroplast of *Zea mays*. Between a-a and the two arrows there are 2:1 relationships between the number of thylakoids in a granum and the number of membranous frets running between grana. ×83,000. (*Source:* From Paolillo and Falk (1966).

staircase where each lamella is turned about 30 degrees from the one below. The interconnection between all the grana in a chloroplast means that the interior of the chloroplast is divided into only two topological compartments, not hundreds of compartments as we might think if we look at only a single thin section. It is absolutely fascinating to consider how this structure is created. Do the thylakoid membranes fuse, bleb, twist, and wind? However they do it, the arrangement of the thylakoids is regulated both environmentally and genetically (Klekowski et al., 1994; Chuartzman et al., 2008).

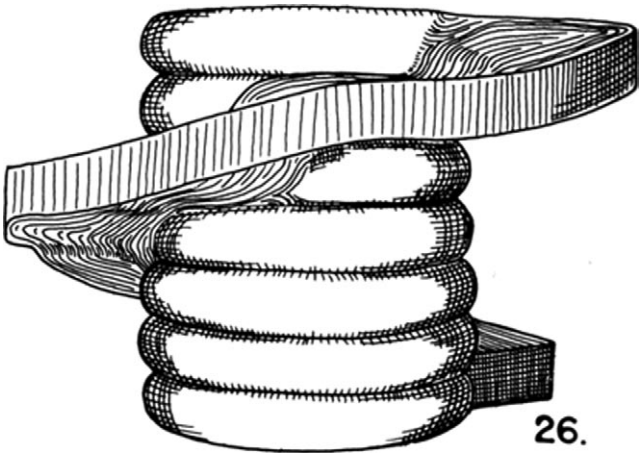


FIGURE 13.9 A three-dimensional model of a single granum showing only one fret. The two additional frets that would normally be attached to the granum are not shown for clarity. (*Source:* From Paolillo and Falk, 1966.)

In a continuation of the battle between chemists and cytologists, this model of chloroplast structure was challenged by Sam Wildman and his associates based on the assumption that cytologists were looking at small samples of dead cells (Spencer and Wildman, 1962; Wildman et al., 1962, 1974, 1980; Honda et al., 1971; Jope et al., 1980). Wildman described a new model of chloroplast structure that contradicted all the previous electron microscopic evidence. They proposed that in living cells, the grana were not arranged in overlapping rows interconnected in all directions, but were arranged in a single plane like a spiral "string of beads." Paolillo and Rubin (1980) observed chloroplasts with the light microscope, and concluded that the discrepancy between the light and electron microscopic images was only apparent and that the data used by Wildman and his coworkers were based on a misunderstanding of the workings of a light microscope. Recently, van Spronsen et al. (1989) have independently confirmed Paolillo's model by observing the autofluorescence of chlorophyll with a confocal microscope (Figure 13.10). More recently, Mehta et al. (1999) and Wildman et al. (2004) showed that in some chloroplasts in some cells of some taxa, the thylakoids are indeed arranged like strings of beads, although this is not a common arrangement.

Providing direct evidence that chloroplasts are the site of photosynthesis, Robert Hill (1937) showed that isolated chloroplasts were able to evolve oxygen when an artificial electron acceptor was added to the isolated chloroplast suspension. The reduction of the electron acceptor is easy to follow spectrophotometrically when the electron acceptor is a dye. This light-stimulated reduction is known as the *Hill reaction*. Given that the oxidation-reduction reactions that took place in the mitochondria depended on cytochromes, it seemed likely that cytochromes would also be involved in these reactions in the chloroplast, and soon such cytochromes were discovered in the chloroplast (Hill and

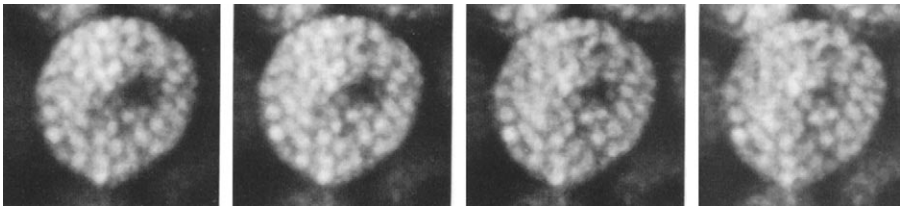


FIGURE 13.10 Successive optical section through a fluorescent chloroplast in a living cell of *Synnema triflorum*. (*Source:* From van Spronsen et al., 1989.)

Scarisbrick, 1951; Hill, 1954). Later, Sakae Katoh (1960, 1995) discovered a copper-containing protein in *Chlorella*, and postulated that it too may be involved in photosynthetic electron transport. Katoh and Takamiya (1961) discovered that this protein could be reduced in the presence of light and grana, and named it plastocyanin because it was a blue-colored protein that was localized in the chloroplast.

After it was discovered that NADP was the source of reducing power for malic enzyme in pigeon liver, the first enzyme discovered that could fix CO_2 (Ochoa et al., 1948), it seemed possible that NADP might be the natural electron acceptor for the light reactions of photosynthesis. Later, Vishniac and Ochoa (1951) found that it was. André Jagendorf (1956) and San Pietro and Lang (1956) showed that the reduction took place in the thylakoids. The NADP reduced by the light reactions serves as a source of reducing power for the so-called dark reactions. Daniel Arnon et al. (1954) discovered that isolated chloroplasts were capable of producing ATP, which is also necessary for the dark reactions. The various membrane-localized protein complexes involved in the light-dependent production of NADP and ATP can be visualized with freeze-fracture electron microscopy (Staehelin, 2003).

13.2 ISOLATION OF CHLOROPLASTS

Haberlandt, Ewart, Mölisch, Hill, and Arnon all attempted to isolate chloroplasts that could perform all the aspects of photosynthesis in vitro with varying amounts of success (Hill and Scarisbrick, 1940; Hill and Whittingham, 1955). In fact, the amount of oxygen given off by the chloroplasts isolated from a moss or *Selaginella* was so small that Haberlandt and Ewart had to use luminescent bacteria in order to detect it. By adding exogenous electron acceptors, Hill was able to get chloroplast preparations that were able to evolve oxygen at a high rate, and eventually Arnon et al. (1954) were able to get chloroplasts that were capable of making ATP in the presence of light. Through the work of many people, the conditions necessary for getting relatively high yields of intact and active chloroplasts were finally found.

In order to isolate chloroplasts, the tissue is homogenized, filtered, and centrifuged at 6000–8000 g for less than 90 seconds to separate the massive chloroplasts from the rest of the cellular components. Since this chloroplast fraction may be contaminated by mitochondria and peroxisomes, it can then be further purified by centrifuging the chloroplasts in a Percoll density gradient at 500 g for 10 minutes. The intact chloroplasts are found as a green band near the bottom of the tube. The most common marker for the chloroplast is chlorophyll and the most common enzyme markers are $NADP^+$-dependent glyceraldehyde phosphate dehydrogenase and ribulose bisphosphate carboxylase.

In order to obtain isolated envelopes, the chloroplasts are gently lysed and layered on a discontinuous sucrose density gradient and centrifuged at 72,000 g for 60 minutes. The deep-yellow carotenoid-containing envelopes collect at the 0.6/0.93 M interface. The envelopes are removed from the interface, resuspended in a 0.3 M sucrose-containing buffer, and centrifuged at 113,000 g for 45 minutes to yield a pellet of envelopes. The marker enzyme for the envelope is a Mg^{2+}-dependent ATPase that is insensitive to DCCD.

The outer and inner membranes of the chloroplast envelope can be separated from each other by putting the chloroplast in a hypertonic medium, which causes the stroma volume to decrease so that the inner and outer envelope membranes separate as a function of the relative impermeability of the inner membrane compared to the outer membrane. The membranes are then ruptured by freeze-thawing and then the two types of membranes are separated by density-gradient centrifugation because their densities are so different (Douce and Joyard, 1990).

To obtain thylakoids, the chloroplasts are lysed and then centrifuged at 6000 g for 5 minutes. The thylakoids, which are in the pellet, can be identified by the presence of ferredoxin: $NADP^+$ oxidoreductase.

13.3 COMPOSITION OF THE CHLOROPLASTS

Lipids make up 58 percent of the dry weight of the chloroplast envelope. The only other membranes that are as rich in lipids are the plasma membranes of Schwann cells and oligodendrocytes that form the myelin sheaths that surround the axonal processes of neurons. Table 13.1 shows the lipid composition of the envelope. Treatment of the intact chloroplast with phospholipase C removes almost all the phosphatidylcholine in the chloroplast, indicating that this lipid, at least, is asymmetrically localized in the P-leaflet of the outer membrane of the envelope.

TABLE 13.1 Lipid composition of the envelope of a chloroplast

Polar Lipid	Percent Dry Weight of Total Lipids
MGDG	20
DGDG	30
TGDG	4
TTGDG	1
SL	6
PC	20
PG	8
PI	1
PE	tr
Fatty Acid Precursor	**Percent by Dry Weight**
16:0	15
16:1	3
16:3	9
18:0	tr
18:1	6
18:2	10
18:3	57
unsat/sat	5.7

Note: MGDG, Monogalactosyldiglyceride; DGDG, digalactosyldiglyceride; TGDG, Trigalactosyldiglyceride; TTGDG, Tetragalactosyldiglyceride; SL, Sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; tr, trace.
Source: From Douce et al. (1973) and Douce and Joyard (1979).

The outer envelope membrane is very permeable and contains a number of proteins (Ferro et al., 2003; Froehlich et al., 2003; Peltier et al., 2004; Bräutigam et al., 2008), including a pore protein that has an equivalent diameter of 3 nm that allows the passage of molecules up to 9–10 kDa (Flügge and Benz, 1984). It also contains a permease that facilitates the transport of lipids that are synthesized by the ER into the chloroplast (Xu et al., 2003). The inner membrane, by contrast, is a typical differentially permeable membrane, and contains a number of proteins involved with metabolite transport. These transporters facilitate the flow of the substrates and products of photosynthesis, as well as the components needed for the synthesis of galactolipids, fatty acids, prenylquinones, and carotenoids (Heber and Heldt, 1981; Douce and Joyard, 1979; Flügge and Weber, 1994).

TABLE 13.2 Lipid composition of the thylakoids

Polar Lipid	Percent Dry Weight of Total Lipids
MGDG	51
DGDG	26
SL	7
PG	9
PI	1
Fatty Acid Precursor	**Percent by Dry Weight**
16:0	8
16:1	5
16:3	13
18:0	tr
18:1	2
18:2	2
18:3	70
unsaturated/saturated	12.2

Source: From Douce et al. (1973).

The lipid composition of the thylakoid membranes has been determined by Douce et al. (1973; Table 13.2). The thylakoids are poor in phospholipids but rich in galactolipids, particularly digalactosyldiglyceride (DGDG) and monogalactosyldiglyceride (MGDG). The thylakoids also contain sulfur-containing lipids, including sulfoquinovosyldiglyceride (SL). MGDG, DGDG, and SL are endemic to the plastids and do not occur in any other organelle type. They do, however, also occur in the membranes of cyanobacteria, providing evidence that these organisms may share a common ancestor with chloroplasts (Douce and Joyard, 1990; see Chapter 15). The distribution of lipids in the thylakoid membranes is asymmetrical. Antibody studies show that phosphatidylglycerol is abundant on the stromal thylakoid leaflet and MGDG and SL are abundant on the lumenal thylakoid leaflet (Murphy, 1986). The thylakoid membranes contain many proteins (Peltier et al., 2002; Majeran et al., 2008), including the protein complexes that are involved with the light reactions of photosynthesis. All the enzymes necessary for the dark reactions of photosynthesis are found in the stroma.

13.4 THERMODYNAMICS AND BIOENERGETICS IN PHOTOSYNTHESIS

Since the chloroplasts are organelles that specialize in the conversion of radiant energy to chemical energy, it is an appropriate time to review the laws of thermodynamics. Heraclitus (540 BCE), who was interested in finding universal laws, proposed that fire was the basic unit of exchange for all things—just like gold was the basic unit of exchange in the marketplace (Hussey, 1995). This concept was further refined into the laws of thermodynamics in the mid-19th century, a revolutionary time when scientists were looking for unifying principles. Schleiden and Schwann came up with the idea that no matter how diverse living organisms are, they are made up of the same basic unit: cells. Darwin was also conceptualizing his theory of evolution by natural selection, indicating that all living organisms, no matter how different they look, are related. Thus, it seemed likely that the various forms of energy could also be related. These scientific discoveries that emphasized the unity of nature also had an influence on economists, including Frederick Engels (1972).

13.4.1 Laws of Thermodynamics

The First Law of Thermodynamics formulated independently by Hermann von Helmholtz, James Joule, and Robert Mayer states that energy cannot be created or destroyed, although it can be converted between different forms (Grove et al., 1867; Lenard, 1933). The Second Law of Thermodynamics, which was formulated by Sadi Carnot, Rudolf Clausius and Lord Kelvin, states that in the process of interconversion, there is a decrease in the ability of the energy to perform work. The decrease in the ability to do work is proportional to the increase in entropy. The Third Law of Thermodynamics, formulated by Walther Nernst, states that the entropy is zero at absolute zero. And the fourth law … ? There isn't a fourth law, and there will never be one according to Nernst, who figured that it took three people to come up with the first law, two to come up with the second, and he was obliged to come up with the third by himself, and thus it follows by extrapolation that nobody could come up with a fourth law (Laidler, 1993).

Michael Faraday (1861) gave a very understandable and clear nonmathematical lecture on the convertibility of energy (then known as forces) to children. Likewise, Hermann von Helmholtz and Robert Mayer wrote clear accounts for the layperson about the "conservation of forces" (Grove et al., 1867). William Thomson and Peter Tait (1862) wrote a very readable account for the layperson on the conservation of energy. In this account, they used the word *energy*, a term introduced into science by Thomas Young (1807). The concept of energy, instead of force, was applied by Rankine. Thomson and Tait then differentiated "kinetic energy" from "potential energy" and coined the term *conservation of energy*. Willard Gibbs (1875–1878) wrote an important, but nearly incomprehensible, treatise on thermodynamics that gave a mathematical framework to relate all the forms of energy to each other and to chemical processes. Luckily, Lewis and Randall (1923) wrote a lively and readable account of their interpretation of Gibbs's idea! In order to get a solid understanding of the biological process of energy conversion, I will use equilibrium thermodynamics to relate the molecular free energies of each step in the conversion process to the one before it and the one after it. According to a fundamental theorem of thermodynamics, at constant temperature and pressure, the change in molecular free energy (ΔE) is composed of two parts, the change in molecular enthalpy ($\Delta H/N_A$) and the change in molecular entropy ($\Delta S/N_A$):

$$\Delta E = \Delta H/N_A - \mathrm{T}\Delta S/N_A \qquad (13.1)$$

The enthalpy of a reaction is determined by burning each reactant and product separately, and determining how much the burning causes the temperature of water to rise. The enthalpy of a chemical is a property of the bonds that make up the chemical itself. Entropy, on the other hand, is a measure of the number of possible states in which a molecule can exist. The number of possible states is, in part, dependent on the possible number of resonant structures that can describe a given molecule (see Chapter 14). It is also dependent on the potential places a molecule can be localized in space. That is, the entropy depends, in part, on the relative disorder of molecules in a given volume, where the state with the maximum entropy is defined by a completely random distribution. Thus, *entropy* is a term that relates a molecule to its environment.

While energy is never created or destroyed, it can be redistributed in space, and this is what happens during every transformation. In order to understand what happens when free energy is transformed from one form of energy to another, we must take into consideration a property known as *entropy* (Clausius, 1879). Entropy, a term that comes from the Greek words for "in transformation," is a measure of the decrease in the amount of free energy available to perform work that occurs during a transformation. For this reason, entropy is a measure of the irreversibility of natural processes (Edsall and Gutfreund, 1983). To get a feel for entropy, consider a given amount of free energy in a system. If the entropy of this system were low, the energy would be concentrated among a few states (or atoms) or in a limited space and can thus be "rounded up" and utilized to do work. By contrast, if the entropy of this system were high, the energy would be partitioned among many states (or atoms) or in a large space and thus can be considered unavailable to do work. Given that the total amount of energy is conserved, when there is an increase in the amount of entropy, there is a decrease or dissipation in the amount of useful energy in a system.

While the Second Law of Thermodynamics allows one to predict the direction in which a reaction will proceed

spontaneously, many physicists believe that the world is fundamentally reversible and thus do not consider the Second Law of Thermodynamics to be a fundamental law. Whether the world is fundamentally reversible or fundamentally irreversible is a recurring topic of debate (Ehrenfest and Ehrenfest, 1912; Steckline, 1983), although an important one since the two views lead to contradictory physical descriptions of the world. Those who prefer an irreversible worldview see the Second Law of Thermodynamics, which states that a system evolves irreversibly toward a state of greater entropy, as being a fundamental law of physics, while those who prefer a reversible worldview see the Second Law of Thermodynamics as an approximate law that is only useful when the system under study is not observed for a long enough period of time for the initial state to recur. Scientists with a reversible worldview give priority to the recurrence theorem of statistical mechanics that states that any process is reversible in theory and can return to its initial condition with a finite probability after a long enough period of time. From the point of view of a cell biologist, the world is fundamentally irreversible since the physico-chemical processes we see in biological systems are always irreversible. For example, cells always get older and not younger, nucleic acid and protein synthesis require the use of a template while degradation does not, and it is impossible to reverse mitosis so that cancer cells disappear.

13.4.2 Molecular Free Energy of Some Photosynthetic Processes

Since photosynthesis involves the absorption of light, I will introduce the equation that relates the energy in a photon of light (ΔE, in J) to its frequency (ν, in s^{-1}) or wavelength (λ, in m). h and c are Planck's constant and the speed of light, respectively:

$$\Delta E = h\nu = hc/\lambda \tag{13.2}$$

This equation was proposed by Max Planck (1920, 1949) in 1900 while he was investigating black-body radiation, extended by Albert Einstein (1905) when he studied the photoelectric effect, used by Niels Bohr (1913) to describe the structure of the hydrogen atom, and directly tested by James Franck (1926) and Gustav Hertz (1926) by bombarding mercury with electrons that had various kinetic energies and observing the color of the light that was emitted. Franck continued his work on the quantum structure of atoms in Germany until Adolph Hitler came to power. Although Franck was a Jew, he was allowed to remain in his position as the Director of the Institute for Physical Chemistry in Göttingen, but only if he dismissed the other non-Aryan students and workers. Not willing to do this, Franck resigned and immigrated to the United States where he began work on the physical processes involved in the light reactions of photosynthesis (Rosenberg, 2004).

In order to quantify the energy transformations that occur during chemical reactions, we need to know the concentrations of each reactant and product under real conditions. However, the energetics of a reaction do not only depend on the absolute amount of the reactants and products, but on their distribution relative to the equilibrium distribution. We often do not know the real concentrations, and thus, in order to compare the molecular free energy of one reaction with that of others, we relate the free energies of a standard state, where the concentrations of all reactants and products are at any given concentration that we can choose. Let us choose 1 M for everything except water, which has a concentration of approximately 55.5 M. The constant K_{std} represents the ratio of products to reactants in the standard state. Consider the following reaction that describes the reversible synthesis of ATP:

$$ADP + P_i \Leftrightarrow ATP + H_2O$$

$K_{std} = [ATP][H_2O]/[ADP][P_i]$ for the synthesis of ATP, and under the conditions that we have conveniently chosen to be standard, $K_{std} = [1][55.5]/[1][1] = 55.5$.

Let K_{eq} represent the equilibrium constant for the synthesis of ATP. K_{eq} describes the concentrations of ATP, ADP, P_i, and H_2O at equilibrium when they started out at the standard. Since the water concentration will always be approximately 55.5 M, it will cancel out when we take the ratio of K_{eq} to K_{std}. Taking water into consideration, the value of K_{eq} is 1.96×10^{-5}. The difference in the free energy between the initial and the equilibrium state is given by the following equation, which is always put in the form of the final state minus the initial state.

$$E_{eq} - E_{std} = kT \ln (K_{eq}/K_{std}) \tag{13.3}$$

Equilibrium thermodynamic measurements depend on the fact that the system is reversible. The molecular free energy released as a reaction goes to equilibrium is equal, but opposite in sign, to the molecular free energy that must be added to push the reaction from equilibrium to the standard state. The amount of energy that must be added to drive the reaction from equilibrium to the standard state is known as the *standard molecular free energy*. It is given by the following equation:

$$\begin{aligned} E_{std} - E_{eq} &= kT \ln (K_{std}/K_{eq}) \\ &= -kT \ln (K_{eq}/K_{std}) \end{aligned} \tag{13.4}$$

If $E_{std} - E_{eq}$ were negative, then the product ATP will be formed spontaneously as the reaction goes to equilibrium, and if $E_{std} - E_{eq}$ were positive, the reactants will be made spontaneously as equilibrium is reached. K_{eq}/K_{std} is equal to the equilibrium concentrations of all the products, reactants, and water, divided by (1 M)(55.5 M)/(1 M)(1 M). K_{eq}/K_{std} is equal to approximately 3.53×10^{-7} for the above

reaction involving ATP. The standard molecular free energy of ATP formation is positive. Consequently, the synthesis of ATP requires molecular free energy (6.1×10^{-20} J), and its hydrolysis yields molecular free energy (-6.1×10^{-20} J).

The actual molecular free energy available to do work by a given reaction depends on how far the products and reactants are from equilibrium in the cell. It is given by the following reaction (Davies et al., 1993):

$$E_{\text{Real}} - E_{\text{eq}} = kT \ln (K_{\text{Real}}/K_{\text{eq}}) \tag{13.5}$$

where $K_{\text{Real}} = [H_2O][ATP]/[ADP][P_i]$ under real or cellular conditions. If K_{Real} is equal to K_{eq}, it will take no energy to synthesize ATP, nor will energy be made available by the hydrolysis of ATP. Since the concentrations of H_2O, ATP, ADP, and P_i in the cell are approximately 55.5 M, 10^{-3} M, 10^{-3} M, and 10^{-2} M, respectively (Mimura et al., 1984; Mimura and Kirino, 1984), then

$$\begin{aligned} K_{\text{Real}} &= [55.5\text{ M}][10^{-3}\text{ M}]/[10^{-3}\text{ M}][10^{-2}\text{ M}] \\ &= 55.5 \times 10^2 \end{aligned} \tag{13.6}$$

and

$$\begin{aligned} E_{\text{Real}} - E_{\text{eq}} &= kT \ln ((55.5 \times 10^2)/(1.96 \times 10^{-5})) \\ &= 8 \times 10^{-20}\text{ J} \end{aligned} \tag{13.7}$$

Thus, under typical cellular conditions it will take 8×10^{-20} J of free energy to synthesize a molecule of ATP, and 8×10^{-20} J will be made available to do work on its hydrolysis. As I will discuss in Chapter 14, ATP is used as an available source of free energy in the cell, in part, because it has very low entropy compared with ADP and P_i, and the concentrations of these molecules are far from equilibrium.

When using Eq. 13.5, the units of K_{Real} and K_{eq} must be identical or else the natural logarithm will be a dimensional number, and the result of the calculation will be dependent on the units used, which is not an attribute of a fundamental mathematical equation. Moreover, eliminating the contribution of water in either the equilibrium reaction or the real reaction could quantitatively change the calculated molecular free energy by a factor of 4 (i.e., ln 55.5). If water is not included in the fundamental equations, then the molecular free energies that are determined for the reactions that depend on the addition or elimination of water may not be comparable to those that do not involve water (Oesper, 1950). In fact, the molecular free energy required for ATP synthesis can be looked at as the free energy needed to pull a water molecule from ADP and P_i in an aqueous environment. The molecular free energy made available by the hydrolysis of ATP can be viewed as the free energy released when a water molecule is added back.

13.4.3 Molecular Free Energy of Oxidation-Reduction Reactions

Energy can also be made available to do work from oxidation-reduction reactions where an electron is passed from one molecule to another (Michaelis, 1930; Johnson, 1949; Cramer and Knaff, 1991). The loss of electrons is known as *oxidation*, and the gain of electrons is known as *reduction*. In order to quantify the energy state of the components involved in oxidation and reduction we cannot use Eq. 13.5 since the electrons do not have an independent existence in solution, and thus their concentration cannot be measured. Therefore, we use a technique that was developed to measure the free energy of a reaction that involves the ionization of a metal (Arrhenius, 1902). Consider the following two reactions:

$$Zn^{2+} + 2e^- \leftrightarrow Zn$$
$$2H^+ + 2e^- \leftrightarrow H_2$$

In these reactions, Zn^{2+} and $2H^+$ are being reduced to form Zn and H_2, respectively. In order to initiate these reactions, a piece of zinc metal is immersed in a 1 M Zn^{2+} solution; and in a separate vessel, a piece of platinum is immersed in a 1 M H^+ solution under 0.1 MPa of H_2 gas. The two solutions are connected with an agar tube, so that the two solutions can remain unmixed, yet electricity can pass between them. The zinc and platinum electrodes are connected by a wire. Because the solution in one vessel will have a different affinity for the electrons than the solution in the other vessel, the electrons will flow from the solution with low affinity to the solution with high affinity. A compound with a high affinity for electrons is known as an *oxidizing agent*, and a compound with a low affinity for electrons is known as a *reducing agent*. The affinity of the components of the solution for electrons can be quantified by inserting a voltmeter in place of the wire between the two electrodes. By definition the vessel that contains 1 M H^+, 1 atm H_2, and a platinum electrode has a standard reduction-oxidation potential ($z\psi^m$) or standard redox potential of 0 V. If the test solution tends to donate electrons to the H^+, the test solution will have a negative standard redox potential ($z\psi^m < 0$), and if it accepts electrons from H_2, the test solution will have a positive standard redox potential ($z\psi^m > 0$). The two electrodes can be placed in a common ionic solution to form an electrical cell. The voltage generated across the two electrodes is equal to the sum of the two voltages generated by the half-cells. The positive pole represents the electron donor and the negative pole represents the electron acceptor.

In order to measure the redox potential of a biological molecule like cytochrome b, a platinum electrode and a reference electrode are placed in a solution of reduced and oxidized cyt b and small redox couples known as *mediators* are added to the solution (Caswell, 1968; Caswell and

Pressman, 1968). The mediators transport electrons from the cytochrome to the electrodes. Since it is not possible to make a cytochrome electrode and use solid cytochrome to define the standard state (as it is for zinc), the standard state is defined as the state where the concentration of the oxidized form and that of the reduced form are equal. Thus, the standard redox potential is called the *midpoint potential*, and together, the oxidized and reduced forms are known as a *redox couple*. The molecular free energy of our test reaction (relative to the molecular free energy of the hydrogen reaction) is given by the following equation:

$$E^m - E^H = (kT \ln([\text{red}]/[\text{ox}]) + nez\psi^m) - (0) \quad (13.8)$$

where $E^m - E^H$ represents the difference in the molecular free energy between the test solution in the standard state (E^m) and the molecular free energy of the hydrogen solution (E^H). $z\psi^m$ is the midpoint potential of the test solution, and n and e represent the number of electrons transported and the elementary charge, respectively.

Any standard state can be chosen—for example, H^+ can be 10^{-7}M (as the biologists prefer it) or 10^0M (which is the chemists' choice). Since $kT \ln([\text{red}]/[\text{ox}]) = 0$ at the standard state, then the molecular free energy of the standard state of the test solution ($E^m - E^H$) is given by the following equation:

$$E^m - E^H = nez\psi^m \quad (13.9)$$

The standard state is defined to be mathematically convenient, but may not reflect the conditions found in the cell. Since the ability of a redox couple to donate electrons to another redox couple will be greater when the concentration of the reduced form of the donor couple is greater than the concentration of the oxidized form of the acceptor couple, we must take the real concentration into account in order to quantify the energetics of redox couples in the cell (Mitchell, 1966). Thus:

$$E^{\text{Real}} - E^m = (kT \ln([\text{red}]/[\text{ox}]) + nez\psi^{\text{Real}}) - nez\psi^m \quad (13.10)$$

where $E^{\text{Real}} - E^m$ represents the difference in molecular free energy between a redox couple in the real state and the molecular free energy of the redox couple in the standard state. This is just another form of the Nernst equation. By definition, $E^m = 0$. The effect of [red]/[ox] on the oxidizing or reducing power of a couple can be quantified by observing the redox couple under equilibrium conditions where $z\psi^{\text{Real}} - z\psi^m = -kT \ln([\text{red}]/[\text{ox}])$ and measuring the voltage necessary to induce each [red]/[ox]. When the electronic forces ($z\psi^{\text{Real}} - z\psi^m$) and the chemical forces ($-kT/\text{ne}$)ln([red]/[ox]) are equal, the couple is in equilibrium with the voltage:

$$-(kT/ne) \ln([\text{red}]/[\text{ox}]) = (z\psi^{\text{Real}} - z\psi^m) \quad (13.11)$$

and

$$z\psi^{\text{Real}} = z\psi^m - (kT/ne) \ln([\text{red}]/[\text{ox}]) \quad (13.12)$$

Thus, when $[\text{red}]/[\text{ox}] = 1$, $z\psi^{\text{Real}} = z\psi^m$. When $[\text{red}]/[\text{ox}] > 1$, $z\psi^{\text{Real}}$ is more negative than $z\psi^m$, and is a stronger reducing agent than it would be at the standard state. When $[\text{red}]/[\text{ox}] < 1$, $z\psi^{\text{Real}}$ is more positive than $z\psi^m$, and thus is a stronger oxidizing agent than it would be at the standard state.

When H^+ are also transferred in the reduction-oxidation reaction, then the complete dependency of the redox potential on concentration, number of electrons, and protons is given by the following equation:

$$z\psi^{\text{Real}} = z\psi^m - (kT/ne)\{\ln([\text{red}]/[\text{ox}]) - m \ln([H^+])\} \quad (13.13)$$

where m is the number of H^+ transferred.

The maximal molecular free energy required for, or released by, the transfer of an electron or a proton between two carriers is given by the following equation:

$$\Delta E = nze(z\psi^{\text{final}} - z\psi^{\text{initial}}) \quad (13.14)$$

where $z\psi^{\text{final}}$ is the real redox potential of the final carrier, and $z\psi^{\text{initial}}$ is the real redox potential of the initial carrier. The real redox potentials depend on the relative concentrations of the reduced and oxidized forms. They are also affected by environmental factors, including ionic strength (Cramer and Knaff, 1991). Since all the relevant facts necessary to determine the real redox potentials are not known, we must be satisfied for the moment with using the midpoint potentials (Table 13.3). I have included the valence of the electron z outside the parentheses in Eq. 13.15 so that this equation is valid for the movement of positive charges ($z > 0$) as well as the movement of negative charges ($z < 0$).

As electrons move from reducing agents to oxidizing agents, free energy is made available; this free energy can be used to translocate protons against their electrochemical difference across a membrane. The energy needed to translocate one H^+ can be calculated from the differences in electrical and chemical potential of H^+ on both sides of the membrane according to the following equation (Nernst, 1923; see Chapter 2):

$$\Delta E(p - e) = kT \ln([H+]_p/[H+]_e) + ze(\psi_p - \psi_e) \quad (13.15)$$

TABLE 13.3 Approximate midpoint potentials ($z\psi^m$, at pH 7) of photosynthetic electron carriers

Redox Couple	$z\psi^m$, at pH 7
Phaeophytin	−0.61
Ferridoxin	−0.43
(½)$NADP^+$/(½)NADPH	−0.32
Q_A	−0.30
Q_B	−0.15
cyt_{b6}	−0.14
cyt_f	0.36
Plastocyanin	0.37
P700 (reduced)	0.35
P700 (oxidized)	0.45
P700 (excited)	−1.20
P680 (reduced)	0.80
P680 (oxidized)	0.90
P680 (excited)	−0.80
Oxygen	0.81

where the subscripts p and e refer to the P- and E-spaces, and the form of the equation depends on how we integrate to get this equation (see Chapter 2). If we integrate from E-space to P-space, we get Eq. 13.15 that tells us the magnitude of the energy gained or lost as a proton moves from the E-space to the P-space. If we want to know the magnitude of the energy gained or lost as a proton moves from the P-space to the E-space, we must use the following equation:

$$\Delta E(e - p) = kT \ln([H+]_e/[H+]_p) + ze(\psi_e - \psi_p) \quad (13.16)$$

Measurement of the Electrical Potential across a Membrane

The electrical potential across a membrane can be determined by measuring the distribution of a lipophilic anion, like tetraphenyl boron (TPB^-) or a lipophilic cation, like tetraphenylphosphonium (TPP^+), both of which permeate the membrane passively. A lipophilic cation is used for membranes with a negatively charged interior, like the inner membrane of the mitochondrion, and a lipophilic anion is used for membranes with a positively charged interior, like the thylakoid. In doing these experiments, the thylakoid membranes are incubated with tracer amounts of TPB^-. Since TPB^- is negatively charged, it will distribute across the membrane in a manner related to the electrical potential of the membrane. For example, if the membrane is uncharged, the TPB^- will be equally distributed on both sides. If the membrane potential is inside-positive, the TPB^- will accumulate inside. The more positive the internal membrane potential, the more TPB^- will accumulate.

At equilibrium, the concentration of TPB^- on both sides of the membrane will be determined precisely by the membrane potential as described by the Nernst equation. The membrane potential (ψ_p) across the thylakoids only amounts to about −0.01 to −0.04 V (lumen = 0 V). The membrane potential of the thylakoid membranes is low as a consequence of the presence of ion transporters, which are capable of neutralizing the charge caused by H^+ accumulation. For example, every time an H^+ enters the thylakoid lumen, a Cl^- also passes through the membrane. The Cl^- neutralizes the charge of the proton, and at the same time, allows the build-up of a large pH difference.

Measurement of the pH Difference across a Membrane

The distribution of a weak acid or weak base between two compartments is a function of the pK_{acid} or pK_{base} of the tracer, and the pH of the two compartments. Thus, we can quantify the chemical potential for protons, which is related to the pH difference across a membrane, by measuring the distribution of a permeant weak base like ^{14}C-methylamine or weak acid like ^{14}C-dimethyloxazolidinedione (DMO), and calculating the internal pH of the membrane from the Henderson-Hasselbalch equation, which was originally derived by Lawrence Henderson (1908) and put in logarithmic form by Karl Hasselbalch. A weak acid is used for membranes with a basic interior, and a weak base is used for membranes with an acidic interior.

A weak base will only pass a membrane when it is uncharged. It will be uncharged in basic compartments and charged in acidic compartments. Thus, there will be a net movement across the membrane from the basic to the acidic compartment. By measuring the amount of tracer in the two compartments, it is possible to determine the pH in both compartments. The Henderson-Hasselbalch equation, used to determine the pOH with a weak base (BOH) could be derived from simple kinetics (Michaelis, 1926):

$$[OH^-] + [B^+] \underset{k_2}{\overset{k_1}{\Leftrightarrow}} [BOH]$$

where k_1 is the on-rate constant for the association of the weak base and k_2 is the off-rate constant for the dissociation of the base into a cation and a hydroxyl ion. At equilibrium, the rate of dissociation of BOH equals the rate of formation of BOH and

$$k_1[OH^-][B+] = k_2[BOH] \quad (13.17)$$

If we define K_b, which is a measure of the ability of a weak base to produce OH^-, as k_2/k_1, then

$$K_{base} = [OH^-][B^+]/[BOH] \quad (13.18)$$

Here we can see that K_{base} is really a dissociation constant and K_{acid}, which is a measure of the ability of a weak acid to produce H^+, is equal to 14 K_{base}. Putting the equation in logarithmic form, we get:

$$\log\ K_{base} - \log[OH^-] = \log\ [B^+]/[BOH] \quad (13.19)$$

After multiplying both sides by -1, we get:

$$-\log\ K_{base} - (-\log[OH^-]) = -\log\ [B^+]/[BOH] \quad (13.20)$$

Since $pX = -\log X$

$$pK_{base} - p[OH^-] = -\log[B^+]/[BOH] \quad (13.21)$$

or

$$pK_{base} - p[OH^-] = \log[BOH]/[B^+] \quad (13.22)$$

If $pK_{base} = p[OH^-]$, then $[BOH] = [B^+]$. If $pK_{base} \gg p[OH^-]$, then $[BOH] \gg [B^+]$. If $pK_{base} \ll p[OH^-]$, then $[BOH] \ll [B^+]$.

The weak base (BOH) can pass across the thylakoid membrane. If the pK_{base} of the weak base is the same in both compartments, then

$$K_{base} = [OH^-]_i[B^+]_i/[BOH]_i = [OH^-]_o[B^+]_o/[BOH]_o \quad (13.23)$$

The log of the relative proportion of B^+ to BOH will be determined by the difference between pK_{base} and $p[OH^-]$. At equilibrium, $[BOH]_i = [BOH]_o$, and thus:

$$[B^+]_i[OH^-]_i = [B^+]_o/[OH^-]_o \quad (13.24)$$

After rearranging terms:

$$[OH^-]_o[OH^-]_i = [B^+]_i/[B^+]_o \quad (13.25)$$

and we can calculate the pH, since pH = 14 − pOH. If the weak base has a pK_{base} that is one unit less than the expected pOH of the compartments, then, according to Eq. 13.22, $[B^+] > [BOH]$, and as a first approximation, the total concentration of radioactive substances measured is approximately equal to $[B^+]$. Using such techniques, the pH of the lumen was found to be about 5 and the pH of the stroma was found to be about 8 (Nicholls and Ferguson, 1992).

Now that we are able to quantify the energetics of photons, chemical reactions, reduction-oxidation reactions, and electrochemical differences, we will use the First Law of Thermodynamics to estimate the work that can be done as one form of energy is converted into another. In only using the First Law of Thermodynamics, we will be assuming that the cells are at equilibrium. I realize that this assumption is ridiculous and that while nonlife may be an equilibrium situation, life is certainly not, and needs a constant input of energy. Albert Claude (1975) wrote, "Life, this anti-entropy, ceaselessly reloaded with energy, is a climbing force, toward order amidst chaos, toward light among the darkness of the indefinite, toward the mystic dream of love, between the fire which devours itself and the silence of the cold." While nonequilibrium thermodynamics is important for a complete description of photosynthesis in living cells, equilibrium thermodynamics is still a powerful tool.

13.5 ORGANIZATION OF THE THYLAKOID MEMBRANE AND THE LIGHT REACTIONS OF PHOTOSYNTHESIS

The light reactions of photosynthesis take place on the thylakoids, which is a specialized membrane system that contains four kinds of protein complexes. These complexes function in the capture of light and the synthesis of ATP and NADPH (Murphy, 1986a,b; Mattoo et al., 1989; Camm and Green, 2004). The energy needed for the synthesis of ATP and NADPH comes from light (i.e., radiant energy). The light is absorbed by two protein-pigment complexes known as *Photosystem I* (PS I) and *Photosystem II* (PS II). The radiant energy is converted to electrical energy, which flows through molecules instead of wire. The electrons flow from the PS II complex to the PS I complex, through the $cyt_{b6\text{-}f}$ complex. As electrons pass through the $cyt_{b6\text{-}f}$ complex, protons are translocated from the stroma to the lumen. Once a proton difference is established, the fourth complex, an ATP synthase, uses the energy of the proton difference to make ATP. Furthermore, the electrons that result from the photochemical process are used to reduce $NADP^+$ to make NADPH.

The protein complexes can be differentially extracted with detergents from the thylakoid. They can also be visualized on the thylakoid membranes using freeze-fracture electron microscopy (see Figure 13.11; Anderson, 1975). The particles have been identified by correlating their presence with the presence or absence of biochemical activities that change with development, or differ between mutants and the wild type. Their presence on either the granal or stromal thylakoids can also be correlated with the biochemical activities of each fraction, and last, the isolated protein

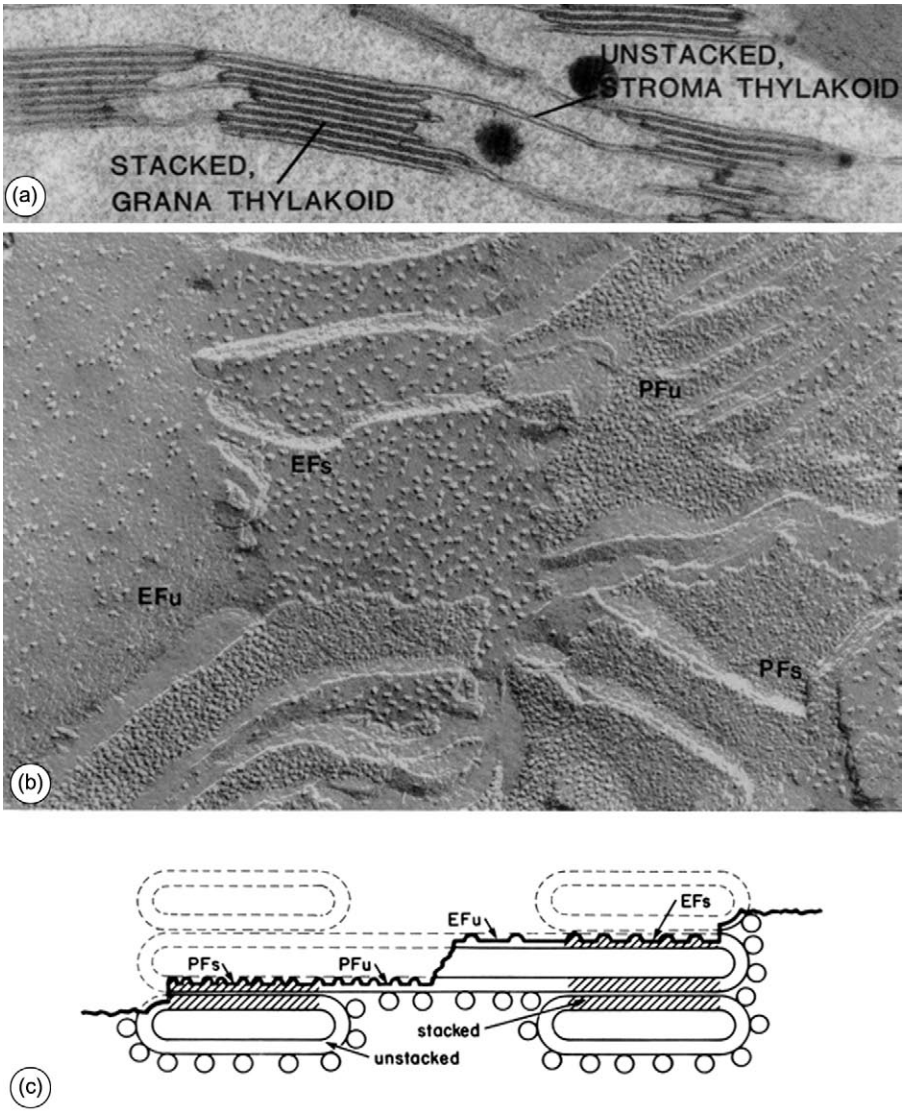


FIGURE 13.11 Thin-section electron micrograph (a), freeze-fracture micrograph (b), and model (c) of the thylakoid membranes of a pea. The membranes in the freeze-fracture micrograph were phosphorylated for 20 minutes in vitro before freezing. EFs and PFs are the E-face (lumen) and P-face (stroma), respectively, of the membranes from the stacked regions. EFu and PFu are the E-face and P-face, respectively, of the membranes from the unstacked regions. (a), ×100,000. (b), ×90,000. (*Source:* From Staehelin and Arntzen, 1983.)

complexes can be put into liposomes and freeze-fractured to see the size of the particles.

PS II particles, which are 8–11 nm in diameter, occur mostly (85%) on the lumenal leaflet of the membrane in the stacked regions of the thylakoids. There are 1200–1700 PS II complex particles per μm^2 in the stacked regions and 300–700 PS II complex particles per μm^2 in the unstacked regions (Staehelin, 1986). PS II mutants do not have this particle. Moreover, the chloroplasts of bundle sheath cells in plants exhibiting C_4 photosynthesis lack both PS II and these particles (Miller et al., 1977; Wrischer, 1989).

PS I complexes, which range in diameter from 10–13 nm, appear mostly (85%) on the stromal leaflet of the membrane in the unstacked regions of the thylakoids. There are about 2100–3300 PS I complex particles per μm^2 in the unstacked regions and 250–400 PS I complex particles per μm^2 in the stacked regions. PS I mutants lack this particle (Miller, 1980; Olive et al., 1983; Staehelin, 1986).

The size of PS I and PS II particles seem to be related to the number of light-harvesting complexes (LHCs) associated with the core complexes (Staehelin, 1986; Sprague et al., 1985). There is also a pool of mobile LHCs that are unbound and appear as 8-nm particles on the stromal leaflet of the thylakoid membrane (Simpson, 1979; Knoetzel and Simpson, 1991). The mobile particles may serve as the adhesion sites that allow the stacking of thylakoid membranes into grana. In the stacked thylakoid membranes, there are about 3600–5100 LHCs per μm^2.

The $cyt_{b6\text{-}f}$ complex is a 8.3-nm particle that is distributed in the stromal leaflet of the thylakoid membrane in both the stacked and unstacked regions. They range in density from 850 to 1300 $cyt_{b6\text{-}f}$ complex particles per μm^2.

The ATP synthase was first imaged as a lollipop-like structure using negative staining (Howell and Moudrianakis, 1967). It appears as a 10-nm particle on the stromal surface of the unstacked region of thylakoids and as a 6.5-nm particle when the membrane is fractured open. The peripheral protein complex, represented by the 10-nm particle, is known as CF_1, and is the hydrolytic portion of the complex. The integral protein complex, represented by the 6.5-nm particle, is known as CF_0, and is the proton-translocating portion (Miller and Staehelin, 1976; Mörschel and Staehelin, 1983). There are approximately 1000–1600 ATP synthase particles per μm^2 in the unstacked regions.

13.5.1 Photosystem Complexes

Charge Separation

Photosystems are pigment-containing protein complexes that contain reaction centers (Reed and Clayton, 1968; Reed, 1969) that convert radiant energy ($h\nu$) into chemical energy. Upon excitation, the pigment (P) becomes a strong reducing agent (P^+) that allows it to pass an electron to a primary acceptor (A), which then becomes reduced (A^-). This process, which takes place within the reaction center, is known as *charge separation* (Kluyver and van Niel, 1956) and is represented in the following reaction:

$$P + A + h\nu \Rightarrow P^+ + A^-$$

This reaction is irreversible as a consequence of the rapid re-reduction of P^+, which occurs as a result of the acquisition of an electron from an electron donor, as well as the rapid reoxidation of A^- that results from the reduction of the next electron acceptor. Both PS I and PS II are oriented in the thylakoid membrane such that the excited electron in the reaction center moves from the lumen side of the membrane to the stromal side of the membrane in an electrogenic manner.

Photosystem II Complex

In the case of PS II, phaeophytin is the primary electron acceptor, and water is the electron donor. The PS II complex can be said to function as a light-driven phaeophytin reductase and a water oxidase according to the following reaction:

$$4h\nu + 2H_2O + 4\text{phaeophytin}_{ox} \xrightarrow{\text{PS II}} O_2 + 4H^+ + 4\text{phaeophytin}_{red}$$

The PS II complex can be subdivided into three major functional units: the PS II core complex, surrounded by the PS II antenna complex (Emerson and Arnodl, 1932a,b; Clayton, 2002), and the water-splitting complex (Ghanotakis and Yocum, 1990). The core complex contains about six polypeptides that bind four chlorophyll a molecules, two of which form the reaction center that has an absorption maximum of 680 nm. These chlorophyll molecules are known as P680. The core also contains two pheophytin, one β-carotene, and one non-heme iron. The β carotenes function as antioxidants that protect the photosystems from damage that may be caused by the free radicals that are formed as a natural result of being subjected to high light irradiances.

Once light is absorbed by P680, the energy of an electron is raised to an excited state and that energy is then passed to phaeophytin. The electron is passed from phaeophytin to a bound form of plastoquinone known as Q_A and then to a freely diffusing form of plastoquinone known as Q_B. After accepting two electrons, Q_B binds two H^+ from the stroma. *Pari passu*, the oxidized P680 becomes a stronger oxidizing agent and strips an electron from water, which causes the formation of H^+ in the lumen and the evolution of $½O_2$. The splitting of water is thought to involve a water-oxidizing complex that is situated in the PS II complex on the lumenal side of the thylakoid (Mayfield, 1991; Ettinger and Theg, 1991).

How much free energy is required to transfer one electron from the reduced form of P680 (0.8 V) to phaeophytin (−0.61 V)? According to Eq. 13.15, it will take 2.3×10^{-19} J, and is thus an endergonic reaction. Is the energy in a photon of 680-nm light sufficient to raise the energy of the electron so that it can reduce phaeophytin? According to Eq. 13.2, there are 2.9×10^{-19} J associated with a photon of 680-nm light so that there is sufficient energy to reduce phaeophytin.

Photosystem I Complex

The PS I complex functions as a light-driven plastocyanin oxidase and a ferredoxin reductase (Golbeck, 1992; Ikeuchi, 1992). PS I is a pigment-containing protein complex that can be subdivided into a core complex and antenna complexes. The core complex, which contains the reaction center, contains seven polypeptides. The reaction center contains two chlorophyll molecules, known as *P700*, which have an absorption maximum at 700 nm. There are approximately 100 chlorophyll a, one β-carotene, two phylloquinone, and three 4Fe-4S centers per P700. The PS I complex passes a single electron from $\text{plastocyanin}_{red}$ to ferredoxin_{ox} with the help of radiant energy according to the following formula:

$$\text{PS I}$$
$$4h\nu + 4\text{Fd}_{\text{ox}} + 4\text{PC}_{\text{red}} \Rightarrow 4\text{Fd}_{\text{red}} + 4\text{PC}_{\text{ox}}$$

By inserting the appropriate values in Eqs. 13.14 and 13.2, we find that one photon of 700-nm light has sufficient energy to excite an electron from P700 to ferredoxin, an iron-sulfur protein. Once ferredoxin gets reduced by the electron from P700, it reduces $NADP^+$. It takes two electrons to reduce $NADP^+$ to NADPH:

$$2\text{H}^+ + 4\text{Fd}_{\text{red}} + 2\text{NADP}^+ \leftrightarrow 4\text{Fd}_{\text{ox}} + 2\text{NADPH}$$

In contrast to PS I, which is an integral membrane complex, ferredoxin and plastocyanin are water-soluble proteins. Ferredoxin is in the stroma, and plastocyanin is in the lumen. The ferredoxin-$NADP^+$ oxidoreductase (Avron and Jagendorf, 1956) is anchored to the stromal side of the thylakoid (Forti, 1999).

13.5.2 Cytochrome$_{\text{b6-f}}$ Complex

Once the cytochromes and plastocyanin were discovered in the chloroplast, their redox potentials and location in the thylakoids were established. The cytochrome$_{\text{b6-f}}$ complex has the appropriate midpoint redox potential to function as a plastoquinone:plastocyanin oxidoreductase according to the following reaction:

$$2\text{PQ}_{\text{B}}{}^{-2} + 4\text{PC}_{\text{ox}} + 4\text{H}^+\text{stroma} \overset{\text{cyt}_{\text{b6-f}}}{\rightarrow} 2\text{PQ}_{\text{B}} + 4\text{PC}_{\text{red}} + 4\text{H}^+\text{lumen}$$

This complex is homologous to the cytochrome b-c complex of mitochondria (see Chapter 14). While the first cytochromes found were given the names a, b, and c, cytochrome f got its name because it is found in *f*ronds (Hill and Scarisbrick, 1951)! The electron transport chain between plastoquinone and plastocyanin transports at least one H^+ from the stroma to the lumen for every electron that moves from plastoquinone to plastocyanin.

Light induces the uptake of protons into the lumen, as determined by measuring the effect of light on the pH of the medium that surrounds isolated chloroplasts (Hinkle and McCarty, 1978). Is the molecular free energy made available by the redox reaction sufficient to translocate a proton across the thylakoid membrane from the stroma to the lumen? Since we do not know the real redox potentials of the various components, we must settle with knowing the midpoint potentials ($z\psi^m$). Given that the midpoint redox potential for Q_B is approximately $-0.15\,\text{V}$ and the midpoint redox potential of plastocyanin is approximately $0.37\,\text{V}$, then for every electron transported, $8.32 \times 10^{-20}\,\text{J}$ of energy is made available to do work according to Eq. 13.14.

Given that $[\text{H}^+]_e = 10^{-5}\,\text{M}$, $[\text{H}^+]_p = 10^{-8}\,\text{M}$, $\psi_e = 0\,\text{V}$, and $\psi_p = -0.02\,\text{V}$, according to Eq. 13.16, it will take $3.2 \times 10^{-20}\,\text{J}$ to transfer an H^+ from the stroma to the lumen. Thus, there is enough energy made available in the transfer of an electron from Q_B to plastocyanin to transfer two protons from the stroma into the lumen. Experiments show that one to two H^+ are transferred across the thylakoid membrane for every electron passed through the cytochrome$_{\text{b6-f}}$ complex (Harold, 1986).

The figures used in the calculations of the molecular free energy of proton transport come from measurements of the bulk pH of the lumen and stroma. It is possible that the local concentration of H^+ around the cytochrome$_{\text{b6-f}}$ complex may vary drastically from the average value, and thus the distribution of protons may be important in understanding the energetics of coupling proton translocation to electron transport (Junge, 2004).

13.5.3 ATP Synthase

The fourth complex in the thylakoid membrane is the ATP synthase, which transduces or couples the potential energy inherent in the transthylakoid proton difference into the chemical energy of ATP (Avron and Jagendorf, 1957; Avron et al., 1958; Krogmann et al., 1959; Good, 1961; Jagendorf, 1961; Avron, 1963; Good et al., 1966). The chloroplast ATPase, also known as the coupling factor, is part of the F_0F_1-type (see Chapter 14) ATPase family and differs from the V-type (see Chapter 7) and P-type (see Chapter 2) ATPases. For over 20 years, it was believed that a high-energy intermediate known as "the squiggle" was responsible for coupling the energy of redox reactions into the chemical energy of ATP. There were, however, some skeptics (see Pasternak, 1993; Williams 1993). Peter Mitchell (1966) proposed an alternative explanation for energy coupling. He proposed that the energy inherent in the electrochemical difference of protons across a membrane could provide the energy for ATP synthesis, but there was no evidence to prove Mitchell's hypothesis. The reaction proposed by Mitchell can be represented by the following formula:

$$\text{ATP synthase}$$
$$\text{ADP} + \text{P}_{\text{i}} + 3\text{H}^+_{\text{stroma}} \Leftrightarrow \text{ATP} + \text{H}_2\text{O} + 3\text{H}^+_{\text{lumen}}$$

Work begun by Geoffrey Hind and André Jagendorf (Hind and Jagendorf, 1963), who showed that ATP formation can be temporally separated from the light requirement, was continued by Jagendorf and Neumann (1965), who showed that light causes a reversible rise in the pH of the medium that surrounded the chloroplasts. This work

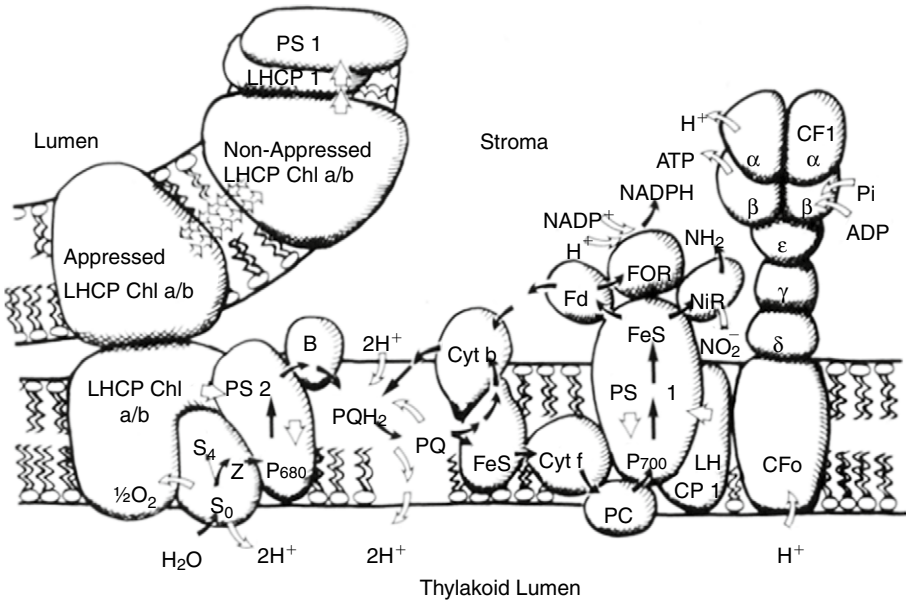


FIGURE 13.12 Diagram of the thylakoid membrane showing the topology of the complexes involved in the light reactions of photosynthesis. (*Source:* From Wellburn, 1987.)

culminated in a publication by Jagendorf and Uribe (1966), in which they revealed that a proton difference across the thylakoid membrane could be used to generate ATP, thus providing the first direct evidence for Peter Mitchell's chemosmotic hypothesis. In order to show that a pH difference across the thylakoid membranes could power ATP synthesis, Jagendorf and Uribe incubated chloroplasts in a permeant acid (i.e., weak acid) to acidify the inside of the chloroplast (pH 3.8) and then transferred the chloroplast to a neutral pH medium (pH 7.0) that contained ADP and P_i. Upon transfer, ATP was synthesized from the energy inherent in the proton difference.

Richard McCarty found that the rate of ATP synthesis is related to the third power of the pH difference across the thylakoid membranes. When viewed in light of kinetic theory (see Chapter 12), this result indicates that the generation of a single ATP probably results from the translocation of three H^+ from the lumen to the stroma through the ATP synthase (Hinkle and McCarty, 1978). Kaim and Dimroth (1999) as well as Junge (2004) believe that ATP synthesis depends on the energy inherent in the membrane potential and not the pH difference. Resolution of this problem will depend on better measurements of the steady-state $\Delta\psi$ and ΔpH in living chloroplasts.

Given that the energy needed to synthesize a single ATP molecule is approximately 8.0×10^{-20} J under physiological conditions (see Eq. 13.6), and the energy made available by an H^+ moving from the lumen to the stroma is 3.2×10^{-20} J (see Eq. 13.16), then it will take the electrochemical potential energy of three H^+ to synthesize one ATP molecule. Thus, McCarty's finding is consistent with the Laws of Thermodynamics (Hinkle and McCarty, 1978).

13.5.4 Light Reactions of Photosynthesis

Light energy is absorbed by the antenna complex of PS II and is passed in a somewhat random fashion to P680 by resonance transfer (Laible et al., 1994). Once the energy reaches P680, an electron is raised to a higher-energy state where it can reduce phaeophytin. Phaeophytin passes the electron to the plastoquinones, which in turn passes it to the cyt_{b6-f} complex. $P680^+$, because it has a more positive redox potential than P680, is able to take an electron from water to refill the hole left by the electron that was ejected by light. One H^+ is produced in the lumen from a water molecule for every photon absorbed by P680. The $cyt_{b6\text{-}f}$ complex oxidizes PQH_2 and reduces PC_{ox}. As the electrons are passed through the cytochrome chain, protons are passed from the stroma to the lumen. Since the electrons take a zigzag course across the membrane, the biochemical pathway of the light reactions was dubbed Z-scheme of photosynthesis (see Figure 13.12; Trebst, 1974), and it is reminiscent of the N-shaped diagram used to depict the redox potentials of the components of the light reactions, when it is turned on its side (Figure 13.13).

When a great-enough proton concentration difference is developed across the thylakoid membrane, the protons move through the CF_0 portion of the ATP synthase, and the energy made available by the collapse of the pH difference is used for the synthesis of ATP from ADP and P_i. In this way, ATP is formed in the light reactions of photosynthesis.

Light is also absorbed by the antenna complex of PS I where the energy is passed by resonance transfer in a random manner to P700. Once it reaches P700 an electron is raised to a higher energy level where it is able to reduce ferredoxin. The $P700^+$ can then take an electron from PC to refill the hole left by the reduction of ferredoxin. The

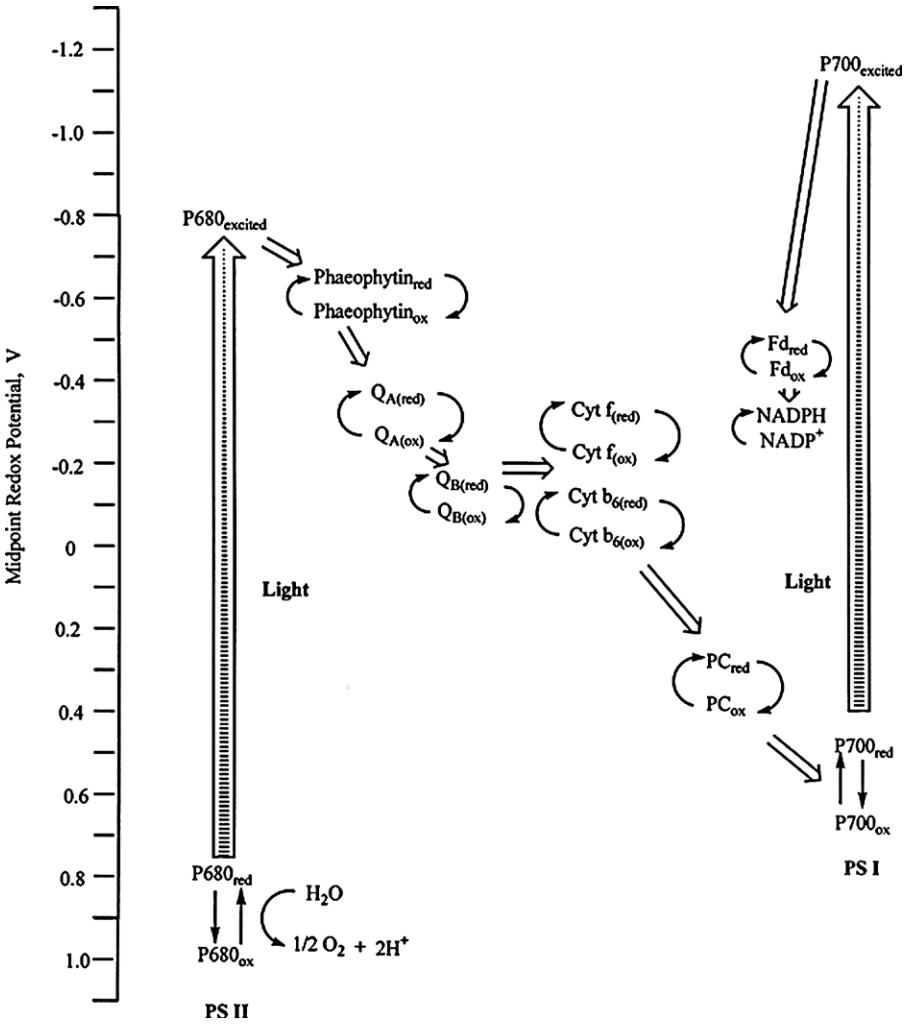


FIGURE 13.13 Diagram of the light reactions of photosynthesis showing the estimated midpoint redox potentials of the various electron transport components.

ferredoxin-$NADP^+$ oxidoreductase is then able to reduce $NADP^+$ to NADPH at the expense of the oxidation of two molecules of ferredoxin and a proton from the stroma. Thus, the formation of NADPH causes an alkalinization of the stroma and this contributes to the pH difference across the thylakoid membrane. The ATP and the NADPH generated in the light reactions are then used to fix CO_2 into glucose in the dark reactions that take place in the stroma. As we will see, it takes two NADPH and three ATP molecules to fix a single CO_2 molecule.

It takes four electrons from PS I to reduce two $NADP^+$ molecules and form two NADPH. It takes four photons from PS II to induce the transfer of four electrons from water to PS I so that the four-electron deficit of PS I can be eliminated. Thus, it takes eight photons to produce two NADPH. Assuming that for every photon absorbed by PS II, one H^+ is released into the lumen from the hydrolysis of water and one H^+ is transferred via the cytochrome pathway, then there will be eight H^+ that can be used for ATP synthesis and thus $^8/_3$ or 2.7 ATPs can be made for every eight photons.

Since, as we will see later, it takes three ATP and two NADPH to fix each CO_2 molecule, the 2.7 ATP to two NADPH stoichiometry produced by the light reactions is not quite right if we look at photosynthesis in isolation. Indeed, this is really a mute point since there are other ATP- and NADPH-requiring reactions in the chloroplast that compete with carbon fixation for these substrates. Nevertheless, it is also possible that the ratio of ATP to NADPH produced by the light reactions is either regulated or variable. A greater amount of ATP could be synthesized if occasionally two H^+ are transferred from the stroma to the lumen with an electron. After all, this is thermodynamically possible. It is also possible that electrons are diverted from ferredoxin to either plastoquinone or the cytochrome complex to transport an H^+ instead of reducing $NADP^+$ in a process called *cyclic photophosphorylation* (Havaux, 1992).

For every four photons absorbed by PS II, one molecule of oxygen is evolved. This ratio can be deduced by measuring the rate of oxygen evolution versus the photon fluence rate and determining the slope of the resultant curve. Many experiments show that one molecule of oxygen is evolved for every eight photons absorbed by the two photosystems together. The oxygen-evolving reaction is as follows:

$$2H_2O + 8 \text{ photons} \Rightarrow 4H^+ + 4e^- + O_2$$

Carbon dioxide is not the only gas fixed by photosynthetic reactions in the biological world. Some bacteria use the products of the light reactions of photosynthesis to fix N_2 (Gest and Kamen, 1949; Kamen and Gest, 1949; Kamen, 1955; Gest, 1994, 1999). Interestingly, nitrogenase, the enzyme that fixes N_2, produces H_2 in the absence of N_2. Imagine running your car on bacterial waste products by combining two volumes of the waste product of H_2-producing bacteria with one volume of the waste product of O_2-producing bacteria to form two volumes of H_2O and 4.75×10^{-19} J of heat per liquid water molecule formed.

Metabolic engineers are working on improving photosynthesis by transforming plants with new versions of genes or by overexpressing the genes that are normally present (Peterhänsel et al., 2008). On the other hand, nature is also the mother of invention, and photosynthesis has served as a model for engineers interested in converting solar energy into chemical energy. They have designed n-type semiconductors made out of titanium dioxide that use the energy of visible light to split water into oxygen and hydrogen with an efficiency of approximately 10 percent (Khan et al., 2002). The hydrogen can be recombined with the oxygen to generate heat and run an engine in which water is the only "waste product."

13.6 PHYSIOLOGICAL, BIOCHEMICAL, AND STRUCTURAL ADAPTATIONS OF THE LIGHT REACTIONS

The organization of the light-harvesting pigments undergoes dynamic adaptations in response to changes in the light quality in order to balance the energy distribution between PS I and PS II. These changes, which are referred to as *State I/State II transitions*, take place when leaves go between bright light and the limiting light conditions that occur when leaves shade each other (Bonaventura and Myers, 1969; Murata, 1969; Glazer and Melis, 1987; Allen, 2003; Chuartzman et al., 2008). Such shade contains a greater proportion of far-red light that is preferentially absorbed by PS I (P700) than does direct sunlight. Shady light has less blue and red because these colors have been absorbed by the chlorophyll in the leaves above it.

State I is the situation when a photosynthetic organism is exposed to the far-red light preferentially absorbed by PS I. A few minutes after being placed in this condition, a lateral movement of light-harvesting complexes takes place that allows more of the absorbed light energy to be directed to PS II, so that PS I and PS II will absorb equal quanta in order to maximize the current flow in the series circuit between PS II and PS I and thus maximize photosynthetic efficiency.

State II is the condition that develops when a photosynthetic organism is exposed to light preferentially absorbed by PS II. Several minutes after a plant is put in this condition, a lateral movement of light-harvesting complexes takes place that allows more of the absorbed light energy to be directed to PS I so that PS I and PS II will absorb equal quanta in order to maximize photosynthetic efficiency.

The lateral movement of the mobile LHCs between the stacked regions that are rich in PS II and the unstacked regions that are rich in PS I is the physical basis for the ability to undergo State I/State II transitions (Staehelin et al., 1982). The ability of the LHCs to pass light energy to the reaction centers by resonance energy transfer decreases with the sixth power of the distance, and consequently the spatial distribution of these complexes with respect to the reaction centers determines which reaction center gets the radiant energy absorbed by the LHCs. Quantitative freeze-fracture microscopy as well as immunological studies show that 20–25 percent of the mobile LHC particles participate in the reversible migration between stacked and unstacked regions (Kyle et al., 1983; D'Paolo et al., 1990).

The position of the mobile LHCs is determined by the phosphorylation state of the complex (Schuster et al., 1986). The portion of the LHC that is exposed to the stroma contains a lot of positively charged amino acids, including lysine and arginine. These positive charges attract the negatively charged lipids and proteins of the thylakoid membranes and cause the thylakoid membranes to stack and form grana. However, when the LHC becomes phosphorylated at the threonine residues near the lysine-/arginine-rich area, this region of the protein becomes negatively charged, and it can no longer support stacking of the thylakoids into grana (Staehelin and Arntzen, 1983; Canaani et al., 1984). Consequently, the mobile LHCs are released from the grana stacks and are free to diffuse within the membrane. When they enter the adjacent unstacked regions, they associate with PS I. The diffusion coefficient of an LHC is approximately $2.5 \times 10^{-16}\,m^2/s$ (Bennett, 1991), depending on the viscosity of the membrane (Haworth, 1983). According to Einstein's (1906) random-walk equation, ($t = x^2/2D$), it will take approximately 0.2, 20, or 2000 seconds to diffuse 10, 100, or 1000 nm, respectively. The movement of the LHCs may also be due, in part, to blebbing and fusion events (Chuartzman et al., 2008).

The phosphorylated LHCs are found preferentially in the unstacked regions of thylakoids (Larsson et al., 1983).

The protein kinase (LHC II protein kinase) that phosphorylates the mobile LHCs is activated when plastoquinone is reduced (PQ_BH_2) (Bennett, 1991). The State I/State II transition is absent in $cyt_{b6\text{-}f}$ mutants. Thus, it is thought that the LHC II protein kinase may be associated with the $cyt_{b6\text{-}f}$ complex, and that the kinase is directly regulated by the redox state of the $cyt_{b6\text{-}f}$ complex, which is regulated by the redox state of PQ (Havaux, 1992). The $cyt_{b6\text{-}f}$ complex may also migrate with the LHC during the state transitions (Vallon et al., 1990). The level of phosphorylation of the LHC depends on the relative activity of the protein kinase and a protein phosphatase (Sun et al., 1989).

In full-sun conditions, when the activity of PS II is high relative to the activity of PS I, there will be a lot of PQH_2 and reduced $cyt_{b6\text{-}f}$, and the kinase will be active. Consequently, the LHC will become phosphorylated and negatively charged and it will migrate to the unstacked regions where it will be near PS I, and it will thus transfer its energy to PS I.

In the shade, PS I will be preferentially activated and the pool of PQH_2 will be relatively small, and thus the kinase will not be activated. Under this condition, the LHC will be dephosphorylated and will thus be positively charged. It will not be able to leave the stacked areas and will thus preferentially pass on its energy to PS II. Here is a case where the real redox state of components, in contrast to the midpoint potential, is important in understanding the regulation of photosynthesis.

The description/explanation of the State I/State II transitions is a beautiful example where the combination of structural, biochemical, biophysical, and physiological experiments have been used hand in hand and in just the right proportion to give a clear understanding of this very important and now extremely interesting biological process. It is not known if the differential stacking that results from the movement of the mobile LHCs explains the differences in chloroplast structure often seen in sun- versus shade-tolerant plants.

13.7 FIXATION OF CARBON

Adolph von Baeyer (1870) proposed that the carbohydrate formed by plants resulted from the direct light-mediated formation of formaldehyde (HCHO), followed by the condensation of six formaldehydes to make sugar. Other chemists and physiologists proffered additional one-step theories (Spoehr, 1926). However, we now know that carbohydrate formation is the result of a cyclic process that takes place in the stroma. The light-mediated synthesis of carbohydrate involves the addition of a one-carbon carboxyl group to a five-carbon receptor molecule. Since the end product of photosynthesis is a carbohydrate, then the carboxylate group must be reduced from the oxidized state (COO^-) to the state of carbohydrate (CH_2O). The reduction requires two NADPH molecules. In theory, one would be used to reduce COO^- to CO and H_2O, and the other would be used to reduce the CO to CH_2O. In practice, many steps and intermediates are involved (Anderson and Beardall, 1991).

Along with Samuel Ruben, Martin Kamen developed a method for producing ^{14}C with the cyclotron in Ernest. O. Lawrence's laboratory and began to work on finding the first products of photosynthesis (Ruben and Kamen, 1940b, 1941; Ruben, 1943; Gest, 2004). However, because of Kamen's liberal leanings, he was dismissed from the laboratory in 1944 (Davis, 1968; Kamen, 1985). Lawrence asked Melvin Calvin, who apparently had the "correct" political views, to continue the project. Calvin and his colleagues combined the investigative power of ^{14}C with the newly developed technique of paper chromatography (Consden et al., 1944) to identify the first products of photosynthesis (Benson and Calvin, 1947; Calvin and Benson, 1948; Bassham and Calvin, 1957; Calvin, 1961; Calvin and Bassham, 1962; Bassham, 2003).

Calvin chose to work on *Chlorella*, a single-celled alga that could be easily cultivated (Bold, 1942; Pringsheim, 1972) and rapidly labeled with $^{14}CO_2$. The reaction could also be stopped rapidly by dropping the cells in boiling methanol, which both killed the cells and extracted the products of photosynthesis. The extracts were loaded onto a paper chromatogram, and each compound moved in a given solvent in a manner that was related to the affinity of the molecule for the polar water absorbed to the cellulose in the paper compared to the nonpolar solvent. In this way, the products could be compared to authentic standards. It could also be determined if the products were phosphorylated by treating the extract chemically or enzymatically to remove the phosphate, and seeing how they move on the chromatogram. In this way, Calvin and his colleagues discovered that within 5 seconds, 3-phosphoglyceric acid was labeled, and suggested that there was a two-carbon acceptor for CO_2 (Calvin, 1952). However, as soon as they increased the temporal resolution of their assay, they found that the first product labeled was a six-carbon molecule formed from the joining of CO_2 to a five-carbon molecule known as ribulose bisphosphate (RuBP).

By following the position of the ^{14}C in the various products over time and analyzing the energetics of each step, Calvin and his colleagues came up with the complete pathway of the photosynthetic carbon-reduction cycle, which is known universally as the *Calvin cycle* (Figure 13.14). According to the Calvin cycle, RuBP is carboxylated to form an unstable six-carbon intermediate that splits to form two molecules of 3-phosphoglyceric acid. Each 3-phosphoglyceric acid molecule is phosphorylated at the expense of ATP to form 1,3-phosphoglyceric acid. Each of these molecules is reduced by a molecule of NADPH to form glyceraldehyde-3-P and P_i. Some of the glyceraldehyde-3-P continues on to form starch or a translocatable sugar, while others are involved in the regeneration of RuBP. The regeneration of RuBP involves the hydrolysis

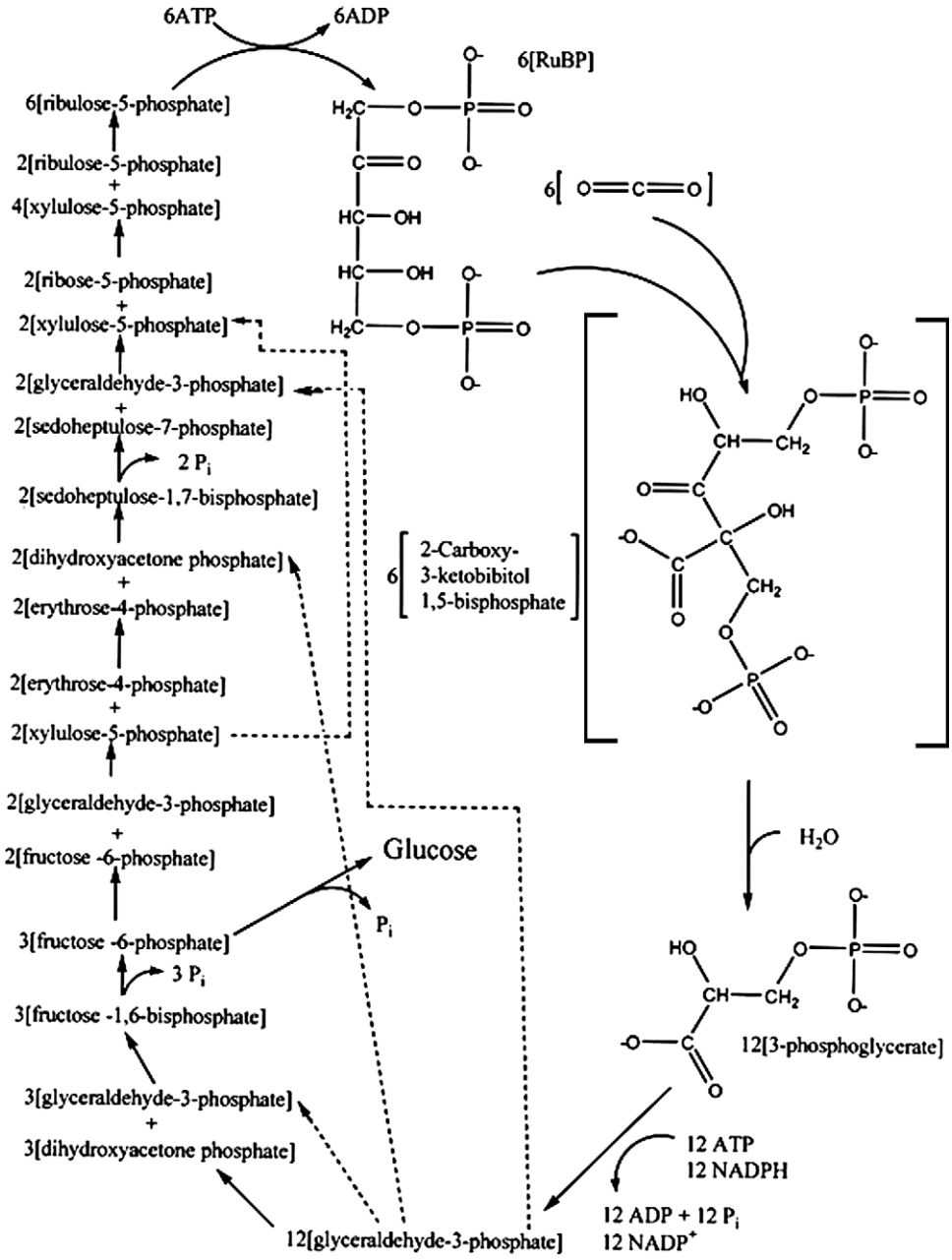


FIGURE 13.14 The Calvin cycle showing the intermediates involved in carbon fixation.

of one ATP for every CO_2 fixed. Thus, two NADPHs and three ATPs are necessary for the fixation of a single CO_2, and the regeneration of the acceptor.

The chemical energy transformed from radiant energy during the light reactions is used for the fixation of CO_2 by rubisco in the stroma. The fixation of CO_2 into a sugar requires three ATP and two NADPH according to the following reaction scheme:

$$2H^+ + CO_2 + 3\,ATP + 3H_2O + 2NADPH \Rightarrow CH_2O + 3P_i + 3\,ADP + 2NADP^+ + H_2O$$

Notice that the fixation of CO_2 also results in the alkalinization of the stroma and thus also contributes to the pH difference across the thylakoid membrane. The production of three ATP and two NADPH required eight photons. Assuming that each photon had a wavelength of 680 nm, it would take 2.3×10^{-18} J to fix a single CO_2, and 48 photons, the energy of which is equivalent to 1.4×10^{-17} J, to synthesize a six-carbon sugar.

Burning a single molecule of glucose completely to CO_2 and H_2O yields a release of heat (enthalpy) equivalent to 4.7×10^{-18} J. If the contribution of entropy to the synthesis of glucose is small, then the enthalpy will be a good indicator of the molecular free energy. Assuming the validity of this approximation, photosynthesis requires an input of 1.4×10^{-17} J of energy to synthesize a molecule that contains 4.7×10^{-18} J of energy. Thus, the photosynthetic process is approximately 34 percent efficient in thermodynamic terms.

Glucose is a highly reactive reducing sugar and will be dangerous for the cell in high concentrations (like plant diabetes). Thus, glucose is either not produced during photosynthesis (Calvin and Benson, 1949), or is immediately converted into nonreducing sugars (e.g., sucrose, raffinose), sugar alcohols (e.g., sorbitol; Zhou et al., 2001, 2002, 2003; Zhou and Quebeddeaux, 2003), or starch. Steviol, the diterpene that is 300 times sweeter than sugar, is produced in the chloroplast of *Stevia rebaudiana* (Kim et al., 1996).

The enzyme that is involved in the fixation of CO_2 was isolated as *Fraction I* protein, originally called *carboxydismutase*, and is now known as *ribulose bisphosphate carboxylase/oxygenase*, or *rubisco* for short (Quayle et al., 1954; Racker, 1955; Jakoby et al., 1956; Weissbach et al., 1956; Wildman, 1979; Lorimer, 1981; Portis, 1992; Spreitzer, 1993; Wildman, 2002). Rubisco catalyzes the attachment of CO_2 to ribulose 1,5-bisphosphate with a V_{max} of 200×10^{-3} mol kg chl^{-1} s^{-1} and a K_m of approximately 5–15 μM (Woodrow and Berry, 1988; Nobel, 1991). Rubisco is an extremely slow enzyme with an off-rate constant of 3 s^{-1}. In order to compensate for this inefficiency, rubisco must be an abundant protein, and can account for 25–75 percent of the leaf protein (Wildman and Bonner, 1947). It represents 50 percent of the chloroplast protein and is the most abundant protein on Earth. In fact, the total protein concentration of the stroma is 225 mg/mL (22.5% protein), which makes the stroma an extremely viscous gel!

Perhaps one reason for the inefficiency of rubisco is that it reached its present state of evolution before plants poured O_2 into the environment. However, given the present atmospheric concentrations of oxygen and carbon dioxide, oxygen is a very effective competitor for the CO_2 binding site (Forrester et al., 1966; Tregunna et al., 1966; Andrews et al., 1971; Bowes et al., 1971; Ogren and Bowes, 1971; Bowes and Ogren, 1972). When rubisco oxidizes RuBP instead of carboxylating it, 3-phosphoglyceric acid and 2-phosphoglycollic acid are formed (Walker, 1992). The carbon lost to 2-phosphoglycollic acid is partially recaptured in the photorespiratory pathway (see Chapter 5).

Some plants have evolved mechanisms to concentrate CO_2 around rubisco, and eliminate the need for the photorespiratory pathway (Kortschak et al., 1965; Hatch and Slack, 1966; Burris and Black, 1976; Nickell, 1993; Hatch, 2002). Organisms that use these pathways fix CO_2 with phosphoenolpyruvate (PEP) carboxylase (Bandurski and Greiner; 1953; Bandurski et al., 1953; Bandurski, 1955; Walker, 1956; Saltman et al., 1956), an enzyme that is insensitive to O_2. The carboxylic acids formed by PEP carboxylase are then decarboxylated by either malic enzyme or phosphoenolpyruvate carboxykinase in such a manner that rubisco is surrounded by its ancestral concentrations of CO_2 and O_2. In the vast majority of C4 plants, the mesophyll cells contain the enzymes involved in the C4 pathway and the bundle sheath cells contain the enzymes involved in the C3 pathway. Between the two cell types there are many plasmodesmata to facilitate the shuttling of the 3-C and 4-C acids between the two cell types (Evert et al., 1977). In some plants, however, the C4 photosynthetic pathway occurs within a single cell with spatially separated granal and agranal chloroplasts (Freitag and Stichler, 2000, 2002; Voznesenskaya et al., 2001, 2002, 2003, 2004, 2005; Edwards et al., 2004; Chuong et al., 2006; Boyd et al., 2007; Lara et al., 2008).

The light-mediated fixation of CO_2 by plants is fundamental for all living things. However, heterotrophic organisms can also fix a small amount of CO_2 (Evans and Slotin, 1940, 1941; Krebs and Eggleston, 1940a; Ruben and Kamen, 1940a; Solomon et al., 1941; Ochoa, 1980). Before the discovery of acetyl CoA (Lipmann et al., 1947), it was thought that the addition of CO_2 onto pyruvic acid, the end product of glycolysis, was necessary for the formation of oxaloacetic acid, and the activity of the citric acid cycle. Severo Ochoa (1952), who studied CO_2 fixation by malic enzyme in pigeon liver, also suggested that "most if not all biochemical reactions are readily reversible and indicate that photosynthetic fixation of carbon dioxide may operate along the same basic patterns established for its fixation in animal tissues."

13.8 REDUCTION OF NITRATE AND THE ACTIVATION OF SULFATE

The readily available energy in the chloroplast makes it an ideal site for the reduction of other molecules besides CO_2 (Anderson and Beardall, 1991). Plants take up sulfate from the soil, and this relatively inert form of sulfur must be activated by the cell before it can be metabolized further (Schmidt and Jäger, 1992). The sulfate is activated by ATP sulfurylases in the chloroplast (Leustek et al., 1994) to form adenosine phosphosulfate (APS) and pyrophosphate. Once activated to form APS, the sulfur can be transferred to many different molecules. Methionine synthesis occurs in the chloroplasts of plants (Ravanel et al., 2004).

The nitrate taken up by plants must be reduced before it can be metabolized. The reduction of nitrate to ammonia takes place in a two-step process: the reduction of nitrate to nitrite and subsequently the reduction of nitrite to ammonia. While the first step takes place in the cytosol (Fedorova et al., 1994), the second reaction takes place in plastids (Guerrero et al., 1981; Solomonson and Barber, 1990;

Ullich et al., 1990). In order to reduce nitrite to ammonia, six electrons are needed. These six electrons come from six ferredoxin molecules reduced in the light reactions. Thus, nitrogen is reduced at the expense of reducing $NADP^+$ that could be used for fixing carbon.

13.9 CHLOROPLAST MOVEMENTS AND PHOTOSYNTHESIS

Plants must balance the harmful effects of light that result in photodamage with the beneficial effects of light that result in photosynthesis (Long et al., 1994; Kasahara et al., 2002; Wada, 2005). Physiological adaptations to these needs include changes in the position of chloroplasts as well as leaf movements. Often the thick cell walls of epidermal cells act like lenses to focus the light (Gausman et al., 1974; Dennison and Vogelmann, 1989; Vogelmann, 1993), and under limiting light conditions, the chloroplasts move to the position of focused light (Stiles, 1925).

The chloroplasts of many plants do not sit passively in the cell, but constantly shift their position in order to optimize photosynthesis. Nowhere is this more spectacular than in the cells of *Mougeotia* (see Figure 10.10 in Chapter 10). The ribbonlike chloroplast in this cell turns broadside to face light of moderate intensity. However, if the intensity of the light is too high, the chloroplast turns so that its thin edge faces the light, presumably preventing damage through photobleaching (Haupt, 1982, 1983).

This photophysiological response is not mediated by the photosynthetic pigments themselves, but by a red light–absorbing pigment called *phytochrome* (or perhaps *neochrome*, which is a phytochrome-phototropin chimera; Suetsuga et al., 2005). Thus red light, absorbed through the photoreceptor pigment phytochrome, is most active in inducing the moderate fluence-turning response. Microbeam irradiation studies indicate that phytochrome is localized on or near the plasma membrane, not on the chloroplast (Bock and Haupt, 1961). In fact, the chloroplast-turning response shows an action dichroism (Haupt, 1960, 1968). That is, the effectiveness of red light depends on the azimuth of polarization of the red light, and only the cells that have their long axis normal to the electrical vector of the light show a response (Figure 13.15). This action dichroism indicates that the photoreceptor pigments are oriented and therefore must be associated with a stable structure like the plasma membrane. The chromophore of the red light–absorbing form of phytochrome is oriented parallel to the cell surface. Interestingly, upon the absorption of red light, the red light–absorbing form of phytochrome, P_r, is converted into the far–red light–absorbing form, P_{fr}, and the chromophore of P_{fr} is oriented normal to the surface of the cell (Haupt, 1970).

Since the flanks of the cell normal to the light rays will absorb more unpolarized light than the flanks parallel with the light rays, there will be more P_{fr} created on the membranes normal to the light rays compared with those parallel with the light rays. Under moderate light conditions, the chloroplast edges tend to move away from the highest concentration of P_{fr} and thus the face of the chloroplast ends up normal to the incident light.

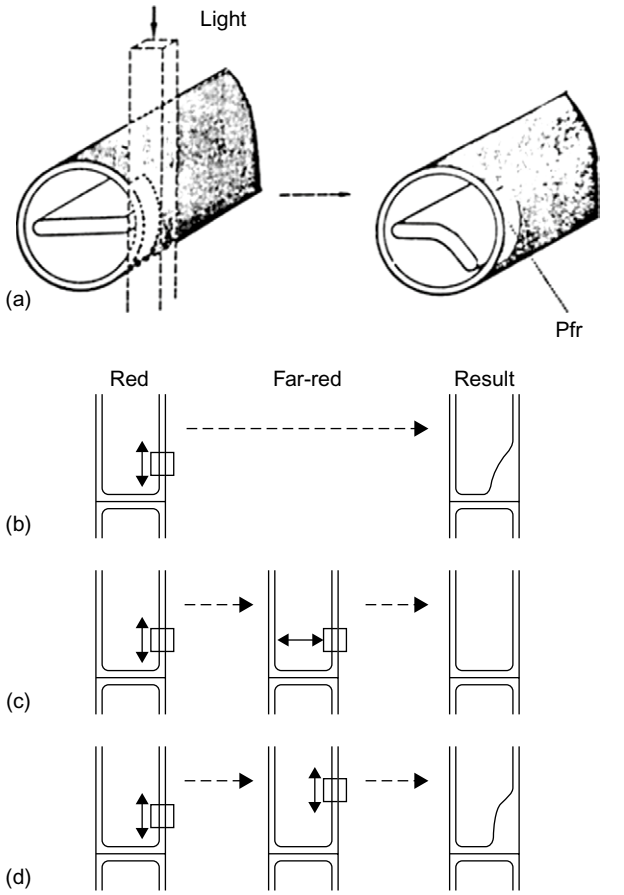


FIGURE 13.15 Microbeam irradiation of a *Mougeotia* cell. Overall view of experiment (a), and individual irradiation protocols (b–d). (b) The cell was irradiated with a red microbeam the light of which vibrated parallel to the long axis of the cell. (c) The cell was first irradiated with a red microbeam vibrating parallel to the long axis of the cell and subsequently irradiated with a far-red microbeam vibrating perpendicular to the long axis of the cell. (d) The cell was first irradiated with a red microbeam vibrating parallel to the long axis of the cell and subsequently irradiated with a far-red microbeam vibrating parallel to the long axis of the cell. The chloroplast moved away from the microbeam in protocols b and d, but not c. (*Source:* From Haupt, 1983.)

How does P_{fr} cause the repulsion of the chloroplast? P_{fr} may increase the length and number of actin microfilaments. In fact, the ability of the chloroplast to remain stationary in the presence of a centrifugal force increases after exposure of a dark-treated cell to red light, indicating that there are more bridges between the chloroplast and the plasma membrane (Schönbohm, 1972). Heavy meromyosin decoration of the links indicates that they are composed of actin microfilaments (Klein et al., 1980).

It seems that the actomyosin system does provide the motive force for chloroplast movement since chloroplast

movement is inhibited by N-ethylmaleimide and cytochalasin B (Schönbohm, 1972, 1975; Wagner et al., 1972). However, microtubules may need to be depolymerized before the chloroplast turns since taxol, a microtubule stabilizer, inhibits chloroplast movement while colchicine speeds it up (Serlin and Ferrell, 1989).

How does P_{fr} cause the activation of the actomyosin system? P_{fr} may act as a protein kinase (Suetsuga et al., 2005). The photoreceptor, through its kinase activity or through another mechanism, may cause an increase in the local concentration of Ca^{2+}. Wagner and Bellini (1976) showed that white light increases the rate of Ca^{2+} influx in *Mougeotia*, and Dreyer and Weisenseel (1979) have shown with $^{45}Ca^{2+}$ autoradiography, that red light induces a net increase in the influx of Ca^{2+}. Serlin and Roux (1984) have shown that the chloroplast edges move away from localized high concentrations of Ca^{2+}, which are applied by putting the bivalent ion-selective ionophore A 23187 on two microneedles on opposite sides of the cell. Ca^{2+} may induce its effect by binding to calmodulin and the calcium-calmodulin complex may then bind to a protein kinase, like myosin light chain kinase, and activate the actomyosin system (Jacobshagen et al., 1986; Roberts, 1989). Calmodulin antagonists inhibit red light–induced chloroplast movement (Wagner et al., 1984; Serlin and Roux, 1984).

There is a splendid array of organisms that show chloroplast movements (Haupt and Scheuerlein, 1990; Haupt, 1999; Wada et al., 2003). The chloroplast-turning response in the single-celled alga *Mesotaenium* is similar to that in *Mougeotia* (Kraml et al., 1988). The streaming chloroplasts of *Vaucheria* aggregate within a microbeam of light (Fischer-Arnold, 1963; Zurzycki and Lelatko, 1969; Blatt and Briggs, 1980; Blatt et al., 1981; Blatt, 1983, 1987). Light-induced chloroplast movements are also seen in the leaves of mosses and *Selaginella* (Haupt, 1982). In these cases, mathematical modeling shows that under various light intensities, the chloroplasts are where they are expected to be in order to maximize photosynthesis (Haupt, 1982).

As we move up the phylogenetic tree, we also find that ellipsoidal chloroplasts in the protonema of *Adiantum* also move toward moderate light and avoid high light (Yatsuhashi et al., 1985, 1987; Wada and Kadota, 1989; Kadota et al., 1989) as well as mechanical stimulation (Sato et al., 1999, 2001a,b). Likewise, the chloroplasts in the angiosperm species of *Vallisneria*, *Lemna*, and *Elodea* show a light-dependent change in orientation (Seitz, 1964, 1967, 1971; Ishigami and Nagai, 1980; Takagi and Nagai, 1986, 1988; Malec, 1994). Under low-light conditions, the chloroplasts are immobilized on the periclinal wall and expose the greatest amount of surface area to the light. High-intensity light causes them to move to the anticlinal walls, where they participate in cytoplasmic streaming and minimize their exposure to the light. Phototropins are the photoreceptor for blue light–induced chloroplast movements (Kagawa et al., 2001; Sakai et al., 2001; Kasahara et al., 2002; Briggs, 2005). While much work has gone into studying the mechanisms involved in these intracellular phototactic movements, it remains a fascinating field of research (Wada, 2005).

13.10 GENETIC SYSTEM OF PLASTIDS

Prokaryotes and eukaryotes both contain DNA; however, the DNA of eukaryotes shows higher levels of organization than that of prokaryotes. This difference served as the initial basis of the distinction between prokaryotes and eukaryotes (Dougherty, 1957). Hans Ris (1961) discovered DNA in the stroma of plastids, and this DNA looked like the naked DNA found in prokaryotes (Ris and Plaut, 1962). Chloroplast DNA is typically circular, has a molecular mass of 85–95 $\times$ 10^6 Da, and is composed of 12–18 $\times$ 10^5 base pairs that make up approximately 50–200 genes (Ohyama et al., 1986; Shinozaki et al., 1986; Sugiura, 2003). The complete sequences of the chloroplast genomes from *Arabidopsis thaliana*, *Astasia longa*, *Atropa belladonna*, *Chaetosphaeridium globosum*, *Chlorella vulgaris*, *Cyanidium caldarium*, *Cyanophora paradoxa*, *Epifagus virginiana*, *Euglena gracilis*, *Guillardia theta*, *Lotus japonicus*, *Marchantia polymorpha*, *Medicago truncatula*, *Mesostigma viride*, *Nephroselmis olivacea*, *Nicotiana tabacum*, *Odontella sinensis*, *Oenothera elata*, *Oryza sativa*, *Pinus thunbergii*, *Porphyra purpurea*, *Psilotum nudum*, *Spinacia oleracea*, *Toxoplasma gondii*, *Triticum aestivum*, and *Zea mays* are known. The DNA may be attached to the inner membrane of the envelope (Whitfield and Bottomley, 1983; Zurawski and Clegg, 1987; Sato et al., 1993). The chloroplast contains its own DNA gyrase (Wall et al., 2004), RNA polymerase (Polya and Jagendorf, 1971), DNA repair mechanisms (Cerutti and Jagendorf, 1993; Cerutti et al., 1992, 1993, 1995), and histonelike proteins (Kobayashi et al., 2002). There has been and continues to be a transfer of genes from the plastids to the nucleus (Huang et al., 2003; Timmis et al., 2004).

The chloroplast genome can be transformed with a gene that encodes a viral antigen that can be used to produce vaccines for animals and humans using solar energy. In this way, plants act as bioreactors to produce vaccines and other therapeutic proteins that can improve human health (Daniell et al., 2001, 2004, 2005; Walmsley and Arntzen, 2003).

The plastid DNA encodes genes that are segregated in a non-Mendelian manner. The non-Mendelian inheritance is typically known as *maternal inheritance* (Anderson, 1923; Darlington, 1944). There are a variety of physical mechanisms that lead to maternal inheritance (Vaughn et al., 1980; Vaughn, 1981, 1985; Hagemann and Schröder, 1989; Mogensen, 1996). For example, in some plants, maternal inheritance of plastids results from the exclusion of plastids from the generative cell, which divides to form the two sperm cells. In these plants, the exclusive presence of plastids in the vegetative cell

and the lack of plastids in the generative cell results from the exclusion of plastids from the portion of the microspore that gives rise to the generative cell. In cases where the plastids enter the generative cell, maternal inheritance can still occur if the plastids or the DNA within them is degraded.

When maternal inheritance depends on the degradation of DNA, the organellar DNA in the female gamete remains intact throughout syngamy while the paternal organellar DNA is degraded either before or after fertilization. In isogamous plants, the male organellar DNA is degraded after syngamy (Kuroiwa et al., 1982; Tsubo and Matsuda, 1984; Coleman and Maguire, 1983; Kuroiwa, 1991), while in anisogamous or oogamous plants, the male organellar DNA is degraded before syngamy (Sun et al., 1988; Kuroiwa and Hori, 1986; Kuroiwa et al., 1988; Miyamura et al., 1987; Corriveau and Coleman, 1988).

More recently, it has been found in conifers and some species of angiosperms that paternal inheritance and biparental inheritance of chloroplast DNA occurs (Szmidt et al., 1987; Chat et al., 1999; Hansen et al., 2007; Hu et al., 2008). Interestingly, in *Wisteria*, the mitochondrial genome can be inherited from the maternal parent while the plastid genome is inherited from the paternal parent, indicating that the mitochondrial and plastid genomes are independently segregated or degraded during the pollination/fertilization process (Trusty et al., 2007).

Both the stroma and the thylakoids contain 70S ribosomes (Chua et al., 1976; Staehelin, 1986; Jagendorf and Michaels, 1990). In the plastids, the ribosomes are directed to the thylakoids by signal peptides (Chua et al., 1973) and by a protein homolog of the signal-recognition particle (SRP; Franklin and Hoffman, 1993).

The plastid genome does not code for all the proteins necessary for the plastid, and thus nuclear-encoded proteins are necessary for chloroplast function. Given that plant nuclei can assimilate exogenous DNA (Hemleben et al., 1975; Blascheck, 1979; Leber and Hemlebem, 1979), the nuclear genes that encode the chloroplast proteins may have been transferred from the original endosymbiont that gave rise to the chloroplast during repeated exposures (Weeden, 1981; see Chapter 15). The nuclear and chloroplast genomes are in a dynamic equilibrium, in terms of evolutionary time scales, and genes continue to move from the chloroplast genome to the nuclear genome (Bubunenko et al., 1994; Kalanon and McFadden, 2008). The coordination of protein, cofactor, pigment, and enzyme production between the chloroplast and the rest of the cell occurs, and it seems likely that some kind of communication takes place between the chloroplast and the nucleus (Ellis, 1977). For example, the nuclear-encoded chlorophyll a/b binding protein is unstable in the absence of chlorophyll (Apel, 1979; Bennett, 1981). In the presence of chlorophyll, the protein concentration increases, without any changes in its mRNA concentration, indicating that there is coordination between the translation and/or stabilization of this protein and chlorophyll synthesis (Klein and Mullet, 1986).

Given that many of the proteins that function in the chloroplast are encoded by the nucleus, there must be a mechanism to import proteins translated on cytosolic ribosomes into the chloroplast. Blair and Ellis (1973) proposed that these must be a protein carrier on the envelope. Chua and Schmidt (1979) proposed that carrier is likely to be found in contact sites where the inner membrane and outer membrane are in contact. The density of contact sites is greatest in developing plastids and decreases in mature chloroplasts (Pfisterer et al., 1982).

The imported polypeptides seemed to require a transit peptide in order to recognize the chloroplast and to be imported into it. When the transit peptide was cleaved from the polypeptide, the polypeptide could not enter the chloroplast (Mishkind et al., 1985). Moreover, if the transit peptide was added to polypeptides that usually did not enter the chloroplast, the latter then did (Lubben et al., 1988). It does not seem as if the primary structure of the transit peptide, once postulated to be the key to chloroplast recognition, is as important as once thought, because as more transit peptides are sequenced, the sequences are more and more divergent from the canonical sequence. It may be that the secondary structure is important (Douce and Joyard, 1990; Flügge, 1990; Willey et al., 1991; Dreses-Werringloer et al., 1991; Cline and Henry, 1996; Bédard and Jarvis, 2005). Indeed, variation around a theme is commonplace in biology. The fact that the cell is compartmentalized and that a specific protein is targeted to one or more compartments is the rule. However, there is variation in the targeting signal of the protein, the receptor and translocator on the targeted organelle, and the intracellular pathway taken to get to the organelle. Indeed, some glycosylated proteins enter the chloroplast through the endomembrane pathway (Chen et al., 2004; Villarejo et al., 2005; Faye and Daniell, 2006; Agne and Kessler, 2007).

Given the variations in the signals used to transport proteins to the chloroplast, there may be a variety of protein translocator complexes. Pain et al. (1988) initially identified a protein that is localized in the contact sites, although this protein turned out to be the phosphate transporter and not the protein transporter (Gray and Row, 1995). A protein translocator complex has been identified through molecular and biochemical studies (e.g., chemical cross-linking to translocated protein, immunoaffinity chromatography using protein A–translocated protein) using detergent-solubilized translocator proteins (Schnell et al., 1994; Caliebe and Soll, 1999; May and Soll, 1999; Bédard and Jarvis, 2005). The *t*ranslocator complexes on the *o*uter and *i*nner *c*hloroplast membranes are known as the Toc apparatus and Tic apparatus, respectively. They are localized at the contact sites (Schnell and Blobel, 1993; Schnell, 1995; Cline and Henry, 1996; Chen et al., 2000; Lopez-Juez and Pyke, 2005).

The first step in protein import is binding to the translocon. This can be detected in vitro in the absence of nucleoside triphosphates and in the presence of cross-linking agents (Perry and Keegstra, 1994; Ma et al., 1996). ATP and GTP are required for the import of proteins into the chloroplast. The ATP may be required for the action of chaperonins that unfold the protein to be translocated, and the GTP is utilized by the GTPase of the Toc complex. Then the Toc and Tic complexes physically associate and the protein is translocated into the stroma in an ATP-dependent manner that may require chaperonins (Gatenby and Ellis, 1990; Dessauer and Bartlett, 1994; Nielsen et al., 1997; Akita et al., 1997; Kouranov et al., 1998). Once a polypeptide enters the chloroplast, the transit peptide, if present, is cleaved off by a stromal processing protease (Keegstra et al., 1989; Bassham et al., 1994; Su et al., 1999). While the transit peptide has a phosphorylation site, removal of the phosphorylation site does not affect its targeting to chloroplasts (Nakrieko et al., 2004). Since the number of genes transferred from the chloroplast to the nucleus has evolved over time, the protein translocation complex itself must have evolved simultaneously in order to import the proteins necessary for chloroplast function (Kalanon and McFadden, 2008).

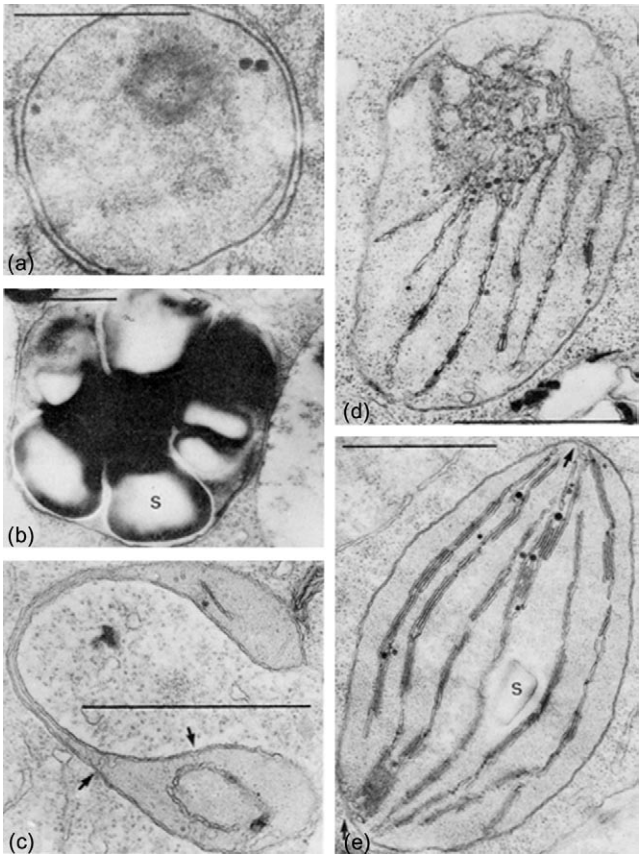


FIGURE 13.16 The development of plastids in light-grown *Phaseolus*: (a) eoplast, (b) amyloplast filled with starch (s), (c) amoeboid plastid, (d) immature chloroplast, and (e) mature chloroplast. Bars, 1 μm. (*Source:* From Wellburn, 1982.)

Polypeptides targeted to the lumen of the thylakoids must cross the envelope and the thylakoid membrane and have two signal sequences (Keegstra et al., 1989). There is more than one protein transport pathway across the thylakoid membranes, since a mutant form of a thylakoid membrane protein inhibits the translocation of thylakoid membrane proteins, but not the lumenal proteins (Smith and Kohorn, 1994; Bédard and Jarvis, 2005).

13.11 BIOGENESIS OF PLASTIDS

All plastids in a plant are formed from proplastids, and they usually contain the same genome (Randolph, 1922; Sirks, 1938; Knudson, 1940; Kuroiwa, 1991). Proplastids are relatively small, spherical organelles (ca. $0.5–1 \times 10^{-6}$ m in diameter) that are present in meristematic cells. In their earliest stages they are called *eoplasts* (see Figure 13.16; Whatley, 1977b, 1978). The proplastids contain nearly no internal membranes and only small amounts of DNA, RNA, ribosomes, and soluble proteins. The proplastid or eoplast then goes through an amyloplast stage where it remains spherical, yet accumulates starch. Then it goes through an amoeboid stage where it takes on a number of different amoeboid-like shapes in the cytoplasm (Senn, 1908; Weier, 1938; Esau, 1944). Eldon Newcomb (1967) has suggested that the amoeboid stage is a "feeding stage" in the development of protein-containing plastids. The amoeboid plastids then enter the pregranal phase. The amoeboid stage can persist in chloroplasts formed in fern gametophytes following X-ray irradiation of the spores (Knudson, 1940; Howard and Haigh, 1968).

When a plant develops in the light, the pregranal stage proplastids usually develop into chloroplasts. However, if the plant is kept in the dark, the proplastids develop into etioplasts, which are smaller than chloroplasts. Their largest diameter is ca. 1–2 μm. The protochorophyll in the etioplasts of dark-grown plants is confined to small optically dense 0.7- to 1.3 μm bodies originally known as *1 μ centers*, and now known as *prolamellar bodies* (Boardman and Anderson, 1964; Virgin, 1981). The prolamellar bodies undergo extensive structural changes as they convert to stromal lamellae and granal stacks (Gunning, 1965). The crystalline membrane region appears to become more and more disorganized as the membrane protrusions appear to become longer and longer. Within 2 minutes after the plastid is exposed to light, the membrane becomes perforated and these membranes persist for about 2 hours, which corresponds to the lag phase of chlorophyll synthesis (Simpson, 1978). Then more and more membranes flow over each other in the miraculous process of forming grana (Wellburn, 1987; Gunning and Steer, 1996). Phytochrome, Ca^{2+}, and cyclic GMP participate in the signal transduction chains that are involved in the greening response (Bowler et al., 1994; Reiss and Beale, 1995; Shiina et al., 1997).

Chloroplasts divide to give rise to other chloroplasts. There is a ring of actin filaments on the cytoplasmic side of the constricted isthmus of the dividing plastids, and indeed plastid division is inhibited by cytochalasin (Mita et al., 1986; Mita and Kuroiwa, 1989; Kuroiwa, 1991). In the red alga *Cyanidioschyzon merolae*, there are two other rings found in the isthmus: one in the intermembranous space between the inner and outer membranes of the envelope and one on the stromal side of the inner membrane. The intermembranous ring does not occur in higher plants.

Osteryoung and Vierling (1995) decided to see if the FtsZ protein is responsible, in part, for chloroplast division based on the role of FtsZ proteins in bacterial division. FtsZ mutants in bacteria form long filaments. This is because the bacteria continue to expand without dividing. The FtsZ mutants no longer form a constricting ring around the midpoint of the bacterium. Interestingly, the FtsZ protein is similar in many ways to tubulin (Lutkenhaus et al., 1980; Bi and Lutkenhaus, 1991; Bermudes et al., 1994; Erickson, 1995; Erickson et al., 1996; Faguy and Doolittle, 1998; Lu et al., 2000: Addinall and Holland, 2002). In plants, both overexpression or silencing of FtsZ genes result in the inhibition of plastid division and the formation of cells with only one to a few large chloroplasts (Pyke et al., 1994; Osteryoung et al., 1998; Strepp et al., 1998; Kiessling et al., 2000; Stokes et al., 2000; McAndrew et al., 2001; Osteryoung and McAndrew, 2001; Vitha et al., 2001; Jeong et al., 2002). FtsZ can be visualized as a ring around the isthmus of the dividing plastids (Mori et al., 2001) and the presence and function of FtsZ in the chloroplast are consistent with the endosymbiotic origin of chloroplasts (Osteryoung and Nunnari, 2003; Miyagishima et al., 2004).

Other classes of mutants also show a reduction in the number of chloroplasts in cells. These mutants include mutations in proteins that are involved in the positioning of FtsZ (Min proteins; Colletti et al., 2000; Kanamura et al., 2000; Dinkins et al., 2001; Itoh et al., 2001; Maple et al., 2002; Reddy et al., 2002; Fujiwara et al., 2008) or mutations in proteins with dynamin-like activity (Pyke and Leech, 1992, 1994; Robertson et al., 1995, 1996; Gao et al., 2003; McFadden and Ralph, 2003; Miyagishima et al., 2003, 2006).

Some of these cytoskeletal-like or rather plastoskeletal proteins may be involved in determining the structure and shape of the chloroplasts (Kiessling et al., 2000; McFadden, 2000). The shape and size of chloroplasts are also regulated by proteins (Maple et al., 2004), including mechanosensitive channels (Haswell and Meyerowitz, 2006).

Thin tubular connections between the plastids as well as thin tubular extensions of the plastids are called *stromatubules* (Kohler et al., 1997a,b; Bourett et al., 1999; Tirlapur et al., 1999; Shiina et al., 2000) or *stromules* (Köhler and Hanson, 2000; Gray et al., 2001; Hanson and Köhler, 2002; Pyke and Howells, 2002; Gunning, 2005). Their shape is determined by microfilaments and microtubules (Kwok and Hanson, 2003). Proteins may move from plastid to plastid in a plastid reticulum through the stromules (Gray et al., 1999, 2001; Köhler et al., 2000; Kwok and Hanson, 2004a,b).

The plastids in many flowers and fruits lack chlorophyll but accumulate tremendous amounts of carotenoids. These chromoplasts attract insects and other animals that may help pollinate or disperse fruits and seeds. Chromoplasts are also responsible for the yellows of the fall foliage (Straus, 1953; Frey-Wyssling and Schwegler, 1965; Israel and Stewart, 1967).

Some plastids in nonphotosynthetic tissues remain clear and function in the storage of macromolecules, including proteins, lipids, or starch. These plastids are known as *proteinoplasts* (Newcomb, 1967), *elaidoplasts* (Wellburn, 1987), and *amyloplasts*. The starch grains in the amyloplasts can be used for taxonomic characters (Reichert, 1919). Many people believe that amyloplast sedimentation is responsible for the perception of gravity (Kiss et al., 1989; Salisbury, 1993; Sack, 1997; Smith et al., 1997; Blancaflor et al., 1998; Fukaki et al., 1998; Kiss, 2000; Volkmann et al., 2000; Fitzelle and Kiss, 2001; Tasaka et al., 2001; Yoder et al., 2001; Schwuchow et al., 2002; Blancaflor and Masson, 2003; Hou et al., 2003; Perbal and Driss-Ecole, 2003; Morita and Tasaka, 2004; Palmieri and Kiss, 2005; Perrin et al., 2005; Mano et al., 2006; Palmieri et al., 2007; Vitha et al., 2007; Shiva Kumar et al., 2008). However, based on the facts that plant cells that do not contain sedimenting amyloplasts still sense gravity (Wayne et al., 1990) and that starchless mutants in higher plants are almost as sensitive to gravity as the wild-type plants (see Figure 13.17; Caspar and Pickard, 1989; Wiese and Kiss, 1999), others do not think that the amyloplasts act as gravity sensors, but as a ballast to enhance the gravitational pressure sensed by proteins at the plasma membrane–

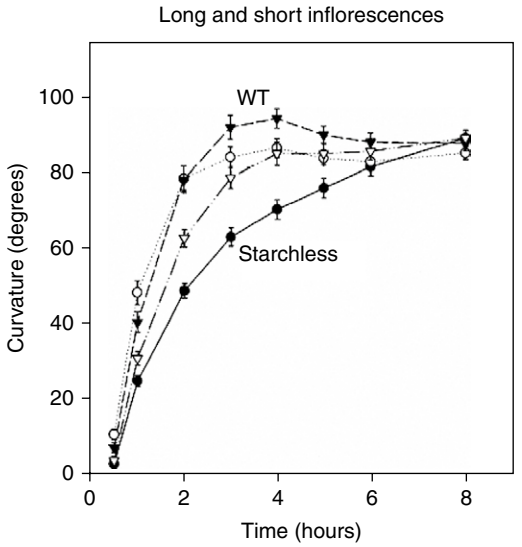


FIGURE 13.17 Gravitropic curvature of the inflorescences of wild-type (filled triangles) and two reduced-starch mutants (open symbols) and a starchless mutant (filled circles) of *Arabidopsis*. (*Source:* From Weise and Kiss, 1999.)

extracellular matrix junction (see Chapter 2; Wayne and Staves, 1996a; Staves, 1997; Staves et al., 1997a,b).

13.12 SUMMARY

The radiant energy of the sun is converted into chemical energy in the chloroplast that supports the life of every animal on the face of the Earth, including humans. The chloroplast converts light into life, both in terms of producing organic matter from carbon dioxide and water and in terms of providing the constant input of free energy necessary to prevent the dissipation of life expected from the Second Law of Thermodynamics. In this chapter, we have discussed the process of photosynthesis from a structural and functional perspective as well as a theoretical and experimental point of view.

The photosynthetic process reminds us that natural selection makes do with the components with which it has to work and selects based on the organism as a whole and not based on a single process. For example, while photosynthesis is critical for all life on Earth, chlorophyll and rubisco are not particularly adapted to collect light and fix CO_2 under the present atmospheric conditions. How does this square with the idea that "nature does nothing in vain," an idea that really goes back to Aristotle's idea of final causes (teleology)? Perhaps a change in a double bond of chlorophyll that could change its absorption spectrum or an amino acid in rubisco that would decrease its affinity for oxygen would harm other processes irrevocably. Thus, in evolution there seems to be a collective bargaining that leads to a unity of the organism.

According to Charles Darwin (1859), natural selection is also relativistic in that "it adapts and improves the inhabitants of each country only in relation to their co-inhabitants." In *The Origin of Species*, Darwin (1859) suggested that absolute perfection is a property of individual creation and not of natural selection, and that "the wonder indeed is, on the theory of natural selection, that more cases of the want of absolute perfection have not been detected."

13.13 QUESTIONS

13.1. What is the evidence that the radiant energy from the sun is converted into chemical energy in the chloroplast?
13.2. What is the mechanism by which the chloroplast converts radiant energy into chemical energy?
13.3. What are the limitations of thinking that the only function of the chloroplast is the conversion of radiant energy into chemical energy?

Chapter 14

Mitochondria

In Chapter 13, I discussed how chloroplasts convert radiant energy to chemical energy in the form of carbohydrates. Carbohydrates are high-energy molecules in thermodynamic terms, however, they are relatively inert and cannot directly provide the energy necessary to fuel cellular processes. Thus, something must act as an intermediary between the carbohydrates and the energy-requiring cellular processes. Throughout the 1940s and 1950s, biologists came to the exciting conclusion that adenosine triphosphate (ATP) is the chemical compound that universally and directly acts as this intermediate. ATP is involved in many cellular responses, including the synthesis of macromolecules (Cori, 1946) and coenzymes (Kornberg, 1950a,b; Schrecker and Kornberg, 1950); the activation of fatty acids (Kornberg and Pricer, 1953), amino acids (Hoagland et al., 1956), and sulfate (Bandurski et al., 1956); cell motility (Szent-Györgyi, 1949b; Hoffman-Berling, 1955; Weber, 1955); the maintenance of the differential permeability of membranes (Ginzburg, 1959); and the transport of ions (Caldwell et al., 1960). The conversion of the chemical energy of carbohydrate into the chemical energy of ATP takes place in the mitochondrion, which Albert Claude (1948, 1975) calls "the real power plants of the cell."

14.1 DISCOVERY OF THE MITOCHONDRIA AND THEIR FUNCTION

This history of mitochondrial studies follows two quite separate paths: one physiological and the other structural. The unification of these two tracks in the 1940s led to a thrilling and satisfying understanding of the energetics of the cell (Claude, 1943, 1975; Lehninger, 1964). First, I will discuss the history of the physiological studies, and then the history of the structural studies.

14.1.1 History of the Study of Respiration

That most organisms require air to survive is common knowledge today, but this need was only discovered serendipitously. Robert Boyle (1662) was conducting experiments to see whether flies and butterflies could fly in air made thin by his newly invented vacuum pump. When he pulled a vacuum in the bell jar in which they were flying, the flies and butterflies fell down and died. He wondered whether they died as a consequence of the fall, or whether the animals themselves became weak because of their need for air. He realized that the air was necessary for life when he repeated his experiments using a lark with a broken wing. Since the lark could not fly, any adverse effects of the lack of air on the lark in the bell jar would not be caused by falling, but would indicate the necessity of air for life. The lark died in the vacuum, and later experiments showed that a mouse died too. Boyle concluded from these experiments that air was necessary for respiration.

His fellow scientists did not believe his conclusions because he did not do the proper control experiment. That is, he did not test whether the mouse could live in the confined space in the bell jar in the absence of "creature comforts," even when there was plenty of air. In response to his critics, Boyle put a mouse in the bell jar overnight, gave it a paper bed and plenty of cheese, and then placed the bell jar by the fireside to keep the mouse warm during the night. In the morning, Boyle observed that the mouse was very much alive, and ate the cheese like a normal mouse. However, as soon as he evacuated the air, the mouse started to die, showing that lack of air, not the lack of "creature comforts," was necessary for life.

Robert Hooke (1726), an assistant of Boyle, showed that the air was taken up by the lungs by demonstrating that a dog could be kept alive if air was continuously blown through the lungs, but not through other parts of the body. Stephen Hales (1727) suggested that air might also be important for plants after performing experiments in which he incinerated plants and found that they were composed of air, which at the time was considered to be an element. Hales also suggested that the leaves might be analogous to the lungs of animals. Joseph Priestley (1774) and John Ingen-Housz (1796) showed that oxygen (i.e., dephlogisticated air) was the vital part of the air that was taken up by the respiration of animals and plants, respectively.

Antoine Lavoisier, the founder of modern chemistry, determined that combustion results from the combination of oxygen with carbon and hydrogen. Lavoisier measured the amount of oxygen in a sample by treating metals with a given sample of air, and measuring how much the mass of the metal increased as a result of oxidation. Lavoisier believed that respiration and combustion were analogous reactions, as he and Armand Séguin (Séguin and Lavoisier, 1789; translated in Fruton, 1972) observed:

> *In respiration, as in combustion, it is the atmospheric air which furnished oxygen and caloric; but since in respiration it is the substance itself of the animal, it is the blood, which furnishes the combustion matter, if animals did not regularly replace by means of food aliments that which they lose by respiration, the lamp would soon lack oil, and the animal would perish as a lamp is extinguished when it lacks nourishment. The proofs of this identity of effects in respiration and combustion are immediately deducible from experiment. Indeed, upon leaving the lung, the air that has been used for respiration no longer contains the same amount of oxygen; it contains not only carbonic acid gas but also much more water than it contained before it had been inspired.*

That is,

$$CH_2O + O_2 \Rightarrow CO_2 + H_2O + \text{heat}$$

where Lavoisier believed that O_2 was a mixture of base of oxygen and caloric. Respiration was defined as a combustion process and measured by the uptake of O_2 and the expulsion of CO_2 and H_2O.

Séguin and Lavoisier (1789) believed that the function of respiration was to produce body heat. Lavoisier and the mathematician Pierre Simon de Laplace collaborated on a set of experiments that showed that the amount of CO_2 produced by an animal is approximately equal to the amount of O_2 consumed. Using a calorimeter, they found that approximately the same amount of ice was melted by the respiration of a guinea pig and the burning of charcoal, which had equal outputs of CO_2. They concluded that the living body produces heat in a manner similar to inanimate objects. Lavoisier never finished his experiments on respiration because he was "politically incorrect" and lost his head in a guillotine during the French Revolution (Rolland, 1926).

Scientists disagreed as to where the conversion of O_2 to CO_2 took place. Was it the lungs as Lavoisier often thought, or was it the blood as Joseph Lagrange proposed (Mendelsohn, 1964)? It turned out that the conversion occurred in both places, as well as in every other part of the organism, as Lazzaro Spallanzani (1803) showed when he isolated various tissues and demonstrated that all tissues were capable of consuming oxygen and giving off CO_2. In 1804, Théodore de Saussure showed that in the dark, plants also take up O_2 and give off CO_2, and thus must have a respiratory mechanism similar to that of animals.

Eduard Pflüger (1872) also believed that every cell respired and that this was true for both plants and animals. Pflüger revealed that when the blood of a frog was replaced by saline, the frog respired just like a normal frog. He also mentioned that insects and plants that have no blood respire too. Pflüger concluded, "Here lies, and I want to state this once and for all, the crucial secret of the regulation of the total oxygen consumption by the organism, a gravity which is entirely determined by the cell itself."

By the end of the 19th century, it was becoming clear that respiration took place in each and every cell, and was thus a cellular process (Krogh, 1916; Haldane, 1922). In 1924, it seemed likely that the uptake of oxygen was associated with the particulate parts of the cytoplasm, which at the time seemed to be the nuclei (Meyerhof, 1924)! Subsequent structural studies showed that the mitochondrion was the respiratory organelle.

14.1.2 History of the Structural Studies in Mitochondria

Mitochondria were first seen in plant cells (*Equisetum*) by Wilhelm Hofmeister in 1851 (see Figure 9.1 in Chapter 9), however, structural studies of mitochondria began in earnest in the late 19th century when mitochondria were seen in many cell types following the introduction of the apochromatic lens (see Figure 14.1; Newcomer, 1940). Excellent apochromatic lenses owe their existence, in part, to the botanist Matthias Schleiden, a teacher of Carl Zeiss. Schleiden personally encouraged Zeiss to develop good microscopes to make it possible to see cellular structure. Zeiss began a microscope company and hired Ernst Abbe to develop a theory of image formation and to design apochromatic lenses from first principles.

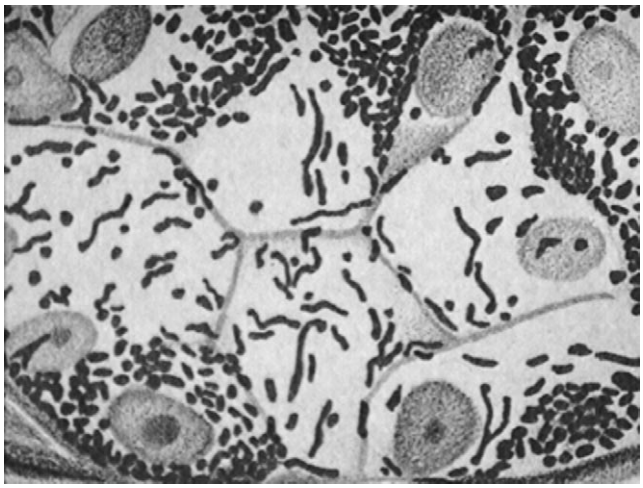


FIGURE 14.1 "Elementary organisms" in the mucilage-secreting cells of the stomach of a cat, fixed with an osmium mixture. (*Source:* From Altmann, 1890.)

The organelles that were later called mitochondria were initially given the following names by the early cytologists: bioblasts, granules, microsomes, plastosomes, eclectosomes, histomeres, chromidia, chondriokonts, chondriosomes, polioplasma, and vibrioden. Of almost 30 terms coined for this structure, only *mitochondria* survived (Cowdry, 1924). Carl Benda (1899), while studying mouse sperm, gave the name "mitochondria" to the cytoplasmic threadlike granules. Benda defined mitochondrion as "granules, rods, or filaments in the cytoplasm of nearly all cells which are preserved by bichromates within a pH range approximately between 4.6 and 5.0, and which are destroyed by acids or fat solvents" (Newcomer, 1940; Goldschmidt, 1956; Sato, 1972). *Mitos* is Greek for thread, and *chondrin* is Greek for small grain. While Altmann, Flemming, Kölliker, Hanstein, Strasburger, and von Valette St. George are considered by various historians to have first discovered the mitochondrion, Richard Altmann made the most complete and accurate descriptions and drawings (see Figure 14.1).

Such a large catalog of names followed from the limitation that the early cytologists could never reach a consensus about what was factual and what was artifactual when they and others observed mitochondria in fixed and stained sections (Fischer, 1899). This should always be kept in mind, not only when doing microscopy, but also when doing any experiments. Moreover, while the arguments were going on about what was fact and artifact, grandiose theories were being put forth to explain the function of these new organelles. For example, Altmann (1890) believed that the mitochondrion was the elementary particle of life and thus named it the bioblast. Others considered it to be the center of protein synthesis, fat synthesis, or the residence of genes. Last, but certainly not least, Kingbury (1912) proposed that the mitochondrion was the respiratory center of the cell.

A major breakthrough that proved that the mitochondrion was really a true organelle and not a preparation artifact came from the work of Leonor Michaelis (1900). At the suggestion of Paul Ehrlich, he tested the ability of the various newly invented fabric dyes to stain living tissue (Beer, 1959). Michaelis showed that the mitochondria in pancreatic exocrine cells were stained selectively and supravitally by a dilute solution of Janus green. Four years later, Meves showed that the mitochondria of plant cells could be visualized with the same dye (Meves, 1904). The staining of mitochondria by Janus green and other vital stains is only transient because the mitochondria reduce the dye and render it colorless (see Lazarow and Cooperstein, 1953). The ability of mitochondria to oxidize and reduce various dyes was what led Kingbury (1912) to propose that the mitochondria may be involved in cellular respiration.

Although Bensley (1953), one of the originators of the technique of differential centrifugation, believed that the mitochondrion did not have the "dignity" of a real organelle, and was at best "a journeyman worker who comes and goes," the question of the reality of mitochondria mostly ended in 1948 when Albert Claude used differential centrifugation to isolate the respiratory particles from rat liver cells. These particles stained with Janus green and thus he identified them as mitochondria and not nuclei as was previously believed (Myerhof, 1924; Brachet, 1985). Further evidence that the particles were mitochondria came from electron microscopic studies, which showed that the particles were membrane-enclosed organelles, identical in appearance to mitochondria found in cells (see Figure 14.2; Hogeboom et al., 1948; Palade, 1952). Kennedy and Lehninger (1949) then discovered that the isolated mitochondria contain all the enzymes involved in the Krebs cycle, and Chance and Williams (1955) found that mitochondria contain the cytochromes involved in electron transport. These were some of the first studies to combine biological chemistry and microscopy, and give incontrovertible support to a hypothesis of function that originated from a morphological observation. At this point, the techniques and ways of thinking heretofore developed independently by biochemists and cytologists coalesced, and the new field of cell biology emerged. The great synthesizers of this time included Christian de Duve, George Palade, and Albert Claude.

The ultrastructure of mitochondria in plants (*Nicotiana*, *Lemna*, *Euglena*), animals, and protozoa was described by George Palade in 1952 and 1953. The mitochondria are cylindrical bodies, 1- to 4-μm long by 0.3- to 0.7-μm wide, which are physically separate from the other organelles (see Figures 14.3 and 14.4; Douce, 1985; Rosamond, 1987). Palade (1953) described the mitochondria as "a system of internal ridges that protrude from the inner surface of the membrane toward the interior of the organelles. . . . In favorable electron micrographs the mitochondrial membrane appears to be double and the cristae appear to be folds of a second, internal mitochondrial membrane." The outer

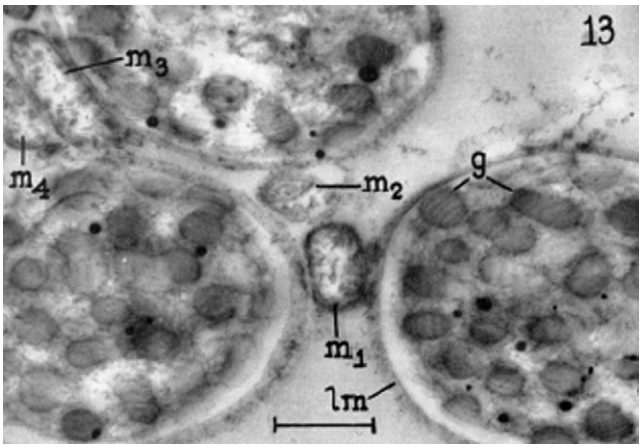


FIGURE 14.2 Thin section of mitochondria in the cytoplasm of a mesophyll cell of tobacco (m_1, m_2, m_3, m_4). The majority of the figure is taken up by chloroplasts that have limiting membranes (lm) and grana (g). (*Source:* From Palade, 1953.)

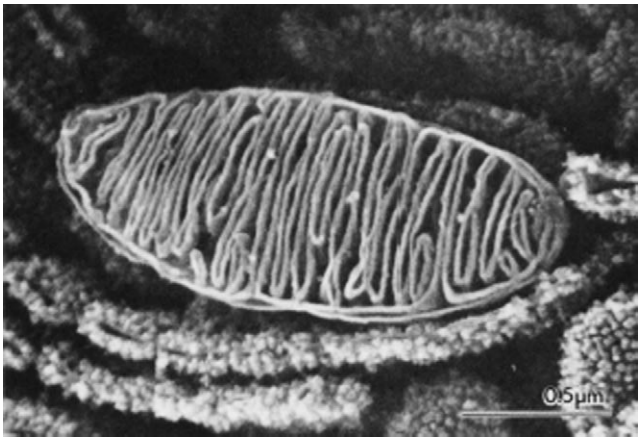


FIGURE 14.3 Scanning electron micrograph of a mitochondrion from a rat pancreatic acinar cell. Note the platelike cristae. (*Source:* From Tanaka, 1987.)

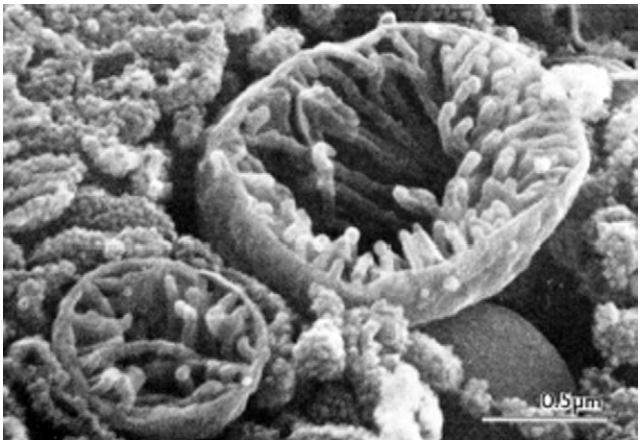


FIGURE 14.4 Scanning electron micrograph of a mitochondrion from a rat pancreatic acinar cell. Note the tubular cristae. (*Source:* From Tanaka, 1987.)

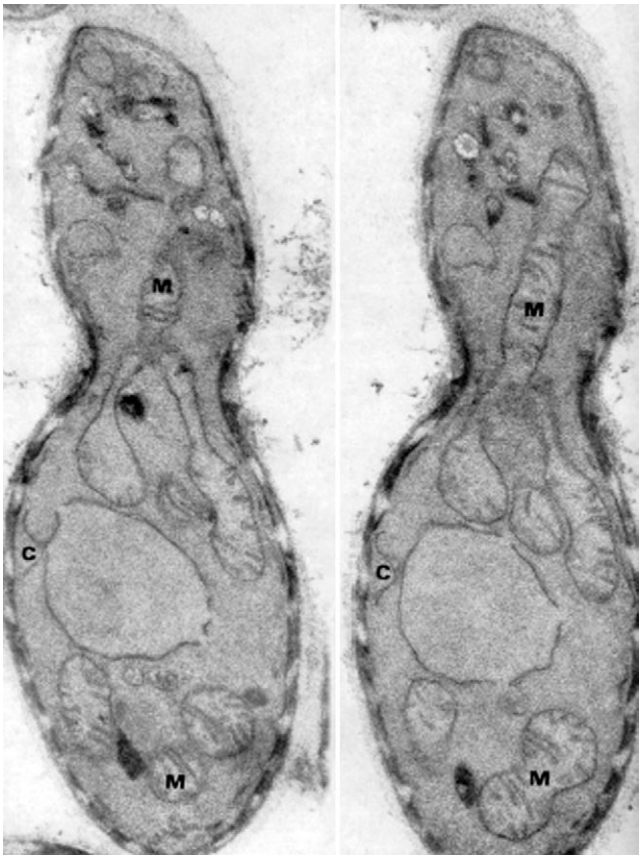


FIGURE 14.5 Two consecutive serial sections of *Pityrosporum* yeast cells that were used to reconstruct the mitochondrial reticulum. C, cytomembranes; M, mitochondrion. (*Source:* From Keddie and Barajas, 1969.) Similar mitochondrial reticula may be found in yeast (Hoffmann and Avers, 1973) and in some root-tip cells of white lupine (Gunning and Steer, 1975).

membrane is approximately 7-nm thick, and the inner membrane, originally called the *cristae mitochondriales*, is approximately 5-nm thick. The two membranes delineate two mitochondrial spaces: the intermembranal space (E-space) and the matrix (P-space). In negatively stained preparations, densely packed, lollipop-like structures can be seen emanating from the P-side of the inner membrane. The lollipop-shaped particles are the F_1F_0-type ATP synthase (Racker, 1965). They have a head that is 9–10 nm in diameter, and a stalk 3.5–4 nm in diameter and 4.5-nm long.

Today, the mitochondria in living cells can be easily observed with vital fluorescent dyes (e.g., Rhodamine 123 and MitoTracker) and green fluorescent protein (GFP; Partikian et al., 1998). Watching mitochondria that have been stained with these dyes show that they are amazingly plastic and mobile organelles (Rosamond, 1987). They appear to fuse, divide, and undergo amoeboid movement as they travel along actin filaments (Grolig, 1990). Vital staining, as well as serial sectioning, shows that mitochondria can even appear as a mitochondrial reticulum (see Figure 14.5; Keddie and Barajas, 1969; Hoffmann and Avers, 1973; Skulachev, 1990). The lengths of mitochondria vary, and the frequency of fusion and division is under genetic control (Kawano et al., 1993). The proteins responsible for mitochondrial shape are being discovered (Youngman et al., 2004).

14.2 ISOLATION OF MITOCHONDRIA

Mitochondria were first isolated from animal cells by Bensley and Hoerr (1934) and from plant cells by Millerd et al. (1951). To isolate mitochondria, the tissues are homogenized, filtered, and centrifuged at 700–1000 g for 10 minutes. The pellet is discarded, and the supernatant is centrifuged at 10,000 g for 20 minutes. The pellet is then resuspended and centrifuged at 75,000–100,000 g for 2–4 hours in a density gradient (Moore and Proudlove, 1988).

Mitochondria have a density of 1.16–1.22 g/ml with a modal value of 1.18 g/ml. The intactness and purity of the isolated mitochondria can be determined with a light microscope after vital staining (Hogeboom et al., 1948) or with an electron microscope. A single mitochondrion can be isolated using laser tweezers (Kuroiwa et al., 1996).

The two mitochondrial membranes can be separated from each other after the mitochondria are lysed in a hypotonic medium. The lysed mitochondria are then layered on top of a discontinuous sucrose gradient and subjected to density-gradient centrifugation. The outer membrane shows up in the 0.3/0.6 M (= 1.04/1.08 g/mL) and 0.6/0.9-M (= 1.08/1.12 g/mL) sucrose interfaces, and the mitoplast—that is, the inner membrane and matrix—is recovered as a pellet below the 1.25 M (= 1.16 g/mL) sucrose layer (Douce, 1985). The matrix can be isolated by osmotically lysing the mitoplasts.

TABLE 14.1 Phospholipid composition (% phospholipid by weight) of sycamore cells and mitochondria

Phospholipid	Cells	Mitochondria
Phosphatidylcholine	47	43
Phosphatidylethanolamine	29	35
Diphosphatidylglycerol[a]	1.8	13
Phosphatidylinositol	16	6
Phosphatidylglycerol	5	3

Source: *From Douce (1985).*
[a]*Also known as cardiolipin.*

14.3 COMPOSITION OF MITOCHONDRIA

The outer membrane of plant mitochondria comprises only 6.8 percent of the mitochondrial protein, while the inner mitochondrial membrane accounts for 30 percent of the total protein. The inner membrane is composed of 60 percent protein and 40 percent lipid on a dry-weight basis, while the outer membrane is composed of 20 percent protein and 80 percent lipid on a dry-weight basis. The outer mitochondrial membrane contains a channel protein called porin that is permeable to molecules that are 10,000 Da or less. It is similar to the pore protein in the outer membrane of the chloroplast envelope.

14.3.1 Proteins

Proteomic analysis shows that the mitochondria are composed of about 3000 proteins (Bykova and Moller, 2006). The proteins used to identify the mitochondria in vitro are those associated with respiration. They include succinate dehydrogenase, cytochrome c oxidase, and fumarase (Hogeboom and Schneider, 1955), as well as several cytochromes, including a, a_3, c, and b_5 (Chance and Williams, 1955). In animal cells, which lack plastids, the enzymes involved in lipid metabolism are present on the outer mitochondrial membrane.

The marker enzymes for the outer membrane are the succinate: cytochrome c oxidoreductase and the NADH: cytochrome c oxidoreductase. The marker enzymes for the inner mitochondrial membrane are malate dehydrogenase and succinate: $K_3Fe(CN)_6$ oxidoreductase.

14.3.2 Lipids

In contrast to the galactolipid-rich chloroplasts, the mitochondria are rich in phospholipids (23–27% by weight). Phospholipids account for as much as 90 percent of the total mitochondrial lipids. Mitochondria contain high concentrations of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as well as diphosphatidylglycerol (DPG = cardiolipin), a lipid that is endemic to mitochondria (Table 14.1). Cardiolipin is also found in the plasma membrane of bacteria, giving support for the hypothesis that mitochondria evolved from bacteria (see Chapter 15).

The lipid compositions of the inner and outer membranes of the mitochondria are distinct. In particular, the inner membrane contains cardiolipin, which is absent from the outer membrane. The inner membrane is also richer in PE and poorer in PC relative to the outer membrane (Table 14.2). The hydrocarbons derived from fatty acids in the outer membrane are slightly more saturated than the hydrocarbons derived from fatty acids in the inner membrane. The phospholipids of plant mitochondria are much less saturated than those from mammalian mitochondria, which exist at 37°C.

14.4 CELLULAR GEOGRAPHY OF MITOCHONDRIA

There are usually hundreds to thousands of mitochondria in a typical plant cell, although they may form a single mitochondrial reticulum (Keddie and Barajas, 1969; Hoffman and Avers, 1973; Skulachev 1990). In sycamore cells, the 250 mitochondria take up 0.7 percent of the protoplast or 7 percent of the cytoplasm (Douce, 1985). By contrast, mitochondria in mammalian liver cells take up to 20 percent of the volume. Mitochondria are typically not randomly arranged, but are often positioned close to the site of carbohydrate storage or ATP utilization. In leaves, mitochondria are often situated next to peroxisomes and chloroplasts; the three organelles in this case function together in the process of photorespiration and carbon recycling. In onion epidermal cells,

TABLE 14.2 Phospholipid composition (% phospholipid by weight) of inner and outer membranes of sycamore and mung bean mitochondria

	Sycamore cells		Mung bean cells	
Lipid	**IM**	**OM**	**IM**	**OM**
Phosphatidylcholine	41	54	29	68
Phosphatidylethanolamine	37	30	50	24
Diphosphatidylglycerol[a]	14.5	0	17	0
Phosphatidylinositol	5	11	2	5
Phosphatidylglycerol	2.5	4.5	1	2

IM, inner membrane; OM, outer membrane.
Source: *From Douce (1985).*

the mitochondria are typically clustered around the nucleus. In plant sperm, the spiral-shaped nucleus is surrounded by a single mitochondrion.

14.5 CHEMICAL FOUNDATION OF RESPIRATION

The lowest-energy form of carbon and hydrogen are the most oxidized states, CO_2 and H_2O, respectively. Reduced-carbon compounds, in the forms of carbohydrate, fats, and proteins, have a certain amount of energy that can be utilized for cellular energy following their oxidation. As Hans Weber (1958) wrote,

> *How is it possible that living creatures are able to utilize invariably the same amount of energy in performing vital work, when they derive this energy from such widely different foodstuffs as protein, carbohydrates, and fats? In order to realize how remarkable this is we need only imagine a diesel engine—a simple structure compared with the living cell—obtaining the necessary energy by consuming in turn gas, diesel oil, coal, or powdered milk.*

While the thermodynamists of the 19th century believed that foodstuffs energized organisms through the production of thermal energy (Grove et al., 1867; Guye, n.d.) or by creating an electrical intermediate (Thomson and Tait, 1862), the energy released in the oxidation of all these compounds is actually conserved in the synthesis of ATP, a molecule that is chemically unrelated to fats, carbohydrates, and lipids, which are the foodstuffs, but is related instead to the nucleic acids.

14.5.1 Fitness of ATP as a Chemical Energy Transducer

Why is ATP the universal energy source? It must have something to do with its chemical properties. Fritz Lipmann (1941) declared that ATP would turn out to be the universal energy currency by virtue of its large enthalpy and designated the bond that bound the terminal phosphate as the "high-energy bond," denoted by "the squiggle." By contrast, Herman Kalckar (1941, 1942) suggested that ATP could serve as the universal energy currency, because of the many resonant structures in which a phosphate molecule could exist. That is, he suggested that entropy was the dominant factor in the selection of ATP as the universal energy currency. Lipmann's view gained widespread acceptance because of its simplicity, yet Kalckar's idea was closer to the truth. I will briefly discuss some of the concepts necessary to understand Kalckar's view.

The molecular free energy of a reaction is a function of the concentrations of the products and reactants. When water takes part in the reaction, the concentration of water must be factored into the equation. In almost all cases, water has been neglected, and thus the published values of dehydration and hydrolytic reactions may be incorrect by a factor of four (= ln 55.5) and should be redetermined (Oesper, 1950).

Free Energy, Enthalpy, and Entropy

The change in molecular free energy that occurs during a reaction (ΔE) is equal to $\Delta H/N_A - T\Delta S/N_A$, and thus the molecular free energy of a reaction depends on both the change in enthalpy and the change in entropy. The change in enthalpy is defined as the ratio of the change in heat of a reaction at constant pressure to the temperature. At equilibrium, $\Delta H/N_A - T\Delta S/N_A = 0$. Reactions that have a negative change in molecular free energy ($\Delta H/N_A - T\Delta S/N_A < 0$) occur spontaneously and are called exergonic. Those that have a positive change in molecular free energy ($\Delta H/N_A - T\Delta S/N_A > 0$) do not occur spontaneously and are called endergonic (Coryell, 1940).

Misconceptions of molecular free energy come, in part, from the fact that enthalpy is often equated with free energy. When enthalpy is equated with free energy, it is assumed that reactions will proceed spontaneously if they are exothermic, and will require energy if they are endothermic. If this were true, ice could not spontaneously melt. Thus, we must take into consideration the entropy of the reaction. Then, if the enthalpy term is positive (endothermic) and the entropy term ($T\Delta S/N_A$) is both positive and greater in magnitude than the enthalpy term, the reaction will be exergonic, and will proceed spontaneously. Likewise, if a reaction is exothermic, it may be endergonic if the entropy term is large and negative.

Typically, the combination of small molecules to make larger molecules requires the addition of free energy. This is because many biosynthetic reactions are dehydrations, and consequently will not proceed spontaneously in the aqueous cellular environment (see Chapter 18). As I will discuss in the following, the molecular free energy made available by the hydrolysis of ATP provides the free energy necessary for these biosynthetic reactions. In order to appreciate this function of ATP, let us study the chemistry of a typical dehydration reaction. Consider the reaction where two carboxylic acids are joined to make an acid anhydride:

$$RCOOH + HOOCR' \Leftrightarrow RCO\text{-}OOCR' + H_2O$$

Due to the high concentration of water in the cell, this reaction tends to go to the left. At equilibrium, the concentration of the carboxylic acids are high and the concentration of the anhydride is low. The reaction also proceeds to the left because the carboxylic acids each have two resonance structures, which tend to stabilize them (Lewis, 1923; Pauling, 1940). The electrons are not as localized in resonating structures as they are in a structure that only has one possible configuration, thus the probability of localizing an electron is low and the entropy of a resonating structure is high. The resonance energy of the carboxylic acid group reduces the overall energy of a molecule by approximately 10^{-19} J, and thus molecules with resonant structures are more stable than they would be if resonance did not occur (Oesper, 1950). In fact, the delocalized nature of the electrons makes it so that organic acids, including acetic acid and fatty acids, cannot enter directly into chemical reactions, but must first be activated.

When two resonating structures are joined, the number of possible resonant structures is diminished, which results in a decrease in the entropy of the product. Thus, in the reaction just shown, the reactants have a lowered free energy due to resonance stabilization (Kalckar, 1942; Dugas, 1996). Given the chemical properties of molecules involved in dehydration reactions, an input of molecular free energy, in the form of ATP, is required to absorb the water molecule and drive these reactions in a cell.

Resonance Structures of Phosphates

Inorganic phosphate has 29 resonance structures (see Figure 14.6; Oesper, 1950). Due to the stabilizing resonance energy of phosphate, the reaction of a phosphate and a carboxylic acid to make a mixed anhydride will tend to proceed to the left spontaneously when placed in water (Lehninger, 1959; Green and Baum, 1970; Becker, 1977):

$$RCOOH + PO_3OH \Leftrightarrow RCOOPO_3 + H_2O$$

Reactions resulting in the formation of polyphosphoanhydrides, including ADP and ATP, also tend to proceed to the left:

$$ADP\text{-}OH + PO_3OH \Leftrightarrow ATP + H_2O$$
$$AMP\text{-}OH + PO_3OH \Leftrightarrow ADP + H_2O$$

These reactions tend to go to the left because the reactants are stabilized by resonance stabilization, and in addition, there is an electrostatic repulsion between the phosphates in the polyphosphate in the compound with the greater number of phosphates. The resonance stabilization accounts for about 70 percent of the change in free energy, and the electrostatic repulsion accounts for about 30 percent (Hill and Morales, 1950, 1951).

It is possible to synthesize almost any molecule in vitro by taking advantage of the Law of Mass Action and increasing the concentrations of the reactants. However, in the cell, this is usually not a viable option for highly endergonic (i.e., irreversible) reactions. In cells, highly endergonic reactions proceed because they are coupled to highly exergonic ones, with no net change in the water concentration.

$RCOOH + PO_3OH \Leftrightarrow RCOOPO_3 + H_2O$	Highly endergonic
$ATP + H_2O \Leftrightarrow PO_3OH + ADP$	Highly exergonic
$RCOOH + ATP \Leftrightarrow RCOOPO_3 + ADP$	Slightly exergonic

Universally, the exergonic reaction used for energetic coupling is the reaction involved in the hydrolysis of ATP. It is, in part, the chemical nature of phosphate that allows it to perform this function. The hydrolysis reaction is given in the following formula:

$$ATP + H_2O \Leftrightarrow ADP + P_i$$

The constant of the reaction, which relates the concentrations of products to the concentrations of reactants, depends on the circumstances. At equilibrium:

$$K_{eq} = [ADP]_{eq}[P_i]_{eq}/([ATP]_{eq}[H_2O]_{eq}) \quad (14.1)$$

In the standard state:

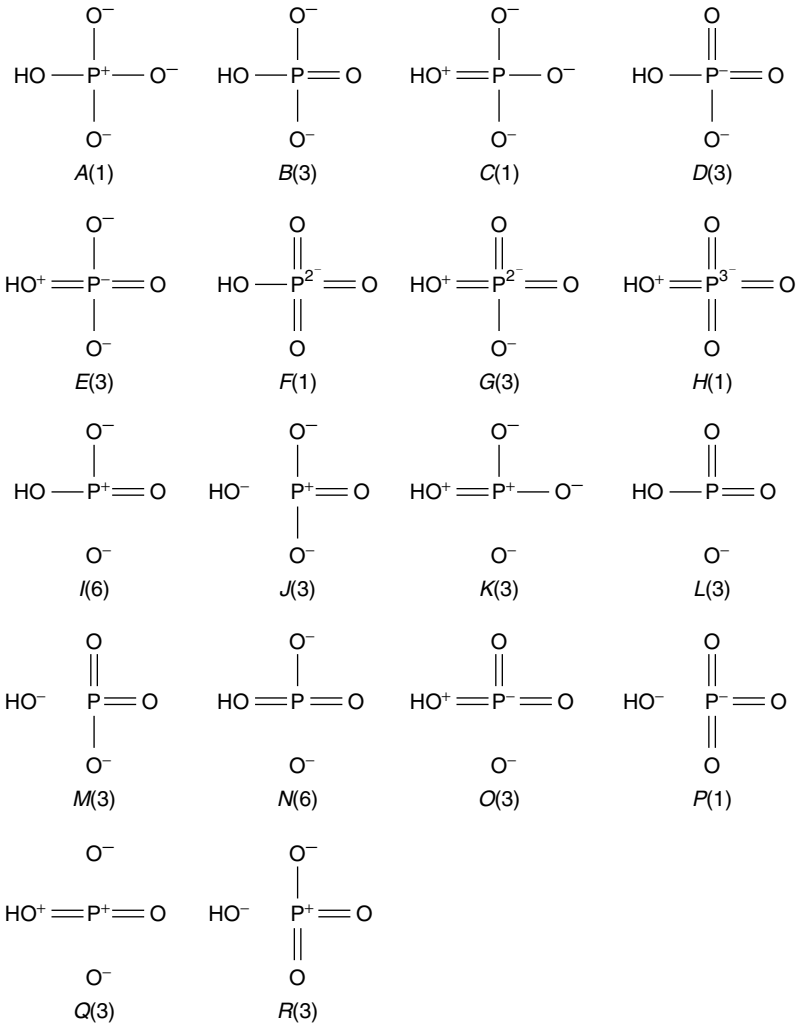


FIGURE 14.6 Resonant structures of phosphate. (*Source:* From Oesper, 1950.)

$$K_{\text{std}} = [\text{ADP}]_{\text{std}}[\text{P}_\text{i}]_{\text{std}}/([\text{ATP}]_{\text{std}}[\text{H}_2\text{O}]_{\text{std}}) = (1\text{ M})(1\text{ M})/((1\text{ M})(55.5\text{ M})) = 1.8 \times 10^{-2} \quad (14.2)$$

Under cellular conditions:

$$K_{\text{Real}} = [\text{ADP}]_{\text{Real}}[\text{P}_\text{i}]_{\text{Real}}/([\text{ATP}]_{\text{Real}}[\text{H}_2\text{O}]_{\text{Real}}) = (10^{-3}\text{ M})(10^{-2}\text{ M})/((10^{-3}\text{ M})(55.5\text{ M})) = 1.8 \times 10^{-4} \quad (14.3)$$

As a function of the chemistry of P_i, the equilibrium constant for the hydrolysis of ATP is large (Burton, 1958). That is, given the equilibrium and standard-state concentrations of ATP, ADP, P_i, and H_2O, K_{eq} for the following reaction is approximately 5.11×10^4. K_{eq} can be calculated by finding values for ΔG° at 25 C, pH 7.4, and 1 mM Mg^{2+} in the *Handbook of Biochemistry and Molecular Biology*, 3rd Edition (Boca Raton, FL: CRC Press, 1989), p. 302.

$$\Delta G^\circ = -8.8\text{ kcal/mol} = -3.668 \times 10^4\text{ J/mol}$$

Thus:

$$E_{\text{std}} = \Delta G^\circ/N_A = -6.11 \times 10^{-20}\text{ J} \quad (14.4)$$

K_{eq} can be calculated from the following equation:

$$E_{\text{eq}} - E_{\text{std}} = kT\ln(K_{\text{eq}}/K_{\text{std}}) \quad (14.5)$$

Since at equilibrium, $E_{eq} = 0$, then

$$E_{\text{std}} = -kT\ln(K_{\text{eq}}/K_{\text{std}}) \quad (14.6)$$

and

$$K_{\text{eq}} = 5.11 \times 10^4$$

The free energy that is made available by the hydrolysis of ATP in the cell depends on how far the real ratio of $[\text{ADP}][\text{P}_\text{i}]/[\text{ATP}][\text{H}_2\text{O}]$ is from the equilibrium ratio. The molecular free energy is given by the following equation:

$$E_{eq} - E_{Real} = kT \ln (K_{eq}/K_{Real}) \quad (14.7)$$

As shown by Tetsuro Mimura and his colleagues, in the living cell, the concentrations of ADP, P_i, ATP, and H_2O are far from equilibrium. They are typically around 10^{-3} M, 10^{-2} M, 10^{-3} M, and 55.5 M, respectively. Thus, K_{Real} for the hydrolysis reaction is about 1.8×10^{-4}, and according to Eq. 14.7, the molecular free energy made available by the hydrolysis of ATP is about 8×10^{-20} J.

The large E_{Real} is a result of two things: The ATP concentration in the cell is much greater than it would be at equilibrium, and at equilibrium, the ATP concentration is very low due to the resonance stabilization of phosphate ions. The resonance stabilization accounts for 70 percent of the free energy of hydrolysis as ATP, ADP, P_i, and H_2O go from the standard state to equilibrium.

Consider the case where there is no resonance stabilization. That is, pretend that resonance and entropy are not important. Then,

$$E_{std}(w/o) = 0.3E_{std} \quad (14.8)$$

$$\begin{aligned} E_{std}(w/o) &= 0.3(-6.11 \times 10^{-20}\ \text{J}) \\ &= -1.83 \times 10^{-20}\ \text{J} \end{aligned} \quad (14.9)$$

At equilibrium, $E_{eq}(w/o) = 0$, and

$$\begin{aligned} E_{eq}(w/o) &= -E_{std}(w/o) \\ &= kT \ln (K_{eq}(w/o)/K_{std}(w/o)) \end{aligned} \quad (14.10)$$

Thus:

$$E_{std}(w/o) = -kT \ln (K_{eq}(w/o)/K_{std}(w/o)) \quad (14.11)$$

Using $E_{std}\ (w/o) = -1.83 \times 10^{-20}$ J, and given that $K_{std}(w/o) = K_{std}$, we can solve Eq. 14.11 to get $K_{eq}(w/o) = 1.54$. With this value we can now calculate what the real molecular free energy made available by the hydrolysis of ATP would be if there were no resonance stabilization.

$$\begin{aligned} &E_{eq}(w/o) - E_{Real}(w/o) = \\ &kT \ln (K_{eq}(w/o)/K_{Real}(w/o)) \end{aligned} \quad (14.12)$$

At equilibrium, $E_{eq}(w/o) = 0$, and

$$E_{Real}(w/o) = -kT \ln (K_{eq}(w/o)/K_{Real}(w/o)) \quad (14.13)$$

Given that $K_{Real}(w/o) = K_{Real}$, and $K_{eq}(w/o) = 1.54$, we can use Eq. 14.13 that $E_{Real}(w/o) = -3.72 \times 10^{-20}$ J. Thus, in the absence of resonance energy, the energy made available by the hydrolysis of ATP would be significantly lower. Even if there were no resonance, the free energy of ATP hydrolysis could be increased to -8×10^{-20} J by raising the concentration of ATP in the cell or lowering the concentration of ADP and/or P_i.

What would $K_{Real}(w/o)$ have to become to increase the energy of hydrolysis of ATP to -8×10^{-20} J in the absence of resonance? Using Eq. 14.12, and assuming $K_{eq}(w/o) = 1.54$, then $K_{Real}(w/o)$ would have to be 5.5×10^{-9}. Using Eq. 14.3, we find that if [ADP] = 10^{-3} M, [P_i] = 10^{-2} M, and [H_2O] = 55.5 M, then the [ATP] would have to increase to 32.76 M. Likewise, if [ATP] remained at 10^{-3} M, the product of [ADP] and [P_i] would have to decrease to 3.05×10^{-10} M.

Thanks to the resonance stabilization of P_i, the hydrolysis of ATP can yield 8×10^{-20} J of energy when [ATP] = 10^{-3} M, [ADP] = 10^{-3} M, and [P_i] = 10^{-2} M.

The concept introduced by Fritz Lipmann (1941) that the phosphoanhydride bond of ATP is a high-energy bond is misleading because it does not take into consideration the resonance stabilization energy. When this is taken into consideration, the phosphoanhydride bond is actually a low-energy bond, and this is the reason it breaks easily (Gillespie et al., 1953).

Due to the dependence of the free energy on both K_{Real} and K_{eq}, other phosphate-containing molecules (e.g., glucose-6-P) would not be as good an energy-transducing molecule as ATP. This is because K_{eq} for glucose-6-P hydrolysis is about 2.6×10^2, approximately 0.1 percent of the equilibrium constant for the hydrolysis of ATP. Thus, if glucose-6-P served as the universal energy intermediate in the cell, the ratio of [glucose][P_i]/[glucose-6-P][H_2O] would have to be 1.3×10^{-7} in order for the hydrolysis of glucose-6-P to release as much energy as the hydrolysis of ATP. That means if the glucose-6-P concentration were 10^{-3} M, a value typical for substrates of energetic reactions, the product of the glucose and P_i concentrations would have to be 7.2×10^{-9} M, too low for glucose to act as an efficient foodstuff. If the product of the glucose and P_i concentrations were 10^{-4} M, the concentration of glucose-6-P would have to be 13.9 M in order to act as a chemical energy transducer. For this reason, it is best not to have one of the carbohydrate intermediates in the glycolytic pathway as the chemical energy transducer. Likewise, it is likely that if the chemical energy–transducing molecule were a component of lipids or protein, the other two most abundant classes of macromolecules in the cell, it too would wreak havoc with the metabolism of these foodstuffs. This may be the reason that a nucleotide became the chemical energy–transducing molecule. Why adenosine is the universal chemical energy–transducing molecule, but the other nucleotides are not, remains a mystery.

The Principle of the Common Intermediate

The free energy released by the hydrolysis of ATP would be useless if it could not be coupled to endergonic chemical reactions. This energy cannot be used in the form of heat, but can only be coupled if there is a transfer of mass between the ATP and a reactant of an endergonic reaction.

This means that there must be a common intermediate in the endergonic and exergonic reaction. This is known as "the principle of the common intermediate." The reaction,

$$\text{Glucose} + P_i \Leftrightarrow \text{Glucose-6-P} + H_2O$$

is endergonic, and the molecular free energy required for the reaction to go to the right is 2.3×10^{-20} J. The hydrolysis of ATP, by contrast, is exergonic and proceeds with a release of free energy of 8.0×10^{-20} J. Thus, the following reactions will proceed simultaneously with a net release of molecular free energy of 5.7×10^{-20} J, if the ATP and glucose are in the same cellular compartment.

$$\text{Glucose} + P_i \Leftrightarrow \text{Glucose-6-P} + H_2O$$
$$ATP + H_2O \Leftrightarrow ADP + P_i$$

The terminal phosphate of ATP will be transferred to the glucose in a reaction catalyzed by hexokinase so that the net reaction is as follows:

$$\text{Glucose} + ATP \Leftrightarrow \text{Glucose-6-P} + ADP$$

In this way, the molecular free energy released by the hydrolysis of ATP can be used to provide the molecular free energy to drive energetically unfavorable reactions.

14.5.2 Glycolysis

The majority of the ATP necessary to drive endergonic reactions is synthesized in the mitochondria. However, since energy cannot be created or destroyed, the synthesis of ATP in the mitochondria requires the input of material. In general, the input of material comes from the glycolytic reactions that occur in the cytosol. Louis Pasteur (1879), while studying wine and beer making, discovered that the breakdown of sugar, known as fermentation, was a general property of all living cells (Fruton, 2006). However, he could only detect fermentation in cells deprived of oxygen, since cells deprived of oxygen metabolize more sugar.

The hydrolysis of glucose is an extremely exergonic reaction. However, it does not occur spontaneously, even though carbon dioxide and water are thermodynamically more stable than glucose and O_2. Glucose is kinetically stable even though it is thermodynamically unstable (Williams, 1961). Thus, while thermodynamics tells us about the possibility of a reaction, it does not tell us anything about its rate (Lewis and Randall, 1923; Glasstone et al., 1941). Thus, glucose will only break down at room temperature in the presence of glycolytic enzymes and/or activated oxygen. An enzyme decreases the activation energy of a reaction. That is, it increases the rate of a reaction by increasing the proportion of substrate molecules capable of entering it (Mehler, 1957).

The Discovery of the Glycolytic Pathway

While Justis von Liebig and Marcelin Berthelot were trying to eliminate the need for calling on "the vital force" for explaining biological reactions (anonymous, 1839), Louis Pasteur (1879) was unable to get ground-up yeast to ferment a sugar solution, and declared that fermentation was a vital action that required living cells (Duclaux, 1920). However, Eduard Buchner (1897) using a German beer yeast instead of a French wine yeast was able to obtain fermentation in vitro when he added sugar to a yeast extract. Actually, this was a lucky find, since Buchner had no interest in glycolysis. He was making a health tonic, and only added the sugar as a preservative when the other antiseptics failed to keep the extract sterile (Harden, 1932). Buchner named the extract zymase, from *zyme*, the Greek word for yeast, and *diastasis*, the Greek word for making a breach. Willy Kühne named all biocatalysts, enzymes, from the Greek words *en zyme*, which mean in yeast.

Unlike the burning of wood, which only takes place at high temperatures, the oxidation of glucose at ambient temperatures depends on the intervention of enzymes and takes place in about a dozen steps. This leads to the production of many intermediates, and thus cellular respiration is also known as intermediary metabolism. It was astonishing for those biochemists who were working on either the study of fermentation in yeast and bacteria or the study of glycolysis in muscle to find that these two seemingly diverse processes taking place in two different kingdoms were, except for the last steps, identical (Pasteur, 1879; Kluyver, 1931; Plaxton, 1996).

The studies of glycolysis and fermentation opened up an entirely new way of doing "biochemistry." Prior to these studies, most chemists felt that biochemistry should be a "super-chemistry" and chemicals should be isolated, but attempts to define their relationship in the cell were rebuked with the words "More matter with less art." Feeling that it was time to say "Enough matter, more art," biochemists, including Arthur Harden (1932), Jan Kluyver (1931), Gustav Embden (Embden and Laquer, 1914), Otto Myerhof (1924), and Jacob Parnas (1910), began to make sense of the copious crowd of newly discovered chemicals that comprised "the chemical zoo" by elucidating the sequence of reactions that occur in living cells.

How could they determine the components and sequence of the pathway in the days before radioactive isotopes and genetic engineering? First, they found inhibitors of CO_2 evolution, including fluoride, and iodoacetate. Then, with classical organic chemical means, they determined which intermediates increased after treatment with the inhibitor. Iodoacetate causes the accumulation of fructose 1,6-bisphosphate, and fluoride causes the accumulation of 2-phosphoglycerate and 3-phosphoglycerate. The compounds were isolated and analyzed by conventional organic analysis (e.g., combustion and enzyme degradation) and volumetric analysis using the manometer developed by Warburg (Dixon, 1952). The chemical of which the concentration increased, after the cells or extract were treated with

an inhibitor, was presumably the intermediate produced just prior to the step blocked by the inhibitor. The scientists working on glycolysis also added putative intermediates to the cells or extracts to see if they stimulated CO_2 evolution. If they did, they were presumably intermediates in the glycolytic pathway. However, not all presumptive intermediates of glucose oxidation turned out to be part of the glycolytic pathway, but part of a parallel pathway, known as the oxidative pentose phosphate pathway (Racker, 1957; Horecker, 1982). This pathway is involved in the synthesis of ribose, an important substrate in the formation of nucleotides (Warburg and Christian, 1931, 1936; Hutchinson, 1964). Once the intermediates were discovered, it became possible to identify the enzymes that utilized and produced them.

The evolution of CO_2 from yeast extracts was not constant, but slowed down over time. Harden and Young (1906) found that the addition of phosphate maintained a high rate of CO_2 evolution. Thus, they guessed that phosphate must combine with glucose, and soon after fructose 1,6-bisphosphate, fructose-6-phosphate, and glucose-6-phosphate were isolated. It was also established that creatine was phosphorylated to creatine phosphate, and ADP was phosphorylated to ATP during glycolysis. From these data, the concept of coupled reactions began to emerge (Myerhof and Lohmann, 1928; Fiske and Subbarow, 1929; Lohmann, 1929; Lehmann, 1935; Myerhof, 1944).

Around the same time, Lundsgaard (1938) discovered that iodoacetic acid, an agent that inhibits glycolysis, has no qualitative effect on muscle contraction. This indicated that a store of energy exists in muscle cells. Lundsgaard noticed that in the poisoned cells, creatine phosphate is broken down at a faster rate than in the normal cells, and proposed that the energy necessary for muscle contraction resulted from the hydrolysis of creatine phosphate. However, with the realization that creatine phosphate does not occur in all cells, while another phosphoanhydride, ATP, does, it seemed likely that ATP may be the universal energy source (Kalckar, 1941, 1942, 1944, 1966; Lipmann, 1941). Kalckar and Lipmann independently concluded that the hydrolysis of ATP provided the molecular free energy necessary to perform all kinds of cellular work, including biosynthetic work, osmotic work, and mechanical work.

Metabolic Channeling in Glycolysis

Much of what we know about enzymes, coenzymes, and biochemical pathways came from the study of glycolysis by luminaries such as the Büchners, Otto Warburg, and Otto Myerhof. Although the glycolytic enzymes can be isolated, purified, and even crystallized, they may not be in soluble form in the cell. In fact, these enzymes are part of the cytomatrix, and consequently even detergent-extracted cells are able to oxidize glucose to pyruvic acid (Schliwa et al., 1987). The fact that some of the glycolytic enzymes are immobilized on the cytoskeleton has been established by FRAP studies (Walsh and Knull, 1987; Pagliaro and Taylor, 1988, 1992; Wang et al., 1996; Azama et al., 2003). Giegé et al. (2003) have shown that in *Arabidopsis*, 3–12 percent of the activities of the glycolytic enzymes are associated with the outer membrane of mitochondria. Clegg (1991) has shown that ^{12}C-glycolytic intermediates do not reduce the amount of $^{14}CO_2$ given off by cells supplied with ^{14}C-glucose, indicating that there is a diffusional barrier between the glycolytic intermediates produced endogenously by the glycolytic pathway, and the intermediates supplied exogenously. All these data are consistent with the concept of metabolic channeling, although this interpretation is not widely held (Ovádi, 1991; Kühn-Velten, 1993; Mathews, 1993; Cascante et al., 1994).

14.5.3 Cellular Respiration

The breakdown of glucose and the formation of two pyruvic acid molecules during glycolysis require nine phosphorylated intermediates, 10 enzymes, and two nucleotide coenzymes, NAD^+ and ATP (see Figure 14.7; Cori, 1942; Myerhof, 1942; Michal, 1999). The net result of these reactions is the production of two molecules of ATP and two molecules of NADH for every glucose molecule oxidized. The production of two ATPs results in the conversion of only a few percent of the chemical energy available from the combustion of a glucose molecule to the chemical energy available from the hydrolysis of ATP. Most of the energy of glucose is still contained in the two pyruvic acid molecules.

The pyruvic acid must leave the cytosol and enter the matrix of the mitochondria in order to generate more ATP (Ochoa, 1943; Bonner and Millerd, 1953; Millerd and Bonner, 1953). Here, the pyruvic acid encounters the pyruvate dehydrogenase complex, which is 20–25 nm in diameter. This complex oxidizes pyruvic acid to acetyl-CoA, and produces one NADH and one CO_2 from each pyruvic acid molecule. The pyruvate dehydrogenase complex requires a variety of "vitamins," including FAD, NAD, CoA, thiamin diphosphate, and lipoic acid (1,2-dithiolane-3-valeric acid; Reed, 2001). Acetyl CoA is the "activated" form of acetic acid (Lipmann, 1971).

Other pathways besides glycolysis enter and leave the Krebs cycle. One such pathway is involved in the breakdown of fats, which results in the formation of acetyl CoA, as shown by Lynen. Moreover, amino acids, the breakdown products of proteins, also enter the Krebs cycle following their deamination.

Biochemistry of the Krebs Cycle

In the matrix of the mitochondrion, acetyl-CoA combines with oxaloacetic acid to form citric acid (Figure 14.8). This step is catalyzed by citrate synthase. The citric acid is then oxidized to isocitric acid in a reaction catalyzed by aconitase. The isocitric acid is further oxidized to α-ketoglutaric acid

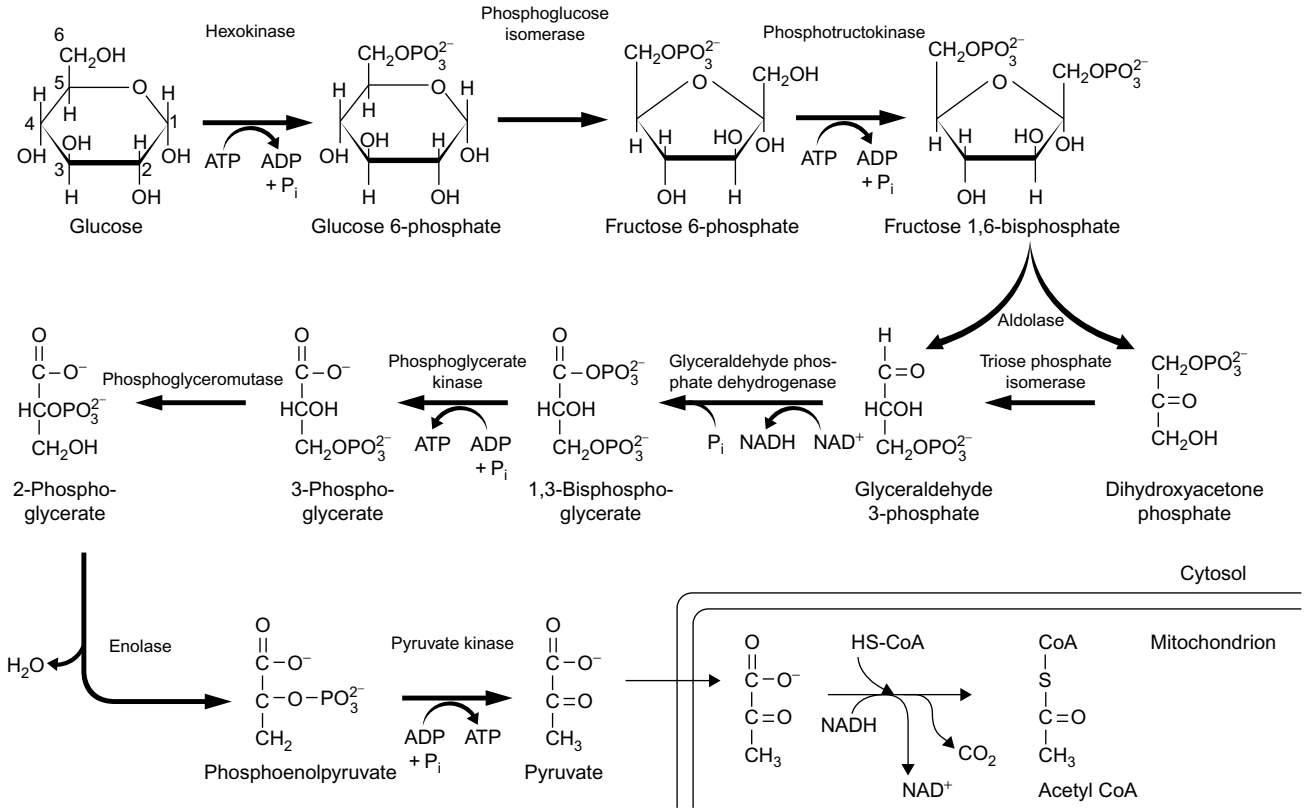


FIGURE 14.7 The glycolytic pathway.

by isocitrate dehydrogenase. This reaction releases CO_2 and forms one molecule of NADH per acetyl-CoA (Krebs, 1950).

The α-ketoglutaric acid then forms succinyl-CoA in a reaction catalyzed by α-ketoglutarate dehydrogenase. This reaction also releases a CO_2 and forms one molecule of NADH per acetyl-CoA. The succinyl-CoA then forms succinic acid in the presence of succinyl-CoA synthetase. This reaction causes the formation of one molecule of ATP per acetyl-CoA. The succinic acid then is converted to fumaric acid by succinic dehydrogenase, a membrane-bound enzyme (Borsook and Schott, 1931; Hogeboom, 1946; Glock and Jensen, 1953; Neufeld et al., 1954; Kearney and Singer, 1956; Avron and Biale, 1957). This reaction causes the formation of one FADH from each molecule of acetyl-CoA. The fumaric acid is then converted into malic acid by the enzyme fumarase. The malic acid is then converted to oxaloacetic acid by the enzyme malate dehydrogenase. This reaction yields one NADH per acetyl-CoA. The oxaloacetic acid is then ready to combine with acetyl-CoA to undergo another round of the cycle. With each round of the cycle, acetyl-CoA is broken down into two CO_2 molecules, three NADH molecules, one FADH molecule, and one ATP. Since two acetyl molecules are derived from each glucose, four CO_2, six NADH, two FADH, and two ATP are produced in the citric acid cycle for each glucose molecule oxidized (Michal, 1999).

Including the one NADH and one CO_2 formed per pyruvate by the pyruvate dehydrogenase complex, a total of six CO_2, eight NADH, two FADH, and two ATP are produced per glucose molecule in the mitochondrion. If we add the two NADH and two ATP formed from each glucose molecule in the cytosol during glycolysis, there is a net yield of six CO_2, 10 NADH, two FADH, and four ATP from each glucose molecule. Only a small percentage of the free energy made available by the oxidation of glucose is conserved in the form of ATP. There is still a lot of energy contained in the reduced bonds of NADH and FADH. Albert Lehninger and his colleagues (Friedkin and Lehninger, 1949; Lehninger, 1949) discovered that the energy conserved in the NADH molecule could be used to produce ATP in the electron transport chain.

Biochemistry of the Electron Transport Chain

The inner mitochondrial membrane contains five kinds of detergent-soluble complexes that are involved in electron transport and ATP synthesis (see Figure 14.9; Michal, 1999):

- Complex I contains NADH-coenzyme Q reductase.
- Complex II contains succinic acid-coenzyme Q reductase.

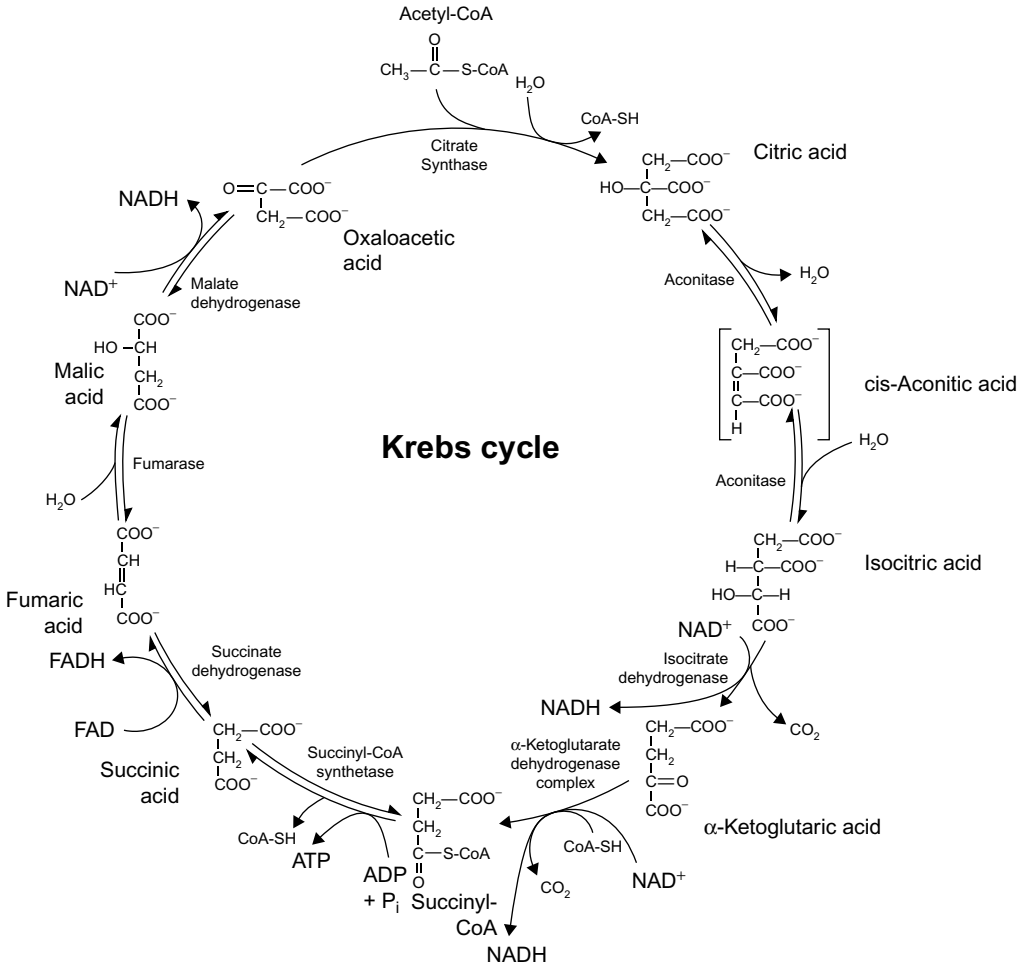


FIGURE 14.8 The Krebs cycle.

- Complex III contains coenzyme QH_2-cytochrome c reductase.
- Complex IV contains cytochrome oxidase.
- Complex V is the ATP synthase.

Complex I is the entry point where NADH produced in the matrix enters the electron transport chain. Complex I is responsible for the transfer of electrons from NADH to ubiquinone. Complex I has a molecular mass of 907 kDa and contains a noncovalently bound flavin mononucleotide (FMN), several iron-sulfur centers, and probably two molecules of ubiquinone, also known as coenzyme Q. This complex spans the membrane; the NADH-binding site is on the matrix side, and the ubiquinone-binding site is in the membrane. The ubiquinone picks up approximately two H^+ for every two electrons it carries and transports them from the matrix to the intermembranal space. The redox potential of the NAD^+/NADH that donates electrons to the complex is −0.32 V, and the redox potential of the ubiquinone is 0 V (Green and Baum, 1970). Thus, the drop across this complex is 0.32 V, and 5.12×10^{-20} J of molecular free energy becomes available to do work for every electron transported (Figure 14.10).

Complex II is the site where FADH, which is produced during the conversion of succinic acid to fumaric acid, enters the electron transport chain (Chance, 1952a,b). Complex II is responsible for the transfer of electrons from FADH to ubiquinone. It contains several non-heme iron centers and has a molecular mass of 130 kDa. This complex is on the matrix side of the membrane and does not translocate protons. The ubiquinone site is in the membrane. The redox potential of the FAD/FADH that donates electrons to the complex is −0.22 V, and the redox potential of the ubiquinone is 0 V. Thus, the drop across this complex is 0.22 V, and 3.52×10^{-20} J of molecular free energy becomes available to do work for every electron transported.

Complex III is responsible for the transfer of electrons from ubiquinone to cytochrome c. The complex contains cytochromes b, c_1, iron-sulfur centers, and ubiquinones. It has a molecular mass of 248 kDa. This complex spans the membrane. The cytochrome c_1 and Fe-S protein are on the intermembranal side. Cytochrome b is in the membrane.

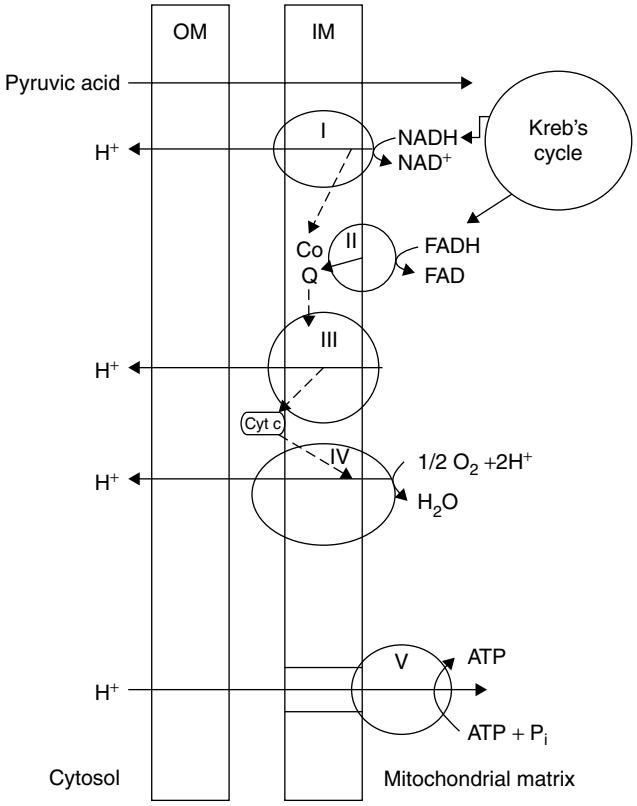


FIGURE 14.9 The inner membrane of the mitochondria showing the topology of the complexes involved in electron transport and ATP synthesis.

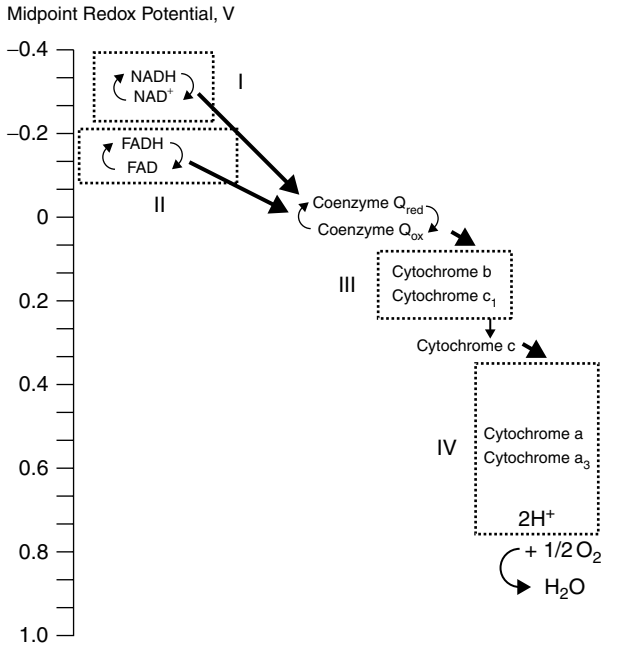


FIGURE 14.10 The estimated midpoint redox potentials of the electron carriers involved in respiration.

This complex translocates $2H^+$ per $2e^-$. Cytochrome c is a water-soluble protein that rapidly transfers electrons from complex III to complex IV. The redox potential of the ubiquinone that donates electrons to the complex is 0 V, and the redox potential of cytochrome c is 0.26 V. Thus, the drop across this complex is 0.26 V, and 4.16×10^{-20} J of molecular free energy becomes available to do work for every electron transported.

Plant mitochondria are able to oxidize cytosolic NADH directly, unlike animal mitochondria. There is an NADH dehydrogenase on the outer surface of the inner membrane that is specific for the β-hydrogen of NADH and feeds electrons directly to complex III. As I will discuss in the following, the oxidation of one molecule of cytosolic NADH then leads to the production of two molecules of ATP. By contrast, each molecule of NADH produced in the matrix of the mitochondria is capable of producing three molecules of ATP.

Complex IV is the terminal complex of the electron transport chain and is often called cytochrome c oxidase. This membrane-spanning complex transfers electrons from cytochrome c to O_2. These electrons bind to protons in the matrix and consequently water is formed along with the slight alkalinization of the matrix. Complex IV contains cytochrome a on the intermembranal side and cytochrome a_3 on the matrix side. It has a molecular mass of 210 kDa. This complex translocates protons with a stoichiometry of $4H^+/2e^-$. The protons are translocated through this complex in the opposite direction than the electrons are. The redox potential of the cytochrome c that donates electrons to the complex is 0.26 V, and the redox potential of the water is 0.82 V. Thus, the drop across this complex is 0.56 V, and 8.96×10^{-20} J of molecular free energy becomes available to do work for every electron transported.

These complexes do not exist in a linear chain in the inner membrane of the mitochondria, and electron transfers are mediated by random collisions between electron donors and electron acceptors. The small lipid-soluble ubiquinone diffuses rapidly in the plane of the membrane, and transfer electrons from complex I and II to complex III. Electrons are transferred between complexes at a rate of one electron transferred per 5–20 ms, the rate of "typical" enzyme reactions. The nonlinear nature is deduced from the observation that the complexes are present in variable ratios. The four electron transport complexes are inserted into the membrane in a fixed orientation so that the protons are all pumped from the matrix (P-space) to the intermembrane space (E-space). This vectorial organization can be demonstrated by using membrane-impermeable probes to specifically label proteins.

The ordered transfer of electrons depends only on the redox potential and the concentrations of the various components. Using Einstein's random-walk equation, Ogston and Smithies (1948) calculated that if the enzymes of the electron transport chain were not confined to the inner membrane of the mitochondria, but were free to diffuse throughout the cell, the rate of respiration would be minis-

cule, thus the "solid-state packing" of the enzymes makes it possible for the cell to produce ATP at a high rate (Ball and Barrnett, 1957; Ziegler et al., 1958; Lehninger, 1959).

Oxidative Phosphorylation

The function of the electron transport chain on the inner membrane is to transport H^+ from the matrix side (P-space) to the intermembranal side (E-space). The proton translocation is electrogenic, thus the matrix side becomes negative relative to the intermembranal side. The free energy inherent in the electrical potential difference across the membrane can be harnessed to make ATP by complex V, the F_1F_0-ATP synthase (Boyer, 1997; Walker, 1997). The ATP synthase synthesizes ATP when protons move passively through its proton channel from the E-space to the P-space. When the ATP synthase is stripped from isolated, inside-out, inner mitochondrial membranes, the inner membranes still oxidize NADH, although they lose their capacity to synthesize ATP. When the ATP synthase is added back to the membranes, they synthesize ATP upon the addition of NADH. However, ATP is only synthesized at the expense of NADH oxidation, when the membrane is intact and impermeable to H^+. Thus, in oxidative ATP synthesis, as in photophosphorylation, the free energy inherent in a proton, by virtue of its position, is used to make ATP. This is why proton ionophores, including dinitrophenol (Loomis and Lipmann, 1948), uncouple ATP synthesis from electron transport in mitochondria and chloroplasts.

Thermodynamics of Oxidative Phosphorylation

The amount of molecular free energy made available by each electron transport complex can be calculated from Eq. 14.14 (Ball, 1944). However, this only gives us an estimate, since the real value will depend on the real concentrations of the reduced and oxidized forms of the couple, and there will be some loss of molecular free energy due to friction.

$$\Delta E = nze(z\psi^{\text{final}} - z\psi^{\text{initial}}) \qquad (14.14)$$

Only the molecular free energy that can be coupled to proton transport will be conserved, and the rest will be dissipated as heat. In order to determine how many protons can be translocated for each pair of electrons that come from NADH or FADH, we must calculate the amount of energy needed to translocate a proton from the matrix side (P-space) of the membrane to the intermembranal side (E-space), using the following equation:

$$\Delta E_{e-p} = kT \ln[H^+]_e/[H^+]_p + ze(\psi_e - \psi_p) \qquad (14.15)$$

The energy needed to translocate a proton across the inner mitochondrial membrane varies from 2.5 to 4.2 $\times$ 10^{-20} J depending on the mitochondria. While the total energy barrier is similar in the chloroplast and mitochondria, the distribution between the chemical potential energy and the electrical energy is different. In mitochondria, the largest contribution to the energy barrier comes from the electrical difference, and it amounts to 2.4 $\times$ 10^{-20} J since the electrical potential across the inner membrane is between -0.126 and -0.250 V, and the E-side is equal to 0 V by convention (Ducet et al., 1983). The pH of the matrix is approximately 7.5, and the pH of the cytosolic side is approximately 7 (Nicholls and Ferguson, 1992), which only accounts for about 5 $\times$ 10^{-21} J, thus it takes approximately 2.4 $\times$ 10^{-20} J to pump a proton. Consequently, complexes I, III, and IV each provide enough molecular free energy to pump at least one proton per electron. This value may vary within a mitochondrion due to shape changes, since the energetics depend on the pH of the mitochondrion, and the pH depends on the volume (Lehninger, 1959).

The energy inherent in the electrical potential difference generated by proton pumping can then be used to synthesize ATP and water from ADP and P_i. We use Eq. 14.16 to calculate the energy made available by a proton moving down its electrochemical potential difference from the intermembranal side (E-space) to the matrix side (P-space). Thus, each proton makes available approximately 3 $\times$ 10^{-20} J of free energy, which is able to do work.

$$\Delta E_{p-e} = kT \ln[H^+]_p/[H^+]_e + ze(\psi_p - \psi_e) \qquad (14.16)$$

The free energy of protons can be used to synthesize ATP from ADP and P_i. According to Eq. 14.17, the energy required for the synthesis of ATP will depend on K_{Real}, which depends on the relative concentrations of ATP, ADP, P_i, and water. Assuming that [ATP], [ADP], [P_i], and [H_2O] equal 10^{-3}, 10^{-3}, 10^{-2}, and 55.5 M, respectively, then the molecular free energy required for the synthesis of ATP is 8 $\times$ 10^{-20} J. However, just as the molecular free energy made available by the hydrolysis of ATP depends on the relative concentrations of ATP, ADP, and P_i, so does the molecular free energy required for ATP synthesis.

$$E_{\text{Real}} = -kT \ln(K_{\text{eq}}/K_{\text{Real}}) = kT \ln(K_{\text{Real}}/K_{\text{eq}}) \qquad (14.17)$$

These thermodynamic calculations reveal that it is possible to make one ATP with approximately three protons. They also tell us that complex I can pump $2H^+/2e^-$, complex II can pump $2H^+/2e^-$, and complex IV can pump $4H^+/2e^-$. Thus, for every NADH oxidized to water, eight protons are transported across the inner membrane. When these protons pass back into the matrix through the ATP synthase, they provide the energy for the synthesis of two to three ATPs. These theoretical determinations are corroborated by experiments performed by Peter Hinkle and his colleagues (Thayer and Hinkle, 1975a,b; Hinkle and McCarty, 1978; Hinkle and Yu,

1979; Berry and Hinkle, 1983; Scholes and Hinkle, 1984; Krishnamoorthy and Hinkle, 1984, 1988; Hinkle et al., 1991).

Why is the stoichiometry of H^+ translocation and ATP synthesis variable or uncertain? Stoichiometric calculations must take into account that mitochondria do not exist in isolation but must take up substrates, including inorganic phosphate and ADP from the cytosol, and export products, including ATP. Each of these processes requires energy and makes use of the proton difference. Another factor that affects the stoichiometry is that the volume of the mitochondria may not be constant. Consequently, the movement of molecules in and out of the mitochondria may result in a swelling or shrinking, which will affect the concentrations of all substances and the concentrations will affect the rates of the reactions.

How many ATPs are generated from each glucose oxidized? Since there are six NADHs coming from the Krebs cycle for each glucose, there will be between 12 and 18 ATPs formed from them. Two NADHs also come from the pyruvate dehydrogenase complex, which will give us four to six more ATPs. The two FADHs formed during the Krebs cycle will yield two ATPs each for a total of four ATPs from FADH. Thus, the mitochondria will produce 20–28 ATPs via the electron transport chain. Including the four ATPs formed by substrate-level phosphorylation during glycolysis and in the Krebs cycle, 24–32 ATPs are made. Lastly, if the two NADHs formed during glycolysis enter the electron transport chain at complex III, then four more ATPs will be made for each glucose. This yields a grand total or 28–36 ATPs per glucose.

Six molecules of molecular oxygen (O_2) are consumed in the oxidation of a six carbon sugar. The overall reaction of respiration is thus:

$$C_6H_{12}O_6 + 6O_2 + n\text{ADP} + nP_i \Rightarrow 6CO_2 + (n + 6)H_2O + n\text{ATP}$$

The Alternative Oxidase

In addition to the cytochrome oxidase chain, plants have an alternative oxidase that is capable of pulling electrons from the reduced quinone pool (Henry and Nyns, 1975) and reducing oxidative stress (Møller, 2001). The alternative oxidase has been purified (Moore and Rich, 1985). The alternative pathway is usually thought to be uncoupled from ATP synthesis so that the electrons can move through the alternate oxidase pathway quickly, and most of the energy of the NADH is given off as heat rather than being conserved in the bonds of ATP. This is how plants in the Araceae family, including skunk cabbage and *Arum*, generate heat to melt the snow around their flowers in the late winter, or use the heat to volatilize substances that attract flies (Church, 1908; James and Beevers, 1950; Knutson, 1974, 1979; Meeuse, 1975; Meeuse and Raskin, 1988; Seymour et al., 1983; Seymour, 2004; Gibernau et al., 2005). The male flowers of *Arum* produce salicylic acid and the generation of heat by the alternative pathway in *Arum* and other plants can be induced by exogenous salicylic acid (Raskin et al., 1987, 1989, 1990; Kapulnik et al., 1992; Raskin, 1992a,b; Minorsky, 2003). It is also possible that in some cases the alternative pathway does produce ATP (Wilson, 1980). The alternative pathway in *Chara*, like that in protozoa, is capable of synthesizing ATP (Takano and Wayne, 2000).

The alternative pathway is expressed in some cells but not in others (Moore and Rich, 1985; Elthon et al., 1989; Guy et al., 1989; Ordentlich et al., 1991). I think that it is fascinating that this pathway is induced by chilling, and as such may function in cellular temperature regulation (Moynihan et al., 1995). Indeed since the generation of ATP is an enzymatic process, the diversion of electrons to the alternative pathway may actually maximize ATP formation by raising the temperature closer to the optimum for maximal enzymatic activity.

Historical Aspects of Cellular Respiration

While most biologists have memorized the Krebs cycle many times, few know how it was discovered. Hans Krebs had just elucidated the ornithine cycle, in which urea is synthesized from carbon dioxide and ammonia (Krebs, 1970, 1981b), and in the 1930s he was ready to tackle the problem of how pyruvate is oxidized to carbon dioxide and water. Krebs was prepared to see everything as a cycle, and the burning of foodstuffs was no exception. Compared with a linear pathway, such a cycle would allow better regulation and integration with the whole cell (Krebs, 1971). Krebs knew that foodstuffs undergo combustion to release energy, as Lavoisier pointed out, and that enzymes typically react with only one or two substrates at a time. Each step releases molecular free energy in the ballpark of 10^{-20} J. Thus, Krebs assumed that the combustion of pyruvic acid to CO_2 and H_2O probably involved many steps, and he decided to search for the intermediates.

As a prelude to Krebs work on oxidation, Albert Szent-Györgyi (1935, 1937) developed a minced pigeon flight muscle system to study the oxidations that may be involved in cellular energetics. Szent-Györgyi added various compounds to the minced muscle to test if any of them were capable of stimulating pyruvate-dependent oxygen uptake. He assumed that the ones that stimulated oxygen uptake would turn out to be the natural intermediates between pyruvic acid and oxygen. Szent-Györgyi tested a number of dicarboxylic acids that were typically found in high concentrations as secondary products in the plants they are named after (e.g., fumaric acid from the fumatory; malic acid from *Malus*; Davy, 1827). Of the chemicals he tried, only succinic acid, fumaric acid, malic acid, and oxaloacetic acid stimulated oxygen uptake. Moreover, the amount of oxidation caused by each dicarboxylic acid was greater than that necessary to oxidize the added compound. Thus, he concluded that the dicarboxylic acids acted as catalysts in the oxidation of pyruvic acid. He also

showed that added dicarboxylic acids could reduce methylene blue, an artificial hydrogen acceptor that mimicked oxygen. However, he believed that the hydrogens of pyruvic acid were passed in order from pyruvic acid to oxaloacetic acid, malic acid, fumaric acid, and succinic acid. At this point, the electrons associated with the hydrogens would enter the electron transport chain.

Meanwhile Martius and Knoop (1937) were studying the oxidation of citric acid in both the test-tube and in liver tissue using the techniques of organic chemistry (Martius, 1982). They showed that citric acid is converted to cis-aconitic acid, then to isocitric acid, and then to α-ketoglutaric acid.

It was already known that α-ketoglutaric acid could be converted to succinic acid. Thus, Krebs realized that if Szent-Györgyi made a mistake in the order of the pathway, then during pyruvic acid oxidation, citric acid could be converted all the way to oxaloacetic acid (Krebs and Johnson, 1937; Krebs and Eggleston, 1940a,b; Krebs, 1940a,b, 1943; Burton and Krebs, 1953; Krebs et al., 1953). Krebs and his colleagues showed that pyruvic acid stimulated both O_2 uptake and citric acid synthesis. They found that under anaerobic conditions, the addition of pyruvic and oxaloacetic acids to muscle suspensions led to the formation of citric acid, which they measured colorimetrically. Thus, Krebs postulated that a cycle was involved in intermediary metabolism, and he called it "the citric acid cycle."

The citric acid cycle was accepted by most but not all biochemists (Breusch, 1939; Thomas, 1939). The evidence for the cycle included the observations that:

1. Succinic, fumaric, malic, oxaloacetic, α-ketoglutaric, citric, isocitric, and cis-aconitic acids stimulate O_2 uptake in pigeon muscle suspensions.
2. Catalytic amounts of these acids stimulate the oxidation of pyruvic acid.
3. The stimulations by catalytic amounts of these acids are prevented by malonic acid, an inhibitor of succinic dehydrogenase.

Malonic acid is an analogue of succinic acid and was known to inhibit O_2 uptake in whole cells (Thunberg 1910; Quastel and Wheatley, 1930). Krebs treated cells with malonic acid and measured how much succinic acid was formed upon addition of the intermediates. The amount of succinic acid was determined by measuring the volume of oxygen taken up in order of oxidize succinic acid to fumaric acid in the presence of added succinic dehydrogenase and an electron acceptor (Krebs and Eggleston, 1940b). The amount of succinic acid formed was equal to the amount of added citric acid, isocitric acid, cis-aconitic acid, or α-ketoglutaric acid. Moreover, in the presence of pyruvic acid, succinic acid was quantitatively formed in malonic acid-treated cells upon the addition of fumaric acid, malic acid, or oxaloacetic acid.

Lastly, Krebs and his colleagues found that pyruvic acid oxidation was blocked in malonic acid-treated suspensions, but this inhibition was overcome by adding oxaloacetic acid in stoichiometric amounts (1:1) but not in catalytic amounts. This occurs because oxaloacetic acid is not regenerated in malonic acid-poisoned preparations as it is in untreated tissues. Because pyruvic acid is oxidized by a cycle, an infinite number of pyruvic acid molecules can be oxidized by just one molecule of oxaloacetic acid.

With the introduction of isotope-labeling techniques, it was found that the ^{14}carbon from the carboxyl group of pyruvate appeared only in the carboxyl group adjacent to the α-keto carbon of α-ketoglutaric acid, and not in both of the carboxyl carbons. This asymmetrical labeling made it seem unlikely that citric acid was the first product of the cycle, because citric acid is a symmetrical molecule, and consequently the labeled carbon in its terminal carboxyl group should appear in either of the two carboxyl carbons of α-ketoglutaric acid. Thus, the cycle was renamed the tricarboxylic acid cycle, and the citric acid was thought to be a branch off of the asymmetrical cis-aconitic acid.

However, Ogston (1948) suggested that a symmetrically labeled molecule like citric acid could give rise to an asymmetrically labeled molecule if the symmetrical molecule binds to an asymmetrical binding site in an enzyme (Bentley, 1978). This model has been elaborated by Mesecar and Koshland (2000) and Koshland (2002). That is, the substrate does not bind to the enzyme in a random position, but in a preferred manner. This seemed to solve the problem (Krebs, 1950), and thus the citric acid cycle, which was renamed the tricarboxylic acid cycle, came to be known as the *Krebs cycle*. According to Lehninger (1975), the asymmetrical action of aconitase comes from a stereochemical attribute (prochirality) of the citric acid molecule itself.

In 1951, coenzyme A was discovered by Fritz Lipmann (1951). He determined that pyruvic acid is decarboxylated to acetyl-CoA, and it is acetyl-CoA that enters the Krebs cycle. Thus, acetyl-CoA combines with oxaloacetic acid to form citric acid. This is the Krebs cycle as we know it today. Proteins and lipids are also oxidized for energy in the Krebs cycle by way of acetyl-CoA. Many reminiscences have been written about the discovery of the pathways involved in carbohydrate metabolism (Kennedy, 2001; A. Kornberg, 2001; Buchanan, 2002; Berg, 2003; H. Kornberg, 2003).

In the 1950s, it was shown that the Krebs cycle also occurs in plants. Harry Beevers and David Walker (1956) showed that all the intermediates of the Krebs cycle stimulated O_2 uptake catalytically in the presence of pyruvic acid in castor bean mitochondria. Raymond and Burris (1953) showed that pyruvic acid-2-C^{14} was incorporated into the tri- and di-carboxylic acids of lupine mitochondria. And Avron and Biale (1957a) showed that malonic acid inhibited O_2 uptake in avocado mitochondria when succinic acid was used as the substrate. Thus, plant and animal mitochondria are similar in the manner that they combust foodstuffs (James, 1933, 1957; Beevers, 1961).

By the way, when Krebs first submitted his paper to *Nature*, he got the following rejection letter: "The editor

of *Nature* presents his compliments to Dr. H. A. Krebs and regrets that as he has already sufficient letters to fill the correspondence column of *Nature* for seven or eight weeks, it is undesirable to accept further letters at the present time." Krebs and Johnson (1937) then submitted their paper to *Enzymologia* where it was accepted.

Now I will discuss how the electron transport chain was discovered. At the relatively low temperature of our body or in plant cells, oxygen does not directly attack carbohydrates. Thus, investigations ensued as to how oxygen was activated. It seemed likely that the catalyst would contain iron, which has a high affinity for oxygen and is able to bind it reversibly. In the mid-nineteenth century it was widely held that the blood was involved in respiration, and, indeed, an iron-containing compound was present in the blood. In fact, the concentration of iron in the blood is so high that the French nobility wore rings made from the iron extracted from the blood of their friends as a keepsake, much as people wear a lock of a loved one's hair in a ring around their finger or in a locket around their neck (anonymous, 1848). In the 1850s, Claude Bernard realized that the color of the blood depended on its state of oxygenation, and he and Felix Hoppe-Seyler independently found that when CO displaced O_2 in arterial blood, the color became even more vivid red. George Gabriel Stokes (1864) concluded that hemoglobin exists in oxidized and reduced states, and these can be distinguished by their absorption spectra.

After the general acceptance that respiration was a cellular phenomenon, respiratory pigments were searched for in many animal tissues. Charles MacMumm in 1884–1886 found a pigment of which the absorption spectrum varied with its oxidation state, just like hemoglobin. David Keilin, using yeast, showed that this pigment consisted of three pigments that he named cytochromes a, b, and c (see Keilin, 1966). Cytochrome c, the soluble cytochrome, was purified by Hugo Theorell in 1936. Oddly enough, none of the cytochromes a, b, or c interacted directly with O_2. Otto Warburg discovered another pigment, which he called the respiratory enzyme because it directly binds O_2 and oxidizes cytochrome. Warburg was a brilliant biochemist, and he was made an honorary Aryan by Hermann Goering so that he could continue his research on cancer in Germany. In contrast to the decision made by James Franck, Warburg decided to remain in Germany while other non Aryan biochemists, including Carl Neuberg, Otto Meyerhof and Hans Krebs were dismissed from their positions.

Warburg was able to isolate this important enzyme using a clever assay. He found that CO inhibited respiration in sea urchins and light reverses the inhibition. Moreover, light caused the release of CO from the respiratory enzyme, and consequently changed its absorption spectrum. In this way, he could assay each fraction to find the one of which the absorption spectrum in the presence of CO varied in the light compared to the dark. Later, Keilin showed that the respiratory enzyme, which had previously been isolated as indophenol oxidase, contains cytochromes a and a_3.

The notion that ATP synthesis is coupled to electron transport reactions comes from the observation that tissue extracts with high rates of O_2 uptake produced many phosphorylated products, and cyanide inhibited both O_2 uptake and phosphorylation (Kalckar, 1937). One of the phosphate acceptors was ADP, the phosphorylation of which resulted in ATP (Needham and Pillai, 1937; Kalckar, 1939). Kalckar suggested that the energy of respiration was conserved in phosphorylated products, and Albert Lehninger and his colleagues (Lehninger, 1949; Friedkin and Lehninger, 1949) showed that ATP is formed when NADH is given to isolated mitochondria.

14.6 OTHER FUNCTIONS OF THE MITOCHONDRIA

The Krebs cycle functions not only in energy conversion, but also in the interconversion of carbon skeletons (Bandurski and Lipmann, 1956). The Krebs cycle has many sites where intermediates can enter and leave, and as such, acts as a distribution center where the supplies and demands for carbon skeletons are analyzed and the carbon skeletons are converted to the required product and sent off (Krebs, 1954). Given the fact that intermediates enter and leave at all points of the cycle, it is unlikely that the enzymes involved in this cycle exhibit any form of metabolite channeling. Trefil et al., (2009) consider the Krebs cycle to be the most likely candidate that marks the conversion between geochemistry and biochemistry in the origin of life.

All mitochondria participate in respiration. However, mitochondria have evolved to perform various functions in a given cell type. There are differences between animal and plant mitochondria, and the DNA of plant mitochondria is much larger than the DNA of animal mitochondria (Douce and Neuburger, 1989). Some of the observed differences may reflect the autotrophic versus heterotrophic lifestyle of the mitochondrial host. However, remember that some plant cells are heterotrophic while others are autotrophic, and, consequently, the mitochondria of each tissue may be specialized. For example, Valerio et al. (1993) found that mitochondria isolated from autotrophic pea leaf cells are capable of hydrolyzing ATP, and in doing so, generate a membrane potential and acidify the medium. Thus, the ATP synthase can "run backwards" in these mitochondria. However, mitochondria isolated from heterotrophic potato tuber cells are unable to "run backwards" (Valerio et al., 1993).

In plant mitochondria, the conversion of glycine to serine in the matrix of the mitochondria is an important aspect in photorespiration, which involves chloroplasts, peroxisomes, and mitochondria (see Chapter 5). Furthermore, the conversion of succinic acid to fumaric acid, and fumaric acid to

malic acid in the matrix of plant mitochondria is an important aspect of the conversion of fats to sucrose, which involves the lipid bodies, peroxisomes, mitochondria, and cytosol (see Chapter 5). The matrix of animal mitochondria contains the enzymes responsible for the β-oxidation of fatty acids, a function that takes place in the peroxisomes of plant cells.

The matrix of plant mitochondria contains the enzymes involved in proline oxidation. These enzymes include proline-5-carboxlic acid dehydrogenase, which catalyzes the conversion of glutamic acid to proline-5-carboxylic acid, and proline dehydrogenase, which catalyzes the conversion of praline-5-carboxylic acid into proline. In several plants, proline is a compatible solute that accumulates in response to salt or water stress. Proline accumulation results from the stimulation of proline synthesis and the inhibition of proline oxidation (Steward et al., 1977). In this way, the mitochondria of plants are involved in the osmotic homeostasis of plant cells.

The inner membranes of plant and animal mitochondria contain the transport proteins that regulate the influx and efflux of substrates and products involved in energy metabolism. They also contain a transport protein involved in Ca^{2+} uptake, and thus the mitochondria participate in cellular calcium homeostasis. The mitochondria are high-capacity, low-affinity calcium stores.

14.7 GENETIC SYSTEM IN MITOCHONDRIA

Mitochondria contain a complete genetic system, including DNA, ribosomes (70S), and the enzymes necessary to synthesize DNA, rRNA, tRNA, and proteins. There are typically 5–10 DNA molecules in the matrix of the mitochondrion. The DNA of liverwort mitochondria is circular or linear and contains 150–2500 kilobase pairs. This is much larger (10–100×) than the circular mitochondrial DNA of animals, which contains 16–19 kilobase pairs. By contrast, the mitochondrial DNA of *Chlamydomonas* is also small, containing 16 kilobase pairs; however, it is linear (Schuster and Brennicke, 1994). In some plants, the mitochondrial genome is organized into three circular "chromosomes" (Palmer and Shields, 1984). Some mitochondrial DNAs have introns (Fox and Leaver, 1981; Conklin et al., 1991; Pruitt and Hanson, 1991).

There is a tendency for mitochondrial genes to move to the nucleus through time. Within the legume family, there are examples where the cox II gene, which codes for a polypeptide of cytochrome oxidase, has been transferred completely, partially, and not at all to the nucleus (Nugent and Palmer, 1991; Covello and Gray, 1992; Schuster et al., 1993; Schuster and Brennicke, 1994). Genetic transfer occurs between all the genomes of a cell: The mitochondrial DNA even has some promiscuous chloroplast sequences (Stern and Lonsdale, 1982; Stern et al., 1983).

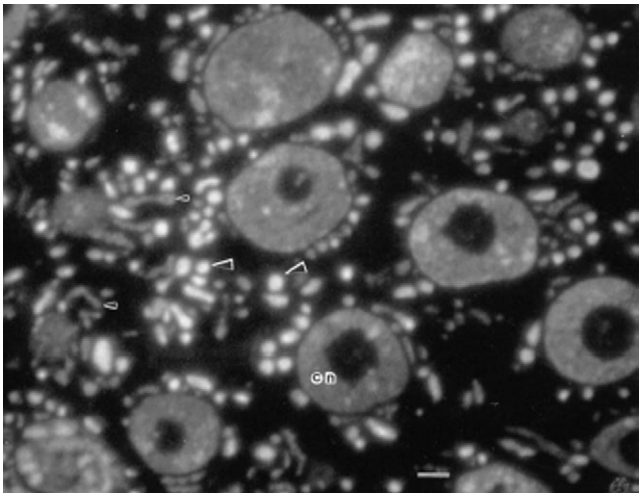


FIGURE 14.11 Cells in the quiescent center of the root of *Pelargonium zonale* stained with 4′-6-diamidino-2-phenylindole (DAPI), a DNA-specific stain. The large arrowheads point to mitochondria, and the small arrowheads point to plastids. Bar, 2μm. (*Source:* From Kuroiwa et al., 1992.)

The mechanism of active DNA transport into mitochondria is currently being studied (Koulintchenko et al., 2003).

The nucleotide sequence of the RNA transcribed in the mitochondria may differ from the DNA template due to editing (Araya et al., 1994), where CGG codons are converted to UGG. Editing was discovered by comparing the sequence of RNA transcripts with the sequence of the gene in question. This further strengthens the notion that life and information transfer is based on integration, and a single sequence, taken in isolation, has little meaning as far as regulation is concerned.

The genetic system of mitochondria is inhibited by the same antibiotics that inhibit bacterial growth. The transcription of mitochondrial RNA is inhibited by acridines, while the transcription of nuclear RNA is inhibited by α-aminitin. Likewise, translation on mitochondrial ribosomes is inhibited by chloramphenicol, tetracycline, and erythromycin, while cytoplasmic ribosomes are inhibited by cycloheximide.

The relative amounts of proteins, as well as the types of proteins synthesized by the mitochondrial genetic system, depend on the organ within which the mitochondria resides (Newton and Walbot, 1985; Rhoades and McIntosh, 1993; Conley and Hanson, 1994; Monéger et al., 1994). Therefore, there must be interesting regulatory mechanisms that control mitochondrial gene expression in a developmentally regulated manner. The mitochondria do not have the capacity to code all their own proteins, and some that are coded by nuclear DNA and synthesized on cytoplasmic ribosomes are imported from the cytosol into the mitochondria.

The presence of a mitochondrial genome allows for cytoplasmic inheritance (see Figure 14.11; Sun et al., 1988) of maternal or paternal mitochondrial DNA. Maternal or paternal inheritance of mitochondrial DNA, like the mater-

nal or paternal inheritance of chloroplast DNA, results from a variety of mechanisms, including the preferential distribution or destruction of mitochondria and its DNA (see Chapter 13).

While accepted today, the concept of cytoplasmic inheritance was frowned upon amid the excitement of the rediscovery of Mendelian inheritance. The concept of an extranuclear genome led to a "cold war in biology" between those who believed that the Mendelian inheritance of nuclear genes can account for all aspects of heredity, and those who believed that there were not only genes in the nucleus, but independent self-replicating and developmentally influential genes in the cytoplasm as well (Darlington, 1944; Srb and Owens, 1952; Waddington, 1956; Lindegren, 1966). The first evidence for cytoplasmic inheritance came from the work of Carl Correns (1909), who showed that the color of the progeny from variegated plants depended on the color of the female and not of the male. This indicated that there were extra chromosomal factors involved in inheritance. Later, Boris Ephrussi (1953) also obtained results from genetic crosses that could not be easily explained by assuming that the nucleus was the sole regulator of heredity. Encouraged by the thought that "we cannot determine the truth of a hypothesis by counting the number of people who believe it," he postulated that there were genetic factors in the mitochondria.

Ephrussi came to this conclusion after observing yeast that formed "petite plaques." It appeared that the gene that caused the petite plaques had an apparently high mutation rate, and the mutation was irreversible. Since the "petite plaques" were smaller than the big plaques only in the presence of oxygen, yet grew at a similar rate in the absence of oxygen, Ephrussi deduced that the factor responsible for plaque size might be in the mitochondria. He suggested that the apparent mutation rate was high due to the unequal segregation of mitochondria that contained the "petite" gene or the "big" gene to the daughter cells. If a daughter cell only got the mitochondria with the "petite" gene, the so-called mutation would show up and be irreversible. Today, we know that these petite mutants lack large sections of DNA in their mitochondria. We also have many examples of cytoplasmic inheritance (Sager and Ryan, 1961).

14.8 BIOGENESIS OF MITOCHONDRIA

Mitochondria divide to give rise to other mitochondria. Prior to division, a spherical mitochondrion elongates, becomes ovoid, and then becomes dumb-bell shaped as the center begins to constrict and the two halves eventually separate (Figure 14.12). Cytochalasin B prevents the elongation, formation of dumb-bell-shaped mitochondria, and division, indicating that these processes require actin (Kuroiwa, 1982). Mitochondrial division also requires FtsZ proteins (Osteryoung and Nunnari, 2003; Miyagishima et al., 2004) and/or dynamin (Arimura et al., 2004b; Mano et al., 2004),

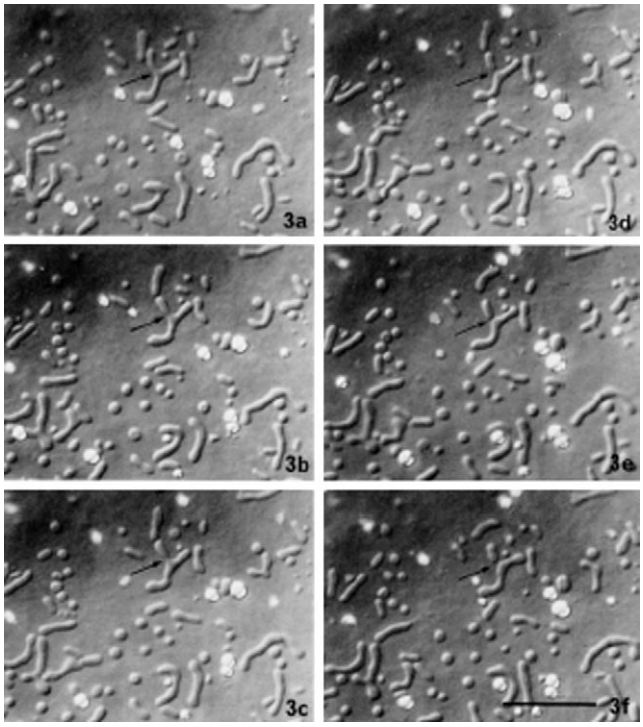


FIGURE 14.12 Differential interference photomicrographs taken 2 minutes apart of dividing mitochondria (arrow) in living cells of *Nitella flexilis*. Bar, 10 μm. (*Source:* From Kuroiwa, 1982.)

where dynamin may have replaced FtsZ as an effecter of mitochondrial division in the evolution of higher plants (Arimura and Tsutsumi, 2002). Mitochondria do not only divide, but they also fuse (Arimura et al., 2004a; Meeusen et al., 2004; Sheahan et al., 2005). The ability to balance the fusion and division processes allows for a temporally and spatially distributable mitochondrial compartment.

While mitochondria are semiautonomous, their activities and numbers have to be coordinated with the activities of the rest of the cell. The continued growth and function of mitochondria require nuclear gene products. If isolated mitochondria are incubated in vitro with radiolabeled mitochondrial proteins, the proteins are taken up into the mitochondria (Kiebler et al., 1993; Moore et al., 1994). These proteins enter at special regions, called contact sites, where the inner and outer membranes touch. Like in the chloroplast, these regions are where the protein translocation complexes exist. A sequence of 12 amino acids on the amino-terminus of a protein is sufficient for the translocation of a protein into the mitochondria. These 12 amino acids can be attached to any protein, and they will direct its import into the mitochondria. The mitochondrial transit peptide forms an amphipathic α-helical structure where the positive charges are lined up on one side of the peptide. This sequence binds with the receptor at the contact site and subsequently the sequence is cleaved by a mitochondrial signal peptidase (Pfanner et al., 1994). A membrane potential across the inner membrane is required for

the penetration of the mitochondrial signal peptide, and ATP hydrolysis is required for the import of the rest of the polypeptide. Chaperonins on both sides of the membrane participate in protein import (Moore et al., 1994; Burt and Leaver, 1994).

The canonical mitochondrial targeting sequence proved to be just one possible example of the many ways that a nuclear-encoded mitochondrial protein can enter the mitochondrion (Bédard and Jarvis, 2005). Some nuclear-encoded proteins involved in transcription and translation in the mitochondria are also involved in these processes in the chloroplast, and the signal sequence on these proteins targets these proteins to both organelles (Millar et al., 2006).

14.9 SUMMARY

In Chapter 13, I discussed how plants transform oxidized carbon and hydrogen into carbohydrate with the input of energy from the sun. In this chapter, I discussed how carbohydrate is converted to a readily usable form of energy in the form of ATP, and also how the carbon skeletons can be used in other biosynthetic reactions. I discussed the details of the grand cycle that is responsible for the conversion of energy and includes the autotrophic organisms and the heterotrophic organisms. This cycle was dubbed the Priestley cycle by Efraim Racker (1957). According to E. J. Conway (1953):

> *In the photosynthetic process … the primary reaction consists in a splitting of water into hydrogen and oxygen atoms. The terminal stages of biological oxidation may be represented as the reforming of water from hydrogen and oxygen ions. The over-all process is a transference of electrons from the hydrogen atoms to oxygen atoms, with the absorption of free energy of the solar radiation bringing about the reverse change. Biological energetics, in all their episodic variety, may then be regarded as the life history of the electrons from hydrogen atoms to water, and all the directed energy of aerobic organisms lies between the splitting and the re-forming of H_2O.*

We must remember that heterotrophic cells do not convert everything into CO_2 and H_2O. This is the role of microorganisms, which are part of the great cycles involved in carbon, oxygen, sulfur, phosphorous, and nitrogen cycles. Although the great coal deposits have resulted from the fact that at certain times in Earth's history, microorganisms have not kept pace with the production of reduced carbon compounds. If microorganisms were not around to decompose organic matter into CO_2 and H_2O, eventually all the CO_2 in the atmosphere would be depleted, and photosynthesis and all life that depends on it would cease. Indeed, along with plants and animals, microorganisms are part of the great cycle of life.

Otto Rahn (1945) wrote in his book *Microbes of Merit*:

> *It may cause some readers a peculiar feeling to realize that the carbon, nitrogen and sulfur atoms which make up the entire living world of today are the same identical atoms which formed the living world of a million years ago, and that our own body may consist of some of the identical atoms which once were part of a dinosaur, or a tree fern of the coal age, or of one of our own ancestors. Only the pattern has changed, but the material from which the organic world has been formed, as long as there has been an organic world, has always remained the same. From dust to dust, as the Bible says; the same clay cast in ever-changing molds.*

14.10 QUESTIONS

14.1. What is the evidence that the mitochondria are the "power plants" of the cell?

14.2. What is the mechanism by which the mitochondria and its components provide energy in the form of ATP to the cell?

14.3. What are the limitations of thinking about the mitochondria exclusively as the power plants of the cell or as the only source of useful energy?

Chapter 15

Origin of Organelles

It is generally considered that eukaryotic cells evolved from prokaryotic cells. The progenitor of the eukaryotic cell may have consisted of a simple genome enclosed by a plasma membrane, and looked much like the prokaryotic cells we see today (Stanier et al., 1963). The plasma membrane of the progenitor probably functioned primarily to maintain the distinction between the inside of the cell and the environment. Over time, however, portions of the plasma membrane may have evolved enzymatic activities, including the ability to synthesize lipids, which would be necessary for the maintenance of the membrane. Such a plasma membrane may have also developed the ability to bind ribosomes, which would facilitate the insertion of nascent proteins into itself. The ancestral cell probably divided by a process involving the invagination of the plasma membrane, followed by its simultaneous breakage and fusion, in a manner that would prevent leakage.

15.1 AUTOGENOUS ORIGIN OF ORGANELLES

As time passed, the progenitor cell may have grown, although doing so would have caused the surface-to-volume ratio to decrease, thus limiting the supply of sufficient material to the interior of the cell. In order to maintain a sufficient surface-to-volume ratio, the plasma membrane may have invaginated to form a series of internal membranes, which led to the endoplasmic reticulum (ER). Such an intermediate can be seen in *Epulopiscium fishelsoni*, an organism with both eukaryotic and prokaryotic characteristics (see Figure 15.1; Montgomery and Pollak, 1988; Angert et al., 1993, 1996; Robinow and Angert, 1998: Schultz and Jørgensen, 2001). The ER probably evolved further, differentiating into tubular and cisternal elements, as well as rough, smooth, and transitional regions. Further elaboration of the ER may have given rise to the Golgi apparatus and the peroxisomal reticulum. Alternatively or simultaneously, the Golgi apparatus may have arisen through invaginations of the plasma membrane by way of an endosomal intermediate. The plasma membrane may have also invaginated in such a way as to sequester the hereditary material, and in so doing, formed a nucleus (Cavalier-Smith, 1975), although it has also been suggested that the nucleus arose from an endosymbiotic event (López-García and Moreira, 2006).

The vacuolar membrane probably also evolved from invaginations of the plasma membrane, where the endosomal compartment forms the intermediate. Perhaps the plasma membrane developed the mechanism of endocytosis in order to take in food in the form of molecules that were too large and polar to cross the plasma membrane. Exocytosis may have evolved along with endocytosis so

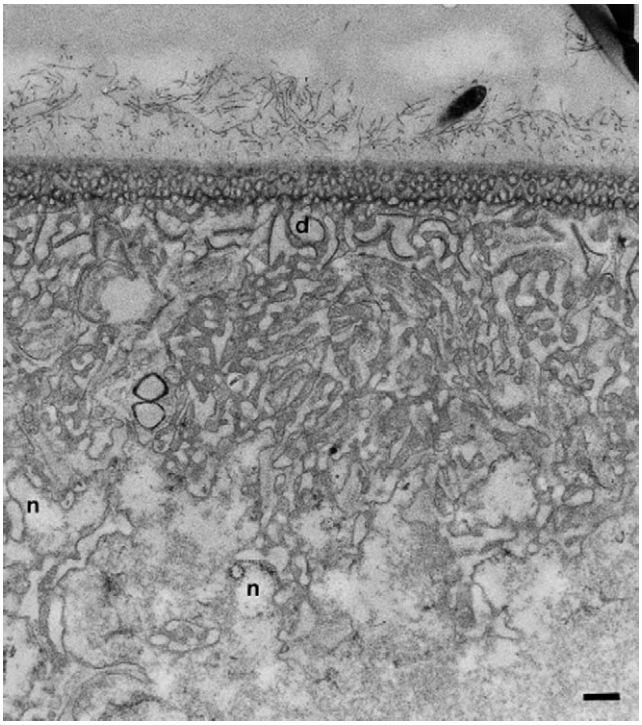


FIGURE 15.1 In the large prokaryotic cells of *Epulopiscium fishelsoni*, ER-like structures arise from invaginations of the plasma membrane. (*Source:* From Robinow and Angert, 1998.)

that a cell could continually take up food without decreasing in size. Perhaps the progenitor cell also developed an endocytotic mechanism that allowed it to take up other cells for food, and to pass them to the vacuole where they would be digested into elementary molecules, including fatty acids, nucleotides, amino acids, and sugars.

The autogenous view of cell evolution suggests that each individual cell is genetically a single individual. What if some of the phagocytosed cells were not digested but developed a symbiotic relationship with the host cell instead? Then each cell would be a colony of evolutionarily distinct individuals. This possibility, which best describes the evolution of what we now call the *chloroplast* and *mitochondrion*, was first proposed by Andreas Schimper (1883). He suggested that plastids might have evolved from cyanobacteria based on his observations that plastids and cyanobacteria have similar morphologies, and the fact that plastids divide to give rise to other plastids.

15.2 ENDOSYMBIOTIC ORIGIN OF CHLOROPLASTS AND MITOCHONDRIA

Schimper's proposal that chloroplasts evolved from cyanobacteria gained support from Constantin Mereschowsky (1905, 1910, 1920) in the early part of the 20th century. Mereschowsky confirmed that chloroplasts share similar physical, metabolic, and reproductive properties with cyanobacteria, and further showed that chloroplasts can survive briefly in cytoplasm, from which the nucleus had been removed.

Following the discovery of mitochondria, it was natural to propose that they too arose endosymbiotically. The way for this idea had already been prepared by Richard Altmann (1890), who believed that the mitochondria, or *bioblasts* as he called them, were not only free-living, but were the fundamental unit of life, living within the lifeless milieu of the cell. Later, Paul Portier (1918) proposed that the mitochondria were not actually a normal component of the cell but bacteria that had been engulfed by the cell and lived within the cell symbiotically. Portier even thought that he had cultured them (Figure 15.2), although his claim was not widely accepted (Lumiére, 1919). By 1922, Ivan Wallin (1922, 1927) proposed that mitochondria, like chloroplasts, evolved from symbiotic bacteria. Moreover, he proposed that the endosymbiotic relationship was important for speciation. He wrote, "Symbionticism insures the origin of new species."

By 1925, the endosymbiotic theory had entered mainstream cell biology textbooks. Edmund B. Wilson (1925) wrote about the endosymbiotic theory:

> *Such an hypothesis is of course unverifiable, and for this reason will to many appear worthless. As a purely speculative construction, however, it seems to the writer to offer possibilities concerning the early evolution of the cell that are worth considering, even though it brings us no nearer to a conception of the origin of life or a comprehension of organic individuality. … To many, no doubt, such speculations may appear too fantastic for present mention in polite biological society; nevertheless it is within the range of possibility that they may some day call for more serious consideration.*

Further support for the endosymbiotic hypothesis came in the 1940s from studies using the newly discovered antibiotics. Luigi Provasoli (1948) and his colleagues treated *Euglena* cells with the antibiotic streptomycin (Lederberg, 1952), and found that this antibacterial agent "cured" the cells of their chloroplasts, providing further evidence for the bacterial origin of chloroplasts (Provasoli et al., 1948). Likewise, the antibiotics, including chloramphenicol, which function as inhibitors of protein synthesis in bacteria, also prevent protein synthesis in chloroplasts and mitochondria, supporting the homology of bacteria, chloroplasts, and mitochondria.

The hypothesis of the endosymbiotic origin of mitochondria and chloroplasts was bolstered by the observations that these organelles contain DNA. The first indication of extranuclear DNA came from the observation by Jean Brachet (1959) that tritiated thymidine, a component of DNA, was incorporated into the cytoplasm as well

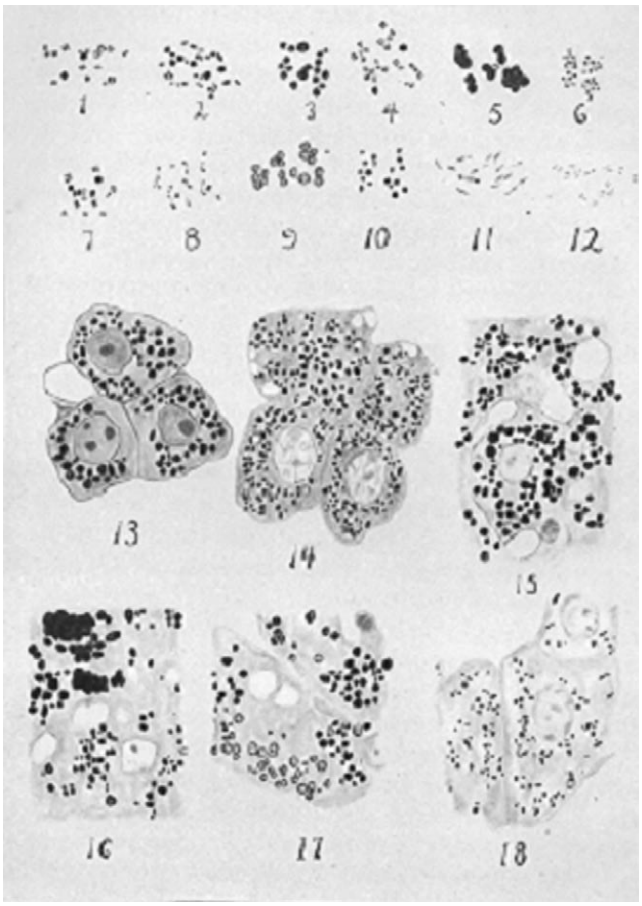


FIGURE 15.2 Camera lucida drawings of mitochondria supposedly cultured from newborn rabbit liver cultured in vitro (1–12) and similar-looking organisms in situ (13–18). (*Source:* From Wallin, 1927.)

as the nucleus. Stocking and Gifford (1959) then showed that tritiated thymidine was taken up into the chloroplast. The DNA in chloroplasts was then visualized by Ris and Plaut (1962) using the electron microscope. They revealed that the chloroplasts contained fibrils of DNA that were similar in appearance to the naked DNA found in prokaryotic cells (Ris and Plaut, 1962). Nass and Nass (1963) later showed that DNA was also present in mitochondria. Ris (1962) suggested that these observations called for a serious reconsideration of the symbiotic hypothesis. Lynn Margulis (1970, 1981), a graduate student at the University of Wisconsin at the time when Hans Ris discovered chloroplast DNA, became very excited about this result and published a synthesis of the endosymbiotic theory and the evidence supporting it. She not only published the idea but also carried on what amounted to a personal crusade for the acceptance of the theory.

There are two polar ways to explain the genomic partitioning between organelles. The autogenous origin hypothesis asserts that the genome of a single individual became distributed between the nucleus and the mitochondria and chloroplasts. By contrast, the endosymbiotic hypothesis professes that the nuclear, mitochondrial, and chloroplastic genomes arose from different cells. Pigott and Carr (1972) performed a series of experiments aimed at distinguishing between these two hypotheses. They carried out a DNA–RNA hybridization experiment using nucleic acids isolated from the nucleus and chloroplast of the same cell, and from a cyanobacterium. They found that the chloroplast DNA hybridized considerably more with the rRNA of free-living cyanobacteria than with the rRNA of the surrounding cytoplasm. This is not what would be expected if the plastid genome differentiated from the nuclear genome. It is, however, what would be expected if chloroplasts had an endosymbiotic origin. A host of protein and nucleic acid sequencing studies support the endosymbiotic origin of chloroplasts and mitochondria (Taylor, 1987; Martin and Müller, 2007; Sapp, 2007). Given the strength of the evidence for the endosymbiotic theory, Weeden (1981) suggests that the reason that the chloroplast and nucleus share responsibility for encoding chloroplast proteins is that many of the genes that encode these proteins have moved from the chloroplast to the nucleus throughout evolution (see Chapter 13).

Additional biochemical evidence lends support to the endosymbiotic origin of chloroplasts and mitochondria: The inner envelope membrane of chloroplasts and the plasma membrane of cyanobacteria both contain monogalactosyldiglyceride and sulfolipid (Douce and Joyand, 1990). Moreover, the inner membrane of mitochondria and the plasma membrane of bacteria uniquely contain cardiolipin (John and Whatley, 1977). Interestingly, chloroplasts and mitochondria have double membranes, and perhaps the inner one represents that of the endosymbiont and the outer one represents that of the host endosome.

There are many candidates for the bacterium that is most closely related to the mitochondrion. John and Whatley (1975, 1977) proposed that mitochondria arose from an aerobic bacterium similar to *Paracoccus denitrificans*, based on the similarity of their respiratory chains. Woese (1977) suggested that mitochondria may have evolved from a bacterium related to purple nonsulfur bacteria (e.g., *Rhodospirillum*) based on the fact that these bacteria have infoldings of the plasma membrane that are similar in appearance to mitochondrial cristae (Figure 15.3). Sequencing data, based on proteins and nucleic acids, support this alliance (Schwartz and Dayhoff, 1978). Like many purple nonsulfur bacteria, the original promitochondrion may have been a nonoxygenic photosynthetic anaerobe. These bacteria are facultative aerobes, and shut down their photosynthetic ability in the presence of O_2. Thus, if they became trapped inside a host cell, they would revert to respiring organelles (Taylor, 1987). The purple nonsulfur bacteria, like mitochondria, have an F_0F_1-type H^+-ATPase.

The splendid variety in the morphologies and pigments of the chloroplasts in the various groups of algae point to the likelihood that chloroplasts arose independently in each major taxon (Raven, 1970). However, it is also possible that the original endosymbiont already contained all the possible pigments, and the chloroplasts in each taxon evolved by the loss of pigments (Löffelhardt et al., 1997). A tremendous effort is now going into sequencing the chloroplast genomes of a variety of algae to distinguish between these hypotheses.

Prochlorococcus marinus, a prokaryotic cell that contains chlorophyll a and chlorophyll b as well as phycobillins, has also been nominated as the candidate that is most closely related to the cyanobacterium that gave rise to the chloroplasts (Hess et al., 1996). *Prochloron*, a prokaryotic cell that contains both chlorophyll a and chlorophyll b but no phycobillins, has been nominated as another possible candidate that is related to the cyanobacterium that gave

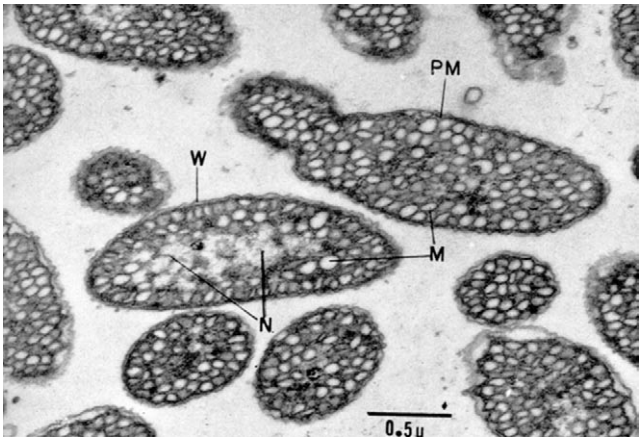


FIGURE 15.3 An electron micrograph of *Rhodospirillum rubrum* showing cells loaded with membrane-bound vesicles (M). N, nucleoplasm; W, extracellular matrix. (*Source:* From Holt and Marr, 1965.)

rise to the chloroplasts of green algae and higher plants (see Figure 15.4; Margulis, 1981). Margulis and Ober (1985) suggest that *Heliobacterium* is a contemporary descendant of the cyanobacterium that gave rise to the chloroplast of the golden-brown algae.

When did these endosymbiotic events take place? Fossil evidence indicates that eukaryotic cells originated 1.4 billion years ago, and the endosymbiotic events would have taken place about then. Since all eukaryotic cells except those of the Archezoa (Cavalier-Smith, 1989a) have mitochondria, the mitochondria probably entered the cells first. Plastids then entered the cells that were to give rise to plants.

15.3 ORIGIN OF PEROXISOMES, CENTRIOLES, AND CILIA

While Margulis (1970, 1981) has been the single most important advocate for the endosymbiotic theory in general, her personal research focuses on the proposal that cilia evolved from the endosymbiotic association of a spirochete-like bacteria (Sagan, 1967). The evidence in support of this hypothesis is the superficial morphological similarities between spirochetes and cilia and the similarities between the proteins FtsZ and tubulin (van Iterson et al., 1967; Margulis et al., 1978; Bermudes et al., 1994; Erickson, 1995). Recently, tubulin genes have been identified in a new division of bacteria known as *verrucomicrobia*, and Li and Wu (2005) have suggested that cilia and flagella may have originated from endosymbiotic events involving bacteria from this division.

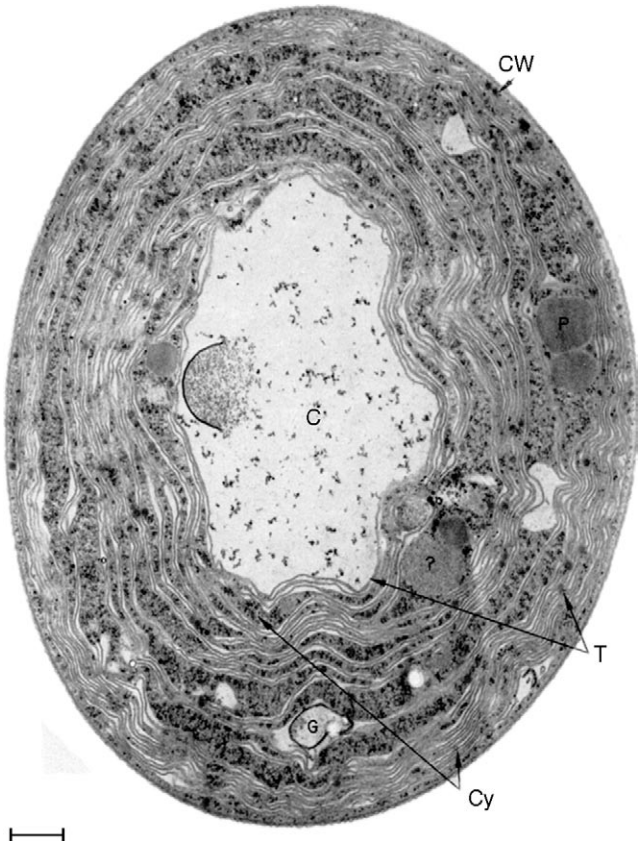


FIGURE 15.4 An electron micrograph of *Prochloron* showing the thylakoid-like membranes. (*Source:* From Whatley, 1977a.)

There is also some evidence that centrioles have evolved from endosymbiotic organisms in that they both propagate by division, although a counterargument is that blepharoplasts, which are identical in structure to centrioles, do not propagate by division, but arise de novo in some cells that will give rise to sperm (Sharp, 1914; Hepler, 1976). Initial support for the endosymbiotic origin of centrioles came from the claim that centrioles contained DNA. However, this claim turned out to be irreproducible, and centrioles do not have DNA (Johnson and Rosenbaum, 1990; Kuriowa et al., 1990; Johnson and Rosenbaum, 1992; Johnson and Dutcher, 1991). However, centrioles do contain RNA (To, 1987), leaving the question of their origin still open.

Unlike chloroplasts, mitochondria, and perhaps centrioles, neither peroxisomes nor cilia contain DNA, RNA, or ribosomes. Thus, if peroxisomes or cilia are the descendents of free-living cells, their entire genetic apparatus has been transferred to the host or was lost. Evidence for the endosymbiotic origin of peroxisomes was supported by their apparent independent growth and division within the cell (Cavalier-Smith, 1989b; Dinis and Mequita, 1994), but evidence that many of the peroxisomal membrane proteins are assembled in the ER argues in favor of an autogenous origin of peroxisomes (Gabalsón et al., 2006; see Chapter 5).

There is evidence that most of the genes that might have been present in the original endosymbiotic pro-mitochondria and pro-chloroplasts have already been transferred to the nucleus (Timmis et al., 2004), and the complete transfer of DNA from an endosymbiont to the host nucleus may be the inevitable fate of all endosymbionts. Such a transfer effectively erases the evidence of the endosymbiotic event. However, the evidence supporting the endosymbiotic origin of peroxisomes, centrioles, and cilia is weak, compared with the evidence supporting the endosymbiotic origin of chloroplasts and mitochondria. When the data at hand do not support a complicated hypothesis, it is more prudent to invoke Occam's razor, which states, "What can be accounted for by fewer assumptions is explained in vain by more" (Gillispie, 1974), and, for the time being, accept the simplest hypothesis (Newton, 1729; Arrhenius, 1915; Northrop, 1961; Galilei, 1962). Since the hypothesis of the autogenous origin of the peroxisome, cilia, and centrioles is simpler and requires fewer assumptions than the endosymbiotic origin hypothesis, I accept it for describing the origin of these organelles. On the other hand, I also believe that everything that is not physically impossible may be possible and that just because a decision is prudent does not mean it is correct.

15.4 ONGOING PROCESS OF ENDOSYMBIOSIS

Symbioses have been going on for the past 1.4 billion years, and are going on all around us. Lichens are symbiotic relationships between algae and fungi. Symbiotic relationships also occur between *Hydra* and *Chlorella*, legumes and *Rhizobium*, and *Paramecium* and *Chlorella* (see Figure 15.5; Pringsheim, 1928; Wichterman, 1986; Lewin, 1992; Reisser, 1992; Tanaka et al., 2002; Kadono et al., 2004). Perhaps even the Apicomplexan parasites, which include *Plasmodium*, the human malarial parasite, resulted from an endosymbiotic relationship with an organism that provided them with a plastid, which is called the *apicoplast* (Wilson et al., 1996; McFadden and Waller, 1997; Dzierszinski et al., 1999; McFadden and Roos, 1999; He et al., 2001; Maréchal and Cesbron-Delauw, 2001; Parsons et al., 2007; Tonkin et al., 2008). The plant partner in this endosymbiosis provides the possibility of preventing or treating malaria with herbicides (Maréchal and Cesbron-Delauw, 2001; Ralph et al., 2004). Symbiotic relationships that give rise to organelles are also still occurring. One example of two organisms caught in the process of one of them becoming a plastid is *Cyanophora paradoxa. Cyanophora* is a photoautotropic protist that contains an obligate symbiotic cyanobacterium known as a *cyanelle* (Löffelhardt et al., 1997; Pfanzagl and Löffelhardt, 1999). Cyanelles function as chloroplasts. However, unlike chloroplasts, they are each surrounded by a vestigial 7-nm-thick peptidoglycan wall. The cyanelles have a reduced circular genome like plastids, and import approximately 80 percent of their proteins from the cytosol (Schenk et al., 1987; Trench, 1991; Steiner et al., 2002).

There are other examples of obligate intracellular symbioses among protists in which the endosymbiont has become an integral part of the host, yet still retains more of its cellular character than do typical chloroplasts. For example, there are small structures in the chloroplasts of *Cryptomonas* and *Chlorachnion* known as *nucleomorphs* that contain DNA (see Figure 15.6; Ludwig and Gibbs, 1987). As long as the nucleus of the endosymbiont continues to control its function, the endosymbiont is considered to be a foreign entity. There is a trend among such symbioses for a mutual dependence between the symbiont and the host, and a concurrent loss of genetic material from both of them (Taylor, 1987; Kuroiwa and Uchida, 1996).

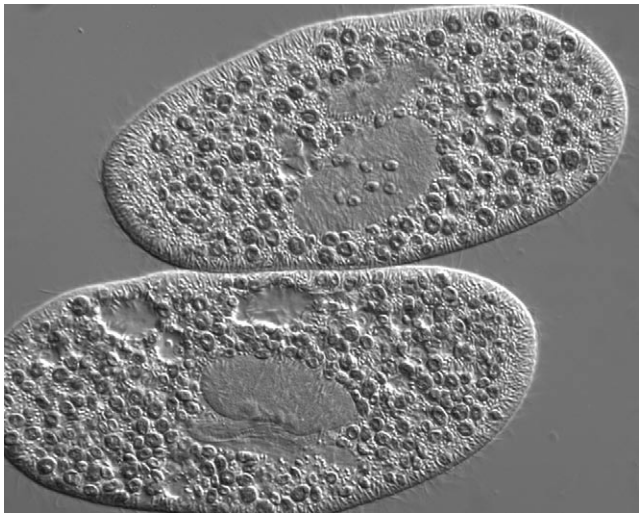


FIGURE 15.5 *Paramecium bursaria*, a symbiotic organism that contains *Chlorella vulgarus*. This symbiotic association has been growing on strictly inorganic media since 1999.

15.5 PRIMORDIAL HOST CELL

While the identity of the primordial host cell remains a mystery, a few candidates for this honor have been proffered. One family of hypotheses state that the *Archaebacteria* are the most likely candidates for the host organism and this organism engulfed *Eubacteria*, which later evolved into mitochondria or chloroplasts (Cavalier-Smith, 1989a). The *Archaebacteria* include the sulfobacteria, halobacteria, and methanobacteria. (The *Eubacteria*,

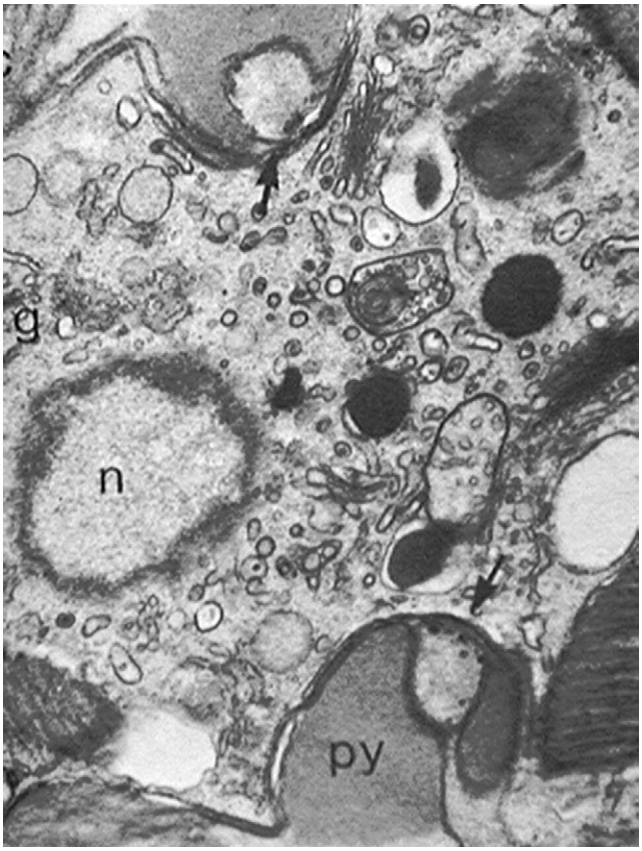


FIGURE 15.6 Nucleomorphs in *Chlorachnion reptans*. In a single cell there is one nucleus (n) and two nucleomorphs (arrows) that are located along the periphery of the cell and adjacent to the pyrenoids (py). c, chloroplasts; m, mitochondria; g, Golgi. ×18,200. (*Source:* From Ludwig and Gibbs, 1987.)

by contrast, include the gram-positive bacteria, *E. coli*, and the cyanobacteria). The *Archaebacteria* hypothesis is based on molecular phylogenetic data, which show that the *Archaebacteria* are more closely related to the eukaryotes than they are to the *Eubacteria* (Balch et al., 1977; Huet et al., 1983; Iwabe et al., 1989; Gogarten et al., 1989; Brown and Doolittle, 1995; Keeling and Doolittle, 1995). Sequence data showing similarities between the H^+-ATPase of *Sulfolobus* and the V-type ATPase of eukaryotic vacuoles indicate that the host cell may be a relative of a contemporary archaebacterium like *Sulfolobus* (Zillig, 1987; Nelson, 1988). The *Archaebacteria* hypothesis is also supported by comparative biochemical research (Martin and Müller, 1998), including Dennis Searcy's (1987), which finds that the thermophilic *Archaebacteria* possess histonelike and actinlike proteins.

An alternative theory, based on comparative cytology, is that a member of the *Archezoa*, a eukaryotic taxon that is characterized by the lack of mitochondria, plastids, peroxisomes, and the Golgi apparatus, was the primordial host cell (Cavalier-Smith, 1987a, 1991; de Duve, 1991). However, others suggest that the *Archezoa* are not primitively "organelle-less," but lost their organelles through evolution (Keeling, 1998; Biagini and Bernard, 2000; Martin, 2000; Seravin, 2001). This interpretation is supported by the finding that nuclear genes, which produce proteins used in the mitochondria, are found in the nuclei of *Archezoa*. However, we do not know whether the genes present in bacteria that had been digested for food were transferred to the nucleus though a horizontal gene transfer mechanism before the evolution of mitochondria (Doolittle, 1998), or if those genes were transferred from mitochondria to the nucleus prior to the loss of the mitochondria (Palmer, 1997). Additional support for the theory that the *Archezoa* "lost" their mitochondria comes from the observation that remnants (50 nm × 90 nm) of mitochondria, with double membranes and that stain with antibodies directed against mitochondrial heat shock protein (Hsp70), can be detected in *Trachipleistophora hominis* (Williams et al., 2002).

According to Lynn Margulis (2001), the original host cell was already a merger between two unrelated prokaryotic organisms. She believes that a merger between an archaebacterium, like *Thermoplasma*, and a eubacterium, like *Spirocheta*, gave rise to the emergent properties of eukaryotic cells.

We do not know the identity of the primordial host cell. Nevertheless, we can take comfort in knowing that *Archaebacteria* are eukaryote-like prokaryotes, the *Archezoa* are prokaryote-like eukaryotes, and that we have narrowed the apparent gap between prokaryotes and eukaryotes. We also know that evolution can be parallel, divergent, or convergent, and that "progressive gradation" or elaboration as well as "progressive degradation" or reduction can take place (Seravin, 2001). Thus, we have a feeling for the missing link, even if we do not know its identity.

15.6 SYMBIOTIC DNA

E. B. Wilson (1925) considered the possibility that the genetic system of the cell, and consequently its development and evolution, could be affected by microorganisms, which we now call *viruses*. The viruses can cause "horizontal" gene transfer between taxa, making us all part of a genetic web of life (Doolittle, 1998; Doolittle et al., 2003; Won and Renner, 2003; Bergthorsson et al., 2004; Mower et al., 2004; Davis et al., 2005; Richardson and Palmer, 2007). The prevalence of organelle-to-organelle gene transfer increases the possibility (Timmis et al., 2004). In the book *The Selfish Gene*, Richard Dawkins (1976) considered the possibility that the majority of nuclear DNA may be nonfunctional and "parasitic," although as time goes on, functions are being discovered for DNA that was previously thought to be nonfunctional (Fire, 2006; Mello, 2006). Will the artificial plasmids produced by genetic-engineering techniques have any unforeseen evolutionary effects on cells?

There may also be symbiotic self-replicating proteins like the prions, which are involved in mad cow disease (Pruisner, 1982) that can be transferred from organism to organism.

15.7 SUMMARY

The eukaryotic cell contains a number of organelles, some of which (nucleus, ER, Golgi apparatus, and vacuoles) may have originated autogenously from the infolding of the plasma membrane. The origins of peroxisomes and centrioles are not clear, although peroxisomes may have also originated from infoldings of the plasma membrane. Centrioles may also have an autogenous origin, but from nonmembranous components. By contrast, the chloroplasts and mitochondria appear to have arisen when bacteria were taken up by phagocytosis, but were not sent to the lysosomal compartment. Instead, they were retained by their host. Thus, throughout evolution these strangers developed a dependence on each other, cooperating in biosynthetic pathways and eventually forming a single organism.

Lewis Thomas (1974) wrote in his delightful book *The Lives of a Cell*:

> *Finally, there is the whole question of my identity, and, more than that, my human dignity. I did not mind it when I first learned of my descent from lower forms of life. I had in mind … ape-men. … I had never bargained on descent from single cells without nuclei. I could even make my peace with that, if it were all, but there is the additional humiliation that I have not, in a real sense, descended at all. I have brought them all along with me, or perhaps they have brought me. … If I concentrate, I can imagine that I feel them; they do not quite squirm, but there is, from time to time, a kind of tingle. I cannot help thinking that if only I knew more about them, and how they maintain our synchrony, I would have a new way to explain music.*

There is something intrinsically good-natured about all symbiotic relations, necessarily, but this one, which is probably the most ancient and most firmly established of all, seems especially equable. … If you are looking for something like natural law to take the place of the "social Darwinism" of a century ago, you would have a hard time drawing lessons from the sense of life alluded to by my chloroplasts and mitochondria, but there it is.

We have come a long way since speculations on the endosymbiotic origin of the mitochondria and chloroplasts were "too fantastic for present mention in polite biological society." Indeed, Cohen (1971) wrote,

The problem [of endosymbiosis] may now be posed as a serious scientific challenge, warranting the most systematic and penetrating exploration. After all, if in the history of science we honor the Copernican revolution as the demonstration that man is not the center of the Universe, what effort should be accorded the proposition that man (and indeed all higher organisms) may be merely a social entity, combining within his cells the shared genetic equipment and cooperative metabolic systems of several evolutionary paths. We suspect that governments should be interested in such a possibility, but they may not yet have heard about it, nor might their responses be readily predictable.

15.8 QUESTIONS

15.1. Is it reasonable to describe eukaryotic cells as a community of genetically distinct individuals that have intermarried throughout time?

15.2. What are the benefits derived by such a multicultural cell?

15.3. What problems must be overcome by such a multicultural cell?

Chapter 16

The Nucleus

16.1 THE DISCOVERY OF THE NUCLEUS AND ITS ROLE IN HEREDITY, SYSTEMATICS, AND DEVELOPMENT

Robert Brown (1831) was the first to describe the structure that we call the *nucleus*. In an aside that was part of a paper devoted to pollination in orchids, Brown described the prominent nucleus as a "single circular areola, generally somewhat more opaque than the membrane of the cell. … This areola, which is more or less distinctly granular, is slightly convex, and although it seems to be on the surface is in reality covered by the outer lamina of the cell. There is no regularity as to its place in the cell; it is not infrequently however central or nearly so." Brown did not mention the nucleus again in the paper.

It is conceivable that others, including Leeuwenhoek, may have seen the nucleus before Brown, and it may have been as unremarkable to them as it was to him. Matthias Schleiden (1845), on the other hand, speculated that the nucleus, which he called the *cytoblast*, was important for the formation of cells. He wrote, "I refer to Robert Brown … who has here, as in so many other instances, opened up a new path of inquiry. He first observed the cytoblast, as a body, frequently present in plants: he did not, however, know its significance in relation to the life of the cell; he called it 'nucleus of the cell.'" According to Schleiden, the cytoblastema was the essence of life, and the cytoblast was the first visible manifestation of the cytoblastema (Figure 16.1). The cytoblast then gave rise to a cell (Figure 16.2). Schleiden's (1838) idea that the nucleus was important in cell formation attracted the attention of a zoologist named Theodor Schwann. Schwann (1884, quoted in Baker, 1988) recounted the surroundings in which he first realized that the cell and its constituents were important for development:

> *One day, when I was dining with Mr. Schleiden, this illustrious botanist pointed out to me the important role that the nucleus plays in the development of plant cells. I at once recalled having seen a similar organ in the cells of the notochord, and in the same instant I grasped the extreme importance that my discovery would have if I succeeded in showing that this nucleus plays the same role in the cells of the notochord as does the nucleus of plants in the development of plant cells.*

Schwann (1838, 1839) promoted the idea that cells, and their structural components (Figure 16.3), were as important in determining the development and morphology of animals as they were in plants. Schleiden (1845) wrote about the importance of the nucleus in cell formation, "If this law is found essential to some plants and animals, this analogy forms a basis for enunciating this mode of formation as a universal law for both kingdoms." On the basis of studies made by Schleiden, a botanist, and Schwann, a zoologist, these two scientists are typically credited as the cofounders of the cell theory. The real founder was Henri Dutrochet (1824), a man who was ahead of his time.

As every introductory biology student knows, the nucleus can be difficult to see in a living cell. In fact, Schleiden (1845), von Nägeli (1844), Hofmeister (1949), and von Mohl (1852) believed that nuclei were absent in the majority of cells, and formed *de novo* just before mitosis. They also believed, however, that in some cells, a nucleus arose from a preexisting nucleus. According to Hugo von Mohl (1852),

> *The … origin of a nucleus, by division of a nucleus already existing in the parent-cell, seems to be much rarer than the new production of them, for as yet it has been observed only in a few cases, in the parent-cells of the spores of Anthoceros, in the formation of the stomates, in the hairs of the filaments of Tradescantia, &c., by myself, Nägeli, and Hofmeister; but it is possible that this process prevails very widely, since, as the preceding statements show, we know very little yet respecting the origin of nuclei.*

The fact that the nucleus is present in all eukaryotic cells at all times was not obvious until Theodor Hartig (1854) and Lord Sidney Godolphin Osborne (1857) noticed that the nuclei in onion and wheat roots were stained by alkaline carmine solutions. Carmine is a dye made from the scale insect *Dactylopius coccus*. Following the observation of carmine-stained nuclei in plants, it was discovered that carmine-stained

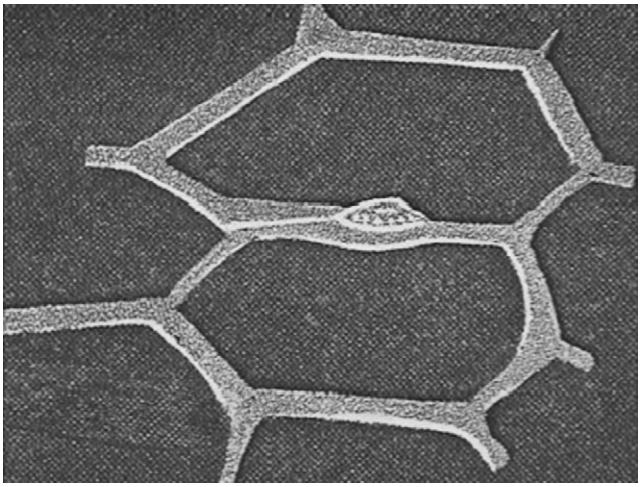


FIGURE 16.1 The cytoblast in cells of *Chaemoedorea schiedeana*. (*Source:* From Schleiden, 1838.)

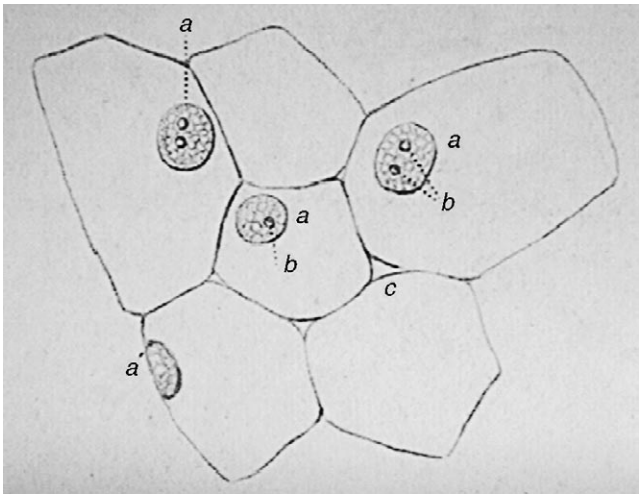


FIGURE 16.3 The nucleus (a) and nucleolus (b) of onion parenchyma cells. (*Source:* From Schwann, 1847.)

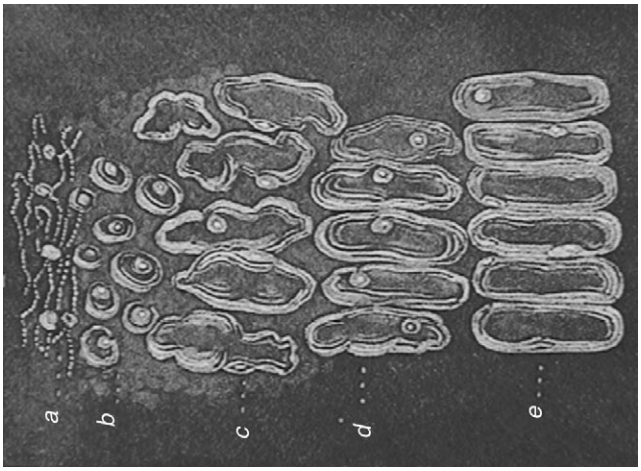


FIGURE 16.2 Free cell formation according to Schleiden. (a) The innermost mass, consisting of gum with intermingled mucous granules and cytoblasts. (b) Newly formed cells, still soluble in distilled water. (c–e) Further development of the cells, which, with the exception of the cytoblasts, may still coalesce, under slight pressure, into an amorphous mass. (*Source:* From Schleiden, 1838.)

nuclei were also common in animal cells (Gerlach, 1858, in Clark and Kasten, 1983), and it became apparent that a nucleus was in each and every animal and plant cell (Virchow, 1860).

As a pioneer of the method of differential staining, Lionel Beale found that resting muscles stain with acid dyes, while active muscles, due to the accumulation of lactic acid, stain with basic dyes. Then it was discovered that the nucleus of active, living organisms also stained with basic dyes, while those of nonliving organisms were apt to stain with acid dyes. Given these data and the theoretical paradigms of the day, it was reasonable to conclude that the more acid an organelle appeared to be, the more vital its activities were. Thus, Lionel Beale (1878) and others (Drysdale, 1874) came to believe that the nucleus must contain the essence of life, or *bioplasm*. Ranke (in Drysdale, 1874) wrote that,

> *… the chief activity of cell chemistry seems to originate in the nucleus. We see the vital activities if the organs run their course with the formation of organic acids, e.g., lactic acid, the production of which is copious in proportion to the heightened activity of the organs. Hence, we see the neutral or slightly alkaline reaction of the muscular or nerve tissues give place, under strongly exerted activity, to an acid one. These chemical transformations of the cell contents originate, as it appears, for the most part, in the nuclei, which in the living cell, exhibit constantly an acid reaction in contradistinction to their alkaline environment. The acid reaction is made known by the property of the nucleus to colour itself red quickly and permanently.*

Prior to the introduction of fixatives and dyes, it seemed that the nucleus appeared and disappeared at various times during the life of a cell. However, the new cytological techniques revealed that the nucleus only seemed to appear and disappear in unstained material because of the lack of contrast, and in actuality, it fragmented into pieces. The fragments were equally distributed to the daughter cells during cell division. Moreover, Eduard Zacharias (1881) showed that the nuclei and their fragments were colored by the stains that he developed to test for the presence of nuclein (see Section 16.4). Since the nucleus was readily stained by colored dyes, its contents were given the name *chromatin* (Fleming, 1882), which means "colored substance." The fragments into which the chromatin divided were given the name *chromosomes*, which means "colored bodies," in a review by Waldeyer (1888; Goldschmidt, 1956).

Eduard Strasburger (1875) visualized the process of nuclear division while he was studying fertilization in *Picea*. He went on to show that nuclear division was not an artifact of fixation since he could also observe it in the living

cells of *Spirogyra*. Strasburger searched for more favorable material than *Spirogyra* to see the process of nuclear division in living cells. He rediscovered the *Tradescantia* stamen hair, which had been previously used by Hofmeister (1849), and found that the process of nuclear division could be readily seen in vivo in these cells because of their leviathan-like chromosomes.

Walther Flemming (1880, 1882) began his cytological studies on salamander larval epithelial cells. He chose salamander larval epithelial cells because they were large and had exceptionally huge nuclei. The chromosomes in salamander cells are all long and thin. That is, the chromosomes are threadlike, and consequently he gave the name *mitosis* to the division of these threadlike chromosomes. As a consequence of the threadlike nature of the salamander chromosomes, Flemming could clearly see that the chromosomes divided longitudinally and not just by a nonquantitative fission process as was suspected by Strasburger, who studied cells that contained mostly globular chromosomes (Hughes, 1959; Baker, 1988).

The process of mitosis so clearly presented by Flemming indicated that the chromatin was not just a homogeneous material as might have been inferred from the results of the chemists Friedrich Miescher and Felix Hoppe-Seyler (see Section 16.4). Indeed, cytologists discovered that chromatin was not just equally distributed to each daughter cell, but the nucleus went through a complicated process to divide the chromosomes in such a way that each somatic cell had not only the same number of chromosomes, but identical copies of the chromosomes (Wilson, 1895). This led to the idea that the chromosomes contained the self-perpetuating information that was necessary to build an organism (Roux, 1883; de Vries, 1889; Weismann, 1893; Nägeli, 1917). E. B. Wilson (1923) described this remarkable finding like so:

> *The cytologist is first struck by the extraordinary pains that nature seems to take to ensure the perpetuation and accurate distribution of the components of the system in cell division, and hence heredity. Nothing is more impressive than the demonstration of this offered by the nucleus of the cell. … To our limited intelligence, it would seem a simple task to divide a nucleus into equal parts. The cell, manifestly, entertains a very different opinion. Nothing could be more unlike our expectation than the astonishing sight that is step by step unfolded to our view by the actual performance. The nucleus is cut in two in such a manner that every portion of its net-like inner structure is divided with exact equality between the two daughter-nuclei, and the cell performs this spectacular feat with an air of complete and intelligent assurance. The net-like framework is spun out into long threads or chromosomes; these are divided lengthwise into exactly similar halves; they shorten, thicken, separate and pass to opposite poles; and from the two groups formed, are built up two daughter-nuclei, while the cell-body divides between them. Such a process seems in some respects to contradict all physical principles; but its meaning has now become perfectly plain. In a general way it means, as Roux pointed out forty years ago, that the nucleus is not composed of a single homogeneous substance, but is made up of different and self-perpetuating components; and it means that these components are strung out in linear alignment in the threads so that they may be divided, or distributed in particular manner, by doubling of the thread.*

While the process of nuclear division was being studied in somatic cells, Strasburger observed that the nucleus of the sperm and egg fused during the process of fertilization in algae, conifers, and flowering plants (Ducker and Knox, 1985). Using the worm *Ascaris megalocephala* var. *univalens*, which only has two chromosomes in the diploid cell, Edouard van Beneden (1883, in Hughes, 1959) observed that the sperm and the egg each contribute exactly one chromosome to the zygote. Thus, irrespective of the discrepancy in the size of the male and female gametes, each parent contributes the identical number of chromosomes. This observation was consistent with the observations by Aristotle in the fourth century BCE. Pierre-Louis Moreau de Maupertuis (1753) and Joseph Kölreuter discovered that each parent contributes equally to the inheritance of the offspring (see Voeller, 1968). Van Beneden also observed a reduction division that resulted in the gametes having half the number of the chromosomes as the parents in order to ensure that the diploid chromosome number remains constant over the generations from parent to offspring. Theodor Boveri and Oscar Hertwig independently discovered the mechanism of how the diploid number of chromosomes gave rise to the haploid number of chromosomes in reproductive cells (Hughes, 1959; Baltzer, 1967; Voeller, 1968).

This was a special kind of process that involved two cell divisions. The first cell division differed from the typical mitotic division because the chromosomes in a cell, which were originally derived from each parent, paired in such a way that the daughter cells received half the number of chromosomes that the mother cell contained. The daughter cells were thus haploid, although they carried two nearly identical copies of each chromosome. The second division did not involve the pairing of chromosomes, which occurred in the previous division, and was thus more similar to the typical mitotic division. Consequently, in 1887, Flemming named the first division *heterotype mitosis*, and the second division *homotype mitosis*. The entire process, including the two divisions, was originally called *maiosis* from the Greek word μειωσιζ for reduction by Farmer and Moore (1905). The term was used in a few publications (Farmer et al., 1905a,b; Moore and Arnold, 1905; Moore and Embleton, 1905; Moore and Walker, 1905). Later, Charles Walker (1907) proposed the term *meiotic phase* as a synonym for heterotype mitosis, and *meiotic division* to describe anaphase of the heterotype mitosis. Misspellings and confusion occurred in the literature, and *meiosis*, a word already in use in the English language to mean understatement (Hughes, 1959), soon replaced the term *maiosis* (Fraser, 1908; Bateson, 1909; Walker, 1910; Farmer, 1913; Sharp, 1921).

Thus, the manner in which the chromatin was distributed from cell to cell in the processes of mitosis in somatic cells, meiosis in reproductive cells, and fertilization of gametes was understood by the end of the 19th century (Weismann, 1891, 1892, 1893; Overton, 1893; Wilson, 1895). For over a century, hereditary factors that could give and shape life were postulated by Gottfried Leibniz, Pierre-Louis de Maupertuis (1753), George-Louis Leclerc Compte de Buffon, John Needham, Herbert Spencer (1864), Charles Darwin (1868), August Weismann (1893), Carl von Nägeli (1914), and others, and given names like *monads, particles, organic particles, elementary molecules, the vegetative force, physiological units, minute granules, gemmules, the idoplasm,* and *biophors*. Following the discovery of chromatin, it seemed self-evident to the 19th-century cytologists that chromatin must be the material basis of the theoretical hereditary devices (Baltzer, 1967).

Hugo de Vries (1889) believed that the chromosomes themselves carried the hereditary factors, which he called *pangens*. In his historical as well as prescient and prophetic work, *Intracellular Pangenesis*, de Vries suggested that a pangen, which was larger than a chemical, but still invisibly small, represented one hereditary character. According to de Vries, each zygote contains a complete set of pangens, and during cell division, each pangen is transmitted to both daughter cells so that each daughter cell contains a complete set. During the formation of gametes, there is a reduction division so that each gamete has half the copies of pangens relative to the somatic cells, and the zygote, which results from the fusion of two gametes, has the same number of copies as the somatic cells. In this book, de Vries urged the emergence of the field of molecular systematics when he wrote,

> *Systematic relationship is based on the possession of like pangens. The number of identical pangens in two species is a true measure of their relationship. The work of the systematist should be to make the application of this measure possible experimentally, by finding the limits of the individual hereditary characters. Systematic difference is due to the possession of unlike pangens.*

Hugo de Vries (1889) also postulated that cell differentiation resulted from the secretion of a specific constellation of pangens from the nucleus to the cytoplasm, where they directed the synthesis of proteins. He wrote,

> *As soon as the moment arrived for certain pangens, which until then had been inactive, to be set into activity, they would obviously pass from the nucleus into the cytoplasm. However, in so doing they would retain their characters, and especially their power to grow and multiply. Only a few like pangens would therefore have to leave the nucleus every time in order, by further multiplication, to impress the characters of which they are the bearers, on a given part of the cytoplasm. This process would repeat itself at every change of function of a protoplast; every time new pangens would leave the nucleus in order to become active.*

De Vries's theory of intracellular pangenesis predicted in essence what was to be discovered 60 years later.

Soon after writing *Intracellular Pangenesis*, de Vries studied hybrids of *Oenothera* and developed a theory that mutations were a cause of speciation. While working on hybridization, de Vries rediscovered Gregor Mendel's work. Mendel's work may have fallen into obscurity as a consequence of the powerful and influential romantic natural philosophy movement in Germany that stressed theory, not experiment. For example, when Mendel presented his results to Carl von Nägeli, Nägeli responded by saying (Chandrashekaran, 1998), "Your results are only empirical data; nothing in them is rational." Ironically, when de Vries, along with Carl Correns and Erich Tschermak, rediscovered the work of Gregor Mendel, de Vries's theory of intracellular pangenesis, which postulated how the genetic factors influenced morphology, fell into obscurity. De Vries (1907) also suggested that a quantitative approach to genetics due to the postulates of pangens and mutations would have a salutary effect on genetics like the postulate of atoms and molecules had on chemistry.

Perhaps de Vries's prophetic work may have fallen into obscurity as a consequence of the appearance of a new and comprehensive textbook by Edmund B. Wilson (1896, 1925). Throughout history, the appearance of comprehensive textbooks, like Euclid's *Elements* and Ptolemy's *Almagest*, have had the effect of making it less necessary for scientists to consult the original literature, and consequently many ideas that are ahead of their time get forgotten. It is also possible that de Vries's ideas were lost as scientists began to specialize, becoming cytologists, geneticists, and developmental biologists.

Interested in unifying the tenets of cytology with those of developmental biology and evolution in order to find the cellular basis for development and heredity, E.B. Wilson marshaled all the known facts and interpretations in these fields into his great book, *The Cell in Development and Inheritance*. He wrote about de Vries's theory of intracellular pangenesis, and posed questions about the relationship between chromosomes, development, and heredity. Wilson's approach had a profound impact on generations of cytologists, embryologists, and cytogeneticists (Agar, 1920; McClung, 1924; Morgan, 1924; Darlington, 1932; Sharp, 1934; Brachet, 1957; Swanson, 1957; Swanson et al., 1967; Uhl, 1996), yet the theory of intracellular pangenesis of de Vries was all but forgotten.

By the close of the 19th century, it was already established that the nucleus contains the hereditary substance that allows life to maintain the appearance of being immortal through the constant succession of individuals, even when the individual organic bodies pass away. It was also postulated that the same substance provided the instructions that were necessary for the formation of the proteins in the cytoplasm that perform the functions that allow the individual cell to be considered alive.

The chromosomal theory of inheritance was not universally accepted around the world. In the 20th century, it was rejected on ideological grounds by Vladimir Ilich Ulyanov (a.k.a. V. I. Lenin) and Josif Vissarionovich Dzhugashvili (a.k.a. Joseph Stalin), who appointed Trofim Lysenko to come up with a theory of heredity that was more in keeping with their Bolshevik philosophy (Lysenko, 1946, 1948, 1954). Genetics and economics are still often interconnected, and experiments that show that the genomes of plants change when they are grown in different environments (Durrant, 1958, 1962; Evans et al., 1966; Timmis and Ingle, 1973; Nagl and Rucker, 1976; Cullis, 1977, 1981, 1990; Natali et al., 1986) are not well received by scientists working in an economy that patents genes as if they were patent medicines.

According to Conrad Hal Waddington (1942), "Genetics … has been the most successful in finding a way of analysing an animal into representative units, so that its nature can be indicated by a formula as we represent a chemical compound by its appropriate symbols." However, he also realized that there are many opportunities during the development of the organism to influence the final outcome of a locus on a chromosome without changing its underlying sequence of DNA. He called the collection of modified loci the *epigenotype* of the organism and named the study of the epigenotype *epigenetics* (Goldberg et al., 2007).

16.2 ISOLATION OF NUCLEI

Nuclei were first isolated from pus cells and sperm in the late 19th century. Currently, nuclei are isolated by homogenizing a tissue and then filtering and centrifuging the homogenate at 1900 g for 10 minutes to pellet the nuclei. The pellet is then resuspended and layered on top of a discontinuous Percoll gradient and subjected to density-gradient centrifugation at 7800 g for 30 minutes. The nuclei are removed from the 25/50 percent interface, and then centrifuged at 1900 g to remove the Percoll (Datta et al., 1985). The purity and intactness are estimated by staining the nuclei with the Feulgen reagent, and assaying for DNA polymerase or RNA polymerase activity (Dunham and Bryant, 1988).

16.3 STRUCTURE OF THE NUCLEAR ENVELOPE AND MATRIX

Much of what we know about the ultrastructure of the nucleus comes from the work of Werner Franke, a cell biologist who also courageously worked to expose the East German program to dope athletes with anabolic steroids (Franke and Berendonk, 1997; Ungerleider, 2001; Gall, 2005; Sitte, 2005).

The nucleus is a large disklike, lobed, or spherical organelle about 10 μm in diameter, the morphology of which depends on the activity of the cell (see Figure 16.4; Mathews, 1899; Franke and Schinko, 1969). The nucleus is surrounded by an envelope composed of two concentric membranes (Dessev, 1992; Hurt et al., 1992) composed of lipids and proteins (Hendrix et al., 1989). The outer nuclear membrane is contiguous with the membranes of the endoplasmic reticulum (ER) and is sometimes regarded as a specialized form of the ER. Perhaps the nuclear envelope is the semi-autonomous source of the endomembranes (see Chapters 5 and 8). The outer nuclear membrane is covered with ribosomes that are engaged in protein synthesis and the proteins that are made on these ribosomes are transported to the space between the inner and outer nuclear membranes. This space, which is an E-space, is contiguous with the lumen of the ER. Like the lumen of the ER, it contains proteins with the carboxy-terminal sequence KDEL as determined by immunolocalization (see Chapter 4; Herman et al., 1990). The outer membrane of the nuclear envelope can be embraced by a network of actin filaments (Seagull et al., 1987; Traas et al., 1987; Schmit and Lambert et al., 1987; Villanueva et al., 2005), and it can act as a microtubule-organizing center (MTOC; see Chapter 11; Mizuno, 1993).

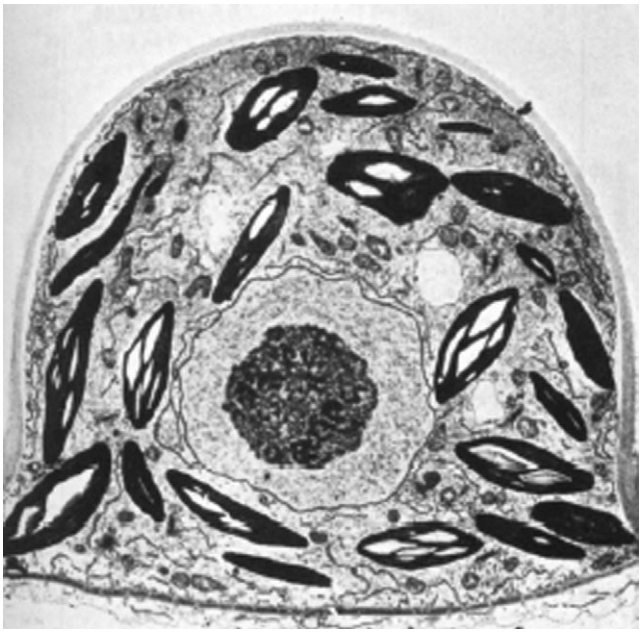


FIGURE 16.4 Electron micrograph of a nucleus from a bud of *Funaria hygrometrica*. ×3900. (*Source:* From Conrad et al., 1986.)

The inner membrane of the nuclear envelope is associated with a network of filaments known as the *nuclear matrix*. The chromatin is embedded in this matrix, which E. B. Wilson (1895) called the *linin*. There are specific regions of the DNA that attach to the matrix (Hall et al., 1991; Breyne et al., 1992; Spiker and Thompson, 1996; Nomura et al., 1997). The matrix is defined operationally as the remains of the nucleus after salt and detergent extraction. Many of the protein components of the nuclear matrix have been characterized (Gosh and Dey, 1986; Nigg,

1988; McNulty and Saunders, 1992; Frederick et al., 1992; Tong et al., 1993; Berezney et al., 1995; Bode et al., 1995; Moreno Díaz de la Espina, 1995).

Because of the large amount of chromatin, the interior of the nucleus is probably too viscous to allow the free diffusion of macromolecules within reasonable time frames (Agutter, 1991, 1995). Thus, the nuclear matrix may function in organizing the interphase nucleus (Heslop-Harrison and Bennett, 1990) and/or transporting RNA to the nuclear pore complex (Xing and Lawrence, 1991). The nuclear matrix may also be important in regulating both DNA replication and RNA transcription. The effect of nuclear structure and the position of genes within the nucleus on gene expression is a particularly fascinating and exciting area of research (Nagl, 1985; Heslop-Harrison et al., 1993; Shaw et al., 1993; Heslop-Harrison, 2000; Lengerova and Vyskot, 2001; Yano and Sata, 2002). The positions of many genes in the interphase nucleus can be found simultaneously using the FISH (fluorescent in situ hybridization) and GISH (genomic in situ hybridization; Heslop-Harrison et al., 1999) techniques.

Many enzymes and proteins that are synthesized in the cytoplasm, but function in the nucleus, including DNA and RNA polymerases and histones, must pass through the nuclear envelope from the cytoplasm into the nucleus. By contrast, mRNA, tRNA, and rRNA associated with protein synthesis must leave the nucleus and enter the cytoplasm. This transport takes place through the pores in the nuclear envelope (see Figure 16.5; Hinshaw et al., 1992; Newmeyer, 1993). Many of the proteins of the nuclear pore complex involved in protein recognition and translocation have been identified (Gruber et al., 1988; Yamasaki et al., 1989; Burke, 1990; Carmo-Fonseca and Hurt, 1991; Scofield et al., 1992).

There is a family of glycoproteins in the nuclear pore complex known as *nucleoporins*. These glycoproteins contain *O*-linked *N*-acetylglucosamine (Snow et al., 1987; Holt et al., 1987), a linkage unlike those made in the ER and Golgi apparatus. Davis and Blobel (1986, 1987) have shown that these proteins are made on cytoplasmic ribosomes and are glycosylated in the cytoplasm.

The importance of the glycoproteins in translocation through the nuclear pore is underscored by the observation that isolated nuclei can transport proteins into them, but not if the nuclei have been pretreated with wheat germ agglutinin, the glycoprotein-binding lectin (Finlay and Forbes, 1990).

The number of nuclear pores varies over time in a single nucleus and it has been observed that transcriptionally active nuclei have more pores than inactive ones. Typically, there are about 11 pores per μm^2, although the nuclear pore density ranges from 5–60 pores per μm^2. The nuclear pores can be distributed regularly or irregularly in the nuclear envelope (Wacker and Schnepf, 1990), and it has been postulated that the distribution of the pores may affect both gene expression and the position of the newly transcribed mRNA (Blobel, 1985).

Each pore is embedded in a large disklike structure that is composed of eight granules and has an eight-fold symmetry (see Figure 16.6; Franke, 1966; Unwin and Milligan, 1982). The pore complex is about 100 nm in diameter and about 15 nm long (Akey, 1989). Filaments extend 60 nm

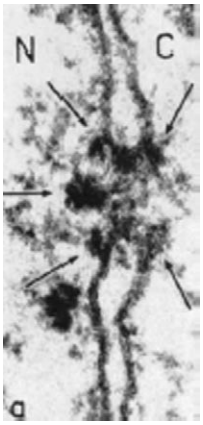


FIGURE 16.5 Nuclear pore of a *Canna generalis* pollen mother cell. ×160,000. (*Source:* From Scheer and Franke, 1972.)

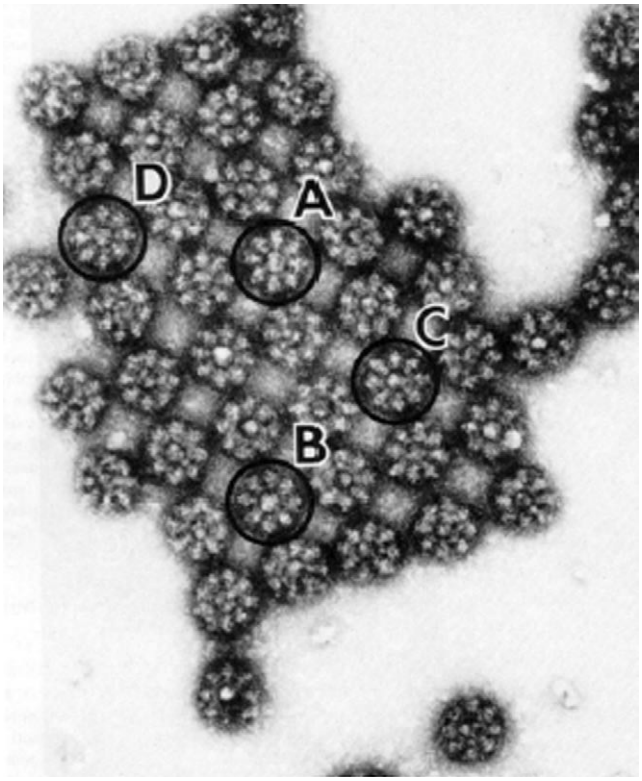


FIGURE 16.6 Nuclear pores on an isolated nuclear envelope from a *Xenopus* oocyte. The pores have been negatively stained and viewed with an electron microscope. A, B, C, and D only refer to pore complexes that were analyzed by image-processing techniques to determine the three-dimensional structure of a pore complex. ×75,000. (*Source:* From Unwin and Milligan, 1982.)

from the nuclear pore complex into the nucleoplasm, forming a basketlike structure (Goldberg and Allen, 1992). There is an aqueous pore in the center of the nuclear pore complex that has an opening of about 9 nm, as determined by measuring the rate of diffusion of fluorescent dyes of different molecular mass from the cytoplasm into the nucleus. A fluorescently labeled protein of 17 kDa equilibrates between the cytoplasm and the nucleus in about 2 minutes. A 44-kDa protein takes about 30 minutes and a 60-kDa protein is hardly able to enter (Lang et al., 1986). The permeability of the nuclear pore depends on the cell type since a 9-kDa dextran, but not a 39-kDa dextran, can pass through the nuclear envelope of starfish oocytes, while in mouse oocytes, a 64.4-kDa dextran can pass through the nuclear envelope, but a 156.9-kDa dextran cannot (Hiramoto and Kaneda, 1988).

Nucleoplasmin is one protein that must enter the nucleus. Nucleoplasmin is a chaperonin that is involved with the assembly of nucleosomes (see Section 16.5). Nuclear proteins, including nucleoplasmin, have been extracted from animal cells and microinjected back into the cytoplasm (Garcia-Bustos et al., 1991; Silver, 1991; Agutter, 1991; Davis, 1992). These proteins return to and accumulate in the nucleus. In fact, if nucleoplasmin is cleaved into a head and a tail region, and the tail region, which can enter the nucleus, is attached to a 20-nm colloidal gold sphere, the colloidal gold is transported into the nucleus, but only when attached to the tail (Figure 16.7). The gold particles are not transported alone. The nuclear pore must recognize the tail protein and open up the pore in a specific manner (Dingwall and Laskey, 1986; Feldherr et al., 1984; Silver and Goodson, 1989). The translocation is coupled to the energy released by the hydrolysis of ATP (Newmeyer and Forbes, 1988).

Proteins that are translocated into the nucleus have a special nuclear import signal or nuclear localization signal. This nuclear localization sequence was first identified in the SV40 virus-encoded protein T-antigen. T-antigen is a 90-kDa protein that must enter the nucleus in order to facilitate viral DNA replication. The T-antigen normally accumulates in the nucleus after it is synthesized in the cytoplasm. However, a single mutation that introduces a threonine instead of a lysine into the protein prevents the import of this polypeptide into the nucleus. A short sequence around this lysine was considered to be the nuclear signal sequence. When a short piece of DNA that codes this region is attached to a gene that codes for a protein that is normally localized in the cytoplasm, the fusion protein is translocated into the nucleus. In this manner, it was determined that the shortest sequence that functioned in nuclear targeting was an eight–amino acid sequence in the middle of the polypeptide (Goldfarb et al., 1986; Kalderon et al., 1984; Lanford and Butel, 1984). Subsequent work showed that over half of the sequenced nuclear proteins have two regions interspersed in each polypeptide that act in concert to target the polypeptide to the nucleus (Restrepo et al., 1990; Carrington et al., 1991; Robbons et al., 1991; Varagona et al., 1991, 1992).

The import of proteins into the nucleus can be regulated through the phosphorylation of amino acids in the targeting sequence. The phosphorylation of an amino acid may affect the ability of the nuclear localization sequence to recognize its receptor. If the protein is one of the transcription factors, this can have a direct effect on gene expression (Moll et al., 1991; Davis, 1992; Hunter and Karin, 1992).

Not only must proteins enter the nucleus, but tRNA, messenger RNA, and ribosomal subunits containing rRNA must leave the nucleus (Mattaj, 1990). When 20-nm colloidal gold particles are complexed to poly-A RNA, rRNA, or tRNA and injected into the nucleus of a frog oocyte, they are transported through the pores into the cytoplasm. However, if they are injected into the cytoplasm, they remain there, indicating that the nuclear pore acts as a rectifier (Clawson et al., 1985; Dworetzky and Feldherr, 1988). The translocation of mRNA has been shown to be dependent on the association of the mRNA with snRNPs (small nuclear ribonucleoprotein, pronounced "snurps"; Davis, 1992; Legrain and Rosbash, 1989).

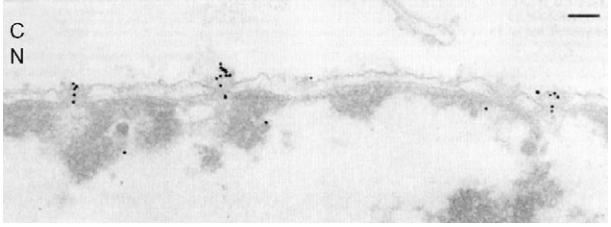


FIGURE 16.7 Colloidal gold particles (10 nm) coated with nucleoplasmin are seen being translocated through the nuclear pore complexes of an isolated rat liver nucleus. C, cytoplasm; N, nucleus. Bar, 100 nm. (*Source:* From Newmeyer and Forbes, 1988.)

16.4 CHEMISTRY OF CHROMATIN

Friedrich Miescher was a physician who was interested in investigating the chemical composition of nuclei (Davidson and Chargaff, 1955; Mirsky, 1955; Fruton, 1972). In 1868, he isolated a compound from the nuclei of pus cells that had unique chemical properties. Unlike the well-characterized albuminous proteins, the new compound was insoluble in water, acetic acid, dilute HCl, and NaCl. When he treated the cells with alcohol to remove the fat, and the pepsin-containing pig gastric mucosa to remove the proteins, something remained in the nuclei. He called the new compound nuclein, which had the chemical formula $C_{29}H_{49}N_9P_3O_{22}$. In 1871, Hoppe-Seyler and his students showed that *nuclei* was ubiquitous, since it was present in yeast as well as the nuclei of snake and bird erythrocytes. Miescher separated from the nuclein a basic proteinaceous substance, which he

named *protamine*, because it was less complex than most proteins. Albrecht Kossel (1884) showed that the protamine was actually a protein called *histone*, which he had already isolated from nuclei (see Kossel, 1928). Richard Altmann (1889) gave the name *nucleic acid* to the protein-free portion of nuclein. In a speculative note submitted along with his other work in 1869, Miescher suggested that the study of individual nucleins might reveal something about the differences between members of a group. However, this note was rejected (Miescher, 1897; Levene and Bass, 1931).

E. B. Wilson (1895) wrote about the possible function of nuclein:

> *The precise equivalence of the chromosomes contributed by the two sexes is a physical correlative of the fact that the two sexes play, on the whole, equal parts in hereditary transmission, and it seems to show that the chromosomal substance, the chromatin, is to be regarded as the physical basis of inheritance. Now, chromatin is known to be closely similar to, if not identical with, a substance known as nuclein ($C_{29}H_{49}N_9P_3O_{22}$, according to Miescher), which analysis shows to be a tolerably definite chemical composed of nucleic acid (a complex organic acid rich in phosphorous) and albumin. And thus we reach the remarkable conclusion that inheritance may, perhaps, be effected by the physical transmission of a particular chemical compound from parent to offspring.*

Indeed, the possibility of testing the hypothesis that nucleic acids were the genetic material seemed possible to Emil Fischer (1914, quoted in McCarty, 1995) when he wrote,

> *With the synthetic approaches to this group we are now capable of obtaining numerous compounds that resemble, more or less, natural nucleic acids. How will they affect living organisms? Will they be rejected or metabolized or will they participate in the construction of the cell nucleus? Only the experiment will give us the answer. I am bold enough to hope that, given the right conditions, the latter may happen and that artificial nucleic acids may be assimilated without degradation of the molecule. Such incorporation should lead to profound changes of the organism, resembling perhaps permanent changes or mutations as they have been observed before in nature.*

At the turn of the 20th century, nucleic acids were isolated from wheat germ, yeast, and thymus in order to study their structures. However, these studies were based on the perspectives of organic chemists with a reductionist approach (Jones, 1920; Feulgen, 1923; Levene and Bass, 1931). Based on degradation studies, the organic chemists considered that nucleic acids were simple macromolecules. When nucleic acids were hydrolyzed, they were broken down into their component parts, which turned out to be the purines (Fischer, 1902), adenine, and guanine, as well as a new class of compounds known as *pyrimidines*. This class included thymine (5-methyl uracil), cytosine, and uracil. Although the organic chemists made outstanding contributions to understanding the components of nucleic acids, they lost sight of the fact that the parent molecule was a macromolecule, and may have a level of complexity that was greater than the complexity of its component parts (Leathes, 1926). Thus, they developed extraction procedures that were suitable for degradation studies, but would not allow them to isolate the native molecule, and to reconstruct correctly the structure of the nucleic acids. Due to the high number of acidic groups, the nucleic acids were considered by chemists to act as intranuclear buffers.

The nucleic acids seemed to be relatively simple molecules. Consequently, the approach used to isolate nucleic acids followed the approach used to isolate other simple materials, and work focused on isolating nucleic acids from a few convenient sources. There was one odd fact though: The nucleic acids of plants seemed to be composed of ribonucleosides, while those of animals were composed of deoxyribonucleosides. Walter Jones (1920) concluded in his monograph on nucleic acids that "there are but two nucleic acids in nature, one obtainable from the nuclei of animal cells, and the other from the nuclei of plant cells." Thus, plant nucleic acids were known as either *yeast nucleic acid* or *ribonucleic acid*, and animal nucleic acids were known as *thymonucleic acid* or *desoxyribonucleic acid*. I would like to stress that the chemists' interpretations of the simplicity of nucleic acids resulted from their preconceived notions of the simplicity of nucleic acids, and thus they developed inadequate extraction procedures.

It appeared to the chemists that nucleic acids were composed of equal quantities of nucleotides, and Phoebus Levene (1921) concluded that nucleic acids were composed of repeating units of four nucleotides. According to this "tetranucleotide hypothesis," one proposed structure for thymonucleic acid was ATCGATCGATCG. The tetranucleotide hypothesis, introduced in 1921, dominated the thinking of scientists for more than 20 years. X-ray diffraction data, based on the X-ray diffraction technique introduced by Max von Laue (1915) and William and Lawrence Bragg (1922, 1924), were used to elucidate the structure of DNA. The data obtained by Astbury and Bell (1938) indicated regular repeats every 0.33–0.34 nm, which they believed were due to the nucleotides, and a 2.7-nm repeat, which they believed indicated that the DNA molecule was composed of a repeating sequence of 8 or 16 nucleotides. William Astbury (1947) concluded that "It seems improbable, too, to judge by the degree of perfection of the X-ray fibre diagram that these four different kinds of nucleotides are distributed simply at random." A nonuniform arrangement of nucleotides would be necessary if the nucleic acids were to function as the genetic material, whereas "the existence of tetranucleotide units, repeated throughout the molecule, would limit the potential number of isomers and hence diminish the possibilities of biological specificity" (Gulland, 1947).

Ideas about the structure of thymonucleic acid also began to change when Signer, Caspersson, and Hammarsten

(1938), using physical techniques, studied the viscosity, rate of diffusion, and rate of sedimentation of thymonucleic acid, and concluded that nucleic acids behaved like thin rods, 300 times longer than they were wide, with molecular masses of between 500 kDa and 1000 kDa. However, due to the strong influence of colloidal chemistry, it was assumed that these large fibers were actually aggregates of thymonucleic acid, and these physical studies, like the chemical studies, gave no impetus for considering DNA to be the genetic material.

Cytologists were obtaining data that conflicted with those of the organic chemists. Using a stain now known as *Feulgen's reagent*, Feulgen and Rossenbeck (1924) discovered that DNA occurred in the nuclei of plants and animals, and not just in those of animal cells. They (quoted in Hughes, 1952) wrote,

> *This gave us great surprise, for the nuclei of the wheat embryo gave the nucleal reaction more intensively than we have ever seen in any animal tissues. This was confirmed in other plants, and so it was demonstrated that the plant nucleus contains nucleal bodies. The old dualism of yeast and thymonucleic acids is thus set aside.*

Conclusions about the importance of DNA based on Feulgen staining were not looked upon favorably because the staining of various cells in the same organism seemed to vary in an unreasonable manner. Approximately two decades later, Mirsky and Ris (1948, 1949; Ris and Mirksy, 1950) determined that the DNA content of nuclei from somatic cells of the same organism is constant, and variations in the ratio of DNA to protein in the nucleus accounted for the variations in the staining.

Prior to the definitive work done by Erwin Chargaff in 1946–1950 there was little reason to even think that nucleic acid was the genetic material based on studies by organic chemists. Indeed, Wendell Stanley (1935) thought that he crystallized the hereditary component of the tobacco mosaic virus when he crystallized its protein. The chemistry of nucleic acids went through a revolution when Erwin Chargaff, "a licensed biochemist," became aware of the genetic evidence that DNA was the hereditary material (Chargaff, 1975). Chargaff was instantly captivated by the experiments performed by Oswald Avery, Colin MacLeod, and Maclyn McCarty in 1944 that indicated that DNA was the genetic material (McCarty, 1985). These experiments were based on the observations of Fred Griffith who discovered in 1928 that when mice were injected with virulent pneumococci that had been heat-killed, along with live avirulent pneumococci, the mice died and their blood contained living virulent pneumococci. Dawson and Sia (1931) found that the transformation of avirulent to virulent pneumatococci could also take place in a test tube. Avery et al. (1944) interpreted all of these results to mean that there was a transfer of genetic material between the bacteria during transformation. Avery et al. (1944) wrote,

> *Biologists have long attempted by chemical means to induce in higher organisms predictable and specific changes which thereafter could be transmitted in series as hereditary characters. Among microorganisms the most striking example of inheritable and specific alterations in cell structure and function that can be experimentally induced and are reproducible under well defined and adequately controlled conditions is the transformation of specific types of Pneumatococcus.*

Avery et al. (1944) fractionated the pneumatocci and meticulously isolated the various fractions and found that the transforming principle was none other than the protein-free DNA fraction. The experiments that pointed to DNA as the genetic material were continued by Rollin Hotchkiss (1995), although not everyone agreed that DNA was the genetic material (Mirsky and Pollister, 1946).

Hearing of Avery's results and believing that DNA was the genetic material, Chargaff began isolating DNA in earnest. He developed the techniques necessary to isolate intact macromolecules of DNA, and used the newly introduced techniques of paper chromatography and UV spectrophotometry to do quantitative work on nucleic acids. Chargaff (1950),

> *… started in our work from the assumption that nucleic acids were complicated and intricate high-polymers, comparable in this respect to the proteins, and that the determination of their structures and their structural differences would require the development of methods suitable for the precise analysis of all constituents of nucleic acids prepared from a large number of different cell types.*

I cannot stress enough how important it was to approach DNA from the mindset that it may be a complicated, information-containing molecule (Butler, 1952). In this respect, Chargaff was a real pioneer. Chargaff showed that contrary to the prediction of the tetranucleotide hypothesis, in native DNA, adenine, guanine, cytosine, and thymine were not present in equimolar amounts. Moreover, he showed that the DNA from different organisms was different, and that indeed, he confirmed the conclusion of Feulgen and Rossenbeck (1924) based on cytochemical data that even plants have DNA (Mirsky, 1953; Chargaff, 1958). Chargaff (1950) further observed: "It is, however, noteworthy … that in all the deoxypentose nucleic acids examined thus far the molar ratios of total purines to total pyrimidines, and also of adenine to thymine and of guanine to cytosine, were not far from 1." Research on nucleic acids had progressed so much that Arthur Hughes (1952), a mentor of Francis Crick's (Olby, 1972), wrote, "In recent years, there has been a tendency to explain every cellular change in terms of nucleic acids."

While Hotchkiss was working on the genetic nature of DNA, and Chargaff was working on its chemical nature and finding that each individual has different DNA molecules, Rosalind Franklin was working in Maurice Wilkins's laboratory to determine the physical structure of DNA

(Franklin and Gosling, 1953; Wilkins et al., 1953; Sayre, 1975). At the same time, Linus Pauling and Robert Corey (1953) were working on the structure of DNA from a theoretical perspective. They had already been successful in applying the concepts of quantum chemistry and the importance of hydrogen bonding to determine the structure of proteins, and they were utilizing this same approach to deduce the structure of DNA from first principles (Pauling, 1972). Pauling and Corey came up with a model of DNA that could be described as a triple helix, with the bases pointing out. Linus Pauling had planned to visit the X-ray crystallographers in Wilkins's laboratory to see the data that would confirm or refute his model, but he was not issued a passport by the State Department due to his liberal politics and concern about the ill effects of nuclear fallout (Pauling, 1963; Watson, 1968; Goertzel and Goertzel, 1995). Thus, he was not privy to the X-ray data obtained by Wilkins et al. (1953) and by Franklin and Gosling (1953).

Unbeknownst to Rosalind Franklin, James Watson (1968) and Francis Crick (1988) did see a report of her unpublished results, and rushed to publish an a posteriori model of DNA that synthesized Avery et al.'s genetic data, Chargaff's chemical data, and the physical data obtained by Wilkins et al. (1953) and Franklin and Gosling (1953). The X-ray diffraction images taken by Franklin were excellent, and, when the DNA was observed in certain conditions of hydration, the images could only be interpreted to result from a structure that has a double helix (Crick, 1954). The double nature was beautiful from a biological and genetic perspective (Watson and Crick, 1953b), and Watson and Crick (1953a) wrote, "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material." Indeed, here was the chemical equivalent of Wilson's (1923) cytological description of the replication and division of the genetic material. It was also an excellent example of Pauling and Delbrück's (1940) hypothesis that complementary structures, as opposed to identical structures (Jordan, 1938, 1939, 1940), will be shown to be involved in autocatalytic processes.

From reading their 1953 paper, it would seem that the double helix sprang out of Watson and Crick's heads just like Athena sprang from the head of Zeus (Watson and Crick, 1953a; Crick, 1974b). Indeed, it even seems like the whole field of molecular biology started with the publication of their paper (Lamanna, 1968; Stent, 1968; Waddington, 1969; Hess, 1970; Weaver, 1970; Wyatt, 1972; Fruton, 1992). Nevertheless, I hope that I have shown that this discovery, like all other discoveries in cell biology, involve the work of many people. Unlike many other discoveries, however, this one has stimulated many people to write about the ethics of James Watson (Chargaff, 1968, 1974, 1978; Pauling, 1973; Sayre, 1975; Donohue, 1976; Stent, 1980; Crick, 1988, 1995).

Watson, like the eugenicists before him, is a man who contemplates ethical issues himself (Galton, 1874, 1872; Pearson, 1905; Davenport, 1910, 1912; Ellis, 1911; Castle, 1916; East and Jones, 1919; East, 1920, 1927, 1931; Jennings, 1925; Pearson and Moul, 1925). He helped launch the Human Genome Project, a large-scale initiative involved in sequencing the entire human genome, in order to better the human condition (Watson and Berry, 2003; McElheny, 2003; Lindee, 2003). In an autobiographical article entitled "Values from a Chicago Upbringing," Watson (1995) wrote of the importance of the human genome project and eugenics:

> *But diabolical as Hitler was, and I don't want to minimize the evil he perpetuated using false genetic arguments, we should not be held in hostage to his awful past. For the genetic dice will continue to inflict cruel fates on all too many individuals and their families who do not deserve this damnation. Decency demands that someone must rescue them from genetic hells. If we don't play God, who will?*

The cell contains a memory of its past and a potential of its future, and this special aspect of life is inseparable from the DNA. Each gene is on the order of about 4–8 nm, as determined by the efficacy of radiation in causing a mutation (Lea, 1947), and a typical diploid nucleus contains about 1–10 pg of DNA. Each gene present in the nucleus is related to the first ancestral gene (see Chapter 18), and has survived in some form throughout the evolution of life. To paraphrase Boris Ephrussi (1953), the nucleus, like Noah's ark, contains two of each gene, one from each parent. Each DNA molecule consists of two long antiparallel chains of deoxyribose phosphates. The deoxyribophosphates are held together with covalent phosphodiester bonds that link the 5′ carbon of one sugar to the 3′ carbon of the next. Each deoxyribose is connected to a nitrogenous base, adenine, cytosine, guanine, or thymine. The adenines pair with the thymines and the cytosines pair with the guanines (Watson and Crick, 1953a; Chargaff, 1955; Crick, 1955; Davidson, 1960; Jordan, 1960). This complementary base pairing results in the formation of a double helix (Herskowitz et al., 1993).

As a result of intrinsic genetic factors, including transposons (McClintock, 1983) as well as environmental mutagens (Klekowski and Berger, 1976; Klekowski and Klekowski, 1982), the DNA in the nuclei can mutate (Sanford, 2008). As is a consequence of these somatic mutations that accumulate in a nucleus over time, the DNA in each nucleus of a long-lived plant may not be necessarily identical (Klekowski, 1971, 1976; Natali et al., 1995; Hallé, 2002). The ability of the plant to keep or lose somatic mutations that take place in the growing tip that gives rise to the rest of the plant depends on the structure of the apical meristem (Klekowski and Kazarinova-Fukshansky, 1984a,b; Klekowski et al., 1989). It is important to remember that this genetic divergence, also known as *genomic plasticity*, is one strategy that long-lived plants use to adapt to a changing environment.

If we assume that a cell needs about 100 proteins to perform the basic functions of life (see Chapter 1), then

the cell would need 100 genes. If each gene coded for a 100,000-Da protein (≈1000 amino acids each), then the DNA would contain about 300,000 base pairs. The smallest genome is in *Mycoplasma genitalium*. This genome consists of 580,000 base pairs and encodes about 470 proteins (Fraser et al., 1995). Even plants with small genomes like *Arabidopsis*, *Quercus*, and *Aesculus* have about 80×10^6 base pairs, which is enough to code for about 27,000 genes (Heslop-Harrison, 1991). Some plants have even more DNA. For example, *Fritillaria* contains $86{,}000 \times 10^6$ base pairs. As a comparison, humans have 3000×10^6 bp of DNA and *E. coli* has 4.7×10^6 bp. It seems paradoxical that there is not any correlation between the amount of DNA (Gall, 1981) and the complexity of the organism. There are many kinds of DNA sequences in a genome. They range from those found in a single copy to those that exist in highly repetitive sequences (Britten and Kohne, 1968; Davidson et al., 1975; Goldberg, 1978, 2001; Peterson et al., 2002).

In Chapter 17, I discuss that the sequence of the base pairs of DNA determines the sequence of nucleotides in microRNA (miRNA), messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA), as well as the sequence of amino acids in proteins. Consequently, clever techniques have been devised to determine the sequence of bases in DNA (Gilbert, 1981; Sanger, 1981). Given the ease of obtaining sequences and the power of computers, the most prominent goal of informatically oriented theoretical biology has become to "compute the organism" (Brenner, 1999; Segal, 2001). Echoing the empty claim of LaPlace, Sidney Brenner declared that "if he had the complete sequence of DNA of an organism and a large enough computer, then he could compute the organism." Likewise, Walter Gilbert claimed "that when we have the complete sequence of the human genome, we will know what it is to be human (see Lewontin, 2000).

A large proportion of biologists are now involved in sequencing the genes of model organisms, including *E. coli*, *S. cerevisiae*, *C. elegans*, *Arabidopsis*, *Drosophila*, and humans. Most gene products are not studied directly, but their function is assigned by comparing the sequence of a gene of interest with the sequence of an *E. coli* or *S. cerevisiae* gene. The activity of sequencing and mapping genes has been given the intentionally nonacademic and magical-sounding name of *genomics* by Thomas H. Roderick (see McKusick and Ruddle, 1987; McKusick et al., 1993). It must be remembered that, while some traits depend exclusively on the sequence of a single gene, the majority of traits depends on many factors, including the genetic background in which the gene is expressed (Grant, 1963; Dobzhansky et al., 1977) and the environment (Lewontin, 2000). Thus, there is not a one-to-one correspondence between a "gene" and a trait; and when we speak of genes we must specify whether we are speaking about sequences of chemicals or of traits (Stadler, 1954).

In the so-called postgenomic period, people are beginning to realize once again that the genome, like the many others things that were considered to be the most fundamental aspect of life, is in itself not the fundamental unit of life (see Chapter 9). Sidney Brenner (2003) writes,

> *... I believe very strongly that the fundamental unit, the correct level of abstraction, is the cell and not the genome. In other words, I've been quoted as saying "forget the genome," you know we don't want to forget it, we'd like to thank all those people for their sterling work and give them all a gold watch and send them home, or better still send them back to the factory to sequence more genomes. But what we've got to do now is to get away from that and look at how we're going to give the true biological picture of it.*

16.5 MORPHOLOGY OF CHROMATIN

In order to evolve, the genome must be able to introduce variability into itself (de Vries, 1905, 1906, 1907). One source of this variability comes from single nucleotide changes in the DNA known as *point mutations*. Point mutations may lead to a protein with altered structures (Pauling et al., 1949). The genome can also evolve through duplication of all the chromosomes (polyploidy). Having two sets of each gene, either from the same parent (autopolyploidy) or from divergent parents (allopolyploidy), makes it possible for one gene to accumulate mutations while the other one produces a functional protein. This "repeat and vary" theme can occur for individual genes as well as for entire genomes. Another source of variability comes from the rearrangement of chromosomes that takes place during the first prophase of meiosis. During this crossing-over process, variation can also result from the mixing of one gene with another. Such mixing may have given rise to the diversity in ion channels and motor proteins. The variation in chromosome structure correlated with the variation seen in the appearance of a species led to the conclusion that chromosomes provide a material basis for the mechanism of heredity and evolution (Morgan et al., 1915; Creighton and McClintock, 1931; Shine and Wrobel, 1976).

Electron micrographs of isolated chromatin show that chromatin looks like "beads on a string" (Figure 16.8). The first electron micrographs that showed a regular repeating

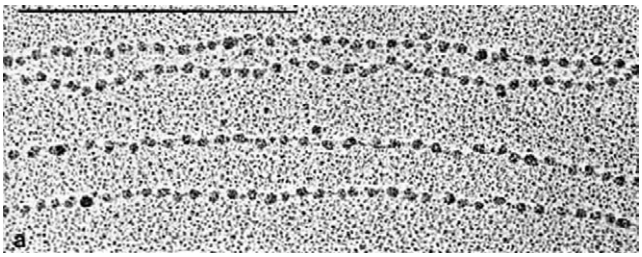


FIGURE 16.8 "Beads on a string" appearance of nucleosomes in *Zea mays* root cells. Bar, 500 nm. (*Source:* From Greimers and Deltour, 1981.)

structure of chromatin were published by Olins and Olins (1974) and not by Christopher Woodcock (1973), due to the luck of the draw when it comes to reviewers. An anonymous reviewer (1973, quoted in van Holde, 1989) of a paper submitted by Woodcock wrote,

> *A eukaryotic chromosome made out of self-assembling 70Å units, which could perhaps be made to crystallize, would necessitate rewriting our basic textbooks on cytology and genetics! I have never read such a naive paper purporting to be of such fundamental significance. Definitely it should not be published anywhere!*

The repeating units, which are 6–8 nm in diameter when viewed by negative staining and 10 nm in diameter when stained positively, are called *nucleosomes*. The nucleosome structure of chromatin had not been seen before 1973 because most protocols used for preparing samples for electron microscopy up until this time involve dehydrating the sample with ethanol. However, ethanol destroys the nucleosomes, and it was not until this step was removed or the chromatin was subsequently rehydrated after ethanol dehydration that nucleosomes could be readily observed (Gigot et al., 1976; Nicolaieff et al., 1976; Woodcock et al., 1976; Frado et al., 1977).

The nucleosomes are composed of some of the histones. The histones are relatively small proteins with a high proportion of positively charged amino acids like lysine and arginine. The positive charges are responsible for the histones binding so tightly to the negatively charged phosphates of DNA. There are five different histones, named, unimaginatively, *H1, H2A, H2B, H3,* and *H4* (Fambrough and Bonner, 1966; Spiker, 1985; Luger et al., 1997; Gardiner et al., 2008). Nucleosomes are assembled with the help of nucleoplasmin, a chaperonin (Laskey et al., 1978; Gatenby and Ellis, 1990).

The repeating structure of chromatin can be confirmed by treating plant or animal chromatin with micrococcal nuclease, and running the digested chromatin through gels. The micrococcal nuclease digests the linker DNA, and consequently bands of integral lengths can be seen when the chromatin is run out on gels (Noll, 1974; Spiker et al., 1983; McGhee and Engel, 1975). An isolated nucleosome consists of a globular core of eight histone molecules (two H2A, two H2B, two H3, and two H4) that is surrounded by 146 base pairs of DNA wrapped in 1.75 turns around the nucleosome. Digestion of chromatin with DNAase I yields DNA fragments, indicating that the DNA is on the outside of the nucleosome. Histone H1 interacts with an additional 20–base pair linker that completes two turns of DNA around the histone core. The amount of DNA around a nucleosome varies. There are 160 base pairs in some fungi and 240 base pairs in some sea urchin sperm (Spiker, 1985). The amount of DNA within the linker varies from organism to organism, from cell type to cell type, and even within different regions of the nucleus. The degree of DNA bending depends on its position within the nucleosome (Hayes and Wolffe, 1992).

The nucleosomes with their associated DNA molecule compose the 10-nm fibers seen in nuclei in electron micrographs. The 10-nm fibers supercoil into a double helix or dinucleosomal ribbon to form the 30-nm fibers seen in interphase nuclei (Woodcock et al., 1984, 1991a,b; Hayes and Wolffe, 1992; Swedlow et al., 1993). The chromatin can be visualized as 10-nm fibers when the ionic strength is lower than about 60 mM, and as 30-nm fibers when the ionic strength is greater than about 60 mM (Pederson et al., 1986). In general, chromatin will only form 30-nm fibers if histone H1 is present (Pederson et al., 1986; Yanagida, 1990). Acetylation of the histones causes the neutralization of the positive charge of lysines in the histones so that the positively charged histones do not bind as tightly to the negatively charged DNA. When the chromatin decondenses, it is more susceptible to degradation by DNase. The histones associated with transcribed genes are more acetylated than those associated with nontranscribed genes are. The acetylation of histone H4 is correlated with the formation of heterochromatin (Jasencakova et al., 2000). Measurements made with laser tweezers show that it takes approximately 15 pN to peel reversibly the DNA from the nucleosomes (Hayes and Hansen, 2002; Brower-Toland et al., 2002). Presumably, the DNA-processing enzymes in eukaryotic cells (e.g., helicase, DNA polymerase, and RNA polymerase) provide the motive force necessary to displace the DNA from the nucleosomes as they perform their better known functions.

Each DNA molecule contains between 50 million and 250 million base pairs and is about 1.7 to 8.5 cm long. The entire human genome is about 2 meters long. If these DNA molecules were only folded into 30-nm fibers, the chromosomes would be about 100 μm long, which is about 10 times longer than the nucleus! Scanning electron micrographs indicate that the 30-nm fibers are folded into looped domains. It appears that the loops contain about 20,000–100,000 base pairs. The long strands of chromatin are not randomly arranged in the nucleus. This was first indicated by the observations of Theodor Boveri (1888), who noticed that the individual chromosome positions in mitotic cells were correlated with the position of the same chromosomes during the preceding cell division. Using biotin-labeled probes, Lichter et al. (1988) showed that each chromosome was restricted to a specific portion of the interphase nucleus (Craft et al., 1999).

The individual genes are not randomly arranged in the interphase nucleus but appear in reproducible positions from one cell division to another. This can be demonstrated using in situ hybridization, where a probe for a given gene hybridizes with the gene in the genome and thus marks the position of that gene (Hillier and Appels, 1989). In some higher eukaryotes, the regions of the chromosomes, called *centromeres* or *kinetochores*, which bind to the spindle fibers, are organized in regions near the poles (Hilliker and Appels, 1989). The nucleolar organizing regions are located

near the nuclear envelope (Hilliker and Appels, 1989; Hernandez-Verdun, 1991). The ends of the mitotic chromosomes, which are known as *telomeres*, can also be located predominantly at the nuclear periphery (Heslop-Harrison, 1991; Rawlins et al., 1991; Abranches et al., 1998; Cowan et al., 2001). It would be awesome to visualize the position of developmentally regulated or functionally associated genes in the nuclei of the cells in which they are expressed, and those in which they are not.

The morphology of the chromatin changes throughout the cell cycle (McLeish and Snoad, 1958). In the interphase nucleus, the chromatin is either highly condensed and it is called *heterochromatin*, or it is much less condensed and it is called *euchromatin* (Figures 16.9 and 16.10). Autoradiographic studies indicate that euchromatin is transcriptionally active, whereas heterochromatin is either less active or inactive (Lafontaine and Lord, 1974; Patient and Allan, 1989).

During prophase, the chromatin begins to condense. By metaphase, the chromatin has condensed approximately 10,000 times; each DNA molecule condenses from about 5 cm long to about 5 μm long. The mitotic chromosomes have very characteristic forms that are invariant for each species (Baum and Appels, 1991). They also can be isolated in large quantities (Dolezel et al., 1992). The lining up and display of the mitotic chromosomes in a species is known as the *karyotype* of that species (Boyle, 1953; Haskell and Wills, 1968; LaCour and Darlington, 1975). The condensed chromosomes observed in mitotic cells represent chromatin that is in a package that is suitable for transport (see Chapter 19).

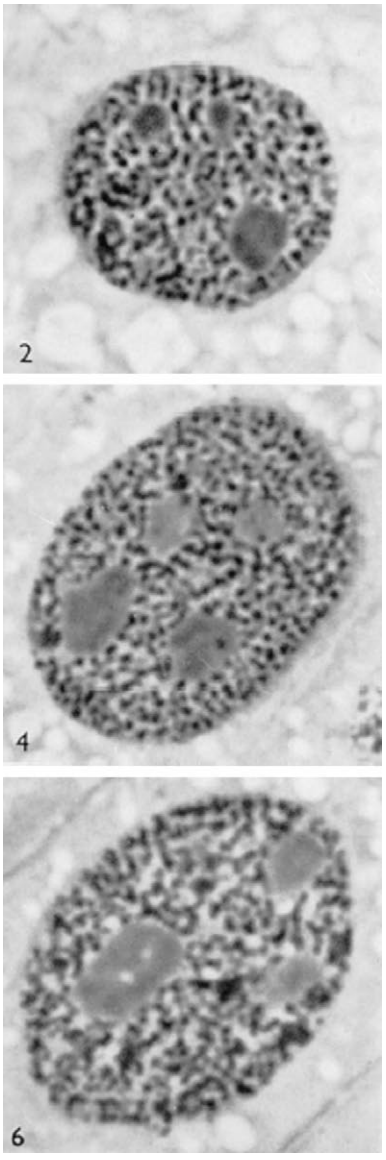


FIGURE 16.9 Phase-contrast micrograph of interphase nuclei of *Allium porrum* in late G1 (2), mid S-phase (4), and G2 (6). ×3800. (*Source:* From Lafontaine and Lord, 1974.)

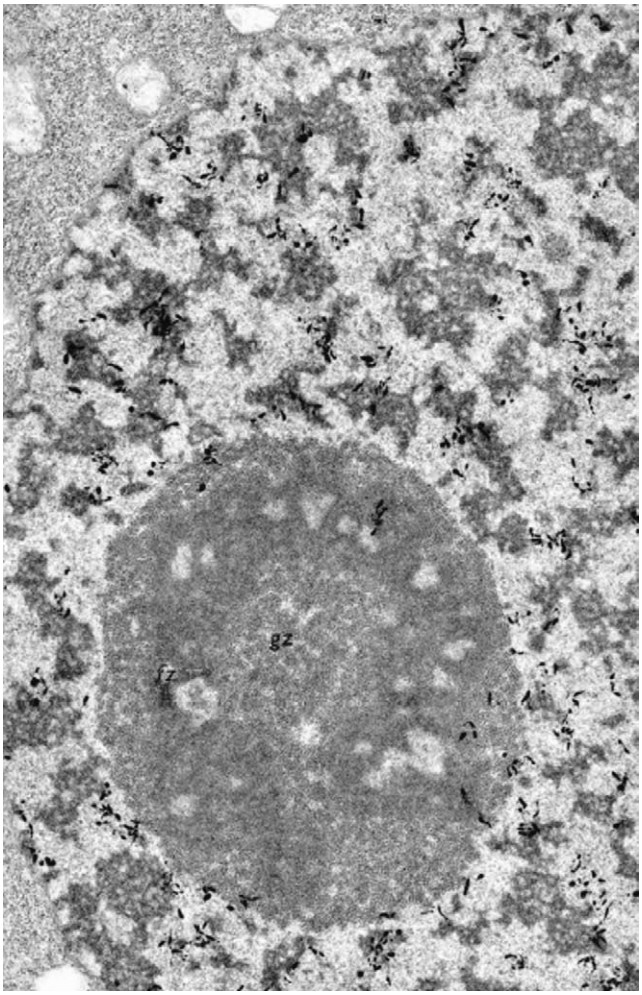


FIGURE 16.10 A radioautograph of the chromatin in a nucleus of *Allium porrum* in early S-phase. Notice that the ^{3}H-thymidine uptake during early S-phase occurs primarily in the lighter areas (euchromatin). ×31,000. (*Source:* From Lafontaine and Lord, 1974.)

16.6 CELL CYCLE

The *cell cycle* is a term that describes the changes that occur through the life cycle of a cell, and, like most cellular processes, it is influenced by environmental conditions (Uchida and Furuya, 1997). The cell cycle is also influenced by circadian rhythms, which ensure that the ultraviolet-sensitive phases of the cell cycle occur at night (Nikaido and Johnson, 2000). The cell cycle is divided into four phases known as G1, S, G2, and M. The nucleus is typically in interphase and the chromatin is decondensed. Interphase is composed of the G1-, S-, and G2-phases. DNA synthesis takes place during the S-phase, and S stands for synthesis. S-phase is preceded by G1-phase and followed by G2-phase, where G stands for gap, since G1 and G2 were named at times when it appeared that nothing was happening during these phases. The G2-phase is followed by mitosis (or meiosis), which is known as M-phase. The newly formed nuclei soon return to the G1-phase. In some cells, notably the antipodal and synergid cells of the female gametophyte, the tapetal cells surrounding the male gametophyte, and the endosperm cells surrounding the developing embryo, multiple rounds of DNA synthesis occur without an intervening M-phase. Endoreduplication results in cells with large polytenic chromosomes with DNA contents around 24,576 times greater than the DNA content of normal haploid chromosomes (Nagl, 1978; Knowles et al., 1990; Polizzi et al., 1998; Sabelli and Larkins, 2007). The genes and proteins involved in regulating the phases and transitions of the cell cycle are particularly well known in yeast (Futcher, 1990), although new ones are being discovered in both yeast and other organisms.

The duration of each phase of the cell cycle can be measured by pulsing the cells with [^{3}H]-thymidine and preparing them for autoradiography (Swanson and Webster, 1985). The durations of the various phases of the cell cycle vary from species to species and from cell type to cell type (Webster, 1979). As an example, in *Vicia faba* meristematic cells, G1 takes about 2.5 hours, S-phase takes about 6 hours, G2 takes about 5 hours, and M takes about 0.5 hours. Most differentiated cells spend the majority of their life in G1, but are still capable of dividing as evidenced by the ability of plants and some animals to regenerate lost parts after wounding (Morgan, 1901; Loeb, 1924). The cell cycle can be best studied in cells, including tobacco BY-2 suspension cultured cells, in which the cell cycle can be readily synchronized (Joubès et al., 2004). While in other cell lines, the mitotic index, which is the percentage of cells undergoing mitosis at the same time, was about 10 percent, in tobacco BY-2 cells, the mitotic index is greater than 70 percent (Nagata et al., 1992, 2006; Nagata and Kumagai, 1999; Nagata, 2004; Inzé, 2007).

What regulates the cell cycle? In 1970, it was found that a protein extract stimulated frog oocytes to divide when it was microinjected into the eggs (Dorée, 1990). The protein in the active extract was called *maturation promoting factor*. Later it was found that a mutant of yeast, called *CDC* (for cell-division cycle), was unable to produce a protein necessary for cell division, and consequently, the mutant stops dead in its tracks during cell division. The CDC protein turned out to be the same as the protein that stimulates frog oocytes to divide. This protein is a protein kinase and occurs in all cell types (John et al., 1987, 1990, 1991; Feiler and Jacobs, 1990; Gorst et al., 1991; Ferreira et al., 1991). In plants, the level of the CDC protein kinase is enriched in meristems, and is thus correlated with the ability of the cell to divide (John et al., 1990; Gorst et al., 1991; John and Wu, 1992). Intracellularly, the CDC protein kinase is enriched in the preprophase band (Mineyuki et al., 1991; Colasanti et al., 1993).

Surprisingly, the CDC protein kinase is always present in dividing cells (John and Wu, 1992), so how does it regulate the cell cycle? The CDC protein kinase is activated by another class of proteins called *cyclins*, and the concentration and type of cyclin change throughout the cell cycle. Each cyclin causes the CDC protein kinase to phosphorylate a different set of proteins necessary for a given phase. Currently, more and more regulatory proteins are being discovered that are involved in regulating the cell cycle. The synthesis, availability, degradation, and phosphorylation states of these proteins are important in initiating each phase of the cell cycle (Nurse, 1990, 2001; John and Wu, 1992; Murray, 1992; Norburg and Nurse, 1992; Kirschner, 1992; Reeves, 1992; Hunt, 2001; Doerner, 2007; Shen, 2007). The genes involved in switching a plant cell over from a mitotic to a meiotic cell cycle, as well as the genes involved in all stages of meiosis including bivalent formation and recombination, are being discovered (Li et al., 2004; Pawlowski et al., 2004, 2007; Pawlowaki and Cande, 2005; Ronceret et al., 2007; Bozza and Pawlowski, 2008).

Cells mature and may even senesce in time. Some of the developmental events that occur as cells age are regulated by extrinsic factors, whereas others are regulated by intrinsic factors. For example, cells may keep track of, in some way, the number of cell cycles, and then initiate differentiation after a particular number of divisions. One way to register the number of cell divisions involves dilution. An initial cell may have a given amount of a particular molecule. Each time the cell divides, the compound will be diluted until there is a subthreshold level, and the cell can no longer divide. This compound or compounds, at least in part, are known to occur at the ends of chromosomes called *telomeres*. After each division, the telomere gets shorter and shorter. When it is no longer there, the cell stops dividing. By contrast, cancer cells produce a protein called *telomerase*. Telomerase is an enzyme that rebuilds the telomeres (Greider and Blackburn, 1984).

Theodor Boveri (1929) wrote that "the somatic cells cease to multiply. From an egoist being, the cell becomes an altruist, in the sense that it proceeds to new division

only when the needs of the whole demand it." While no one knows how cells tell time, we know that some gene on the human chromosome #1 causes cells to lose their immortality. It is known that when human chromosome #1 is transferred into an immortal line of Syrian hamster cells, the cells acquire a limited lifespan (Suguwara et al., 1990; Pereira-Smith and Smith, 1985). On the other hand, oncogenes make products that cause the cell to be immortal (Groves et al., 1991). Likewise, *Agrobacterium* causes a cancerlike condition in plants (Smith and Townsend, 1907; Schilperoort, 1970).

The senescence of organisms may be a manifestation of the senescence of cells (McCormick and Campisi, 1991). This idea comes from studies in mammals, including humans, where the maximum number of divisions in culture cells is directly proportional to the lifespan of the species and inversely proportional to the age of the donor. Moreover, cells derived from donors with heritable premature aging syndromes usually senesce after fewer divisions than cells derived from people the same age who do not have the syndrome in question. The causes of programmed cell death, known as *apoptosis*, are being vigorously studied (Ellis et al., 1991; Groover et al., 1997).

16.7 CHROMOSOMAL REPLICATION

Chromosomal replication includes the replication of the double-stranded DNA molecule that resides in the chromosome, and the assembly of a new set of chromosomal proteins (Bloch and Godman, 1955; Stillman, 1989; Virshup, 1990; Groth et al., 2007). DNA replication depends on the ability of bases to undergo complementary base pairing in a process known as *DNA templating*. DNA synthesis is "semi-conservative," and each strand acts as a template for a new strand (Watson and Crick, 1953c; Meselson and Stahl, 1957; Delbrück and Stent, 1957; Taylor et al., 1957; Sueoka, 1960). During DNA templating, adenine pairs with thymine, and cytosine pairs with guanine. In order for this process to occur, the two strands of the DNA helix must be separated. The DNA helix is unwound by an enzyme called *DNA helicase*. DNA helicase unwinds the DNA helix at the expense of ATP hydrolysis, so DNA helicase is a mechanochemical enzyme (Tuteja, 2003).

Once the DNA helicase unwinds the double helix, other proteins known as *helix-destabilizing proteins* or *single-strand DNA-binding proteins* bind to the single-stranded DNA and stabilize the opened strands without covering up the bases. This allows time for complementary base pairing. Once the DNA helix is unwound, the replication fork is created. The new DNA is synthesized semi-conservatively in the tines of the fork. Once base pairing is accomplished to form a new 5′ to 3′ strand on the template 3′ to 5′ strand, an enzyme known as *DNA polymerase* connects the internal phosphate of the nucleotide at the 5′ end of the nucleotide to the OH group of the 3′ end of the last nucleotide in the polymer at the expense of the hydrolysis of the pyrophosphate from the newly added nucleotide (Kornberg, 1960, 1961, 1989). The pyrophosphate made in dividing cells can be used by the vacuolar H^+-translocating pyrophosphatase. Moreover, it is possible that this pyrophosphatase can drive the biosynthetic reactions involved in DNA synthesis by using up the end product.

DNA polymerase only works in the 5′ to 3′ direction at a rate of about 50 nucleotides per second. So, how does the newly formed DNA on the other strand, which would appear to need to be synthesized in the 3′ to 5′ direction, get polymerized? The DNA in the "lagging strand" is synthesized in the 5′ to 3′ direction in a complicated manner that involves RNA to some extent. After DNA templating, DNA polymerase moves from the 5′ to the 3′ end, creating replication intermediates that are about 100–200 nucleotides long in eukaryotic cells. These fragments are called *Okazaki fragments* and are joined together by an enzyme known as *DNA ligase* to form a continuous chain of DNA.

Interestingly, autogradiographic studies show that, even though replication results in identical DNA molecules in each chromatid, during mitotic division (see Chapter 19), chromatids containing DNA strands of identical age segregate as a single unit in mammalian (Lark et al., 1966), plant (Lark, 1967), and fungal cells (Rosenberger and Kessel, 1968). This indicates that the template strands of DNA in all the chromatids are somehow connected with each other. In fact, in filamentous fungal cells, where it is possible to follow the movement and positioning of individual nuclei, Rosenberger and Kessel (1968) have shown that the chromatids that contain the template DNA are preferably positioned at the tip of the hypha.

Replication begins at specific sites known as the *replication origins* (Van't Hoff, 1975, 1985, 1988; Van't Hoff and Bjeeknes, 1977, 1981; Van't Hoff et al., 1978, 1987a,b; Van't Hoff and Lamm, 1992; Haaf, 1996; Mullmann-Diaz et al., 1996; Bryant et al., 2001; Quélo and Verbelen, 2004). Replication forks originate at replication origins (Virshop, 1990). Replication origins, which are about 300 nucleotides long, form a structure known as the *replication bubble*. Many copies of an initiator protein bind to the replication origin to form a complex that then binds the DNA helicase and positions it correctly. Subsequently, the replication enzymes, including DNA polymerase, form complexes known as *primosomes*, and eventually two complete primosomes move in opposite directions along the two forks that are formed in one replication origin. The nuclear matrix may provide the necessary framework for replication to take place in eukaryotic cells. In fact, it is possible that the replication sites are fixed on the nuclear matrix and the unreplicated DNA is spooled through these sites (Pardee, 1989).

The existence of multiple replication origins and their bidirectional movement can be visualized by pulsing cells with [^{3}H]-thymidine, extracting the DNA in a relatively

unfragmented state, and processing it for radioautography. Radioactive portions of DNA alternate with nonradioactive portions, indicating multiple sites of origin. Longer pulses result in longer-labeled DNA strands, indicating that the replicated segments of DNA are gradually being extended. Differential time pulses or pulses with various specific activities indicate that the DNA replicates in both directions, and eventually the whole strand of chromatin appears labeled (Swanson and Webster, 1985).

How does DNA replication take place in the presence of nucleosomes? Structural changes in chromatin may take place at the sites that are undergoing replication (Nagl et al., 1983; Nagl, 1982, 1985). There is a transient disruption of the nucleosomes located ahead of the replication fork and a transfer of the parental histones from these nucleosomes to the newly formed DNA strands. The nucleosomes on the nascent strand are then completed by the addition of newly synthesized histones (Groth et al., 2007). While the two DNA strands must inherit the nucleosomes in an organized manner in order to pass on the posttranslational modifications of the histones that define inherited epigenetic states, it is still unknown how the two strands inherit the nucleosomes (Pardee, 1989).

If all the replication origins were activated simultaneously, it would take on the order of 1 hour to complete DNA replication. However, S-phase in Chinese hamster cells takes 6–8 hours. This is because the 20–80 replication origins in a chromosome are not activated simultaneously. There seems to be a definite hierarchy in which different regions of the genome are replicated (Stubblefield, 1975). This was determined by pulsing synchronized cells with a thymidine analog at different times during S-phase, and then observing its distribution. The chromatin that corresponds to the G-C–rich bands of mitotic chromosomes replicate during the first half of S-phase, while the chromatin that corresponds to the A-T–rich bands in mitotic chromosomes replicate during the last half of S-phase. The heterochromatin that resides near the centromere replicates very late during S-phase (Lima de Faria and Jaworska, 1968).

Using DNA probes for specific genes it is possible to determine the time of replication of any gene. In these experiments, nonsynchronous cells are pulsed with a thymidine analog, and the cells are separated by their size, which corresponds to their age. The younger cell's DNA would have been labeled early in S-phase, and the older cell's DNA would have been labeled late in S-phase. The thymidine analog containing DNA is then separated from the rest of the DNA. Brown et al. (1987) showed that the housekeeping genes that are active in all cells are replicated very early in S-phase, while genes that are only active in a few cell types are replicated early in the cells in which they are expressed and later in other cell types.

This seems to be a very reasonable method of replicating DNA. First, the vital DNA is replicated, then the DNA that will be expressed in the cell in its specialized state is replicated, and finally the DNA that won't ever be expressed in that cell is replicated. So why is S-phase so complicated? Perhaps the orderly turning on and off of replication units influences chromatin structure. The various resulting chromatin structures may then determine, in part, which genes are expressed in various cell types.

16.8 TRANSCRIPTION

Some cells isolated from plants are capable of giving rise to complete plants, and thus maintain complete copies of the genome. These cells are called *totipotent* (Steward et al., 1958; Thomas and Davey, 1975). Subsequently, a nucleus isolated from an intestinal cell of a frog and implanted into an enucleated egg cell of a frog was shown to be capable of directing the complete development of a frog (Gurdon, 1962; Gurdon and Uehlinger, 1966; Davidson, 1968). Today, clones of dogs and cats as well as sheep and cows are manufactured by injecting a totipotent somatic nucleus into an enucleated cell. There have also been claims that human beings have also been cloned in the same manner. I assume that all cells are totipotent, although for technical reasons this has not always been realized. If all cells are totipotent and contain a full complement of genes, then cell differentiation must result from the differential expression of those genes.

The chromatin of actively transcribing DNA is different from that of quiescent DNA, as evidenced from experiments in which the DNA in vertebrate cells was treated with DNAse I. In each cell, about 10 percent of the genome is digested, and the pattern of digestion varies in different cell types. The DNA that is degraded in each cell type corresponds to the genes transcribed in that cell type. Thus, transcribed genes are in a conformation susceptible to nuclease digestion (Vega-Palas and Ferl, 1995; Li et al., 1998). Long stretches of plant and animal DNA lack nucleosomes, and these regions contain nuclease-hypersensitive sites (Elgin, 1990; Grunstein, 1990). These stretches may represent locations where a sequence-specific protein displaced a nucleosome (Murray and Kennard, 1984; Spiker et al., 1983), or perhaps the nucleosomes may not form in these regions as a result of the DNA sequence itself (Elgin, 1990). Sawyer et al. (1987) found a greater DNase sensitivity of the legumin gene in actively transcribing cotyledons compared to leaves where the gene is not expressed. The structure of the chromatin in a cell type expressing a given gene differs from the chromatin structure of the gene in nonexpressing cell types (Vega-Palas and Ferl, 1995; Li et al., 1998).

The type of attachment of the nucleosome to the DNA can be modified as a result of the chemical modification of the histones. The histones can be chemically modified in a number of ways, including methylation, acetylation, and phosphorylation (Hayes and Wolffe, 1992; Hansen and Ausio, 1992; Morse, 1992; Prymakowska-Bosak et al., 1999), and these posttranslational modifications may influence the ability of the transcribing RNA polymerase to access the associated DNA template (Clark and Felsenfeld, 1992),

perhaps by influencing the ability of a group of nuclear proteins known as the *nonhistone proteins, acidic proteins, high-mobility group proteins, or transcription factors* (Liu et al., 1999; Chen et al., 2002) to bind to the DNA and initiate transcription. The addition of nucleosomes has been shown to inhibit transcription in vitro and the inhibition is reversed when transcription factors are added (Jackson, 1991).

The physical position of a gene may also affect its expression. While the experiments performed by Gregor Mendel (1865) indicated that genes assorted independently, further work on mice by Darbishire (1904) and sweet peas by Bateson et al. (1906) indicated that not all genes segregated independently; that is, some were linked. Genetic studies on *Drosophila* performed by members of Thomas Morgan's laboratory indicated that some genes were more closely linked to each other than to other genes. Morgan postulated that genes were linearly arranged on the chromosome, and the degree of genetic recombination between chromosomes is a reflection of the distance between the genes (Morgan, 1911, 1924; Morgan et al., 1915; Castle, 1919). Harriet Creighton and Barbara McClintock working with maize, and Curt Stern working with *Drosophila*, provided cytological evidence that showed that during meiosis, chromosomes do exchange material and this crossing over is the cytological basis of genetic recombination (McClintock, 1930; Creighton and McClintock, 1931; Stern, 1931; Kass, 2003).

Alfred Sturtevant (1913, 1951) performed genetic crosses between different mutants of *Drosophila* in order to construct maps of the chromosomes that showed the position of the known genes and soon he began to find that gene expression depended on the position of the gene (Sturtevant, 1925; Painter, 1939). Carl Swanson (1957) wrote in *Cytology and Cytogenetics*,

> *Early concepts of the gene, especially those relating to its particulate nature, were derived from crossing over data and from radiation studies. But a greater appreciation of the gene as a functional unit of inheritance and of the chromosome as an organized structure, has come from an analysis of position effects. Discovery of this phenomenon by Sturtevant [1925] has been followed by numerous analyses in Drosophila [Green and Green, 1949; Lewis, 1950, 1951, 1952, 1955; Green, 1954, 1955a,b], Oenothera [Catchside, 1947], and maize [McClintock, 1951, 1953], and it is becoming increasingly evident that the genetically determined phenotype is not only dependent upon the gene itself but upon the nature of the chromatin adjacent to it. The gene, therefore, even if it is of a particulate nature, is not an isolated unit operating simply in conjuction with other genes; it is actively influenced by them.*

The fact that gene expression depends on the position of the gene may in part relate to the physical arrangement of chromatin throughout the nucleus (Nagl, 1985; Bloom and Green, 1992; Reyes et al., 2002), and this may depend in part on the attachment of chromatin to the nuclear matrix (Bode et al., 1995). The fact that an enhancer or silencer sequences thousands of base pairs away from a gene can regulate the expression of that gene supports the idea that the physical conformation of chromatin is important for the regulation of gene expression (Tariq et al., 2002). Moreover, some transformed plants express a given gene while others do not, suggesting that the position of the inserted gene may be as important as its sequence in determining a phenotype.

The three-dimensional structure of chromatin may be important if stresses can cause the activation of a gene. When RNA polymerase opens a DNA helix that is anchored at both ends, tension will develop and the DNA molecule will tend to form supercoiled loops to relieve the tension. A moving RNA polymerase will tend to create positive superhelical tension in front of it and negative superhelical tension behind it. The positive tension may facilitate the opening of nucleosomes that may be required for transcription. If this occurs, the position of a gene will be important for its expression. We must begin to appreciate DNA as a three-dimensional structure instead of a linear sequence, just as we have already begun to appreciate the three-dimensional structure of small organic molecules and organelles (Wollaston, 1808; LeBel, 1874; van't Hoff, 1874, 1967).

Richard Goldschmidt (1951) considers that if we define a gene as the hereditary material that determines a character of the organism, it is impossible to look at a gene in isolation of where it is in a chromosome. It is possible that every gene affects every other gene and DNA sequences may not only code for proteins and regulatory RNAs, but may also influence the physical properties of the chromatin that allow certain genes to be either expressed or silenced. Concerned with the relationship of genes to the whole organism, Barbara McClintock wondered, "Should one gene mutate by what mechanisms are adjustments made in every part of the complex whole?" (see Fedoroff, 1992).

The synthesis of RNA requires RNA polymerase. Eukaryotes have three different RNA polymerases, called *RNA polymerase I, II,* and *III*. They each synthesize different kinds of RNAs. Polymerase I synthesizes the large ribosomal RNAs, polymerase II synthesizes messenger RNAs and microRNAs, and polymerase III synthesizes tRNAs and the 5 S ribosomal RNA. Unlike bacterial RNA polymerase, eukaryotic RNA polymerases do not bind directly to DNA, but must first bind to a transcription factor that recognizes a given promoter sequence (Pabo and Sauer, 1992). Each of the RNA polymerases recognizes a different set of transcription factors. The bacterial RNA polymerase is a mechanochemical enzyme that is capable of exerting a force of about 14 pN (Yin et al., 1995), almost 10 times greater than the force exerted by the cytosolic motor proteins. Thus, if the eukaryotic polymerases are similar we can assume that the nucleus is extremely viscous and the physical stresses that may be important for gene expression exist in the nucleus.

Promoters are sequences of DNA, upstream from the transcribed region of the gene, which regulate the binding of RNA polymerase necessary for transcription. Some

promoters cause the frequent initiation of transcription while others are more pokey. As long as the position of the gene allows its transcription, the efficiency of transcription depends on the sequence of the promoter. RNA synthesis involves the opening of the two DNA strands to form an open complex. The RNA polymerase works by extending the newly formed polymer in the 5′ to 3′ direction. The RNA polymerase continues to add the ribonucleotides at a rate of about 30 nucleotides per second until it reaches a termination or stop signal (Cook, 1990). Once it reaches the stop signal, the newly synthesized RNA and the DNA separate from the RNA polymerase.

Transcription can be visualized in detergent-treated nuclei with the electron microscope. Among the normal nucleosome configuration of the chromatin, other globular particles are observed. These particles are RNA polymerase complexes that are attached to a trailing RNA molecule. Usually, RNA polymerase particles are seen as single units, indicating that most genes are transcribed infrequently into RNA so that one polymerase finishes before another one begins. However, sometimes many RNA polymerase particles can be seen attached to a gene, indicating that it is transcribed at a high frequency.

The majority of the RNA transcripts synthesized by RNA polymerase II are called *heterogenous nuclear RNA* (hnRNA). The 5′ end of an hnRNA transcript is immediately capped by the addition of a methylated guanine. Capping occurs after approximately 30 nucleotides have been polymerized. The methylguanosine cap may facilitate RNA export from the nucleus (Hamm and Mattaj, 1990; Hamm et al., 1990). The 3′ end of the hnRNAs is characterized by a poly-A tail. There are two sequences that signal the cleavage of the 3′ end. One sequence, AAUAAA, is on the RNA and signals that the RNA should be cleaved at a site 10–30 nucleotides downstream from this sequence, and there is another sequence that is downstream of the cleavage site. The poly-A tail, which includes 100–200 adenylic acid residues, is added by a separate poly-A polymerase to the 3′ end after cleavage. The poly-A tail regulates translation of the RNA transcript (Jackson and Standart, 1990). The newly made hnRNA associates with proteins to form spherical particles about 20 nm in diameter. These heterogenous nuclear ribonucleoprotein particles can be isolated and treated with ribonucleases at a concentration that just breaks the linker RNA.

The processing of hnRNA into messenger RNA requires the removal of long sequences, known as *introns*, from the middle of the RNA molecule (Gilbert, 1978). The introns can be visualized in the electron microscope using the R-loop technique. With this technique, mRNA is isolated and purified from a cell. This RNA is then mixed with a cloned double-stranded DNA from a genomic library. The mRNA displaces one of the strands of DNA where their sequences match, and thus forms a DNA–RNA duplex. In the regions of the DNA where no matches with the mRNA occur, loops of double-stranded DNA form. The loops represent introns and the bound regions represent exons, sequences present in both the DNA and mRNA.

Processing involves the cutting out of introns and the annealing of exons in a process known as *RNA splicing*. Splicing occurs in spherical particles approximately 40–60 nm in diameter known as *spliceosomes* (Reed et al., 1988). A spliceosome contains several small nuclear ribonucleic acids (snRNAs), which have sequences complementary to those near the splice site, as well as a host of small ribonuclear protein particles (snRNPs), which facilitate the removal of introns. In some cases, the splicing may be accomplished by the nucleophilic attack by the 2′OH group of an adenosine in the intron on the 5′-phosphate of an exon. This step breaks the RNA molecule. The 3′OH produced by the first step then performs a nucleophilic attack on the 3′ phosphate of the adjacent exon. This step results in the release of the intron and the ligation of the two exons. The functions of many of the spliceosome proteins and snRNAs have been tested by reconstituting spliceosomes in vitro (Cech, 1986; Gall, 1991; Guthrie, 1996). Splicing, known as trans-splicing, can even occur between two separate RNA molecules. Trans-splicing occurs in 10–15 percent of the mRNAs of *C. elegans*, and further supports the contention that a gene is not necessarily a single linear sequence of DNA.

Contrary to the central dogma, which states that information flow moves linearly from DNA to RNA to protein, RNA can also influence the expression of DNA. The realization that RNA can influence the expression of DNA came from deeply analyzing the unexpected results that plant biologists were obtaining when they transformed plants with gene sequences with apparently known functions (Dougherty and Parks, 1995). While overexpression of sense mRNA was supposed to increase the expression of a particular gene product and the expression of antisense RNA was supposed to decrease its expression, in many cases, both strategies seemed to downregulate the expression of the gene. This was particularly evident in the attempts to enhance the colors of flowers that resulted in colorless flowers (see Chapter 7; Napoli et al., 1990; van der Krol et al., 1990). De Carvalho et al. (1992) suggested that the suppression of gene expression caused by overexpression of sense RNA may occur at the posttranscriptional level.

The fact that plants transformed with sense RNA for a viral protein became resistant to the virus suggested that the plant cell could recognize too much RNA, incapacitate the viral RNA, and prevent it from replicating in the cell (Lindbo et al., 1993). But how?

Plant cells have a mechanism to eliminate RNA viruses using an RNA-dependent RNA polymerase (Schiebel et al., 1993a,b). This polymerase randomly copies RNA, making 10–75 nucleotide copies. These small RNAs bind to other RNAs in the cell and target their demise (Dougherty and Parks, 1995; Baulcombe, 2008).

The fact that microRNAs, or miRNAs, have the ability to regulate development and physiology naturally was discovered in *C. elegans* (Lee et al., 1993; Reinhardt et al., 2000). MicroRNA is formed when RNA is transcribed in the nucleus by polymerase II from regions of DNA that do not code for proteins—the so-called junk DNA. These RNAs are then processed by a ribonuclease III–like nuclease to form miRNAs, which are approximately 18–25 nucleotides long (Kurihara and Watanabe, 2004). In the cytosol, these miRNAs pair with complementary regions of mRNA to inhibit their translation or target their degradation by an enzymatic process that utilizes enzymes known as *dicer* and *slicer* (Fire, 2006; Mello, 2006). Natural miRNAs that are capable of regulating development were discovered in plants by noticing that mutants that show an overproliferation of meristems, loss of polarity, or conversion of determinant to indeterminate structures had reduced levels of mRNA (Carrington and Ambros, 2003; Bonnet et al., 2004; Yoo et al., 2004; Sunkar and Zhu, 2004). MicroRNAs are also important in the regulation of nutrient uptake (Grennan, 2008).

Using a technique known as *posttranscriptional gene silencing, quelling*, or *RNA interference* (RNAi), double-stranded microRNAs can be used as a tool to test the function of a given gene product. This is done by treating a cell with a double-stranded miRNA with a given sequence so that it will silence or interfere with any gene that contains the complementary sequence to either strand. In this way, artificial miRNAs have been very successful in testing the function of a given gene product in a given response (Iseki et al., 2002). RNA interference techniques are a good way to test the importance of a particular class of gene products that are encoded by a family of genes and high-throughput methods are under way to test the function of many classes of genes in many responses (Waterhouse and Helliwell, 2002).

The fate of the primary RNA transcripts can be followed by treating the cells with a pulse of tritiated uridine and following its fate. While the primary RNA transcripts are about 6000 nucleotides long, the mature mRNA molecules are about 1500 nucleotides long. The posttranscriptional processing of the hnRNA takes about 30 minutes. After 30 minutes, the transcripts begin to leave the nucleus, although only about 5 percent of these newly synthesized mRNA ever leaves the nucleus and the rest are degraded. Let us not always assume that natural selection acts to make every process in the cell efficient. Natural selection, like collective bargaining, must depend on compromise.

The mature mRNA is recognized by the nuclear pore complex and transported to the cytoplasm. The spliceosome proteins are stripped off in the nucleus and are never found in abundance in the cytoplasm. The spliceosome may contain special targeting sequences that directly target it to the nuclear pore. The mature mRNA is transferred from the spliceosome to the nuclear pore from which it is transported out of the nucleus to the cytoplasm where it is translated (Goldfarb and Michaud, 1991). The mRNA may move along the cytoskeleton to specific locations in the cytoplasm with the aid of cellular motors (Sundell and Singer, 1991).

Differentiated cells contain only a portion of the proteins the genome is capable of producing. Therefore, gene expression is selective. In a given cell, only a portion of the DNA is transcribed into functional mRNA (transcriptional control). Even if the gene is transcribed, only a small proportion transcribed into RNAs survives the RNA-processing steps (RNA-processing control). Perhaps only some of these mRNA are transported out of the nucleus (RNA-transport control), and once these mRNA get to the cytoplasm, they must be translated. This step too is regulated (translational control). Even if the protein is synthesized, it may need to be covalently modified or activated by ions (posttranslational control) in order to act, and if it is an enzyme, its substrates must be available. Thus, many steps are involved in regulating the expression of a gene and its product.

Selectivity requires the differential presence of only a few regulatory proteins (Singh, 1998). If one initial cell gives rise to two cells that differ in only one gene regulatory protein, and these cells divide and give rise to daughter cells, which differ in another protein, then the four cells will each have a different complement of proteins. According to this scheme, 10 different regulatory proteins can determine the differentiation of more than 1000 cell types, including the parental cells. Of course, as long as cells have the appropriate receptors (see Chapter 12), variations in extrinsic factors, including light, hormones, oxygen, gravity, and tissue stress, can result in differential gene expression.

16.9 NUCLEOLUS AND RIBOSOME FORMATION

A eukaryotic cell must synthesize about 10 million copies of each ribosomal RNA molecule to make the 10 million ribosomes it will need throughout its life. This is facilitated by the fact that a cell contains multiple copies of ribosomal genes that reside in a cluster known as the *nucleolar organizer region*. The nucleoli, in which the ribosomes are made (Perry, 1969), form in this region at the end of mitosis and disappear again at prophase. The transcription of rRNA genes can be easily visualized in spread chromatin using the electron microscope (Figure 16.11). The transcription of these genes by RNA polymerase I looks like a Christmas tree. The tip of the tree represents the point where transcription is initiated. The base of the tree is created by the detachment of the RNA polymerase and its transcript.

Each gene produces the same primary transcript, known as *45 S rRNA*, which is about 13,000 nucleotides long. The

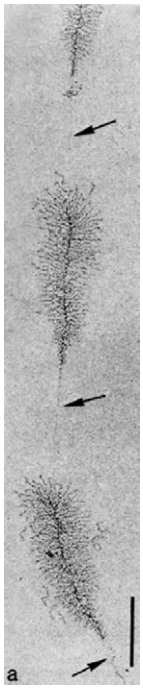


FIGURE 16.11 Transcriptional unit of rDNA of a nucleolus isolated from *Acetabularia cliftonii*. Arrows indicate the spacer regions. Bar, 1 μm. (*Source:* From Franke et al., 1976.)

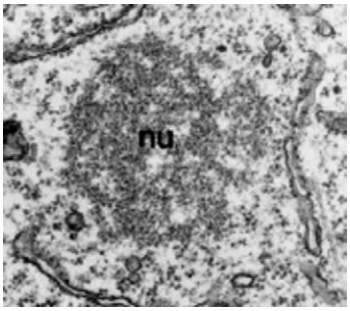


FIGURE 16.12 Electron micrograph of a nucleolus (nu) of *Funaria hygrometrica* that has been extruded intact into the cytoplasm. ×35,300. (*Source:* From Conrad et al., 1986.)

45 S transcript is cleaved to yield one copy of 28 S rRNA, one copy of 18 S rRNA, and one copy of 5.8 S rRNA. The remaining part of the primary transcript is degraded in the nucleus. The splicing of rRNA involves a nucleolus-specific small nucleoprotein particle (Fisher et al., 1991).

Ribosome formation requires a fourth rRNA, the 5 S rRNA. It is transcribed from another set of tandemly arranged genes. This rRNA is synthesized by RNA polymerase III. It is not known why this rRNA is in a separate cluster and transcribed separately. The 5 S rRNAs are made outside the nucleolus and imported into the nucleolus for ribosome formation.

The 28 S, 5.8 S, and 5 S transcripts combine with about 50 polypeptides to form the large 60 S ribosomal subunit. The 18 S transcript combines with about 30 polypeptides to make the 40 S ribosomal subunit. The large 60 S and the small 40 S subunits form the complete 80 S ribosome. The packaging of the rRNA with the polypeptides takes place in the nucleus, in a specialized structure called the *nucleolus* (Warner, 1990; Hernandez-Verdun, 1991). The nucleolus is not surrounded by a membrane and is topologically part of the nuclear P-space. However, it appears to contract, expand, and undergo amoeboid movement, indicating it has different matrix properties than the nucleus (Figure 16.12).

The nucleolus, which is formed by the rRNA genes, contains large loops of DNA from several chromosomes that contain the 45 S rRNA genes (Brown and Shaw, 1998). If an rRNA gene is added to a genome, a new nucleolus forms (Karpen et al., 1988). If a nucleolar organizing center is ablated with a laser, the remaining nucleolar organizing centers tend to exhibit higher activity, indicating that a nucleolus is capable of sensing the absence of another one, and compensating for the loss (Berns et al., 1981). The increased activity of the remaining nucleolar organizing region may also be the result of an increased concentration of substrate due to the decreased competition for nucleotides.

Ribosomes are also composed of proteins (Liljas, 1991), which enter the nucleolus from the cytoplasm. These proteins have a specialized signal (Underwood and Fried, 1990) in addition to the usual nuclear import signal (Hernandez-Verdun, 1991). The joining of the ribosomal polypeptides with the primary rRNA transcript can be seen in the electron microscope. The protein–rRNA combination appears as a large particle at the 5′ end of ribosomal RNA transcript.

Currently, the issue of exactly where rRNA transcription occurs is unresolved. Scheer and Benavente (1990) think that transcription takes place in the pale-staining fibrillar region of the nucleolus because this region is stained by anti-polymerase I antibodies (Scheer and Benavente, 1990). However, Hernandez-Verdun (1991) believe that the polymerases that are detected by the antibodies can only be in the free form and are not attached to DNA and are not actively involved in transcription. Thus, Hernandez-Verdun (1991) and Jordan (1991) believe that the dense fibrillar regions are involved in transcription. This is a fascinating argument because we do not know whether or not the antibody competes with the substrate, and so either interpretation is valid (Fisher et al., 1991; Jordan et al., 1992). Work that is more recent suggests that the lightly staining fibrillar region may be the site of transcription of rRNA. Using in situ hybridization techniques, Shaw et al. (1993) performed experiments that show that there is an inverse relationship between transcriptional activity (determined by the amount of antisense rRNA bound) and the amount of dense fibrillar regions in the nucleolus. Thus, they believe that transcription does not take place in the densely staining fibrillar regions. Also using in situ hydridization, Olmedilla et al. (1993), using tritiated uridine, found that rDNA was

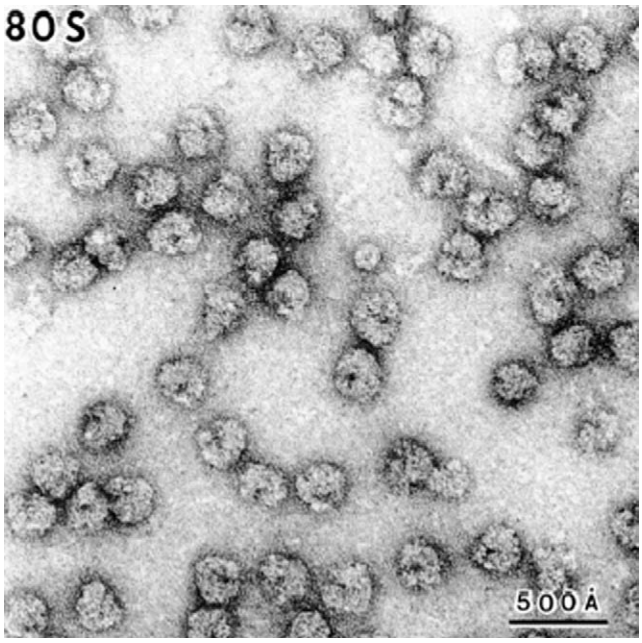


FIGURE 16.13 Electron micrograph of negatively stained 80 S ribosomes from *Dictyostelium discoideum*. (*Source:* From Boublik, 1987.)

synthesized in the dense fibrillar regions. By looking at the incorporation of bromo-UTP and observing it with fluorescence microscopy, Dundr and Raska (1993), Hozak et al. (1994), Melcak et al. (1996), and Thompson et al. (1997) have shown that rRNA synthesis takes place in the dense fibrillar region. On the whole, it seems that rRNA is synthesized by RNA polymerase I in the dense fibrillar regions.

The accumulation of the two subunits of the ribosome gives certain regions of the nucleolus a granular appearance. Pulse chase experiments with tritiated uridine show that the process of ribosome formation and export to the cytoplasm takes about 60 minutes.

The last step in ribosome maturation takes place in the cytoplasm, making it impossible for functional ribosomes to come in contact with the hnRNA in the nucleus. In Chapter 17, I discuss the involvement of the ribosomes (Figure 16.13), tRNA, and mRNA in protein synthesis.

16.10 SUMMARY

The nucleus is the organelle that contains the DNA. The DNA molecule, which is the only molecule that is unique in every living being, contains the hereditary information that is passed from generation to generation, and contains the information necessary for the development of each living being. Each cell has a certain potential that is determined by the genes that are expressed in that cell. However, there is not necessarily a direct precursor-product relationship between a gene, as defined as a DNA sequence, and a trait. There are many factors that determine which genes are ultimately expressed. These factors may include the three-dimensional position of the gene in the genome and in the nuclear matrix, the presence of regulatory factors including protein transcription factors and microRNAs, the activity of the spliceosomes, the ability of the mRNA to recognize and be transported through the nuclear pores, the movement of the mRNA through the cytosol to specific sites, the translation of the mRNA at these sites, and the pH, Ca^{2+}, protein kinase, and phosphatase activities as well as protease activity at these sites that may modify the activity of the gene product. Thus, there are more than a few processes to elucidate before we are able to understand the role DNA plays in determining the individuality of a cell or an organism (Chargaff, 1997).

16.11 QUESTIONS

16.1. What is the evidence that the nucleus is the organelle involved in heredity and development?
16.2. What are the mechanisms by which the nucleus and its constituents participate in heredity and development?
16.3. What are the limitations of thinking about the nucleus as the sole regulator of heredity and development?
16.4. What are the limitations of thinking about the sequence of DNA as the sole means of directing heredity and development?

Chapter 17

Ribosomes and Proteins

17.1 NUCLEIC ACIDS AND PROTEIN SYNTHESIS

With the realization that there was a division of labor in cells, and that the chromatin in the nucleus carried the instructions for many vital processes that took place in the cytoplasm, the question arose of how the genetic material coded for the proteins was involved in these processes (de Vries, 1889; Beadle and Tatum, 1941a,b; Tatum and Beadle, 1942; Dounce, 1952; Beadle, 1945, 1955, 1958; Tatum, 1958; Beadle and Beadle, 1966; Horowitz, 1985; Perkins, 1992). Immediately following the unveiling of the double-helix model for the structure of DNA by Watson and Crick, George Gamow (1954) suggested that amino acids may bind directly to the DNA double helix in a lock-and-key manner that is specified by the sequence of three bases, and thus the DNA would directly act as a template for protein synthesis. While the proposal that a sequence of three out of four bases would be required to code for each of the 20 amino acids was correct; the idea that DNA acted directly as the template had already been shown to be untenable by cytological data.

In the 1930s and 1940s, Jean Brachet and Torbjörn Caspersson established that RNA is correlated with protein synthesis. Caspersson (1950) used ultraviolet (UV) microspectrophotometry combined with ribonuclease digestion to localize the RNA in the cell. In complementary experiments, Brachet (1957, 1985) observed the distribution of RNA by staining cells that had been treated with or without ribonuclease with the basic dye methyl green-pyronine. They both found that RNA was most abundant in cells that were rapidly growing, like onion meristematic cells or the imaginal discs of *Drosophila* larvae, and further studies indicated that there was a correlation between RNA quantity and protein synthesis. That is, in a given organism, RNA was abundant in cells that synthesized large quantities of protein, but was scarce in cells that did not. The DNA amount, by contrast, was the same in all of the cells of the organism. Moreover, when cells were challenged with various physiological conditions that affected their rate of protein synthesis, the quantity of RNA changed in parallel with the rate of protein synthesis. By contrast, the DNA quantity remained constant.

The lack of a direct connection between DNA and protein synthesis was underscored by microsurgical experiments in which the nucleus was removed from the cell. The DNA-containing nucleus can be readily removed from a variety of cell types, including the giant alga *Acetabularia* (Hämmerling, 1953; Brachet, 1957). The enucleated stalks of *Acetabularia* live for several weeks and are capable of synthesizing proteins. They even undergo partial regeneration and differentiation of the cap. When a nucleus of another species of *Acetabularia* is inserted in the enucleated cell, the cap is completely regenerated although it has characteristics of the two species. Thus, substances remained in the enucleate cell that were products of the nucleus, but stand between gene and character. Some evidence, although it was not compelling, suggested that this substance was RNA (Brachet, 1957, 1985).

It seemed reasonable to biochemists that the template for proteins should be at least as large as the protein, and the only possible candidates for the template present in the protoplasm of all cells was protein, DNA, and RNA. In 1952, Alexander Dounce (1952) proposed a mechanism whereby RNA functioned as a template for protein synthesis. In his model, three adjacent nucleotides encoded for each amino acid. Dounce went on to say that a three-letter code could account for all the amino acids known to occur in proteins, and moreover, if there is "complete freedom of choice in arranging the order of four nucleotides … a sufficient number of nucleic acids could theoretically exist to account for the large variety of proteins in nature." It was unlikely that protein was the template for making more protein according to Dounce (1956), who wrote,

> *My interest in templates, and the conviction of their necessity, originated from a question asked me on my Ph.D. oral examination by Professor J.B. Sumner. He enquired how I thought proteins might be synthesized. I gave what seemed the obvious answer, namely, that enzymes must be responsible. Professor Sumner then asked me the chemical nature of*

enzymes, and when I answered that enzymes were proteins or contained proteins as essential components, he asked whether these enzyme proteins were synthesized by other enzymes and so on ad infinitum.

As I previously described, DNA did not seem to be the direct template, and further experiments supported this conclusion. When cells were treated with X-ray or UV irradiation, DNA synthesis was inhibited, but these treatments had little effect on RNA or protein synthesis. On the other hand, treatments that interfered with RNA, including the addition of analogues of uracil, inhibit protein synthesis in vitro (Spiegelman, 1956). However, these in vitro experiments, where DNA or RNA were either selectively added or removed from broken cells, had ambiguous effects on protein synthesis (Spiegelman, 1956; Gale, 1957).

As I previously mentioned, Watson and Crick's (1953a) paper on the structure of DNA stimulated George Gamow to wonder how the information coded in DNA in the form of a sequence of four different bases could be translated into a sequence of 20 amino acids that make up a protein (see Gamow and Ycas, 1967; Gamow, 1970). While Gamow (1954) proposed a triplet code, it was not clear if the code was overlapping or not. Taking a theoretical approach to determine whether or not the code was overlapping required the best computers of the day (Gamow and Metropolis, 1954). Many mathematically inclined scientists from the Manhattan Project, including Richard Feynman, Nicholas Metropolis, Stanislaw Ulam, Edward Teller, and John von Neumann, contributed to cracking the code (Gamow et al., 1956). Gamow and Ycas (1955) used the computer at Los Alamos to perform a statistical analysis that compared the relative abundance of nucleotides in RNA with the relative abundance of amino acids in proteins, much as one would compare the relative abundance of letters in a code to determine which ones were vowels. They concluded from this analysis that there was a triplet code, and it was not overlapping.

Gamow and his colleagues provided further support for a nonoverlapping code by studying the sequence of amino acids in proteins, and concluded that there were more combinations of adjacent amino acids possible than could be accounted for by an overlapping code (Gamow et al., 1956). After rejecting all possible overlapping codes, Gamow and Ycas (1955) wrote, "Thus it appears more probable that the number of determining nucleotides exceeds by a factor of 3 the number of amino acid residues in the synthesized protein, so that neighboring residues do not share determining nucleotides." Brenner (1957) and Crick et al. (1957, 1961) concurred with the concept of a three-base, nonoverlapping code. The fact that hemoglobin from people with sickle-cell anemia only differed by one amino acid from the wild-type hemoglobin (Pauling et al., 1949) supported the idea that the code was not overlapping. Nevertheless, experimental support for a nonoverlapping code came from studies that showed that mutagenesis of tobacco mosaic viruses usually causes a single amino acid substitution in any given part of the protein (Tsugita and Fraenkel-Conrat, 1960). If the code were overlapping, it would be likely that two or more adjacent amino acids would be substituted in each mutant.

Progress in understanding the relationship between nucleic acids and proteins advanced along another tact when Albert Claude (1943a,b) used differential centrifugation to isolate the microsome fraction from cells (see Chapter 4). He found that this fraction contained approximately 40–50 percent of the total cellular RNA, and also stained with basic dyes. Claude concluded that the ribonucleic acid was responsible for binding the basic dyes, and thus the microsomes were the basophilic substance seen by cytologists. The importance of the microsome fraction in protein synthesis was demonstrated by Boorsook et al. (1950) and Elizabeth Keller (1951), who injected rats with radioactive leucine and found that the microsomal fraction contained the highest specific activity. Palade (1955, 1958) suggested that the small particulate components on the endoplasmic reticulum (ER), and not the ER itself, were the cause of the basophilic staining observed by the cytologists, and consequently may be involved in protein synthesis.

These particles that were known as the small particulate component, Palade's granules, opaque particles, ergastoplasmic particles, and ribonucleoprotein particles were given the "pleasant-sounding name," ribosome at the first symposium of the Biophysical Society (Roberts, 1958). Ribosomes can be found attached to the ER, nuclear envelope, actin cytoskeleton (Yang et al., 1990; Davies et al., 1991; You et al., 1992; Durso et al., 1996), or free in the cytosol.

17.2 PROTEIN SYNTHESIS

Many reactions in the cell are reversible, and, in their search for simplicity, Max Bergmann and Joseph Fruton postulated that the well-characterized enzymes involved in protein degradation would also prove to be involved in protein synthesis, although during protein synthesis the reaction would run backwards (van't Hoff, 1903; Bergmann and Fruton, 1944; Linderstrøm-Lang, 1952; Brachet, 1957; Fruton, 1955, 1957; Fruton and Simmonds, 1958; Zamecnik, 1960). These experiments did not involve the use of a linear template, and consequently highly branched proteinlike molecules were synthesized.

With the discovery of ^{14}carbon, and a means to produce it in copious amounts (Ruben and Kamen, 1940), biochemists had a way of studying protein synthesis in preparations that contained high background levels of protein. Unfortunately, the early in vitro studies using radioactive alanine and glycine gave ambiguous results, since the amino acids were not only incorporated into protein, but participated in intermediary metabolism, including the glucose-alanine cycle, gluconeogenesis, purine synthesis, and porphyrin synthesis

(Siekevitz, 1952; Zamecnik, 1969; Elliott and Elliott, 1997). As unreliable as these experiments turned out to be, they suggested that adenosine triphosphate (ATP) may be important for protein synthesis, since amino acid incorporation required oxygen and was inhibited by dinitrophenol.

In vitro experiments on protein synthesis became more productive after Elizabeth Keller introduced radioactive leucine, because leucine is not involved in as many nonprotein-synthetic pathways as alanine and glycine. Moreover, leucine is abundant in proteins (Keller et al., 1954). The incorporation of radioactive leucine into protein was inhibited by ribonuclease, indicating that RNA is important for protein synthesis. Leucine incorporation also required ATP (Zamecnik and Keller, 1954). Fritz Lipmann (1941) had already suggested that amino acids would not readily react with each other to form peptide bonds unless they were activated, and suggested that ATP may be involved in the activation of amino acids. Indeed, Mahlon Hoagland, Elizabeth Keller, and Paul Zamecnik (1956) showed that when $^{32}P_iP_i$ was added to the soluble fraction of liver, ^{32}P-ATP was formed, and this exchange reaction was stimulated by adding amino acids. The active component in the soluble fraction was probably a protein since it was heat-labile, nondialyzable, and could be precipitated at pH 5. While Hoagland et al. were running the reaction backwards for the convenience of assaying the enzyme, they concluded that the enzyme in the pH 5 fraction actually activates an amino acid by hydrolyzing ATP, and forming an enzyme-aminoacyl-AMP complex and P_iP_i (Hoagland, 1990). Competition experiments showed that there was a different enzyme to activate each amino acid. These enzymes are now known as aminoacyl-tRNA synthetases. Each aminoacyl-tRNA synthetase is specific for a particular amino acid (Moras, 1992).

Thus, Zamecnik's group discovered that protein synthesis could take place in vitro, as long as ATP and the pH 5 fraction were added to the microsomes. Or could it? It turned out that when purified ATP was used in place of the crude source, amino acids were not incorporated into protein (Zamecnik, 1969). Keller and Zamecnik (1956) discovered that guanosine triphosphate (GTP) or guanosine diphosphate (GDP), in addition to pure ATP, was necessary for protein synthesis, and thus the crude source of ATP must have contained guanylate nucleotides. I will discuss the function of GTP below.

Littlefield et al. (1955) and Littlefield and Keller (1957) discovered that a ribonucleoprotein particle can be separated from the rest of the microsomal fraction by treating the microsomes with sodium deoxycholate, and centrifuging the mixture at about 100,000 g. The ribonucleoprotein particles that were in the precipitate were capable of performing protein synthesis in the presence of ATP and GTP. The fact that a large volume of unlabeled amino acids added at the end of the assay did not reduce the amount of label incorporated into protein indicated that ATP- and GTP-dependent protein synthesis in the ribonucleoprotein particles was irreversible.

Robert Holley (1957, 1968) wondered: If activation of the amino acids was the first step in protein synthesis, what was the second? He surmised that the enzyme-aminoacyl-AMP complex might bind to a molecule X forming an aminoacyl-X complex and releasing the enzyme and AMP. Holley assumed that the two reactions could be described like so:

$$\text{enzyme} + \text{amino acid} + \text{ATP} \Leftrightarrow \text{enzyme-aminoacyl-AMP} + P_iP_i$$

$$\text{enzyme-aminoacyl-AMP} + X \Leftrightarrow \text{aminoacyl-X} + \text{enzyme} + \text{AMP}$$

Holley conjectured that these two reactions were reversible and coupled, and thus he could discover X by adding various fractions to the assay system. The assay system included the pH 5 precipitate (Hoagland et al., 1956), alanine as the amino acid, and radioactive AMP. Holley fractionated the cytosol using a column as tall as the USDA building on the Cornell University campus, in which he was working. Holley assayed various soluble fractions for X by running the coupled reactions in the presence of radioactive AMP. He found a soluble fraction that catalyzed the alanine-dependent formation of radioactive ATP. ATP formation was inhibited by RNAse, indicating that the intermediate X was a soluble ribonucleic acid. These results were extended by Hoagland et al. (1957, 1958) and Ogata and Nohara (1957) for the activation of leucine and alanine, respectively. The soluble RNA was eventually given the name transfer RNA, which is abbreviated to tRNA.

Apparently unaware of much of the data described above, Francis Crick (1958) came up with the "Sequence Hypothesis" and the "Central Dogma." In the hypothesis and the dogma, Crick said that the sequence of nucleic acids codes for the sequence of amino acids in a protein, and information is passed from nucleic acid to nucleic acid or from nucleic acid to protein, but never from protein to nucleic acid. The ribosomes contain an RNA that acts like a template for protein synthesis. The amino acids are carried to the template by small RNA molecules, which he called adapters. Crick (1958) went on to say,

> *It will be seen that we have arrived at the idea of common intermediates without using the direct experimental evidence in their favour; but there is one important qualification, namely that the nucleotide part of the intermediates must be specific for each amino acid, at least to some extent. It is not sufficient, from this point of view, merely to join adenylic acid to each of the twenty amino acids. Thus one is led to suppose that after the activating step, discovered by Hoagland … , some other more specific step is needed before the amino acid can reach the template.*

Crick finished this paper with the following: "I shall be surprised if the main features of protein synthesis are not discovered within the next ten years." When I first read Crick's 1958 paper, I was under the mistaken impression that this

paper formed the foundation of the field of protein synthesis. Now I realize that it, like Watson and Crick's (1953a) paper, in which they wrote that their theoretical model of DNA "must be regarded as unproved until it has been checked against more exact results" and that they "were not aware" of Franklin's more exact results, did not present an accurate version of the history of science.

It was initially assumed that the RNA in the ribosomes provided the template for protein synthesis. This came to be known as the "one gene-one ribosome-one protein hypothesis." However, evidence accumulated that indicated that the ribosomal RNA did not function as a template, and there may be another kind of RNA that acts as an intermediate between the DNA and proteins. The first evidence came when Volkin and Astrachan (1956) discovered that when *E. coli* were infected with the T2 phage in the presence of ^{32}P, the radioactive phosphorous was immediately incorporated into RNA. Although they did not know the function of this RNA, they postulated that it may be a new species since its base composition was different than that of the average RNA in the cell. By 1958, Astrachan and Volkin tentatively suggested that this RNA may be involved in protein synthesis. Using yeast, Ycas and Vincent (1960) found that the RNA that was newly labeled had a base composition that was very similar to yeast DNA, except that in the RNA, uridylic acid replaced the thymidylic acid in DNA.

Further evidence hinted at the possibility that there was a transient RNA intermediate involved in gene expression. In a series of experiments, known in unromantic jargon as the "blender experiments," Riley et al. (1960) allowed bacteria to mate, and disrupted the mating process at various times. They found that enzyme formation began in the recipient bacterium within 2 minutes of the time of injection of a gene from the donor bacterium. Riley et al. felt that this was too short of a time to build a ribosome, which is what would be expected according to the one gene-one ribosome-one protein hypothesis. They suggested that the gene produced an unstable RNA intermediate in the recipient bacterium that moved to the preformed ribosomes and acted as a template for protein synthesis. Jacob and Monod (1961) dubbed this unstable RNA, which accounts for about 1–5 percent of the total cellular RNA, messenger RNA, or mRNA. Ribosomal RNA (rRNA) makes up the majority of the cellular RNA. Subsequent studies showed that rapidly labeled mRNA molecules could be found in the ribosomes (Brenner et al., 1961; Gros et al., 1961; Loening, 1962; Risebrough et al., 1962; Watson, 1963).

Meanwhile, Marshall Nirenberg and his associates (Nirenberg and Matthaei, 1961) added synthetic poly-U RNA to a cell-free protein synthetic system and discovered that the RNA acted catalytically as a template in the formation of polyphenylalanine. This synthetic mRNA also proved helpful in determining that aggregates of ribosomes or polyribosomes typically bound to the mRNA (Barondes and Nirenberg, 1962; Spyrides and Lipmann, 1962). However, the big breakthrough with synthetic RNA came when Nirenberg and his colleagues (Nirenberg and Leder, 1964; Bernfield and Nirenberg, 1965), as well as Gobind Khorana (1961, 1968, 1976) and his associates, used synthetic mRNAs to crack the genetic code. Nirenberg's group added synthetic RNAs to a cell-free system and analyzed which ^{14}C-aminoacyl-tRNAs bound to the ribosomes. When they used a synthetic poly-U message, the phenylalanine-tRNA bound. Likewise, lysine-tRNA and proline-tRNA bound when poly-A and poly-C, respectively, were used as the synthetic message. Khorana's group synthesized RNA with various known sequences of repeating dinucleotides, trinucleotides, and tetranucleotides, and analyzed the resultant polypeptides that were formed until they figured out the codes for each amino acid. In this way, the two groups cracked the genetic code.

Work on the structure of tRNA continued during this miraculous decade of research in molecular biology, and by 1965, Robert Holley and his associates developed techniques of nucleic acid sequencing and sequenced the entire alanine tRNA from yeast. One end of the tRNA contains the terminal adenine, and binds to the carboxyl group of the amino acid in an exchange reaction that released AMP and the aminoacyl-tRNA synthetase. The OH group of the COOH of the amino acid binds to the 3′-OH group of the ribose. Another region of the folded tRNA molecule contains a sequence of three nucleotides, known as an anticodon, which is complementary to and thus can bind with the three-nucleotide codon of an mRNA.

Thus, during gene expression, the DNA coding for a gene is transcribed into RNA, and after this RNA is processed, the subsequent mRNA leaves the nucleus and attaches to a ribosome in the cytosol. The small subunit of the ribosome contains one binding site for mRNA and two for tRNA. One tRNA binding site is called the peptidyl-tRNA binding site (P-site). This site holds the tRNA that is linked to the growing end of the polypeptide chain. The other tRNA binding site is called the aminoacyl-tRNA binding site (A-site), and it holds the incoming aminoacyl-tRNA. The anticodons of the tRNAs pair with the codons of the mRNA in these sites. The P- and A-sites are so close that the two tRNAs form base pairs with adjacent codons. The pairing of codons and anticodons allows the formation of a polypeptide chain according to the sequence of the mRNA molecule. The tRNAs then act as translators of the genetic code from the nucleic acid sequence to the amino acid sequence from gene to polypeptide.

The initiation of protein synthesis is mediated in the small subunit by a group of polypeptides known as initiation factors. A special aminoacyl-tRNA is required for this step, and in eukaryotic ribosomes it is always methionine-tRNA. The methionine-tRNA first binds to the small subunit of the ribosome where it is put in the correct position in the P-site by a protein known as a eukaryotic initiation factor. *Pari passu*, the small subunit binds an mRNA that it recognizes by the 7-methylguanosine residue at the 5′ end. The small subunit of the ribosome, containing the methionine-tRNA, then moves down the mRNA in search of the

AUG codon (start codon). Then several polypeptides are released from the small subunit and the small and large subunit bind together, thus completing the formation of the ribosome. Protein synthesis continues as the next aminoacyl-tRNA binds to the A-site.

The polypeptide chain then elongates in a cycle that consists of three steps. Firstly, an aminoacyl-tRNA, which is bound to an elongation factor–GTP complex, binds to the vacant A-site. In order for elongation to proceed, the GTP must be hydrolyzed. This will only happen if the GTP is positioned correctly in the A-site for a sufficient amount of time. The GTP will only remain in the A-site for a long enough time if there is perfect matching between the codon and anticodon (at least for the first two nucleotides). Remember from Chapter 12 that

$$k_{\text{off}} = K_d(k_{\text{on}}) \tag{17.1}$$

and

$$\text{the half time of a reaction} = (\ln\ 2)/k_{\text{off}} \tag{17.2}$$

If the match is not perfect (the K_d is too large), and thus the binding strength is not sufficiently great, then the aminoacyl-tRNA complex is released from the ribosome before the GTP is hydrolyzed. If the codon–anticodon matching is correct, the tRNA molecule remains bound to the mRNA and the GDP-elongation factor complex is released from the ribosome.

The requirement for a correct binding before the GTP is hydrolyzed results in a proofreading mechanism and ensures the correct synthesis of a protein (Crick et al., 1957). In the next step of protein elongation, the carboxyl end of the polypeptide chain is uncoupled from the tRNA molecule in the P-site and linked to the amino group of the aminoacyl-tRNA in the A-site to form a peptide bond. This step is catalyzed by peptidyl transferase, which is a ribozyme, an enzyme made of RNA (Noller et al., 1992).

Subsequently, the free tRNA that was in the P-site is released from the ribosome as the peptidyl-tRNA that was in the A-site is translocated to the P-site as the ribosome moves three nucleotides down the mRNA (or the mRNA moves along a stationary ribosome). This translocation is also coupled to the hydrolysis of GTP. Thus, the addition of a single amino acid to a protein requires the hydrolysis of two GTP molecules. Counting the ATP that is necessary to activate the carboxylic acid group of the amino acid before it is attached to the tRNA molecule, the hydrolysis of three nucleoside triphosphates is necessary for the addition of each amino acid to a protein, and consequently, protein synthesis is an energy-intensive process. Soon after the process of protein synthesis was elucidated, the dynamic process was demonstrated in a dance that was filmed in 1971. The film entitled, "Protein Synthesis: An Epic on the Cellular Level" can be seen at http://www.youtube.com/watch?v=u9dhO0iCLww.

The ribosome moves along the mRNA in the 5′ to 3′ direction. A protein is synthesized stepwise from its amino-terminal end to its carboxy-terminal end. The growing peptide moves through a tunnel in the large subunit of the ribosome. This tunnel accommodates approximately 35–39 amino acids as determined by protease-protection assays (Blobel and Sabatini, 1970; Bernaeu and Lake, 1982; Yonath et al., 1987). Approximately 10 amino acids are incorporated into a protein per second, and thus an average protein is synthesized in about 20–60 seconds.

Since the "average" amino acid has a molecular mass of 110 Da, on the average 1.1 kDa of protein is synthesized per ribosome per second. The time it takes to synthesize one protein with a molecular mass, M_r (in Da), is given by the following equation:

$$\text{Time} = M_r/1100\ \text{Da/s} \tag{17.3}$$

Highly expressed proteins are not synthesized by a single ribosome, but by many ribosomes simultaneously (Galau et al., 1977). They appear as polyribosomes or "polysomes" in electron micrographs and density gradients.

Protein synthesis is terminated when the ribosome reaches a stop codon (UAA, UAG, or UGA). Cytosolic proteins known as release factors bind to the stop codon when it is in the A-site. This binding causes the peptidyl transferase to add a water molecule instead of an amino acid to the peptidyl-tRNA, and the carboxyl group of the polypeptide chain is freed from the tRNA and released from the ribosome. The ribosome then releases the mRNA and splits into its two subunits. Protein synthesis is inhibited by cycloheximide and puromycin.

If the amino-terminus of the nascent protein has a signal sequence, the ribosome will be translocated to the surface of the ER; otherwise, it will remain either free in the cytosol or bound to the cytoskeleton. The mitochondria and chloroplasts also have their own ribosomes (Tao and Jagendorf, 1973; Chua et al., 1973, 1976; Bhaya and Jagendorf, 1985; Staehelin, 1986; Friemann and Hachtel, 1988). The ribosomes in the chloroplast and mitochondrion are smaller than the ribosomes in the cytosol, and like the ribosomes in prokaryotes, they are inhibited by chloramphenicol and not by cycloheximide.

The central dogma asserted that all the information necessary for protein folding must be in the amino acid sequence itself that is coded by the gene (Crick, 1958, 1974a). Indeed, this idea seemed likely at the time since, in vitro, many isolated proteins had been denatured, refolded, and renatured spontaneously due to the small difference in free energy between the native and denatured state. However, many and perhaps most proteins require chaperonins to fold correctly in vivo, where the concentration of a protein may be higher than it is in a test tube. Without chaperonins, proteins would bind to each other in a dysfunctional manner in the crowded conditions found in cells (Anfinsen, 1973; Hartl et al., 1992; Ellis, 1996).

Chaperonins have been identified biochemically by adding the putative chaperonin to a solution of denatured enzymes or structural proteins and testing whether or not

there is an increase in the number of functional enzyme molecules or normal protein structures compared to the test tubes in which the proteins were able to self-assemble. By doing such experiments, in turned out that the assembly of significant protein complexes, like nucleosomes and rubisco, required chaperonins. Chaperonins are involved in the folding of newly synthesized proteins as well as in the unfolding and refolding of proteins that are translocated through membranes. Consequently, chaperonins are found in most, if not every, compartment of the cell.

17.3 PROTEIN ACTIVITY

Enzymes are catalysts that accelerate the rate of reactions as long as the free energy of the products is greater than the free energy of the reactants. The free energy released depends on the concentrations of reactants and products, as well as the dissociation constant of the enzyme for the substrates (Michaelis and Menton, 1913; Haldane, 1930; Lineweaver and Burk, 1934). Enzymes catalyze chemical reactions by reducing the activation energy necessary for bringing the substrates together in the correct orientation so that a reaction can take place. Enzymes also reduce the activation energy by creating the right environment in the active site for the reaction to take place. For example, to dehydrate substrates, enzymes create a water-free, hydrophobic pocket out of amino acids, including tryptophan, phenylalanine, glycine, alanine, valine, leucine, isoleucine, proline, and methionine. About one-third of enzymes contain metal ions in their active sites that are able to orient substrates. In order to hold Ca^{2+} in the active site, the enzymes may contain negatively charged amino acids, including aspartic acid and glutamic acid. By contrast, Zn^{2+} and Mg^{2+} are bound by nitrogen-containing amino acids, including asparagine, histidine, and glutamine. Once in the active site, metal ions often act as electrophiles, accepting electrons so that two substrates can bond (Glusker et al., 1999). Enzymes are remarkable in that they work at ambient temperatures. Chemical reactions that take place in the vats of the chemical industry typically require high temperatures and pressures to occur at acceptable rates.

Enzymes can be extremely specific for a given substrate and even discriminate between optical isomers (Czapek, 1911; Euler, 1912; Pfeiffer, 1955). In 1894, Emil Fischer (quoted in Clark, 1952) wrote about enzymes and substrates: "The one may be said to fit into the other as a key fits into a lock." While today we take for granted that the majority of enzymes are proteins, this was a revolutionary idea when James Sumner (1926, 1927) announced that he crystallized urease from jack bean and found that it was a protein. Richard Willstätter (1927) and J. B. S. Haldane (1930) suggested that the real nonproteinaceous enzyme was probably just absorbed to a protein colloid in the crystal and would eventually be separated from it (Sumner, 1933). Eventually all the enzymes that were crystallized turned out to be proteins, and every schoolchild knew that "all enzymes are proteins although not all proteins are enzymes." Revolutionary history repeated itself in the 1980s, when Sidney Altman and Tom Cech independently proved that not all enzymes are proteins—some, in fact, were made out of RNA (Guerrier-Takada et al., 1983; Guerrier-Takada and Altman, 1984; Cech, 1985, 1988; Altmann, 1990; Noller et al., 1992). In whichever chemical class an enzyme belongs, I can empathize with Arthur Kornberg (1989) who wrote, "I never met a dull enzyme."

Crick (1958) wrote, "Watson said to me, a few years ago, 'The most significant thing about the nucleic acids is that we don't know what they do.' By contrast the most significant thing about proteins is that they can do almost anything." Crick went on to say,

> *Biologists should not deceive themselves with the thought that some new class of biological molecules, of comparable importance to the proteins, remains to be discovered. This seems highly unlikely. In the protein molecule Nature has devised a unique instrument in which an underlying simplicity is used to express great subtlety and versatility; it is impossible to see molecular biology in proper perspective until this peculiar combination of virtues has been clearly grasped.*

Proteins are intimately involved in every aspect of cellular life. Proteins form the ion channels and pumps in the membranes; the cytoskeleton and the motors to move organelles through the cell; the enzymes involved in carbohydrate, fat, protein, and nucleic acid metabolism; and energy production. Protein activity is not invariant, but can be regulated by varying the rate of synthesis, degradation, and/or substrate availability. Protein activity can also be regulated through the binding of ions or small molecules (e.g., Ca^{2+}, cAMP, GTP) or the covalent modification of the protein (e.g., phosphorylation/dephosphorylation, acylation, glycosylation, prenylation, and acetylation; Gruhler and Jensen, 2006). These posttranslational modifications cause a change in the charge or hydrophobicity/hydrophilicity of the protein that result in a change in the structure of the protein. These kinds of modifications can control protein activity because the structure of a protein determines its function. It is especially important to understand how the activity of an enzyme that controls the rate-limiting step in a biochemical pathway is controlled (Blackman, 1905).

17.4 PROTEIN TARGETING

Once a protein is synthesized it must be targeted to its correct location in the cell (Blobel and Sabatini, 1971; Blobel and Dobberstein, 1975a,b; Schekman, 1985; Chrispeels and Staehelin, 1992; Schatz and Dobberstein, 1996). This intracellular sorting is accomplished by the presence of a signal peptide or a signal patch (Blobel, 1980). A signal

peptide is a linear stretch of 15–60 amino acid residues and is often but not always removed from the mature protein once translocation into the targeted organelle is completed. Signal peptides direct proteins from the cytosol to the ER, to the vacuole, to the peroxisomes, to the mitochondria, to the chloroplasts, and to the nucleus. A signal patch is a three-dimensional arrangement of atoms on the protein surface that forms once the protein folds up. The amino acids that form this patch may be widely separated from one another in the linear sequence, and they generally remain in the mature protein.

The importance of the individual signal peptides for protein targeting has been demonstrated by putting the signal sequence on another peptide using genetic-engineering techniques (Chua and Schmidt, 1978, 1979; Huisman et al., 1978; Garoff, 1985; White and Scandalios, 1988). The targeting of a protein can then be controlled artificially. The import of the protein into the targeted organelle is then assayed using immunoprecipitation, immunocytochemistry, or reporter molecules like green fluorescent protein (GFP), which can be placed into the transported protein using genetic-engineering techniques that allow the protein of interest to be autofluorescent (Cutler et al., 2000; Tian et al., 2004; Koroleva et al., 2005; Goodin et al., 2007; Shemer et al., 2008; Thompson and Wolniak, 2008). Such experiments clearly show that the peptide is necessary and sufficient for correct targeting. However, they do not discern whether the primary sequence, the secondary structure, or the chemical or physical properties are important for specificity. A variety of signals may target different proteins to the same compartment (Hunter et al., 2007). Each organelle has its own complex that can recognize and translocate the targeted protein (Soll and Schleiff, 2004; Bédard and Jarvis, 2005; Kalanon and McFadden, 2008; Koenig et al., 2008).

17.5 PROTEIN–PROTEIN INTERACTIONS

The binding of or interaction between two polypeptides or two domains of a single protein can be visualized by engineering a fluorescent peptide into each of the two polypeptides of interest. In order to characterize the proximity or change in proximity of the two polypeptides, which may give an indication of the protein activity, the ability of the second fluorescent peptide to fluorescence in response to excitation of the first fluorescent peptide is assayed (Dixit et al., 2006; Shaw, 2006; Goodin et al., 2007). The transfer of energy between the first and second fluorescent peptide is dependent on the sixth power of the distance between the two fluorescent peptides. The transfer is known as Förster (or fluorescence) resonance-energy transfer. The interaction between two peptides can also be monitored using the bimolecular fluorescence complementation technique, whereby one engineers one part of a bioluminescent protein like luciferase into one polypeptide and another part of the same bioluminescent protein into another polypeptide and sees if the two regions are close enough to each other to luminescence under the appropriate conditions (Chen et al., 2008).

Protein–protein interactions can also be analyzed by other techniques, including the yeast two-hybrid system and the split-ubiquitin system (Braun and Schmitz, 2006; Liu et al., 2007; Rahim et al., 2008). In the split-ubiquitin system, two parts of ubiquitin are expressed by two different engineered fusion genes. Each gene is an engineered fusion of a part of the ubiquitin gene, a reporter gene, and a gene for a protein of which the interaction with another protein will be tested. If the two proteins interact, then the ubiquitin parts will be brought close enough together to allow the binding of a ubiquitin-specific protease and the release of the reporter protein. The field of computational cell biology is emerging to quantify and describe the complex interaction of proteins within the cell in space and time (Slepchenko et al., 2002).

17.6 PROTEIN DEGRADATION

The rapid turnover of molecules in biological tissues was inferred by Peter Mark Roget (1834) while he was studying regeneration. However, these observations were forgotten. The introduction of stable and rare isotopes of H, O, N, and S by Rudolf Schoenheimer (1942) made possible the astonishing results that "all constituents of living matter, whether functional or structural, of simple or of complex constitution, are in a steady state of flux." Prior to his work, it seemed reasonable that mature living matter was like a machine that used nutritive substances as fuel to run the machine and the excretions were the part of the food that were not used. After all, most cells and organisms spend most of their life at a constant size. Schoenheimer's work indicated that the living matter was constantly being built up and broken down, and each molecule had a lifetime that was less than that of the cell or organism itself (Schoenheimer and Rittenberg, 1938).

All proteins are eventually degraded by proteases. Proteases exist in many organelles, including the ER, nucleus, mitochondrion, and chloroplast, and in the cytosol as well as in the lysosomal-vacuolar compartment (Dice, 1990; Goldberg, 1990). The lysosomal-vacuolar compartment is probably involved in the degradation of endocytosed extracellular proteins and intracellular proteins under times of stress. Otherwise, most selective degradation probably takes place in the cytoplasm (Ciechanover and Gonen, 1990). The various proteases hydrolyze the substrate proteins into free amino acids. As recycled amino acids, they can participate again in protein synthesis. The activities of some proteases are activated by calcium ions (Moriyasu and Tazawa, 1987; Reddy et al., 1994; Lid et al., 2002; Moriyasu and Wayne, 2004).

All polypeptides in eukaryotes are synthesized with a methionine at the amino-terminal end. The methionine is

often cleaved by a specific aminopeptidase. The amino-terminal amino acid determines the stability of the cytosolic proteins. If the amino-terminal amino acid is Met, Ser, Thr, Ala, Val, Cys, Gly, or Pro, the cytosolic polypeptide will be stable and long-lived. However, if the amino-terminal amino acid is not one of the above amino acids, the cytosolic protein is targeted to be degraded in the ubiquitin-dependent pathway (Rechsteiner, 1987; Ciechanover and Gonen 1990). In this pathway, many molecules of the small protein ubiquitin are covalently attached to the target protein. The ubiquitin-linked proteins are then degraded by an ATP-dependent protease that exists in a multienzyme complex known as the proteasome (Arrigo et al., 1988; Goldberg, 1990; Schliephacke et al., 1991; Vierstra, 1993, 2003; Moriyasu and Malek, 2004; Pickart, 2004). Phytochrome is degraded by this pathway (Shanklin et al., 1987).

17.7 STRUCTURE OF PROTEINS

The primary sequence of a protein is determined by a number of constraints. First and foremost, the catalytic portion must bind the substrate and release the product with appropriate binding constants and reaction rates. Secondly, other sites on the protein must either bind or not bind the regulatory molecules that are present in the cell so that the protein is activated or inactivated at the appropriate times. Thirdly, the protein must be soluble in the compartment that it is active in—for example, membrane (lipid), stroma (pH 8), vacuole (pH 5). Lastly, a protein must have the correct targeting sequences to get it to the correct compartment. Any of these constraints may compete with the other constraints, and consequently cells and organisms may have many variations of a given enzyme. Such varieties of enzymes are known as isozymes. Proteins function in the milieu of a given compartment. Consequently, an enzyme that exists in two very different compartments may need different amino acid sequences to perform identical functions, whereas enzymes in the same compartment may perform very different functions, yet have similar amino acid sequences.

The structure of proteins can be deduced from the X-ray diffraction pattern. In the 1920s, only six years after Sirs William and Lawrence Bragg deduced the structure of sodium chloride and other simple substances, R. O. Herzog and W. Jancke took the first X-ray diffraction photographs of the protein-rich samples of hair, horn, muscle, silk, and tendon. This work was followed up by W. T. Astbury, C. H. Bamford, W. E. Hanby, F. Happey, and Max Perutz (Pauling et al., 1955). The structures of many proteins and protein complexes, including the H^+-ATPase (Kühlbrandt et al., 2002), a K^+ channel (MacKinnon, 2003), the reaction center of a photosystem (Diesenhofer and Michel, 1988; Huber, 1988), and a protein-translocating channel (Van den Berg et al., 2004), are being determined in order to relate their structure to their function and regulation.

17.8 FUNCTIONS OF PROTEINS

There are typically tens of thousands of genes in an organism that encode proteins with various functions. Currently, there is an emphasis on discovering a vast array of mutants that are unable to undergo a given process and guess the function of the protein encoded by the gene of interest using computer databases. Indeed, as technology advances, the rate of discovery of genes postulated to be directly involved in a given process coming from experiments done in silico seems to double each year or two following a biotechnological analogue of Moore's Law for integrated circuits, which states that the number of components in an integrated circuit will double every year or two (Moore, 1965). However, conclusions about the function of a protein drawn from in silico work must be confirmed with conclusions drawn from functional assays done in vitro.

Throughout this book, I have emphasized the importance of well-defined in vitro functional assays in creating the understanding that we have concerning the activity of a given protein in cellular processes, including membrane transport, plasmodesmatal selectivity, secretion, motility, signaling, photosynthesis, glycolysis, respiration, replication, transcription, translation, mitosis, cell plate formation, and growth. I look forward to the day when the identification of mutants is routinely complemented with a well-defined in vitro functional assay of the wild-type and mutated-gene products.

17.9 TECHNIQUES OF PROTEIN PURIFICATION

Plant cells, particularly those involved in protein storage, have provided a rich source for purified proteins (Osborne, 1924; Sørensen, 1925; Chibnall, 1939). These studies demonstrated the variety and individuality of proteins. The proteins, which reside in a primarily water-rich protoplasm, were originally classified by their solubilities. The albumins are soluble in water; the globulins are insoluble in water but soluble in salt solutions; the prolamins are insoluble in water, but soluble in 50–90 percent ethanol; and the glutelins are insoluble in neutral solutions, but soluble in dilute acid or base (Chibnall, 1939; Bidwell, 1974).

In order to study proteins, they should be purified to homogeneity. In order to purify a protein, we must first decide on the source of the protein. Do we want the protein from a particular cell type, or do we want large amounts of protein but do not care which cell type it comes from? Do we think that there may be isozymic differences in the proteins in different cell types, and consequently we must separate the cells before we isolate the protein? Do we want to isolate the protein from a given organelle and therefore must first isolate the organelle? We may also opt to isolate a gene expressed in a given cell type and then express the protein in bacteria using molecular-cloning techniques.

Once we decide on a source, we must solubilize the protein. We must homogenize the organ, tissue, or cell. If we want to isolate a protein from a given organelle, we must use centrifugation and/or aqueous two-phase partitioning to isolate the given organelle or membrane first. While we homogenize the starting material, we must make sure that the protein is stable, so we must include in our homogenization medium a buffer with the correct pH, ion activity, ionic strength, and protease inhibitors. We must perform the purification at temperatures that minimize damage due to proteolysis.

In order to differentially purify a protein to homogeneity, we can precipitate it, chromatograph it, or ultracentrifuge it (Svedberg, 1937; Sumner and Somers, 1943). Starting in 1926, proteins were purified to homogeneity by finding, with great patience, the conditions that allowed them to crystallize out of a solution (Northrup et al., 1948). It is again becoming popular to crystallize proteins and apply biophysical techniques to determine the structure of the protein at atomic resolution. Remember, in order to ensure that we are isolating the intact protein, we must perform a functional assay at each stage of purification (Racker, 1985).

The field of proteomics strives to identify all the proteins in a cell, tissue, or organ at a given point in development or following a treatment (Patterson, 2004a,b; Heazlewood and Miller, 2006; Isaacson and Rose, 2006; Rajjou et al., 2006; Sun et al., 2009). Complex mixtures of hundreds to thousands of proteins can be analyzed by mass spectrometry and their identity determined from the protein sequence predicted by data sets produced as a result of genome sequencing. The proteomic approach has the advantage of listing all the proteins in a given compartment that may interact with each other in order to accomplish a given function. While the various types of mass spectrometers used for proteomic studies are excellent in identifying proteins, particularly in plants of which the genome has been sequenced, it is a fundamental truism that in order to get meaningful data from the analysis, it is necessary to solubilize all the proteins of interest, to protect them from degradation, and to prevent proteins present in unwanted compartments from contaminating the sample.

17.10 PLANTS AS BIOREACTORS TO PRODUCE PROTEINS FOR VACCINES

Certain plants can annoy various people as a result of the production of proteins that function well for the plant but act as allergens that induce an immune response in people (Cosgrove et al., 1997; Yennawar et al., 2006; Valdivia et al., 2007a,b). Charlie Arntzen has long thought of taking advantage of the ability of plants to synthesize immunogenic proteins by using plants as bioreactors to produce vaccines against viruses that plague humans and animals. Arntzen and others have transformed plants and plant cells with genes that encode proteinaceous antigens against a number of viruses, including the Newcastle virus and the hepatitis B virus. The proteinaceous antigens produced by the "plant cell factories" have been purified from whole plants and cultured plants cells and used to immunize humans and animals (Mason et al., 1992, 2002; Raskin et al., 2002; Ma et al., 2003; Walmsley and Arntzen, 2003; Goldstein and Thomas, 2004; Arntzen et al., 2005; Sunil Kumar et al., 2005; Twyman et al., 2005; Vitale and Pedrazzini, 2005; Kim et al., 2007; Pascual, 2007; Santi et al., 2008).

17.11 SUMMARY

Richard Vierstra (2003) describes the life of a protein in terms of birth, taxes, and death. This chapter follows the life history of proteins from their synthesis (birth), through the regulation of their activity (taxation), to their degradation (death), and even to their isolation and characterization. I have described the experiments that showed that nucleic acids act as a template for protein synthesis; the experiments showing that RNA may be an intermediate between DNA and protein synthesis; the experiments done to determine the mechanism of protein synthesis; and the experiments done to show that protein synthesis takes place in ribosomes. I have also shown that the expression of a single gene product that is a single polypeptide requires the integrated action of many proteins, and by extension, the coordinated action of many genes. Thus, a change in the sequence or expression of a single gene could influence the correct expression of many other genes. These experiments, which were aimed at understanding the "language of life," involved the work of many people.

The physicists who entered genetics, including Francis Crick and George Gamow, like the colloidal chemists before them, did not take the work and perspective of the cytologists and licensed biochemists seriously. This resulted in the creation of untenable hypotheses and many missed opportunities for advancement upon previous data. It seems to me that to understand the "language of life" people trained in many disciplines must respect each other and speak a common language.

17.12 QUESTIONS

17.1. What is the evidence that proteins are involved in the functions of the cell mandated by the genes?
17.2. What are the limitations about thinking of proteins as the only catalysts in the cell?
17.3. Why are there so many amino acids, and what are the properties of the amino acids that are important for protein function, targeting, interaction, etc.?

Chapter 18

The Origin of Life

We dance round in a ring and suppose,
But the Secret sits in the middle and knows.

—Robert Frost, "The Secret Sits"

One of the attributes of life is the ability to store and express hereditary information. In Chapters 16 and 17, I discussed DNA, RNA, and proteins—three classes of molecules that are necessary for the storage and expression of genetic information. In this chapter, I discuss the origin of life primarily in terms of the origin of these three classes of molecules, which make up the genetic apparatus.

18.1 SPONTANEOUS GENERATION

Where did life come from, and how can we answer this question? The obvious answer is to look in the Bible, although we would not get a clear answer since two alternative creation stories are given in Chapters 1 and 2 of *Genesis* (Airy, 1876; Paine, 1880). We could also turn to the writings of the great philosophers; for example, Aristotle, who synthesized the teachings of the times into a theory of life that envisioned that living beings can either come from other living beings or be formed spontaneously. Aristotle believed that plants originated spontaneously from the earth: Frogs and mice sprang up from mud; fireflies came from the morning dew; and mosquitoes, maggots, flies, fleas, bed bugs, and lice came from manure, the slime of wells, human excrement, decaying meat, and other filth. Actually, this conclusion is supported by casual observations of the world (Virgil, 1952). Even van Helmont had a recipe for producing mice by combining human sweat with wheat germ and leaving them alone in a jar for 21 days (Oparin, 1938; Fothergill, 1958; Horowitz, 1958). However, in 1668, Francesco Redi saw things differently. Redi showed that maggots did not appear in meat when he placed the meat in a jar, and carefully covered it with muslin. In fact, he noticed that maggots did not arise spontaneously, but only developed when flies were allowed to lay their eggs on the meat.

The belief in spontaneous generation of large plants and animals began to wane throughout the 17th and 18th centuries, due in part to observations on sperm by Antony van Leeuwenhoek (1678, 1699a,b, 1700, 1701; Cole, 1930; Castellani, 1973; Ruestow, 1983, 1996) and on embryo development by William Harvey, Marcello Malpighi, and Pierre-Louis Moreau de Maupertuis (Maupertuis, 1753; Needham and Hughes, 1959). However, with the discovery of microbes by Leeuwenhoek (1677), the belief in the spontaneous generation of microorganisms became the standard belief because the microbes seemed to appear out of nothing (Farley, 1977). The apparent spontaneous generation of microorganisms was confirmed experimentally when John Needham (1749) boiled mutton gravy, stoppered it, and found that microbes grew in the boiled broth.

Lazzaro Spallanzani (1769, 1784) repeated Needham's experiment and showed that if you boiled chicken broth *extensively* before you stoppered it tightly, microbes would not appear in the broth. They only appeared after the stopper was opened. Thus, it appeared that microbes only seemed to arise spontaneously because they were ubiquitous. They were either already in any preparation that had not been properly sterilized or were capable of contaminating any preparation that they could enter. Spallanzani's supporters believed that he had shown that spontaneous generation was impossible, whereas Needham's supporters believed that Spallanzani had only shown that microbes need air.

In the middle of the 19th century, Louis Pasteur performed the critical experiment. With his now-famous swan-shaped flasks that allowed air, but not microbes, to pass, Pasteur showed that as long as a solution is properly sterilized (e.g., pasteurized) and airborne contaminants excluded, no microbes were generated in the broth, even when air was able to freely pass through the long neck. He concluded that there is no such thing as spontaneous generation of microbes (Dubos, 1960). John Tyndall (1898) showed that filtration also prevented the "spontaneous" appearance of organisms (Arrhenius, 1908).

If living organisms cannot originate spontaneously, and the early Earth was a molten ball incapable of supporting

life, then how did they originate on Earth? Some scientists, including H. E. Richter, Lord Kelvin (King, 1925), Hermann von Helmholtz (1881), Svante Arrhenius (1908), Fred Hoyle (1983; Hoyle and Wichramasinghe, 1981), and Francis Crick (1981), realizing that no one has yet created life in the laboratory, suggested that life cannot be created, but must come from existing life. If life can only originate from life, then life on Earth must have originated in outer space and come to Earth on meteorites in the form of cosmozoa, microbes, spores, or seeds. This theory is called *panspermia*, which means seeds everywhere. Arrhenius (1908) wrote, "The Universe in its essence has always been what it is now. Matter, energy, and life have only varied as to shape and position in space." This sounds very much like—conservation of life!

Even if the panspermia theory is true, we are still faced with the question of how living organisms originated in the universe. So although it is possible that life on Earth originated on another planet in another solar system or another galaxy, I will use Occam's razor and assume that life on Earth originated from lifeless matter on Earth itself. This does not mean that life did not also arise from lifeless matter elsewhere in the universe, and the arguments I make apply to the origin of life anywhere (Muller, 1973a; Levy et al., 2000).

18.2 CONCEPT OF VITALISM

The notion that life arose from lifeless matter will seem more outrageous to many readers than the idea of special creation, since there seems to be an enormous gap between living and nonliving matter. However, as I discussed in Chapter 1, it is impossible to devise a classification system to separate the living from the lifeless, and, in this book, I take the view that there is a continuum between lifeless matter and living beings. Artificial classification systems designed to separate animate from inanimate molecules generally have been found wanting. For example, in the past century, chemists thought that the organic molecules present in living beings were different from the inorganic molecules present in rocks. The term *organic* was originally coined by Torbern Bergman in 1780 to indicate one of the three main classes of organizations based on complexity:

- The first class was composed of minerals, a relatively simple group.
- The second class was composed of the pure chemicals extracted from living organisms. These chemicals were considered more complex than minerals, but not living.
- The third class, designated organic, included the constituents of the fluids and tissues of plants and animals.

Jöns Jacob Berzelius, a leading authority in chemistry at the beginning of the 19th century, believed that organic compounds had properties that were determined by the *vital force*, a term coined by Friedrich Medicus in 1774. The concept of a vital force has arisen many times throughout history to explain how organic molecules were made. Galen called it the *pneuma* or "breath of life." Aureolus Phillipus Theophrastus Bombastus von Hohenheim, who called himself Paracelsus, called it the "vital spirit" or *Archeus*. In order to distinguish chemical reactions that take place in solution or in minerals (and depend solely on electrical forces) from those reactions that take place in living organisms (and depend on the vital force), Berzelius divided chemistry into two branches: inorganic and organic.

On account of the requirement for a vital force for the synthesis of organic compounds, Berzelius believed that inorganic compounds could be prepared in the laboratory, but organic compounds could only be formed in living organisms. Berzelius wrote (quoted in Needham, 1971):

> *In living, as compared with inanimate nature elements appear to obey quite different laws. The products of their mutual reactions are quite different from those in the sphere of inorganic nature. The discovery of the cause of this difference of the behaviour of the elements in living and non-living nature would furnish the key to the theory of organic chemistry.*

In 1828, Friedrich Wöhler synthesized urea from ammonium cyanate. He was clearly excited about this result and recognized the importance of this synthesis in relation to vitalistic thought when he wrote to his colleague and former teacher, Berzelius: "I live in the daily, nay hourly, intense hope of receiving a letter from you, however I will no longer wait but instead write you again because I cannot, as it were, hold my chemical water and must tell you that I can make urea, without a kidney and without any animal be it human or dog" (see Wallach, 1901). Of course, the urea was actually synthesized by a living organism, Wöhler himself! Almost 50 years later, August Hofmann (1876) wrote of this work:

> *Wöhler had demonstrated the possibility of building up from its elements this very urea, the formation of which, up to that period, had been supposed to take place exclusively under the influence of vitality—an experiment very memorable, since it removed at a single blow the artificial barrier which had been raised between organic and inorganic chemistry.*

Louis Pasteur realized that many natural organic molecules, including sugars, starch, quinine, morphine, and tartaric acid, were optically active, while many inorganic molecules and organic molecules synthesized in the laboratory were not (Findlay, 1948). Inspired by this realization, Pasteur proposed a new distinction between living and nonliving matter (Meierhenrich, 2008). He proposed that only living organisms could synthesize asymmetric molecules and that optically active molecules could never be synthesized in the laboratory. Pasteur said, "This important criterion [molecular asymmetry] constitutes perhaps the only sharply defined difference which can be drawn at the present time between the chemistry of dead and of living matter" (quoted

in Dubos, 1960). However, this idea was dropped when, in 1860, William Henry Perkin and Baldwin Francis Duppa synthesized racemic acid, a mixture of optically active forms of tartaric acid from optically inactive succinic acid (Hudson, 1992). Later, Vladimir Vernadsky (1944) proposed that living organisms could be distinguished from nonliving matter by their ability to discriminate among different isotopes. This classification too was shown to be artificial when scientists found that they could separate the isotopes using physical techniques, including diffusion, centrifugation, and mass spectrometry (Urey, 1939). Assuming that there are no clear distinctions between living and lifeless matter, I will discuss how living matter may have arisen from nonliving matter on Earth. I will start at the very beginning, with the origin of the universe.

18.3 THE ORIGIN OF THE UNIVERSE

Our present idea of the origin of the universe is intimately connected with our concept of its size. In Aristotle's time it was believed that Earth was the center of a spherical universe (White, 1913; Thiel, 1957). In the third century BCE, Eratosthenes measured the diameter of Earth by noticing that during the summer solstice, the rays from the sun went straight down a well in Syene, while at the same time, about 500 miles away in Alexandria, the sun's rays struck the side of a well, making a 7-degree angle with the vertical. This angle was too large to be explained by a flat Earth, in which case the sun's rays would hit Alexandria at approximately the same angle that they hit Syene. Assuming then that Earth was spherical, and the circumference is equal to both πd and 360°, Eratosthenes set up the relationship: $7°/360° = 500/\pi d$ to determine the circumference and diameter of Earth. He found that the circumference was approximately 25,000 miles, and the diameter was approximately 8000 miles (Asimov, 1971). Except for the writings of a few minor clergymen and the members of the Flat Earth Society, knowledge that Earth was round was commonplace from ancient times through the present (White, 1877, 1913; Draper, 1898; Russell, 1981; Principe, 2006; Garwood, 2008).

Using the diameter of Earth and the rules of trigonometry, Hipparchus, in the second century BCE, estimated the distances of the sun and moon from Earth to be 1210 and 59 times the radius of Earth, respectively (Hirshfeld, 2001). However, without sensitive screw micrometers (which were invented by Joseph Fraunhöfer in 1820) to measure the tiny angles that occur when measuring large distances, it was not possible to measure the distance to the stars, which all seemed to be equally far from Earth. Thus, the ancient Greeks believed that the universe was a finite sphere where the edge consisted of a layer that contained the fixed stars (see Ptolemy, 1984).

Nevertheless, in the 17th century, Isaac Newton (1728) and Christiaan Huygens (1698, in Munitz, 1957) independently made attempts to measure the distance to the stars by using the Principle of Uniformity of Nature and provisionally assuming that the sun was a star and the distant stars had the same intrinsic brightness as the sun. They assumed that the stars only appeared dimmer than the sun due to their distance. Using the inverse square law, which states that the apparent brightness decreases with the square of the distance, Huygens estimated that Sirius, the brightest star, is 28,000 times farther from Earth than the sun is. (This was an overestimate because Sirius has an intrinsic brightness that is about 25 times as bright as that of the sun.) Throughout the 18th century, telescope designs improved, and astronomers, including William Herschel, began to see more distant stars, which had not been seen with the older telescopes. With each improvement of the telescope, the known universe became larger and larger (Shapley, 1926; de Sitter, 1932). By the 19th century, technology had advanced sufficiently to allow Friedrich Bessel, Thomas Henderson, and Otto Struve to measure the distances to the closest stars using triangulation techniques by relating the apparent positions of the stars to the diameter of Earth's orbit (Jeans, 1930).

Our present understanding of the size of the universe came unexpectedly from the study of Cepheids, which are stars of which the brightness varies in a regular and periodic manner. While Harlow Shapley was studying the spectra of these variable stars in the beginning of the 20th century, he noticed that the spectra were blue-shifted when the Cepheid was at its brightest and red-shifted when it was at its dimmest (Shapley, 1943). Normally the spectrum of stars is red-shifted when a star is moving away from us and blue-shifted when it is approaching. The Cepheids seemed to do both. Shapley thought that perhaps it was more likely that the Cepheids were expanding and contracting in a regular manner, a behavior that would give the observed patterns of spectral shifts. This is in fact what happens. The variable light originating from pulsars is produced by a different mechanism involving a rapidly spinning neutron star that emits electromagnetic radiation in jets from its poles (Gold, 1968, 1969; Hewish et al., 1968; Sullivan, 1964; Harrison, 1981; Taylor, 1993; Hulse, 1993; McNamara, 2008).

In 1908, Henrietta Leavitt discovered a relationship between the average apparent brightness of a group of Cepheids in the Magellanic Clouds and the periodicities with which they winked (Leavitt, 1908; Pickering, 1912; Johnson, 2005). That is, the greater the average apparent brightness of the Cepheid, the longer the period. Since all the Cepheids in the Magellanic Clouds are approximately the same distance from Earth, Einer Hertzsprung assumed that the period of each one was probably related to its intrinsic brightness. Hertzsprung studied Cepheids that were closer to Earth as determined from published records of their apparent tangential movement. He compared the apparent brightness of the near and far Cepheids with similar periods. By assuming that the intrinsic brightnesses of

Cepheids with the same period are the same, he could estimate the distance to the Cepheids in the Magellanic Clouds by assuming that the difference in the apparent brightness of Cepheids with similar periods was due to a difference in their distance from Earth (Leuschner, 1937). Using the same method, Shapley (1943) mapped the galaxies in the universe by using the period-distance relationship of Cepheid stars found in the various galaxies, and concluded that the universe was very large.

Meanwhile, Vesto Slipher measured the spectral shifts in a number of galaxies. Using the Doppler Principle, he concluded that, as a rule, the galaxies were receding from Earth at tremendous velocities. Edwin Hubble (1937) noticed that the recession velocities of galaxies were proportional to their distance from Earth, and concluded that the universe was expanding. This result was consistent with one formulation of the Theory of General Relativity (de Sitter, 1932; Einstein, 1961). Hubble determined the constant of proportionality between the recession velocity and the distance from Earth. The proportionality constant, which is known as *Hubble's constant*, is 1.58×10^{-18} km s^{-1}/km. If we assume that prior to the large-scale expansion of the universe all the galaxies were clumped together, we can estimate the age of the universe from the reciprocal of Hubble's constant. Using this estimate, the universe began 20×10^9 years ago. Actually, the relationship between recession velocity and distance is not linear, and is influenced by the mass-density of the universe, a number that is not known with certainty (Guth, 1997). In addition, it is difficult to measure both the recession velocity and the distance of a given galaxy, and consequently, the Hubble constant is continually being refined (Ferris, 1997). Taking everything into consideration, the best guess for the age of the universe is 13 billion years. This is far older than the estimates made by biblical scholars including Moses Maimonides, and Archbishop Ussher, who determined, by tracing back the lineages given in various ancient translations of the Bible(s), that the universe was created between 6000 and 4000 bce (many authors, 1747; Lamarck, 1802; Wallace, 1844; Ussher, 1658, 1854). According to John Lightfoot, another biblical scholar, "heaven and earth, center and circumference, were created together" and "this work took place and man was created by the Trinity on the twenty-third of October, 4004 b.c., at nine o'clock in the morning" (see White, 1913).

How was the universe formed? The current consensus among cosmologists is that 13 billion years ago, space and time, as well as all the matter and energy contained in the universe, came into being in one gigantic explosion. This theory, proffered by George Gamow, Ralph Alpher, and Robert Herman, is called the *Big Bang Theory* (Alpher et al., 1948; Gamow, 1952; Marateck, 2008), a moniker given by Fred Hoyle in order to mock this cosmological creation theory. Hoyle believed that the universe was eternal and thus could not have had a beginning. Although I will only discuss the Big Bang Theory since it is strongly supported by the discovery of the cosmic microwave background radiation (Penzias and Wilson, 1965; Peebles, 1993), there is an alternative cosmological theory, known as the *Steady-State Theory*, based on the idea of continuous creation (Hoyle, 1953; Bondi, 1960; Alfvén, 1966; Bondi et al., 1995).

According to the modern version of the Big Bang Theory (Lederman and Teressi, 1993; Guth, 1997), at time zero, the universe exploded from a infinitesimally tiny and infinitely hot point. There was only one thing, and this force/particle traveled in this infinitesimally small space with an energy of kT, where T equals infinity. Of course, this infinitesimally small point could not exist inside anything, because that would be something. So according to the Big Bang Theory, and *Genesis* for that matter, in the beginning there was a unity, a singularity, a primeval atom (Lemaître, 1950). Some people may call it *God*, others *love, intelligence, the spirit of life*, or *consciousness* (Spinoza, 1492; Ray, 1691, 1692; Grew, 1701; Kafatos and Nadeau, 1990). It is also called the *unified field*, for which Albert Einstein spent his life searching. Whatever we call it, the violent explosion caused the universe to expand, and as a consequence of the expansion, the universe began to cool. Approximately 10^{-43} seconds after the Big Bang, the universe had already cooled to 10^{32} K. At this temperature, the single particle in the universe no longer had enough energy to prevent its splitting into two particles, and when it split there was not enough energy (kT) to fuse the two split particles together (Weinberg, 1979). Thus, the particles that carry the gravitational force separated from the particles that carry the grand unified (GUT) force. This "phase change" is not so different from the changes that occur when water separates from steam, or ice separates from water. The energy of a given particle is typically expressed in electron volts (eV). The energy of a particle can be related to temperature with the following identity:

$$eV = kT \tag{18.1}$$

The universe continued to expand and by 10^{-35} seconds after the creation of the universe, the temperature cooled to 10^{26} K, which is low enough to allow the separation of the GUT particle into particles that carry the electroweak and the strong force. Ten nanoseconds after the creation of the expanding universe, the temperature cooled to 10^{16} K, and the particles that carry the electroweak force separated into particles that carry the weak force and the electromagnetic force. Between 1 microsecond and 1 millisecond after the creation of the universe, the temperature cooled to 10^{11} K, and at this lowered temperature, it became possible for protons, neutrons, electrons, and other elementary particles and their antiparticles to persist. Determining the behavior of particles at various temperatures after the Big Bang comes from extrapolating from the results of experiments done in particle accelerators that mimic these early conditions. The times are obtained from estimations of the rate of expansion of the universe. If we assume that the First

Law of Thermodynamics was true at the Big Bang, then we can see clearly how gravitational, electrical, and nuclear energy are convertible.

Three minutes after the Big Bang, the universe cooled to 10^9 K, which allowed the formation of hydrogen and helium nuclei (Alpher et al., 1948; Schramm and Turner, 1998). Even at this temperature, the nuclei in this plasma collided with each other with energies (kT) that were too high to allow the formation of atoms. Ten thousand years after the creation of the universe, the universe cooled to 10^4 K, which allowed the association of electrons with nucleons, and hydrogen and helium atoms were formed. Thus, in accordance with Einstein's equation:

$$E = mc^2 \tag{18.2}$$

energy was converted into mass as we know it (Einstein, 1946). Currently, the temperature of the universe is about 3 K (Penzias and Wilson, 1965; Peebles, 1993; Smoot and Davidson, 1994; Mather and Boslough, 1996).

About 10.5–12 billion years ago, the atoms began to coalesce into dense areas as a result of gravitational attraction. The aggregation of these atoms gave rise to stars and galaxies. As the atoms in the stars were pulled together as a result of gravitational attraction, the gravitational energy was transformed into radiant energy, and the masses of helium and hydrogen ignited to become glowing stars. The high temperatures and pressures developed inside the stars provided the energy necessary to fuse the hydrogen into helium and other light elements, including carbon, nitrogen, oxygen, sulfur, and phosphorous—the elements so important for life (Hoyle, 1979). The fusion of hydrogen into helium, one of the reactions that causes sunlight, takes place in a cyclic reaction involving the catalytic participation of carbon, nitrogen, and oxygen (Prout, 1815, 1816; Bethe and Critchfield, 1938; Bethe, 1939, 1968, 2003; Weizsäcker, 1949; Brock, 1985). Eventually these first-generation stars exploded, sending fragments of dust into the universe. The energy of the explosion formed the heavier elements, including Na, Mg, Ca, Fe, and Co, which were spread over the universe in the form of cosmic dust (Oparin, 1964; Salpeter, 1974, 2002; Mason, 1992).

Approximately 4.6 billion years ago, on the edge of a spiral galaxy known as the Milky Way, a rotating cloud of gas and dust known as a *nebula* collapsed and began to spin faster and faster, just like a figure skater does, according to the Law of Conservation of Angular Momentum. The center of the cloud became so massive and dense, it collapsed under gravitational pressure and ignited the gasses within it to form a star, which we call our sun. Around the sun, other dust particles clumped together into what we now call the *planets*. One of these clumps formed our home planet, Earth (Urey, 1979; Struve, 1962; Cameron, 1979; Kelley, 1988).

The age of Earth was estimated in the past few centuries by estimating how long it would take a fresh water ocean to become salty given the present salt concentration and the rate of salt transport to the oceans by estimating how long a molten body would take to cool given the diameter of Earth and the temperature difference between the Earth and sky or by estimating the time it would take for the sediments carried by rivers to reach their current heights given the rate of sediment flow (Lyell, 1872; Kelvin, 1897; Rutherford, 1909; Jeans, 1930; Lindley, 2004). Currently, the age of Earth is determined by comparing, in ancient rocks, the quantity of radioactive isotopes with the quantity of their decay product (Jeans, 1930; Libby, 1952; Dalrymple, 1969; Lewis, 2000). For example, potassium-40 decays to argon-40 by electron capture with a half-life of 1.3×10^9 years, and an analysis of the ratios of ^{40}K to ^{40}Ar in meteorites found on Earth indicate that their age is between 4.5 and 4.6 billion years old. Analysis of ^{87}Rb and ^{87}Sr ratios from lunar rocks gives approximately the same age. Analysis of $^{142}Nd/^{144}Nd$ ratios that result from the radioactive decay of ^{146}Sm to ^{142}Nd indicates that the oldest known rocks on Earth are 4.28 billion years old (O'Neil et al., 2008). Hence, the entire solar system is approximately 4.3–4.6 billion years old.

18.4 GEOCHEMISTRY OF THE EARLY EARTH

Four and a half billion years ago Earth was becoming fully formed, although it was extremely hot and essentially oceanless and atmosphereless. Heat was primarily generated by radioactive decay, although some heat may have been due to gravity pulling Earth's components together. As a result of gravity, the dense nickel and iron sank to the core while the lighter rocky materials containing aluminum, silicon, calcium, magnesium, sodium, and potassium floated to the surface (Siever, 1979). Earthquakes, volcanism, and impacts caused gasses in rocks to be released, probably producing an atmosphere of H_2O, CO_2, N_2, as well as CO, CH_4, NH_3, and H_2S. The gravitational attraction of Earth was not great enough to hold onto the lightest elements, including H_2 and He_2, and thus most of the original atmosphere of hydrogen and helium was lost (Mendeleev, in Oparin, 1924). The loss of hydrogen does not mean that the atmosphere became oxidizing, because there was no molecular oxygen in the atmosphere yet. The accumulation of molecular oxygen (O_2) only occurred after the origin of life and the evolution of the photosynthetic mechanisms. It is still a mystery whether or not the early atmosphere was oxidizing, reducing, or, most likely, something in between.

Water from outgassing reacted with CO_2 in the air to produce carbonic acid. Returning to Earth as rain, the carbonic acid probably leached Ca^{2+} and Mg^{2+} from rocks and formed limestone and dolomite. In this way, the CO_2 was removed from the atmosphere and precipitated in sediments. Atmospheric CO_2 would have acted as a greenhouse gas to keep the early Earth warm; thus knowledge of the CO_2 concentration would be useful in determining the

climate (Arrhenius, 1908). While the actual concentration during the formation of Earth is not known, the amount of CO_2 in the atmosphere was determined by the balance between outgassing and precipitation (Walker, 1983; Kasting and Ackerman, 1986).

From the formation of Earth 4.6 billion years ago until approximately 3.8 billion years ago, Earth may have been bombarded with meteorites or fragments of rocks that were not included in the initial process of planet formation. Any one of these impactors may have hit with so much energy that it would have vaporized any organic molecules or living organism that may have already formed (Sleep et al., 1989). The idea that Earth was hot at this time is currently being challenged by investigators looking at zircon formed during this period that would not have been able to form if Earth were hot (Hopkins et al., 2008). It is not clear whether the zircons formed during this time represent microenvironments on Earth or generalized conditions. Thus, if Earth were hot from 4.6 to 3.8 billion years ago, attempts at the creation of life, at least in locations far from the zircons, would have been frustrated by the enormous energy provided by the impactors, and life neither could have formed nor continued (Sleep et al., 1989). According to Tommy Gold (1992, 1999), thermophilic microbes may have originated in the igneous rocks deep within a hot planet using the abundant quantities of primordial methane (natural gas) as an energy source. Gold also contends that the CO_2 present in the early earth atmosphere was more likely a product of oxidation by these thermophilic microbes using methane as a food source than the abiotic source of carbon for the bottom of the food chain. In fact, Gold suggests that the remains of these methane-fueled chemotrophic microbes or the molecules they secreted may be the source of deep oil deposits.

Some of the oldest known rocks, which are 3.5 billion years old, formed on Earth contain fossils that resemble cyanobacteria and stromatolites (Schopf, 1992; Tice and Lowe, 2004). Thus, prokaryotic-like cells evolved between 3.8 and 3.5 billion years ago, only 300 million years after what may have been repeated sterilizations of the planet by impactors from space. Eukaryotic cells originated approximately 1.4 billion years ago.

18.5 PREBIOTIC EVOLUTION

Life as we know it requires carbon-containing compounds, and we must ask: What was the source of the organic compounds that made up the first life on Earth? It is possible that organic compounds, including urea, formaldehyde, amino acids, purines, sugars, etc., came from asteroids, comets, or meteorites, and these compounds have been found in meteorites (Chamberlin and Chamberlin, 1908; Kvenvolden et al., 1970, 1971; Cronin et al., 1988; Chyba, 1990a,b; Chyba et al., 1990; Chyba and Sagan, 1987, 1988, 1992; McKee, 2005), and cyanide and acetylene have been found by NASA's Spitzer Space Telescope surrounding a star named IRS 46 in a galaxy 3 billion light years away from Earth (*www.nasa.gov/vision/universe/starsgalaxies/spitzer-20051220.html*). However, according to Matthias Schleiden (1853), Charles Darwin, Eduard Pflüger (see Arrhenius, 1908), and Frederick Engels (1972), it is likely that prebiotic chemical evolution took place on Earth. The idea of prebiotic chemical evolution was taken up by Jacques Loeb, who in 1916 proposed that proteins could be produced from CO_2 and nitrogen.

Alexander Oparin (1924) strongly pushed the idea of prebiotic evolution, perhaps due to its consistency with the socialist philosophy of dialectic materialism that has no place for God. John Burdon Sanderson Haldane, another socialist, felt prebiotic evolution was also the mechanism that best explains the origin of life. Haldane (1929) wrote,

> *Now, when ultra-violet light acts on a mixture of water, carbon dioxide, and ammonia, a vast variety of organic substances, including sugars and … proteins are built up. … In this present world, such substances, if left about, decay—that is to say, they are destroyed by micro-organisms. But before the origin of life they must have accumulated till the primitive oceans had reached the consistency of hot dilute soup.*

"Impressed by the precise way in which all biological phenomena, when carefully investigated, turned out to be in accordance with physical laws, including those of chemistry, and not to involve any special vital principles", John Bernal (1951, 1962, 1967), who was also a socialist interested in the material basis of life, turned his interest in using X-rays to study crystal structure to the idea that in prebiotic times, a self-replicating inorganic crystal may have given rise to life itself.

In 1951, experiments on prebiotic evolution began when Melvin Calvin and his associates succeeded in fixing carbon dioxide into a more reduced, organic form. They irradiated a mixture of water and carbon dioxide in a closed chamber with a helium ion beam from a cyclotron. This resulted in the formation of formic acid and formaldehyde (Figure 18.1). Formic acid, like all organic acids, has the structure, RCOOH, where R = H and formic acid is the first acid in a homologous series. Formaldehyde, like all aldehydes, has

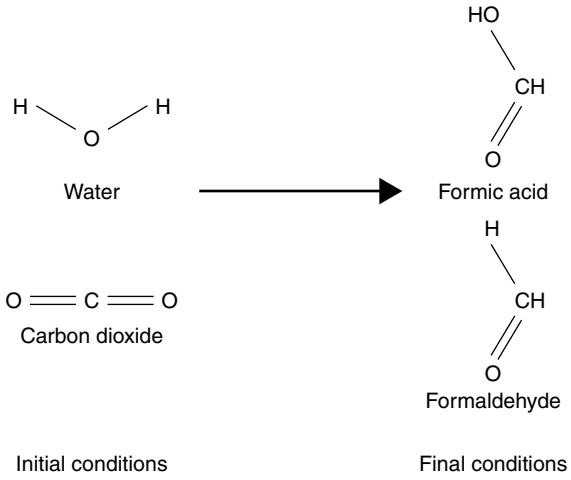


FIGURE 18.1 The synthesis of formic acid and formaldehyde.

the structure RCHO, where R = H and formaldehyde is the first aldehyde in the homologous series. This experiment represented the first accepted production of organic molecules under presumed prebiotic conditions (Garrison et al., 1951; Fox, 1959; Miller and Urey, 1959b; Calvin and Calvin, 1964; Calvin, 1969; Fox and Dose, 1972; Miller and Orgel, 1974).

At about this same time, Harold Urey (1952a,b), who had been studying the atmosphere of Jupiter, wrote that the atmosphere of the early Earth, like that of Jupiter's, may have been reducing, and thus may have consisted largely of hydrogen, methane, ammonia, and water. He suggested that Calvin's experiment be repeated using a reducing, not an oxidizing, atmosphere. Stanley Miller, a graduate student of Urey's, created an apparatus designed to mimic this presumed early-Earth condition. A gaseous mixture of methane, ammonia, hydrogen, and water was connected to a flask of boiling water. The steam created by the boiling water caused the gasses to move past electrodes, the electrical discharges of which simulated lightning in the atmosphere. A cold-water jacket caused molecules to condense and fall out of the "atmosphere." The reaction was allowed to run for a week, after which the solution, which had become deep red, was analyzed. Miller had succeeded in producing not only the formic acid and formaldehyde formed in the experiment of Garrison et al. (1951), but since he included nitrogen, he could also form hydrogen cyanide, which can combine with water and aldehydes to form amino acids (Miller, 1953, 1955; Miller and Urey, 1959a,b; Bada and Lazcano, 2003; Lazcano and Bada, 2004).

In fact, glycine and alanine were created in these experiments. This exciting result indicated that amino acids may have been present on the early Earth before the advent of life. How was glycine made in Miller's experiment? It is likely that hydrogen cyanide and formaldehyde combined with water to form glycine via an amino nitrile intermediate. A more complex aldehyde yields more complex amino acids. For example, substituting acetaldehyde (CH_3CHO) for formaldehyde (HCHO) in the reaction results in the production of alanine (Figure 18.2).

Under prebiotic conditions, amino acids can polymerize into polypeptides without the aid of enzymes or a template (Flores and Ponnamperuma, 1972). The peptide bonds between the amino acids occur as a result of dehydration reactions. Even more complex structures like proteinoid microspheres can form under prebiotic conditions. Proteinoids are large, branched molecules produced when amino acid mixtures containing large amounts of aspartic acid, glutamic acid, or lysine are heated without water. When these dry proteinoids are placed in warm water and allowed to cool, microspheres are produced (Fox and Dose, 1972; S. Fox, 1988). Such proteinoid microspheres may have joined together with phospholipids, which can also be synthesized under prebiotic conditions (Hargreaves et al., 1977), to form the first plasma membranes in a process of self-assembly (Deamer and Fleischaker, 1994). The proteinoid microspheres look similar to the microspheres found in rocks that are 3.8 billion years old (Strother and Barghoorn, 1980). Ernest Just (1939) wrote in his book *The Biology of the Cell Surface*, "In the differentiation of ectoplasm [plasma membrane] from the ground-substance we thus must seek the cause of evolution." And E. Newton Harvey (1952) wrote that "by their membrane ye shall know them."

Nucleic acids can also be synthesized under early-Earth conditions (Orgel, 1973; Miller and Orgel, 1974). The purines turned out to be relatively easy to synthesize, and Juan Oró (1960, 1976) and Sanchez et al. (1968) have shown that under prebiotic conditions, adenine can be formed from hydrogen cyanide. Adenine is made according to the simple overall reaction: 5 HCN ⇔ adenine (Figure 18.3). Thus, HCN, a poison to aerobic organisms today, may have been the giver of life billions of years ago. Ponnamperuma et al. (1963a) showed that adenine can be formed by electron irradiation of methane, ammonia, and water. Ribose and other sugars can also be made under similar conditions by the overall reaction: 5 × formaldehyde (CH_2O) ⇔ ribose ($C_5H_{10}O_5$). The adenine and ribose can lose a single water molecule and form adenosine (see Figure 18.4; Ponnamperuma et al.,

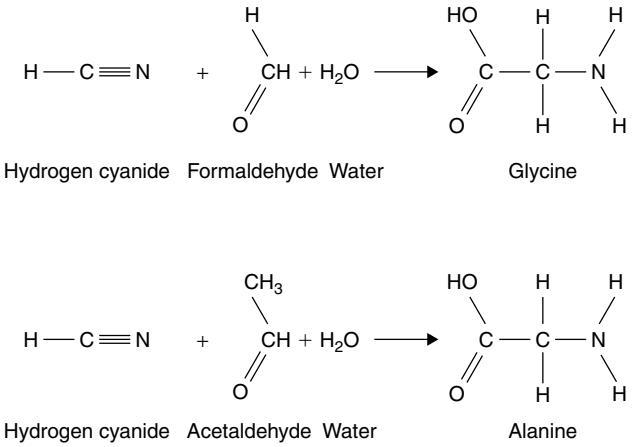


FIGURE 18.2 The synthesis of glycine and alanine.

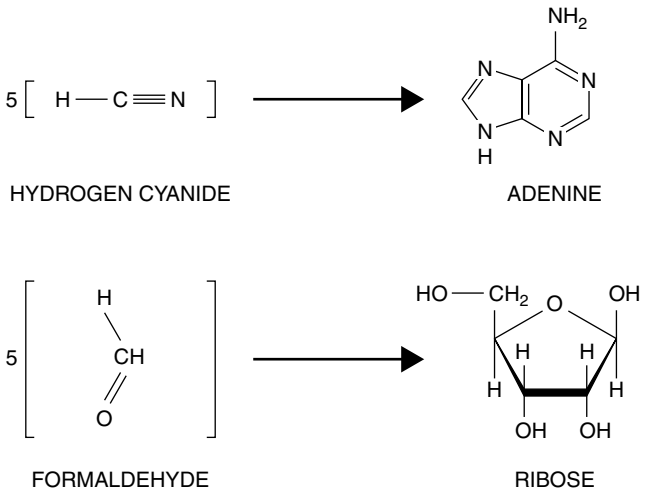


FIGURE 18.3 The synthesis of adenine and ribose.

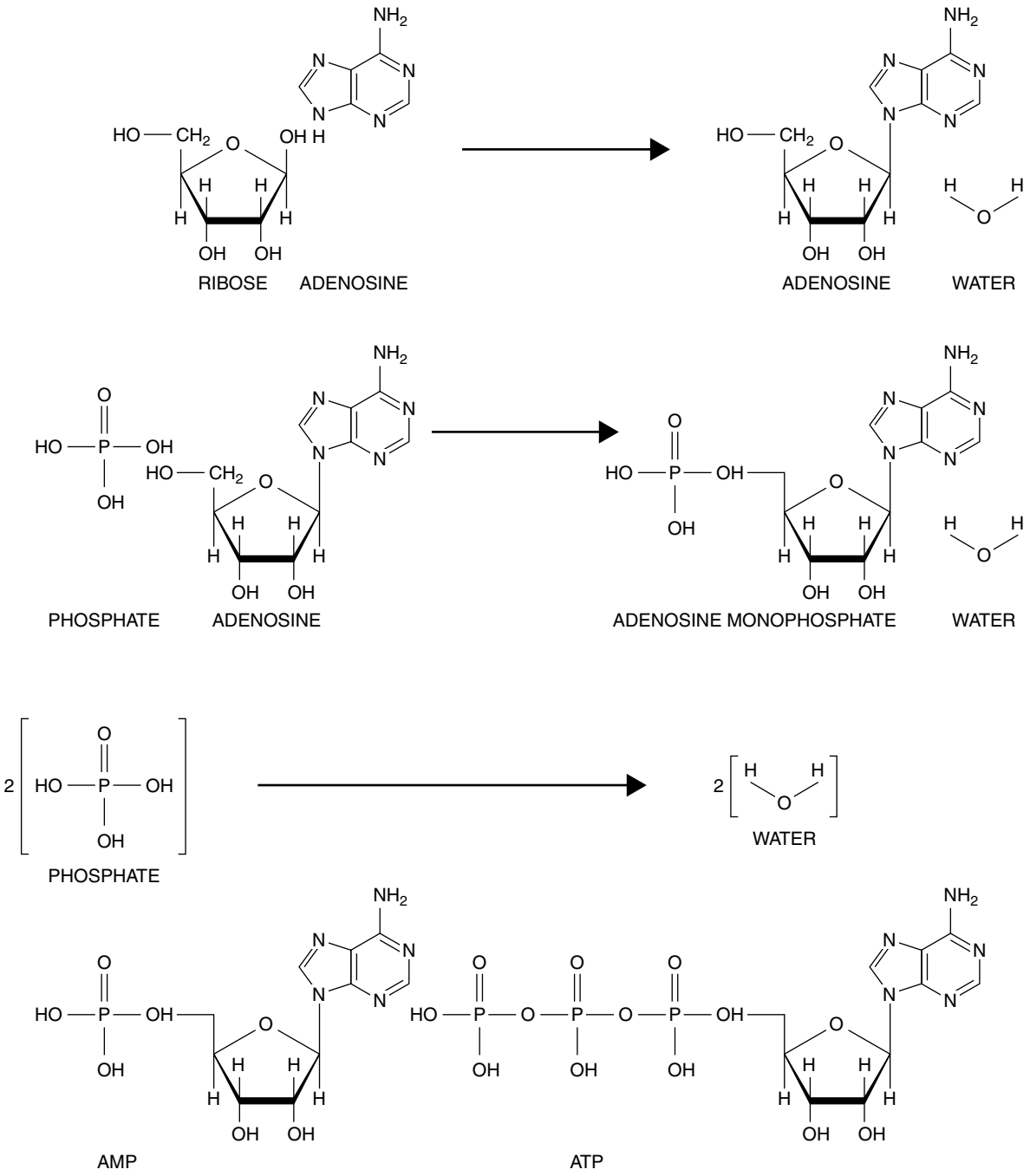
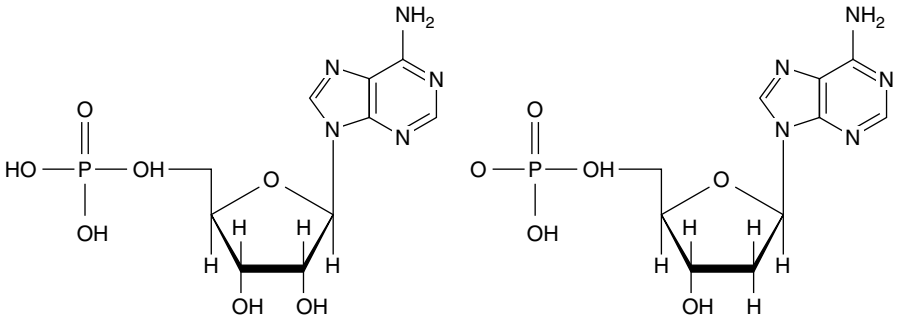


FIGURE 18.4 The synthesis of adenosine, adenosine monophosphate, and ATP.

1963b; Fuller et al., 1972). When inorganic phosphate is added to the prebiotic mix, adenosine trisphosphate (ATP) is formed (Ponnamperuma et al., 1963c).

The pyrimidines uracil and cytosine have been formed from cyanoacetaldehyde and urea by Robertson and Miller (1995). Shapiro (1999) worries that the small amount of any cytosine formed under prebiotic conditions would break down faster than it was formed. As a consequence, before he died, Stanley Miller found a way to increase the yield of cytosine and uracil formed under presumed prebiotic conditions (Cleaves et al., 2006). He succeeded by concentrating the precursors by eutectic freezing (Sanchez et al., 1966; Orgel, 2004). That is, Miller and his colleagues froze a dilute solution of precursors, and as the water in the solution crystallized, the concentrations of the precursors in the solution between the ice crystals rose to concentrations



ADENOSINE 5' MONOPHOSPHATE (5' ADENYLIC ACID)

DEOXYADENOSINE 5' MONOPHOSPHATE (5' DEOXYADENYLIC ACID)

FIGURE 18.5 The structure of adenosine 5′ monophosphate and deoxyadenosine 5′ monophosphate.

high enough to form pyrimidines. By including phosphate in the presumed early-Earth conditions, nucleoside monophosphates, including adenosine monophosphate, guanosine monophosphate, cytidine monophosphate, thymidine monophosphate, and uridine monophosphate, can also be formed (Ponnamperuma and Mack, 1965). Nicotinamide, a coenzyme that contains adenine, is also formed under presumed prebiotic conditions (Cleaves and Miller, 2001).

Urea is formed under prebiotic conditions (Lohrmann, 1972). This is important since urea catalyzes many reactions. For example, adenosine 2′,3′-phosphate forms in a urea-catalyzed reaction (Lohrmann and Orgel, 1971). The adenosine 2′,3′-phosphate will polymerize via a dehydration reaction to form ribonucleic acids with a 3′,5′ linkage (i.e., RNA; Verlander et al., 1973). A reduction of the 2′ hydroxy group of ribose would result in deoxyribose, and the polymerization of deoxyribonucleotides would result in DNA (Figure 18.5). Peptide nucleic acids, which are more stable than RNA alone, are also formed under prebiotic conditions (Nelson et al., 2000).

Only molecules with more than one functional group are capable of polymerizing. Monomers with at least two functional groups, including amino acids, nucleotides, and sugars, are capable of polymerizing into macromolecules as a result of dehydration reactions (Staudinger, 1961; Kenyon and Steinman, 1969). Ester bonds, like those found in DNA and RNA, result when a bond is formed between an acid group and an alcohol group upon the removal of water. A dehydration between two hydroxyls in sugars results in the type of glycosidic bond found in polysaccharides such as starch or cellulose. A dehydration between a phosphate and a pentose results in the type of phosphodiester bonds found in nucleotides. A dehydration between the amino group of one amino acid and the carboxyl group of another results in the type of peptide bond found in proteins. The importance of dehydrations in forming larger molecules cannot be underestimated. In order for these dehydrations to take place, nonaqueous microenvironments must be formed temporally or spatially.

The structures of organic molecules commonly found in cells are given in Figures 18.6–18.11. In the experiments described previously, which are performed under early-Earth conditions, the yields of organic molecules and macromolecules are low. The yields depend greatly on the reducing power of the atmosphere used, and the presence of activating agents (Chang et al., 1983; Schlesinger and Miller, 1983). A reducing atmosphere not only enhances the synthesis of organic compounds, but minimizes the breakdown, since oxidation is typically responsible for the breakdown of organic molecules (Wald, 1955). The yields also depend on the energy available in the form of light, heat, lightning, cosmic rays, etc., and the availability of dehydrating conditions (Ponnamperuma, 1972; R. Fox, 1988; Miller, 1992). While the probability of various molecules coming together to form a living organism is low, it only had to happen once. During a long enough time and with a large enough number of mixtures, every possible combination will eventually occur and improbable combinations eventually occur (Guye, no date; Bohm, 1961). As Herodotus (ca. 450 BCE) said, "If one is sufficiently lavish with time, everything possible happens." And as Émile Borel (1913), Arthur Eddington (1929), and Sir James Jeans (1930) suggested, with enough time, a million monkeys could type all the volumes that exist in the British Museum.

Where did these molecules come together? Charles Darwin guessed that life began in a "warm little pond." In 1871, he wrote to his friend, Joseph Hooker, "But if (and oh! What a big if!) we could conceive in some warm little pond, with all sorts of ammonia and phosphoric salts, light, heat, electricity, etc. present, that a protein compound was chemically formed ready to undergo still more complex changes" (quoted in Harrison, 1981). J. B. S. Haldane thought life arose in a hot dilute soup, but Stanley Miller (1992) feels that since it is the ratio of synthesis to decomposition that is important for the accumulation of molecules, and since many biologically important molecules are stable at ambient temperatures but decompose readily at warmer temperatures, life most likely arose in a cold

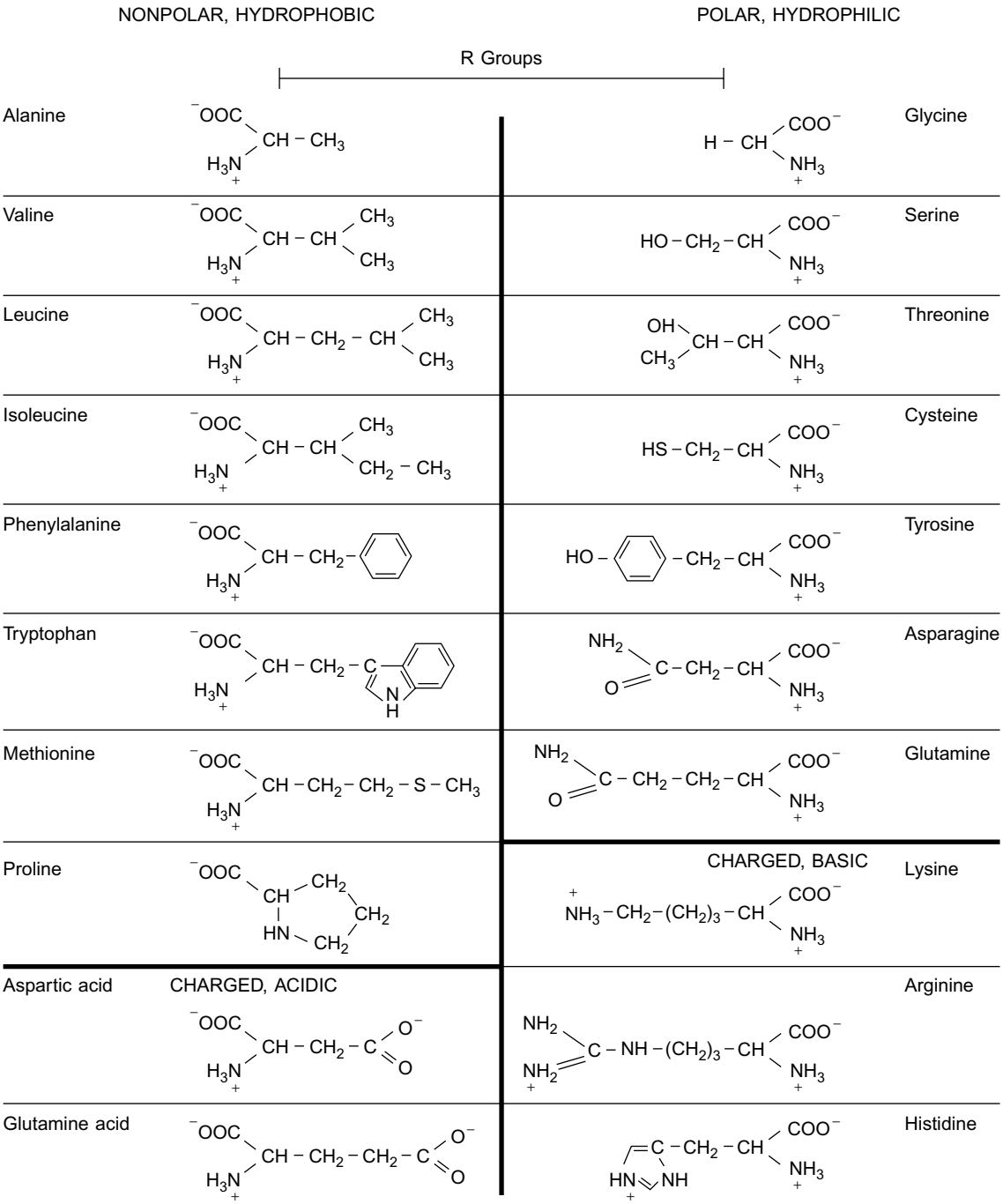


FIGURE 18.6 The structures of a variety of organic compounds found in living cells (amino acids).

concentrated soup. T. H. Huxley thought he discovered the primeval albuminous ooze and named it *Bathybius Haeckelii*. However, this species turned out to be gypsum precipitated by alcohol (see Arrhenius, 1908). This organic–inorganic combination may still be important in the origin of life. Bernal (1951, 1967), Broda (1975), and Arrhenius et al. (1997) think it is more likely that submerged claylike rocks facilitated the formation of living molecules due to their ability to adsorb and concentrate molecules. The clay would promote dehydration reactions by bringing molecules together close enough to from a bond.

There seems to be a thin line that separates chemistry from biology. Jacobius van't Hoff (1878, quoted in de Vries, 1889) wrote,

> *From the chemical properties of carbon it appears that this element is able, with the help of two or three others, to form the numberless bodies which are necessary for the manifold*

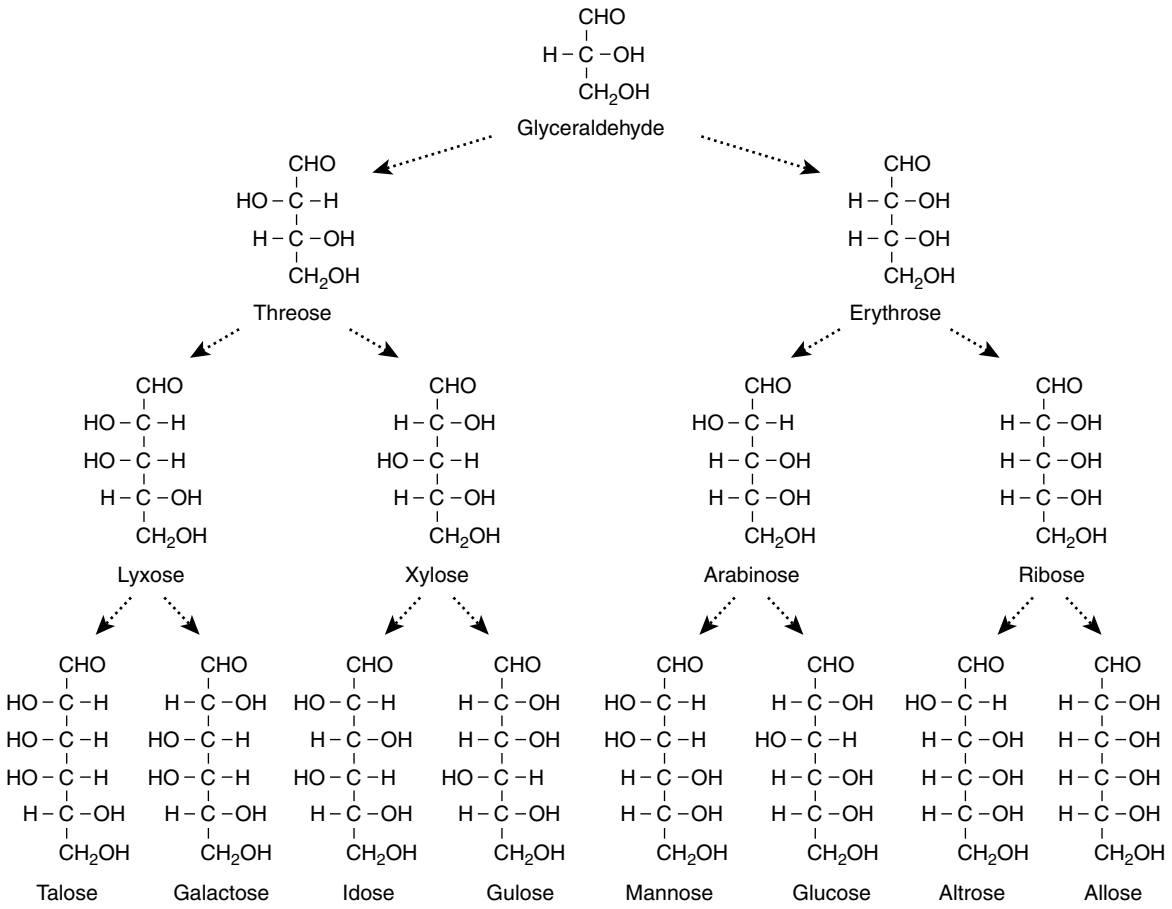


FIGURE 18.7 The structures of a variety of organic compounds found in living cells (sugars).

> *needs of a living being; from their almost equal tendency to combine with hydrogen and oxygen, follows the capacity of the carbon-compounds to be adapted alternately for processes of reduction and oxydation as the simultaneous existence of a vegetable and an animal kingdom requires. … Therefore, one does not go too far in assuming that the existence of the vegetable and animal world is the enormous expression of the chemical properties which the carbon-atom has at the temperature of our earth.*

The importance of the chemical properties of carbon for producing life as we know it is further explained and extolled by Ernst Haeckel (1866), Lawrence Henderson (1913), John Edsall and Jeffries Wyman (1958), and George Wald (1963).

18.6 THE EARLIEST DARWINIAN ANCESTOR AND THE LAST COMMON ANCESTOR

Stimulated by Charles Darwin's *Origin of Species*, Ludwig Boltzmann (1886), a strong proponent of the reality of atoms, combined his interests in physics and biology and proposed that life began with the formation of self-replicating complexes of atoms (Broda, 1983). In order for life to evolve, it must replicate with a high yet finite degree of fidelity (Fitsch and Margoliasch, 1967; Ohta and Kimura, 1971; Ulam, 1972; Muller, 1973b; Stebbins, 1982; Szostak et al., 2001). However, given the complexity of the current genetic apparatus, it is unlikely that the genetic apparatus arose all at once. How then did the first self-replicating molecular structure arise? The first self-replicating, information-bearing structure of which the replication was not quite perfect, and was thus capable of evolving through natural selection, is known as the earliest Darwinian ancestor (Bloch and Staves, 1986).

One candidate for the earliest Darwinian ancestor, alluded to in *Genesis* (2:7), is clay (Cairns-Smith, 1982; Cairns-Smith and Hartman, 1986). Clays are inorganic microcrystalline particles approximately 10 μm in diameter that are made out of hydrated aluminum silicates and other assorted cations and anions. As crazy as this idea sounds, clays are capable of replicating themselves. Normally, the composition of a clay crystal that forms *de novo* is determined by the relative abundance of ions in a solution. However, if a suspension of a given charge is seeded with crystals of differing charge, the growing crystals are typical of the seeding clays rather than the suspension. This

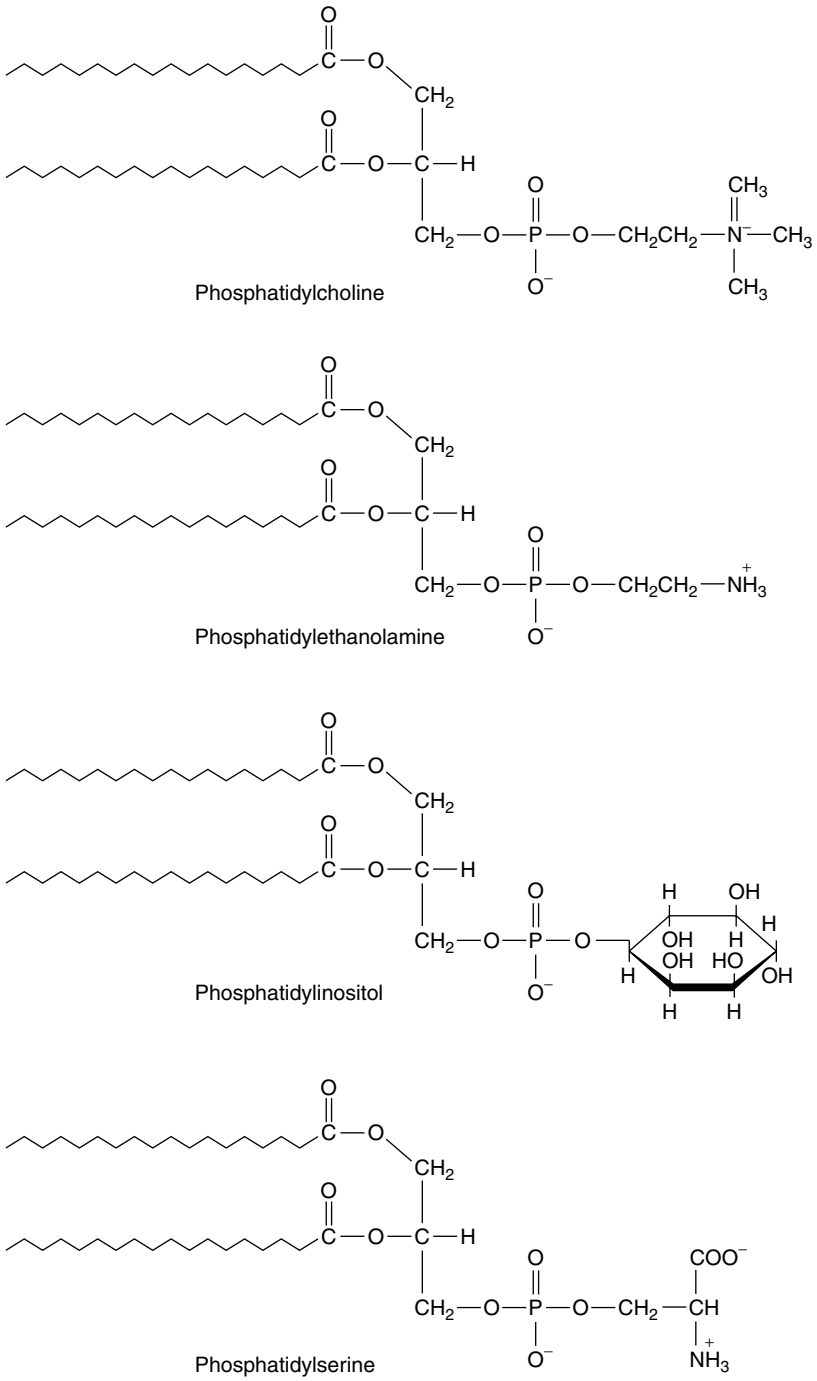


FIGURE 18.8 The structures of a variety of organic compounds found in living cells (lipids).

is because the activation energy of the nucleating process is greater than the activation energy of the growing process (Weiss, 1981; as it is for the polymerization of actin or tubulin as discussed in Chapters 10 and 11).

The parental clay used by Weiss had a phenotype that distinguished it from the clay that would have formed in the solution in the absence of the "seed clay." The parental clay had the proper redox potential to reduce Co^{3+} to Co^{2+}. In Weiss's experiments, this phenotype was transferred unchanged to the descendants for 22 generations.

Clays can have many other catalytic properties that are important for life (Pinnavaia, 1983). For example, clays can facilitate important biochemical processes, including the polymerization of activated amino acids. The presence of clays also increases the length of polypeptides formed under presumed prebiotic conditions (Paecht-Horowitz et al., 1970; Paecht-Horowitz, 1976; Paecht-Horowitz and Eirich,

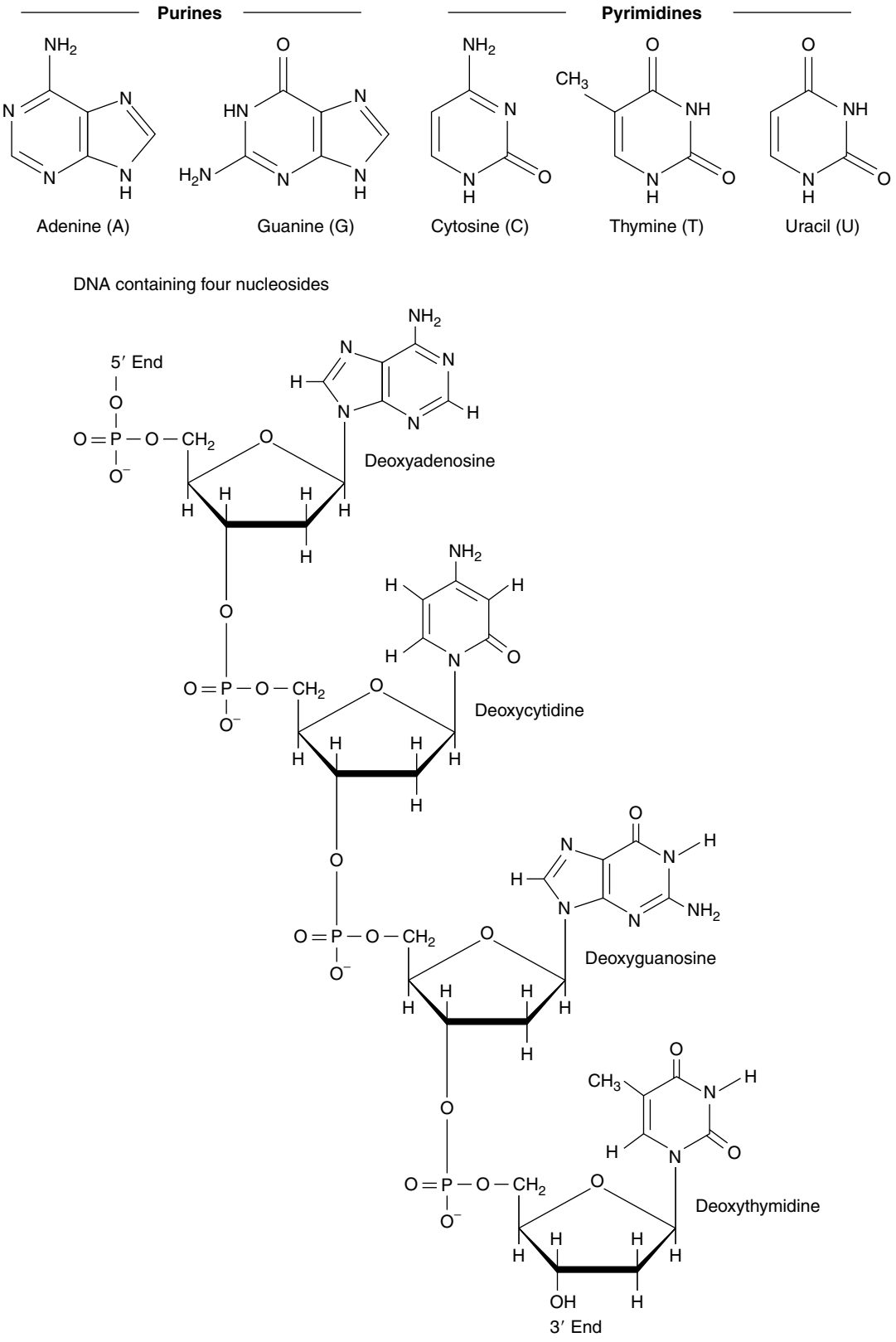


FIGURE 18.9 The structures of a variety of organic compounds found in living cells (purines, pyrimidines, nucleotides).

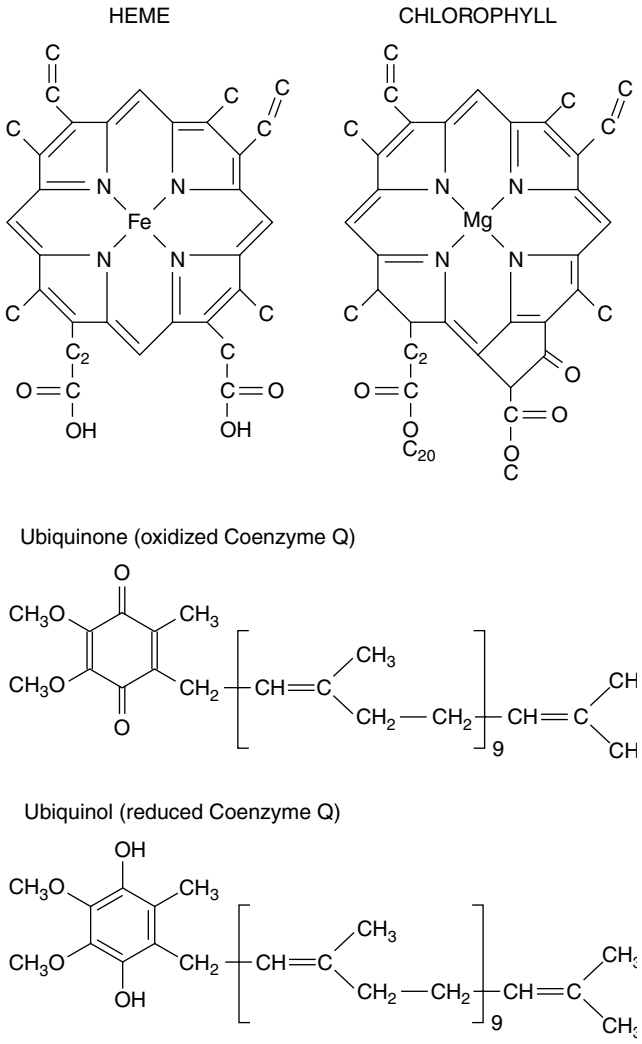


FIGURE 18.10 The structures of a variety of organic compounds found in living cells (tetrapyrrols and quinones).

1988; Böhler et al., 1996; Ferris et al., 1996). Clays are currently used to facilitate a number of industrial syntheses.

The clays may have facilitated the formation of organic molecules in prebiotic conditions. These organic molecules, in turn, might have facilitated the growth of the clays by acting as proteinaceous glues or organic acid-based ion buffers or chelators (e.g., polygalacturonic acid). The clays may have bound nucleotides, including NADH, NADPH, FADH, ATP, UTP, and CTP, the functions of which were to activate amino acids or carbohydrates, etc. through group transfer so that the activated molecules could have participated in complex synthetic reactions. A given sequence of charge density on a clay might have resulted in the binding and ordering of a particular linear sequence of nucleotide coenzymes that might have resulted in the performance of sequential reactions. The sugar phosphates of closely bound nucleotides might have esterified to form a backbone so that the macromolecular complex could have performed sequential reactions free in solution.

Clays have the additional life-generating ability to accelerate vesicle (0.5–30μ in diameter) formation from fatty acid containing micelles by about 100-fold (Hanczyc et al., 2003). The clay acts catalytically, since the generated membrane surface area is 50-fold greater than the maximum possible surface area of the clay. The vesicles have the ability to retain fluorescently labeled RNA associated with the clay.

A sequence of clay-bound nucleotides might have contained the information necessary to form a polymer and to allow a sequence of reactions. As an added bonus, however, the nucleic acid polymer would have the ability to bind with a "complementary nucleotide" through the formation of hydrogen bonds, and form an intermediate template so that it could reproduce itself. If it could reproduce faster than the clays reproduced, the nucleic acids would outcompete the clays for the replicating function. This is what Graham Cairns-Smith (1982) calls *genetic takeover*. Thus, the first Darwinian ancestor may have gone from a clay-based replicating system to a nucleotide-based replicating system. Eventually the nucleotides left the evolutionarily challenged clays behind, and the nucleotide-based genetic code went through its own evolutionary development.

Manfred Eigen and his colleagues suggest that RNA, and not clay, is the most likely candidate for the earliest Darwinian ancestor (Eigen et al., 1981). RNA is a good candidate because it is both an information-bearing molecule, which has the ability to replicate itself due to its endogenous polymerase and ligase activities (Guerrier-Takada et al., 1983; Inoue and Orgel, 1983; Cech, 1986a,b; Doudna and Szostak, 1989), as well as a molecule with other enzymatic activities, for example, the ability to polymerize amino acids (Noller et al., 1992; Piccirilli et al., 1992). Peptide nucleic acids may have served as a precursor to RNA (Nelson et al., 2000).

David Bloch, Mark Staves and their associates figured that they might be able to find the primeval RNA sequence if they scanned the sequences of the ribosomal and transfer RNAs from phylogenetically diverse organisms, including archaebacteria, eubacteria, yeast, and bovine mitochondria, to find an area rich in nucleotide matches. Using this molecular archeological approach, they found such sequences (Bloch et al., 1983, 1985). Since there were no differences in the matches found among intraspecific or interspecific searches, the matches probably reflect ancient similarities derived from common origins rather than a more recent convergence of the molecules. They believe the length of the ancestral RNA was nine bases long (Nazarea et al., 1985; Bloch, 1986, 1988). Eigen et al. (1981) have shown that short sequences of RNA that are allowed to replicate together in a test tube compete for nucleotides, and are thus subject to Darwinian evolution (Eigen and Schuster, 1979; Dyson, 1985; Eigen, 1992, 1993).

RNA may have also provided the mechanism for polypeptide formation (Dounce, 1952; Lacey and Mullins, 1983; Lacey et al., 1988, 1990a,b, 1991, 1992, 1993; Wickramasinghe et al., 1991; Wickramasinghe and Lacey,

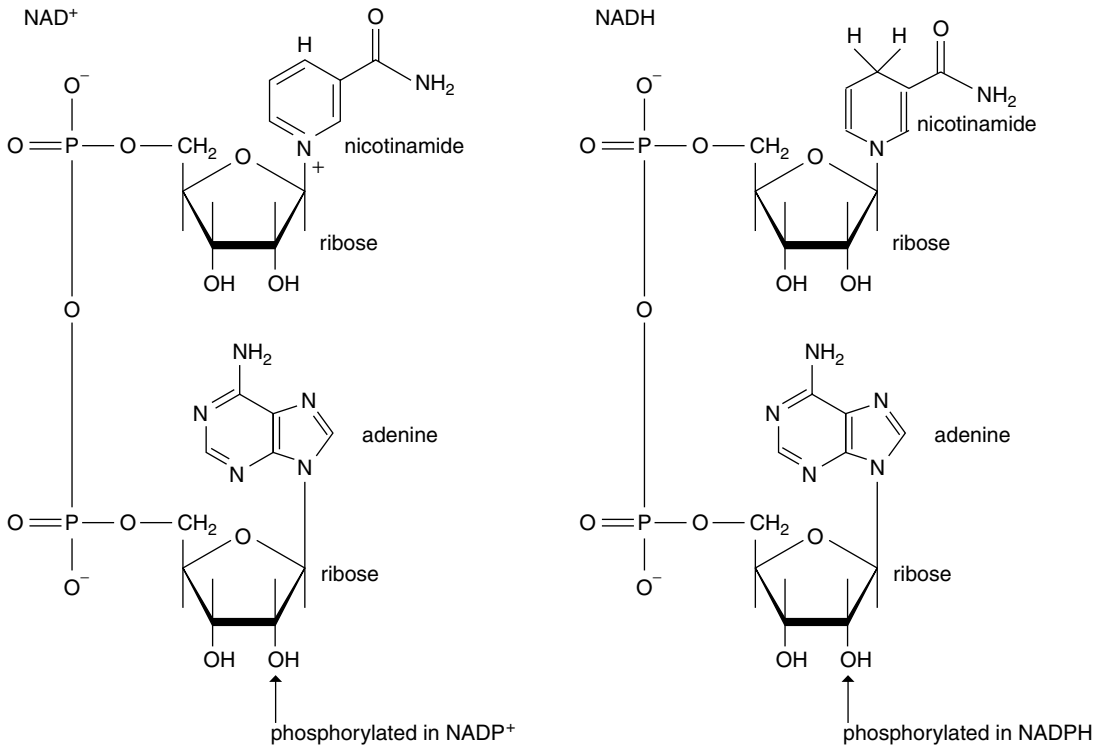


FIGURE 18.11 The structures of a variety of organic compounds found in living cells (coenzymes) (*Continued*).

1994; Bailey, 1998). In fact, a single ribonucleoside, adenosine monophosphate, which is the 3′ terminus of all tRNAs, is capable of catalyzing the formation of polypeptides from activated L-amino acids. This process is uncoded and results in the formation of peptides of which the composition is determined solely by the availability of amino acids. In this process, activated amino acids react with the 2′ hydroxyl of AMP. After binding, L-amino acids migrate to the 3′ position, leaving the 2′ position open for further attack by another amino acid. Because of their proximity, a peptide bond forms between the adjacent amino acids and this process occurs indefinitely. However, D-amino acids do not migrate to the 3′ hydroxyl but remain at the 2′ position. This prevents the addition of another amino acid onto the AMP, and consequently polymerization is terminated. In all living organisms, polypeptide formation is catalyzed in ribosomes by RNA (Noller et al., 1992; Piccirilli et al., 1992). Such catalytically active RNAs are known as *ribozymes*.

The possibility also exists that the earliest Darwinian ancestor was a protein. Lee et al. (1996) have shown that a 32–amino acid–polypeptide sequence, typically found in a nuclear transcription factor, is capable of replicating itself when supplied with amino acids. Moreover, a prion, which is a modified cellular protein (e.g., PrP) that causes infectious neurodegenerative diseases such as mad cow disease, is able to replicate in the absence of nucleic acids. Each strain-specific phenotype is encoded by the tertiary structure of the protein (Telling et al., 1996; Prusiner, 1997a,b; Hegde et al., 1998; Legname et al., 2004). The replication of the yeast prion requires a heat-shock protein (Serio et al., 2000; Shorter and Lindquist, 2004).

Whether clay, proteins, or RNA was the earliest Darwinian ancestor, the genetic apparatus probably evolved from RNA alone, into the trinity of molecules that carry the information of life: DNA, RNA, and protein (Woese, 1967; Crick, 1968). DNA would be selected for over RNA as an informational molecule, in part, because its stability is greater than that of RNA due to the reduction of the 2′OH to 2′H. The 2′OH is able to perform a nucleophilic attack on the phosphorous atom participating in the phosphodiester bond with the 3′ carbon (Lilley, 2003). This results in the breakage of the phosphodiester bond that links the 3′ carbon of one ribose with the 5′ carbon of the next, and causes the formation of a 2′3′ cyclic phosphate on the 3′ ribose.

Proteins, on the other hand, out perform RNA in enzymatic functions, and thus would be selected through natural selection, due perhaps to the variety of functional groups found in the amino acid monomers compared to the nucleotides. These functional groups are capable of interacting with many different atoms, bonds, and molecules. Eventually, RNA not only provided the link between the coding function of DNA, and the catalytic function of proteins, but also became intimately involved in the regulation of gene expression through small nonprotein-coding RNAs (Fire et al., 1998; Ambros, 2008; Baulcombe, 2008).

While the earliest Darwinian ancestor may have evolved into the genetic apparatus of cells, other processes are also necessary for life. Throughout this book, I have discussed

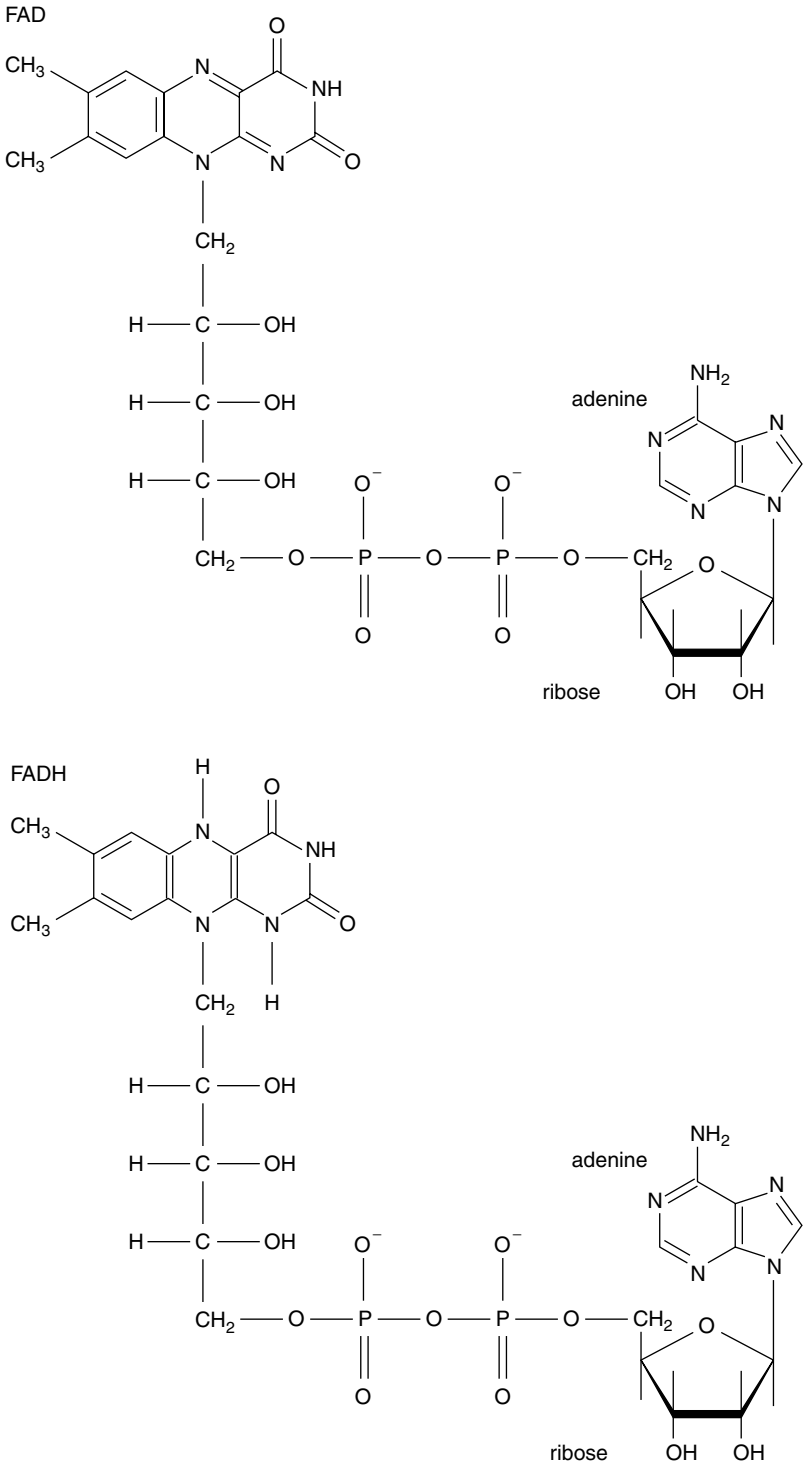


FIGURE 18.11 (Continued)

how various organelles make life possible. In terms of making environmental energy available for cellular work, the chloroplast converts radiant energy into the chemical energy of carbohydrate, as well as the chemical energy of readily usable activated nucleotides, including ATP and NADPH; the mitochondria convert the chemical energy of organic macromolecules into the chemical energy of the activated nucleotide ATP. The plasma membrane, due to its capacitance, converts differences in ionic concentrations on both sides of the membrane into electrical energy that can be used for secondary transport and for signaling in response to changes in environmental conditions. The membranes, as well as the solutions they surround, provide the surfaces and volumes necessary for catalysts to perform chemical

reactions. In order to visualize the original cell, we must imagine the minimum components necessary to perform the functions necessary for life. Currently, Albert Libchaber and his associates are trying to create artificial life by adding a given mRNA to cytosolic homogenates enclosed within an artificial lipid bilayer and expressing one gene at a time, while Craig Venter and his associates at Synthetic Genomics (*www.syntheticgenomics.com/about.htm*) are mutating the individual genes in the genome of *Mycoplasma genitalium*, which has 517 genes, in order to discover the minimal genome necessary for life (Hutchison et al., 1999). Since genes that are individually dispensable may not be simultaneously dispensable, the scientists at Synthetic Genomics would like to create an artificial chromosome to find the minimal genome. Synthetic Genomics is also adding laboratory-made genomes to cells in order to produce novel organisms capable of synthesizing metabolites that can be used for biofuels or pharmaceuticals.

The earliest Darwinian ancestor may have formed symbioses with other prebiotic entities that may have been good at performing both endergonic and exergonic chemical reactions. The endergonic reactions may have been performed by prebiotic chemicals (e.g., porphorins) that were capable of capturing radiant energy and reducing sulfur with the aid of mineral catalysts. Perhaps the molecules involved in these bioenergetic processes passed the excited electrons from one molecule to another by making use of an iron intermediate. Perhaps these entities associated with others that were able to make lipids. Perhaps a lipid bilayer formed on the surface of a puddle, and wave action or the falling of a rock induced the formation of a liposome that encapsulated the other entities to form a protocell. The membrane surrounding this protocell may have been able to allow the passage of necessary nutrients into the cell, while still being capable of maintaining an ionic difference. Perhaps the polypeptides created by clays or from a nucleic acid template could have facilitated the transport functions. At some point, the protocell would have to be able to divide and reproduce. At this stage it probably formed the last common ancestor of all organisms (Deamer, 1997; Woese, 1998). William Kirby (1853), describing Lamarck's view of the origin of the first cell, wrote,

> *We know, by observation, that the most simple organizations, whether vegetable or animal, are never met with but in minute gelatinous bodies, very simple and delicate; in a word, only in frail bodies almost without consistence and mostly transparent. These minute bodies he supposes nature forms, in the waters, by the power of attraction; and that next, subtile and expansive fluids, such as caloric and electricity, penetrate these bodies, and enlarge the interstices of their agglutinated molecules, so as to form utricular cavities and so produce irritability and life, followed by a power of absorption, by which they derive from without.*

The similarities in molecules, mechanisms, metabolic pathways, and structures in living organisms point to a single common ancestor (Horowitz, 1945; Granick, 1957; Gaffron, 1960; Blum, 1962; Lipmann, 1965; Ycas, 1974; Jensen, 1976; Morowitz, 1992; Dyer and Obar, 1994). Throughout history, the idea of common descent was espoused in one form or another by Empedocles (see Lambridis, 1976), Pierre-Louis Moreau de Maupertuis (1753), Jean Lamarck (1809), Robert Chambers (n.d.), Patrick Matthew, W. C. Wells, Herbert Spencer, and others (see Darwin, 1859–1882). Matthias Schleiden (1853) wrote,

> *This view, that the whole fullness of the vegetable world has been gradually developed out of a single cell and its descendants, by gradual formation of varieties, which became stereotyped into species, and then, in like manner, became the producers of new forms, is at least quite as possible as any other, and is perhaps more probable and correspondent than any other, since it carries back the Absolutely Inexplicable, namely the production of Organic Being, into the very narrowest limits which can be imagined.*

Charles Darwin (1859) presented evidence that since variation could be acted upon by artificial selection, evolution must take place as a gradual result of natural selection. Richard Goldschmidt (1933, 1940, 1955), who was skeptical of the well-established belief in Darwin's theory of the gradual origin of species by natural selection, offered an alternative theory for the origin of species based on comparative morphology and genetics. He proposed that new species evolve through drastic changes that result from a mutation in a gene that influences the relative rates of various developmental processes. Such a change would create "hopeful monsters which would start a new evolutionary line if fitting into some empty environmental niche" (Gould, 1977). A minute change in the DNA that encodes a transcription factor, an element in a signal transduction cascade or a regulatory RNA, may provide the mechanism that leads to such a drastic change and a new evolutionary line (Kirschner and Gerhart, 2005).

18.7 DIVERSITY IN THE BIOLOGICAL WORLD

Charles Darwin (1859–1882) believed that "All the members of whole classes are connected together by as chain of affinities." Darwin (1860) wrote in *The Origin of Species*,

> *It is interesting to contemplate a tangled bank, clothed with many plants of many kinds, with birds singing on the bushes, with various insects flitting about, and with worms crawling through the damp earth, and to reflect that these elaborately constructed forms, so different from each other, and dependent upon each other … have all been produced by laws acting around us. … There is grandeur in this view of life, with its several powers, having been originally breathed by the Creator into a few forms or into one; and that whilst this planet has gone cycling on according to the fixed law of gravity, from*

so simple a beginning endless forms most beautiful and most wonderful have been, and are being evolved.

This is a good time to put this book down, walk outside, and marvel at the beauty and wonder of nature (Agassiz, 1863; Burroughs, 1908; Muir, 1911). Aldo Leopold (1949) wrote in *A Sand County Almanac*,

It is a century now since Darwin gave us the first glimpse of the origin of species. We know now what was unknown to all the preceding caravan of generations: that men are only fellow-voyagers with other creatures in the odyssey of evolution. This new knowledge should have given us, by this time, a sense of kinship with fellow creatures; a wish to live and let live; a sense of wonder over the magnitude and duration of the biotic enterprise.

Think about the biochemical, genetic, structural, and physiological unity that underlies the apparent diversity. All living things are related, yet unique. Each living being contains unique molecules of deoxyribonucleic acid that probably never existed before or will ever exist again. I marvel in the richness of the tapestry, the tightness of the weave, the beauty with which each thread complements every other one. It is amazing that even as we look at smaller and smaller pieces of the tapestry of nature, the weave still looks just as fine, elaborate, harmonious, and beautiful. However, even if we did not have an eye for the beauty and poetry of nature, or the innocence of children, nature's treasure house is important to cell biologists because it provides a vast array of organisms, each one specialized to fit in its niche, and because of this, each species has a distinct set of traits that make it amenable to the study of a given biological process (Krogh, 1929; Krebs, 1975; Wayne and Staves, 1996b). Many of these organisms hold the key to help us understand biology in a whole new light or to cure a disease of mankind.

Liberty Hyde Bailey (1915), Aldo Leopold (1949), and others developed a system of ethics based on the interrelationships between organisms and the land (Leopold, 2004). Leopold (1949) wrote, "A thing is right when it tends to preserve the integrity, stability, and beauty of the biotic community. It is wrong when it tends otherwise." Pierre Teilhard de Chardin (1966) felt that the connections were even greater in that the whole universe was living. Do we have any indication that life may actually be supra-organismal? According to Albert Szent-Györgyi (1948),

In our discussion we have passed several levels of organization from electrons … [to] the whole rabbit, but I doubt whether the list is herewith complete. Everyone knows this much of biology—that one rabbit could never reproduce itself, and if life is characterized by self-reproduction, one rabbit could not be called alive at all, and one rabbit is no rabbit, and only two rabbits are one rabbit, and so we may go on calling in the end only the whole of living nature alive.

18.8 THE ORIGIN OF CONSCIOUSNESS

I have discussed how the original quantum particle evolved into atoms, how atoms gave rise to molecules, how molecules gave rise to self-replicating systems, and how self-replicating systems gave rise to cellular life. In each stage of the evolution of life in the universe, new and surprising properties emerged from the combination of previous entities (Bergson, 1911; Morgan, 1923, 1926, 1933). Louis de Broglie (1946) maintains that thought is an essential condition for the progressive evolution of the human race. Some cells may specialize in higher functions of thought and self-identity.

A small group of large spindle-shaped cells has been discovered in the brains of humans and primates (Nimchinsky et al., 1999; Allman et al., 2001; Hof et al., 2001; Allman et al., 2002). These cells may be involved in self-identity and self-awareness. When these cells are damaged, people become "vegetables." These cells are less active in depressed people, disappear in people afflicted with Alzheimer's disease, and are more active in people with manic disorders. These cells alone are probably not sufficient to make us human (Bullock, 2003).

What is the relationship between the origin of consciousness and the origin of life (Drysdale, 1874; Wythe, 1880; Troglodyte, 1891; Shapley, 1958, 1967; Sinnott, 1961; Luria, 1973; Broda, 1983; Crick, 1994; Cairns-Smith, 1996; Margulis, 2001)? I discussed in Chapter 12 how the processes involved in cell signaling in single cells could provide a material basis for the concepts of consciousness and morality. I discussed that action potentials and electrical signaling provide a means of communication in plant as well as animal cells. In Chapter 3, I discussed how plasmodesmata in plants and gap junctions in animal cells provide another means to integrate the activities between cells. Although truly amazing, is it not possible that when you put together billions of cells that are specialized for communication that consciousness is a natural outcome?

George Wald (1963) captured this awe and rational thinking when he spoke in front of the president of the United States and said:

We have been told so often and on such tremendous authority as to seem to put it beyond question, that the essence of things must remain forever hidden from us; that we must stand forever outside nature, like children with their noses pressed against the glass, able to look in, but unable to enter. This concept of our origins encourages another view of matter. We are not looking into the universe from outside. We are looking at it from inside. Its history is our history; its stuff, our stuff. From that realization we can take some assurance that what we see is real.

Judging from our experience upon this planet, such a history that begins with elementary particles, leads perhaps inevitably toward a strange and moving end; a creature that knows, a science-making animal that turns back upon the process that generated him and attempts to understand it. Without his like, the universe could be, but not be known, and that is a poor thing.

Surely this is a great part of our dignity as men, that we can know, and that through us matter can know itself; that

beginning with protons and electrons, out of the womb of time and the vastness of space, we can begin to understand; that organized as in us, the hydrogen, the carbon, the nitrogen, the oxygen, those 16 to 20 elements, the water, the sunlight—all, having become us, can begin to understand what they are, and how they came to be.

Throughout this book, I have treated cells as if they act as mechanical machines that obey the laws of physics and chemistry. Alan Turing (1992a) and John von Neumann (1958; Neumann and Burks, 1966) predicted that computers would be able to think faster than humans and even replicate. In the future, will silicon-based machines become better thinkers than carbon-based machines and accomplish all the requirements of life?

18.9 CONCEPT OF TIME

Any discussion of evolution is predicated on the assumption that time flows in a linear manner as Issac Newton assumed in *Principia*. What is the true nature of time? Throughout human existence, our concept of time has wavered between the idea of absolute and real linear flowing time and the idea that time, like space, is nothing more than an arbitrary axis in the four-dimensional space–time continuum we call our universe (Newton, 1946; Minkowski, 1908; Whitrow, 1961).

Aristotle even questioned the reality of time, realizing that the past and the future have duration but no existence, while the present has existence but no duration (Physics Book IV in Barnes, 1984). According to many religious philosophies, time had a dual nature, and by achieving enlightenment, people could transmigrate from the world of flowing time to the eternal timeless world of the spirit (Macey, 1987). In ancient times, philosophers generally considered time to be a cyclic process as opposed to a linear flow, and in this scenario, the world would periodically undergo a recreation. While the Hindus and Buddhists believed that the universe evolved while undergoing a cyclic process of dissolution and recreation, the Stoics believed that in each recreation, the world and all its events would be identical. Saint Augustine, however, reasoned that time must flow in a linear fashion, for if it did not, the crucifixion of Christ would not be a unique event. However, Saint Augustine also proposed that time was a mental construct.

In constructing his system of the world, Isaac Newton (1946) postulated that time is absolute, and it flows linearly and independently of both mind and matter. He wrote,

Absolute, true, and mathematical time, of itself, and from its own nature, flows equably without relation to anything external, and by another name is called duration: relative, apparent, and common time, is some sensible and external (whether accurate or unequable) measure of duration by the means of motion, which is commonly used instead of true time; such as an hour, a day, a month, a year.

Disagreeing with Newton, Immanuel Kant proposed that time is not a characteristic of physical reality, but is only an outcome of the way humans make the world intelligible. He came to this conclusion based on the idea that he could imagine that there were other species that could experience space and time differently from the way human beings do. According to Kant, human beings structure perceptions in such a way that time appears to flow along a mathematical line. Kant's belief in the illusion of linear time was dismissed in the 19th century when geologists, including Charles Lyell (1872), and biologists, including Jean Lamarck (1802) and Charles Darwin (1859), used the concept of absolute linear time to make sense of the evolutionary processes they were proposing. In a recent move that deemphasizes the putative linear nature of time, biologists interested in the relationships between taxa have minimized the concept of the linear flow of time in their conclusions and have developed cladograms to represent the similarities between taxa without committing to any evolutionary processes that are coexistent with a given direction or duration of time.

Henri Poincaré reintroduced Kant's rejection of absolute time based on the fact that it takes a finite time for information from an event to be perceived by an observer, and, as a result, two observers will perceive the order of events depending on their spatial position relative to the events. Albert Einstein (1905, 1926, 1961) expanded this view in his theories of special and general relativity. In his Theory of Special Relativity, Einstein concluded that the measurement of time depends on the velocity of the observer, and the order of events seen by one observer may not be the order of events seen by another. Einstein came to this conclusion by assuming that the speed of light was constant, and any visual information that came to the observer would take a finite time. Thus, an event that took place at a given instant near the observer would seem to take place simultaneously with another event that occurred at a distant place much earlier, if the light rays that formed the images of the two events arrived at the observer at the same time (Bondi, 1964).

Given the strength of relativity theory in describing the world, a reasonable person could conclude that there is no absolute, linear flow of time, and everything that we consider to be past, present, and future was created at once and coexists as coordinates in the four-dimensional space–time continuum. Given this scenario, the flow of time and thus evolution is an illusion. On the other hand, if one accepts the evolution of life as an objective fact, a reasonable person could conclude that there is reason to look for an alternative to the Theory of Special Relativity (see Appendix 2).

18.10 SUMMARY

In this chapter, I discussed the role of astronomy and astrophysics in determining that the universe had a beginning. This means that life too must have had a beginning, and consequently, as biologists, we must consider the origin

of life. Astronomy also teaches us how to make a series of approximations in a complicated system that is not so easily isolated, and subject to experimental measurements and tests. Thus, there are many similarities in the methods used to study the heavens (astronomy) and life (biology).

In this chapter, I have also stressed the chemical nature of life as I was discussing the origin of life. I would like to relate an anecdote told by the biochemist Arthur Kornberg (1989) in his book *For the Love of Enzymes*. In it, he tells of the importance of chemistry to medical science. It goes like this:

> *Physicians are inclined to action. There is the story, often retold, of a surgeon who, while jogging around a lake, spotted a man drowning. He pulled off his clothes, dove in, dragged the victim ashore, and resuscitated him. He resumed his jogging, only to see another man drowning. After he dragged the second one out and got him breathing again, he wearily resumed his jogging. Soon he saw several more drowning. He also saw a professor of biochemistry nearby, absorbed in thought. He called to the biochemist to go after one while he went after another. When the biochemist was slow to respond, he asked him why he wasn't doing something. The biochemist said: "I am doing something. I'm desperately trying to figure out who's throwing all these people in the lake."*

Kornberg goes on to say,

> *This parable is not intended to convey a disregard of fundamental issues among physicians nor a callousness among scientists. Rather, it portrays the reality that, in the war on disease, some must contribute their special skills to the distressed individual while others must try to gain the broad knowledge base necessary to outwit both present and future enemies.*

In this chapter, I discussed the prebiotic origin of organic chemicals. This can be compared with the mechanism used by autotrophic plant cells to produce the carbon skeletons necessary for life of all other cells. Since autotrophic plant cells directly or indirectly provide the carbon skeletons to all heterotrophic cells, the biblical conclusion that "All flesh is grass" is not far from correct. Now we also know that all life is also made up of a little clay and a smattering of stardust. Is it possible that carbon-based life is making it possible for silicon-based life to evolve on Earth, just like clay-based life may have made it possible for carbon-based life to evolve?

18.11 QUESTIONS

18.1. What is the strongest evidence for the origin of life from nonliving molecules?

18.2. What is the weakest part of the evidence for the origin of life from nonliving molecules?

Chapter 19

Cell Division

The gifts of the microscopes to our understanding of cells and organisms are so profound that one has to ask: What are the gifts of the microscopist? Here is my opinion. The gift of the great microscopist is the ability to THINK WITH THE EYES AND SEE WITH THE BRAIN.

—Dan Mazia, 1996

19.1 MITOSIS

In Chapters 16 and 17, I discussed the events that take place during interphase when the chromatin is in a metabolically active condition, undergoing replication and transcription. In this chapter, I concentrate on the events that take place during the mitotic phase, where RNA synthesis stops and protein synthesis slows down to about 25 percent of its normal rate. In fact, many cell activities, including cytoplasmic streaming and pinocytosis, stop.

Mitosis was first defined by Walther Flemming (1882) as a mode of indirect nuclear division that provides the daughter nuclei with identical chromosomes (Figure 19.1).

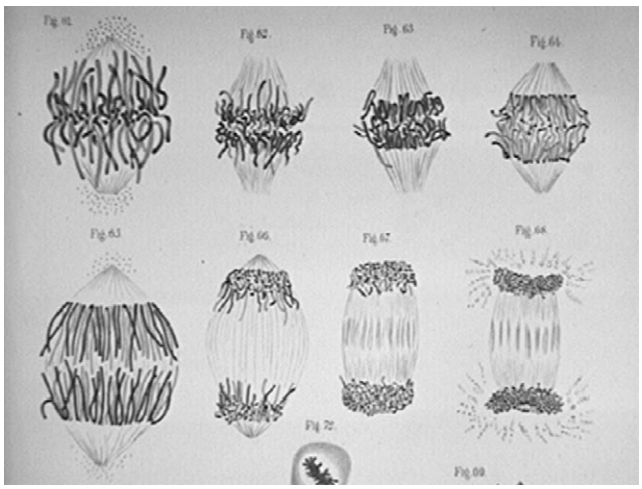


FIGURE 19.1 Mitosis in *Lilium croceum*. (*Source:* From Fleming, 1882.)

Mitosis is part of the process where the cell changes from oneness to twoness. While mitosis involves the equal division of chromosomes between two daughter cells, I will not discuss the chromosomes themselves as much as I will discuss the structural and motile elements that may be involved in bringing them together to the metaphase plate and then distributing them to the daughter cells. Daniel Mazia (1961) considered the chromosomes to be like the corpse at the funeral where everybody expects it to be there, but nobody expects it to play an active role. When we study the movements of chromosomes, we must remember the admonitions of Aristotle in *The Movement of Animals* (Barnes, 1984). That is, the movement of one part depends on the nonmovement of an adjoining part. Thus, in order to have motion, there must be an anchor that is capable of providing resistance to motion. Archimedes realized the importance of an anchor when using a lever when he said, "Give me a place to stand—and I shall move the world" (see Heath, 1897). In mitosis, it is possible that separate proteins act as the lever and the anchor, although a given protein may have dual functions. That is, a cross-bridging protein may act as a motor to induce motion at one time, and as an anchor, against which another motor may exert a force at another time.

The segregation of the genome occurs in all dividing cells; however, mitosis only occurs in eukaryotes. In prokaryotes, genomes are segregated by the growth of the plasma membrane between the attachment points of the two replicated DNA molecules. The process of mitosis is far more complex and it has gone through evolutionary change in each eukaryotic kingdom. In general, the segregation of the genome depends less and less on membranes and more and more on spindle fibers as one progresses up the evolutionary ladder (Kubai, 1975, 1978). The dinoflagellates provide examples of intermediates between typical prokaryotic and eukaryotic genome division (Kubai and Ris, 1969). Knowing that there are both similarities and differences in the structure of the mitotic apparati in various organisms, we might expect that there also will be similarities and differences in the mechanisms involved in mitosis.

Mitosis is a continuous process (Figures 19.2 and 19.3). For convenience, however, it is divided into six phases: prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis. Karyokinesis, or the division of the nucleus, is typically, but not always, followed by cytokinesis, where the cytoplasm is divided into two. It should always be kept in mind that mitosis is a three-dimensional process. Charles Walker (1907) wrote in *The Essentials of Cytology*, "It is of the greatest importance to the right understanding of the phenomenon of mitosis that all mental concepts of the various phases of the process should be definitely three dimensional." Consequently, Walker included in the book a series of stereopticon cards, which gives one three-dimensional views of mitosis.

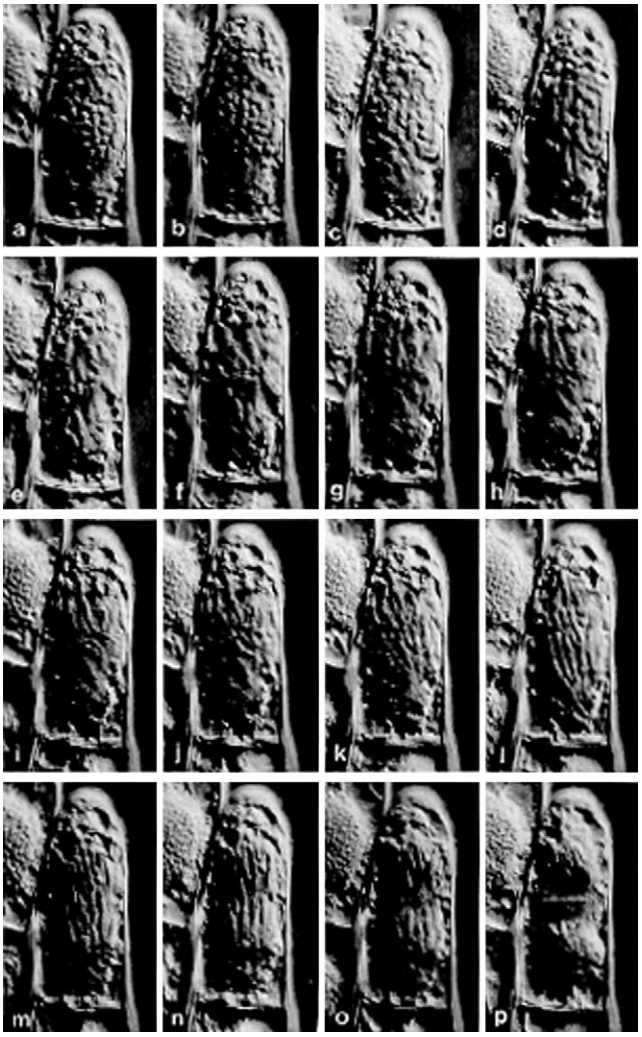


FIGURE 19.2 Time-lapse photographs of mitosis in a stamen hair cell of *Tradescantia*. The entire sequence was shot in 52 minutes. ×680. (*Source:* From Hepler, 1985.)

Mitosis has been traditionally studied in two types of cells. One type, which is good for morphological studies, is optically clear and has few large chromosomes (Bajer and Mole-Bajer, 1972; Reider and Khodjakov, 2003). The other type, which is good for biochemical studies, can be easily synchronized and grown in large quantities (Zachleder and van den Earle, 1992). Currently, much work, using organisms with small genomes, is being done to discover the genes that code for the proteins that are important for mitosis (Endow et al., 1994). In order to establish a context in which the biochemical and genetic studies can be understood, I will discuss the morphology and movements that occur during each phase of mitosis.

19.1.1 Prophase

Prophase begins as the chromatin starts to condense from its metabolically active state into well-defined chromosomes that are more easily moved through the cytoplasm. Each chromosome is composed of two sister chromatids. Each sister chromatid contains one of the two strands of DNA that result from the semi-conservative DNA replication process that took place during the previous S-phase (see Chapter 16). Some of the proteins that are required for sister chromatid cohesion have been identified (Kerrebrock et al., 1995; Kitajima et al., 2004; Marston et al., 2004; Salic et al., 2004). Typically, each somatic cell has an even number of homologous chromosomes, one set from each parent. (Unfortunately, textbooks often show diagrams of

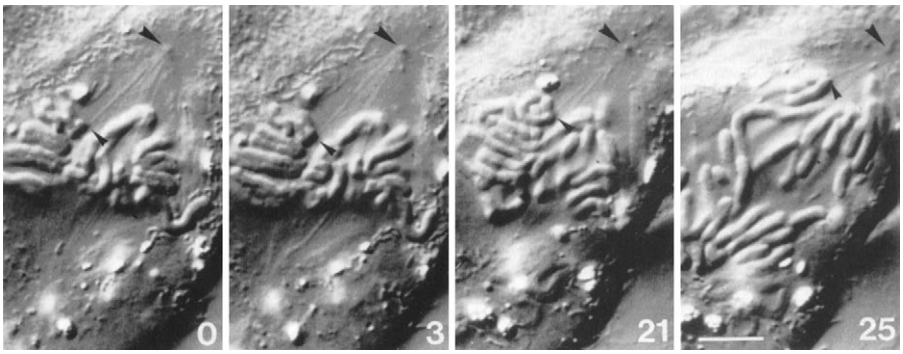


FIGURE 19.3 Mitosis in cultured newt lung cells visualized by Nomarski differential interference contrast microscopy. Metaphase (0 minutes and 3 minutes); anaphase (21 minutes and 25 minutes). The arrow heads point to the centrosome and one kinetochore. Bar, 10 μm. The time in minutes since metaphase is given by the numbers. (*Source:* From Mitchison and Salmon, 1992.)

mitotic cells with an odd number of chromosomes, as if each parent contributed unequally to the offspring.)

During prophase, the mitotic spindle begins to form outside the nuclear envelope from microtubule organizing centers (MTOCs), which are sometimes called *centrosomes* or *centrospheres* (see Chapter 11). In the fungi, the centrosomes are known as *spindle pole bodies* (Aist and Williams, 1972). The centrosome of animal cells typically contains a centriole, and the centriole has been often, although erroneously, considered to be the organizer of the spindle during prophase for over 100 years. In 1895, E. B. Wilson wrote, "It seems certain that the centriole is in many cases a definite morphological body lying within the reticulated centrosphere. … There are, however, many grounds for accepting the view that the centriole, though a frequent, is not a necessary element of the centrosphere."

Evidence that the centriole is not required for spindle formation or mitosis comes from the observation that most plant cells do not have centrioles, although they still form mitotic spindles and undergo mitosis (Mazia, 1961; Inoué, 1964; Pickett-Heaps, 1969a, 1971). Moreover, when the centriole of animal cells is destroyed by laser irradiation, the spindle still forms, and the cell undergoes mitosis. By contrast, irradiation of the pericentriolar material prevents spindle formation, indicating that it is something in the centrosome besides the centriole that is required for spindle formation (Berns et al., 1981). Experiments aimed at forming spindles in vitro indicate that the factors necessary for spindle formation include dynein, dynactin, a dynein-activating protein, and kinesin (Merdes and Cleveland, 1997).

Thus, the MTOC, and not the centriole, contains the fundamental substances necessary to initiate the prophase spindle. The MTOC can have a diverse array of shapes, and in many plant cells, the MTOC may be dispersed along the nuclear envelope (Wick et al., 1981; Mazia, 1984; Mizuno, 1993). The microtubules generated from this MTOC form an optically clear zone around the nucleus (Bajer and Molè-Bajer, 1972; Vandré and Borisy, 1989). The division of the MTOC is another sign that a cell is going from a state of oneness to a state of twoness.

The mitotic apparatus can exist in a multitude of forms. It can appear spindle shaped, barrel shaped, or almost amorphous (Vejdovský, 1926–1927; Belar, 1929; Mazia, 1961; Ambrose and Cyr, 2007). Forms that are even more diverse can appear in hybrids (Darlington and Thomas, 1937; Walters, 1958). Ivor Cornman (1944) suggested that each chromosome has an independent spindle, and Mottier (1903) and Bungo Wada (1966) suggested that each more or less independent spindle joins together to form a bipolar mitotic apparatus of which the shape depends on the shape of the cell. Thus, the concept of independent spindles explains the lack of bipolar organization that exists in the mitotic apparatus of long, thin pollen tubes (Sax and O'Mara, 1941; Palevitz and Cresti, 1989; Liu and Palevitz, 1991), the oblique metaphase plate that occurs in polyploid wheat (Wada, 1966), and other short squat cells with large chromosomes (Palevitz and Hepler, 1974a). Smirnova and Bajer (1993) think that multiple, short-lived nucleating sites may regulate spindle formation. Perhaps the diverse organizations of spindles identifiable in the biological world also represent varying degrees of unity of chromosomes that came together into the same genome by various mechanisms, including duplication and hybridization. Interestingly, when two independent spindles form in a cell created by cell fusion techniques, the two spindles show some degree of coordination (Rieder et al., 1997).

During spindle formation in prophase, the poles move away from the nuclear envelope as the microtubules grow. The separation of the poles, which results in the formation of the bipolar spindle, requires a motive force. The pushing force may be provided by a kinesin-related protein because it has been shown that immunodepletion of a kinesin-related protein prevents bipolar spindle formation (Sawin et al., 1992a,b). A kinesin-related protein may not be the only motor protein involved in spindle formation, since injection of dynein antibodies also inhibits spindle formation during prophase (Vaisberg et al., 1993). These data indicate that spindle formation may be a result of the balanced actions of oppositely directed motors. Spindle orientation also depends on kinesin and dynein in some cells; in others, it depends on actin-related proteins (Stearns, 1997).

During prophase, the viscosity of the nucleus in the regions surrounding the chromosomes is still fairly low, and small particles move in all directions. Just before prometaphase the spindle undergoes a marked contraction and the oscillations of the particles become linear in the direction of the poles (Molè-Bajer, 1958). The viscosity of the nucleus at this stage is about 0.282 Pa s (Alexander and Rieder, 1991). The nucleolus usually disappears during prophase, although it may persist throughout mitosis in some cases (Pickett-Heaps, 1970; Vaughan and Braselton, 1985).

19.1.2 Prometaphase

The onset of prometaphase is marked by the breakdown of the nuclear envelope. While the nuclear envelope typically breaks down and becomes indistinguishable from the endoplasmic reticulum (ER), the integrity of the nuclear envelope is not completely lost since the nuclear envelope–ER complex forms a loose sheath that encircles and isolates the mitotic apparatus from the rest of the cell (Wada, 1966; Hepler, 1980, 1989b). Nuclear pores are no longer seen at this stage, but fenestrated lamellae, which look like nuclear pores without the pore complex, are prevalent (see Figure 4.6 in Chapter 4). It is not known whether the proteins of the pore complexes associate with specific parts of the chromosomes during mitosis. If this happens, the nuclear pores may be positioned next to specific genes during a given stage in development in each cell type. The nuclear

envelope never breaks down in many lower plants, animals, and fungi, and consequently, the spindle forms inside the nucleus (Wise, 1988; Rose, 2007).

If and when the nuclear envelope breaks down, the spindle microtubules permeate the nuclear region and attach to the kinetochores. The nuclear envelope–ER complex also permeates the spindle and runs coparallel with the microtubules that attach to the kinetochores (Hepler and Wolniak, 1984).

The spindle fibers typically attach to a visibly constricted region of the chromosome known as the *primary constriction*, *kinetochore*, or *centromere* (Sharp, 1934). The terms *kinetochore* and *centromere*, along with 25 other terms, define the part of the chromosome that is attached to a spindle fiber (Schrader, 1944). The terms were defined to mean the same structure, and consequently, I will use them interchangeably. However, there is a tendency in the literature to refer to the DNA and protein in the primary constriction as the centromere and kinetochore, respectively. In some monocots, as well as in the arthropods, the chromosomes do not have a visible primary constriction, and the microtubules attach along the whole length of the chromosome. In this case, each of the chromatids has a diffuse kinetochore.

Localized kinetochores appear to be crescent-shaped, trilaminar plates or diffuse balls in the primary constriction that have similar staining properties as centrioles (Schrader, 1936). Kinetochores can be isolated from mitotic chromosomes, and the proteins that make up the kinetochore are being identified (Brinkley et al., 1989; Brinkley, 1990; Mole-Bajer et al., 1990; Compton et al., 1991; Liu and Palevitz, 1992; McEwen et al., 1993; Starr et al., 1997). When a localized kinetochore is in the middle of a chromosome, the chromosome is said to be metacentric; when it occurs between a short arm and a long arm, the chromosome is said to be *acrocentric*, and when it occurs at the very end, the chromosome is said to be *telocentric*.

Van Beneden and Neyt (Haydon et al., 1990) first proposed in the late 1880s that the chromosomes attach to fibers generated by the spindle poles. The attachment process results in rapid, irregular oscillations of the chromosomes. This frenzied movement, which is called *metakinesis*, takes place at a rate of 2.5 μm/s. As a result of this movement, the chromosomes become drawn out to fine points at their kinetochores and then relax as they continue to interact with the spindle. Initially, the kinetochore of each chromosome moves toward one or the other pole. By the end of four to six of these rapid oscillations, the arms of the chromosomes have been moved to the metaphase plate where they extend perpendicular to the central spindle (Pickett-Heaps et al., 1980; Pickett-Heaps, 1991; Nagele et al., 1995).

Tippit et al. (1980) interpret the frenzied movement of chromosomes to indicate that microtubules radiate from the pole and capture the kinetochore. Usually one kinetochore of a pair is captured by a microtubule before the other. The attachment of a microtubule from a pole leads to the movement of the chromosome toward that pole. Eventually the other kinetochore of the pair interacts with a microtubule from the other pole and the chromosome undergoes a series of linear oscillations between the two poles until the chromosomes align along the metaphase plate, equidistant between the two spindle poles. If both kinetochores of the same chromosome interact with two microtubules from the same pole, the chromosomes undergo unstable oscillations with a maximal velocity of 2.5 μm/s. During these unstable oscillations one of the kinetochores is able to interact with microtubules from the other pole, and the chromosome then undergoes stable oscillations (0.5 μm/s). During the stable oscillations, additional microtubules attach to the kinetochore and a discrete kinetochore spindle fiber is built. If chromatids are separated by laser microsurgery so that each "chromosome" has only one kinetochore, the chromosome still congresses to the metaphase plate as long as it is captured by microtubules from both poles (Khodjakov et al., 1997). This means that a single kinetochore may have two independent domains, and the motile function of each domain acts independently.

Using newt lung cells, which have a particularly optically clear nuclear region, Haydon et al. (1990) have shown that the microtubules do radiate from the centrosome during prometaphase and then capture kinetochores. These microtubules exhibit dynamic instability (see Chapter 11). They elongate at a rate of 0.24 μm/s and shorten at a rate of 0.26 μm/s. Each microtubule repeatedly elongates toward the chromosome and catastrophically shortens until it finds a kinetochore within its "casting range." When a microtubule attaches to the kinetochore, the chromosome moves poleward (Figures 19.4 and 19.5). According to Rieder and Alexander (1990),

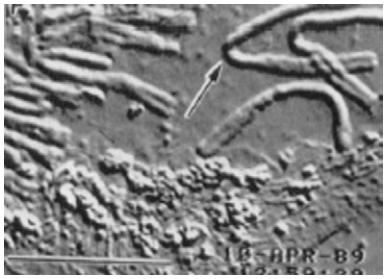


FIGURE 19.4 Nomarski differential interference micrograph of a newt lung cell undergoing prometaphase movement. Bar, 20 μm. (*Source:* From Rieder and Alexander, 1990.)

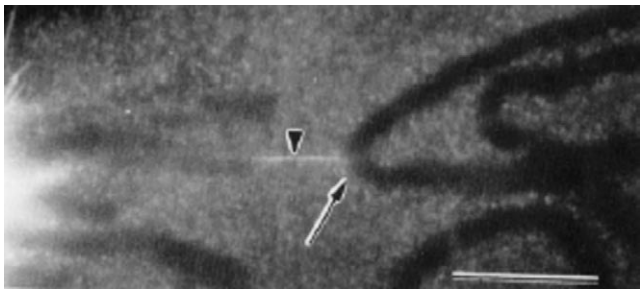


FIGURE 19.5 Immunofluorescence micrograph showing a single microtubule that attached to a kinetochore during prometaphase movement in newt lung cells. Bar, 10 μm. (*Source:* From Rieder and Alexander, 1990.)

The MTs [microtubules] can therefore be envisioned to be analogous to a stationary "fisherman" who casts radially about in search of fish in the surrounding water. As with chromosomes in NPs [Newt pnematocytes] hungry fish not within the range of casts will not be caught until either the fisherman moves closer to the fish or the fish wanders closer to the fisherman. Our data indicates that the "casting" range of the NP centrosome is seldom >50 μm.

After the microtubule captures the kinetochore, the kinetochore moves along its surface (Rieder and Alexander, 1990). The microtubule does not terminate at the kinetochore, but some distance past it, indicating that poleward movement of the chromosome does not have to be coupled to microtubule disassembly. Moreover, this observation indicates that the motor is either on the surface of the microtubule, on the corona of the kinetochore, or both. Rieder and Alexander suggest that dynein provides the force to move the chromosomes poleward at the observed speeds of 0.4–0.9 μm/s. Dynein has been localized in the kinetochores (Pfarr et al., 1991; Steuer et al., 1990), and antibodies directed against dynein inhibit prometaphase movements (Wise and Bhattacharjee, 1992). Other kinetochore proteins may also be involved in prometaphase movement (Bernat et al., 1990; Cooke et al., 1990; Simerly et al., 1990; Lombillo et al., 1995b; King et al., 2000). Laser microsurgery experiments show that the kinetochore that follows the leading kinetochore to the pole does not exert any pushing force (Khodjakov and Rieder, 1997). These data indicate that, in these cells, each kinetochore can exist in two states: the pulling state and the neutral state.

Gorbsky and Ricketts (1993) found an antibody that recognizes a phosphoprotein in kinetochores. In mid-prometaphase, when the chromosomes are oscillating rapidly, some chromosomes show staining on both sister kinetochores, and others show staining on only one sister kinetochore. It turns out that in the case where one kinetochore is stained, only the leading kinetochore is stained. Gorbsky and Ricketts conclude that dephosphorylation prevents the activity of a minus end–directed motor (i.e., dynein) or a minus end–directed kinesin.

Currently there are two hypotheses to explain how the chromosomes move away from the pole during prometaphase. One is that the polymerization of microtubules from the poles push the chromosomes away as the microtubules polymerize (Haydon et al., 1990). The alternative hypothesis is that kinesin-related proteins provide the plus end–directed force. These hypotheses are not mutually exclusive. The first hypothesis is supported by the observation that chromosome oscillations are inhibited in vivo by the microtubule-stabilizing agent taxol, and that chromosome fragments are pushed away from the closest pole (Ault et al., 1991; Rieder, 1991). The polar ejection forces do not occur in all cells. In fact, in *Haemanthus* endosperm cells, chromosome fragments are pushed toward the closest pole during prometaphase. The poleward force is inhibited by colchicine. Thus, in contrast to vertebrate cells, where polar ejection forces are typical, in *Haemanthus*, polar ejection forces are absent, and microtubule-dependent, kinetochore-independent poleward forces exist (Khodjakov et al., 1996).

The hypothesis that chromosomes move away from the poles due to the action of a kinesin-related protein is supported by the observations that kinesin-related proteins are present in kinetochores of mitotic cells (Sawin et al., 1992b), and that kinesin-related genes appear to regulate mitosis and meiosis (Rieder, 1991; Roof et al., 1992; Hoyt et al., 1992). However, if in fact the kinesin only acted as a bridge between the kinetochore and a kinetochore MT (Desai and Mitchison, 1995), then the polar ejection force could be the only pushing force, and the prometaphase motor in the kinetochore would have only a pulling and a neutral state (Khodjakov and Rieder, 1996; McEwen et al., 1997).

During prometaphase, the microtubules that become attached to kinetochores are more stable than non-kinetochore microtubules (Cassimeris et al., 1988; Huitorel and Kirschner, 1988; Salmon, 1989). Cooling, heating, cell lysis, high pressure, and treatment with colchicine causes the non-kinetochore microtubules to depolymerize before the kinetochore microtubules. If biotinylated tubulin is microinjected into the cell at prometaphase, non-kinetochore microtubules become fully labeled within 1 minute, whereas kinetochore microtubules take 10 minutes to become fully labeled (Mitchison et al., 1986). These data indicate that the tubulin dimers in non-kinetochore microtubules turn over more quickly than the tubulin dimers in kinetochore microtubules.

Current data indicate that the kinetochores themselves do not initiate the majority of the microtubules under normal conditions in vivo (Bergen et al., 1980; Summers and Kirschner, 1979), although the possibility remains that they initiate one or two. At one time it appeared that the kinetochore was the primary site of microtubule initiation based on the observations that microtubules are attached to the kinetochores in normal cells (Harris and Bajer, 1965); the kinetochore has similar staining properties as the centriole (Schrader, 1936); microtubules can grow from the kinetochore in vivo (Carothers, 1936; Pollister and Pollister, 1943) and in vitro (McGill and Brinkley, 1975; Snyder and McIntosh, 1975; Telzer et al., 1975; Gould and Borisy, 1978); and kinetochores nucleate microtubules in vivo in cells that had been previously treated with microtubule depolymerizing agents and later released from inhibition (Ris and Witt, 1981). However, when the concentration of tubulin in the various experiments is taken into consideration, it seems that the kinetochores are only capable of initiating microtubules under conditions where the concentration of free tubulin dimers is greater than would be expected under normal conditions in vivo. In normal mitotic cells, the poles and nuclear envelope are the main, if not the only, sites of mitotic microtubule initiation or stabilization (Salmon, 1989). However, chromosomes are necessary for spindle formation since the removal of the

chromosomes with micromanipulators prevents the formation of the spindle (Zhang and Nicklas, 1995).

The kinetochores are capable of organizing microtubules that have been initiated elsewhere, and therefore, Euteneuer and McIntosh (1981) suggest that the kinetochores should still be considered MTOCs, but the concept of MTOCs should be broken up into two parts: microtubule-initiating sites and microtubule-positioning sites. Sometime after prometaphase, centrosomes become dispensable, since they can be removed by micromanipulation and mitosis still proceeds normally (Hiramoto and Nakano, 1988).

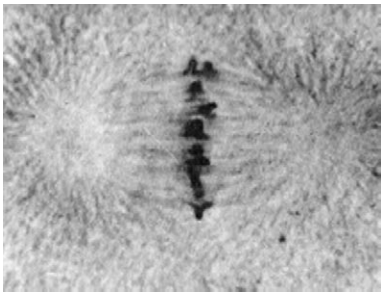


FIGURE 19.6 Photomicrograph of metaphase in the zygote of *Toxopneustes*. (*Source:* From Wilson, 1895.)

19.1.3 Metaphase

Metaphase begins when the chromosomes line up in a plane midway between the two poles (Walker, 1907; see Figures 19.6 and 19.7). The formation of a metaphase plate is not essential for mitosis, and in cells, particularly those of the fungi, the chromosomes do not line up on a metaphase plate (Aist and Williams, 1972). The mitotic apparatus is fully developed during metaphase. While the fibers in the mitotic apparatus were readily observed in fixed and stained cells, they were not readily observed in living cells, and were considered a fixation artifact (Schrader, 1934). The reality of the spindle fibers began to be appreciated when they could be seen in living cells with polarized light microscopy (Schmidt, 1939; Schmitt, 1939; Swann, 1951a,b, 1952; Inoué and Dan, 1951; Fuseler, 1975), and the spindle could be isolated as a semi-functional unit (Mazia, 1956). It was not until the introduction of electron microscopy, however, that we could really get an appreciation of how the fibers were arranged in the mitotic apparatus. We know that in general, the spindle is mainly composed of two kinds of microtubules—those that run from the pole to the kinetochore, which are called *kinetochore microtubules*, and the others that do not attach to kinetochores (Heath, 1981; McDonald, 1989). This distinction is less than clear, since the microtubules join together and appear as "fir trees" (Bajer and Molé-Bajer, 1986).

The arrangement of microtubules varies from spindle to spindle (Pickett-Heaps, 1969a; Kubai, 1975; Fuller, 1976). Paweletz (1967) finds that the non-kinetochore microtubules do not run continuously from pole to pole but different sets of microtubules are formed from each pole and their plus ends overlap in the middle. On the other hand, Ding et al. (1993) show that some microtubules are continuous from pole to pole. Likewise, three-dimensional reconstructions of serial-sectioned spindles indicate that the kinetochore microtubules are continuous between the kinetochore and the spindle pole in some cells (McDonald et al., 1992; Ding et al., 1993), but not in others (Schibler and Pickett-Heaps, 1987).

The orientation of the microtubules can be inferred from the observations by Ritter et al. (1978) who observed microtubules grow from the poles to the kinetochores in

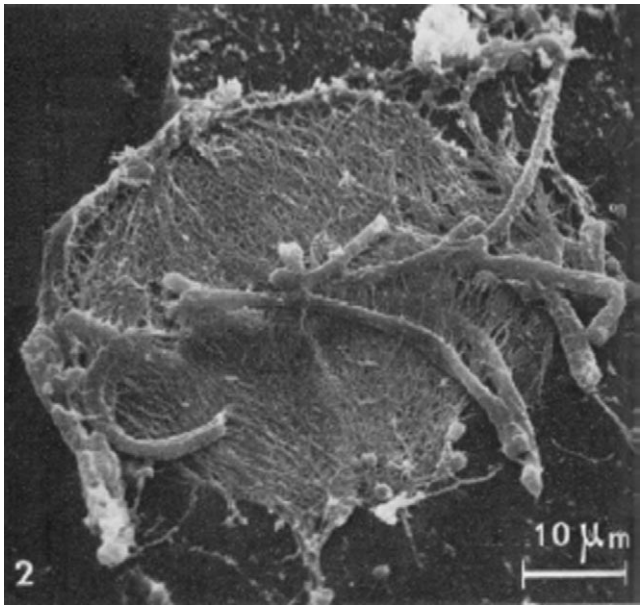


FIGURE 19.7 Scanning electron micrograph of metaphase in the endosperm cells of *Haemanthus katherinae*. (*Source:* From Heneen and Czajkowski, 1980.)

Barbulanympha. This would suggest that the minus end is situated at the centrosome and the plus end extends toward the kinetochore. Euteneuer and McIntosh (1980, 1981) and McIntosh et al. (1980) have confirmed this polarity of microtubules in PtK_1 and *Haemanthus* cells using neurotubulin to decorate the microtubules. When they add neurotubulin to fixed material it forms hooklike projections from the microtubules that are clockwise when we are looking from the plus end. Euteneuer and McIntosh (1981) find that 90–95 percent of the microtubules between the kinetochores and the pole are oriented with their plus end toward the kinetochore and their minus end toward the pole. The polarities of the microtubules in each half-spindle are opposing. Using dynein to decorate the microtubules of the meiotic spindle of the clam *Spisula*, Telzer and Haimo (1981) also show that the majority of the microtubules in the half-spindle have the same polarity; that is, the minus end is oriented toward the pole and the plus end is oriented toward the kinetochore.

During metaphase, the microtubules of the spindle are not static but continuously exchange subunits with the pool of free tubulin dimers. This can be seen by microinjecting fluorescently labeled tubulin into the cell and observing the redistribution of fluorescence after photobleaching (Salmon et al., 1984; Wadsworth and Salmon, 1986; Hush et al., 1994). These experiments show that the turnover time for the microtubules in the spindle is about 30 seconds. These experiments confirm that the kinetochore microtubules are more stable than the non-kinetochore microtubules during metaphase as they are during prometaphase (Mitchison et al., 1986).

During metaphase, as in prometaphase, the attachment of microtubules to the kinetochore does not prevent tubulin exchange at the plus end of the kinetochore microtubule. Mitchison et al. (1986) microinjected biotinylated tubulin during metaphase, and fixed the cells at various times after microinjection. They found that within 1 minute biotinylated tubulin was incorporated into the kinetochore microtubules next to the kinetochore. Wise et al. (1986) found the same thing at the light microscope level using fluorescently labeled tubulin. Thus, tubulin exchange occurs at the plus ends of microtubules stabilized by the kinetochore.

The haphazard metakinetic movements of the chromosomes that occur during prometaphase give rise to the apparently ordered positioning of chromosomes midway between the two poles. This position is known as the *metaphase plate* (see Figures 19.6 and 19.7). How do the chromosomes congress exactly to this position? Van Beneden (1883), Cornman (1944), and Östergren (1949, 1951) proposed that the fibers, which appear between the kinetochores and the poles, exert force on the chromosomes. The tensile force (F, in N) produced by elastic fibers, according to Hooke's Law, depends on the spring constant (K, in N/m) and length (x, in m) of the contractile fiber:

$$F = -Kx \tag{19.1}$$

Since the tensile force is proportional to the length, the longer the fiber, the greater the force available to pull the chromosome to the pole. If a chromosome was not on the metaphase plate, the traction fiber on one side would be longer than the traction fiber on the other side, and the longer fiber would pull with greater force until the chromosome lined up midway between the poles. Currently, there is a consensus that the microtubules make up the traction fibers. However, this conclusion may be provisional, and microtubules may only play a part in the tensile system (Pickett-Heaps et al., 1997).

Östergren (1949, 1951) believed that the traction fibers were made out of protein subunits that were themselves contractile. However, it now seems more likely that the tension may be due to the presence of plus end–directed motors that are anchored in the spindle matrix near the pole. If the motors bound to microtubules in a spatially periodic manner, then the force they generated would be proportional to the length of the spindle fiber (Mitchison, 1989; Salmon, 1989).

The tension that holds the chromosomes at the metaphase plate has been demonstrated in experiments in which the kinetochore microtubules are irradiated with an ultraviolet or laser microbeam that severs the microtubule–kinetochore connection. Upon microbeam irradiation, the distance between the nonirradiated kinetochore of the pair and the spindle pole becomes shortened, indicating that the chromosome was under tension (McNeill and Berns, 1981; Leslie and Pickett-Heaps, 1983; Hays and Salmon, 1990; Skibbins et al., 1995). The proteins involved in sister chromatid cohesion are also necessary for the full development of tension (Salic et al., 2004).

Arthur Forer (1965) helped pioneer the technique of optically identifying and analyzing the movements of microtubules in the mitotic apparatus of living cells. Forer's technique, which is presented in a paper that uniquely uses peace signs for symbols in some of the figures, has undergone many modifications to ensure that we can visualize the kinetochore microtubules and not the other elements of the spindle. Mitchison (1989), who has helped pioneer the modifications, injected a type of tubulin into the cell that becomes fluorescent upon relatively low light irradiation. He then activates the fluorescence in a small area of the spindle with a microbeam. Mitchison found that the fluorescent bar moves poleward during metaphase at a velocity of 0.4–0.6 μm/min. He concluded that the forces that cause the poleward flux might also cause the tension in the metaphase spindle (Figure 19.8).

The fact that the tension is necessary to provide stability to the metaphase plate can be demonstrated by micromanipulation of the meiotic chromosomes of grasshopper spermatocytes (Nicklas and Koch, 1969; Henderson and Koch, 1970). When both kinetochores of a chromosome are experimentally attached with a microneedle to one pole, the chromosome oscillates rapidly. However, when the chromosome fibers are restrained with a microneedle, the unstable oscillations stop and the chromosome becomes stable. Thus, the kinetochores cannot intrinsically distinguish between the two poles, but respond to the tension normally imposed on them (Nicklas and Staehly, 1967; Nicklas, 1967). The bipolar orientation of kinetochores at the metaphase plate, which ensures the equal distribution of the hereditary material, is the most stable configuration.

R. Bruce Nicklas (1997) and his colleagues have shown that not only do the cells sense tension, but the tension directly affects the phosphorylation state of a protein in the kinetochore. Li and Nicklas (1995) have shown that when a univalent chromosome is present in a meiotic cell, the cell does not enter anaphase for 5–6 hours. However, if the chromosome is artificially held under tension with microneedles, the cell enters anaphase in less than an hour. When the chromosome is not under tension, a protein in the kinetochore is phosphorylated. However, holding the univalent under tension with a microneedle causes a dephosphorylation of the kinetochore protein (Nicklas et al., 1995). Tension indirectly results in the

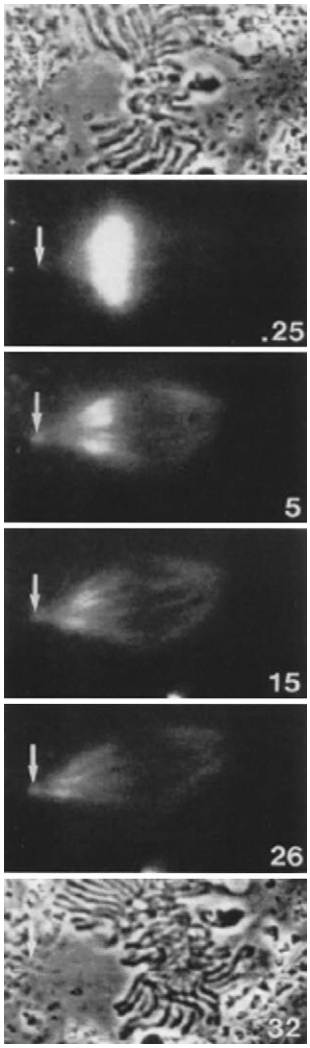


FIGURE 19.8 The visualization of tubulin in newt lung cells by fluorescence microscopy following a photoactivation pulse. The tubulin that has incorporated into the spindle fibers is photoactivated and can then be visualized to move toward the centrosome (arrows) during metaphase. The numbers in the corner indicate time following photoactivation. The top and bottom phase-contrast micrographs show the cell in metaphase and early anaphase, respectively. (*Source:* From Mitchison and Salmon, 1992.)

dephosphorylation of these proteins. The greater the tension to which a kinetochore is exposed, the greater the number of microtubules that are attached to that kinetochore. Once a sufficient number of microtubules attach to the kinetochore, the attachment-sensitive proteins become dephosphorylated (Nicklas et al., 2001). Here is an excellent demonstration of how physical energy can be converted into chemical changes in biological systems.

Errors in the attachment of chromosomes to the spindle could lead to cells with missing or extra chromosomes. If this were to occur during crucial stages of development, birth defects could occur (Nicklas et al., 2001). The attachment-sensitive proteins act as a checkpoint during mitosis and meiosis. Unattached kinetochores have a phosphoprotein known as *Mad2* that binds to the attachment-sensitive protein. Upon attachment of the kinetochore to the spindle, the phosphoprotein is dephosphorylated and Mad2 detaches from the dephosphorylated protein. Anaphase will not commence until all the Mad2 proteins have been lost from the kinetochores. It appears that the loss of the Mad2 protein acts as a "proceed to anaphase" signal that prevents errors in attachment from occurring during mitosis and meiosis (Nicklas et al., 2001).

19.1.4 Anaphase

Anaphase begins abruptly as the paired kinetochores of each chromosome split (Pauliulis and Nicklas, 2000). However, microtubule-mediated tension is not required for chromosome separation since the chromatids separate synchronously from each other even when the microtubules in the spindle have been depolymerized by colchicine (Beams and King, 1938; Levan, 1938; Nebel and Ruttle, 1938; Walker, 1938; Shimamura, 1939; Berger and Witkus, 1943; Molè-Bajer, 1958). The separation of sister chromatids may be due to the activation of a protease that degrades proteins that hold together sister chromatids (Earnshaw and Bernat, 1991; Cooke et al., 1987; Uhlmann et al., 2000; Peters 2002), topoisomerase II (Shamu and Murray, 1992), a change in the charge of the chromosomes (Kamiya, cited in Molè-Bajer, 1958), or to a heretofore unrecognized element of the spindle matrix. Following the longitudinal splitting of the chromosomes, they continue to separate at a rate of 0.2–9 μm/min, which is one of the slower manifestations of cell motility (Barber, 1939; Ris, 1943, 1949; Hughes and Swann, 1948; Aist and Williams, 1972) and on the order of the speed of continental drift (Wegener, 1966; Forer, 1978) and fingernail growth.

Chromosome segregation can be effected by two kinds of movements that distinguish two substages of anaphase: anaphase A and anaphase B. During anaphase A, the chromosomes move to the poles, and during anaphase B, the two poles move apart. The two forms of anaphase occur to different extents in various cell types, and in plants anaphase B is rarely seen. Sometimes anaphase A precedes anaphase B, sometimes anaphase B precedes anaphase A, and sometimes they occur simultaneously (Fuseler, 1975; Hayashi et al., 2007). Intact microtubules are required for both types of anaphase movements (Inoué, 1952; Inoué and Sato, 1967; Sato et al., 1975). Interestingly, when the chromosomes are removed with micromanipulators from dividing cells, "anaphase" still continues, indicating that the chromosomes themselves are not necessary for anaphase movement (Zhang and Nicklas, 1996).

In order to determine the forces that are necessary to move chromosomes to the pole at the observed velocity, we must determine whether inertial forces or viscous forces predominate. To do this, we must estimate the Reynolds number (*Re*; see Chapter 9):

$$Re = (w\ v\ \rho)/\eta \tag{19.2}$$

Given that the observed velocity (v) of chromosome movement is 1.6×10^{-8} m/s, the characteristic length (w) of the chromosome is approximately 10^{-5} m, the density (ρ) of the cytoplasm is 1.0145×10^{3} kg/m^3, and the viscosity (η) of the cytoplasm for chromosomes is about 1 Pa s, therefore, the Reynold's number is about 3×10^{-10}. Thus, any inertial forces will be minuscule, and only viscous forces need to be considered.

The force necessary to move the chromosomes through the cytoplasm can thus be determined with Stokes' Law (see Chapter 9):

$$F_v = w\ v\ \eta \tag{19.3}$$

where w is the size factor for nonspherical objects and is considered to be $25–59 \times 10^{-6}$ m for chromosomes (Nicklas, 1965). Thus, when w is 59×10^{-6} m, then $F_v = 9.4 \times 10^{-13}$ N, which is approximately 1 pN.

This is approximately the force produced by a single molecule of dynein, kinesin, or myosin, and thus a single motor protein would be sufficient to move a chromosome to the pole (Forer, 1969). Indeed, some chromosomes are attached to only one microtubule (Church and Lin, 1985; Nicklas and Kubai, 1985).

While each chromosome only requires about 1 pN to move to the pole, the force that is available to pull the chromosomes to the pole may be much larger. The fact that chromosomes, which have been experimentally shortened by X-ray irradiation, move to the pole at the same rate as normal chromosomes can be interpreted to mean that the motor that pulls the chromosomes is capable of sensing the load and adjusting its force (Nicklas, 1965). Indeed, during anaphase, meiotic chromosomes with two centromeres, known as *bridge chromosomes*, are pulled to the two poles at a rate that is identical to the rate that the other chromosomes are pulled, indicating that the motive force is capable of pulling chromosomes at a constant velocity, even when the load is greater (Cornman, 1944; Östergren et al., 1960). The force that is exerted on a kinetochore by a spindle fiber has been measured with the aid of glass microneedles (Nicklas, 1983). These experiments show that chromosome motion is unaffected by pulling on a chromosome with a force of 100 pN. The velocity decreases linearly as the force is increased and falls to zero at 700 pN. Above this level of force, the chromosomes will unravel (Houchmandzadeh et al., 1997). Thus, the actual force is about 100 times greater than was expected. How can we explain this?

It is possible that the actual force produced by the motor is regulated by the load that the motor has to pull. Thus, as the load is increased with a microneedle, the force increases up to a maximum of 700 pN. Thus, the motor may be able to sense the load and try to maintain a constant velocity. It could do this by regulating the number of motor proteins involved or regulating such properties as the length of the displacement, the rate of ATP hydrolysis, or other things that affect the duration of the mechanochemical cycle.

It is also possible that the discrepancy between the actual force and the calculated force results from the presence of a governor (Nicklas, 1983). The governor effectively increases the viscous force. The kinetochore microtubules may be the governor, and their slow rate of depolymerization may limit the movement of the chromatids, no matter how hard the motor works.

David Begg and Gordon Ellis (1979) used micromanipulators to push a chromosome toward the pole in anaphase. When they did this, the chromosome waited until the other chromosomes caught up to it before it continued to travel to the pole. According to Inoué (1981), "The chromosomes behave as though they were all being reeled in to the pole by individual fishing lines each attached to the kinetochore, but all sharing a common reel." Thus, any theory of anaphase motion must be able to account for the possibility that the chromosomes can move to the pole in an interdependent fashion.

There has been no dearth of theories introduced to explain anaphase motion. Wilson (1925) summarizes the rise and fall of many of the early theories, including the magnetic and electrical theories based on the fact that the spindle "vividly recalls the arrangement of iron filings about the two poles of a magnet." I will discuss a few hypotheses. Any theory must be consistent with the cytological observations that (1) chromosomes move together to the poles as an "anaphase plate" at a constant velocity, and thus the motive force equals the viscous force, resulting in no net force, or else the chromosomes would accelerate; and (2) the kinetochores lead the way to the poles. Like every generalization, however, there are exceptions (Darlington, 1932; Metz, 1936), and until the problem is solved we never know whether the exceptions to the generalizations are just red herrings, or whether they provide the insight necessary to discover the underlying mechanism.

Although currently, the mechanism(s) responsible for anaphase motion during mitosis and meiosis are not known, cell biologists continue to experiment and piece together information in order to fully understand the mechanisms, just as Walther Flemming did when he wrote the following words in 1880: "If, for the time being, we assemble and compare observations on the life processes of the cell, we do so with the hope that they will and must aid in the ultimate insights and physical explanation of the same phenomena. Without this conviction I would have no basis for sitting at my microscope any longer." The mitotic puzzle, the process that ironically is described both as cell *multiplication* and cell *division*, is one of the most intriguing aspects of cell biology, and will never lose its appeal.

The Sliding Filament Hypothesis

After the mechanism of ciliary beating (see Chapter 11) was described it was natural to look for cross-bridges that

may represent the dynein-like mitotic motor responsible for anaphase motion (Hepler et al., 1970). Once such cross-bridges were found, McIntosh et al. (1969) proposed that sliding occurs between antiparallel kinetochore and non-kinetochore microtubules driven by dynein. This model was supported by experiments that showed that vanadate and other inhibitors of dynein inhibit anaphase motion in permeabilized PtK_1 cell models (Cande and Wolniak, 1978; Cande, 1982a,b). It is not supported by experiments that show that antibodies directed against dynein do not inhibit anaphase motion (Vaisberg et al., 1993).

In order to describe anaphase motion, the sliding filament hypothesis requires that the non-kinetochore and kinetochore microtubules be antiparallel. When Euteneuer and McIntosh (1980, 1981) and Telzer and Haimo (1981) discovered that 90–95 percent of the microtubules in each half-spindle have identical polarities, this model lost favor. However, since one pair of antiparallel microtubules would be sufficient to pull a chromosome to the pole, and it is possible that the kinetochore initiates the formation of one or two microtubules, for some cells, this model is still a contender. The sliding filament hypothesis has found support in explaining anaphase B movement since the microtubules are often antiparallel at the zone of microtubule overlap midway between the two poles (Cande and McDonald, 1985, 1986; Masuda and Cande, 1987; Wordeman and Cande, 1987; McIntosh and Koonce, 1989). However, in order to push the poles apart, the motor would have to be a plus end–directed motor like kinesin. Laser microbeam studies in fungal and mammalian cells indicate that anaphase B is predominantly powered by dynein-mediated pulling forces on the astral microtubules that radiate from the poles, and the overlap region actually slows down movement (Aist and Berns, 1981; Aist et al., 1991, 1993; Waters et al., 1993).

Microtubule Depolymerization and Poleward Flux Theories

The mitotic spindle is a dynamic structure that reversibly breaks down when exposed to elevated hydrostatic pressures (Pease, 1946) and microtubule-depolymerizing drugs, including colchicine (Inoué, 1952). While observing the mitotic spindle of *Chaetopterus* with polarization microscopy, Shinya Inoué (1953, 1959, 1964) noticed a remarkable and completely unexpected phenomenon. He observed that as he increased the temperature of the stage, the amount of anisotropy of the mitotic spindle increased. Since a completely random specimen is isotropic, and anisotropy typically indicates that the specimen is ordered, an increase in the anisotropy of the specimen means an increase in order. This was surprising, because typically, increasing the temperature increases the disorder in a system.

This unusual effect of temperature on protein had been observed before with muscle protein and the tobacco mosaic virus protein. It turns out that protein monomers have a shell of water around them, just as ions do (see Chapter 12), and this bound water prevents the protein monomers from interacting among themselves and forming a polymer. The removal of the bound water requires an input of energy, which comes from raising the temperature. As the temperature increases, the shell of water surrounding the protein monomers is removed and hydrophobic interactions between the protein monomers themselves can take place. Inoué proposed that the fibers in the spindle were made out of proteins, and the fibers formed when the protein subunits polymerized.

Inoué quantified his work in the following way: He assumed that the maximum anisotropy occurred when all the subunits were polymerized. The anisotropy of a specimen is measured with a polarizing microscope by determining the phase of the light passing through the specimen in one direction relative to the phase of the light passing through the specimen in an orthogonal direction. Let's call the total concentration of the protein subunits A_o and the concentration of polymerized subunits B. B is proportional to the amount of anisotropy (Γ, in nm) measured with the polarizing microscope. When all the subunits are polymerized $A_o = B$, and when all the subunits are unpolymerized $B = 0$, the free subunits can be calculated as $A_o - B$. Inoué assumed that polymerization occurred according to the following reaction:

$$A_o - B \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\Leftrightarrow}} B$$

where

$$K_{\text{eq}} = [B]/[A_o - B] \tag{19.4}$$

Inoué determined the concentration of polymerized subunits at a given temperature by measuring the amount of anisotropy of the spindle at that temperature. The maximal amount of anisotropy he obtained was an estimate of the total concentration of subunits, and he calculated the concentration of free subunits at a given temperature by subtraction.

The degree of polymerization is determined by the equilibrium constant (K_{eq}), which represents the ratio of polymerized to free subunits ($[B]/[A_o - B]$). The standard molecular free energy (E_{std}) relative to the energy that occurs at equilibrium ($E_{\text{eq}} = 0$) when a subunit is added to the polymer can be calculated from the following equations (see also Chapter 13):

$$\begin{aligned} E_{\text{eq}} - E_{\text{std}} &= kT \ln (K_{\text{eq}}/K_{\text{std}}) \\ &= kT \ln \{([B]/[A_o - B])/(1/1) \end{aligned} \tag{19.5}$$

$$\begin{aligned} E_{\text{std}} - E_{\text{eq}} &= -kT \ln (K_{\text{eq}}/K_{\text{std}}) \\ &= -kT \ln \{([B]/[A_o - B])/(1/1) \end{aligned} \tag{19.6}$$

At standard pressure, the change in standard molecular free energy (relative to the equilibrium state) can be related

to the change in standard molecular enthalpy ($\Delta H_{\text{std}}/N_A$, in J/molecule), and to the change in standard molecular entropy ($\Delta S_{\text{std}}/N_A$, in J molecule^{-1} K^{-1}) according to the following equation:

$$\Delta E_{\text{std}} = \Delta H_{\text{std}}/N_A - (T/N_A)\Delta S_{\text{std}} \tag{19.7}$$

Therefore,

$$\begin{aligned}\Delta E_{\text{std}} &= \Delta H_{\text{std}}/N_A - (T/N_A)\Delta S_{\text{std}} \\ &= -kT \ln([B]/[A_o - B])\end{aligned} \tag{19.8}$$

which can be rewritten as a linear equation:

$$\begin{aligned}&-\ln([B]/[A_o - B]) \\ &= (\Delta H_{\text{std}}/kN_A)(1/T) - \Delta S_{\text{std}}/kN_A\end{aligned} \tag{19.9}$$

If we plot $\ln([B]/[A_o - B])$ versus $(1/T)$, we get a van't Hoff plot, where the slope is equal to $(\Delta H_{\text{std}}/kN_A)$ and the y-intercept is equal to $\Delta S_{\text{std}}/kN_A$. Thus, at a given temperature we can estimate $\Delta H_{\text{std}}/N_A$ and $(T/N_A)\Delta S_{\text{std}}$. Inoué determined the standard molecular enthalpy of polymerization at room temperature to be 1.9×10^{-19} J/subunit and the product of the standard molecular entropy and the temperature to be 2.1×10^{-19} J/subunit. Thus, the standard molecular free energy made available by the polymerization reaction is 2×10^{-20} J. Since $(T/N_A)\Delta S_{\text{std}}$ is greater than $\Delta H_{\text{std}}/N_A$, polymerization of the subunits is an entropy-driven process. Thus, Inoué concluded that the subunits of the spindle fibers were surrounded by bound water and the removal of the bound water caused an increase in entropy. This increase in entropy provided the molecular free energy necessary to drive the polymerization reaction. Moreover, he proposed that the controlled depolymerization of the spindle fibers provided the motive force that pulled the chromosomes to the poles during anaphase. These experiments are remarkable in light of the fact that they were done approximately 20 years before the discovery of tubulin.

After the discovery of microtubules with the electron microscope, Inoué and Sato (1967) recast the dynamic equilibrium hypothesis in terms of microtubules (Sato et al., 1975; Inoué, 1981). The force exerted by a microtubule that is either polymerizing or depolymerizing is given by the following equation:

$$\left|E_{\text{Real}}/x\right| = \left|F\right| = \left|(kT/x)\ \ln([C_T]k_{\text{on}}/k_{\text{off}})\right| \tag{19.10}$$

where x is the length that the microtubule changes on the addition or loss of a dimer, E_{Real} is the molecular free energy of the polymerization reaction under cellular conditions, E_{Real}/x is the force exerted by the polymerization reaction under cellular conditions, $[C_T]$ is the concentration of tubulin, k_{on} is the on-rate constant for the addition of tubulin, k_{off} is the off-rate constant for the loss of tubulin, and $[C_T]k_{\text{on}}/k_{\text{off}}$ is equal to K_{eq}. Assuming $x = 0.61$ nm, approximately the length of a tubulin dimer, Inoué and Salmon (1995) estimated that the pushing force ($|F|$) is 0, 16, 31, or 46 pN when $[C_T]k_{\text{on}}/k_{\text{off}} = 1$, 10, 100, and 1000, respectively, and the pulling force ($|F|$) is 0, 16, 31, or 46 pN when $[C_T]k_{\text{on}}/k_{\text{off}} = 1$, 0.1, 0.01, and 0.001, respectively. Thus, a change in $[C_T]$, k_{on}, and/or k_{off} will result in a force either toward or away from the poles (Cohn et al., 1986).

Koshland et al. (1988) tested the ability of microtubule depolymerization to generate force in vitro. They have shown that chromosomes can move from the plus end to the minus end of isolated microtubules in the absence of ATP. Coue et al. (1991) estimated the force generated by microtubule depolymerization using *Tetrahymena* cells that had been lysed and extracted such that the radial arrays of microtubules remained. They then perfused a solution containing isolated chromosomes by the microtubules until some bound to the microtubules. They then decreased the tubulin concentration so that the microtubules would depolymerize. The depolymerizing microtubules pulled the chromosomes. This movement took place in the absence of ATP and in the presence of vanadate, indicating that motor proteins like kinesin or dynein were not responsible for the movement. Coue et al. (1991) calculated the force exerted by depolymerization to be greater than 1 pN by measuring the depolymerization-mediated movement against a buffer that flowed in the opposite direction.

When microtubules depolymerize in vitro, the typical motor proteins, including kinesin, kinesin-related protein, and dynein, facilitate the minus end–directed movement of microspheres in the absence of ATP, indicating that they may not act as a mechanochemical ATPase, but can act as a bridge between the kinetochore and the depolymerizing microtubules (Desai and Mitchison, 1995; Lombillo et al., 1995b).

The dynamic equilibrium model has been extended to include the properties of microtubules that lead to dynamic instability (see Chapter 11). Microtubules show catastrophic changes in length that are a result of the differing stability of microtubules capped with either guanosine triphosphate (GTP)-tubulin or guanosine diphosphate (GDP)-tubulin. The GTP is spontaneously hydrolyzed over time so that the minus end, the end closest to the spindle pole, is composed entirely of GDP-tubulin, while the plus end, the end attached to the kinetochore is composed of GTP-tubulin. The GTP-tubulin readily binds new monomers of GTP-tubulin and the microtubule grows from the plus end. However, if the rate of GTP hydrolysis exceeds the rate of polymerization, then the plus end will be capped with a GDP-tubulin, which does not bind GTP-tubulin well, and a catastrophic depolymerization at the plus end will ensue. The model then states that the microtubule depolymerizes at the kinetochore in a catastrophic manner and pulls the chromosome along with it.

Shelden and Wadsworth (1992) injected biotinated tubulin into the spindle of PtK_1 cells, and observed with the electron microscope that it was incorporated into the kinetochore microtubules at the kinetochore. When biotin-labeled tubulin

was injected into living cells during anaphase, the chromosomes changed their movement from going toward the poles at 1 μm/min to moving away from the poles at 0.5 μm/min. Moreover, they observed that the kinetochore was compressed during the reversal, indicating that the reversal force was applied at or near the kinetochore. These data support the original hypothesis of Inoué and Sato (1967) that the force for chromosome motion is generated by kinetochore microtubule disassembly. Shelden and Wadsworth (1992) propose that the rate and direction of chromosome-to-pole motion are regulated by the concentration of tubulin subunits at the kinetochore, but it is unclear whether the microtubule depolymerization acts as the motor or the governor (Nicklas, 1965, 1975). During polymerization and depolymerization, the kinetochores remain attached to the plus ends of the microtubules (Hyman and Mitchison, 1990; Coue et al., 1991) because the electrostatic or hydrophobic interaction energy between the kinetochore and the microtubule is greater than the thermal energy necessary for the diffusion of the chromosome away from the microtubule.

In order to determine whether the depolymerization of microtubules at the kinetochore is limiting for anaphase motion, Sawin and Mitchison (1991a,b) labeled cell extracts from *Xenopus* eggs with photoactivatable tubulin. During anaphase, the distance between the bright band arising from the photoactivated fluorescent tubulin and the chromosomes does not change while the bright band of tubulin moves poleward at a rate of 2.9 μm/min, similar to the rate of chromosome movement to the pole. This poleward flux was inhibited by AMP-PNP and unaffected by vanadate. Since AMP-PNP inhibits both poleward flux and kinesin-related proteins, and since both poleward flux and kinesin-related proteins are insensitive to vanadate, Sawin and Mitchison propose that the poleward flux is driven by a kinesin-related protein. Kinesin-related proteins move along microtubules from the minus end to the plus end in an ATP-dependent manner. Thus, if kinesin-related proteins were immobilized in the spindle matrix, they would pull the microtubule toward the pole (Mitchison and Sawin, 1990). Thus, it seems that depolymerization at the minus end combined with a kinesin-related motor is responsible for anaphase motion in *Xenopus* eggs. Higher-resolution studies show that depolymerization of MTs at the plus end contributes a little to the movement of chromosomes to the pole (Maddox et al., 2003).

Mitchison and Salmon (1992) used the same technique to study newt lung cells. They find that in these cells, the chromosomes move to the pole at a rate of 1.7 μm/min, while poleward flux occurs at a rate of only 0.44 μm/min, indicating that in these cells, poleward flux can only account for about one-quarter of the motive force, and the majority of the force necessary for anaphase motion must be provided at the kinetochore. Likewise, Zhai et al. (1995) find that more than two-thirds of the force necessary to move chromosomes during anaphase in PtK_1 cells must be provided at the kinetochore. This force could be provided by depolymerizing microtubules or by an MT-associated motor. By contrast, poleward flux occurs at the same rate as chromosome movement in tobacco BY-2 cells, indicating that poleward flux provides the lion's share of the force necessary for anaphase movement in these cells (Dhonukshe et al., 2006).

The Motile Kinetochore Model

Cytoplasmic dynein has been localized in kinetochores (Pfarr et al., 1990; Steuer et al., 1990; Lombillo et al., 1995a,b; Wordeman and Mitchison, 1995) and is capable of moving a chromosome from the plus end to the minus end of a microtubule. The observation that anaphase can be inhibited by inhibitors of dynein in PtK_1 cells (Cande and Wolniak, 1978; Cande, 1982a,b) indicates that dynein is important for anaphase motion. Dynein is capable of producing velocities around 1 μm/s. However, anaphase motion is 1–3 μm/min. Perhaps the slow velocity at anaphase results from the possibility that microtubule depolymerization at the kinetochore limits the velocity that dynein is capable of producing. According to this model, the kinetochore microtubules, due to their slow rate of depolymerization, act as a governor and the dynein molecules are the motor.

The motile kinetochore model is supported by observations by Hiramoto and Nakano (1988) on sand dollar eggs and Nicklas (1989) on spermatocytes. They have come up with a novel approach to test whether the kinetochores are passively pulled to the poles by traction forces or whether they actively move down the microtubules. They cut the spindle with a microneedle by pressing the spindle between the microneedle and a coverslip. When they separate the chromosomes from the pole in this way, the chromosome still moves to within 0.5 μm of the ends of the microtubules during anaphase, indicating that the motor must be in or near the kinetochore.

Actin

Arthur Forer (1969, 1978, 1988) has long held the view that, until proved otherwise, actin is the universal cellular motile protein and most likely participates in any given motile process, just as ATP is the universal energy currency and DNA is the universal genetic molecule (see Chapter 10). This has not been a very popular view since cytochalasins (Schmit and Lambert, 1988), anti-actin antibodies (Molè-Bajer et al., 1988), or phalloidin (Schmit and Lambert, 1990) do not typically inhibit chromosome motion during anaphase. However, cytochalasin B inhibits mitosis in mycoplasma (Ghosh et al., 1978) and Pickett-Heaps et al. (1996) and Sampson et al. (1996) have shown that in the cells of *Oedogonium*, actin microfilaments are coaligned with kinetochore microtubules and cytochalasin inhibits mitosis in these cells. Cytochalasin also inhibits meiosis in crane-fly spermatocytes (Forer and

Pickett-Heaps, 1998). In these cells, anti-actin and anti-myosin (2,3 butanedione 2-monoxine) drugs inhibit poleward flux during metaphase (Silverman-Gavrila and Forer, 2000, 2001). Thus, the actomyosin system may provide the force for anaphase movement in some cells.

Double Insurance and Functional Redundancy

There are probably multiple mechanisms that account for the coordinated movement of chromosomes during anaphase in all cells. Metz (1936) wrote,

> *Considering the fundamental nature of the process of mitosis and the essential uniformity of its results throughout both plants and animals it is only to be expected that a mechanism would exist which would insure its correct operation under widely varying conditions. A single agency could hardly be effective to this extent, and it seems only natural that in most organisms definite insurance should be provided by the presence of additional agencies. One is reminded here of Spemann's principle of "double insurance" in the regulation of development of the embryo. I suspect that in mitosis we have not only double, but probably multiple insurance and that ultimately several distinct agencies will be identified here, any one of which could perhaps bring about the necessary chromosomes movements alone if the others failed to act.*

Arthur Hughes (1952) wrote, "Mitosis seems to me to be the supreme example in science of diversity within a unity." The study of mitosis is truly the study of beauty. In fact, according to Samuel Taylor Coleridge, in Roman times, the definition of beauty was "multitude in unity."

I have described many ways that chromosomes could be pulled to the poles. They could be pulled by sliding microtubules by kinesin-related proteins pulling the microtubules to the pole while they were depolymerizing at the minus end. They could be pulled by depolymerization of microtubules at the plus end, with or without an active mechanochemical ATPase. It is also possible that the microtubules act only as a governor and the actomyosin system may provide the motive force. Perhaps each force-generating reaction takes part to a greater or lesser extent in the mitotic or meiotic apparatus of each cell type, and their relative roles represent the balance reached in each cell to have equal division in a nucleus composed of chromosomes, which may have come together by various mechanisms, including duplication and hybridization.

Pickett-Heaps et al. (1996, 1997) have stressed that cell biologists have concentrated too long on the microtubules in the spindle and should pay more attention to the other proteins in the spindle matrix. Forer (1969) argues that the microtubules make up only a few percent of the volume of the spindle. Perhaps the nuclear matrix continues to contribute to chromatin structure and the position of chromosomes during mitosis and the nuclear membrane-matrix-chromatin association never completely breaks down but reforms to make the spindle matrix. This spindle matrix may reform into the nuclear matrix during telophase.

19.1.5 Telophase

Telophase begins when the daughter chromatids arrive at the poles, the kinetochore microtubules disappear, and the nuclear envelope reforms around each daughter nucleus (Benavente, 1991). Nuclear lamins may specifically interact with chromatin to promote nuclear envelope reassembly (Glass and Gerace, 1990). The chromatin begins to decondense and the nucleoli reappear. During telophase, all the nuclear proteins must be rounded up and brought back into the nucleus (see Chapter 16).

19.2 REGULATION OF MITOSIS

Lewis V. Heilbrunn (1956) postulated that Ca^{2+} regulated mitosis after seeing the gelation or contraction that occurs during mitosis and equated it to the gelation seen in Ca^{2+}-dependent blood clotting. The Ca^{2+} hypothesis seemed likely when Weisenberg (1972) showed that Ca^{2+} caused the depolymerization of microtubules. This observation has been repeated in a variety of systems, both in vivo and in vitro (Kiehart, 1981; Keith, 1987; Cyr, 1991). Hepler and his colleagues (Wick and Hepler, 1980, 1982; Wolniak et al., 1980, 1981; Hepler and Wolniak, 1983, 1984), as well as Silver et al. (1980), showed that Ca^{2+}-sequestering membrane systems exist in the mitotic apparatus, and mitosis is inhibited by antibodies and inhibitors directed against the Ca^{2+}-sequestering system (Silver, 1986). Molecular genetic studies have shown that calmodulin is necessary for mitosis (Ohya and Anraku, 1989; Sun et al., 1991).

Ca^{2+} is required for mitosis since calcium channel blockers inhibit mitosis, injection of Ca^{2+} into the cell affects mitosis, and the cellular concentration of Ca^{2+} varies during mitosis. Thus, according to Jaffe's Rules (see Chapter 12), Ca^{2+} is involved in the mitotic regulatory system, but we are still trying to find out how (Kao et al., 1990; Hepler, 1992, 1994).

19.3 ENERGETICS OF MITOSIS

All movement requires energy and chromosome motion is no different—in theory. However, the energetics of mitosis have been very difficult to sort out. Indeed, the first experiments on the energetics of mitosis done by Otto Warburg were harbingers of the difficulties that have been encountered. Otto Warburg (1931) showed that normal cells required respiration for mitosis while rapidly dividing cancerous cells got their energy from the energetically inefficient glycolytic reactions and not the efficient respiratory reactions (see Krebs, 1981a). While Amoore (1962, 1963),

Hepler and Palevitz (1986), Spurck and Pickett-Heaps (1987), and Armstrong and Snyder (1987) have tested the effect of metabolic inhibitors on mitosis, the data are conflicting, and there is no general conclusion.

In order to see how difficult this problem is, I will only consider the molecular entropy contribution to the free energy and not even discuss ATP. Let's assume that the concentration of tubulin increases so that microtubules spontaneously form on MTOCs. Let's assume the microtubules eventually attach to kinetochores. Those microtubules will be present as long as the tubulin concentration remains high. Imagine that a tubulin-binding protein appears that begins to sequester the tubulin subunits. As the free concentration of tubulin falls, the microtubules will depolymerize at a rate that depends on the concentration of tubulin. The depolymerization is capable of pulling the chromosomes to the pole. So while the molecular free energy is important for any motile process, we have to seriously consider how the increase in molecular entropy generated by a variety of molecules may influence molecular free energy used to move the chromosomes to the poles.

19.4 DIVISION OF ORGANELLES

In order for cells to pass on their organelles, the organelles must grow by taking up newly synthesized proteins, enlarge their membrane surface area, divide, and be distributed between the daughter cells (Hoepfner et al., 2005). Interestingly, as a reflection of their evolutionary past, these requirements are of differential importance for the various organelles in the cell. For example, the plastids, mitochondria, and presumably the ER are semi-autonomous organelles, of which the loss during division would be fatal. By contrast, the Golgi apparatus, its associated membranes, the plasma membrane, the endosomal compartment, and the vacuolar compartment are all derived organelles and can be regenerated from the ER. While the preponderance of current data indicate that the peroxisomes should be included in the class of organelles that do not have to divide prior to cell division, but can be derived *de novo* in the daughter cells, the jury is still out (see Chapter 5; Lazarow, 2003; Kunau, 2005; Schekman, 2005).

During cell division, all the organelles are typically distributed to the daughter cells (Wilson, 1925; Cleary et al., 1992; Langhans et al., 2007; Nebenführ, 2007; Sano et al., 2007). While it is unknown how most of the organelles divide, actin and other motile proteins, including FtsZ and dynamin, mediate the division of the plastids and mitochondria, which are part of the autonomous organelles (see Chapters 13 and 14).

19.5 CYTOKINESIS

Typically, but not always, karyokinesis is followed by cytokinesis. Since they do not always occur together, I will consider them to be related but independent processes. I would like to mention that mitosis and cytokinesis are a *sine qua non* for differentiation of some cell types since gamma-irradiated wheat embryos that are incapable of dividing are unable to differentiate guard cells, subsidiary cells, and trichomes—cells that typically differentiate following an asymmetrical division (Foard and Haber, 1961; Haber et al., 1961).

19.5.1 Cell Plate Formation

Melchior Treub (1878) first described cytokinesis in the fixed and stained epidermal cells of orchid ovules. Eduard Strasburger, in his *Textbook of Botany* (Strasburger et al., 1912), called the newly formed cell wall the *cell plate*. It appeared that the cell plate arose from a system of fibrils that was called the *phragmoplast* by Leo Errera[1] (1888). Becker (1932, 1938) saw that the vesicles at the cell plate accumulated stain like vacuoles do, and concluded that the cell plate formed from the fusion of petite vacuoles. Using polarization microscopy, Becker (1938) and Inoué and Bajer (1961) showed that the phragmoplast existed in living cells.

With the advent of electron microscopy, Buvat and Puissant (1958), Porter and Caulfield (1958), and Porter and Machado (1960) observed cells in the process of cell plate formation and concluded that the phragmoplast fibers are real, the membranes of the vesicles become the new plasma membrane, and the contents of the vesicles form the new extracellular matrix. Later, Whaley and Mollenhauer (1963) and Frey-Wyssling et al. (1964) concluded that the vesicles involved in cell plate formation were derived from the Golgi apparatus. With the advent of glutaraldehyde fixations, it became clear that the phragmoplast was composed of microtubules (see Figure 19.9; Hepler and Newcomb, 1967; Hepler and Jackson, 1968; Bajer and Jensen, 1969). The phragmoplast has also been visualized using green fluorescent protein (GFP; Granger and Cyr, 2000b; Hasezawa et al., 2000) and fluorescent microtubule-associated proteins (Smertenko et al., 2000; Twell et al., 2002).

Typically, the microtubules of the phragmoplast begin as the remaining non-kinetochore microtubules at the end of anaphase, but this is not necessarily always the case (Baskin and Cande, 1990). For example, in the algae that are classified in the lines that did not give rise to higher plants, a phragmoplast does not exist; rather, a phycoplast exists. A phycoplast is a group of microtubules that run parallel, instead of perpendicular, to the developing cell plate between the two daughter nuclei in telophase (Pickett-Heaps, 1972, 1975). The independence of the spindle microtubules and the phragmoplast microtubules is underscored by the observation that irradiating the spindle microtubules with an ultraviolet (UV) microbeam prevents

[1]Leo Errera (1894) wrote *The Russian Jews: Extermination or Emancipation?* David Nutt, London.

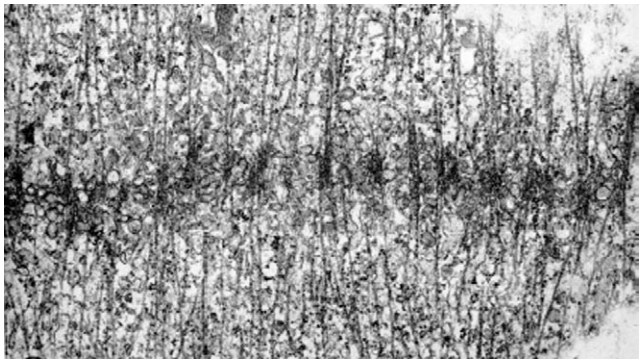


FIGURE 19.9 Microtubules at an early stage of cell plate formation in *Haemanthus katherinae*. ×29,000. (*Source:* From Hepler and Jackson, 1968.)

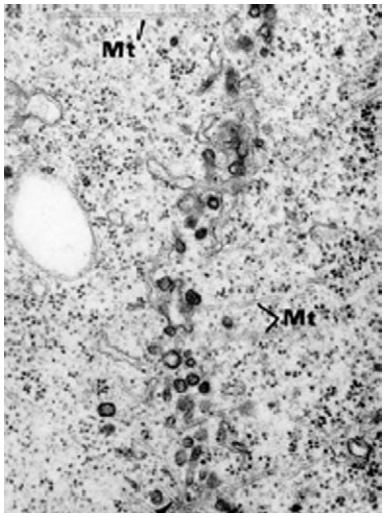


FIGURE 19.10 Early stage of cell plate formation in *Phaseolus vulgaris*. ×38,000. (*Source:* From Hepler and Newcomb, 1967.)

spindle formation but has no effect on phragmoplast formation (Wada and Izutsu, 1961). In addition, chloral hydrate or ethidium bromide inhibit spindle formation and function but have no effect on the phragmoplast (Gunning, 1982), and last, the phragmoplast can form in cytoplasmic drops of endosperm that lack nuclei (Bajer and Allen, 1966).

Bohumil Nemec (1899) noted that the fibers that are perpendicular to the cell plate seem to have free ends, indicating that they may originate at the cell plate. In modern terms, this would make the cell plate area an MTOC. In 1964, Inoué irradiated either the distal ends of the microtubules or the ends that were embedded in the cell plate. Using polarization microscopy to assay the integrity of the microtubules, he found that when he irradiated the distal ends, the proximal ends survived, whereas when he irradiated the proximal ends, the whole microtubule disappeared. This indicated that the end embedded in the cell plate was the growing or plus end. Euteneuer and McIntosh (1980) confirmed this interpretation of the polarity of the phragmoplast microtubules using neurotubulin to decorate the phragmoplast microtubules. Thus, the phragmoplast is composed of two sets of interdigitated microtubules with opposite polarities. Fluorescent microscopy also shows that the plus ends of the microtubules are embedded in the cell plate (Asada et al., 1991, 1997). These data allow us to conclude that the phragmoplast is an MTOC, but the microtubules grow from the center, pushing out the minus ends of the growing microtubules. This contrasts with the microtubule-MTOC relationship in the centrosome and basal body, where the minus ends of the microtubules are embedded in the recognizable microtubule organizing structure.

Typically, the phragmoplast begins in the center of the cell and vesicles move toward the cell plate parallel to the microtubules. Kinesin-like proteins provide the motive force (Lee and Liu, 2000; Liu and Lee, 2001; Lee et al., 2001). Just as the 64-nm-diameter vesicles fuse to form a vesicular-tubular structure, the microtubules in that area begin to shorten and appear C-shaped in cross-section (Lambert and Bajer, 1972; Samuels et al., 1995; Otegui et al., 2001). New microtubules are added to the centrifugal side of the cell plate at a rate of about $8000\,\text{min}^{-1}$. The phragmoplast appears lens shaped as it moves centrifugally since microtubules are polymerizing on the outside edge of it, are maximally polymerized in the center of the moving phragmoplast, and are depolymerizing at the inner edge. Actin microfilaments are also present in the developing cell plate (Kakimoto and Shibaoka, 1987; Staiger and Schliwa, 1987; Palevitz, 1987; Mole-Bajer and Bajer, 1988; van Lammeren et al., 1989; Schopfer and Hepler, 1991).

As the Golgi vesicles move toward the cell plate, their stainability changes, indicating that chemical changes are taking place as they "mature." The Golgi-derived vesicles form tubular, irregularly branched, or star-shaped bodies with fuzzy-coated arms in the plane of the cell plate as they fuse (see Figures 19.10–19.13; Jones and Payne, 1978; Samuels et al., 1995; Staehelin and Hepler, 1996; Seguí-Simarro et al., 2004, 2007). Observations of the developing cell plate in cells that have been fixed by freeze-fixation show details of the variety of membrane morphologies that occur in the initial and later stages of cell plate formation (Figures 19.12 and 19.13). The Golgi-derived vesicles have the endoxyloglucan transferase necessary for the formation of the cell plate (Yokoyama and Nishitani, 2001). Phragmoplastin, a member of the dynamin GTPase family of proteins involved in membrane tubule formation and the pinching off of vesicles, is specifically localized in the cell plate (Dombrowski and Raikhel, 1995; Gu and Verma, 1996; Kang et al., 2003; Hong and Verma, 2007).

The centrifugal microtubules of the phragmoplast form as the centrifugal microtubules depolymerize. The depolymerization of the centripetal microtubules is inhibited by brefeldin A, which indicates that a mechanism exists that ties together the formation of vesicles with the depolymerization of the microtubules that bring those vesicles to the cell plate (Yasuhara and Shibaoka, 2000). That is, the microtubules bring the vesicles to the cell plate and the

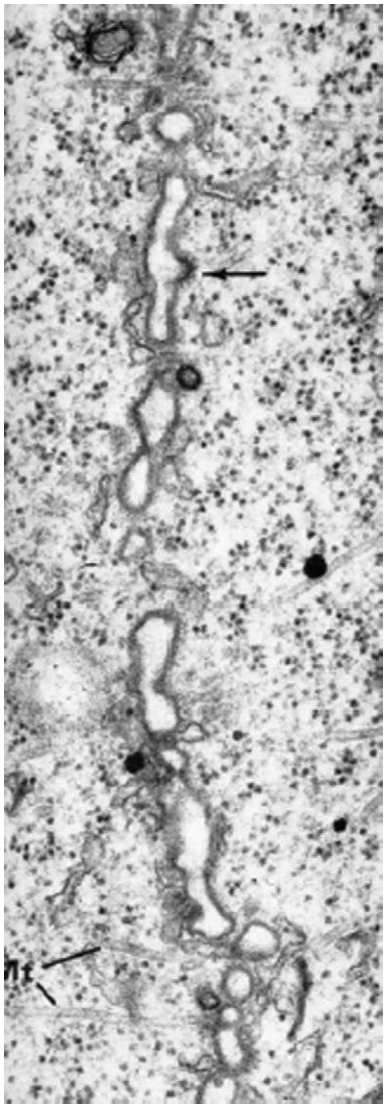


FIGURE 19.11 Mid-stage of cell plate formation in *Phaseolus vulgaris*. ×62,000. (*Source:* From Hepler and Newcomb, 1967.)

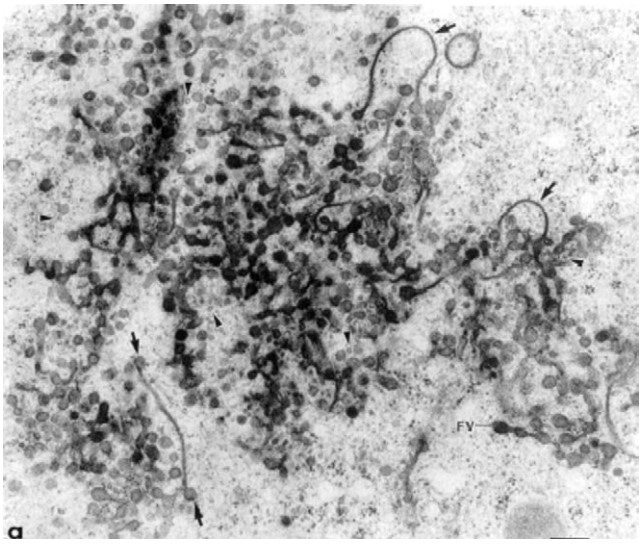


FIGURE 19.12 *En face* view of fusing cell plate vesicles in a tobacco root-tip cell. The arrows show fuzzy-coated tubular membrane structures that are continuous with vesicle-like structures. FV, fusing vesicle. Bar, 250 nm. (*Source:* From Samuels et al., 1995.)

vesicles keep the microtubules there. The membranes of the ER are also evident in the developing cell plate (Porter and Machado, 1960; Hepler, 1982; Gupton et al., 2006) where they give rise to the desmotubule of the plasmodesmata (see Figures 3.3 and 3.5 in Chapter 3).

The first wall products detectable in the cell plate are callose (Fulcher et al., 1976; Northcote et al., 1989; Jones and Payne, 1978) and hemicelluloses (Moore and Staehelin, 1988), although this generalization may turn out to be an oversimplification, and the first products may be cell-type specific. The callose remains only until the cell plate fuses with the parental wall and then disappears. The polymerization of callose may provide the motive force for cell plate growth (Samuels et al., 1995), or it may not be functional, but only a consequence of the high [Ca^{2+}] that may exist in the developing cell plate. The hemicellulose is persistent. Pectins do not seem to be incorporated into the wall at cell plate formation, but only later in development (Moore and Staehelin, 1988). This, however, may turn out to depend on the cell type.

When the cells are challenged with caffeine, an agent that interferes with Ca^{2+} homeostasis, the vesicles move to the cell plate as they do in untreated cells (see Figure 19.14), however, remarkably, after the vesicles come together to form the cell plate, they do not fuse to form the extensive tubulo-vesicular network seen in normal cells (see Figure 19.13), but scatter in the cytoplasm. Consequently, the cell becomes binucleate (see Figure 19.15; Lopez-Saez et al., 1966b; Paul and Goff, 1973; Bonsignore and Hepler, 1985; Hepler and Bonsignore, 1990; Samuels and Staehelin, 1996). While caffeine may affect vesicle fusion and the formation of the vesico-tubular network, it has no effect on the cytoskeletal elements of the phragmoplast (Valster and Hepler, 1997).

Cell plate formation depends, in part, on actomyosin since inhibitors of myosin and agents that affect actin polymerization retard or inhibit the lateral expansion of the cell plate (Valster et al., 1997; Hepler et al., 2002; Molchan et al., 2002).

19.5.2 Isolation of Cell Plates

Hiroh Shibaoka (1992) has developed a system to isolate cell plates for biochemical work. He isolates spindles from synchronized tobacco BY-2 suspension cells. After the cells are synchronized, cells are isolated and treated with cellulase and pectinase to make protoplasts. Then, after a 90-minute incubation with the enzymes, most of the cells are

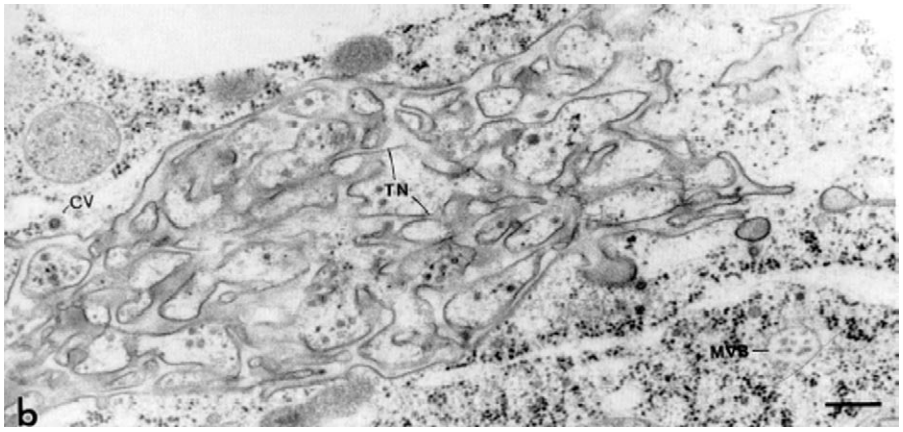


FIGURE 19.13 A tangential section through a tobacco BY-2 cell plate showing the extensiveness of the tubular network of membranes. CV, coated vesicle; TN, tubular network; MVB, multivesicular body. Bar, 250 nm. (*Source:* From Samuels et al., 1995.)

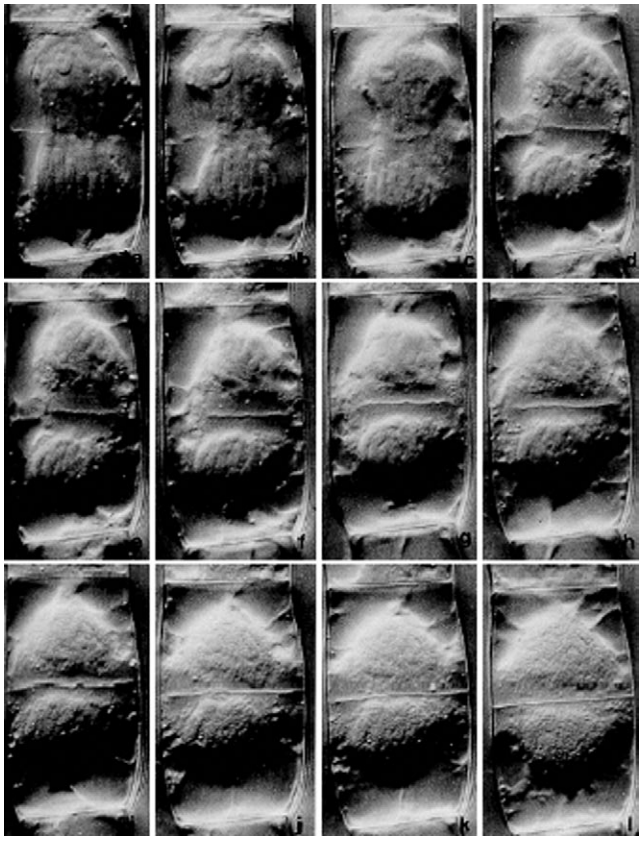


FIGURE 19.14 Time-lapse photographs of cell plate formation in a stamen hair cell of *Tradescantia*. The entire sequence was shot in 27 minutes. The vesicles begin to fuse in the midzone of the cell (c), and thereafter, the cell plate grows centrifugally. (*Source:* From Bonsignore and Hepler, 1985.)

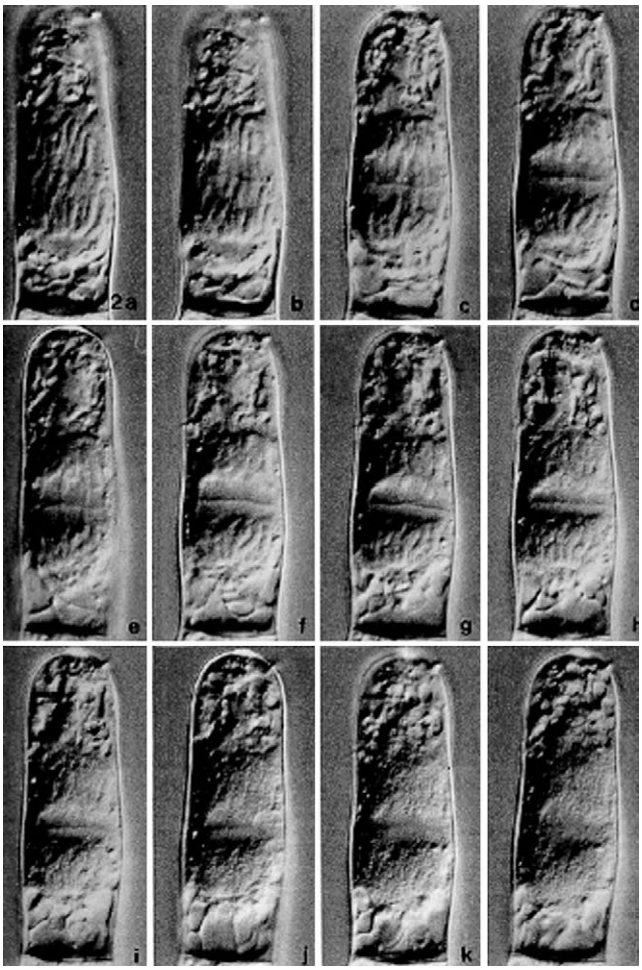


FIGURE 19.15 Time-lapse photographs of cell plate formation in a caffeine-treated stamen hair cell of *Tradescantia*. The entire sequence was shot in 32 minutes. The cell plate begins to form just as it does in the control cells. However, the vesicles never fuse and the cell plate dissolves, leaving a binucleate cell. (*Source:* From Bonsignore and Hepler, 1985.)

in anaphase or telophase. The protoplasts are then washed free of enzymes and resuspended in a buffer in which they are lysed by gently pushing them through a 16-μm nylon mesh. The cell plates are then concentrated by centrifugation at 170 *g* for 2 minutes and resuspended in the same buffer. Using isolated cell plates, Kakimoto and Shibaoka

(1992) have studied enzymes involved in carbohydrate synthesis and microtubule motility. A protein kinase has been found in the cell plate (Nishihama and Machida, 2001; Nishihama et al., 2001).

19.5.3 Orientation of the Cell Plate

Cell division in plants plays an important role in cell differentiation, and often a change in the plane of cell division or a change in the symmetry of cell division is one of the first signs that a cell will differentiate (Lindsey, 2004; Schmit and Nick, 2008). The genetically determined orientation or symmetry of cell division may also be important in the development of form in plants and plant organs. For example, the fruits of cucurbids and tomatoes that come from plants that differ by a single gene have tremendous variations in shape (Sinnott et al., 1950). Sinnott (1960) proposed that there are "shape genes" that control the orientation of the cell plate and/or the polarity of cell growth. What determines the orientation of the cell plate?

In general, the preprophase band predicts the position where the developing cell plate will fuse with the parent cell wall (Pickett-Heaps and Northcote, 1966a,b; Hardham and Gunning, 1980; Gunning, 1982, 1992). It is thought that microfilament arrays radiate from the preprophase band to the nucleus, which provides a mechanism for the orientation of the cell plate (Valster and Hepler, 1997).

However, the preprophase band does not occur in all cell types that have predetermined planes of cell division, and prior to an asymmetric division, the nucleus may migrate to the site of cell division hours before the formation of the preprophase band (Pickett-Heaps, 1969b; Gunning, 1982). Thus, the preprophase band may not directly be involved in determining where the cell plate fuses with the parental wall, but may be a consequence of an earlier polarizing event.

If not the preprophase band, what determines where the cell plate forms and the orientation of the developing cell plate? Sinnott and Bloch (1940, 1941) coined the term *phragmosome* to describe the cytoplasmic structure providing the earliest possible indication of the plane of cell division. They found that in vacuolate cells, the nucleus moves from a parietal position to a central position and strands radiate in a three-dimensional network from the nucleus, but eventually, the strands move into a two-dimensional arrangement, into a plane that predicts the future plane of the cell plate. They emphasized that the cytoplasm, not the nucleus, is involved in determining the plane of cell division.

When cells are wounded the phragmosome aligns perpendicular to the wound (Lloyd, 1991a,b). Venverloo et al. (1980) studied phragmosome formation after stimulating epidermal cells to divide in the periclinal direction by excising a part of the leaf. They found that the nucleus moves from a peripheral part of the cell to the center about 14 hours before the onset of prophase. Then the number of transvacuolar strands increases, generating a three-dimensional system of transvacuolar strands that radiates from the nucleus. A few hours prior to prophase, the strands become two-dimensional in the periclinal plane. Again, the formation of the phragmosome is a gradual process that precedes karyokinesis. Moreover, the phragmosome precedes the preprophase band by several hours (Vanderloo et al., 1980). Panteris et al. (2004) have found that the earliest sign indicating the position of the future cell plate in vacuolate cells is an actin- and ER-rich region that rings the cortical cytoplasm. This cortical cytoplasmic ring (CCR) persists through the cell cycle and its formation is inhibited by actin and myosin antagonists.

The cytoplasmic strands that radiate to the nuclei contain microtubules and are under tension (Hahne and Hoffman, 1984; Goodbody et al., 1991). D'Arcy Wentworth Thompson (1959) predicted that the cell plate formed under conditions of minimal tension. Now we know that each strand of the phragmosome is indeed under tension. It appears that the strands may initiate anywhere, but then move along the cortex until they reach the position of minimum tension. Since the strands are contractile and under tension, according to Hooke's Law ($F = -Kx$), the position that would lead to minimum tension is where the distance from the nucleus to the plasma membrane is the shortest. Since the corners of a cubic cell are farther away from the nucleus than the sides, a new cell plate does not form in the vertex of three cells, and consequently, each vertex has a maximum of three, but not four, walls. Thus, cells are packed in a tissue the way that bricks are placed in a wall—alternating to give maximum strength. It will be important to determine whether the cytoplasmic strands themselves, the microtubules, the actin filaments, and/or the plasma membrane are sensing and responding directly to the various tensions, stresses, and strains (Pickett-Heaps et al., 1999).

For more than 100 years, it has been observed that the cell plate usually forms so as to assume an orientation that results in its occupying the minimum area (Gunning, 1982; Furuya, 1984). Can we be more mechanistic about this observation? There is evidence that the plane of cell division is regulated by the mechanical forces to which the dividing cell is subjected. French and Paolillo (1975) observed that the guard cells that developed on the capsule, under the calyptra of *Funaria* and *Physcomitrium*, always divide longitudinally in the anticlinal plane. However, if the calyptra is removed, thus relieving the capsule of compressive forces, the guard cell mother cells divide in any anticlinal plane, from transverse to longitudinal, and the stomatal complexes appear random.

Philip Lintilhac (1974) and Lintilhac and Jensen (1974) investigated the effects of mechanical stress in developing embryos by combining theoretical models with classic anatomical techniques. They conclude that the cell plate forms in the only plane that is completely free from

shearing stresses, perpendicular to the direction of the maximal principal stress (either compression or tension). Indeed, Lintilhac and Vesecky (1980, 1981, 1984) show that when a mechanical stress is applied to callus tissue the new cell plate forms perpendicular to the source of the compressive stress. Thus, within an organ, the orientation of the division plane depends in part on the mechanical forces set up by the entire organ. By contrast, Lynch and Lintilhac (1997) find that in protoplasts, the new cell plate forms primarily parallel to the principal compressive stress. While the callus cells tend to divide perpendicular to the compressive stress and the protoplasts divide parallel to the compressive stress, in both situations, the cell plate is free from shearing stresses. Indeed, the behavior of the part depends on the physical influence of the whole (Heitler, 1963).

How may the mechanical forces actually control the orientation of the cell plate? Perhaps the mechanical energy is converted into electrical energy that may orient the cell plate. Mary Jane Saunders (1986a,b) finds that a cell-generated ionic current enters the site that predicts the position of cell plate fusion with the parental cell in *Funaria* protonemata. There is more evidence that ionic currents are involved in regulating the orientation of the cell plate (Harold, 1990).

Light as well as other factors, including gravity and the site of sperm penetration, can determine the plane of cell division in the zygotes of the Fucaceae. Lionel Jaffe (1977, 1979, 1980, 1981, 1990, 2004, 2005) and Franklin Harold (1990) proposed that all of these stimuli act by inducing a transcellular current. The photoreceptor responsible for the light-induced polarization is probably localized in the plasma membrane since the direction of the cell plate depends on the azimuth of polarization of the light (Jaffe, 1958).

Jaffe (1966) was interested to test if the zygotes of *Fucus* were polarized by an electrical event, but needed a way to measure the tiny currents and voltages that exists in cells. In order to measure whether or not the zygotes are electrically polarized by light, he placed lots of zygotes in series in a tube. They then secreted a jelly that held them firmly in the tube. He then exposed them to unilateral light. Each cell, like a cell in a battery, contributed a tiny transcellular electrical potential that summed up to something that Jaffe could measure. Since the cells were electrically polarized, he assumed that the zygotes passed a current through themselves. The rhizoidal and thalloidal ends acted like negative and positive poles, respectively. After measuring the voltage generated by the eggs and the resistances of the eggs, Jaffe calculated that each egg generates a current of approximately 2 pA through itself.

Jaffe and his associates then developed the vibrating electrode in order to measure the magnitude and direction of the current. Concurrently, they also determined the ionic composition of the current and found that the electrical current consists mainly of K^+ and Cl^-, but appears to be activated by an initial influx of Ca^{2+} (Robinson and Jaffe, 1973, 1975; Chen and Jaffe, 1978, 1979; Nuccitelli, 1978; Speksnijder et al., 1989; Kühtreiber and Jaffe, 1990), and by cyclic guanosine monophosphate (cGMP) (Robinson and Muller, 1997). This current, as well as the rotation of the cell plate, is disrupted by cytochalasins (Brawley and Robinson 1985; Allen and Kropf, 1992), indicating that actin, which is only observed in the cortex (Kropf et al., 1989), organizes the current. In the zygotes of the Fucaceae and the protonemata of *Funaria*, an electrical current is associated with cell plate formation. However, an important difference exists in these two systems: In the Fucaceae, the current is perpendicular to the plane of the forming cell plate; in *Funaria*, it is parallel.

What causes the cell plate to become oriented in a certain position is still one of the most fascinating mysteries in plant cell biology. It appears to involve actin, mechanical stress, and ionic currents. We could tentatively assume that stress due to growth causes a strain in the plasma membrane, which activates mechanosensitive Ca^{2+} channels, which in turn may activate mechanochemical ATPases (myosin?) that rotate the spindle poles so that they are perpendicular to the direction of growth and so that there is equal tension from both sides. Since maximal growth causes maximal strain in the membrane, the new cell plate will form perpendicular to the direction of growth. In isodiametric cells, the influx of Ca^{2+} would be almost the same everywhere, and the cell plate is random.

The developing cell plate does not always form perpendicular to the parental cell walls. Sometimes it is oblique (Saunders and Hepler, 1981), sometimes lens shaped, sometimes funnel shaped, and sometimes it can even take on a cylindrical shape so that the daughter cells form concentric cylinders (Bierhorst, 1971; Gunning, 1982). These uncommon shapes are common in guard cell mother cells and antheridia. In *Allium* guard cell mother cells, the cell plate begins to form in a transverse orientation but rotates to take its final place in a longitudinal orientation (see Figure 10.11 in Chapter 10; Palevitz and Hepler, 1974a). The reorientation is inhibited by cytochalasins (Palevitz and Hepler, 1974b; Palevitz, 1980), although cytochalasin has no effect on the formation of a normal cell plate (Mole-Bajer and Bajer, 1988). Cytochalasin also prevents the normal orientation of the cell plate that gives rise to the subsidiary cells (Cho and Wick, 1990, 1991). An antimyosin drug inhibits the normal positioning of the cell plate in *Tradescantia* (Molchan et al., 2002). In general, the actomyosin system affects the orientation but not the formation of the cell plate. Some of the genes involved in cell plate orientation and asymmetric cell divisions are being identified (Smith, 2001; Falbel et al., 2004; Sack, 2004).

Plants are exceptional organisms for studying the orientation of the division plane (Wright and Smith, 2007). Particularly impressive are the divisions that give rise to two-dimensional growth from one-dimensional growth in moss and fern gametophytes (Miller, 1968, 1980b; Stetler

and DeMaggio, 1972; Davis et al., 1974; Stockwell and Miller, 1974; Dyer, 1979; Raghavan, 1979; Cooke and Paolillo, 1980; Miller, 1980b; Saunders and Hepler, 1981, 1982; Cooke and Racusen, 1982; Racusen and Cooke, 1982; Racusen et al., 1988; Racusen, 2002). The plane of cell division is also important in animals. Early studies in experimental embryology showed that the cleavage plane of a singled-celled embryo is correlated with the future development of the two protoplasmic parts (Roux, 1888), and one way to ensure that the two daughter cells formed by a dividing cell are different is to produce an asymmetrical division (Bisgrove and Kropf, 2007). An asymmetrical division precedes the formation of many differentiated cell types, including rhizoids in ferns (Murata and Sugai, 2000) and trichomes and guard cells in the epidermis of angiosperms (Nadreau and Sack, 2003; Lucas et al., 2006). The placement of the division plane is just beginning to be studied in animal cells (White and Borisy, 1985; Canman et al., 2003).

19.6 SUMMARY

Mitosis is the process in which one cell divides into two. Typically, nuclear division (karyokinesis) is followed by the division of the whole cell (cytokinesis). In order to ensure an equal distribution of the hereditary material during nuclear division, numerous motile processes occur, including metakinesis, congression to the metaphase plate, and anaphase motion. Each motile process involves force-generating reactions, and the establishment of an anchor, against which force can be generated. In general, it is believed that tubulin-, actin-, dynein-, and kinesin-related proteins are involved in force generation and anchoring. It is becoming clear how these proteins induce the various mitotic movements in individual cell types. However, a unified description of mitosis is not yet at hand.

I also described the process of cytokinesis and how it involves the fusion of Golgi-derived vesicles to form the cell plate. The formation of the cell plate is influenced by a portion of the cytoskeleton known as the phragmoplast. The orientation of the cell plate is also dependent on the cytoskeleton. It is also influenced by ionic currents passing through the plasma membrane.

We are still searching for a unified theory of mitosis that takes into consideration all the experimental and observational data and can describe and explain the great diversity observed in different organisms undergoing mitosis. Such a theory will take into consideration the various physicochemical mechanisms or "means" (which must obey the laws of physics) to attain the same "end" (equal division of hereditary material).

19.7 QUESTIONS

19.1. Do you think that the concept of double insurance and functional redundancy is valuable in understanding biological systems and planning and interpreting experiments? Why or why not?

19.2. What limits the various means a cell can use to accomplish a specific end, like the duplication and division of the hereditary material?

Chapter 20

Extracellular Matrix

Something there is that doesn't love a wall,
That sends the frozen-ground-swell under it,
And spills the upper boulders in the sun,
And makes gaps even two can pass abreast.…

There where it is we do not need the wall:
He is all pine and I am apple orchard.
My apple trees will never get across
And eat the cones under his pines, I tell him.
He only says, "Good fences make good neighbors."

—Robert Frost, "Mending Wall"

20.1 RELATIONSHIP OF THE EXTRACELLULAR MATRIX OF PLANT AND ANIMAL CELLS

In the 17th century, the presence of thick walls in wood and cork made it possible for cells to be readily identified in plants (Hooke, 1665). The presence of a thick wall was the original *sine qua non* for defining a cell, and cells were defined and characterized by their walls and not their contents. The fact that the extracellular matrix of most animal cells is thin or nonexistent was an impediment to the realization that animals were also composed of cells.

Most cells, to a greater or lesser degree, secrete macromolecules into the surrounding medium. These macromolecules amalgamate into an organized structure, which has been called a cell wall, an extracellular matrix, an apoplast, a periplast, slime, a glycocalyx, or a cell covering (Okuda, 2002; Niklas, 2004). Although plant cells generally have a thicker extracellular matrix than animal cells, there is a continuum between the two extremes. Some plant cells, like those of *Dunaliella*, have a thin or nonexistent extracellular matrix, and some animal cells, like those of the tunicates, have an extracellular matrix composed of cellulose (Huxley, 1853a; Hall and Saxl, 1960, 1961). Among plant biologists, the terms *cell wall* and *extracellular matrix* are used interchangeably, and the continuum of cell walls is usually referred to as the *apoplast*. Each term carries with it a slightly different shade of meaning. The term *cell wall* emphasizes the supporting role of the structure, and accentuates the apparent gulf between plant and animal cells. The term *extracellular matrix* emphasizes the primary importance of the plasma membrane and not the cell wall as an active barrier to diffusion in separating the protoplasm from the external environment. The term *extracellular matrix* also emphasizes the lively and dynamic aspects of the region external to the plasma membrane, and has never carried the connotation of being a dead part of cells, as does the term *apoplast*, which comes from the Greek for "without form." The use of the term *extracellular matrix* has been productive in understanding the unity of nature while still allowing an appreciation of its diversity. This is not a new view. Thomas Huxley (1853), in his essay "The Cell-Theory," considered the extracellular matrix of plant and animal cells to be homologous, and gave them a common name: *periplast*.

Young plant cells are surrounded by a thin extracellular matrix that prevents them from lysing when placed in dilute solutions typical of lakes, streams, and soils. By contrast, animal cells with a thin extracellular matrix will lyse unless they are surrounded with a solution that is isotonic with the cell. In fact, the differences in the ionic basis of action potentials in plant and animal cells can be traced to the fact that plant cells, unlike animal cells, are not typically bathed in solutions containing high concentrations of NaCl (Wayne, 1994; Johnson et al., 2002). In order to live on land or in dilute aqueous environments, plant cells have evolved specializations in the extracellular matrix (including the hollow tracheary elements), while animal cells have evolved specializations in the circulatory systems. While the extracellular matrix allows plant cells to live in dilute environments, it contains numerous fixed charges that accumulate considerable amounts of essential nutrients in a nonosmotic form (Grignon and Sentenac, 1991; Gabriel and Kesselmeier, 1999). Thus, the cell carries a suitcase around itself that contains the necessary nutrients found in its ancestral seas.

In the meristems of plants, the extracellular matrix begins to form during cytokinesis (see Chapter 19). The

FIGURE 20.1 The primary wall of *Zea mays* showing a pit field through which the plasmodesmata traverse. ×15,000. (*Source:* From Mühlethaler, 1950.)

primary cell wall is approximately 100 nm thick (Roberts, 1989, 1990, 1994), and is only slightly rigid so that the cell can still expand. The primary cell wall contains even thinner regions, known as *primary pit fields*, through which plasmodesmata pass (see Chapter 3; Figure 20.1). Correlated with the cessation of expansion, the secondary cell wall begins to be deposited either by thickening the primary cell wall (intussusception) and/or by apposition, which is the deposit of new layers of wall material approximately 10 μm thick between the plasma membrane and the primary cell wall (Figure 20.2). The primary and secondary cell walls are defined based on their order of formation (Esau, 1965). The secondary wall is specialized for mechanical support, and often, cells surrounded by a secondary wall are dead at maturity. The thick-walled support tissue, including sclerenchyma tissue, composed of fiber cells with thick secondary walls, and collenchyma tissue, composed of cells with thick primary walls, is distributed within the plant body so as to minimize the effect of mechanical stress on the plant due to wind and other factors (Bower, 1925). In fact, Venning (1949) and Walker (1960) have shown that exposure to wind causes an increase in the amount of collenchyma tissue in a plant. The correlation between thick cell walls and dead cells has historically caused plant biologists to view the cell wall as dead wood instead of the dynamic organelle it is (Bolwell, 1993; Kieliszewski and Lamport, 1994; Carpita et al., 1996; He et al., 1996; Somerville et al., 2004).

The extracellular matrix of plants thickens during development. This allows each cell to generate an internal hydrostatic pressure or turgor pressure in the order of 0.1–1 MPa, which provides the cell with a certain amount of rigidity and mechanical strength. Consequently, plants can grow tall and wide in order to maximize their ability to intercept the sun's rays for photosynthesis. While a thick extracellular matrix is extremely useful for a phototrophic

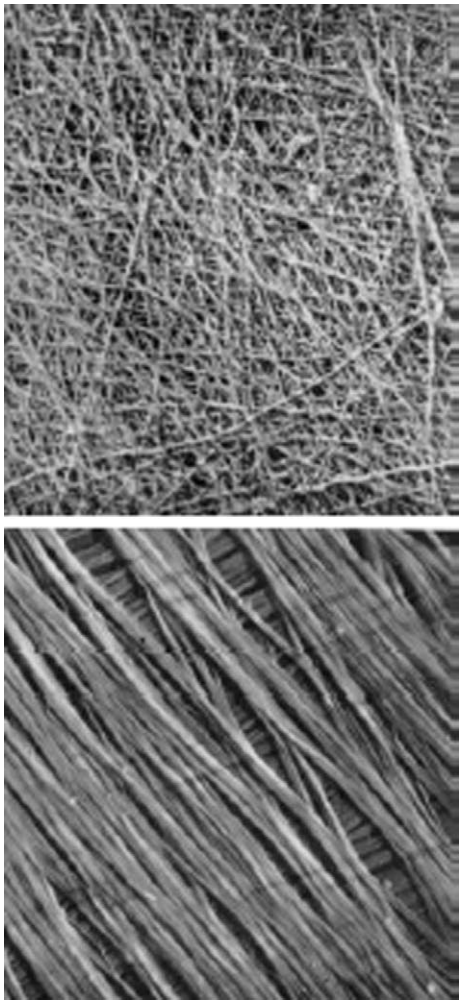


FIGURE 20.2 Surface view of an early stage (top) and a late stage (bottom) of development of the extracellular matrix of *Valonia ocellata*. Top, ×5450; bottom, ×8200. (*Source:* From Steward and Mühlethaler, 1953.)

organism, it prevents plants from moving, thereby making them susceptible to attack by predators.

It is a general biological principle that crises lead to opportunities, and consequently species evolve by natural selection (Darwin, 1859). The very extracellular matrix that prevents plants from moving has evolved a number of properties that allow it to function in the defense of plant cells. In some cases, the extracellular matrix becomes extremely thick in order to prevent the entrance of pathogens (Aist, 1977; Israel et al., 1980; Kobayashi et al., 1997). In other cases, it acts as a lookout at the frontier of the cell, and signals the appearance of a pathogen to the rest of the cell (Bergey et al., 1996; Brady and Fry, 1997; Gelli et al., 1997; Stratmann and Ryan, 1997; Xing et al., 1997). In this role, fragments of the extracellular matrix known as *elicitors* are released by the hydrolytic enzymes of pathogens and signal the rest of the cell to form antiseptic compounds known as *phytoalexins* (Darvill and Albersheim, 1984; Ryan and Farmer, 1991; Zhao and Last, 1996). The extracellular matrix of epidermal

cells in the aerial portion of the plant are clad with a cuticle, which contains cutin and wax that prevents pathogen attack (Cutler et al., 1982; Kerstiens, 1996; Riederer and Muller, 2006). Agrobacterium-mediated transformation also depends on a functional extracellular matrix (Zhu et al., 2003).

The extracellular matrix of plant cells can be considered a keeper of positional information, and as such can be involved in many aspects of cell physiology and development in addition to those mentioned above. The influence of the extracellular matrix can be tested with cell wall mutants and/or by treating cells with cell wall enzymes and their inhibitors (Hofmannová et al., 2008; Paredez et al., 2008). Such studies have shown that the extracellular matrix is involved in organizing the actin and microtubular cytoskeletons (Akashi et al., 1990; Ryu et al., 1997; Fisher and Cyr, 1998; Paredez et al., 2008). It is also required for positioning the nucleus (Katsuta and Shibaoka, 1988), for cell division (Meyer and Herth, 1978; Schindler et al., 1989), for adhesion (Kaminskyj and Heath, 1995; Henry et al., 1996), for appressorium formation (Corrêa et al., 1996), for fixation of the embryonic axis (Kropf et al., 1988), for gravity sensing (Wayne et al., 1992; Staves, 1997), and for embryo formation (Barthou et al., 1999). Likewise, the extracellular matrix of animal cells is involved in parasite interactions (Finley, 1990), signaling during fertilization (Snell, 1990), adhesion (O'Rourke and Mescher, 1990), motility (Zetter and Brightman, 1990), differentiation (Ginsberg et al., 1992; Dansky and Werb, 1992), and gene expression (Slavkin, 1972; Slavkin and Greulich, 1975; Juliano and Haskill, 1993; Sastry and Horwitz, 1993). The thick extracellular matrix of endosperm cells, in which plasmodesmata were first discovered by Tangl (1879), serves as a source of carbon for developing embryo (Otegui, 2007).

There are many fascinating structural specializations of the extracellular matrix that result in extraordinary colors in plants (Fox and Wells, 1971; Lee, 2007). The iridescent blue colors of the leaves of *Selaginella willdenowii* and of the fern *Danaea nodosa* and the "brilliant blue fruits" of rudraksha (*Elaeocarpus angustifolius*) are caused by the interference of light resulting from the multilayered nature of the extracellular matrix in their outer epidermal walls. The blue color of the leaves of the blue spruce results from the selective scattering of short-wavelength light from the small waxy particles that cover the leaves. While the function of these extracellular matrix specializations in the epidermal cells of leaves is not well understood, the striated cuticle in petals that occurs in over 50 percent of angiosperm taxa acts as a diffraction grating and produces colors that may be important as a visual cue for bumblebees during pollination (Whitney et al., 2009).

20.2 ISOLATION OF THE EXTRACELLULAR MATRIX OF PLANTS

In order to isolate the extracellular matrix, tissues are homogenized (Fry, 1988; Harris, 1988), and either centrifuged at 250–1000 g for 5–20 minutes to pellet the extracellular matrix, or filtered through a mesh with pores 5–10 μm in diameter, and the material that is pelleted in the centrifuge tube or trapped in the filter is washed and saved. The purity of the extracellular matrix fraction is assayed by observing it with a light microscope.

20.3 CHEMICAL COMPOSITION AND ARCHITECTURE OF THE EXTRACELLULAR MATRIX

The composition and architecture of the extracellular matrix of plants has been of interest to humanity for millennia due to its importance in the clothing, paper, food, and building industries (Kidd, 1852; Cross and Bevan, 1895; Heuser, 1944; Percival, 1950). Plant cell walls were also used in the manufacture of celluloid and rayon (R. M. Roberts, 1989) and currently plant cell walls and the enzymes that degrade them are important for understanding what causes rot and for the production of cellulosic biofuels (Mandels and Reese, 1965; Reese, 1976; Mousdale, 2008; Rubin, 2008; Somerville, 2008). Consequently, the extracellular matrix of the plants as a whole, and not the extracellular matrix of individual cells, has been studied. Thus, we have a pretty good idea about the "average extracellular matrix," but we are just beginning to understand how unique the extracellular matrices of individual cells are. In general, the plant extracellular matrix is approximately 60 percent water and the dry matter is composed primarily of polysaccharides, including cellulose, hemicelluloses, and pectins (Bauer et al., 1973; Keegstra et al., 1973; Talmadge et al., 1973; Carpita and McCann, 2002; Popper and Fry, 2003).

According to Malcolm Brown (2004), more than 10^{11} tons of cellulose is produced each year, making it the most abundant macromolecule on Earth. Cellulose is a long linear chain of at least 500 glucose molecules linked together with β-1,4 glycosidic bonds. Adjacent cellulose molecules are held together by hydrogen bonds, and approximately 60–90 cellulose molecules, all with the same polarity, are held together to form a cellulose microfibril 4.5–8.5 nm in diameter (Preston, 1939; Burgess, 1985; McCann et al., 1990; Wolters-Arts et al., 1993). The cellulose microfibrils are crystalline and are birefringent when viewed under polarized light (see Figure 11.17 in Chapter 11 and Figure 20.3). Cellulose accounts for approximately 25–30 percent of the dry weight of the extracellular matrix (Burgess, 1985).

Hemicelluloses are a heterogeneous group of branched polysaccharides composed of a linear backbone of a β-1,4-linked homopolymer of a sugar (e.g., glucose), from which short side chains of other sugars (e.g., xylose, galactose, fucose) protrude. The hemicelluloses include xyloglucans and arabinoxylans. The backbone of the hemicelluloses bind tightly but noncovalently to the surface of each microfibril, thus cross-linking them via hydrogen bonds (Hayashi,

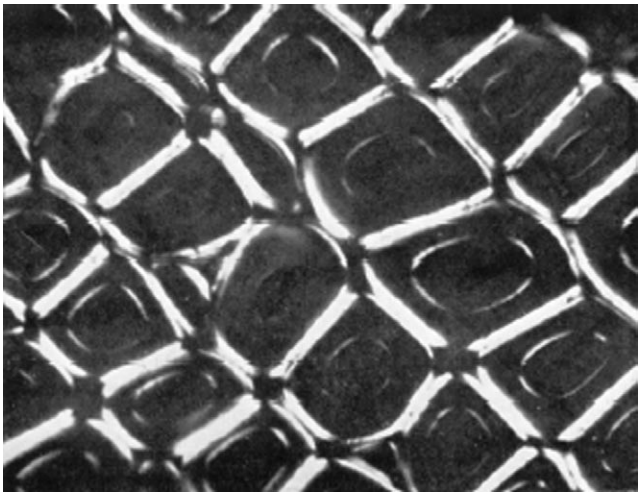


FIGURE 20.3 Transverse section of the tracheids of *Pinus radiate* photographed with a polarizing microscope. Due to the alignment of microfibrils, the extracellular matrix is birefringent, and thus appears bright on a dark background. (*Source:* From Preston, 1952.)

1989, 1991; McCann et al., 1990). The arabinoxylans bind to each other by means of coupled phenols. Hemicelluloses account for about 15–20 percent of the dry weight of the extracellular matrix (Burgess, 1985).

Pectins are a heterogeneous group of branched molecules that contain a backbone of many α-1,4-linked negatively charged galacturonic acid residues. The pectins include polygalacturonic acid and rhamnogalacturonans (Hayashi, 1989; Varner and Taylor, 1989; Shea et al., 1989; Vreeland et al., 1989; Knox et al., 1990). The pectins bind to the cellulose microfibrils via ester bonds and to each other by means of calcium bridges or glycosidic bonds. The carboxyl groups of the galacturonic acid residues bind cations and act as a storage place for these cations (Proseus and Boyer, 2006a; Carpita, 2007). Interestingly, the binding affinity of pectins for calcium ions may be modified by the tension on the pectins (Proseus and Boyer, 2007). Pectins make up about 35 percent of the dry weight of the extracellular matrix (Burgess, 1985). A borate ester is necessary for cross-linking rhamnogalacturonan II so that it forms dimers (O'Neill et al., 1996, 2001; Ishii et al., 2001).

Proteins account for 5–10 percent of the dry weight of the extracellular matrix of plants. Many of the major proteins are glycoproteins and have a high proportion of attached sugars. The most well-known extracellular matrix protein is a protein named *extensin*, which was prematurely named since it has no relationship to cell extension (Lamport, 1965). About 30 percent of the amino acids of extensin and related proteins are hydroxyproline (Cassab and Varner, 1988; Adair and Appel, 1989; Langan and Nothnagel, 1997). Collagen, a major component of the animal extracellular matrix, is also rich in hydroxyproline. The plant extracellular matrix also contains many nonstructural proteins, particularly glycosidases, that are involved in the building and restructuring of its architecture (Cassab and Varner, 1988; Fischer and Bennett, 1991; del Campillo and Lewis, 1992), and proteins involved in interacting with the plasma membrane (Pennell et al., 1989; Schindler et al., 1989; Sanders et al., 1991; Quatrano, 1990; Wagner et al., 1992; Pickard and Ding, 1992, 1993; Lord and Sanders, 1992; He et al., 1997; Caderas et al., 2000; Kim et al., 2000; Rose et al., 2000; Catalá et al., 2001; Madson et al., 2003; Yokoyama et al., 2004). There are also hundreds of cell wall–related genes (Schindelman et al., 2001; Vanzin et al., 2002; Lao et al., 2003; Yokoyama and Nishitani, 2004) and the proteins they encode (Robertson et al., 1997; Bayer et al., 2006; Isaacson and Rose, 2006; Jarnet et al., 2008).

Why is the sugar coating surrounding each cell so diverse in terms of its composition of sugars and glycosidic linkages? In order to be in a position to answer these questions, the structure of the plant extracellular matrix surrounding each cell should be thought of in the same way that Hermann Staudinger (1961) viewed the structure of macromolecules; Emil Fischer, James Sumner, and Frederick Sanger viewed the structure of proteins (Fruton, 1972); and Erwin Chargaff (1950, 1978) viewed the structure of nucleic acids. That is, begin the isolation procedure with the notion that the extracellular matrix is an extremely complex macromolecule, involved in positional information, of which the activity varies with the arrangement of monomers.

Keith Roberts, Bruce Knox, and Andrew Staehelin have changed our ways of looking at the extracellular matrix. They have taught us that the extracellular matrix has both a history and a geography by demonstrating immunocytochemically that the extracellular matrix varies from cell to cell, from one side of the cell to the other, and during the development of the cell (see Figure 20.4; Moore and Staehelin, 1988; Stafstrom and Staehelin, 1988; Moore et al., 1991; Knox, 1992a,b; Lynch and Staehelin, 1992; Levy and Staehelin, 1992). The chemical composition of individual cells can now be studied with infrared microscopes, which can identify the various chemical bonds found in carbohydrates (McCann et al., 1997; Himmelsbach et al., 1999; Carpita et al., 2001). Many studies are showing how dynamic the extracellular matrix is and how the chemical composition of the extracellular matrix changes with the environment (Iraki et al., 1989) and during development (Gorshkova et al., 1997; Peña and Carpita, 2004; Peña et al., 2004; Dunn et al., 2007). Thus, the models of wall architecture derived from grinding up the whole plant serve as a first approximation of the architecture of the extracellular matrix, but an understanding of the role of the extracellular matrix in growth and development will depend on an understanding of the extracellular matrix of the cell in question.

As a first approximation, the extracellular matrix of plants can be considered to be a gel-like matrix of hemicelluloses, pectins, and proteins surrounding cellulose microfibrils just like reinforced concrete is composed of steel wires embedded in cement (see Figure 20.5; Varner and Lin, 1989; McCann et al., 1990). However, in reality, the

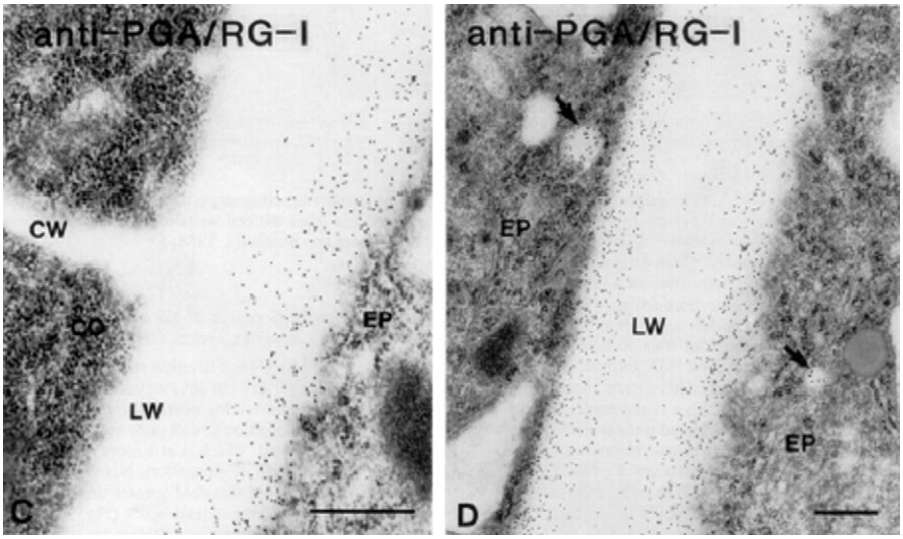


FIGURE 20.4 (c) An antibody to pectins labels the portion of the extracellular matrix next to the epidermal cell (EP) but not next to the cortical cell (CO). (d) An antibody to pectins labels the portion of the extracellular matrix next to the epidermal cells (EP), but not the portion midway between the two epidermal cells. Bars, 500 nm. (*Source:* From Lynch and Staehelin, 1992.)

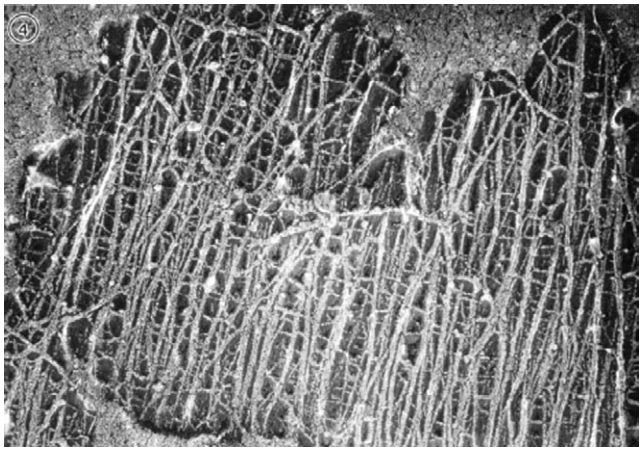


FIGURE 20.5 The extracellular matrix of a root-tip cell from *Zea mays* revealed by deep etching. Regularly spaced cross-bridges that attach the cellulose microfibrils are visible. The smooth area on the left is the plasma membrane. ×213,000. (*Source:* From Satiat-Jeunemaitre et al., 1992.)

gel-like matrix is also made out of approximately 20- to 40-nm-long macromolecules that can cross-link the cellulose microfibrils (McCann et al., 1990). This means that a 100-nm-thick extracellular matrix has about three layers of cellulose microfibrils.

Like the extracellular matrix of plant cells, the extracellular matrix of animal cells is also composed of high–molecular mass polysaccharides, including hyaluronan and cellulose (Huxley, 1898; van Daele et al., 1991; Kimura and Itoh, 1995), and proteins like collagen, elastin, fibronectin, laminin, and vitronectin (Sanders et al., 1991). However, in the extracellular matrices of animal cells, there is typically a greater proportion of proteins than polysaccharides.

20.4 EXTRACELLULAR MATRIX–PLASMA MEMBRANE–CYTOSKELETAL CONTINUUM

While the extracellular matrix is on the E-side of the plasma membrane, it is not totally isolated from the internal contents of the cell. The plasma membranes of fungal, plant, and animal cells are attached to the proteins in the extracellular matrix by protein–protein interactions (Ruoslahti, 1988). The integral plasma membrane proteins that recognize and bind various amino acid sequences in the proteins of the extracellular matrix are known as *integrins* (Schindler et al., 1989; Haas and Plow, 1994). Arg-Gly-Asp (RGD) is one of the sequences in the extracellular matrix proteins that is recognized by integrins, and the binding of integrins with these extracellular matrix proteins can be inhibited by adding oligopeptides containing the sequence RGD (Humphries, 1990).

The cytoplasmic domain of integrins can interact with the peripheral membrane proteins on the cytosolic side of the membrane known as the membrane skeleton (Steck, 1974; Luna and Hitt, 1992). The *membrane skeleton* is composed of proteins, including spectrin and ankyrin, which can link directly and/or indirectly to cytoskeletal elements, including actin, tubulin, and intermediate filament proteins (Bennett, 1990a,b; Magee and Buxton, 1991; Turner and Burridge, 1991; Quaranto and Jones, 1991; Gens et al., 1996, 2000; Pickard, 2008). There is evidence that the extracellular matrix–plasma membrane–cytoskeletal continuum also exists in plant cells since the organizations of microtubules and microfilaments are altered following the digestion of the extracellular matrix (Akashi et al., 1990; Melan, 1990; Masuda et al., 1991; Ryu et al., 1997).

The physical interaction of integrins with the extracellular matrix of animal cells allows the transmission of

stresses throughout the cell, and from the outside to the inside, and from the inside to the outside. According to the tensegrity model (see Chapter 11), these stresses can lead to morphogenesis (Ingber and Folkman, 1989a,b; Williamson, 1990; Ingber, 1991) by influencing signal transduction chains (Schwartz et al., 1995; see Chapter 12) that involve increases in intracellular Ca^{2+} (Wacholtz et al., 1989), changes in intracellular pH (Schwartz et al., 1991), and increases in tyrosine phosphorylation (Juliano and Haskill, 1993; Kornberg et al., 1991, 1992).

20.5 BIOGENESIS OF THE EXTRACELLULAR MATRIX

20.5.1 Plasma Membrane

I will use the biogenesis of the extracellular matrix as a pedagogical tool to briefly review most of the concepts covered in the previous chapters. If one were to go through this book backward, this chapter could serve as an introduction to the rest of the cell since there is a relationship between the extracellular matrix and the organelles that make up the protoplast (Newcomb, 1963). The cellulose microfibrils in the extracellular matrix are synthesized from UDP-glucose by the cellulose-synthesizing complexes on the plasma membrane. The existence of an enzymatic cellulose-synthesizing complex was first postulated by Roelofsen (1958) and was later discovered by Brown and Montezinos (1976), Mueller et al. (1976), and others (Robenek and Peveling, 1977; Mueller and Brown, 1980; Wada and Staehelin, 1981; Hogetsu, 1983; Herth and Weber, 1984; Reiss et al., 1984; Emons, 1985; Schmid and Meindl, 1992) using freeze-fracture electron microscopy. In freeze-fracture micrographs, it appears as a single globule on the E-face of the plasma membrane and as a rosette of six particles, 8 nm in diameter, arranged in a hexagon on the complementary P-face. The cytoplasmic domain of the cellulose-synthesizing complex has been observed in plasma membrane ghosts isolated from protoplasts. The cellulose-synthesizing complexes are associated with the ends of cellulose microfibrils, and appear as hexagonal structures 45–50 nm in diameter and extending 30–35 nm into the cytoplasm (Bowling and Brown, 2008).

The organization of the cellulose-synthesizing complex determines the size and shape of the resulting cellulose microfibrils (Okuda et al., 2004; Saxena and Brown, 2005). The complex occurs with a density of approximately 0.5–5/μm^2 and binds antibodies to a cellulose synthase polypeptide (Kimura et al., 1999), confirming that it is indeed the cellulose-synthesizing complex. Fluorescence studies in living cells show that the cellulose-synthesizing complex moves along the plasma membrane at a rate of 330 nm/minute—a rate that is equivalent to the polymerization of 300–1000 glucose monomers per glucan chain per minute (Lloyd, 2006; Paredez et al., 2006).

The rosette arrangement of particles occurs on the plasma membrane of higher plants and the algae in the evolutionary line that gave rise to higher plants; for example, *Nitella* (Hotchkiss and Brown, 1987), *Chara* (McLean and Juniper, 1986), and *Coleochaete* (Okuda and Brown, 1992). In the other algae, as well as in bacteria and *Dictyostelium*, the cellulose-synthesizing complex on the E-face is typically linear, and the length of the complex seems to be correlated with the number of cellulose molecules that make up the cellulose microfibril (Robinson and Preston, 1972; Brown and Montezinos, 1976; Brown et al., 1976; Zaar, 1976; Peng and Jaffe, 1976; Willison and Brown, 1978; Quader and Robinson, 1981; Itoh et al., 1984; Brown, 1985; Mizuta, 1985; Itoh, 1992). The structure of the cellulose-synthesizing complex in tunicates is also linear (Kimura and Itoh, 1996).

Evidence that the linear terminal complexes are involved in cellulose synthesis comes from the observations that the addition of EDTA, congo red, or Tinopal (Calcofluor) causes the inhibition of cellulose synthesis and the perturbation or disappearance of the linear terminal complexes (Montezinos and Brown, 1979; Robinson and Quader, 1981; Itoh et al., 1984).

The integrity of the plasma membrane is important for cellulose synthesis. The plasma membrane participates in maintaining a submicromolar concentration of intracellular Ca^{2+} in the cytosol that is necessary for cellulose synthesis. Calcium ionophores, which increase the concentration of intracellular-free Ca^{2+}, inhibit the synthesis of cellulose, and stimulate the synthesis of callose, a β-1,3 glucan (Quader and Robinson, 1979). Heinrich Kauss (1987) proposed that the same enzyme complex is involved in the synthesis of both polymers, but it synthesizes cellulose under submicromolar Ca^{2+} conditions and callose under micromolar Ca^{2+} conditions. The transient increase in the intracellular Ca^{2+} concentration may function as a component of the signal transduction chain that signals an attack so that the cell can seal itself off from the rest of the plant with wound callose (Kauss, 1987; Delmer, 1991).

The wound callose response is inhibited by the Ca^{2+} channel blockers, nifedipine and La^{3+}. Furthermore, treatment of the cell with fungal wall products that induce the callose response causes the total Ca^{2+} concentration of the cell to rise by 100 μM. Thus, the wound callose response satisfies all three of Jaffe's Rules for implicating Ca^{2+} as a second messenger in the elicitor-induced formation of callose (see Chapter 12).

Another fascinating observation concerning callose synthesis is that a membrane potential is required for the polymerization of UDP-glucose into callose by a crude membrane fraction from bacteria (Bacic and Delmer, 1981; Delmer, 1987). The membrane potential was established across the membrane vesicles by adding K^+ and valinomycin, a potassium ionophore, which allowed K^+ to diffuse across the membrane and establish a diffusion potential across the membrane in response to the imposed

concentration difference (see Chapter 2). If these experiments were to be repeated in the presence of Ca^{2+}/EGTA buffers that maintain a submicromolar concentration of Ca^{2+}, it is possible that cellulose would be formed *in vitro* instead of callose. While genetic evidence suggests that callose synthase and cellulose synthase come from independent genes (Jacobs et al., 2003; Nishimura et al., 2003; Somerville, 2006), the idea of assaying the function of membrane proteins in the presence of a membrane potential and physiological ion concentrations and even asymmetries is still valuable when it comes to looking at enzymatic processes from a cellular perspective.

Nevertheless, hard work on isolating a cellulose synthase from higher plants has come to fruition and the various genes that encode cellulose-synthesizing enzymes as well as the cellulose synthase itself have been isolated and characterized (Delmer, 1991, 1999; Mayer et al., 1991; Amor et al., 1991; Delmer et al., 1991; Kudlicka et al., 1995, 1996; Pear et al., 1996; Kudlicka and Brown, 1997; Arioli et al., 1998; Carpita and Veraga, 1998; Blanton et al., 2000; Richmond and Somerville, 2000; Holland et al., 2000; Carpita et al., 2001; Nobles et al., 2001; Peng et al., 2001; Vergara and Carpita, 2001; Wu et al., 2001; Gillmor et al., 2002; Pagant et al., 2002; Read and Bacic, 2002; Roberts et al., 2002; Kurek et al., 2003; Tanaka et al., 2003; Roberts and Roberts, 2004; Saxena and Brown, 2005; Somerville, 2006; Lu et al., 2008; Taylor, 2008; Wang et al., 2008). Karl Niklas (2004) and Malcolm Brown (2004) emphasize the importance of horizontal transfer of the cellulose-synthesizing genes, from eubacteria to the nucleus of eukaryotic plant cells, in the evolution of land plants.

20.5.2 Cytoskeleton

The orientation of the cellulose microfibrils is thought to be regulated, in part, by microtubules, since microtubule orientation is parallel to, and correlated with, the orientation of cellulose microfibrils. Moreover, microtubule orientation predicts the orientation of newly deposited cellulose microfibrils. Last, agents that inhibit microtubule polymerization or organization affect cellulose microfibril orientation. Remember that the majority of the data favor the hypothesis that microtubules control the cellulose-synthesis complex and orient microfibrils (Baskin, 2001; Gardiner et al., 2003; Wasteneys and Collings, 2006; Rajangam et al., 2008), but there is strong evidence for alternative hypotheses (see Chapter 11).

20.5.3 Endomembrane System

The synthesis of hemicellulose takes place predominantly or even exclusively in the Golgi apparatus (Zhang and Staehelin, 1992; Lynch and Staehelin, 1992; Staehelin et al., 1992). Many of the (100–1000) enzymes required for hemicellulose synthesis, including glucosyl, xylosyl, fucosyl, and arabinosyl transferases, have been localized in the Golgi apparatus (Ray et al., 1969; Gardiner and Chrispeels, 1975; Green and Northcote, 1978; James and Jones, 1979; Ray, 1980; Hayashi and Matsuda, 1981; Urbanowicz et al., 2004). The resulting hemicelluloses are transported to the plasma membrane through the secretory pathway. Perhaps the same vesicles that contain the cellulose-synthesizing complexes also contain the hemicelluloses and pectins. Since the hemicelluloses and pectins in each cell type may be different, the enzymatic composition of the Golgi apparati in these cells must also be different. Moreover, the position of the Golgi body or the delivery of Golgi-derived vesicles to various sides of the cell must be regulated in cells that are surrounded by an extracellular matrix that is not uniform all the way around the cell. We do not know what controls the position of the Golgi apparatus in these cases.

The synthesis of extracellular matrix proteins like extensin begins when the gene becomes transcriptionally active. Then RNA polymerase II transcribes the DNA into hnRNA that subsequently binds to RNA-binding proteins and SNURPs to form spliceosomes. The introns are then removed from the hnRNA to make mRNA and the 5′ end is capped with methylguanosine and the 3′ end is polyadenylated. The protein–mRNA complex then interacts with the nuclear pores and moves into the cytoplasm (see Chapter 16).

The mRNA for extensin must bind to the ribosomes, and, as translation begins, the nascent polypeptide must bind the signal recognition particle and move to the ER where the signal recognition particle binds to its receptor (see Chapter 4). Subsequently, the amino-terminus containing the signal peptide is inserted through a protein-translocating channel of the endoplasmic reticulum (ER) and enters the secretory pathway, where it moves to the Golgi apparatus. Extensin is glycosylated, and the *O*-linked arabinosylation of extensin begins in *cis*-Golgi cisternae (Moore et al., 1991). Double immunolabeling experiments with colloidal gold show that extensin moves through the entire Golgi apparatus and can be processed in the same Golgi stack as xyloglucan (Moore et al., 1991). Eventually, the extracellular matrix proteins end up in the lumen of a secretory vesicle (see Chapter 8).

The vesicles that bud off the Golgi cisternae or the *trans*-Golgi network (TGN) move through the viscous cytosol (see Chapter 9) with the aid of a mechanochemical ATPase (see Chapters 10 and 11) to the plasma membrane. The vesicles fuse with the plasma membrane and contents are secreted into the extracellular matrix. Of course, if too much plasma membrane is added relative to the wall material needed, the membrane is recycled by endocytosis (see Chapter 8). Moreover, the extracellular matrix is dynamic, and is also undergoing turnover (Labavitch, 1981; Gorshkova et al., 1997). We do not know how the exocytotic and endocytotic vesicles that contain the cellulose-synthesizing complexes, hemicelluloses, pectins, and proteins are regulated, related, or coordinated.

20.5.4 Self-Assembly of the Extracellular Matrix

The final assembly of the extracellular matrix takes place in the extracellular matrix itself and there are enzymes endemic to the extracellular matrix that can facilitate the assembly (Fry, 2004). The extracellular matrix varies in space, and thus the endemic enzymes of the extracellular matrix must be localized in various regions of the extracellular matrix. Moreover, the extracellular matrix changes during cell development, and thus the enzymatic component must also vary temporally. This kind of spatiotemporal complexity has been elegantly demonstrated in developing transfer cells (Talbot et al., 2007; Vaughn et al., 2007). The ubiquity and importance of the extracellular matrix along with its structural complexity and unique and dynamic constellation of enzymes really give it organellar status.

20.6 PERMEABILITY OF THE EXTRACELLULAR MATRIX

The extracellular matrix is not solid and amorphous, but contains numerous aqueous pores and, like other polysaccharide networks (Laurent, 1995; Ogston, 1995), acts as a sieve through which water and polar molecules can easily diffuse when the extracellular matrix is hydrated (Kamiya et al., 1962, 1963). The extracellular matrix becomes limiting to water movement when it is dehydrated and evaporation is the driving force for water movement (Tazawa and Okazaki, 1997). The average sizes of pores in semidehydrated extracellular matrices were originally estimated to be approximately 3–5 nm in diameter (Carpita et al., 1979; Miller, 1980; Carpita, 1982), while those in hydrated extracellular matrices were estimated to be approximately twice as large (Tepfer and Taylor, 1981; Baron-Epel et al., 1988a; Shepherd and Goodwin, 1989; Meiners et al., 1991a; McCann and Roberts, 1991). Therefore, when the extracellular matrix is hydrated, its permeability is typically greater than that of the plasma membrane and can be ignored as a first approximation when measuring the permeation of solutes into or out of the cell (see Chapter 2).

The original experiments to determine pore size of the extracellular matrix were done in 100–300 mol/m^3 solutions that contained solutes that were either small enough to pass through the extracellular matrix and plasmolyze the cell, or were too large to pass through the extracellular matrix and caused it to crinkle (cytorhysis). Either way, the 0.1- to 0.3-M solutions dehydrated the extracellular matrix during the experiment, and thus provided a minimum estimate of the average pore size (Carpita et al., 1979; Miller, 1980a; Capita, 1982).

Melvin Schindler and his colleagues performed experiments involving fluorescence redistribution after photobleaching in order to study the permeability of the extracellular matrix of soybean cells in a more sensitive manner (Meiners et al., 1991a). Although these cells were also plasmolyzed, and thus the extracellular matrix was dehydrated, they find that the average pore size is between 8.6 and 8.8 nm. The minimum estimate of the pore size of the extracellular matrix may not only depend on the technique used to measure it, but it may also vary from cell type to cell type and depend on the physiological state of the cell (Read and Bacic, 1996; Titel et al., 1997; Beretovsky et al., 2001; Proseus and Boyer, 2005).

Tepfer and Taylor (1981) used a clever technique to estimate the porosity of the hydrated extracellular matrix. They ground it into a powder, loaded it into a typical chromatography column tube, and used the powder as the matrix of a gel exclusion column. Then proteins of various sizes were added to the top of the column. If a protein was too large to enter the "pore space," it eluted in the void volume—that is, it was not retarded by the column. Proteins that were retarded must have traveled through the tortuous porous pathways. Using this technique, Tepfer and Taylor found that proteins less than 7 nm in diameter were retarded by the column, and thus most of the pores must have an average diameter of less than 7 nm. While the pore size is likely to vary from cell to cell and during the development of a single cell, the extracellular matrix of living cells is porous enough to allow the movement of proteins (Stafstram and Staehelin, 1988; Kandasamy et al., 1989, 1991; Hoson et al., 1991; Inouhe and Nevins, 1991).

The permeability of the plasma membrane can be tested directly using patch-clamping techniques after removing the extracellular matrix enzymatically, mechanically, or by using lasers and forming protoplasts or spheroplasts (Martinac et al., 1990; Kaiser et al., 1998; Roberts et al., 1999). The pressure difference across the membranes of wall-less cells is different from the pressure across the membranes of walled cells. In addition, the extracellular matrix proteins and carbohydrates may directly influence the ion channels, as would the ionic and osmotic (matrix potential) properties of the extracellular matrix. We always must consider the relationship of the part to the whole.

20.7 MECHANICAL PROPERTIES OF THE EXTRACELLULAR MATRIX

Most studies on the mechanical properties of the extracellular matrix have been carried out on plant tissues and organs (Geitman, 2006), and consequently, average mechanical properties of the extracellular matrix have typically been measured. This limits the ability to resolve the subtle and transient mechanical properties that control the growth of a given cell. Progress is being made, however, in measuring the mechanical properties of the extracellular matrix of single cells (Wang et al., 2004; Wei et al., 2006).

The extracellular matrix of plant cells is almost as strong as steel. The strength of a material can be quantified by its

elastic modulus and its tensile strength. The elastic modulus (M, in N/m^2) is a measure of the amount of stress (in N/m^2) required to produce a doubling of the length of a substance. The greater the elastic modulus, the greater the amount of stress is necessary to obtain a given strain. A doubling of the length is equivalent to unit strain, where *strain* is defined as the change in length divided by the initial length.

$$\text{elastic modulus} = \text{stress/strain, when strain} = 1 \qquad (20.1)$$

The tensile strength is the amount of stress needed to break a material. The tensile strength is generally less than the elastic modulus of a given material, and consequently, the elastic modulus is obtained by extrapolation from a plot of strain versus stress, and thus assumes that the ratio of strain to stress is proportional. The elastic modulus is typically greater than the tensile strength since most materials break before they double in length. The elastic modulus and tensile strength of a cotton fiber are $6–12 \times 10^9$ and $0.25–0.8 \times 10^9$ N/m^2, which is not that much less than the elastic modulus and tensile strength of steel, which are 200×10^9 and 10^9 N/m^2, respectively (Mark, 1967; Siegel, 1967; Harris, 1980; Niklas, 1992).

Building a plant out of materials with high elastic moduli and tensile strengths allows plants to withstand many mechanical stresses. It is not only the mechanical properties of the walls, but the spatial arrangement of cells with an extra-thick cell wall within the plant that allows the organ to withstand and/or recover from such forces as those experienced by plants on a windy day, or the force on a branch caused by the weight of the fruits it bears (Roget, 1834; Farmer, 1913; Niklas, 1992).

The high tensile strength of the extracellular matrix protects the protoplast from lysing and allows the plant to survive in a dilute aqueous environment. When the extracellular matrix is removed, as in the case of protoplasts, the protoplast will burst unless placed in an iso-osmotic medium. This is because the tensile strength of a plasma membrane is approximately 175 N/m^2 (Nobel, 1983). When the extracellular matrix is present, the cell takes up water, but can only swell to a limited extent. It stops taking up water when the difference in the osmotic pressure becomes balanced by the hydrostatic or turgor pressure that develops within the cell (see Chapters 7 and 12).

The high turgor pressure that results from the osmotic difference between the outside of the cell and the inside, combined with the high tensile strength of the extracellular matrix, provide the plant with mechanical strength and rigidity. This strength allows plants to stand tall and wide, spreading their leaves to maximize solar light capture and thus photosynthetic processes that take place within the chloroplast (see Chapter 13). Thus, in general, the exceedingly stiff extracellular matrix is very useful for an autotrophic organism, but the resultant immobility would be a hindrance to a heterotrophic organism.

On the other hand, the high tensile strength of the extracellular matrix will impede growth, which can be looked at as a type of movement. Therefore, mechanisms must exist to loosen the extracellular matrix to permit cell expansion. Cell expansion results when the pressure exerted by the protoplast causes a stress in the extracellular matrix that is great enough to overcome the strength of the load-bearing bonds between the polymers in the extracellular matrix. That is, sufficient stress is needed to shear the elements of the extracellular matrix past each other. The extracellular matrix acts as if it were composed both of elastic and viscous components, both of which may be important for growth. How do we measure the viscoelastic properties of the extracellular matrix that limit cell expansion? We would like to measure the parameter, known as the *viscoelastic extensibility* (Φ, in Pa^{-1} s^{-1}), which relates the volumetric relative rate of strain (dV/Vdt, in s^{-1}) to the stress (σ, in N/m^2) according to the following equation:

$$\Phi = (dV/Vdt)/\sigma \qquad (20.2)$$

The viscoelastic extensibility is the reciprocal of the viscosity of the wall. It is possible to determine it by measuring the volumetric relative rate of strain after imposing multiaxial stresses of various magnitudes (Kamiya et al., 1963; Richmond et al., 1980), or by measuring the stress at various volumetric relative rates of strain. However, there are no adequate techniques to measure the viscoelastic extensibility in many cell types, and consequently, people somewhat arbitrarily measure the relationship between stress and strain in just one dimension (Masuda, 1978; Gertel and Green, 1977; Probine and Preston, 1961, 1962; Probine and Barber, 1966; Métraux and Taiz, 1977, 1979; Métraux et al., 1980; Taiz, 1984; Cosgrove, 1993b; Burgert and Fratzl, 2006). In order to estimate the mechanical properties of the wall, one can measure strain under constant stress, the stress relaxation properties, or stress under various strains. Induced changes in strain or stress should be gradual as opposed to sudden, in order to best mimic physiologically relevant conditions (Wei and Lintilhac, 2003). Each of these techniques measures unidirectional properties of the extracellular matrix, when in reality, the three-dimensional viscoelastic extensibility, which relates the volumetric rate of strain to various stresses, is needed (Baskin, 2005).

Even so, potentially important mechanical properties can be determined by measuring strain under constant stress. With this technique, a weight is attached to a dead strip of extracellular matrix, and the strain or percent change in length is measured over time (Figure 20.6). Initially there is an instantaneous percent change in length, followed by a time-dependent percent change in length known as *creep*. Creep is constant with the logarithm of time. After a period of time, the weight is removed and the percent change in length decreases almost instantaneously to a constant level. The irreversible percent change in

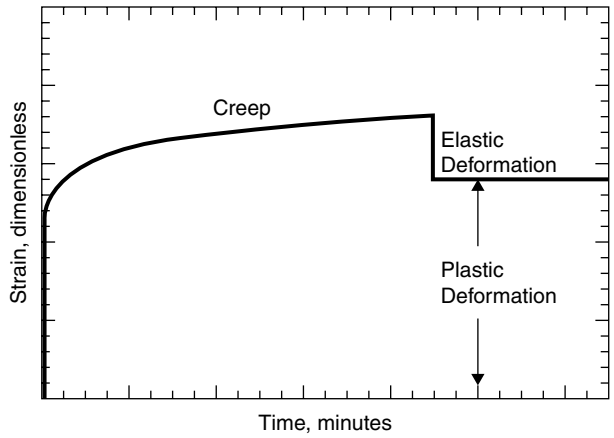


FIGURE 20.6 A representative time course of strain of a dead extracellular matrix. A constant stress was applied to the cell wall and it extended. The log-linear portion is known as *creep*. When the stress is removed, the wall shrinks again. The irreversible extension is known as the *plastic deformation* and the reversible extension is known as the *elastic deformation*.

length is known as the *plastic deformation*, and the reversible percent change in length is called the *elastic deformation*. The initial rate of creep is correlated with growth conditions of the cell, and thus growth is considered by some to be a metabolically sustained creep process.

Métraux and Taiz (1978) and Richmond et al. (1980) have measured the strain under constant stress in the walls from growing cylindrical cells of *Nitella*. They find that, ceteris paribus, at a given stress, the amount of creep as well as the amounts of plastic and elastic deformations are greater in the longitudinal direction than in the transverse direction. They also find that the relationship between stress and strain is nonlinear, and the extracellular matrix has a yield value (Figure 20.7). The yield value in the transverse direction is about twice as large as the yield value in the longitudinal direction. As I will discuss later that the stress in the transverse direction is twice as great as the stress in the longitudinal direction. As long as the deformation at a physiologically relevant stress is greater in the longitudinal direction than in the transverse direction, the cell will elongate. Under physiological pressures, the elastic modulus and the viscoelastic extensibility in the longitudinal direction are 4–7 times greater than they are in the transverse direction.

A second and complementary way to measure the potentially important mechanical properties of the wall is to stretch rapidly a dead piece of wall by applying a weight. The wall is then held at that constant length (isometric) and the stress in the wall is determined by dividing the force measured with a force transducer by the cross-sectional area of the wall. Over a short period of time, the stress decreases while the length stays the same (Figure 20.8). The stress relaxation time is different in tissues with different growth rates, and is inversely correlated with the rate of growth when measured before or after the application of auxin (Hoson, 1991).

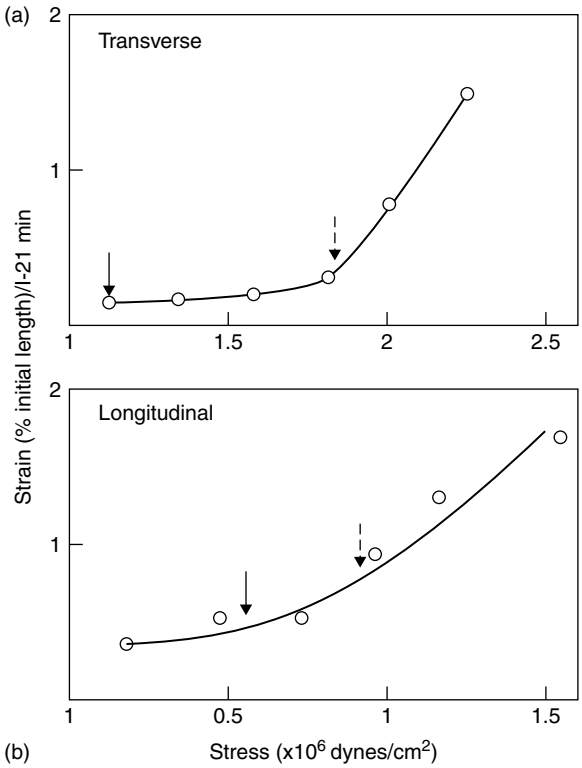


FIGURE 20.7 A rate of strain versus stress curve for transversely extended (a) and longitudinally extended (b) extracellular matrices of *Nitella axillaris*. The solid and broken arrows indicate the in vivo stresses when the turgor pressure equals 0.5 and 0.8 MPa, respectively. (*Source:* From Métraux and Taiz, 1978.)

Other potentially important mechanical properties of the extracellular matrix can be determined by measuring stress under various strains. This is done by attaching a dead segment to an instrument known as an Instron™ (Cleland, 1971a). This instrument has a force transducer, and the stress can be calculated by multiplying the cross-sectional area of the wall by the measured force. The force is measured as the extracellullar matrix is lengthened at a constant rate. Once a predetermined force is reached, the extension is stopped and the clamps are returned to their original distance. Then the wall is lengthened again at a constant rate (Figure 20.9). The first lengthening includes both elastic and plastic extension, while the second lengthening includes only elastic extension. The total compliance extensibility (strain/stress, in Pa^{-1}) is obtained from the initial slope of the curve from the first extension. The elastic component of the total compliance of extensibility is obtained from the initial slope of the curve from the second extension. Lastly, the plastic component of the total compliance of extensibility is obtained by subtracting the elastic component from the total extensibility. The plastic component of a wall preparation is correlated with the growth rates of the tissues from which the wall came. The fact that the slope of the second extension is different from that of the first indicates that the

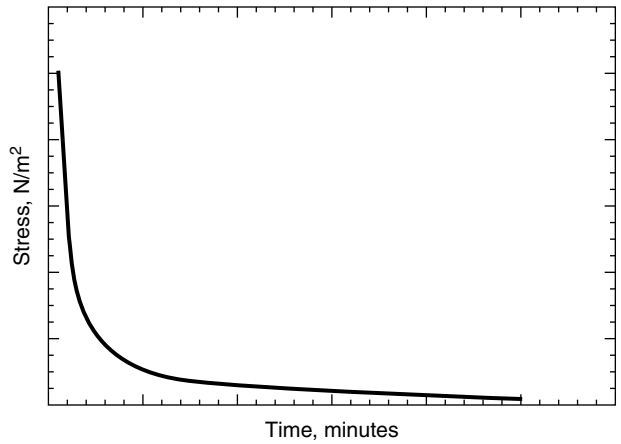


FIGURE 20.8 A stress-relaxation curve. A wall is stretched and held at a constant length. The stress is measured. Over time the wall relaxes and the stress decreases.

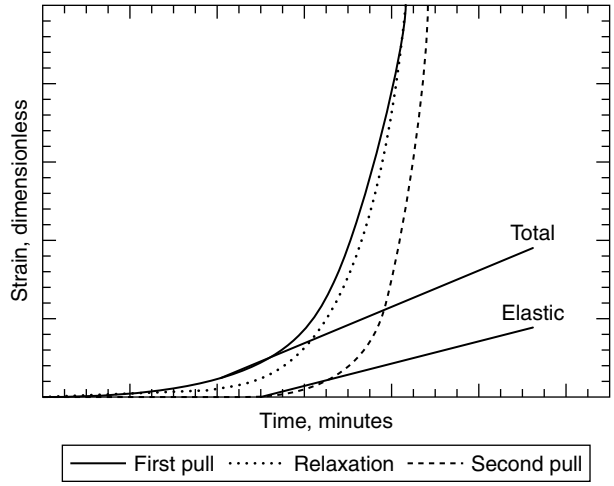


FIGURE 20.9 A curve that represents the results from an experiment where a wall is lengthened at a constant rate and the stress in the wall is measured. The slope of the first pull gives the total compliance of extensibility (in Pa^{-1}), and the slope of the second pull gives the elastic compliance of extensibility. The plastic compliance of extensibility is obtained by subtraction.

measurements of extensibility depend on the previous history of the wall—that is, how much it has been extended already. Thus, measurements of mechanical properties of the cell must be interpreted carefully. Each one of the three techniques just mentioned gives information about some of the mechanical properties of the polymeric architectural system found in the wall. In theory, each one of the mechanical properties allows one to know something about the molecular arrangements and degree of cross-linking of polymers with known mechanical properties in the wall.

20.8 CELL EXPANSION

Long-term growth is a complex process that depends on a number of factors in addition to those involved in cell wall loosening. Continual cell expansion is a hormone-modulated, energy-dependent process that requires the synthesis of RNA, protein, and cell wall–directed carbohydrates; the delivery of proteins and carbohydrates to the extracellular matrix; the uptake of water across the plasma membrane and vacuolar membrane into the vacuole; and the maintenance of the turgor pressure of the cell and tension in the extracellular matrix above the yield threshold (Sachs, 1882, 1887; Jost, 1907; Davenport, 1908; Vanderhoef, 1985; Vanderhoef and Stahl, 1985; Cleland, 1971a; Wei and Lintilhac, 2003, 2007; Wei et al., 2006; Fricke and Chaumont, 2006; Proseus and Boyer, 2006c, 2007; Verbelen and Vissenberg, 2006). Growth can be defined many ways, but I will define it simply as a change in the volume of the cell in a given time. A change in volume per unit time is a rate of change in position (in three dimensions), and thus it follows from Newton's Second Law that a force is needed to change the rate of movement.

20.8.1 Forces, Pressures, and Stresses and Their Relationship to Strain

At a constant growth rate, the inertial force exactly balances the viscous force, and the net force is zero. When the acceleration is zero and the cell expands at constant velocity, the inertial force due to turgor is exactly balanced by the viscous force that results from the viscosity of the extracellular matrix. The inertial force results from the hydrostatic pressure in the cell acting against the wall. The force exerted by the hydrostatic pressure is three-dimensional, but for convenience can be resolved into two components: one that pushes on the end walls and thus acts parallel to the long axis of the cell, and one that pushes perpendicular to the long axis of the cell. The force against the end walls provides a shearing force on the extracellular matrix along the length of the cell. The viscous force results from the viscosity of the extracellular matrix. As a consequence of the low Reynolds number, growth stops immediately when the inertial force stops (see Chapter 9).

If we initially assume that the extracellular matrix is a Newtonian fluid, the velocity of movement closest to the site of generation of the motive force will be the highest and it will decrease farther and farther away from the protoplast. The higher the viscosity of the extracellular matrix, the smaller the velocity gradient in the extracellular matrix will be. The higher the viscosity, the more uniformly the extracellular matrix will extend. If the extracellular matrix were thixotropic, then there will be no movement until the shearing stress is great enough to overcome the yield value (see Chapter 9). While the micrographs of cell walls are consistent with their being non-Newtonian fluids, there is currently a paucity of rheological data to determine directly if many cell walls are non-Newtonian (Métraux and Taiz, 1978; Richmond et al., 1980; Taguchi et al., 1999;

Okamoto-Nakazato, 2002; Proseus and Boyer, 2006b). In order to determine the magnitude of the shearing stress in the extracellular matrix we must consider the forces that cause it.

The forces involved in growth depend on the geometry of the cell. For simplicity, I will consider a cylindrical cell of radius (r) and length (l). This cell has a wall with a thickness (x). The inertial force against the end wall of the cell is equal to the hydrostatic pressure, (P_t), times the area of the end wall, (πr^2), and is given by the following equation:

$$F_i = (P_t)(\pi r^2) \tag{20.3}$$

That is, the inertial force against the end wall equals $(P_t)(\pi r^2)$, and, in the absence of acceleration the inertial force is exactly balanced by the viscous force. If we want to calculate the shearing stress (σ_L) induced by the viscous force, we must determine the area over which the viscous force acts. This area is equal to the cross-sectional area of the cylindrical wall ($2\pi rx$), and consequently:

$$F_v = \sigma_L(2\pi rx) \tag{20.4}$$

Many people confuse the quantities of mass (kg), force (N, kg m s^{-2}), and pressure or stress (Pa, N/m^2, kg m^{-1} s^{-2}). In order to differentiate between force and shearing stress, or force and pressure, consider two wires of identical lengths made of the same material, one with a radius of 1 mm and the other with a radius of 3 mm. Experiments show that we must add a force (e.g., hang a weight) to the second wire that is nine times as heavy as the weight that we must add to the first to get identical changes in length. Since the two wires are made of the same material and have the same elastic modulus, we must have added equal stresses in order to get equal strains. This could only be true if the stress is equal to the force divided by the area on which the force acts.

Thus, the shear stress parallel to the long axis of the cell (σ_L) is given by the following equation:

$$\sigma_L = (F_v)/(2\pi rx) \tag{20.5}$$

and since at constant growth rate, $F_i = F_v$:

$$\sigma_L(2\pi rx) = (P_t)(\pi r^2) \tag{20.6}$$

and

$$\sigma_L = (P_t)(\pi r^2)/(2\pi rx) = P_t r/(2x) \tag{20.7}$$

Thus, the shearing stress that acts on the extracellular matrix is proportional to the turgor pressure and the radius of the cell. It is inversely proportional to twice the thickness of the extracellular matrix.

Let us also consider the hydrostatic pressure that pushes perpendicularly to the extracellular matrix along the long axis of the cell. Again, let us assume that the extracellular matrix moves outward at a constant velocity with respect to time. Again, the inertial force due to turgor equals the viscous force that is a result of the viscosity of the extracellular matrix.

Consider that the inertial force perpendicular to the long axis of the cell acts to split the cell in two longitudinally, and thus the inertial force is equal to the turgor pressure times the area against which it pushes. While we really need calculus to show this (see Appendix 3), for now assume that the total inertial force is equal to $P_t(2rl)$. At constant velocity, the viscous force that resists the inertial force must be equal to $P_t 2rl$. The stress (σ') in the extracellular matrix in the direction perpendicular to the long axis of the cell is equal to this force divided by the area over which it acts, which is approximately equal to $2xl$, where x is the thickness of the extracellular matrix on each side of the hemicylinder and l is the length of the cell (Castle, 1937; Preston, 1952; Nobel, 1974). Thus, $F_v = (\sigma')2xl$, and since, at constant velocity, $|F_i| = |F_v|$, then:

$$(\sigma_\perp)2xl = (P_t)(2rl) \tag{20.8}$$

and

$$\sigma_\perp = (P_t)(2rl)(2xl) = (P_t)(r)/(x) \tag{20.9}$$

Thus, the perpendicular stress that acts on the extracellular matrix is proportional to the turgor pressure and the radius of the cell. In contrast to the shearing stress, it is inversely proportional the thickness of the extracellular matrix and not inversely proportional to twice the thickness.

I have shown that $\sigma_L = P_t r/(2x)$ and $\sigma' = P_t r/x$, and consequently:

$$\sigma_\perp = 2\sigma_L \tag{20.10}$$

That is, for a given turgor pressure, the tangential stress is twice as great as the longitudinal stress.

The strain is proportional to the stress and, according to Eqs. 20.11 and 20.12, the elastic modulus (M) is the proportionality constant that relates the strain to the stress. Consequently, the linear extension rates parallel and perpendicular to the long axis of the cell will be given by the following equations:

$$\text{strain}_\perp = \sigma_\perp/M \tag{20.11}$$

$$\text{strain}_L = \sigma_L/M \tag{20.12}$$

and since $\sigma' = 2\sigma_L$,

$$\text{strain}_\perp = 2(\text{strain}_L) \tag{20.13}$$

Likewise, the rate of strain in the transverse and longitudinal directions are given by the following equations:

$$\text{rate of strain}_{\perp} = \sigma_{\perp}\Phi \tag{20.14}$$

$$\text{rate of strain}_{L} = \sigma_{L}\Phi \tag{20.15}$$

and since $\sigma' = 2\sigma_L$:

$$\text{rate of strain}_{\perp} = 2(\text{rate of strain}_{L}) \tag{20.16}$$

Thus, if the elastic moduli or viscoelastic extensibilities are the same in the tangential and longitudinal directions, then the cell should grow twice as wide as it grows long. Since cells typically elongate, the elastic modulus and viscoelastic extensibility perpendicular to the long axis of the cell must be greater than the same parameters parallel to the long axis of the cell.

In fact, this is true, and the reason the elastic modulus and viscoelastic extensibility are unequal in the two directions is that the cellulose microfibrils in the wall are not arranged randomly but transversely around a growing cell. This can be observed with both polarization and electron microscopy (Frey-Wyssling, 1959; Green 1954, 1958a,b, 1961, 1962, 1963).

In a young cell, the cellulose microfibrils are deposited in a transverse orientation perpendicular to the long axis of the cell. Consequently, close to the plasma membrane, the microfibrils are transverse to the direction of cell expansion. As we move out into the extracellular matrix, we encounter older microfibrils, and these have become obliquely oriented as a result of growth. Still farther out, the oldest microfibrils are passively reoriented longitudinally as a result of growth. Thus, the elastic modulus in each layer of the extracellular matrix could theoretically be different (Green, 1960; Frey-Wyssling and Mühlethaler, 1965; Frey-Wyssling, 1953, 1959). Consequently, due to the differences in orientation, as well as potential differences in the numbers and types of cross-bridges between the microfibrils, each layer could influence growth differently (Baskin, 2005).

The orientation of cellulose microfibrils, which may be dependent on the orientation of microtubules (see Chapter 11), is thought to regulate the direction of cell growth in response to the motive force provided by turgor pressure. Thus, randomly arranged microfibrils will give rise to a roughly spherical cell like a cortical parenchyma cell. Cells that have transversely arranged microfibrils will elongate in a direction perpendicular to the direction of the microfibrils, and will give rise to elongate cells like those in the procambium. The orientation of cellulose microfibrils thus determines the direction of growth, and consequently, the shape of the cell. While the polarity of growth depends on the orientation of cellulose microfibrils, the rate of elongation is not invariantly related to the orientation of cellulose microfibrils (Paolillo, 2000; Baskin, 2005). This is reasonable when we take into consideration the fact that cellulose microfibrils are often involved in the two sometimes-opposing functions of determining the polarity of growth and of mechanical support.

The mechanical properties of the wall are only one of the physical factors involved in cell expansion. The irreversible expansion of cells depends on two interdependent physical processes: the uptake of water and the yielding of the extracellular matrix (Lockhart, 1965; Lockhart et al., 1967; Cleland, 1958, 1971a,b, 1981; Yamamoto et al., 1970; Masuda, 1978a,b; Cosgrove, 1986, 1987; Hayashi, 1991; Kutschera, 1991; Hoson, 1992; Ortega, 1985, 1990; Proseus et al., 1999). According to one model of cell expansion, growth occurs when the extracellular matrix is elastically stretched by the protoplast as a result of hydrostatic pressure. This gives rise to a stress in the load-bearing bonds. Irreversible growth results when the load-bearing bonds in the extracellular matrix break and allow the elastic components in the wall to relax. This relaxation allows a reduction in the stress in the extracellular matrix. Since the stress in the wall and the hydrostatic pressure constitute equal and opposite forces, the hydrostatic pressure and the wall stress decrease simultaneously. The decrease in the hydrostatic pressure or turgor pressure reduces the water potential of the cell. This gives rise to a water influx, which increases the cell volume and extends the extracellular matrix, and the load-bearing bonds form in a new place. The cycle repeats itself until the extracellular matrix is no longer extensible. Thus, like the Krebs and Calvin cycles, growth is also cyclic and there is a growth cycle.

This growth cycle only describes short-term growth that takes place in time scales less than an hour. Long-term growth requires the expression of genes and the synthesis on new wall material to make new load-bearing bonds. Without these processes, growth would stop (Masuda, 1990). There may be feedback loops between the extracellular matrix and the cytoplasmic components involved in the synthesis and positioning of the polymers in the extracellular matrix. Thus, there may be a growth cycle that involves the whole cell.

James Lockhart (1965) made a mathematical model to describe the dynamic balance between wall yielding, which tends to dissipate turgor, and the uptake of water, which tends to restore turgor. I will present the derivation of the Lockhart equation. This equation describes how the growth rate depends on the viscoelastic extensibility of the wall (Φ, in $Pa^{-1}\ s^{-1}$), the difference in the osmotic pressure inside and outside the cell (Π), the yield value of the wall (Y, in N/m^2), and the hydraulic conductivity of the plasma membrane (L_p, in $m\ s^{-1}\ Pa^{-1}$). L_p is equal to the osmotic permeability coefficient P_{os} (in m/s; see Chapter 2) times ($\bar{V}_w/RT$), where $\bar{V}_w$ is the partial molar volume of water (see Chapter 12). Growth is positively correlated with Φ, Π, and L_p, and negatively correlated with Y. It is possible that one or another parameter becomes dominant in controlling growth

at different times in the growth process, in different cells in an organ, or in different plants. Therefore, in each tissue type it is necessary to find out which cells limit growth, and in that cell type, it is necessary to find out which parameter is limiting. This is another specific example of Blackman's (1905) law of limiting factors, which states that "When a process is conditioned as to its rapidity by a number of separate factors, the rate of the process is limited by the pace of the 'slowest' factor."

The rate in which a cell grows depends on how big it is at the start of the measurement. Thus, I will use the elemental rate of growth, which accounts for the initial size of a cell (Lockhart, 1971). The elemental rate of growth is a "specific growth rate" or "relative growth rate," and is defined to be $(1/V)(dV/dt)$. The elemental rate of volume growth (in s^{-1}) is a function of the viscoelastic extensibility of the extracellular matrix (Φ), and the difference between the turgor pressure (P_{ti}) and the yield value (Y) of the extracellular matrix as defined in the following equation:

$$(1/V)(dV/dt) = \Phi(P_{ti} - Y) \tag{20.17}$$

The elemental rate of volume growth is also dependent on hydraulic factors according to the following equation:

$$(1/V)(dV/dt) = A\, L_p(P_{wo} - P_{wi})/V \tag{20.18}$$

where V is the cell volume (in m^3), t is the time (in s), A is the surface area of the cell (in m^2), L_p is the hydraulic conductivity (in m s^{-1} Pa^{-1}), and $(P_{wo} - P_{wi})$ is the water potential difference between the outside and the inside of the cell. Since

$$\begin{aligned} P_{wo} - P_{wi} &= (P_{to} - sP_{\pi o}) - (P_{ti} - sP_{\pi i}) \\ &= sP_{\pi i} - sP_{\pi o} - P_{ti} = s\Pi - P_{ti} \end{aligned} \tag{20.19}$$

where P_{to} and P_{ti} are the hydrostatic pressures outside and inside the cell, respectively (P_{to} is defined as 0 by definition); $P_{\pi o}$ and $P_{\pi i}$ are the osmotic pressures outside and inside the cell, respectively; $\Pi = (P_{\pi i} - P_{\pi o})$; and s is the reflection coefficient for the solutes. Thus:

$$(1/V)(dV/dt) = A\, L_p(s\Pi - P_{ti})/V \tag{20.20}$$

and

$$P_{ti} = s\Pi - (dV/dt)(1/AL_p) \tag{20.21}$$

Substituting $P_{ti} = s\Pi - (dV/dt)(1/AL_p)$ into

$$(1/V)(dV/dt) = \Phi(P_{ti} - Y)$$

we get:

$$(1/V)(dV/dt) = \Phi(s\Pi - Y - (dV/dt)(1/AL_p)) \tag{20.22}$$

which is equivalent to:

$$\begin{aligned} dV/dt &= V\Phi\,(s\Pi - Y - (dV/dt)(1/AL_p)) \\ &= V\Phi\,(s\Pi - Y) - V\Phi(dV/dt)(1/AL_p) \end{aligned} \tag{20.23}$$

Bring all the terms with dV/dt to the left side of the equation:

$$dV/dt + (dV/dt)(V\Phi/AL_p) = V\Phi(s\Pi - Y) \tag{20.24}$$

Using the distributive rule:

$$(dV/dt)(1 + (V\Phi/AL_p)) = V\Phi(s\Pi - Y) \tag{20.25}$$

After solving for dV/dt, we get:

$$dV/dt = V\Phi(s\Pi - Y)/(1 + (V\Phi/AL_p)) \tag{20.26}$$

and the elemental growth rate is given by the following equation:

$$dV/(Vdt) = \Phi(s\Pi - Y)/(1 + (V\Phi/AL_p)) \tag{20.27}$$

The foundation of the Lockhart equation is the assumption that the growth rate results from a difference between the force exerted on the wall due to turgor and its restraint due to the mechanical properties of the wall. It is clear from Eqs. 20.26 and 20.27 that a change in any number of factors that influence turgor and the mechanical properties of a cell has the potential of influencing the growth rate and elemental growth rate. These rates will increase when Φ, s, Π, and/or L_p increases, and when Y decreases. Potentially, each of these variables can be affected by a stimulus, including hormones and light. Thus, experiments performed to elucidate the mechanism of how stimuli influence growth must not only show which factors are modulated, but they also must show which ones remain constant.

The growth equation and the others we have used in this book were derived with a few assumptions from a limited number of examples, and any of the equations given in this book may have to be modified or expanded to efficiently describe a given situation and to have predictive value. These equations should not be used as laws, but as approximations that simplify the information that we know and allow us to plan experiments and consider the effect of more than one factor at a time. Most scientific arguments start because one group may have tested one parameter and assumed the others were constant, while another group tested a different parameter assuming that another set of

parameters were constant. These equations help us to see that more than one group may be partially right, and that there may be more than one factor that influences a process. Indeed, the synthesis comes from combining the thesis with the antithesis. Deriving an equation by ourselves reminds us of the assumptions that we have included in the equation and the information that we have left out. The experiments, however, can guide experiments, and they can help refine the equation (Einstein, 1950).

The words of T. H. Huxley (1890) give us perspective on the value of a quantitative approach and its limits. He wrote in an essay, "On the Physical Basis of Life":

> *But the man of science, who, forgetting the limits of philosophical inquiry, slides from these formulae and symbols into what is commonly understood by materialism, seems to me to place himself on a level with the mathematician, who should mistake the x's and y's with which he works his problems, for real entities—and with this further disadvantage, as compared with the mathematician, that the blunders of the latter are of no practical consequence, while the errors of systematic materialism may paralyse the energies and destroy the beauty of a life.*

The growth equation derived above describes the growth of cylindrical single cells, like the internodal cells of *Nitella* or *Chara*. It has also been used to describe the growth of cylindrical organs. However, the application of this equation to multicellular organs depends on the assumption that to some degree, all the cells of the organ are identical, or the internal cells provide the pressure for growth, while the outer epidermal cell wall resists growth. The growth of a multicellular organ is a complicated problem. Throughout the organ there are a number of cell types in each tissue (epidermal, cortical, pith, phloem, or xylem) that have different wall architectures, thicknesses, extensibilities, etc. In sunflower hypocotyls, for example, the pith and cortical cells have a very thin extracellular matrix, and thus their turgor pressure will keep them extended. This may cause the thick-walled epidermal cells to be under tension. Then the tension in the epidermal cells may keep the cortical and pith cells under compression and prevent water uptake.

The tissue stresses in an organ can be readily observed by peeling the epidermis and seeing that the isolated epidermis contracts approximately 20 percent, whereas the inner tissues expand immediately due to the water uptake, which was prevented by the tissue compression. In sunflower hypocotyls, growth appears to be driven by water uptake into the compressed parenchyma tissues, and limited by the extensibility of the epidermal cells (Kutschera, 1991). Thus, there is a supracellular level of control that is not included in the growth equation.

Auxin is a hormone that promotes growth in most plants, and the possibility remains that auxin may affect one or all of the parameters in the growth equation and to a different degree in each cell type or in each plant. Robert Cleland and David Rayle have provided a wealth of evidence that auxin acts on the plasma membrane proton-pumping ATPase that acidifies the extracellular matrix, thus activating enzymes that break hydrogen bonds between polymers (Cleland and Rayle, 1977; Rayle and Cleland, 1977). The breakage of the load-bearing bonds increases extensibility and/or decreases the yield value (Taguchi et al., 1999). Auxin may act directly on the Golgi apparatus or other parts of the secretory pathway to cause the secretion of new extracellular matrix material (Ray, 1977). Auxin may stimulate elongation growth by acting directly on microtubule orientation and keeping microtubules in a transverse orientation for a longer period of time, particularly in the outer epidermal wall (Bergfeld et al., 1988; Nick et al., 1990; Mayumi and Shibaoka, 1996). Auxin may also increase the hydraulic conductivity of the plasma membrane (Kang and Burg, 1971; Boyer and Wu, 1978; Loros and Taiz, 1982). And lastly, auxin may act directly on gene expression by activating transcription of growth-limiting mRNAs and/or translation of growth-limiting proteins (Key, 1969; Ulmasov et al., 1997). These effects are not mutually exclusive, and auxin may influence one or more in a given cell at the same time (Vanderhoef et al., 1977).

Dan Cosgrove (1993a,b,c) and his colleagues (McQueen-Mason et al., 1992, 1993, 2006; Li et al., 1993; Cosgrove et al., 2002) discovered some enzymes that may be activated by an auxin-induced acidification of the extracellular matrix. Cosgrove and his associates developed a functional assay to look for the enzymes necessary for extension. First, they isolated extracellular matrices from either cucumber hypocotyls or oat coleoptiles. The native walls extend in response to low pH (4.5) under constant stress. However, when the walls are heat-treated they fail to extend. Proteins were then isolated from other walls, fractionated, and tested to see if they cause extension of the heat-treated walls. In this way, Cosgrove and his associates isolated expansins, which are proteins that induce extension, with a pH optimum of 4.5. Expansins may bind to xylans and induce extension by transiently breaking hydrogen bonds between xyloglucans and cellulose (Whitney et al., 2000; Davis, 2006). There is a web site devoted to these interesting proteins (www.bio.psu.edu/expansins/), and it includes many references. It covers the evolutionary origin, mode of action, and the role of expansins in various developmental responses, including rachis elongation in semiaquatic ferns (Kim et al., 2000), elongation of wheat coleoptiles (Gao et al., 2008), and cell wall disassembly during fruit ripening (Rose and Bennett, 1999; Rose et al., 2000).

Interestingly, localized application or expression of expansins induces the generation of leaf primordia in apical meristems, indicating that localized expansion of the cell wall is capable of triggering morphogenesis (Fleming et al., 1997; Pien et al., 2001), a result that would have pleased Paul Green (1996, 1997), who pioneered the role of physical mechanisms in initiating morphogenesis at the apical meristem (Silk, 2000).

In some cases, auxin increases the extensibility of the wall and expansins may be the enzymes involved in increasing the extensibility in response to auxin. Other studies show that auxin also decreases the yield threshold of the wall (Okamoto et al., 1989, 1990; Mizuno et al., 1993). Okamoto and Okamoto (1995), Okamoto et al. (1997), and Okamoto-Nakazato et al. (2000a,b, 2001, 2002) have isolated two proteins from the extracellular matrix of *Vigna*: one that is involved in regulating extensibility and one that is involved in regulating the yield threshold (Figure 20.10). They call the extracellular matrix–localized protein that regulates the yield threshold of the wall *yieldin* (Okamoto-Nakazato, 2002).

Gibberellin is another hormone that affects plant growth (Stowe and Yamaki, 1959), and a lack of gibberellin results in the dwarf phenotype observed by Mendel (Lester et al., 1997). Gibberellin and auxin do not act independently in inducing growth in plants and organs (Yang et al., 1996), and consequently it is difficult to separate the cellular effects of each hormone. Hiroh Shibaoka has pioneered studies aimed at elucidating the cellular mechanisms of gibberellin-induced growth. Shibaoka (1972) first noticed that gibberellin promoted elongation growth at the expense of stem thickening and the lateral expansion of cells, and concluded that gibberellin controls the direction of expansion of plant cells.

Shibaoka (1972) studied the effect of gibberellin in stem segments of the azuki bean. The control segments were incubated in auxin and the treated segments were incubated in auxin plus gibberellin. The gibberellin-treated segments elongated over the controls in the absence of cell division. Shibaoka postulated that gibberellin caused the microtubules to orient transversely to the cell axis. As a result, the newly synthesized cellulose microfibrils were also laid down transversely to the cell axis. Consequently, the cell expanded longitudinally, but not transversely. Shibaoka showed that colchicine inhibited gibberellin-induced growth. An inhibitor of gibberellin biosynthesis causes swelling of cells and a randomization of microtubules (Mita and Shibaoka, 1983, 1984a,b). Shibaoka and his colleagues observed that the microtubules and the cellulose microfibrils were transversely oriented in gibberellin-treated cells (Shibaoka, 1974; Takeda and Shibaoka, 1981a,b). Although Shibaoka (1994) originally believed that auxin did not affect microtubule orientation in azuki beans like it does in other plants, Mayumi and Shibaoka (1996) found that auxin is required for the gibberellin-induced suppression of the reorientation of microtubules from a transverse to a longitudinal arrangement.

Growth is not always evenly distributed along a cell, and in *Nitella*, growth occurs predominantly near the proton pumps, which are organized into bands (Dorn and Weisenseel, 1984). Growth is not always straight either. One side of a cell can grow faster than the other side. This can be observed clearly in the large internodal cells of characean algae or the sporangiophores of *Phycomyces* when these cells bend in response to light and gravity (Bergman et al., 1969; Staves et al., 2005). In these cases, either the viscoelastic extensibility or the yield value for the wall on each side must be different as the turgor pressure that drives growth is the same on both sides. Of course, differential growth also occurs in the phototropic and gravitropic responses of the organs of higher plants, but in these cases, it is not clear whether the cells on two sides of an organ grow differentially or if the bending of an organ is due to the summation of the differential growth of each cell (Tomos et al., 1989; Cosgrove, 1997; Evans and Ishikawa, 1997).

There are many other specialized forms of growth, including the polar growth that occurs in root hairs, pollen tubes, and fungal hyphae. Tip growth allows these cells to "search out" the nutrients or cell types necessary for growth and/or reproduction. In order to orient themselves, tip-growing cells are capable of sensing tiny gradients in chemical, electrical,

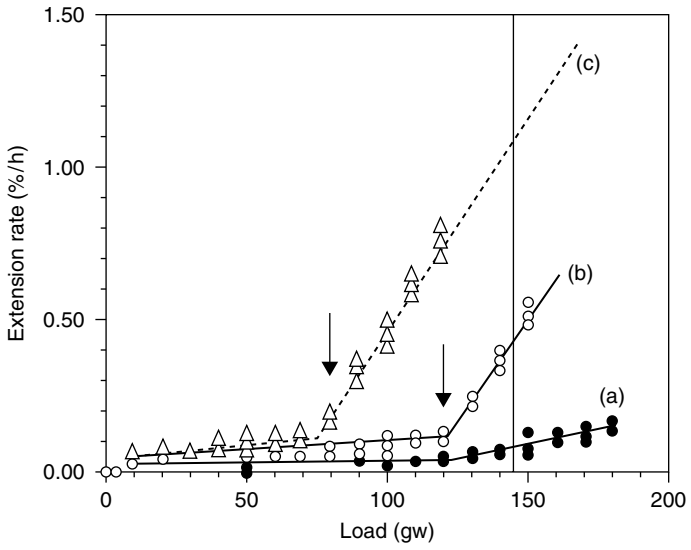


FIGURE 20.10 The demonstration of the function of yieldin. (a) extension at pH 6.2; (b), extension at pH 4; and (c), extension at pH 4 plus yieldin. (*Source:* From Okamoto-Nakazato, 2002.)

and mechanical stimuli. Tip growth is dependent on an influx of Ca^{2+} into the tip (Schnepf, 1986; Miller et al., 1992; Kühtreiber and Jaffe, 1990; Franklin-Tong et al., 1993, 1996; Malhó et al., 1994; Pierson et al., 1994, 1996; Malhó and Trewavas, 1996; Lancelle et al., 1997; Messerli and Robinson, 1997). Polar growth depends on the actin-mediated transfer of Golgi-derived vesicles filled with wall material to a growing tip. Let's not forget that there is even wall-to-wall communication between a tip-growing pollen tube and the pistil. In this case, the pistil produces an extracellular matrix glycoprotein that stimulates the growth of pollen tubes and attracts them to the ovules (Cheung, 1995; Cheung et al., 1995).

We can use the Lockhart equation, repeated below, to get a glimpse of the difficulty facing people using a reductionist approach to cell biology based on the study of mutants:

$$dV/(Vdt) = \Phi(s\Pi - Y)/(1 + (V\Phi/AL_p)) \quad (20.28)$$

When one sees a mutation that affects growth, one could imagine that the mutation could affect the extensibility, the turgor pressure, the reflection coefficient, the osmotic pressure, the yield value, or the hydraulic conductivity. That is not all: Imagine that the yield threshold of the wall is directly affected by the enzyme yieldin. How would a mutation in transcription, RNA processing, translation, translocation at the ER, membrane trafficking, vesicle transport, synthesis of the substrate for yieldin, etc. affect the yield value of the cell wall? Likewise, imagine that the extensibility of the wall is directly affected by the enzyme expansin. Since there are numerous isozymes of each cell wall protein, one must know if the mutation affects the expression in the cell in question. Cell biologists must be able to recognize the relationships between the part and the whole in all aspects of "informatic" and "-omic" analysis.

20.9 SUMMARY

The extracellular matrix is a dynamic organelle that surrounds the cell. It is a matrix of polysaccharide and protein polymers that prevents cell lysis and allows the development of a hydrostatic pressure within the cell that provides mechanical support for the plant. The extracellular matrix contains its own cadre of enzymes to assemble and rearranges itself to allow for growth. The extracellular matrix has a history and a geography, and may act as an informational macromolecule that encodes three-dimensional positional information.

20.10 EPILOG

Now that I have discussed the components of wood, and by extension, the properties of logs, I will end this book with the "epilog." I am awed by how much we know about the cell, and how much is understood in terms of the cellular mechanisms that make life possible. When I realize how much there still is for me to learn about the cell, it is hard to stop writing this book. But I must publish what I know and what I think I know or else I will have created the cellular version of "The Madonna of the Future" (James, 1962).

I hope that this book has fulfilled James Clerk Maxwell's goal as a teacher. He wrote (quoted in Mahon, 2003),

> *In this class, I hope you will learn not merely results, or formulae applicable to cases that may possibly occur in our practice afterwards, but the principles on which those formulae depend, and without which the formulae are mere mental rubbish. I know the tendency of the human mind is to do anything rather than think. But mental labour is not thought, and those who have with labour acquired the habit of application often find it much easier to get up a formula than to master a principle.*

Maxwell also wrote,

> *My duty is to give you the requisite foundation and to allow your thoughts to arrange themselves freely. It is best that every man should be settled in his own mind, and not be led into other men's ways of thinking under the pretence of studying science. By a careful and diligent study of natural laws I trust that we shall at least escape the dangers of vague and desultory modes of thought and acquire a habit of healthy and vigorous thinking which will enable us to recognise error in all the popular forms in which it appears and to seize and hold fast truth whether it be old or new.*

Throughout this book, I have emphasized the unity of nature and knowledge (Kidd, 1852; Butcher, 1891; Bohr, 1963; Schrödinger, 1996). I have used the teachings of mathematicians, physicists, and chemists to help us understand the nature of life, and the mechanisms cells use to transform matter and energy into life. I have often emphasized the relationship between the part and the whole, and how the combination of parts leads to a whole with new emergent properties, which are based on but were not found in the properties of the component parts. In this respect, I have discussed the formation of atoms from elementary particles, the formation of molecules from atoms, the formation of macromolecules from monomers, the formation of cells from macromolecules, the formation of multicellular organisms from cells, and their continual development of multicellular organisms into rational, feeling organisms that are capable of understanding and appreciating the wonder, beauty, and spirit of the universe (Bergson, 1911). One theme of *Zorba the Greek*, written by Bergson's Ph.D. student, Nikos Kazantzakis (1953), is that every day, each of us repeats this evolutionary process as we convert food into fat, work, or spirit. The proportion we convert into each product describes to some extent who we are. Physiology and behavior recapitulate phylogeny.

If there truly is a unity of knowledge and nature, an understanding of the nature of life from the biological point of view should be able to help us appreciate, value, and understand higher levels of the organizations of life—where the entire

organism is a part of a family, a town, a state, a nation, and a world (Priestley, 1771; Boveri, 1929; Browne, 1944; Emerson, 1947; Thoreau, 1947; Jefferson, 1955; Picken, 1960; Hamilton et al., 1961; Olson, 1965; Claude, 1975; Cohen, 1997; Elwick, 2003). Albert Szent-Györgyi (1957a, 1963a, 1970) defined ethics as the set of rules that makes living together possible in these higher levels of organization of life.

At every level I have studied in biology, there is a relationship between the part and the whole. Every process can be seen as a compromise between what is optimal for the part and what is optimal for the whole. Society is the same way, albeit more complicated than a cell. Yet we must look at every situation in terms of the complementary relationship between the part and the whole. As citizens of the same planet, we must not only live for ourselves, but we must ask what we can do for the freedom of humankind (Kennedy, 1961), dedicate ourselves to the struggle for human rights (Carter, 1979), be tolerant of people with different values, and occasionally rise above principle (Martin, 1988)—that is, we must live and let live. As President Kennedy said at the Commencement Address at American University in Washington on June 10, 1963:

> *So, let us not be blind to our differences—but let us also direct attention to our common interests and to the means by which those differences can be resolved. And if we cannot end now our differences, at least we can help make the world safe for diversity. For, in the final analysis, our most basic common link is that we all inhabit this small planet. We all breathe the same air. We all cherish our children's future. And we are all mortal.*

Now I have shared will you my personal outlook on cell biology. I have discussed my views on the origin of cells, the mechanisms of how cells transform matter and energy into life, and the relationship of cells to organisms and even higher levels of organization. I have presented cell biology to the best of my ability. I still have much to learn, so I encourage you to question all my conclusions. After all, this book is only a snapshot of my current understanding of plant cell biology—an understanding that continually grows, develops, and evolves much like the plants from which the cells come. According to Szent-Györgyi (1964), "Books are there to keep the knowledge in while we use our heads for something better. … So I leave knowledge, for safe-keeping, to books and libraries and go fishing, sometimes for fish, sometimes for new knowledge."

In this book, I have presented cell biology to you from my own point. I encourage you to find your own point of view. I am but one of the blind men described by the poet John Godfrey Saxe, who studied elephants. You can be another, and all together, with our diverse viewpoints, we may understand the cell of life. God save us from scientists who lack the courage to find their own viewpoints. For if Richard Feynman (1955) is correct in believing that the greatest value of science is the "freedom to doubt," then a healthy science depends on the courage and convictions of the individuals (Conklin, 1923; Blackett, 1935; Einstein et al., 1936; Rutherford, 1936; Brady, 1937; Stark, 1938; Bernal, 1939, 1949; Szent-Györgyi, 1943, 1972; Sax, 1944; Baker, 1945; Lysenko, 1946, 1948, 1954; Zirkle, 1949; Kennedy, 1964; Fermi, 1971; Marshall, 1982a,b; Kamen, 1985; King, 1992; Williams, 1993a; Krogmann, 2000; Levi-Montalcini, 1988). Today, people are discussing the socio-politico-economic influence of scientists on each other and the need for a Declaration of Academic Freedom (Rabounski, 2005).

I have tried to present to you the fashionable (Stevens, 1932; Chargaff, 1976; Bauer, 1994) and the unfashionable theories and experiments in cell biology. Science requires both (Wangensteen, 1947). According to Richard Feynman (1965),

> *… possibly the chance is high that the truth lies in the fashionable direction. But, on the off-chance that it is in another direction—a direction obvious from an unfashionable view …—who will find it? Only someone who has sacrificed himself by teaching himself … from a peculiar and unusual point of view; one that he may have to invent for himself. I say sacrificed himself because he most likely will get nothing from it, because the truth may lie in another direction, perhaps even the fashionable one.*

So be yourself, trust yourself, be an explorer or a tourist, but either way, take responsibility for your assumptions, your conclusions, and your actions. I look forward to hearing your views, and your criticisms of mine. For now, dear reader, we must end our journey together through the cell, and I will close this book with the words Mathias Schleiden (1853) used to conclude his beautiful book, *Poetry of the Vegetable World*:

> *A gentle sound trembles through the fragrant evening air. The bell of the native village calls him home, returned after restless travel over the great God's World, after rich impressions, exciting adventures, pressing hardships, and strange delights, back to rest, to that, which, in spite of all intervening things, he never does nor can forget, the paradise of childhood, the house of his parents, his mother's arms.*

20.11 QUESTIONS

20.1. What is the relationship between the extracellular matrix and the plasma membrane?
20.2. What is the relationship between the extracellular matrix and the nucleus?
20.3. What is the relationship between the extracellular matrix and the endoplasmic reticulum?
20.4. What is the relationship between the extracellular matrix and the Golgi apparatus?
20.5. What is the relationship between the extracellular matrix and the endosomal compartment?
20.6. What is the relationship between the extracellular matrix and the cytoskeleton?
20.7. What is the relationship of the extracellular matrix, the plasma membrane, nucleus, endoplasmic reticulum, Golgi apparatus, vacuole, and cytoskeleton to growth?

Appendix 1

SI Units, Constants, Variables, and Geometric Formulae

1. SI Units

Concept	Unit name	Units	Symbol
Basic Units			
length	meter		m
mass	kilogram		kg
time	second		s
electric current	ampere		A
temperature	Kelvin		K
amount of substance	mole		mol
Derived Units			
electrical potential	volt	$kg\ m^2\ s^{-3}\ A^{-1}$	V
force	Newton	$kg\ m\ s^{-2}$	N
energy, work	Joule	N m	J
pressure, stress	Pascal	$N\ m^{-2}$	Pa
electric charge	Coulomb	A s	C
capacitance	Farad	C/V	F
resistance	Ohm	V/A	Ω
conductance	Siemens	A/V	S

2. Constants

Symbol	Name	Approximate value
π	Pi	3.14
N_A	Avogadro's number	6.02×10^{23} mol^{-1}
μ_o	magnetic permeability of vacuum	$4\pi \times 10^{7}$ N/A^2
ε_o	electrical permittivity of vacuum	8.85×10^{12} F/m
$c = (\varepsilon_o\mu_o)^{-1/2}$	speed of light	3×10^{8} m/s
Da	Dalton, atomic mass unit (amu)	1.66×10^{-27} kg
e	elementary charge	1.60×10^{-19} C
$F = eN_A$	Faraday constant	9.65×10^{4} C/mol
g	gravitational acceleration	9.8 m/s^2
h	Planck's constant	6.63×10^{-34} J s
$\hbar = h/2\pi$	reduced Planck's constant	1.05×10^{-34} J s
k	Boltzmann's constant	1.38×10^{-23} J/K
$R = kN_A$	universal gas constant	8.31 J mol^{-1} K^{-1}
w	Wien coefficient	2.89784×10^{-3} m K
σ_B	Stefan-Boltzmann's constant	5.67×10^{-8} J K^{-4} m^{-2} s^{-1}

3. Variables

Symbol	Name	Units
a	acceleration	m/s^2
a_w	relative activity of water	dimensionless
A	area	m^2
c'	local speed of light	depends on relative velocity
C	concentration	mol/m^3
C_{sp}	specific capacitance	F/m^2
D	diffusion coefficient	m^2/s
E	molecular free energy	J
EFR	energy fluence rate	J m^{-2} s^{-1}
f	fluidity	Pa^{-1} s^{-1}
F	force	N
G	conductance	S
H	molar enthalpy	J/mol
i	ionization coefficient	dimensionless

Symbol	Name	Units
I	current	A, C/s
J	flux	mol m^{-2} s^{-1}
k	rate constants	defined for each equation
k	angular wave number	m^{-1}
K	constants	defined for each equation
K	partition coefficient	dimensionless
L_p	hydraulic conductivity	m s^{-1} Pa^{-1}
m	mass	kg
M	elastic modulus	N/m^2
M_r	molecular mass	Daltons
n	refractive index	dimensionless
n	quantity of matter	dimensionless
P	permeability coefficient	m/s
P	pressure	Pa
P_t	hydrostatic pressure	Pa
P_w	water potential	Pa
P_π	osmotic pressure	Pa
q	charge	C
Q	volume flow	m^3/s
r	radius	m
R	maximal radius	m
R	resistance	Ω
S	molar entropy	J mol^{-1} K^{-1}
t	time	s
T	absolute temperature	K
u	mobility coefficient	m^2 J^{-1} s^{-1}
u'	electrical mobility coefficient	m^2 V^{-1} s^{-1}
u	energy density per unit wavelength	J/m^4
U	energy density	J/m^3
v	velocity	m/s; other units are possible when talking about reaction velocities
V	volume	m^3
$\bar{V}_w$	partial molar volume of water	m^3/mol
w	length	m

(*Continued*)

Symbol	Name	Units
x	length	m
Y	yield value	N/m^2
z	valence	dimensionless
α	P_{Na}/P_K	dimensionless
β	P_{Cl}/P_K	dimensionless
ε	relative permittivity	dimensionless
ρ	density	kg/m^3
ρ	photon density	photons/m^3
σ	shearing stress	N/m^2
σ	cross-section	m^2
η	viscosity	Pa s
λ	wavelength	m
ν	frequency	s^{-1}
γ	rate of shear	s^{-1}
Π	osmotic pressure difference	Pa
Φ	viscoelastic extensibility	$Pa^{-1}\ s^{-1}$
ψ	electrical potential	V
Ψ	wave function	depends on type of wave
Δ	indicates a finite difference between two quantities	
d, ∂	when used in calculus indicates an infinitesimally small difference between two quantities	

4. Geometric formulae

Circle
- Radius = r
- Diameter = $2r$
- Circumference = $2\pi r$
- Area = πr^2

Sphere
- Radius = r
- Diameter = $2r$
- Area = $4\pi r^2$
- Volume = $(4/3)\pi r^3$

Right circular cylinder
- Length = x
- Radius = r
- Area of curved portion = $2\pi rx$
- Area of two ends = $2\pi r^2$
- Total area = $2\pi rx + 2\pi r^2$
- Volume = $\pi r^2 x$

Square
- Length = x
- Perimeter = $4x$
- Area = x^2

Cube
- Area = $6x^2$
- Volume = x^3

Appendix 2

A Cell Biologist's View of Non-Newtonian Physics

Ask not what physics can do for biology, ask what biology can do for physics.

—Stanislaw Ulam (quoted in Knight, 2002)

Cells live in the world of neglected dimensions between the world of macroscopic physics and the world of microscopic physics. Studying physico-chemical processes in such a world has its advantages and disadvantages. One disadvantage of working in this world of neglected dimensions is that it is not easy to assume that a given subset of physical laws can be neglected in order to model biological processes and solve the equations easily. One advantage of working in the world of neglected dimensions is that a cell biologist has the opportunity to look for fundamental laws that are applicable to microscopic systems as well as macroscopic systems, and thus help to unify macrophysics and microphysics. Such laws could provide a parsimonious toolbox for modeling and solving a wide range of physico-chemical problems.

Although cells and the particles within them do not travel anywhere near the speed of light, as a cell biologist, I have gained a perspective to suggest why charged particles do not travel faster than the speed of light. In this appendix, I present a hypothesis that light itself in the form of a dilatant photon gas prevents charged particles from moving faster than the speed of light. It may be right, it may be wrong, but it is definitely thought provoking.

Newton's Second Law ($F = m\ dv/dt$) implies that any particle with mass (m) can be accelerated to any velocity (v) in time (t) by the application of a large enough constant force (F). However, experience shows that particles do not accelerate to infinite velocity and the amount of force required to accelerate a charged particle increases nonlinearly as its velocity asymptotically approaches the speed of light (c). This indicates that there must be a non-Newtonian process that decreases the effectiveness of the constant force (F). The non-Newtonian process may increase the particle's mass (m) nonlinearly, nonlinearly influence its reckoning of the duration of time the force is administered, or add a nonlinear velocity-dependent resistance to the acceleration as a particle's velocity approaches the speed of light.

According to Einstein's Special Theory of Relativity, the duration of time (dt) is relative. Consequently, according to an observer in the inertial frame of a moving particle, the force applied to the particle is attenuated because a moving particle experiences a constant force for a shorter duration of time (dt_{proper}) than does the experimenter who is at rest with respect to the force (dt_{improper}). According to the Special Theory of Relativity, the duration of time differs in a velocity-dependent manner according to the following equation:

$$dt_{\text{proper}}/dt_{\text{improper}} = \sqrt{(1 - v^2/c^2)} \qquad \text{(A2.1)}$$

The question for the physico-chemically minded cell biologist is: Is the non-Newtonian behavior observed for particles moving at speeds approaching the speed of light best explained by invoking the relativity of space and time given by the Special Theory of Relativity, or by invoking the presence of a resisting force that causes the moving particle to experience a nonlinear relationship between force and acceleration? A cell biologist is at an advantage in discovering a resisting force that causes a moving particle to respond to a constant force in a nonlinear manner as a result of working in the world of neglected dimensions.

Ever since Antony van Leeuwenhoek (1677) observed the incessant movement of animalcules and Bonaventura Corti (1774) and Giovanni Amici (1818) observed rotational cytoplasmic streaming in giant algal cells, cell biologists have realized that movement is one of the fundamental attributes of life (Huxley, 1890). In order to understand the physico-chemical mechanisms that make life itself possible, cell biologists have studied the movement of ions through channels (see Chapter 2); the movement of small molecules through plasmodesmata (see Chapter 3); the movement of proteins from their site of synthesis to their site of action (see Chapters 4 and 17); the movement of membrane vesicles, tubules (see Chapters 6, 7, and 8), and organelles

(see Chapters 13 and 14) throughout the viscous cytoplasm (Chapter 9); the movement of polymerases along the DNA that comprises the hereditary material (see Chapter 16); the movement of the hereditary material during mitosis (see Chapter 19) and meiosis when it is packaged in the bodies suitable for transport; the movement of the polymers of the extracellular matrix that allow cell growth (see Chapter 20); and the movement of proteins, including myosin, dynein, and kinesin, which convert chemical energy into mechanical energy to facilitate the movement of organelles and membrane vesicles along the cytoplasmic tracks known as microfilaments (see Chapter 10) and microtubules (see Chapter 11). Each one of these cellular movements, as well as others not listed here, involves a motive force that overcomes a nonnegligible resistive force. The study of these movements gives a cell biologist a special talent in identifying resistances. Moreover, since the resistance of the cytoplasm and the extracellular matrix is nonlinear, a cell biologist has profound experience in understanding non-Newtonian physics (Seifriz, 1936, 1938b; Kamiya, 1950; Métraux and Taiz, 1978; Kamitsubo et al., 1988; Okamoto-Nakazato et al., 2000a,b, 2001; Okamoto-Nakazato, 2002).

When one studies the movement of a vesicle or a chromosome through the cytoplasm or nucleoplasm, one must ask, "What is the nature of the cytoplasmic or nucleoplasmic space through which the vesicle or chromosome moves?" Likewise, when one studies the movement of a particle through a space, one must also ask, "What are the properties of the space through which the particles move?" Let us consider the movement of an electron through space where the motive force is provided by an electric field. At any temperature above 0 K, the space consists of a radiation field composed of photons. The photons can be considered to have a black-body distribution (Figure A2.1).

An electron moving through a photon gas in which the radiation relative to the center of momentum of the radiation field is distributed according to Planck's radiation law, experiences the photons as being Doppler shifted. The photons that collide with the front of the moving electron will be blue shifted and the photons that collide with the back of the moving electron will be red shifted (Figure A2.2).

Since the electron is moving at a velocity (v) relative to the center of momentum of the radiation field, I will describe the radiation experienced by the moving particle with an original relativistic wave equation. It describes the propagation of light waves between inertial frames moving relative to each other at velocity v. This new relativistic wave equation is given by:

$$\partial^2\Psi/\partial t^2 = cc'(\sqrt{(1 - v/c)}/\sqrt{(1 + v/c)})\partial^2\Psi/\partial x^2 \qquad \text{(A2.2)}$$

where $v > 0$ when the source and observer move away from each other, and $v < 0$ when the source and observer move toward each other. Different aspects of the speed of a light wave are represented by c and c'. The parameter c, which is absolute and independent of the velocity of the source or the observer, gives the speed of the wave through space and is equal to the square root of the reciprocal of the product of the electric permittivity (ε_o) and the magnetic permeability (μ_o) of the vacuum. By contrast, c' is

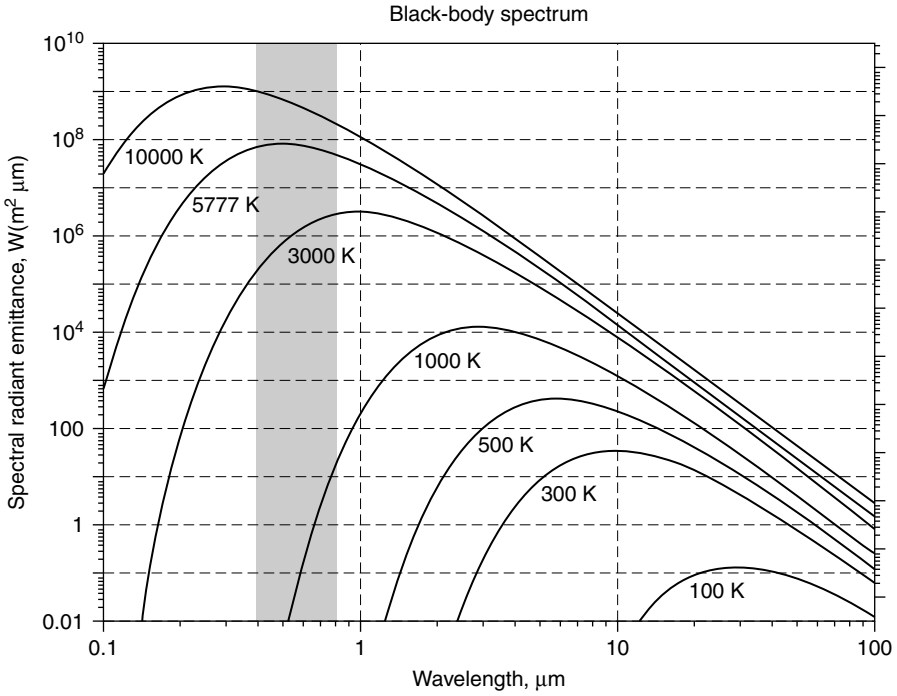


FIGURE A2.1 Black-body distribution of radiation at various temperatures. The gray band indicates the visible wavelengths.

local and depends on the relative velocity of the source and observer. c' gives the ratio of the angular frequency (ω) of the source in its inertial frame to the angular wave number (k) observed in any inertial frame ($c' = \omega_{source}/k_{observer}$). The ratio of c' to c is equal to the ratio of the angular frequencies in the inertial frame of the source and the inertial frame of the observer ($c'/c = \omega_{source}/\omega_{observer}$). When there is no relative motion between the source and the observer, $v = 0$, $cc'\,(\sqrt{(1 - v/c)}\,/\sqrt{(1 + v/c)}) = c^2$, and the new relativistic wave equation reduces to d'Alembert's or Maxwell's wave equations.

By introducing the perspicuous correction factor $(\sqrt{(1 - v/c)}\,/\sqrt{(1 + v/c)})$ in order to ensure the invariance of this new relativistic wave equation, I obtain the relativistic Doppler equation naturally as the dispersion relation:

$$\begin{aligned} k_{observer} &= \pm k_{source}[\sqrt{(1 - v/c)}/\sqrt{(1 + v/c)}] \\ &= \pm k_{source}[(1 - v/c)/\sqrt{(1 - v^2/c^2)}] \end{aligned} \quad \text{(A2.3)}$$

The experimental observations of Ives and Stillwell (1938) on the displacement of the spectral lines of hydrogen with velocity confirm the utility and validity of the new relativistic wave equation.

The linear momentum of a photon is given by $\hbar\boldsymbol{k}$, where $\hbar$ is Planck's constant (h) divided by 2π. As a consequence of the relativistic Doppler effect, the linear momentum of the radiation perceived by an electron moving with speed v is velocity dependent and is given by:

$$\begin{aligned} \hbar\boldsymbol{k}_{observer} &= \hbar\boldsymbol{k}_{source}[\sqrt{(c - v)}/\sqrt{(c + v)}] \\ &= \hbar\boldsymbol{k}_{source}[(1 - v/c)/\sqrt{(1 - v^2/c^2)}] \end{aligned} \quad \text{(A2.4)}$$

For convenience, I will use the absolute value of the velocity and split Eq. A2.4 into two equations—one for an electron moving parallel relative to the waves propagating from the source, and one for an electron moving antiparallel relative to the waves propagating from the source. The momentum of the light experienced by an electron traveling parallel to light propagating from the source is:

$$\hbar\boldsymbol{k}_{electron} = \hbar\boldsymbol{k}_{source}(1 - v/c)/(1 - v^2/c^2)^{1/2} \quad \text{(A2.4a)}$$

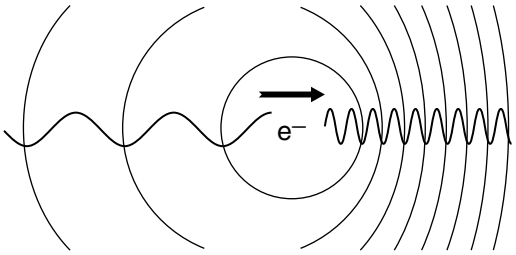


FIGURE A2.2 An electron moving through a photon gas consisting of a black-body distribution of radiation experiences the photons as being Doppler shifted. The photons striking the front of an electron are blue shifted and the photons striking the rear of the electron are red shifted.

Equation A2.4a is identical to the equation that describes the Compton effect, where $\hbar\boldsymbol{k}_{source}$ is the momentum of a photon before a collision, and $\hbar\boldsymbol{k}_{electron}$, the momentum transferred to the electron, is equivalent to the momentum lost by the photon after a collision (Compton, 1924). The momentum of the light experienced by a particle traveling antiparallel to light propagating from the source is:

$$\hbar\boldsymbol{k}_{electron} = \hbar\boldsymbol{k}_{source}(1 + v/c)/(1 - v^2/c^2)^{1/2} \quad \text{(A2.4b)}$$

Equation A2.4b is identical to the equation that describes the inverse Compton effect, where $\hbar\boldsymbol{k}_{source}$ is the momentum of a photon before a collision and $\hbar\boldsymbol{k}_{electron}$, the momentum transferred from the electron, is equivalent to the momentum gained by the photon after a collision (Feenberg and Primakoff, 1948).

Assume that the moving electron interacts with one photon from the front and one photon from the back. In this case, there is a net momentum transferred from the electron to the radiation field and the vector of the momentum transferred to the radiation field is antiparallel to the velocity vector of the electron and is given by:

$$\begin{aligned} \hbar\boldsymbol{k}_{electron} &= \hbar\boldsymbol{k}_{source}[(1 - v/c) - (1 + v/c)]/ \\ &\qquad (1 - v^2/c^2)^{1/2} \\ &= \hbar\boldsymbol{k}_{source}(-2v/c)/(1 - v^2/c^2)^{1/2} \end{aligned} \quad \text{(A2.5)}$$

The average decrease of momentum ($m_o\boldsymbol{v}$) experienced by a moving particle upon colliding with one photon in an isotropic radiation field would be:

$$\begin{aligned} \hbar\boldsymbol{k}_{electron} &= -\tfrac{1}{2}\,\hbar\boldsymbol{k}_{source}(2v/c)/(1 - v^2/c^2)^{1/2} \\ &= -\hbar\boldsymbol{k}_{source}(v/c)/(1 - v^2/c^2)^{1/2} \end{aligned} \quad \text{(A2.6)}$$

where the negative sign indicates that the momentum of the electron moving through the radiation field decreases.

Since the "average photon" can strike the moving particle at any angle from 0 to $\pm\pi/2$ with differing effectiveness, the average transfer of momentum[1] from the radiation field to the particle is $\hbar\boldsymbol{k}_{source}(1/4)(v/c)/(1 - v^2/c^2)^{1/2}$ for a single collision. Consequently, at all temperatures greater than absolute zero, contrary to Newton's First Law, all bodies will slow down, in principle. In an adiabatic radiation field, this will result in an increase in temperature and an increase in the peak angular wave number of the photons in the radiation field. In an isothermal radiation field, this will result in an expansion of the field. Since an isotropic radiation field exists at all temperatures above absolute zero,

[1] Assuming isotropy, where the linear momentum coming from any direction $\hbar\boldsymbol{k}(\theta,\phi) = \hbar\boldsymbol{k}(0,0)$, the total linear momentum coming from all directions is $\hbar\boldsymbol{k} = \int_0{}^2\pi\ \int_0\pi\ \hbar\boldsymbol{k}(\theta,\phi)\ \sin\theta\ d\theta\ d\phi = 4\pi\hbar\boldsymbol{k}(0,0)$. The total linear momentum coming from all directions per unit area per unit time is $\hbar\boldsymbol{k}\ \int_0{}^2\mathrm{p}\ \int_0\mathrm{p}/2\ \cos\theta\ \sin\theta\ d\theta\ d\phi = \hbar\boldsymbol{k}/4$.

Newton's First Law is only valid for charged particles at absolute zero. By taking the thermodynamics of the radiation field into consideration, I will recast Newton's Second Law in an alternative form that applies to charged particles[2] moving at velocities close to the speed of light and that parallels Einstein's Special Theory of Relativity.

The velocity-dependent relativistic Doppler-shifted momentum of the radiation field provides the basis for a velocity-dependent counterforce ($\boldsymbol{F}_{\mathbf{Dopp}}$) on a particle accelerated by a constant applied force ($\boldsymbol{F}_{\mathbf{app}}$).

$$\boldsymbol{F}_{\mathbf{app}} + \boldsymbol{F}_{\mathbf{Dopp}} = mdv/dt \tag{A2.7}$$

where $\boldsymbol{F}_{\mathbf{app}}$ and $\boldsymbol{F}_{\mathbf{Dopp}}$ are antiparallel.

The force exerted by the radiation field on a moving particle is a function of the collision rate between the moving particle and the photons in the field. The collision rate (dn/dt) depends on the photon density (ρ), the speed of the particle (v), and the cross-section of the photon (σ) according to the following equation:

$$dn/dt = \rho v \sigma \tag{A2.8}$$

The photon density is a function of the absolute temperature. The absolute temperature on Earth and in the cavity of some accelerators, including the LINAC at Stanford University, is close to 300K, and the absolute temperature of the cosmic microwave background radiation and in the cavity of other accelerators, including the LINAC at Jefferson Laboratory, is 2.73K.

Assuming a black-body distribution of energy, the photon density can be calculated from Planck's black-body radiation distribution formula. According to Planck (1949a), the energy density per unit wavelength interval (u) is given by:

$$u = (8\pi hc/\lambda^5)(1/(\exp[hc/\lambda kT] - 1)) \tag{A2.9}$$

The peak wavelength can be obtained by differentiating Eq. A2.9 with respect to wavelength or by simply using Wien's distribution law:

$$\lambda_{\text{peak}} = 2.89784 \times 10^{-3}\ \text{mK}/T = w/T \tag{A2.10}$$

where w is called the *Wien coefficient* and is equal to 2.89784×10^{-3} mK. The peak wavelengths (λ_{peak}) in 300 K and 2.73 K radiation fields are 9.66×10^{-6} m and 1.87×10^{-3} m, respectively. The energies of photons with these wavelengths are given by Planck's equation:

$$E = hc/\lambda_{\text{peak}} \tag{A2.11}$$

and are 2.06×10^{-20} J/photon and 1.06×10^{-22} J/photon for the peak photons in a 300 K and 2.73 K radiation field, respectively. The total energy density (U) of a radiation field can be determined by integrating Eq. A2.9 over wavelengths from zero to infinity.[3]

$$\begin{aligned} \mathrm{U} &= \int u\, d\lambda = \int (8\pi hc/\lambda^5)\\ &\quad (1/(\exp[hc/\lambda kT] - 1))\, d\lambda \\ &= (8\pi k^4 T^4/c^3 h^3) \int x^3 [\exp(x) - 1]^{-1}\, dx \\ &= (8\pi k^4 T^4/c^3 h^3)(\pi^4/15) \\ &= (8\pi^5 k^4/15 c^3 h^3)(T^4) \\ &= 7.57 \times 10^{-16}(T^4) \end{aligned} \tag{A2.12}$$

The quantity 7.57×10^{-16}, known as the radiation constant, is equal to $(4\sigma_B/c)$, where σ_B represents the Stefan-Boltzmann constant. The total energy densities of radiation fields with temperatures of 300 K and 2.73 K radiation are 6.13×10^{-6} J/m^3 and 4.02×10^{-14} J/m^3, respectively. The photon densities (ρ) in 300 K and 2.73 K radiation fields, which are obtained by dividing Eq. A2.12 by Eq. A2.11, are 2.98×10^{14} photons/m^3 and 3.79×10^{8} photons/m^3, respectively.

Although light is often modeled as an infinite plane wave or a mathematical point, the phenomena of diffraction and interference indicate that a photon has neither an infinite nor a vanishing width (Lorentz, 1924; Wayne, 2009). I assume that a photon has a finite wave width as well as a wavelength and that the geometrical cross-section[4] (σ) of a photon is given by:

$$\sigma = \pi r^2 \tag{A2.13}$$

where r is the radius of the photon. The radius of a photon can be estimated using a mixture of classical and quantum reasoning following the example of Niels Bohr—by making use of the fact that all photons have the same quantized angular momentum ($L = \hbar$), independent of their wavelength, and that classically, angular momentum is equal to $m\omega r^2$.

[2] This way of thinking also occurs to neutral particles, including neutrons and neutrinos, with a magnetic moment that may form an electrical dipole that can couple to the radiation field.

[3] Let $x = hc/\lambda kT$; $\int x^3[\exp(x) - 1]^{-1}\, dx = \pi^4/15$.

[4] While these geometrical cross-sections appear large, using Eq. A2.13, the geometrical cross-section calculated for a 10-MeV photon is 1.23×10^{-27} m^2 or 0.123 barn, within the range of the experimentally determined photon cross-sections. The cross-section is typically a measure of the probability that any given reaction will occur, and the total cross-section is a measure of the probability that all possible reactions will occur. The cross-sections for individual processes that make up the total cross-sections vary by many orders of magnitude and may be less than, equal to, or greater than the geometrical cross-section. Here, I assume that a charged particle in thermal equilibrium with the black-body radiation field has a resonance for photons in the radiation field with every possible angular wave number, and thus the probability of an electron interacting with the radiation field is unity. Consequently, the effective cross-section equals the geometrical cross-section.

By using $E = mc^2 = \hbar\omega$ and assuming the equivalent mass (m) of a photon is given by $\hbar\omega/c^2$, then its radius (r) will be equal to $\sqrt{(\hbar/m\omega)} = \sqrt{(\hbar c^2/\hbar\omega^2)} = \sqrt{(c^2/\omega^2)} = c/\omega = 1/k = \lambda/2\pi$, which is the reciprocal of the angular wave number.[5] Thus, the geometrical cross-section, which is related to its angular wave number, is given by:

$$\sigma = \pi(1/k)^2 = \pi(\lambda/2\pi)^2 = \lambda^2/4\pi \qquad \text{(A2.14)}$$

According to this reasoning, the cross-sections of thermal (300K) photons and microwave (2.73K) photons are $7.43 \times 10^{-12}\,m^2$ and $2.78 \times 10^{-7}\,m^2$, respectively.

According to Eq. A2.8, the collision rate (dn/dt) between a moving electron and photons in an isotropic thermal or microwave radiation field is dependent on the velocity of the electron. After factoring in the photon densities and the cross-section of the peak photons, I find that the collision rate is equal to (2214.14 collisions/m)v and (105.36 collisions/m)v for 300 K and 2.73 K radiation fields, respectively, where v is the velocity of the electron relative to the observer. For a given velocity, the collision rate increases with the temperature of the radiation field. In a 300 K radiation field, at speeds approaching the speed of light, the collision rate will be about $6.64 \times 10^{11}\ s^{-1}$, while it will be about $3.16 \times 10^{10}\ s^{-1}$ for a 2.73 K microwave radiation field.

The velocity-dependent counterforce ($\boldsymbol{F}_{\mathbf{Dopp}}$) exerted by the radiation field is given by the product of the collision rate and the average velocity-dependent momentum of a photon in the radiation field:

$$\begin{aligned}\boldsymbol{F}_{\mathbf{Dopp}} &= -(\rho\sigma v)(\hbar k_{\text{source}})(¼)(v/c)/(1 - v^2/c^2)^{½} \\ &= -(\rho\sigma h/4\lambda_{\text{source}})(v^2/c)/(1 - v^2/c^2)^{½}\end{aligned} \qquad \text{(A2.15)}$$

At $v = 0$, there is no net counterforce and the average momenta ($h/4\lambda_o$) of photons in a thermal radiation field and a microwave radiation field are 1.72×10^{-29} kg m/s and 8.86×10^{-32} kg m/s, respectively, and an electron will exhibit Brownian motion in the photon gas. Tables A2.1 and A2.2 give the velocity-dependent counterforce exerted by black-body distributions of thermal radiation (300K) and microwave radiation (2.73K). The influence of temperature and velocity on the counterforce is presented in Figure A2.3. The nonlinear temperature-dependent coefficient of friction (r) of the radiation field is given by $\boldsymbol{F}_{\mathbf{Dopp}}/v = (\rho\sigma h/4\lambda_o)(v/c)/(1 - v^2/c^2)^{½}$ and the power dissipated by the radiation field is given by $v\boldsymbol{F}_{\mathbf{Dopp}}$ (Figure A2.4). The dissipated power will increase the temperature and/or increase the volume of the radiation field. Such an effect may have been important in the expansion of the universe.

TABLE A2.1 Counterforce produced by thermal background radiation (300°K)

Velocity ($\times 10^{-8}$ m/s)	$(\rho\sigma h/4\lambda_o)$ ($\times 10^{26}$ Ns/m)	$(v^2/c)/(1 - v^2/c^2)^{½}$ ($\times 10^{-9}$ m/s)	F_{Dopp}
2.9	3.80	1.11	4.22×10^{-17} N
2.99	3.80	3.98	1.51×10^{-16} N
2.997	3.80	12.1	4.60×10^{-16} N
2.9979	3.80	74.0	2.81×10^{-15} N
2.99792	3.80	172.0	6.54×10^{-15} N

TABLE A2.2 Counterforce produced by cosmic microwave background radiation (2.7°K)

Velocity ($\times 10^{-8}$ m/s)	$(\rho\sigma h/4\lambda_o)$ ($\times 10^{30}$ Ns/m)	$(v^2/c)/(1 - v^2/c^2)^{½}$ ($\times 10^{-9}$ m/s)	F_{Dopp}
2.9	9.33	1.11	1.04×10^{-20} N
2.99	9.33	3.98	3.71×10^{-20} N
2.997	9.33	12.1	1.13×10^{-19} N
2.9979	9.33	74.0	6.90×10^{-19} N
2.99792	9.33	172.0	1.60×10^{-18} N

We can define the product of ρ and σ as the linear photon density (ρ_L): Replace h with $e^2/\varepsilon_o c\alpha$, using the definition of the fine-structure constant (α), substitute μ_o for $1/\varepsilon_o c^2$, and replace λ_{source} with w/T to get:

$$\boldsymbol{F}_{\mathbf{Dopp}} = -[\rho_L T e^2 \mu_o/4w\alpha]\,(v^2)/(1 - v^2/c^2)^{½} \qquad \text{(A2.16)}$$

This form of the counterforce shows explicitly that the counterforce depends on the temperature, the square of the charge of the moving particle, and the fine-structure constant, which quantifies the strength of the interaction

[5] This calculation can also be based on the fact that light radiated from an object provides that object with linear momentum parallel to the direction of radiation (Lewis, 1908). Semi-classically, the equivalent momentum of the photon of light is equal to mv. Since the photon travels at the speed of light (c), its equivalent momentum is given by mc. According to quantum theory, the momentum of the photon is given by $\hbar\boldsymbol{k}$. By equating the classical and quantum descriptions of momentum, the equivalent mass of a photon is given by the absolute value of $\hbar k/c$, which is equal to $\hbar\omega/c^2$. Friedrich Hasenöhl derived the relationship $E \approx mc^2$, entirely based on classical reasoning making use of Maxwell's light pressure and equating the Poynting vector to the momentum vector multiplied by c^2 (Lenard, 1933; Pauli, 1958).

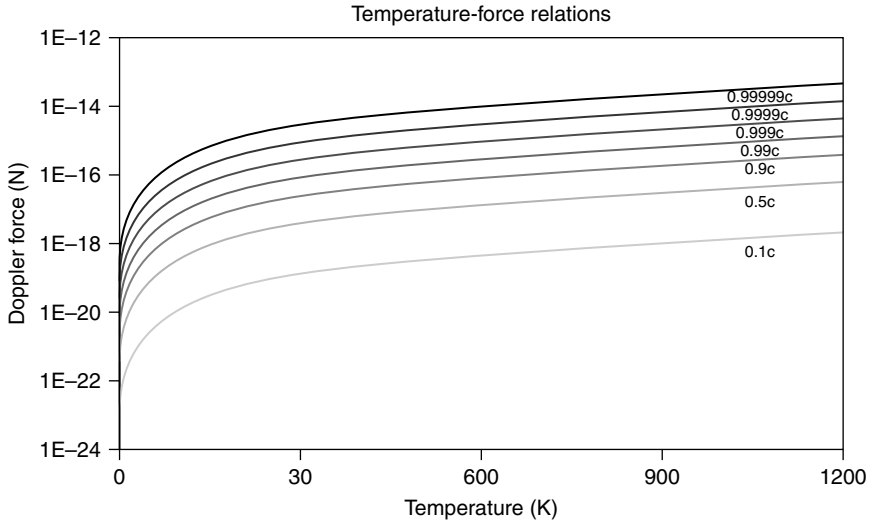


FIGURE A2.3 The effect of temperature on the magnitude of the Doppler force acting on an electron moving at various velocities relative to the speed of light.

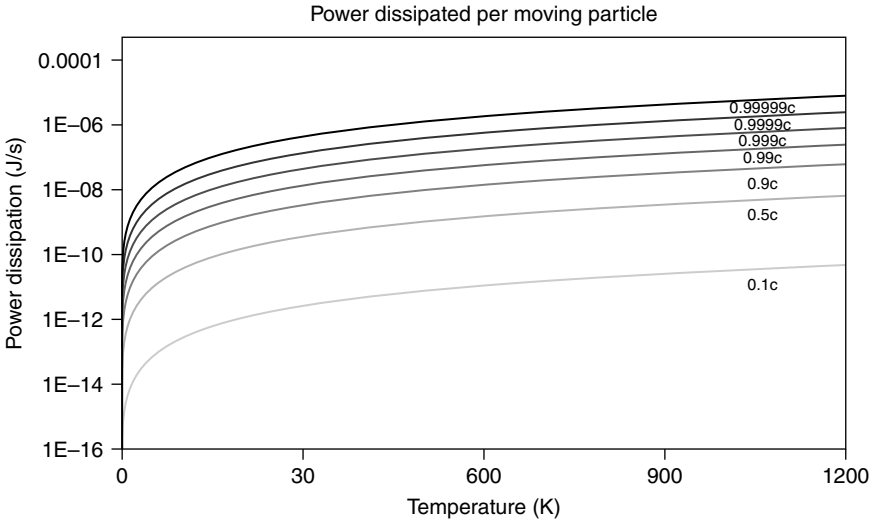


FIGURE A2.4 The effect of temperature on the power dissipated by an electron moving at various velocities.

between a charged particle and the radiation field. The counterforce vanishes as either the charge of the moving particle or the temperature goes to zero.

The equation of motion that accounts for the temperature and velocity-dependent resistance due to the optomechanical Doppler effect is:

$$\boldsymbol{F}_{\mathbf{app}} - [\rho_L Te^2 \mu_o / 4w\alpha]\,(v^2)/(1 - v^2/c^2)^{1/2} = m_o\, dv/dt \qquad \text{(A2.17)}$$

Equation A2.17, which is based on thermodynamics, contrasts with Planck's (1906) relativistic version of Newton's Second Law:

$$\boldsymbol{F}_{\mathbf{app}} = (d/dt)[m_o v(1 - v^2/c^2)^{-1/2}] \qquad \text{(A2.18)}$$

Depending on the assumptions, Eq. A2.18 can take the following forms:

$$\boldsymbol{F}_{\mathbf{app}} = m_o[(1 - v^2/c^2)^{-1/2}](dv/dt) \qquad \text{(A2.19)}$$

$$\boldsymbol{F}_{\mathbf{app}} = m_o[(1 - v^2/c^2)^{-3/2}](dv/dt) \qquad \text{(A2.20)}$$

Equations A2.17, A2.19, and A2.20 all predict that the force-velocity relationship for a given interval of time will be nonlinear and thus non-Newtonian. That is, the optomechanical Doppler effect model and the Special Theory of Relativity both predict that the force necessary to accelerate a constant mass electron from rest to velocity v in 1 second will be velocity dependent. However, the optomechanical

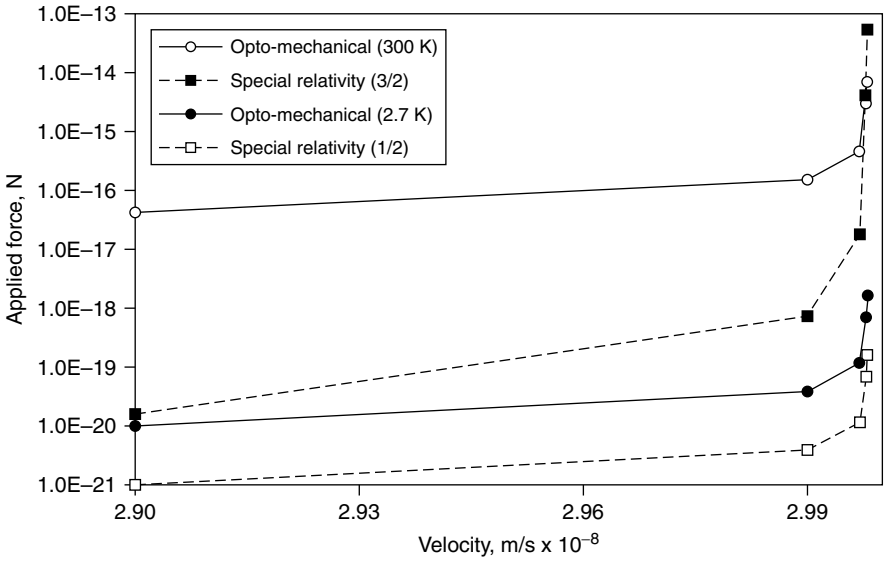


FIGURE A2.5 Prediction of how much force will be necessary to accelerate an electron to a given velocity at a given temperature according to the temperature-dependent optomechanical Doppler theory and the temperature-independent Theory of Special Relativity.

Doppler effect model, in contrast to the Special Theory of Relativity, further predicts that the force necessary to overcome the friction resulting from the optomechanical Doppler effect will be temperature dependent. The predictions given by the optomechanical Doppler effect equation solved for 2.73 K and 300 K and both equations of Special Relativity are given in Figure A2.5. Notice that the temperature-independent relativistic equation (A2.20) approximates Eq. A2.17 for temperatures between 2.73 K and 300 K.

Is the counterforce exerted by the thermal radiation field in an accelerator on Earth sufficient to limit the speed of an electron to the speed of light? Yes, if one takes the approach of a cell biologist and *assumes* that time and/or mass are not local quantities, and that a nonlinear relationship between a constant force exerted for a given time and velocity reveals that the electron with a constant mass must be traveling through a space that contains something that can resist the movement of the electron in a velocity-dependent manner. The optomechanical properties of photons are such that they could provide the velocity-dependent counterforce that would prevent an electron from accelerating beyond the speed of light (Wayne, 2009). There is evidence already that the cosmic microwave background radiation is mechanically active in that intergalactic electrons striking the cosmic microwave background radiation provide the source of the high-energy cosmic X-rays resulting though the inverse Compton effect (Feenberg and Primakoff, 1948).

The amount of applied constant force in a given time needed to accelerate an electron to a given velocity predicted by the equation of motion that includes the optomechanical Doppler effect differs from the amount of applied constant force in a given time needed to accelerate an electron to a given velocity predicted by the Theory of Special Relativity. These differences should make it possible to test the temperature-dependent, optomechanical resistance of the radiation field by performing experiments similar to those done by Kaufmann, Bucherer, Neumann, Jones, Guye, Rogers, and Bertozzi (1964), in order to determine the effect of velocity on mass under defined temperatures. To this end, it would be interesting to compare the dynamics of electron acceleration in the linear accelerators at Jefferson Laboratory that runs at 2 K and the Stanford Linear Accelerator that runs at 300 K, both of which reach energies in the GeV range.

In 1905, when the Special Theory of Relativity was published, Einstein (1905) had just conceived the idea of the quantum of radiation. It would be another 12 years before he would publish his ideas on the transfer of linear momentum as opposed to inertia between radiation and matter. Thus, it is no surprise that the effect of the linear momentum of the background radiation was not taken into consideration in the Special Theory of Relativity (Einstein, 1961). However, as I have shown, at temperatures greater than 0K, the radiation field acts as a dilatant, shear-thickened, viscous "non-Newtonian solution" or optical molasses analogous to sand, cornstarch, Kevlar, and Silly Putty®. Oddly enough, the concept of dilatancy was developed by Osborne Reynolds (1885, 1886) when he was contemplating the nature of the luminous ether.

The cell biologist's optomechanical picture presented here indicates that light acts as an ultimate speed limit to any particle because, at velocities approaching the speed of light, the radiation field itself (composed of light *sensu latu*) is more than a Newtonian photon gas, but is a dilatant gas that becomes infinitely viscous as the velocity of a moving particle approaches the speed of light. That is,

the radiation field provides a velocity-dependent optomechanical counterforce that prevents charged particles from accelerating as they reach the speed of light. This cell biologist's explanation contrasts with that given by the Special Theory of Relativity, which asserts that either time or mass is relative and local quantities that depend on velocity, and consequently, the force acting on an accelerating electron, are not as efficient as the same force acting on a stationary electron, and the force becomes less and less efficient as the velocity of the electron increases. The optomechanical version of Newton's Second Law, developed from the perspective of a plant cell biologist and given in this appendix, applies to microscopic and macroscopic physical processes.

Appendix 3

Calculation of the Total Transverse Force and Its Relation to Stress

In order to show that the transverse stress ($\sigma_{\perp}$) is equal to $(rP_t)/x$ and that the magnitude of the tangential force is equal to $(P_t)2rl$, where P_t is the turgor pressure of the cell, I need to use calculus. Consider a cylindrical cell to be composed of two hemicylinders. In spite of the turgor pressure inside acting to push them apart, each hemicylinder is held together by two tiny pieces of the extracellular matrix with a combined area of $2xl$ (Figure A3.1). The total force that pushes the hemicylinders apart at any one point is the product of the area of the flat face of each hemicylinder ($2rl$) and the effective pressure that is pushing against this surface area. That is:

$$F = (2rl) \text{ effective pressure} \qquad \text{(A3.1)}$$

The turgor pressure acting on the wall can be resolved into two components: one that is parallel to the axis of the cylinder ($P_{\text{longitudinal}}$) and one that is perpendicular to the axis of the cylinder ($P_{\text{transverse}}$). Only the perpendicular component will be effective in pushing the two hemicylinders apart. Given the definition of sine, $P_{\text{transverse}}$ is related to P_t by the following equation:

$$P_{\text{transverse}} = \tfrac{1}{2} P_t \sin\theta \qquad \text{(A3.2)}$$

where θ is the angle between the turgor pressure vector and the flat portion of the hemisphere. At θ equal to either 0 or π, $P_{\text{transverse}}$ will vanish; at $\pi/2$, it will be equal to $\tfrac{1}{2} P_t$ and will be maximally effective in pushing the two hemicylinders apart. At all other angles, $P_{\text{transverse}}$ will have intermediate values according to Eq. A3.3. The ½ is included in Eq. A3.2 because we are only considering the portion of the turgor pressure exerted by one-half of the cylinder on the other:

$$F_i = (2rl)\, \tfrac{1}{2} P_t \sin\theta = (rl)\, P_t \sin\theta \qquad \text{(A3.3)}$$

To get the total inertial force exerted from $\theta = 0$ to $\theta = \pi$, we must integrate Eq. A3.3 with respect to θ from

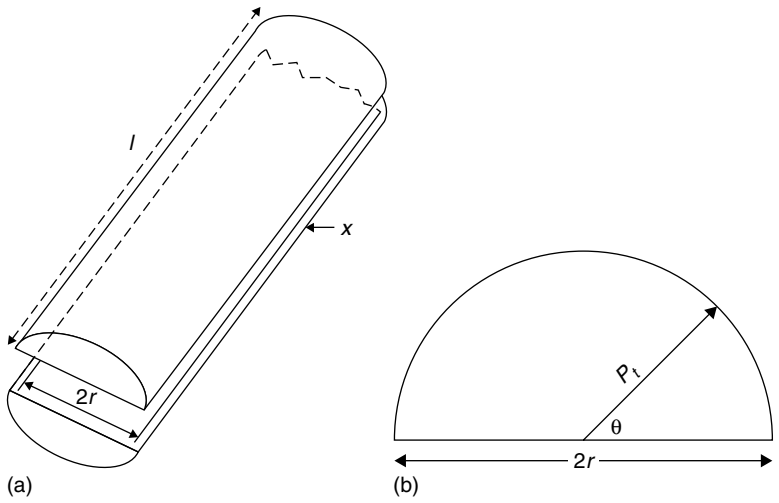


FIGURE A3.1 (a) The geometry of a cell presented as two hemicells. *l*, length; *r*, radius; *x*, thickness of wall. (b) The cross-section of a hemicell showing the position of θ, the angle between the flat portion of the hemicell and the radial vector.

0 to π. The total force pushing the hemispheres apart is thus:

$$F = rl\,P_t \int_o^{\pi} \sin\theta\, d\theta \tag{A3.4}$$

Since $\int \sin\theta = -\cos\theta$, Eq. A3.4 becomes:

$$F = -rl\,P_t\,[\cos(\pi) - \cos(0)] \tag{A3.5}$$

Since $\cos(\pi) = -1$ and $\cos(0) = 1$, Eq. A3.5 becomes:

$$F = 2rl\,P_t \tag{A3.6}$$

When the velocity of movement is constant, the inertial force must be equal to the viscous force. As described in Chapter 20, the viscous force that resists the inertial force is given by:

$$F_v = \sigma_\perp 2xl \tag{A3.7}$$

where x is the thickness of the extracellular matrix and l is the length of the hemicylinder. Thus, at constant velocity:

$$F_i = 2rl\,P_t = F_v = \sigma_\perp 2xl \tag{A3.8}$$

and after solving for $\sigma_\perp$, we get

$$\sigma_\perp = rP_t/x \tag{A3.9}$$

As shown in Chapter 20, the transverse stress is twice the longitudinal stress, yet cells tend to elongate instead of bulge as a result of the transverse orientation of cellulose microfibrils in the extracellular matrix.

Appendix 4

Laboratory Exercises

LABORATORY 1: INTRODUCTION TO THE LIGHT MICROSCOPE

The purpose of this and the next two laboratory exercises is to introduce you to the light microscope so that you will feel empowered to make detailed and accurate observations of plant cells. You will learn various types of microscopy that will help you to see different aspects of cell structure and cellular processes. See Wayne (2009) for detailed explanations of the microscopy methods presented here. We will begin with a historical approach that will provide you with the opportunity to be a virtual witness to the observations made by the founders of cell biology.

Bright-Field Microscopy: Establish Köhler Illumination on the Olympus BH-2

Get a prepared slide and put it on the microscope stage. Focus on the specimen with the coarse and fine focus knobs. Close down the field diaphragm and focus an image of the field diaphragm on the specimen plane by raising or lowering the substage condenser. Adjust the interpupillary distance by moving the oculars together or apart until you find the position that gives you the most comfortable binocular vision. Adjust the diopter by looking at the specimen with your right eye through the right ocular and focusing using the fine focus control. Then look at the same image point with your left eye through the left ocular, and without using the coarse and fine focus knobs, rotate the diopter adjustment ring until the image point is in clear focus for your left eye.

The interpupillary distance adjustment and the diopter adjustment allow you to set up the microscope for the distance between your eyes and the difference in focal length of your two eyes, respectively. Open the field diaphragm so that it almost fills the field and center it with the centering screws on the substage condenser. Open and close the aperture diaphragm so that you optimize resolution and contrast. Remove the ocular and look down the microscope tube. At the position where the resolution and contrast are optimal, the light will fill about 80 percent of the width of the optical tube. All microscope work from here on in will be done with the room lights off. You can use a flashlight to find things, prepare specimens, and write in your notebook. Set the light path selector one stop so that 80 percent of the light goes to the camera or two stops so that 100 percent of the light goes to the camera. Take a picture of your specimen.

Measurements with a Microscope

To measure the length of an object, insert an ocular micrometer in the ocular and place the stage micrometer on the stage. Carefully focus the stage micrometer. Both the stage micrometer and the ocular micrometer will be in focus and sharply defined. Turn the ocular so that the lines of the eyepiece are parallel to each other.

Determine how many intervals on the ocular micrometer correspond to a certain distance on the stage micrometer and then calculate the length that corresponds to one interval of the ocular micrometer. For example, if 35 intervals correspond to 200 μm (0.2 mm), then one interval equals (200/35) = 5.7 μm. This value is specific for each objective. Calibrate the ocular micrometer for each objective on your microscope. Put the calibration in a convenient place in your laboratory notebook.

Find the field-of-view number on the ocular. Divide the field-of-view number by the magnification of the objective to get the diameter (in mm) of the field of view. Remember to divide this distance again by the magnification introduced by any additional intermediate pieces. List the diameter of the field for each objective next to its calibration.

Take a picture of the stage micrometer using each objective. The photographs of the stage micrometers can be used as convenient rulers to measure the size of objects in photomicrographs taken under the same conditions as the stage micrometer.

Observations of Cells and Organelles Using Bright-Field Microscopy

Cells were first observed and described by Robert Hooke in his book *Micrographia* in 1665. Make a thin hand section of a piece of cork, as Robert Hooke did. Mount it in a drop of lens cleaner on a Gold Seal microscope slide and cover it with a Gold Seal #1½ cover glass (*www.tedpella.com/*). Keep the lids closed so the slides and cover glasses remain dry and dust-free. The lens cleaner helps eliminate air bubbles. What is the most prominent structure of the cell? Can you see why Hooke chose to name the building blocks of plants by using the word to describe the austere dwelling of a monk? Adjust the aperture diaphragm to optimize resolution and contrast. Take a picture of the cork.

When you take photomicrographs, make a note of the specimen identity, the type of objective you use, its numerical aperture, its magnification, the total magnification, and the type of microscopy (e.g., bright-field). Also, note the camera and/or film type and the exposure adjustment. How do you set the exposure adjustment? If the specimen is completely distributed throughout the bright background, set the exposure adjustment knob to 1×. If the specimen covers approximately 25 percent of the bright background, set the exposure adjust to 0.5× to make the exposure longer. If the specimen covers less than 25 percent of the bright background, set the exposure adjust to 0.25× to make the exposure even longer.

Nehemiah Grew compared the cells of the root of *Asparagus* in his book *The Anatomy of Plants* in 1682 to the froth of beer. Repeat Grew's observations on the root of *Asparagus* by making transverse hand sections with a sharp razor blade. What is the most prominent structure of the cell? Can you see why cell biology was known as cytology, which means the study of hollow places, for such a long time? Adjust the aperture diaphragm to optimize resolution and contrast and take a picture of a cross-section of an *Asparagus* root.

Robert Brown discovered the nucleus, an organelle he serendipitously found in the seemingly hollow cavities of the cell, while he was studying pollination. Brown described the nucleus in the epidermal cells of orchids in 1831 in a paper entitled "On the Organs and Mode of Fecundation in Orchideae and Asclepiadeae." Repeat Brown's observations of the nucleus by putting an epidermal peel from the top of a vanilla orchid leaf on a drop of water on a microscope slide and observing it with the Olympus BH-2 microscope set up for Köhler illumination. Can you see the nucleus?

Now, I will teach you a trick. Instead of using Köhler illumination, we will use oblique illumination, which was readily available to microscopists in Brown's time. To obtain oblique illumination with your microscope, slightly rotate the substage condenser turret until a pseudo-relief image appears. The nucleus in each cell will appear as prominent as they did to Robert Brown. Do you remember how difficult it was to see the nucleus in high school, freshmen biology, and just a minute ago, before you knew about oblique illumination? Document your observations with photographs.

The bright-field microscope is optimal for visualizing colored objects. Pull off a young leaf of a dark-adapted *Elodea* with forceps and place it on a drop of water on a slide. Carefully lower a coverslip on top of the specimen and observe the epidermal cells with a microscope. Unlike most epidermal cells, the epidermal cells of *Elodea* contain chloroplasts that are easy to see because of their natural pigmentation. Notice the positions of the chloroplasts and take a photograph. What happens to the chloroplasts over time as they become irradiated by the microscope light? Photographically document the changes that occur in response to light.

In order to get a feeling for the diversity in the form of chloroplasts, make slides of *Spirogyra* (ER-15-2525), *Zygnema* (ER-15-2695), and *Micrasterias* (ER-15-2345), all of which are green algae available from Carolina Biological Supply (*www.carolina.com*). Document your observations with photographs.

In order to visualize the vacuole, peel off the epidermis from the lower side of a *Rhoeo discolor* leaf by snapping the leaf and pulling it so that epidermal peels stick out of the broken ends of the leaf. The vacuole of *Rhoeo* contains anthocyanins, which makes it a convenient specimen for observation. Mount one epidermal peel in water and another one in 0.5-M sucrose (17.1 g/100 mL H_2O). Observe the epidermal cells that were mounted in water. Estimate the percentage of an epidermal cell that is taken up by the vacuole. Observe the protoplasts in the plasmolyzed cells in the sucrose solution. How can you explain the various shapes of the protoplasts in these cells? This plasmolysis technique can be used quantitatively to determine the osmotic pressure of the cell (Bennet-Clark et al., 1936) and the osmotic permeability coefficient for water (Stadelmann and Lee, 1974). Can you figure out how?

Observations of Cells Using Dark-Field Microscopy

Focus on the diatoms on a diatom exhibition slide or a diatom test plate. These slides are available from Klaus D. Kemp, Microlife Services (*www.diatoms.co.uk/pg.htm*). Raise the dark-field condenser on the Olympus BH-2 microscope so that it almost touches the bottom of the slide. Turn the substage condenser turret to DF. Open the field diaphragm only until all the specimens in the field are evenly illuminated, and observe the diatoms. Do some of the diatoms appear colored? Are they still colored when viewed with bright-field microscopy? Can you guess the cause of the colors? Document your observations with photographs.

Antony van Leeuwenhoek (1679), a linen draper, made microscopes in his spare time and discovered animalcules or "living atoms" with his microscopes. Leeuwenhoek was able to see the single-celled organisms with his simple microscope because he used dark-field illumination. Repeat Leeuwenhoek's observations by putting a drop of pond water, a drop from a soil-water mixture, a drop of pepper-water, or a drop of hay infusion on a microscope slide and cover it with a cover glass. Observe the animalcules that Leeuwenhoek saw 300 years ago. If you have a slowly moving organism in the preparation, document your observations with photographs.

LABORATORY 2: PHASE-CONTRAST, POLARIZATION, AND DIFFERENTIAL INTERFERENCE CONTRAST MICROSCOPY

While the bright-field microscope is optimal for colored objects, transparent objects appear almost invisible when observed under conditions that maximize the resolving power of the microscope. That is when the aperture formed by the diaphragm is large. The phase-contrast microscope, the polarizing microscope, and the differential interference contrast microscope can be used to introduce contrast in transparent living specimens. The polarizing microscope has the added advantage in that it can allow the user to differentiate between macromolecules with different spatial orientations. The purpose of this laboratory is to learn how to set up a microscope for phase-contrast, polarization, and differential interference microscopy and to gain experience in observing the nature of transparent cells with these contrast-generating techniques.

Observation of Cells with Phase-Contrast Microscopy

Before you obtain cells to view with phase-contrast microscopy, put a drop of water on a microscope slide so that the cells you will obtain will remain hydrated. You can make a peel of the epidermis from the convex side of the bulb scale by cutting out a 1 cm × 1 cm piece of a bulb scale that is four or five bulb scales deep into the onion. Snap the bulb scale so that most of it breaks in two. The epidermal layer will not break clean. Pull this layer back with forceps and place it on a drop of water on a microscope slide. To make a peel of the epidermis on the concave side of the bulb scale, remove a bulb scale that is four or five layers deep within the onion. Make a checkerboard pattern of cuts with a razor blade on the concave side of the bulb scale. Pick up several 3 mm × 3 mm squares of epidermal tissue with forceps and place the epidermal tissue sections on the drop of water.

Set up Köhler illumination in the Olympus BH-2 microscope. Observe an onion epidermal cell with bright-field optics using the 10× phase-contrast objective lens. Make sure that the condenser ring is in the 0 position. The cells are virtually invisible and the contrast is best when the specimen is slightly defocused. Contrast can also be enhanced by closing down the aperture diaphragm. Although the contrast increases, diffraction rings and lines are produced that obscure the round and linear details, respectively, that make up the specimen.

Phase-contrast microscopy can be used to observe transparent cells with both high resolution and high contrast. Turn the substage condenser turret to the 10 position in order to observe the cells with phase-contrast microscopy. In general, the number on the turret matches the magnification of the objective lens. Center the phase ring in the substage condenser turret by removing one ocular and inserting the centering telescope. Focus the centering telescope so that the phase ring and the phase plate are in focus. Use the phase annulus-centering screws to center the phase ring and align it with the phase plate. Remove the centering telescope and replace the ocular. Repeat for the 40× phase objective.

Observe the onion epidermal cells with the 40× PL phase-contrast objective. Are the epidermal cells on the convex side different from the epidermal cells from the concave side? The cells certainly do not appear hollow. Do you see the cytoplasm circulating throughout the cell? Which organelles move and which stay still? Can you see structure within the nucleus? What is the three-dimensional shape of the nucleus? Can you see mitochondria? Can you see the peripheral endoplasmic reticulum? Document your observations with photographs.

Look at the pollen tubes of periwinkle (*Catharanthus roseus*) that have been growing on a modified Brewbaker-Kwack medium for about 2 hours. Notice the fountain, reverse fountain, or rotational streaming of the cytoplasm in the pollen tube. Notice the muscular nature of the cytoplasm and the variety of organelles that are visible within it. Document your observations with photographs.

To make 10 mL of modified Brewbaker-Kwack medium, mix together 3 g sucrose, 1 mg boric acid, 2 mg $MgSO_4 \times 7H_2O$, 1 mg KNO_3, 3 mg $Ca(NO_3)_2 \times 4H_2O$, and 58 mg 2-(*N*-morpholino)ethanesulfonic acid (MES). Add 7 mL distilled water and titrate to pH 6.5 with 1 N NaOH. Bring up to 10 mL with distilled water.

Place a segment of a root of *Hydrocharis* or *Limnobium* in a drop of water on a slide and look at the root hairs. Notice the fountain streaming of the cytoplasm in the root hair, the muscular nature of the cytoplasm, and the variety of organelles within it. Document your observations with photographs.

Observation of Cells with Polarization Microscopy

The polarizing microscope allows one to see anisotropic objects with high contrast. Set up the Olympus BH-2

microscope for bright-field microscopy and then insert the polarizer and the analyzer. Leave the first-order wave plate in the out position.

Obtain a lightly stained prepared slide of bordered pits or make your own specimen out of a pine board. Observe the bordered pits under crossed polars with the polarized light microscope with and without the first-order wave plate. Document your observations with photographs, making sure to document the orientation of the polarizer, the analyzer, and the slow axis (z') of the first-order wave plate. The cellulose microfibrils that make up the bordered pit are positively birefringent. How are they oriented in the bordered pit? Estimate the retardation of the bordered pits using the Michel-Lévy color chart.

Grind a cube of potato in water with a mortar and pestle. Pipette a drop of the starch grain solution on a microscope slide and cover with a cover glass. Observe the starch grains under crossed polars with the polarized light microscope with and without the first-order wave plate. Document your observations with photographs, making sure to document the orientation of the polarizer, the analyzer, and the slow axis (z') of the first-order wave plate. The starch molecules that make up the starch grains are positively birefringent. How are the starch molecules oriented in the grain? Using the Michel-Lévy color chart, estimate the retardation of the starch grain. If you like, prepare a thin section of the potato to see the starch grains in situ.

Using forceps, mount thin strands of herring sperm DNA in a drop of lens cleaner on a microscope slide. Observe with a polarized light microscope under crossed polars with and without the first-order wave plate. Document your observations with photographs, making sure to document the orientation of the polarizer, the analyzer, and the slow axis (z') of the first-order wave plate. The strands of DNA are negatively birefringent. Do the strands, the physical axes of which are parallel to the slow axis of the first-order wave plate, show additive colors (blue) or subtraction colors (yellow-orange)? Put a lot of DNA on the slide and observe retardation-dependent colors with and without the first-order wave plate. Herring sperm deoxyribonucleic acid is available from Sigma Aldrich (*www.sigmaaldrich.com/*).

Make a thin transverse hand section of an *Asparagus* root using a razor blade. Mount it in distilled water. Observe it with a polarized light microscope with and without the first-order wave plate. Document your observations with photographs. What can you say about the orientation of the positively birefringent cellulose microfibrils? Using the Michel-Lévy color chart, estimate the retardation of the walls.

Make an epidermal peel from the bottom of a vanilla leaf. Mount it in distilled water. Observe the stomata with a polarized light microscope with and without the first-order wave plate. How are the positively birefringent cellulose microfibrils arranged in the guard cells of the stomatal complex? Using the Michel-Lévy color chart, estimate the retardation of the walls. Document your observations with photographs.

Differential Interference Contrast Microscopy (Demonstration)

Mount a *Tradescantia* stamen hair from the second-youngest bud in water on a slide and focus on the hair cells using bright-field optics and Köhler illumination. If you are lucky you will be able to see mitosis and/or cytokinesis in the cells at or close to the tip of the hair. Use the SPLAN objectives. Slide in the polarizer that is attached to the substage condenser. Slide in the differential interference contrast beam-recombining prism with the built-in analyzer and first-order wave plate. Rotate the substage condenser turret to the red number that matches the magnification of the objective lens. Vary the contrast by turning the knob on the beam-recombining prism. Which color gives the best contrast? How does the direction of shear influence the image? Optically section the cells. Document your observations with photographs.

LABORATORY 3: FLUORESCENCE MICROSCOPY

The purpose of this laboratory is to learn how to set up a microscope for fluorescence microscopy in order to visualize transparent cells with optimal resolution and contrast. You should begin to get a sense of the absolute and relative size of each organelle, their shapes, their number, and their distribution.

Setting up Köhler Illumination with Incident Light

Before you begin, set up Köhler illumination for both the transmitted light and the incident light on the Olympus BH-2. To set up Köhler illumination for the incident mercury light:

1. Turn on the Hg lamp.
2. Place a slide on the stage.
3. Open the shutter slider all the way.
4. Rotate the iris diaphragm (A) and the field diaphragm (F) counterclockwise so they are open maximally.
5. Close down the field diaphragm so that you can just see the edges. Center the field diaphragm by adjusting the centering screws. Open the field diaphragm.
6. Center the lamp carefully and gently with the two lamp-centering screws, until the center of the field is maximally bright.
7. Adjust the collector lens with the focusing handle until the field is maximally bright and evenly illuminated.

8. You may find that you get better contrast with some specimens and even-enough illumination by focusing the lamp on the image plane.

Observe Organelles (e.g., Mitochondria and/or Peroxisomes) in Tobacco Cells Transformed with Organelle-Targeted GFP

Tobacco plants that have been transformed with the gene for green fluorescent protein (GFP) combined with sequences that code for the targeting of the GFP to the various organelles have been prepared. For each plant, make several hand sections of a piece of tobacco leaf in water or make epidermal peels of the leaf. Place the sections or peels in a drop of water on a microscope slide. Using the blue excitation cube, look for the organelles in the epidermal hairs. For each organelle, estimate its size and describe its distribution in the cell. Estimate how many of each organelle exists in the cell. Do the proportion and distribution vary in the various cells that make up the hair? Document your observations with photographs.

Visualizing Organelles with Fluorescent Organelle-Selective Stains

To visualize the mitochondria of onion epidermal cells, place epidermal peels in a droplet of 1 to 10 μg/mL rhodamine 123 on Parafilm in a covered petri dish in the dark for 30 to 60 minutes. Transfer epidermal peels to distilled water in a petri dish and let them sit overnight in the dark. Place the epidermal peels on 0.05 percent n-propylgallate and observe the mitochondria using the green excitation cube. What can you say about the size, distribution, and motility of the mitochondria? Document your observations with photographs.

Place an unstained onion epidermal peel on a drop of water and observe it with fluorescence microscopy. What do you see? Since you did not stain the cells, anything you see is due to autofluorescence. Are the mitochondria autofluorescent? If so, then, how do you know that the rhodamine 123 selectively stained the mitochondria? If not, you can be more certain that the rhodamine 123 selectively stained the mitochondria.

To take micrographs of bright fluorescent specimens scattered over a dark field, you will typically have to set the exposure meter so that the camera takes shorter exposures. If the staining is too intense and there is too much background staining, dilute the working solution down to 1:50 with distilled water. As a rule, as one gets to know the specimen better, one requires less stain and consequently achieves better selectivity and contrast.

In order to observe the endoplasmic reticulum of onion epidermal cells, make a stock solution of $DiOC_6(3)$ by dissolving 1 mg of $DiOC_6(3)$ in 1 mL ethanol and dilute the stock solution with water (1:1000) to make the working solution. Then prepare several pieces of onion epidermis and mount them on a drop of staining solution. Wait 5 to 10 minutes. Remove the staining solution with a pipette and immediately replace it with 0.05 percent n-propylgallate. Observe the endoplasmic reticulum using the blue excitation cube. Describe its structure. Document your observations with photographs.

In order to observe the lipid bodies or spherosomes in onion epidermal cells, make a 1-mg/mL stock solution of Nile Red in acetone and dilute the stock solution to make a 1 μg/mL working solution. Then prepare several pieces of onion epidermis and mount them on a drop of staining solution. Wait 5 to 10 minutes. Remove the staining solution with a pipette and immediately replace it with 0.05 percent *n*-propylgallate. Observe the spherosomes using the blue excitation cube. Document your observations with photographs.

It is important to shut off the mercury lamp when you finish with your observations, since the lamp has a limited lifetime and may explode if left on for extended periods when you leave the laboratory. One should never turn a hot mercury lamp back on until it has cooled.

LABORATORY 4: PHOTOMICROGRAPHY

The purpose of this laboratory is to learn to take photomicrographs that capture the content of the specimens in the most artistic way possible. It also gives you a chance to observe new kinds of cells and reobserve the cells you have already seen now that you have experience with many types of light microscopy.

The procedures are as follows:

1. Go to the conservatory and choose plants that interest you to use as specimens, bring in your own research materials, or use the materials listed below:
   - Cork
   - *Asparagus* root
   - Orchid epidermis
   - Beetroot cells
   - *Elodea* leaves
   - Onion epidermis
   - Diatoms (from a drainage ditch and prepared slides)
   - Fern sporangia
   - *Tradescantia* or *Setcresea* stamens
   - *Catharanthus* pollen
   - Cotton hairs
   - Herring sperm DNA
   - Potato starch grains
   - Circular-bordered pits (*Chamycyperus* or *Pinus*)
   - Iris leaf
   - *Tradescantia* stem
   - *Impatiens* stem

- African violet leaves
- Tobacco plants transformed with green fluorescent protein targeted to the nucleus, endoplasmic reticulum, mitochondria, or peroxisomes.

2. Prepare each specimen and set up each type of microscope optimally. Photograph each specimen, taking into consideration the composition, exposure, resolution, and contrast.
3. Take your time. You have 2 weeks to make your own *Atlas of the Plant Cell*. Your photographs may also be included in making this year's Plant Cell Biology class calendar.

LABORATORY 5: MEMBRANE PERMEABILITY

The purpose of this laboratory is to develop the technical and mathematical skills necessary to describe membrane permeability, a biophysical aspect of cells.

Determination of the Osmotic Permeability Coefficient of the Plasma Membrane of *Chara corallina*

In this lab, we will measure the osmotic permeability coefficient of the plasma membrane of *Chara corallina* using the transcellular osmosis technique.

Movement of water across a membrane occurs when there is a difference in the water potential (Ψ, in Pa) on the two sides of the membrane. When the difference in water potential is due to a difference in the osmotic pressure of the solutions on both sides of the membrane, the movement of water is known as *osmosis*. The rate of osmotic water movement across a membrane (J_v, in m^3/s) is proportional to the difference in the water potential outside the cell (Ψ_o) and inside the cell (Ψ_i). The rate of osmotic water movement through a membrane is also proportional to the surface area of the membrane (A, in m^2).

$$J_v \propto A(\Psi_o - \Psi_i)$$

The coefficient that relates the osmotic water flow to the difference in the water potential is the hydraulic conductivity (L_p, in m s^{-1} Pa^{-1}):

$$J_v = L_p A(\Psi_o - \Psi_i)$$

The water potential can be represented as the difference between the hydrostatic pressure (P) and the osmotic pressure (π). Consequently,

$$\Psi_o = P_o - \pi_o$$

and

$$\Psi_i = P_i - \pi_i$$

The hydrostatic pressure inside the cell is commonly called the *turgor pressure* of the cell. The hydrostatic pressure outside the cell is equal to zero by convention, and consequently:

$$\Psi_o = -\pi_o$$

The difference in water potential between the outside of the cell and the inside of the cell is:

$$\Psi_o - \Psi_i = -\pi_o - (P_i - \pi_i) = \pi_i - \pi_o - P_i$$

If we define $\pi_i - \pi_o$ as $\Delta\pi$, then the above equation becomes:

$$\Psi_o - \Psi_i = \Delta\pi - P_i$$

The most general way to measure the hydraulic conductivity coefficient is to subject the cell to plasmolysis and to measure the change in volume of the protoplast induced by an osmotic pressure difference across the plasma membrane. However, since the shrinking of the plasma membrane that occurs during plasmolysis may affect the water-transporting properties of the plasma membrane itself, it is desirable to find a method to measure water movement without plasmolyzing the cell. Transcellular osmosis (TCO) is such a method (Osterhaut, 1949; Kamiya and Tazawa, 1956; Kiyosawa and Ogata, 1987; Wayne and Tazawa, 1988; Wayne and Tazawa, 1990). In order to perform transcellular osmosis, a cell is partitioned into two hydraulically isolated compartments A and B (Figure A4.1). The difference in water potential across the plasma membrane in chamber A is:

$$\Psi_A = \pi_a - \pi_A - P_i$$

where π_a is the osmotic pressure of the cell part in chamber A and π_A is the osmotic pressure of the solution in chamber A. P_i is the turgor pressure of the cell. The turgor pressure, which moves at the speed of sound, equalizes throughout the cell rapidly and is the same in the cell part in chamber A and the cell part in chamber B. The difference in water potential across the plasma membrane in chamber B is:

$$\Psi_B = \pi_b - \pi_B - P_i$$

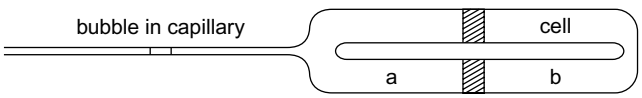


FIGURE A4.1 A chamber for transcellular osmosis.

where π_b is the osmotic pressure of the cell part in chamber B and π_B is the osmotic pressure of the solution in chamber B. If π_A is set to zero,

$$\Psi_A = \pi_a - P_i$$

The water potential difference between the outside of the plasma membrane in chamber A and the outside of the plasma membrane in chamber B is:

$$\Psi_A - \Psi_B = (\pi_a - P_i) - (\pi_b - \pi_B - P_i)$$
$$\Psi_A - \Psi_B = \pi_a - P_i - \pi_b + \pi_B + P_i$$
$$\Psi_A - \Psi_B = \pi_a - \pi_b + \pi_B$$

and if we assume that initially $\pi_a - \pi_b = 0$, then

$$\Psi_A - \Psi_B = \pi_B$$

At the start of transcellular osmosis, the difference in the water potential across the plasma membrane is equal to the osmotic potential of the solution placed in chamber B. Thus:

$$J_v = K\ \pi_B$$

where K is a coefficient that takes into consideration the area of the cell and the hydraulic conductivity coefficient of the plasma membrane. The osmotic pressure (π_B) is given by the following equation:

$$\pi_B = RTc$$

where R is the universal gas constant 8.31 J mol^{-1} K^{-1}, T is the absolute temperature in K, and c is the concentration of the osmoticum in mol/m^3. Since J = Nm and Pa = N/m^2, the universal gas constant can be given in pressure units instead of energy units and is equal to 8.31 Pa m^3 mol^{-1} K^{-1}. Calculate the osmotic pressure of a 100-mM sorbitol solution.

If we assume that the cell is partitioned into equal halves in the two chambers and that the L_p for water movement into the cell (endosmosis in chamber A) is equal to the L_p for water movement out of the cell (exosmosis in chamber B), then

$$K = A\ L_p/2$$

where A (in m^2) is the surface area of the cell in either chamber. Therefore:

$$L_p = 2J_v/A\pi_B$$

To measure the hydraulic conductivity coefficient of the plasma membrane of *Chara corallina*, measure the length (l) of an isolated light-green internodal cell, approximately 4 cm long, with a ruler. Measure the diameter (d) of the cell under the microscope using a calibrated ocular micrometer. Calculate the surface area of the cell in either chamber by assuming the cell is a cylinder and using the equation:

$$A = \pi dl$$

Fill chamber A of the transcellular osmosis apparatus with distilled water, put grease in the area between chambers A and B, and grease the block that will separate the two chambers. Blot the surface of the internodal cell with toilet paper until it is almost dry and place it in the silicone grease-filled groove of the transcellular osmosis chamber. Place the cell so that equal lengths are in chambers A and B. Cover the cell part in the groove and apply the chamber cover in such a way that there are no air bubbles in the chamber and the water meniscus will be visible in the calibrated capillary tube.

Add distilled water to chamber B and let the cell equilibrate. Put the chamber under the Olympus CH microscope so that the meniscus in the calibrated capillary tube is visible with a 10× objective lens. For a clear image of the meniscus, put a drop of water on the capillary above the meniscus and put a tiny piece of a coverslip on top of the water. When the meniscus is stable, replace the water in chamber B with 100 mM sorbitol. With the help of your laboratory partner and a stopwatch, measure the rate that the meniscus moves across a calibrated ocular micrometer from 6 seconds to 18 seconds after the addition of 100 mol/m^3 sorbitol.

The rate that the water moves in this time can be calculated. First, calibrate the ocular micrometer with the stage micrometer using the 10× objective to determine the number of meters per unit.

Next, calibrate the volume of water moved per unit distance the meniscus moves along the ocular micrometer. This is done my measuring with a ruler the length between the calibrated volume marks (e.g., 0.01 mL = 15 mm). Determine the number of cubic meters per meter along the calibrated capillary tube.

To determine the volume flow (J_v) of the water (in m^3/s), we use the following equation:

$$J_v = \text{(units/s)(meters/unit)(meters cubed/meter length)}$$

where units/s is measured between 6 and 18 seconds.

The hydraulic permeability coefficient can be calculated from the volume flow, the surface area of the cell part in one chamber, and the osmotic pressure used to instantiate water flow according to the following equation:

$$L_p = 2J_v/A\pi_B$$

By expressing the difference in the osmotic pressure across the membrane as a difference in the water concentration, the osmotic permeability coefficient can be expressed as a permeability coefficient known as the *osmotic permeability coefficient* (P_{os}, in m/s).

$$P_{os} = (RT/\bar{V}_w)\, L_p$$

where $\bar{V}_w$ represents the partial molar volume of water and is equal to $1.818 \times 10^{-5}\,m^3/mol$. How does the osmotic permeability coefficient compare to other permeability coefficients you can find in the literature?

Measurement of the Reflection Coefficient of Nonelectrolytes

In order to measure the hydraulic conductivity coefficient in *Chara corallina*, we need to use a nonpermeating solute like sorbitol to induce the water movement. In essence, the sorbitol is not transmitted through the membrane but is reflected by it. The ability of a membrane to reflect a solute is known as the membrane's *reflection coefficient* (σ, dimensionless) for that solute (Staverman, 1951; Owen and Eyring, 1975; Steudle and Tyerman, 1983). By comparing the reflection coefficients of a membrane, one gets a feeling for the permeability of that membrane. The reflection coefficient for a solute can be determined by taking the ratio of the volume flow induced by the solute to the volume flow induced by the same concentration of sorbitol according to the following equation:

$$\sigma_{solute} = J_{v\text{-solute}}/J_{v\text{-sorbitol}}$$

Determine the volume flow of water induced by 100-mol/m^3 solutions of the solutes given in the following table:

Solute	Molecular Mass (g/mol)	Density (g/ml)	Radius (nm)
Diethylamine	73.14	0.707	—
Butyl alcohol	74.12	0.803	—
Glucose	180.16	—	0.35
Glycerol	92.09	1.261	—
Ethanol	46.07	0.785	—
Propanol	60.10	0.785	—
Methanol	32.04	0.791	—
Ethylene glycol	62.07	1.113	—
Urea	60.06	1.335	—
Thiourea	76.12	—	—

How do your results compare with Overton's Rules? Can the reflection coefficients be explained in terms of the lipophilicity of the molecules or the relative size of the molecules compared to the size of aqueous pores in the membrane?

The volume of a molecule (in mL) can be approximated with the following formula:

volume = (molecular mass)/
(density × Avogadro's number)

The volume in meters cubed can be determined by dividing the volume in mL by 10^6. The radius of the molecule can be determined by assuming that the molecule is spherical and that the radius cubed = $(3/4\pi)$(volume of the molecule). Estimate the radii of the molecules used to measure the reflection coefficient. What is the relationship between the reflection coefficient and the radius of each molecule?

Determination of the Permeability of Living Cells to Acids and Bases

The anthocyanins in the vacuole of the lower epidermal cells of *Rhoeo* can be used as an intrinsic pH indicator to report on the pH of the vacuole. When we observe a color change in the vacuole, we can infer that the H^+ concentration in the vacuole has changed. When these changes occur in response to the application of acids and bases to the solution surrounding the cell, we can infer that the acids or bases must have crossed the plasma and vacuolar membranes. We can get a sense of the permeability of membranes to acids and bases by following the rate in which the color changes in response to the addition of a given acid or base to the external solution.

Prepare a number of strips of the lower epidermis of *Rhoeo* and float them on distilled water. The vacuole is reddish purple when anthocyanin is protonated and blue when it is unprotonated. It is protonated below its pK_a and unprotonated above it. Is the vacuole basic or acidic?

Place two strips into 0.25 N KOH and six into 0.025 N NH_4OH. What happens? Record the time it takes for the anthocyanin to turn blue.

After the strips have turned blue in the 0.025 N NH_4OH solution, transfer three strips from the 0.025 N NH_4OH solution to water and then transfer them to the 0.025 N acetic acid solution. At the same time, transfer three strips from 0.025 N NH_4OH to 0.025 N HCl. What happens? Record the time it takes for a color change to take place.

After the color change is completed, transfer the strips from the acid solutions into the water and then transfer them back to the 0.025 N NH_4OH solution. Describe and interpret your results. What can you say about the permeability of the membrane to acids, bases, and H^+? How do your results relate to the chemiosmotic theory?

Observation of Spatial Heterogeneity of the Proton Pump in the Plasma Membrane of *Chara*

The plasma membrane is not homogeneous throughout, but is differentiated into regions with different transporter

protein activities. The plasma membrane of *Chara corallina* cells is differentiated into regions that have a net proton efflux and regions that have a net proton influx (Spear et al., 1969; Lucas and Smith, 1973). The acid and alkaline regions of *Chara corallina* can be visualized by placing cells on 1 percent agar containing the following components: 0.1 mM KCl, 0.1 mM $NaHCO_3$, 0.1 mM NaCl, 0.1 mM mM $CaCl_2$, 0.1 mM phenol red, and titrated to pH 7.2 with NaOH. The phenol red is a pH indicator that turns yellow where the pH is acidic and red where the pH is basic. Describe the possible distribution of transporters on the plasma membrane that would give the observed color pattern.

How could you use regions of these agar plates far from the cells to measure the diffusion coefficient of protons through the agar?

LABORATORY 6: CELL DIVERSITY AND CELL MOTILITY

The purpose of this laboratory is to observe a variety of single-celled organisms to get a feel for the diversity among cells. Each of these cells is at once a single cell and a complete organism, and in these capacities performs all the functions of life.

One attribute of life is spontaneous movement. We will see many motile processes, including swimming powered by cilia and flagella, cytoplasmic streaming based predominantly on the microfilament and/or microtubule system, as well as endocytosis, exocytosis, and vesicle movements that take place as a part of feeding.

Use the following materials:

- *Paramecium bursaria*
- *Amoeba proteus*
- *Chaos carolinensis*
- *Volvox globator*
- Green *Hydra*
- *Vorticella*
- *Stentor*
- *Acetabularia*
- *Caulerpa*
- *Bursaria truncatella*
- *Micrasterias*
- *Rhodospirillum rubrum*
- *Dictyostelium discoideum*
- *Spirogyra grevilleana*
- *Alternaria alternata*
- *Coleochaete scutata*
- *Caulerpa*

These organisms can be obtained from Carolina Biological Supply (*www.carolina.com/*).

Observation of Cells with Your Microscope

Using the hanging-drop method with depression slides, or flat slides with two strips of fishing line supporting the coverslip, observe the variety of single-celled organisms provided. You may slow down their movements by adding a drop of Protoslo to your preparation.

Look at *Vorticella* and watch it move. See its spasmoneme? Can you see its cilia? Can you see the organelles move inside? Look at *Stentor* and *Bursaria*. Look at how *Amoeba proteus* and *Chaos carolinensis* move across the slide. Look at the pseudopods. Can you see their organelles move inside? Look at *Volvox*. How does it move? Can you see the protoplasmic strands that connect all the cells together? Look at *Dictyostelium discoideum*. How does it move?

Look at *Micrasterias*. Imagine the cellular processes that must take place to generate the elaborate shape of each semicell!

Look at *Spirogyra grevilleana*. Isn't the chloroplast beautiful? Can you see saltational movements in the cytoplasm? Can you find the suspended nucleus?

Look at *Coleochaete scutata*. Notice how the cells form a tissue.

Look at *Rhodospirillum rubrum*, a photosynthetic, purple, nonsulfur bacteria. This bacterium has infoldings of the membrane that are similar in appearance to the cristae of mitochondria and may be related to the bacteria that gave rise to mitochondria.

Look at *Alternaria alternata*. Can you see the spores?

Look at *Paramecium bursaria*. Can you see the *Chlorella* cells? Can you see the contractile vacuole? Can you see the cytoplasmic streaming? Can you see them conjugating?

Look at a drop of pond water. What other fascinating organisms and types of movements can you see?

Observation of Giant Cells Using the Dissecting Microscope

Look at *Acetabularia*, which is a large single-celled and uninucleate green alga. The cells can be up to 20 cm long and have beautiful cytoplasmic streaming. *Acetabularia* has a rapid wound-healing response that allows one to remove the nucleus by cutting off the rhizoid with a pair of scissors. A nucleus from another species can then be implanted in the enucleated species to test the role of nuclear determinants in the generation of form. From such studies done with *Acetabularia* in the 1930s, Hämmerling demonstrated that molecules involved in the genetic determination of the phenotype of the cap are produced in the nucleus and transported to the cytoplasm.

Look at *Hydra*. We have two forms of *Hydra*: one is transparent and the other is green. The green one is an endosymbiotic association between *Hydra* and *Chlorella*. Notice how the translucent *Hydra* buds. Notice how both

types of *Hydra* respond to mechanical stimulation. Try making a hanging-drop mount of the *Hydra* so that you can look at it with your microscope.

Look at *Caulerpa*. You are looking at a single cell that has differentiated into leaflike, stemlike, and rootlike structures, showing that, at least in this case, the differentiation of the organism into cells is not necessary for complex development.

Using Cytoskeletal Inhibitors to Probe Cell Motility

Use inhibitors of the cytoskeleton to test whether microtubules or microfilaments are involved in swimming, cytoplasmic streaming, and/or vacuole contraction in *Paramecium bursaria*. The contribution of actin microfilaments can be tested by treating the cells with cytochalasin D (10 μM) or latrunculin B (30 nM). The contribution of microtubules can be tested by treating the cells with colchicine (5 μM or 5 mM) or oryzalin (10 μM).

Observing Actin Filaments in Onion Epidermal Cells

In order to observe actin microfilaments in onion epidermal cells, prepare 2 mL of the staining solution by mixing 1.8 mL of Part A with 0.2 mL of Part B. To make 10 mL of Part A, add 5.5 mL of a 100 mM stock solution of piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 7.0), 0.055 mL of a 10 percent stock solution of Triton X-100 (to permeabilize the cells), 0.55 mL of a 100 mM stock solution of $MgCl_2$, 0.275 mL of a stock solution of ethylene glycol tetraacetic acid (EGTA, pH 7), 0.165 mL of a 100 mM stock solution of dithiothreitol (DTT), 0.165 mL of a 100 mM stock solution of phenylmethylsulfonyl fluoride (PMSF), 0.275 mL of 200 mM Na^+ phosphate buffer (pH 7.3), and 0.44 g NaCl. To make 10 mL of 200 mM Na^+ phosphate buffer (pH 7.3), mix together 2.3 mL of 200 mM monobasic sodium phosphate and 7.7 mL of 200 mM dibasic sodium phosphate. Part B consists of a 3.3 mM stock solution of rhodamine-labeled phalloidin dissolved in methanol.

Place several peels of the onion epidermis in the staining solution for 10 minutes in a warm place (35°C). Mount the epidermal peels in phosphate-buffered saline (PBS) that contains 0.05 percent (w/v) n-propylgallate. To make PBS, add 0.85 g NaCl, 0.039 g $NaH_2PO_4 \times H_2O$, 0.0193 g $Na_2HPO_4 \times 7H_2O$, and enough distilled water to bring the solution up to 100 mL. Observe the actin microfilaments using the green excitation cube. Document your observations with photographs.

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References

Ever since the publication of Euclid's *Elements* and Ptolemy's *Almagest*, textbooks have had the unfortunate effect of stifling the search for the original papers cited within. In order to prevent this, I have provided a somewhat comprehensive bibliography to facilitate the readers research into the original literature. I am particularly indebted to the staff of Mann Library, Kroch Library and the University Annex, for finding, storing, and making it possible for me to read these references.

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