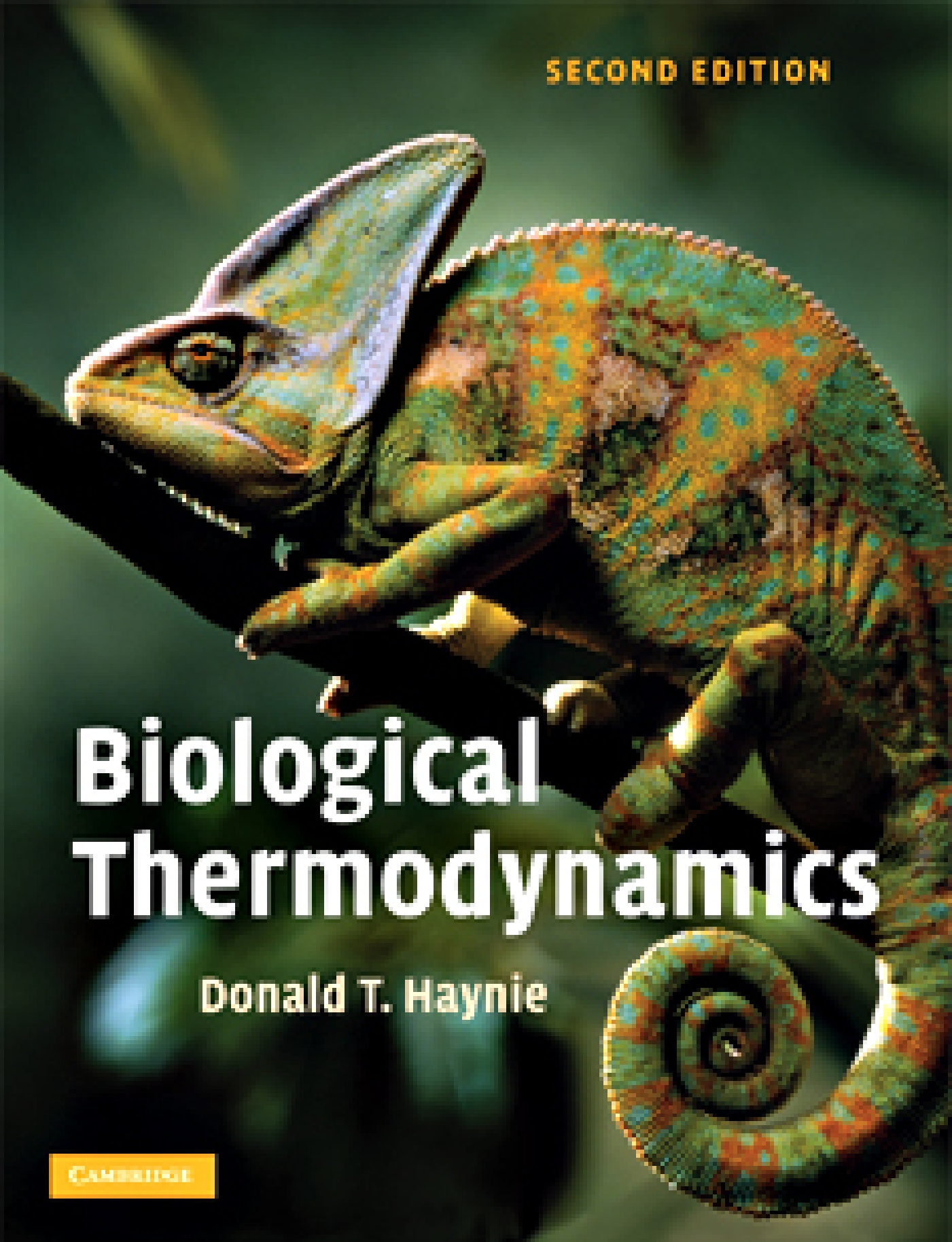
SECOND EDITION



Biological Thermodynamics

Donald T. Haynie

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Biological Thermodynamics

This inter-disciplinary guide to the thermodynamics of living organisms has been thoroughly revised and updated. Providing a uniquely integrated and notably current overview of the subject, the second edition retains the refreshingly readable style of the first edition and serves as an eminently useful introduction to the study of energy transformation in the life sciences. *Biological Thermodynamics* is a particularly accessible means for biology, biochemistry, and bioengineering undergraduate students to acquaint themselves with the physical dimension of their subject. Graduate students, too, will find the book useful. The emphasis throughout the text is on internalizing basic concepts and sharpening problem-solving skills. The mathematical difficulty increases gradually by chapter, but no calculus is required. Topics covered include energy and its transformation, the First and Second Laws of thermodynamics, the Gibbs free energy, statistical thermodynamics, binding equilibria, and reaction kinetics. Each chapter comprises numerous illustrative examples taken from different areas of biochemistry, as well as a broad range of exercises and references for further study.

Reviews of the first edition:

> In my opinion, the author has covered a traditionally "boring field" with vivid description and interesting examples. My overall impression is that this book is comprehensive, illustrative and up-to-date ... and I would certainly recommend it to my students.
>
> Professor Yigong Shi, Department of Molecular Biology, Princeton University, USA

> ... an outstanding supplement to the treatment offered in most textbooks of biochemistry ... very rewarding for students majoring in biochemistry, biophysics, or biotechnology
>
> Professor Frank Vella, Department of Biochemistry, University of Saskatchewan, Canada

> ... a very readable and informed introduction to energy transformation at several levels of biological organization: molecules, cells, and multicellular organisms ... a good introduction to the new field of biological thermodynamics and represents an important contribution to the literature.
>
> Dr. Lloyd Demetrius, Department of Organismic and Evolutionary Biology, Harvard University, USA, and Max Planck Institute for Molecular Genetics, Berlin, Germany

Don Haynie is the Co-Founder and Chief Scientist of Artificial Cell Technologies, Inc., Director of the Bionanosystems Engineering Laboratory and Research Professor of Biochemistry and Biophysics at Central Michigan University, and a Clinical Professor at the University of Connecticut School of Medicine. Former members of his research group are at Harvard Medical School, King's College London, Schering-Plough, and Pacific Nanotechnology. He has held academic appointments at The Johns Hopkins University, the University of Oxford, the University of Manchester Institute of Science and Technology, and Louisiana Tech University, in departments of biophysics, biomolecular sciences, biomedical engineering, chemistry, and physics. He has taught thermodynamics to biology, biochemistry, and engineering students world-wide.

Weblink to Don Haynie's site:
http: //www.biologicalthermodynamics.com.

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Second edition

Donald T. Haynie

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In memory of
BUD HERSCHEL

The trouble with simple things is that one must understand them very well

ANONYMOUS

Contents

Preface to the second edition

Interest in the biological sciences has never been greater. Today, biology, biochemistry, biophysics, and bioengineering are engaging the minds of young people in the way that physics and chemistry did thirty, forty, and fifty years ago. There has been a massive shift in public opinion and in the allocation of resources for university-based research. Breakthroughs in genetics, cell biology, and medicine are transforming the way we live, from improving the quality of produce to eradicating disease; they are also stimulating pointed thinking about the origin and meaning of life. Growing awareness of the geometry of life, on length scales extending from an individual organism to a structural element of an individual macromolecule, has led to a reassessment of the principles of design in all the engineering disciplines, including computation. And a few decades after the first determination at atomic resolution of the structures of double-stranded DNA and proteins, it is becoming increasingly apparent that both thermodynamic and structural information are needed to gain a deep sense of the functional properties of biological macromolecules. Proteins, nature's own nanoscale machines, are providing inspiration for innovative and controlled manipulation of matter at the atomic scale.

This book is about the thermodynamics of living organisms. It was written primarily for undergraduate university students; mostly students of the biological sciences, but really for students of any area in science, engineering, or medicine. The book could serve as an introductory text for undergraduate students of chemistry or physics who are interested in biology, or for graduate students of biology or biochemistry who did their first degree in a different subject. The style and depth of presentation reflect my experience of learning thermodynamics as an undergraduate student, doing graduate-level research on protein thermodynamics at the Biocalorimetry Center at Johns Hopkins University, teaching thermodynamics to biochemistry undergraduates in the Department of Biomolecular Sciences at the University of Manchester Institute of Science and Technology and to pre-meds at Johns Hopkins, discussing thermodynamic properties of proteins with colleagues in

the Oxford Centre for Molecular Sciences, and developing biomedical applications of nanofilms and nanowires in the Institute for Micromanufacturing and Center for Applied Physics Studies at Louisiana Tech University.

My sense is that an integrated approach to teaching this subject, where the principles of physical chemistry are presented not as a stand-alone course but as an aspect of biology, has both strengths and weaknesses. On the one hand, most biological science students prefer to encounter physical chemistry in the context of learning about living organisms, not in lectures designed for physical chemists. On the other hand, applications-only courses tend to obscure fundamental concepts. The treatment of thermodynamics one finds in general biochemistry textbooks compounds the difficulties, as the subject is usually treated separately, in a single chapter, with applications being touched on only here and there in the remainder of the text. Moreover, most general biochemistry texts are written by scientists who have little or no special training in thermodynamics, making a coherent and integrated presentation of the subject that much more difficult. A result is that many students of the biological sciences complete their undergraduate study with a shallow or fragmented knowledge of thermodynamics, arguably the most basic area of all the sciences and engineering. Indeed, many scientists would say that the Second Law of Thermodynamics is the most general idea in science and that energy is its most important concept.

It is hardly difficult to find compelling statements in support of this view. According to Albert Einstein, for example, "Classical thermodynamics . . . is the only physical theory of universal content concerning which I am convinced that, within the framework of applicability of its basic concepts, will never be overthrown." Einstein, a German–American physicist, lived 1879–1955. He was awarded the Nobel Prize in Physics in 1921 and described as "Man of the Century" by *Time* magazine in late 1999. Sir Arthur S. Eddington (1882–1944), the eminent British astronomer and physicist, has said, "If your theory is found to be against the Second Law of Thermodynamics I can give you no hope; there is nothing for it but to collapse in deepest humiliation." C. P. Snow, another British physicist, likened lack of knowledge of the Second Law to ignorance of Shakespeare, to underscore the importance of thermodynamics to basic awareness of the character of the physical world. And M. V. Volkenstein, member of the Institute of Molecular Biology and the Academy of Sciences of the USSR, has written, "A physical consideration of any kind of system, including a living one, starts with its phenomenological, thermodynamic description. Further study adds a molecular content to such a description."

The composition and style of this book reflect my own approach to teaching thermodynamics. Much of the presentation is informal and qualitative. This is because knowing high-powered mathematics is often quite different from knowing what one would like to use

mathematics to describe. At the same time, however, a firm grasp of thermodynamics and how it can be used can really only be acquired through numerical problem solving. The text therefore does not avoid expressing ideas in the form of equations where it seems fitting. Each chapter is imbued with *l'esprit de géométrie* as well as *l'esprit de finesse*. In general, the mathematical difficulty of the material increases on the journey from alpha to omega. Worked examples are provided to illustrate how to use and appreciate the mathematics, and a long list of references and suggestions for further reading are given at the end of each chapter. In addition, each chapter is accompanied by a broad set of study questions. These fall into several categories: brief calculation, extended calculation, multiple choice, analysis of experimental data, short answer, and "essay." A few of the end-of-chapter questions are open-ended; it would be difficult to say that a "correct" answer could be given. This will, I hope, be seen as more of a strength of the text than a weakness. For the nature of the biological sciences is such that some very "important" aspects of research are only poorly defined or understood. Moreover, every path to a discovery of lasting significance has its fair share of woolly thinking to cut through.

Several themes run throughout the book, helping to link the various chapters into a unified whole. Among these are the central role of ATP in life processes, proteins, the relationship between energy and biological information, and the human dimension of science. The thermodynamics of protein folding/unfolding is used to illustrate a number of key points. Why emphasize proteins? About 50% of the dry mass of the human body is protein, no cell could function without protein, a logical next step to knowing the amino acid sequence encoded by a gene is predicting the three-dimensional structure of the corresponding functional protein, and a large portion of my research activity has involved peptides or proteins. I also try to give readers a sense of how thermodynamics has developed over the past several hundred years from contributions from researchers of many different countries and backgrounds.

My hope is that this text will help students of the biological sciences gain a clearer understanding of the basic principles of energy transformation as they apply to living organisms. Like a physiologically meaningful assembly of biological macromolecules, the organization of the book is hierarchical. For students with little or no preparation in thermodynamics, the first four chapters are essential and may in some cases suffice for undergraduate course content. Chapter 1 is introductory. Certain topics of considerable complexity are dealt with only in broad outline here; further details are provided at appropriate points in later chapters. The approach is intended to highlight both the independence of thermodynamics from biological systems and processes and applicability of thermodynamics to biology; not simply show the consistency of certain biological processes with the laws of thermodynamics. The second and third chapters discuss the First and

Second Laws of thermodynamics, respectively. This context provides a natural introduction to two thermodynamic state functions, enthalpy and entropy. Chapter 4 discusses how these functions are combined in the Gibbs free energy, a sort of hybrid of the First and Second Laws and the main thermodynamic potential function of interest in biology. Chapter 4 also elaborates several basic areas of physical chemistry relevant to biology. In Chapter 5, the concepts developed in Chapter 4 are applied to a wide range of topics in biology and biochemistry, the aim being to give students a good understanding of the physics behind the biochemical techniques they might use in an undergraduate laboratory. Chapters 4 and 5 are designed to allow maximum flexibility in course design, student ability, and instructor preferences. Chapters 6 and 7 concern molecular interpretations of thermodynamic quantities. Specifically, Chapter 6 introduces and discusses the statistical nature of thermodynamic quantities. In Chapter 7 these ideas are extended in a broad treatment of macromolecular binding, a common and extremely important class of biochemical phenomena. Chapter 8, on reaction kinetics, is included for two main reasons: the equilibrium state can be defined as the one in which the forward and reverse rates of reaction are equal, and the rate of reaction, be it of the folding of a protein or the catalysis of a biochemical reaction, is determined by the free energy of the transition state. In this way inclusion of a chapter on reaction kinetics gives a more complete understanding of biological thermodynamics. Finally, Chapter 9 touches on a number of topics at the forefront of biochemical research where thermodynamic concepts are of striking and relatively general interest.

A note on units. Both joules and calories are used throughout this book. Unlike monetary exchange rates and shares on the stock exchange, the values of which fluctuate constantly, the conversion factor between joules and calories is constant. Moreover, though joules are now more common than calories, one still finds both types of unit in the contemporary literature, and calories predominate in older but still useful and sometimes very interesting publications. Furthermore, the instrument one uses to make direct heat measurements is a called a calorimeter not a joulimeter! In view of this it seems fitting that today's student should be familiar with both types of unit.

Three books played a significant role in the preparation of the text: *Introduction to Biomolecular Energetics* by I. M. Klotz, *Foundations of Bioenergetics* by H. J. Morowitz, and *Energy and Life* by J. Wrigglesworth. My own interest in biophysics was sparked by the work of Ephraim Katchalsky (not least by his reflections on art and science!) and Max Delbrück,[1] which was brought to my attention by my good

[1] Delbrück played a key role in the development of molecular biology and biophysics. Raised in Berlin near the home of Max Planck, Nobel Laureate in Physics, Delbrück was, like Planck, son of a professor at Berlin University, and one of his great-

friend Bud Herschel. I can only hope that my predecessors will deem my approach to the subject a helpful contribution to thermodynamics education in the biological sciences.

The support of several other friends and colleagues proved invaluable to the project. Joe Marsh provided access to historical materials, lent me volumes from his personal library, and encouraged the work from an early stage. Paul C. W. Davies offered me useful tips on science writing. Helpful information was provided by a number of persons of goodwill: Rufus Lumry, Richard Cone, Alan Eddy, Klaus Bock, Mohan Chellani, Bob Ford, Andy Slade, and Ian Sherman. Van Bloch was a steady and invaluable source of encouragement and good suggestions on writing, presenting, and publishing this work. I thank Chris Dobson. Alan Cooper, Bertrand Garcia-Moreno Esteva, and Terry Brown, and several anonymous reviewers read parts of the text and provided valuable comments. I wish to thank my editors, Katrina Halliday and Ward Cooper, for the energy and enthusiasm they brought to this project, and Beverley Lawrence for expert copy-editing. I am pleased to acknowledge Tariq, Khalida, and Sarah Khan for hospitality and kindness during the late stages of manuscript preparation. I am especially grateful to Kathryn, Kathleen, and Bob Doran for constant encouragement and good-heartedness.

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grandfathers was Liebig, the renowned biochemist. Delbrück studied astronomy and physics. Having obtained relatively little background in experimental physics, he failed his Ph.D. oral exam in the first attempt. Nevertheless, he went on to study with Niels Bohr in Copenhagen and Wolfgang Pauli in Zürich, each of whom was recognized for contributions to quantum theory by a Nobel Prize in Physics. In 1937 Delbrück left Germany for the USA; his sister Emmi and brother-in-law Klaus Bonhoeffer (brother of the theologian Dietrich) stayed behind, working in the German Resistance against the Nazi regime. Delbrück became a research fellow at Caltech and devoted himself to the study of bacterial viruses, which he regarded as sufficiently simple in hereditary mechanism for description and understanding in terms of physics. There are reasons to believe that Delbrück was a significant source of inspiration for some of Richard Feynman's remarks in his 1959 talk, "Plenty of Room at the Bottom," which has come to play a seminal role in the development of nanotechnology (see Haynie *et al.*, 2006, *Nanomedicine: Nanotechnology, Biology, and Medicine*, **2**, 150–7 and references cited therein). Delbrück was awarded the Nobel Prize in Medicine or Physiology in 1969 for his work on bacteriophages.

Raven (Missouri Botanical Garden), Gamal Rayan (University of Toronto), Alison Roger (Warwick University), Stan Sandler (University of Delaware), Yigong Shi (Princeton University), Ernest W. Tollner (Georgia State University), and Jin Zhao (Penn State University). Above all these I thank my wife, for love and understanding.

D. T. H.
15th September, 2007
New Haven, Connecticut

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Chapter 1

Energy transformation

A. Introduction

Beginning perhaps with Anaximenes of Miletus (fl. *c.* 2550 years before present), various ancient Greeks portrayed man as a microcosm of the universe. Each human being was made up of the same elements as the entire cosmos – earth, air, fire, and water. Twenty-six centuries later, and several hundred years after the dawn of modern science, it is somewhat humbling to realize that our view of ourselves is fundamentally unchanged.

Our knowledge of the matter of which we are made, however, has become much more sophisticated. We now know that all living organisms are composed of hydrogen, the lightest element, and of heavier elements like carbon, nitrogen, oxygen, and phosphorus. Hydrogen was the first element to be formed after the Big Bang. Once the universe had cooled enough, hydrogen condensed to form stars. Then, still billions of years ago, the heavier atoms were synthesized by nuclear fusion reactions in the interiors of stars.[1] We are made of "stardust."

Our starry origin does not end there. For the Sun is the primary source of the energy used by organisms to satisfy the requirements of life (Fig. 1.1).[2] Some organisms acquire this energy (Greek, *en*, in + *ergon*, work) directly; most others, including humans, obtain it indirectly. Even chemosynthetic bacteria that flourish a mile and a half beneath the surface of the sea require the energy of the Sun for life. They depend on plants and photosynthesis to produce oxygen needed for respiration, and they need the water of the sea to be in

[1] The 1967 Nobel prize in physics went to Hans Bethe for work in the 1930s on the energy-production mechanisms of stars. Bethe is said to have solved problems not by "revolutionary developments" but by "performing the simplest calculation that he thought might match the data. This was the Bethe way, or as he put it: 'Learn advanced mathematics in case you need it, but use only the minimum necessary for any particular problem'."

[2] Recent discoveries have revealed exceptions to this generalization. See Chapter 9.

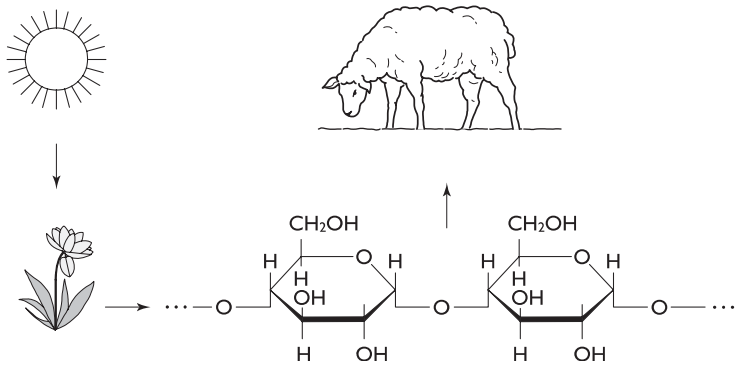

Fig. 1.1 A diagram of how mammals capture energy. The Sun generates radiant energy from nuclear fusion reactions. Only a tiny fraction of this energy actually reaches us, as we inhabit a relatively small planet and are far from the Sun. The energy that does reach us – *c.* 5×10^{18} MJ yr^{-1} (1.7×10^{17} J s^{-1}) – is captured by plants and photosynthetic bacteria, as well as the ocean. (J = joule. This unit of energy is named after British physicist James Prescott Joule, 1818–1889). The approximate intensity of direct sunlight at sea level is 5.4 J cm^{-2} min^{-1}. Energy input to the ocean plays an important role in determining its predominant phase (liquid and gas, not solid), while the energy captured by the photosynthetic organisms (only about 0.025% of the total; see Fig. 1.2) is used to convert carbon dioxide and water to glucose and oxygen. It is likely that all the oxygen in our atmosphere was generated by photosynthetic organisms. Glucose monomers are joined together in plants in a variety of polymers, including starch (shown), the plant analog of glycogen, and cellulose (not shown), the most abundant organic compound on Earth. Animals, including grass-eaters like sheep, do not metabolize cellulose, but they are able to utilize other plant-produced molecules. Abstention from meat (muscle) has increased in popularity over the past few decades, but in most cultures humans consume a wide variety of animal species. Muscle tissue is the primary site of conversion from chemical energy to mechanical energy in the animal world. There is a continuous flow of energy and matter between microorganisms (not shown), plants (shown), and animals (shown) and their environment. The sum total of the organisms and the physical environment participating in these energy transformations is known as an *ecosystem*.

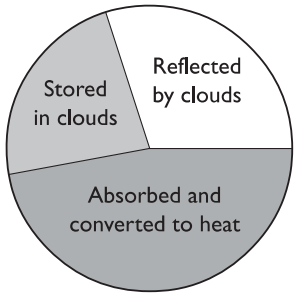

Fig. 1.2 Pie plot showing the destiny of the Sun's energy that reaches Earth. About one-fourth is reflected by clouds, another one-fourth is absorbed by clouds, and about half is absorbed and converted into heat. Only a very small amount ($\ll$1%) is fixed by photosynthesis.

the liquid state in order for the plant-made oxygen to reach them by convection and diffusion.[3] Irrespective of form, complexity, time or place, all known organisms are alike in that they must capture, transduce, store, and use energy in order to live. This is a key statement, not least because the concept of energy is considered the most basic one of all of science and engineering.

How does human life in particular depend on the energy output of the Sun? Green plants flourish only where they have access to

[3] The recent discovery of blue-green algae beneath ice of frozen lakes in Antarctica, for example, has revealed that bacteria can thrive in such an extreme environment. Blue-green algae, also known as cyanobacteria, are the most ancient photosynthetic, oxygen-producing organisms known. For polar bacteria to thrive they must be close to the surface of the ice and near dark, heat absorbing particles. Solar heating during summer months liquifies the ice in the immediate vicinity of the particles, so that liquid water, necessary to life as we know it, is present. During the winter months, when all the water is frozen, the bacteria are "dormant." See Chapter 3 on the Third Law of Thermodynamics.

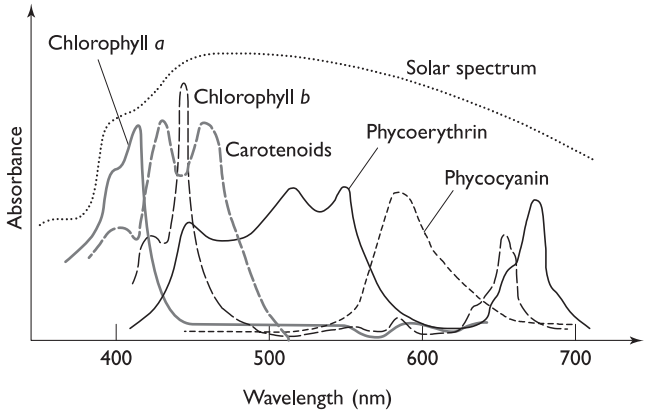

Fig. 1.3 Absorption spectra of various photosynthetic pigments. The chlorophylls absorb most strongly in the red and blue regions of the spectrum. Chlorophyll a is found in all photosynthetic organisms; chlorophyll b is produced in vascular plants. Plants and photosynthetic bacteria contain carotenoids, which absorb light at different wavelengths from the chlorophylls.

light. Considering how green our planet is, it is interesting that much less than 1% of the Sun's energy that manages to penetrate the protective ozone layer, water vapor, and carbon dioxide of the atmosphere, actually gets absorbed by plants (Fig. 1.2). Chlorophyll and other pigments in plants act as molecular antennas, enabling plants to absorb the light particles known as photons over a relatively limited range of energies (Fig. 1.3). On a more detailed level, a pigment molecule, made of atomic nuclei and electrons, has a certain electronic *bound* state that can interact with a photon (a *free* particle) in the visible range of the electromagnetic spectrum (Fig. 1.4). When a photon is absorbed, the bound electron makes a transition to a higher energy but less stable "excited" state. Energy captured in this way is transformed by a very complex chain of events.[4] What is important here is that the relationship between wavelength of light, λ, photon frequency, ν, and photon energy, E, is

$$E = hc/\lambda = h\nu, \tag{1.1}$$

where h is Planck's constant[5] (6.63×10^{-34} J s) and c is the speed of light *in vacuo* (2.998×10^{8} m s^{-1}). Both h and c are fundamental constants of nature. Plants combine trapped energy from sunlight with carbon dioxide and water to give $C_6H_{12}O_6$ (glucose), oxygen, and heat. In this way solar energy is turned into chemical energy and stored in the form of chemical bonds, for instance the chemical bonds of a glucose molecule and the $\beta(1 \rightarrow 4)$ glycosidic bonds between glucose monomers in the long stringy molecules called

[4] There is a sense in which living matter engages electromagnetic theory, says Hungarian Nobel laureate Albert von Nagyrapolt Szent-Györgyi, how it "lifts one electron from an electron pair to a higher level. This excited state has to be of a short lifetime, and the electron drops back within 10^{-7} or 10^{-8} s to ground state giving off its energy in one way or another. Life has learned to catch the electron in the excited state, uncouple it from its partner and let it drop back to ground-state through its biological machinery utilizing its excess energy for life's processes." See Chapter 5 for additional details.

[5] Named after the German physicist Max Karl Ernst Ludwig Planck (1858–1947). Planck was awarded the Nobel Prize in Physics in 1918.

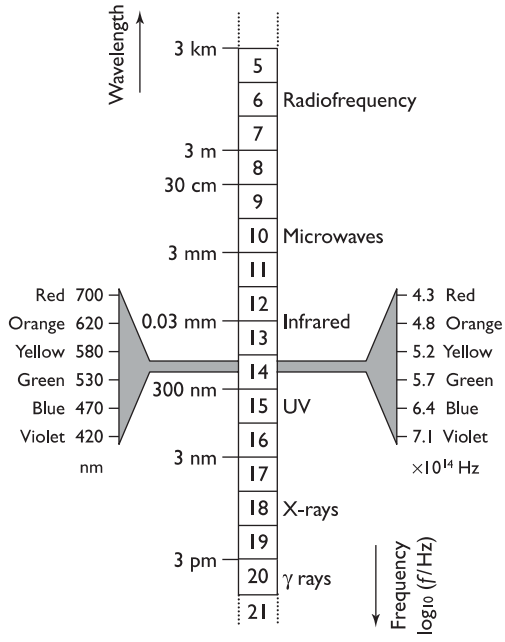

Fig. 1.4 The electromagnetic spectrum. The visible region, the range of the spectrum to which the unaided human eye is sensitive, is expanded. As photon wavelength increases (or frequency decreases), energy decreases. The precise relationship between photon energy and wavelength is given by Eqn. (1.1). Photon frequency is shown on a $\log_{10}$ scale. Redrawn from Fig. 2.15 in Lawrence *et al.* (1996).

cellulose (see Fig. 1.1). Cellulose is the most abundant organic compound on Earth and the repository of over half of all the carbon of the biosphere.

Herbivorous animals like pandas and omnivorous animals like bears feed on plants, using the energy of digested and metabolized plant material to manufacture the biological macromolecules they need to maintain existing cells of the body or to make new ones.[6] Mature red blood cells, which derive from stem cells in the bone marrow in accord with the genetic program stored in DNA and in response to a hormone secreted by the kidneys, are stuffed full of hemoglobin. This protein plays a key role in an animal's utilization of plant energy, transporting from lungs (or gills) to cells throughout the body the molecular oxygen needed to burn plant "fuel." The energy of the organic molecules is released in animals in a series of reactions in which glucose, fats, and other organic compounds are oxidized (burned) to carbon dioxide and water, the starting materials, and heat.[7] Animals also use the energy of digested food for locomotion, maintaining body heat, generating light (e.g. fireflies), fighting off infection by microbial organisms, and reproduction (Fig. 1.5). These biological processes involve a huge number of

[6] The giant panda is classified as a bear (family Ursidae) but it feeds almost exclusively on bamboo. Its digestive system is that of a carnivore, however, making it unable to digest cellulose, the main constituent of bamboo. To obtain the needed nourishment, the adult panda eats 15–30 kg of bamboo in a day over 10–12 h.

[7] This chain of events is generally "thermodynamically favorable" because we live in a highly oxidizing environment: 23% of our atmosphere is oxygen. More on this in Chapter 5.

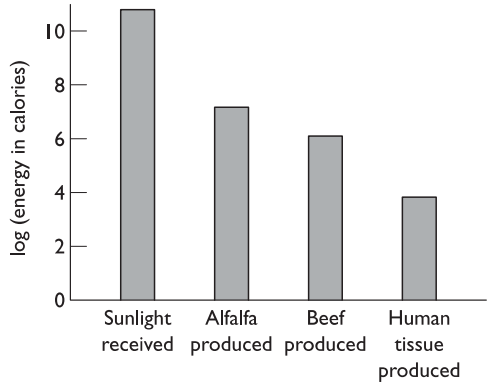

Fig. 1.5 Log plot of energy transformation on Earth. Only a small amount of the Sun's light that reaches Earth is used to make cereal. Only a fraction of this energy is transformed into livestock tissue. And only part of this energy is transformed into human tissue. What happens to the rest of the energy? See Chapters 2 and 3. A calorie is a unit of energy that one often encounters in older textbooks and scientific articles (where 1 cal = 1 calorie) and in food science (where 1 cal = 1000 calories). A calorie is the heat required to increase the temperature of 1 g of pure water from 14.5 °C to 15.5 °C. 1 calorie = 1 cal = 4.184 J *exactly*. Based on Fig. 1–2 of Peusner (1974).

exquisitely specific biochemical reactions, each of which requires energy to proceed.

The energy transformations sketched above touch on at least two of the several requirements for life as we know it: *mechanisms* to control *energy* flow, for example, the membrane-associated protein "nanomachines" involved in photosynthesis; and *mechanisms* for the storage and transmission of biological *information*, namely, polynucleic acids. The essential role of *mechanisms* in life processes implies that *order* is a basic characteristic of living organisms. Maintaining order in the sort of "system" a living creature is requires significant and recurring energy input. A remarkable and puzzling aspect of life is that the structures of the protein enzymes which regulate the flow of energy and information in and between cells are encoded by nucleic acids, the information storage molecules. The interplay of energy and information is a recurring theme in biological thermodynamics, indeed, in all science, engineering, and technology. The preceding discussion also suggests that energy flow in nature bears some resemblance to the movement of currency in an economy: energy "changes hands" (moves from the Sun to plants to animals . . .) and is "converted into different kinds of currency" (stored as chemical energy, electrical energy, etc.). This is another recurring theme of our subject.

A deeper sense of the nature of energy flow can be gained from a bird's-eye view of the biological roles of adenosine triphosphate (ATP), the small organic compound that is known as "the energy currency of the cell." This molecule is synthesized from solar energy in outdoor plants and chemical energy in animals. The detailed mechanisms involved in the energy conversion processes are

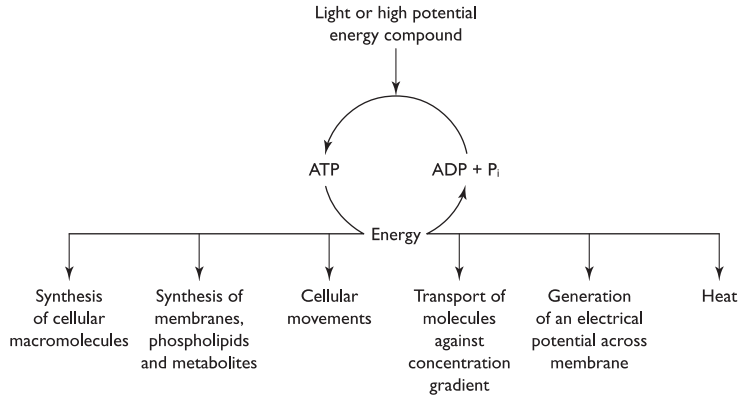

Fig. 1.6 ATP "fuels" an amazing variety of interconnected cellular processes. In the so-called ATP cycle, ATP is formed from adenosine diphosphate (ADP) and inorganic phosphate (P_i) by photosynthesis in plants and by metabolism of "energy rich" compounds in most cells. Hydrolysis of ATP to ADP and P_i releases energy that is trapped as usable energy. This form of energy expenditure is integral to various crucial cellular functions and is a central theme of biochemistry. Redrawn from Fig. 2–23 of Lodish *et al.* (1995).

complex and extremely interesting, but they do not concern us here. The important point is that once it has been synthesized, ATP plays the role of the main energy "currency" of biochemical processes in all known organisms. ATP provides the chemical energy needed to "power" a huge variety of biochemical process, for example, muscle contraction. ATP is involved in the synthesis of deoxyribonucleic acid (DNA), the molecular means of storing and transmitting genetic information between successive generations of bacteria, nematodes, and humans. ATP is also a key player in the chemical communications between and within cells. ATP is of basic and central importance to life as we know it (Fig. 1.6).

Now let's return to money. Just as there is neither an increase nor a decrease in the money supply when money changes hands: so in the course of its being transformed, energy is neither created nor destroyed. The total amount of energy is *always* constant. This is a statement of the First Law of Thermodynamics. The money analogy has its limitations. Some forms of finance are more liquid than others, and cash is a more liquid asset than a piece of real estate, but even though the total energy in the universe is a constant, the energy transformations of life we have been discussing certainly can and do indeed affect the relative proportion of energy that is available in a form that a living organism will find *useful*. This situation arises not from defects inherent in the biomolecules involved in energy transformation, but from the nature of our universe itself.

Let's check ourselves before going further. We have been going on about energy as though we knew what it was; we all have at least a vague sense of what energy transformation involves. For instance, we know that it takes energy to heat a house in winter (natural gas, oil, combustion of wood, solar energy), we know that energy is required to cool a refrigerator (electricity), we know that energy is used to start an automobile engine (electrochemical) and

keep it running (gasoline). But we still have not given a precise definition of *energy*. We have not said *what* energy is. A purpose of this book is to discuss what energy is with regard to living organisms.

B. Distribution of energy

Above we said that throughout its transformations energy was conserved. The proposition that *something* can change and stay the same may seem strange, indeed highly counterintuitive, but we should be careful not to think that such a proposition must be untrue. We should be open to the possibility that some aspects of physical reality might differ from our intuitive, macroscopic, day-to-day experience of the world. There, the something that stays the same is a quantity called the total energy, and the something that changes is how all the energy is *distributed* – where it is found and in what form. A colorful analogy is provided by a wad of chewing gum. The way in which the gum molecules are distributed in space depends, first of all, on whether the stick is in your mouth or still in the wrapper! Once you've begun to work your tongue and jaw, the gum changes shape a bit at a time, or quite dramatically when you blow a bubble. But the *total amount* of gum is *constant*. The analogy does not imply that energy is a material particle, but it does suggest that to the extent that energy resembles matter, knowing something of the one might provide clues about the other.

The money–energy analogy helps to illustrate additional points regarding energy distribution. Consider the way a distrustful owner of a busy store might check on the honesty of a certain cashier at the end of the day. The owner knows that m_b dollars were in the till at the beginning of the day, and, from the cash register tape, that m_e dollars should be in the till at the end of trading. So, obviously, the intake is $m_e - m_b = \Delta m$, where "Δ," the upper case Greek letter *delta*, means "difference." But knowing Δm says nothing at all about how the money is distributed. How much is in cash? Checks? Traveller's checks? Credit card payments? Let's keep things simple and assume that all transactions are in cash and in dollars. Some might be in rolls of coins, some loose in the till, and some in the form of banknotes of different denomination. When all the accounting is done, the different coins and banknotes should add up to Δm, if the clerk is careful and honest. A simple formula can be used to do the accounting:

$$\begin{aligned}\Delta m = {} & \$0.01 \times (\text{number of pennies}) + \$0.05 \times (\text{number of nickels}) \\ & + \cdots + \$10.00 \times (\text{number of ten dollar bills}) \\ & + \$20.00 \times (\text{number of twenty dollar bills}) + \cdots\end{aligned} \tag{1.2}$$

The formula can be modified to include terms corresponding to coins in rolls:

$$\begin{aligned}\Delta m = {} & \$0.01 \times (\text{number of pennies}) + \$0.50 \times (\text{number of rolls of} \\ & \text{pennies}) + \$0.05 \times (\text{number of nickels}) + \$2.00 \times (\text{number of} \\ & \text{rolls of nickels}) + \cdots + \$10.00 \times (\text{number of ten dollar bills}) \\ & + \$20.00 \times (\text{number of twenty dollar bills}) + \cdots\end{aligned} \tag{1.3}$$

A time-saving approach to counting coins would be to weigh them. The formula might then look like this:

$$\begin{aligned}\Delta m = {} & \$0.01 \times (\text{weight of unrolled pennies})/(\text{weight of one penny}) \\ & + \$0.50 \times (\text{number of rolls of pennies}) + \$0.05 \\ & \times (\text{weight of unrolled nickels})/(\text{weight of one nickel}) \\ & + \$2.00 \times (\text{number of rolls of nickels}) + \cdots + 10.00 \\ & \times (\text{number of ten dollar bills}) + 20.00 \times (\text{number of} \\ & \text{twenty dollar bills}) + \cdots\end{aligned} \tag{1.4}$$

The money analogy is useful for making several points. One, the set of numbers of each type of coin and banknote is but one possible distribution of Δm dollars. A different distribution would be found if a wisecrack paid for a \$21.95 item with a box full of nickels! (Fig. 1.7.) One might even consider it possible to *measure* the distribution of the Δm dollars by considering the relative proportion of pennies, nickles, dimes, and so on. Two, given a particular distribution of Δm dollars, there are still many different *ways of arranging* the coins and banknotes. For example, there are many possible orderings of the fifty pennies in a roll (the number is $50 \times 49 \times 48 \ldots 3 \times 2 \times 1$). The complexity of the situation increases when we count coins of the same type but different date as "distinguishable" and ones of the same type and same date as "indistinguishable." Three, the more we remove ourselves from scrutinizing and *counting* individual coins, the more *abstract* and theoretical our formula becomes. As the ancient Greek philosopher Aristotle[8] recognized quite a long time ago, the basic nature of scientific study is to proceed from observations to theories; theories are then used to explain observations and make predictions about what has not yet been observed. A theory will be more or less abstract, depending on how much it has been developed and how well it works. And four, although measurement of an abstract quantity like Δm might not be very hard (the manager could just

[8] Aristotle (384–322 BC) was born in northern Greece. He was Plato's most famous student at the Academy in Athens. Aristotle established the Peripatetic School in the Lyceum at Athens, where he lectured on logic, epistemology, physics, biology, ethics, politics, and aesthetics. According to Aristotle, minerals, plants, and animals are distinct categories of being. He was the first philosopher of science.

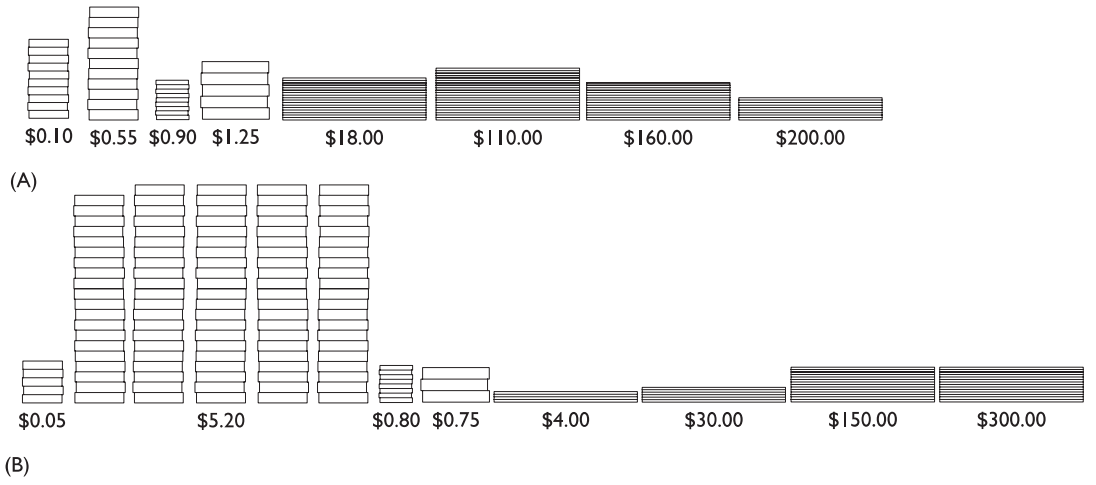

Fig. 1.7 Two different distributions of money. The columns from left to right are: pennies ($0.01), nickels ($0.05), dimes ($0.10), quarters ($0.25), one dollar bills ($1.00), five dollar bills ($5.00), ten dollar bills ($10.00) and twenty dollar bills ($20.00). Panel (A) differs from Panel (B) in that the latter has a larger number of nickels. Both distributions represent the same total amount of money. Small wonder that the world's most valuable commodity, oil, is also the key fuel for communication in the form of domestic and international travel. When the first edition of this book was published, in 2001, the average retail price of gasoline in the USA was about $1.20 per US gallon. At the time of writing the present edition, in 2007, it is about $3.00. The price is much higher in European countries, where individual consumers pay a big tax on fuel.

rely on the tape if the clerk were known to be perfectly honest and careful), determination of the contribution of each relevant component to the total energy could be a difficult and time-consuming business – if not impossible, given current *technology* and *definitions* of thermodynamic quantities.

As we have seen, a quantity of energy can be distributed in a large variety of ways. But no matter what forms it is in, the total amount of energy is constant. Some of the different forms it might take are chemical energy, elastic energy, electrical energy, gravitational energy, heat energy, mass energy, nuclear energy, radiant energy, and the energy of intermolecular interactions. Although all these forms of energy are of interest to the biological scientist, some are clearly more important to us than others; some are relevant only in specialized situations. In living organisms the main repositories of energy are macromolecules, which store energy in the form of covalent and non-covalent chemical bonds, and unequal concentrations of solutes, principally ions, on opposite sides of a cell membrane. Figure 1.3 shows another type of energy distribution. For a given amount of solar energy that actually reaches the surface of our planet, more photons have a wavelength of 500 nm than 250 or 750 nm. The solar spectrum is a type of energy distribution. According to the kinetic theory of gases, which turns up at several places in this book, the speeds of gas molecules are distributed in a certain way, with some speeds being much more probable than

Table 1.1. *Energy distribution in cells. Contributions to the total energy can be categorized in two ways: kinetic energy and potential energy. There are several classes in each category*

Kinetic energy	Potential energy
Heat or thermal energy – energy of molecular motion in all organisms. At 25 °C this is about 0.5 kcal mol^{-1}.	*Bond energy* – energy of covalent and non-covalent bonds, for example a σ bond between two carbon atoms or van der Waals interactions. These interactions range in energy from as much as 14 kcal mol^{-1} for ion–ion interactions to as little as 0.01 kcal mol^{-1} for dispersion interactions; they can also be negative, as in the case of ion–dipole interactions and dipole–dipole interactions.
Radiant energy – energy of photons, for example in photosynthesis. The energy of such photons is about 40 kJ mol^{-1}.	*Chemical energy* – energy of a difference in concentration of a substance across a permeable barrier, for instance the lipid bilayer membrane surrounding a cell. The magnitude depends on the difference in concentration across the membrane. The greater the difference, the greater the energy.
Electrical energy – energy of moving charged particles, for instance electrons in reactions involving electron transfer. The magnitude depends on how quickly the charged particle is moving. The higher the speed, the greater the energy.	*Electrical energy* – energy of charge separation, for example the electric field across the two lipid bilayer membranes surrounding a mitochondrion. The electrical work required to transfer monovalent ions from one side of a membrane to the other is about 20 kJ mol^{-1}.

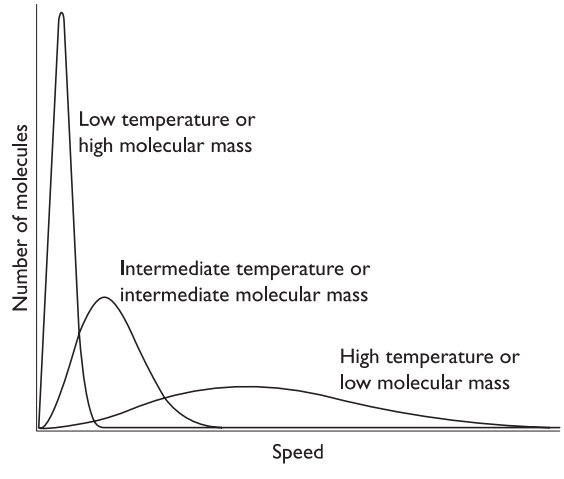

Fig. 1.8 The Maxwell distribution of molecular speeds. The distribution depends on particle mass and temperature. The distribution becomes broader as the speed at which the peak occurs increases. Based on Fig. 0.8 of Atkins (1998).

others (Fig. 1.8). In general, slow speeds and high speeds are rare, near-average speeds are common, and the average speed is related to temperature. A summary of some forms of energy of interest to biological scientists is given in Table 1.1.

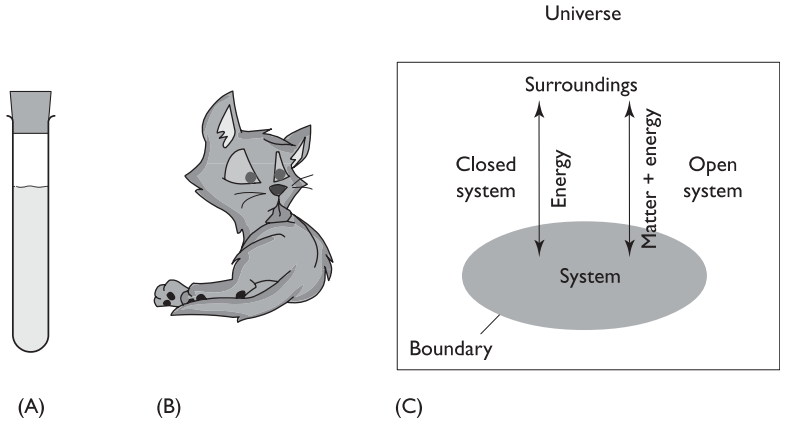

Fig. 1.9 Different types of system. (A) A closed system. The stopper inhibits evaporation of the solvent, so essentially no matter is exchanged with the surroundings (the air surrounding the test tube), but heat energy can be exchanged with the surroundings through the glass. (B) An open system. All living organisms are open systems. A cat is a rather complex open system. A simplified view of a cat is shown in Fig. 1.10. (C) A schematic diagram of a system.

C. System and surroundings

We need to define some important terms. This is perhaps most easily done by way of example. Consider a biochemical reaction that is carried out in aqueous solution in a test tube (Fig. 1.9A). The *system* consists of the solvent, water, and all chemicals dissolved in it, including buffer salts, enzyme molecules, the substrate recognized by the enzyme, and the product of the enzymatic reaction. The system is defined as that part of the universe chosen for study. The *surroundings* are simply the entire universe excluding the system. The system and surroundings are separated from each other by a *boundary*, in this case the test tube.

A system is at any time in a certain thermodynamic *state* or condition of existence (which types of molecule are present and in what amount, the temperature, the pressure, *etc.*). A system is said to be *closed* if it can exchange heat with the surroundings but not matter. That is, the boundary of a closed system is impermeable to matter. A leaky tire and a dialysis bag in a bucket of solvent – objects permeable to small molecules but not to large ones – are not closed systems! In our test tube illustration, as long as no matter is added during the period of observation, and as long as evaporation of the solvent does not contribute significantly to any effects we might observe, the system can be considered closed. Moreover, the system will be closed even if the biochemical reaction we are studying results in the release or absorption of heat energy; energy transfer between system and surroundings can occur in a closed system. Another example of a closed system is Earth itself. Our planet continually receives radiant energy from the Sun and gives off heat, but because Earth is neither very heavy nor very light, the planet exchanges practically no matter with its surroundings. By contrast, black holes have such a large gravitational attraction that little or nothing can escape, but asteroids have no atmosphere.

Box 1.1 Hot viviparous lizard sex

Viviparous reptiles bear their offspring live. Skinks are any of the more than 1000 lizard species which constitute the family Scincidae. Present in tropical regions across the globe, these lizards are particularly diverse in Southeast Asia. Some species lay eggs; others give birth to fully developed progeny. *Eulamprus tympanum* is a medium-sized viviparous scincid lizard which inhabits alpine regions in southeastern Australia. Mothers actively thermoregulate to stabilize the temperature of gestation. The litter size is 1 to 5 young. Recently, researchers in Australia found that the developing embryos of *E. tympanum* are subject to temperature-dependent sex determination. In other words, the mother can influence the sex of her offspring and sex ratios in wild populations. Warmer temperatures give rise to a higher percentage of male progeny, the fraction of females falling from nearly 3/5 in the field to 9/20 at 25 °C, 1/4 at 30 °C, and 0 at 32 °C. In the laboratory, females provided with unlimited conditions for thermoregulation maintain a body temperature of 32 °C and produce male offspring only, whereas in the field, equal sex ratios result from natural gestation. The warmer temperatures of lower altitudes could yield a preponderance of male young and the eventual inability of those populations to procreate. Global warming could drive *E. tympanum* into extinction. In early 2007 climatologists announced that the recent drought in Australia was likely to lead to an increased average temperature of several degrees across the continent for the next several years.

What if matter can be exchanged between system and surroundings? Then the system is said to be *open*. An example of an open system is a cat (Fig. 1.9B). It breathes in and exhales matter (air) continually, and it eats, drinks, defecates and urinates periodically. In barely sufferable technospeak, a cat is an open, self-regulating and self-reproducing heterogeneous system. The system takes in food from the environment and uses it to maintain body temperature, "power" all the biochemical pathways of its body, including those of its reproductive organs, and to run, jump and play. The system requires nothing more for reproduction than a suitable feline of the opposite sex. And the molecular composition of the eye is certainly very different from that of the gut; hence, heterogeneous. In the course of all the material changes of this open system, heat energy is exchanged between it and the surroundings, the amount depending on the system's size and the difference in temperature between its body and the environment. A schematic diagram of the internal structure of this open system is shown in Fig. 1.10. Whether the living system is a cat, crocodile, baboon or bacterium, it is an open system. It seems that it can only be the case that all living systems that have ever existed have been open systems.

To wrap up this section, an *isolated* system is one in which the boundary permits neither matter nor energy to pass through. The system is constant with regard to material composition and energy. A schematic diagram of a system, surroundings and boundary are shown in Fig. 1.9C.

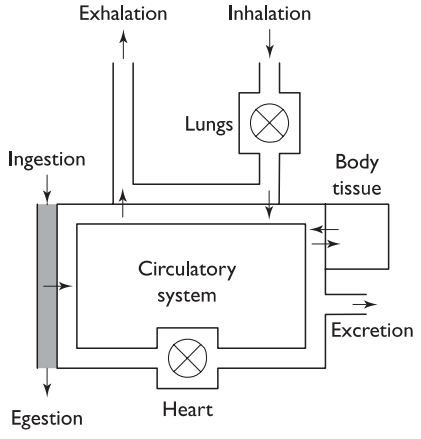

Fig. 1.10 The plumbing of a higher animal. Food energy, once inside the body, gets moved around a lot. Food is digested in the gut and then absorbed into the circulatory system, which delivers it to all cells of the body. The respiratory system plays a role in enabling an organism to acquire the oxygen it needs to burn the fuel of food. Again, the circulatory system is involved, providing the means of transport of respiratory gases. When energy input to the body exceeds output (excretion + heat), there is a net increase in weight. In humans and other animals, the ideal time rate of change of body weight, and therefore food intake and physical activity, varies with age and physical condition. Based on Fig. 1–5 of Peusner (1974).

D. Animal energy consumption

Now let's take a more in-depth look at the relationship between food, energy, and life. We wish to form a clear idea of how the energy requirements of carrying out various activities, for instance walking or sitting, relate to the energy available from the food we eat. The discussion is largely qualitative, but a formal definition of *heat* will be given.

Energy measurements can be made using a calorimeter. Calorimetry has made a big contribution to our understanding of the energetics of chemical reactions, and there is a long tradition of using calorimeters in biological research. In the mid seventeenth century, pioneering experiments by Robert Boyle (1627–1691) in Oxford demonstrated the necessary role of air in combustion and in respiration. Taking a breath is more like burning a piece of wood than many people suspect. About 120 years later, in 1780, Antoine Laurent Lavoisier (1743–1794) and Pierre Simon de Laplace (1749–1827) used a calorimeter to measure the *heat* given off by a live guinea pig. On comparing this heat with the amount of oxygen consumed, the Frenchmen correctly concluded that respiration is a form of combustion. Nowadays, a so-called bomb

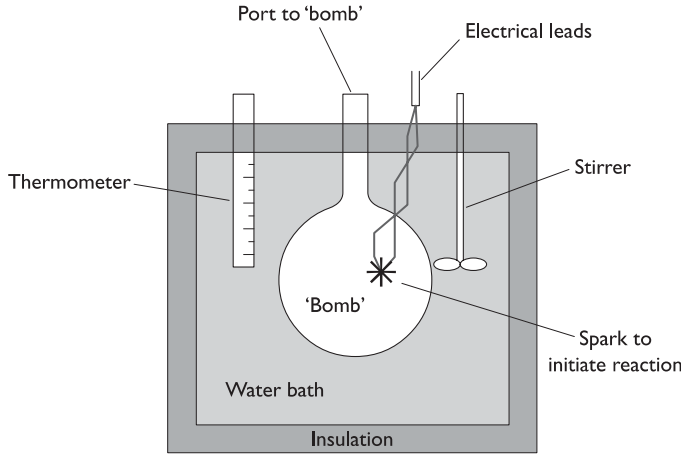

Fig. 1.11 Schematic diagram of a bomb calorimeter. A sample is placed in the reaction chamber. The chamber is then filled with oxygen at high pressure (>20 atm) to ensure that the reaction is fast and complete. Electrical heating of a wire initiates the reaction. The increase in water temperature resulting from the combustion reaction is recorded, and the temperature change is converted into an energy increase. The energy change is divided by the total amount of substance oxidized, giving units of J g^{-1} or J mol^{-1}. Insulation helps to prevent the escape of the heat of combustion, increasing the accuracy of the determination of heat released from the oxidized material. Based on diagram on p. 36 of Lawrence *et al.* (1996).

calorimeter[9] (Fig. 1.11) is used to measure the heat given off in the oxidation of a combustible substance like food, and nutritionists refer to tables of combustion heats in planning a diet.

The study of energy transformations is called thermodynamics. It is a hierarchical science – the more advanced concepts assume knowledge of the more basics ones. To be ready to tackle the more difficult but more interesting topics in later chapters, let's use this moment to develop an understanding of *what* is being measured in the bomb calorimeter. We know from experience that the oxidation (burning) of wood gives off *heat*. Some types of wood are useful for building fires because they ignite easily (e.g. splinters of dry pine); others are useful because they burn slowly and give off a lot of heat (e.g. oak). The amount of heat transferred to the air per unit volume of burning wood depends on the density of the wood and its *structure*. The same is true of food. Fine, but this has not told us what heat is.

It is the nature of science to define terms as precisely as possible and to formalize usage. Accepted definitions are important for minimizing ambiguity of meaning. What we need now is a

[9] But one of many different kinds of calorimeter. The instrument used to measure the energy given off in an atom smasher is called a calorimeter. In this book we discuss a bomb calorimeter, isothermal titration calorimeter, and differential scanning calorimeter.

definition of *heat*. Heat, or thermal energy, q, is a form of kinetic energy; that is, energy arising from motion. Heat is the change in energy of a system that results from a temperature difference between it and the surroundings. For instance, when a warm can of Coke is placed in a refrigerator, it gives off heat continuously until reaching the same average temperature as all other objects in the fridge, including the air. The heat *transferred* from the Coke can to the air is absorbed by the other things in the fridge. Heat is said to *flow* from a region of higher temperature, where the average speed of molecular motion is greater, to one of lower temperature.

The flow of heat does indeed remind us of a liquid, but it does not necessarily follow, and indeed we should not conclude, that heat is a material particle. Heat is rather a type of energy transfer. Heat makes use of *random* molecular motion. Particles that exhibit such motion (*all* particles!) are subject to the usual mechanical laws of physics. A familiar example of heat transfer is the boiling of water in a saucepan. The more heat applied, the faster the motion of water. The bubbles that form on the bottom of the pan give some indication of how fast the water molecules are moving. This is about as close as we get under ordinary circumstances to "seeing" heat being transferred. But if you've ever been in the middle of a shower when the hot water has run out, you will know what it's like to *feel* heat being transferred! By convention, $q > 0$ if energy is transferred *to* a system as heat, if the total energy of the system increases by way of heat transfer. In the case of a cold shower, and considering the body to be the system, q is negative.

Now we are armed for another look at the oxidation of materials in a bomb calorimeter and the relationship to nutrition. The heat released or absorbed in a reaction is measured as a change in temperature; calibration of an instrument using known quantities of heat can be used to relate heats of reaction to changes in temperature. One can plot a standard curve of temperature *versus* heat, and the heat of oxidation of an unknown material can then be determined experimentally. Table 1.2 shows the heats of oxidation of different foodstuffs. Evidently, and important for physiology, some types of biological molecule give off more heat per unit mass than others. Some idea of the extent to which the energy obtained from food is utilized in various human activities is given in Table 1.3.

Animals, particularly humans, "consume" energy in a variety of ways, not just by eating, digesting and metabolizing food. For instance, most automobiles of the present day run on octane, and electrical appliances depend on the generation of electricity. The point is that energy transformation and consumption can be viewed on many different levels. As our telescopic lens becomes more powerful, the considerations range from one person to a family, a neighborhood, city, county, state, country, continent, surface of the earth, biosphere, solar system, galaxy ... As the length scale decreases, the microscope zooms in on an organ, a tissue,

Table 1.2. | *Heat released upon oxidation to CO_2 and H_2O*

Substance	Energy yield			
	kJ (mol^{-1})	kJ (g^{-1})	kcal (g^{-1})	kcal (g^{-1} wet wt)
Glucose	2 817	15.6	3.7	—
Lactate	1 364	15.2	3.6	—
Palmitic acid	10 040	39.2	9.4	—
Glycine	979	13.1	3.1	—
Carbohydrate	—	16	3.8	1.5
Fat	—	37	8.8	8.8
Protein	—	23	5.5	1.5
Protein to urea	—	19	4.6	—
Ethyl alcohol	—	29	6.9	—
Lignin	—	26	6.2	—
Coal	—	28	6.7	—
Oil	—	48	11	—

D-glucose is the principal source of energy for most cells in higher organisms. It is converted to lactate in anaerobic homolactic fermentation (e.g. in muscle), to ethyl alcohol in anaerobic alcoholic fermentation (e.g. in yeast), and to carbon dioxide and water in aerobic oxidation. Palmitic acid is a fatty acid. Glycine, a constituent of protein, is the smallest amino acid. Carbohydrate, fat and protein are three different types of biological macromolecule and three different sources of energy in food. Metabolism in animals leaves a residue of nitrogenous excretory products, including urea in urine and methane produced in the gastrointestinal tract. Ethyl alcohol is a major component of alcoholic beverages. Lignin is a plasticlike phenolic polymer that is found in the cell walls of plants; it is not metabolized directly by higher eukaryotes. Coal and oil are fossil fuels that are produced from decaying organic matter, primarily plants, on a time scale of millions of years. The data are from Table 2.1 of Wrigglesworth (1997) or Table 3.1 of Burton (1998). See also Table A in Appendix C.

Table 1.3. | *Energy expenditure in a 70 kg human*

Form of activity	Total energy expenditure (kcal h^{-1})
Lying still, awake	77
Sitting at rest	100
Typewriting rapidly	140
Dressing or undressing	150
Walking on level, 2.6 mi/h	200
Sexual intercourse	280
Bicycling on level, 5.5 mi/h	304
Walking on 3 percent grade, 2.6 mi/h	357
Sawing wood or shoveling snow	480
Jogging, 5.3 mi/h	570
Rowing, 20 strokes/min	828
Maximal activity (untrained)	1440

The measurements were made by indirect calorimetry. Digestion increases the rate of metabolism by as much as 30% over the basal rate. During sleep the metabolic rate is about 10% lower than the basal rate. The data are from Table 15–2 of Vander, Sherman and Luciano (1985).

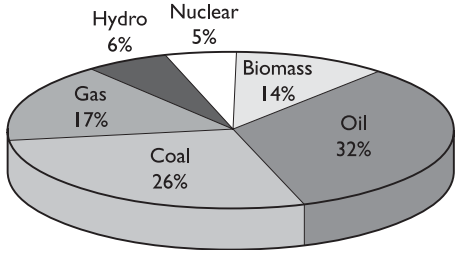

Fig. 1.12 Global human energy use. In 1987 the total was about 4×10^{20} J yr^{-1}. Energy production and consumption have increased substantially since then, but the distribution has remained about the same. The rate of energy consumption is about four orders of magnitude smaller than the amount of radiant energy that is incident on Earth each year (see Fig. 1.1). Note also that *c.* 90% of energy consumption depends on the products of photosynthesis, assuming that fossil fuels are the remains of ancient organisms. Redrawn from Fig. 8.12 in Wrigglesworth (1997).

cell, organelle, macromolecular assembly, protein, atom, nucleus, nucleon, quark ... Figure 1.12 gives some idea of humankind's global energy *use* per sector. Comprehensive treatment of all these kinds and levels of energy would be impossible, if not in principle than definitely in the space of 400 pages. Our more modest focus is basic principles of energy transformation in the biological sciences.

Box 1.2 In praise of cow pies and grass

Interest in improving air quality and reducing dependence on foreign energy sources are playing a key role in the development of solar power and biofuels. A biofuel is any fuel that is derived from biomass – living and recently living biological matter which can be used as fuel for industrial production. Two examples of biofuels are plant material and some metabolic byproducts of animals, for instance, dried cow dung. In contrast to petroleum, coal, and other such natural energy resources, biofuel is renewable. Biofuel is also biodegradable and relatively harmless to the environment, unlike oil. Like oil and coal, the biomass from which a biofuel is derived is typically a form of stored solar energy. The carbon in plants is extracted from the atmosphere, so burning biofuels does not result in a net increase in atmospheric carbon dioxide. Plants specifically grown for use as biofuels include soybean, corn, canola, flaxseed, rapeseed, sugar cane, switchgrass, and hemp. Various forms of biodegradable waste from industry, agriculture, and forestry can also be converted to biogas through anaerobic digestion by microorganisms. Fermentation yields ethanol and methanol. Currently, most bioenergy is consumed in developing countries, and it is used for direct heating rather than electricity production. But the situation is changing rapidly, and industrialized countries are actively developing new technologies to exploit this key resource. In the USA, for example, which has lagged behind some European countries in promoting the development of alternative fuel sources, there is a push towards replacing 75% of oil imports by 2025. Development of biofuel technologies is certain to play a role in the

attempt to reach this lofty goal. "With recent advances in industrial biotechnology, the United States can achieve the goal of producing 35 billion gallons of renewable fuel by 2017," said Jim Greenwood in 2007. Greenwood is the current CEO of the Biotechnology Industry Organization, which represents more than 1100 biotechnology companies, academic institutions, state biotechnology centers and related organizations across the United States and 31 other nations. In the European Union it has been decided that at least 5.75% of traffic fuel in each member state should be biofuel by 2010. Which nations will succeed in attaining this objective? The race is on to develop inexpensive means of preparing liquid and gas biofuels from low-cost organic matter (e.g. cellulose, agricultural waste, sewage waste) at high net energy gain.

E. Carbon, energy, and life

We close this chapter with a brief look at the relationship of *energy* and *structure* in carbon, a key atom of life as we know it. The elemental composition of the dry mass of the adult human body is roughly 3/5 carbon, 1/10 nitrogen, 1/10 oxygen, 1/20 hydrogen, 1/20 calcium, 1/40 phosphorus, 1/100 potassium, 1/100 sulfur, 1/100 chlorine, and 1/100 sodium (Fig. 1.13). We shall see these elements at work in later chapters of the book. The message of the moment is that carbon is the biggest contributor to the weight of the body. Is there is an energetic "explanation" for this?

Maybe. Apart from its predominant structural feature – extraordinary chemical versatility and ability to make asymmetric molecules – carbon forms especially stable single bonds. N–N bonds and O–O bonds have an energy of about 160 kJ mol^{-1} and 140 kJ mol^{-1}, respectively, while the energy of a C–C bond is about twice as great (345 kJ mol^{-1}). The C–C bond energy is moreover nearly as

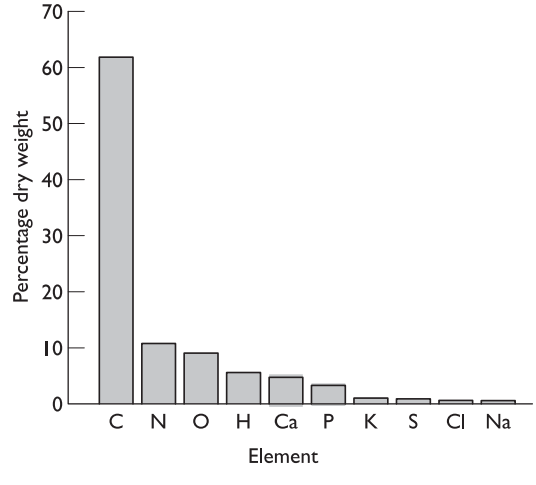

Fig. 1.13 Composition of the human body after removal of water. Protein accounts for about half of the dry mass of the body. On the level of individual elements, carbon is by far the largest component, followed by nitrogen, oxygen, hydrogen and other elements. It is interesting that the elements contributing the most to the dry mass of the body are also the major components of air. Based on data from Freiden (1972).

great as that of a Si–O bond. Chains of Si–O are found in great abundance in the silicate minerals that form the crust of Earth, and one might guess therefore that silicates could support life in distant solar systems, if not elsewhere in our own. Although this possibility cannot be excluded, we can say that Si–O is unlikely to be as useful for life as C–C because it is practically "inert." The predominant importance of carbon in the molecules of life is likely to be the rule throughout the universe rather than the exception here on Earth.

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G. Exercises

1. What *is* energy? Give the etymology of *energy*. When did *energy* acquire its present scientific meaning? (Hint: consult the *Oxford English Dictionary* and any good encyclopedia of physics.)

2. Some primitive religions teach that the celestial bodies we call stars (or planets) are gods. This view was common in the ancient Greek world, and it was espoused by Thales of Miletus (fl. 6th century BC), one of the greatest thinkers of all time. Needless to say, ancient Greeks knew nothing about nuclear fusion in stars, though they were certainly aware that the Sun is much larger than it appears to the unaided eye and that plants need light and water to grow. Explain briefly how the belief that stars are gods is remarkably insightful, even if polytheism and animism are rejected on other grounds.

3. Equation (1.1) shows E as a *continuous* and *linear* function of λ^{-1}; the energy spectrum of a *free* particle is not characterized by *discrete*, step-like energy levels. A continuous function is one that changes value smoothly; a linear function is a straight line. Consider Eqn. (1.1). Does it suggest a fundamental limit to the magnitude of the energy of a photon? In contrast, the electronic *bound* state with which a photon interacts in photosynthesis is restricted to certain energy *levels*, and these are determined by the structure of the pigment molecule and its electronic environment; electromagnetic radiation interacts with *matter* as though it existed in small packets (photons) with *discrete* values. All of the energy levels of the bound electron are below a certain threshold, and when this energy level is exceeded, the electron becomes a free particle. What effect could an electron's becoming a free particle have on the plant? What prevents high-energy photons from the Sun from doing this to plants?

4. Chlorophylls absorb blue light and red light relatively well, but not green light (Fig. 1.2). Why are tree leaves green in summer, some other color in autumn, and brown in late autumn?

5. The wavelength of blue light is about 4700 Å; that of red light, about 7000 Å. (1 Å = 10^{-10} m; the ångström is named in honor of the Swedish physicist Anders Jonas Ångström (1814–1874)). Calculate the energy of a photon at these wavelengths. About 7 kcal mol^{-1} is released when ATP is hydrolyzed to ADP and inorganic phosphate (under standard state conditions). Compare the energy of the photons absorbed by plants to the energy of ATP hydrolysis (1 mole = 6.02×10^{23}).

6. In the anabolic (biosynthetic) reduction–oxidation reactions of plant photosynthesis, 8 photons are required to reduce one molecule of CO_2. 1 mol of CO_2 gives 1 mol of carbohydrate (CH_2O). What is the maximum possible biomass (in g of carbohydrate) that can be produced in 1 hour by plants receiving 1000 μE s^{-1} of photons of a suitable wavelength for absorption? Assume that 40% of the photons are absorbed. 1 E = 1 einstein = 1 mol of photons (the einstein is named in honor of the theoretical physicist of the same name). The atomic masses of H, C and O are 1, 12 and 16, respectively.

7. The energy of oxidation of glucose to H_2O and CO_2 is –2870 kJ mol^{-1}. At least 2870 kJ mol^{-1} are needed to synthesize glucose from H_2O and CO_2. How many 700 nm photons must be absorbed to fix one mole of CO_2? If the actual number needed is 3 to 4 times the minimum, what is the efficiency of the process?

8. Devise your own analogy for energy conservation and distribution. Indicate how the analog resembles nature and where the similarity begins to break down.

9. Give three examples of distributions: a spatial distribution, a temporal distribution, and a spatio-temporal distribution.

10. Give three examples of a closed system. Give three examples of an open system.

11. Describe the preparation of a cup of tea with milk in terms of energy transformation.

12. Describe an astronaut in a spaceship in terms of open and closed systems.

13. The optimal growth temperatures of almost all known organisms are between the freezing and boiling points of water at 1 atm pressure. Notable exceptions are marine organisms that live in the sea a few degrees below 0 °C (sea water remains liquid in such cases because salt and increased pressure reduce the freezing point; see Chapter 4). Homeothermic organisms maintain an almost constant body

temperature, independent of the temperature of the environment. Human beings are an example, as are horses and cats. Fluctuations about the average temperature of these organisms are generally less than 1 °C. All such organisms have an average temperature between 35 and 45 °C; a narrow range. Most birds strictly regulate their body temperatures at points between 39 and 44 °C. In some bird species, however, body temperature can vary by about 10 degrees centigrade. Poikilotherms, which include reptiles, plants, microorganisms, show even less temperature regulation. Eubacteria and archaebacteria exhibit the greatest range of growth temperatures of all known organisms. Describe how a reptile might regulate its temperature. What about a plant?

14. Calculate the heat energy released by complete burning of an 11 g spoonful of sugar to carbon dioxide and water (Table 1.2).

15. Banana skins turn brown much more rapidly after the fruit has been peeled than before. Why?

16. Human daily energy requirement. A metabolic rate is a measure of energy consumption per unit time. Basal metabolic rate (BMR) is measured after a 12 h fast and corresponds to complete physical and mental rest. A 70 kg man might have a BMR of 80 W. A very active man might have a BMR three times as large. Calculate the minimal daily energy requirement of a man who has a BMR of 135 W.

17. The energy of protein catabolism (degradation) in living organisms is different from the energy of protein combustion in a calorimeter. Which energy is larger? Why?

18. Consider a 55 kg woman. Suppose she contains 8 kg of fat. How much heavier would she be if she stored the same amount of energy as carbohydrate?

19. Student A spends 15 h day^{-1} sitting in the classroom, library, student cafeteria or dormitory. Another half-hour is spent walking between the dorm and lecture halls, and an hour is used for walking in the morning. Assume that lying down "costs" 5.0 kJ min^{-1}, sitting, 5.9 kJ min^{-1}, standing, 8.0 kJ min^{-1}, and walking, 13.4 kJ min^{-1}. Calculate Student A's daily energy requirement. Student B's routine is identical to Student A's except that his hour of exercise is spent watching television. Calculate the difference in energy requirements for these two students. Referring to Table 1.2, calculate the mass of fat, protein or carbohydrate Student A would have to ingest in order to satisfy her energy needs. How much glucose does Student A need for daily exercise? List the underlying assumptions of your calculations.

20. In nuclear fusion, two ^{2}H (deuterium) atoms combine to form helium and a neutron.

$$^2\text{H} + ^2\text{H} \rightarrow ^3\text{He} + \text{n}$$

The mass of ^{2}H is 2.0141 atomic mass units (a.m.u.), the mass of ^{3}He is 3.0160 a.m.u., and the mass of a neutron is 1.0087 a.m.u. (1 a.m.u. $= 1.6605 \times 10^{-27}$ kg). Perhaps the most famous mathematical formula in the history of civilization on Earth is $E = mc^2$, where m is mass in kg, c is the speed of light in m s^{-1}, and E is heat energy in J. The relationship between matter and energy is a recurring theme in this book. Show that the heat released on formation of one mole of helium atoms and one mole of neutrons from two moles of deuterium atoms is about 3.14×10^8 kJ.

21. Worldwide energy production (WEP) was 320 quadrillion (320×10^{15}) Btu (British thermal units; 1 Btu = 1.055 kJ) in 1987. By 1996, it had increased by 55 quadrillion Btu. Give the magnitude of energy production in 1996 in joules and the percentage increase ($[(\text{WEP}_{1996} - \text{WEP}_{1987})/\text{WEP}_{1987}] \times 100$). Calculate the average annual rate of increase in WEP between 1987 and 1996. In 1996, the USA produced 73 quadrillion Btu, more than any other country. Compute the contribution of the USA to WEP in 1996. Only about 0.025% of the Sun's radiant energy that reaches Earth is captured by photosynthetic organisms. Using the data in the legend of Fig. 1.1, calculate the magnitude of this energy in kJ s^{-1}. Find the ratio of WEP_{1996} to the Sun's energy captured by photosynthetic organisms. Assuming that $173\,000 \times 10^{12}$ W of the Sun's radiant energy reaches Earth and is then either reflected or absorbed, calculate the total energy output of the Sun.[10] (Diameter of Earth = 12 756 km; area of a circle $= \pi \times (\text{diameter}/2)^2$; surface area of a sphere $= 4 \times \pi \times (\text{diameter}/2)^2$; mean distance of Earth from Sun $= 149.6 \times 10^6$ km). Using your result from the previous problem, calculate the number of moles of ^{2}H consumed when a heat this large is released. Calculate the energy equivalent of the Earth (mass $= 5.976 \times 10^{27}$ g). Compare the mass energy of Earth to the radiant energy of the Sun that reaches Earth in one year.

22. It is said that energy is to biology what money is to economics. Explain.

[10] 1 W = 1 J s^{-1}. A unit of power, the watt is named after Scottish inventor James Watt (1736–1819).

Chapter 2

The First Law of Thermodynamics

A. Introduction

To gain a good understanding of the laws of thermodynamics, it will help to develop an appreciation of the meaning of the words *law* and *thermodynamics*. Let's take a moment to think about these words before launching into a detailed discussion of how we might unpack the content of how the laws can be formulated. We are aided in this quest by the nature of science itself, which unlike ordinary prose and poetry aims to give words a more or less precise definition.

We are familiar with the concept of law from our everyday experience. Laws are rules that we are not supposed to break; they exist to protect someone's interests, possibly our own, and there may be a penalty to pay if the one who breaks a law gets caught. Such are civil and criminal laws. Physical laws are similar but different. They are similar in that they regulate something, namely how matter behaves under given circumstances. They are different in that violations are not known to have occurred, and they describe what is considered to be a basic property of nature. If a violation of a physical law should ever seem to have occurred, you will think first that the experiment has gone wrong at some stage, and second that maybe the "law" isn't a law after all.

Here's an example. Galileo,[1] like Copernicus,[2] believed that the orbits of the known planets were circles; the circle being the shaper of perfection and perfection being of the heavens. This view was inherited from Aristotle. Galileo also thought that the motion of

[1] Galileo Galilei, Italian astronomer and physicist, lived 1564–1642. His model of the Earth, the Moon, the Sun and planets was based on that of Copernicus, who had proposed a Sun-centered planetary system in his *De Revolutionibus Orbium Coelestium* (1543). Galileo is widely considered the father of modern science, because he emphasized the role of observations and experimentation in the discovery of new aspects of nature.

[2] Nicolaus Copernicus (1473–1543) held an ecclesiastical position in a church in Poland and was fascinated by astronomy.

celestial objects like planets was qualitatively different from the motion of terrestrial objects like cannonballs and feathers. But in fact, the orbits of planets are ellipses, not circles,[3] and the mechanical laws of planetary motion are fundamentally the same as those of a missile flying through the air on a battlefield, an object rolling down an inclined plane, and an apple falling to the ground in an orchard.[4] The point is not that Galileo was poor at science: his contributions to science have played an extremely important role in its development. Rather, the point is that what was considered a "law" was later shown not to be a law. (We can also see that at times a great does not get it quite right, in the best cases not through an inherent unwillingness to give all due consideration to available evidence, but because the evidence needed to change a perspective simply did not exist and was not yet sufficiently compelling.) There are many related examples one could cite from the history of science. It is the nature of human awareness of the physical world to develop in this way. It borders on the inhumane to assess the scientific ability of people who lived in a previous age by the standards and knowledge of today.

Whereas a human can break a law intentionally or unwittingly, a basic assumption of science is that a particle cannot break a law of physics. Particle motion is *governed* by the laws of physics (even if we don't know what those laws are). An important fact for us is that no violation of a law of thermodynamics is known to have occurred in nearly two hundred years of research in this area. Because of this many scientists, for example, Einstein, consider the laws of thermodynamics to be the laws of physics least likely to be overturned or superseded by further research. The laws of thermodynamics are generally described as the most general concepts of all of modern science. It behoves the biologist to be familiar with the basic principles of thermodynamics because they are of such basic importance. In view of all this, we might begin to suspect that the concepts we shall discuss are very deep and that considerable study and thought will be the price to pay for mastery of them. Thus has it ever been with basic things.

Energy has been around, well, since "the beginning," but the word *thermodynamics* was not coined until 1840, from the Greek roots *therme*, heat, and *dynamis*, power. The same roots appear in *thermometer* (a device to measure temperature, or heat) and *dynamite* (a powerful explosive). We can guess, then, that thermodynamics

[3] This was demonstrated by the German astronomer Johannes Kepler (1571–1630). In fact, though, the orbit of Earth is remarkably close to circular.

[4] As shown by the English mathematician, natural philosopher, and alchemist Isaac Newton (1642–1727). Sir Isaac is perhaps the greatest scientist of all time. His voluminous writings show that he was apparently as interested in theology and alchemy as in mathematics and natural philosophy, i.e. science. Thomas Jefferson, principal author of the Declaration of Independence and third president of the USA, owned a copy of one of Newton's lesser known works, *Observations upon the Prophecies of Daniel*.

will have to do with heat energy and power or movement. In fact, this branch of physics is concerned with energy storage, transformation, and dissipation. Thermodynamics aims to describe and relate – in relatively simple mathematical terms – the physical properties of systems of energy and matter. Thermodynamics has very much to do with molecular motion.

You might not think so, but you will certainly know something about thermodynamics. If not from having studied physics before starting university, then from having seen what happens when a pan of water is heated on the stove! At first, when the temperature of still water is about 25 °C, nothing seems to be happening; the eye does not detect any motion. But when heat is applied, motion becomes more significant and indeed readily apparent, so that by the time the boiling point is reached the water is moving about rather violently! So you do know something about thermodynamics, even if you don't normally think about it in the framework of today's physics, and a lot was known about thermodynamics well before the word was invented. There is not space to say much about the history of thermodynamics here, but it is worth mentioning that the principles of this science grew out of practical attempts in the nineteenth century to understand how to make a steam engine work as efficiently as possible and why heat is generated when one drills the bore of cannon, not academic speculation on universal law. This suggests that there may be value in avoiding being too prescriptive about how scientific knowledge should develop.

Like Kepler's laws of planetary motion and Newton's laws of mechanics, there are three laws of thermodynamics (plus one). There is a good deal about the first two of them here and in Chapter 3; they form the core of classical thermodynamics. Discussion of the First and Second Laws also provides the necessary context for introducing concepts that underlie the concept of free energy, a useful tool in the biological sciences (Chapters 4 and 5). The Third Law of Thermodynamics is of less immediate importance to biologists, but we'll touch on it at the end of Chapter 3, showing how it raises some very interesting questions about the nature of living organisms. For the purposes of our present subject, the chief practical value of studying the laws of thermodynamics is that they provide insight into how biological systems work and a framework for designing experiments, testing hypotheses, and explaining results.

We're ready for the First Law of Thermodynamics. But before investigating it, let's take one minute to go over the so-called Zeroth Law. The function of the Zeroth Law is to *justify* the concept of temperature and the use of thermometers (two things most of us are accustomed to take for granted!), and it is included here to provide a broader conceptual foundation to our subject. The form of the Zeroth Law is identical to that of a famous logical argument known at least as early as the ancient Greeks. It goes like this: if $\alpha = \beta$ (one premise), and $\beta = \gamma$ (another premise), then $\gamma = \alpha$

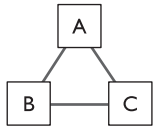

Fig. 2.1 The Zeroth Law of Thermodynamics. If three systems, A, B and C, are in physical contact, at equilibrium all three will have the same temperature. The concept of equilibrium is discussed in depth in Chapter 4.

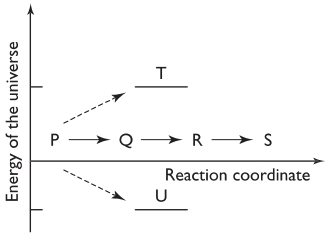

Fig. 2.2 The First Law of Thermodynamics. The total energy of the universe is constant, no matter what changes occur within. This principle also applies to an isolated system. Moreover, it is no less applicable to an open system or a closed system, as long as a complete account can be made of energy exchanged with the surroundings.

(conclusion). The Zeroth Law is built on this *syllogism*, or logical argument consisting of three propositions. It involves the concept of thermal equilibrium, that two objects A and B are in contact and at the same temperature.[5] The Zeroth Law states that if A is in thermal equilibrium with B, and B is in equilibrium with object C, then C is also in thermal equilibrium with A (Fig. 2.1). Simple! In Chapter 1 we touched on how temperature is a measure of the average speed of molecules in a gas. And now that we have the Zeroth Law, we are free to use the concept of temperature as much as we like.

The First Law is a conservation law: energy can be changed from one form to another, but in all its transformations energy is neither created nor destroyed (Fig. 2.2). There is a close resemblance to the conservation of matter, according to which the total amount of matter in a chemical reaction is a constant. The First Law of Thermodynamics is empirical in nature; it cannot be derived from more basic principles. Unlike the Pythagorean theorem,[6] for example, which can be derived from the most basic principles of Euclidean geometry,[7] there is no *mathematical* proof that the First Law of Thermodynamics is right. So then why should you believe it? *Sed solum ego ipse dixi*? Some might question an appeal to "authority" in scientific circles. We accept the First Law on a number of different bases, a most important and necessary one being that it is based on the experience of many, many researchers. The First Law has been tested many times, and as far as anyone knows, it has not been violated even once. It works. It's simple. It makes sense. That alone does not *prove* that the First Law is *true*, but it does at least give a good reason for thinking that it is probably a pretty good description of nature. So we believe in the First Law of the thermodynamics.

Despite its lack of a rigorous mathematical foundation, the First Law is the basis of *all* quantitative accounts of energy, regardless of form. The First Law makes energy the most scientific important concept in physics. And to the extent that physics is the basis of all of science and engineering, energy is the most important scientific concept in these technical areas. We saw in the previous chapter, the energy of a system can be converted from one form to another and distributed in a myriad of ways. And now we assume that energy is

[5] That thermal equilibrium is characterized by the equality of a single parameter (temperature) for all systems was first stated by Joseph Black (1728–1799), a Scottish chemist and physician.

[6] Named after Pythagoras (*c.* 580–500 BC), a mathematically inclined pre-Socratic religious philosopher. The Pythagorean theorem is $a^2 = b^2 + c^2$, where a, b and c are the lengths of the sides of a right triangle. Clay tablets unearthed in present-day Iraq prove that various combinations of integer which satisfy the algebraic equation were known a millennium before Pythagoras was born, historically on the island of Samos, very close to present-day Turkey. A more intuitive, geometrical proof of the theorem requires no knowledge of algebra. The theorem boasts more different proofs than any other theorem of mathematics – literally hundreds of different ones.

[7] The Greek mathematician Euclid lived *c.* 300 BC. His *Elements of Geometry* was the standard work on the subject until other types of geometry were invented in the nineteenth century.

not created or destroyed. The energy of a system plus surroundings is constant in time. For example, you can turn the chemical energy of an aphrodisiac into heat by a series of bodily actions that are better left to the imagination than described in writing or expressed in mathematical formulae, but the amazing thing is that throughout all the underlying changes, the total energy remains the same.

Box 2.1. Thermogenic oscillations keep the home fire burning

Homeotherms like mammals display adaptive thermogenesis. Detected exposure to cold by the brain leads to the activation of efferent pathways which control energy dissipation through the sympathetic nervous system. Mammals maintain body temperature by constant metabolism in cells throughout the body and by circulation of the blood. Plants, by contrast, lack a nervous system and are generally regarded as poikilotherms; most plants are unable to control their own temperature. Some cold-tolerant plants can, however, acclimatize to reduced temperature. So-called thermogenic plants have metabolic pathways that increase the temperature of a particular organ or tissue in response to cold. This group of plants includes the lotus (*Nelumbo nucifera*), a sacred symbol of perpetual regeneration in various religions. The lotus maintains its receptacle temperature between 30 °C and 36 °C during the 2–4 day sequence of anthesis, during which the flower bud opens and is fully functional. A perhaps more prosaic thermogenic plant is the skunk cabbage (*Symplocarpus foetidus*), a species of arum lily whose Japanese name, *Zazen-sou*, means Zen meditation plant, and whose English name comes from its bad smell and cabbage-like leaves. Skunk cabbage blooms in the early spring and maintains the temperature of the female spadix, the spike-like flowering stalk, at close to 20 °C for about a week, even when the ambient air temperature drops below the freezing point of water. Recently, researchers in Japan have shown that the thermogenic oscillations of the skunk cabbage are induced by a change in spadix temperature, through chemical reactions in the cells' mitochondria. The oscillations have a period of around 60 min, and the threshold is less than 0.9 °C, the greatest precision known among plants. There is also some evidence that the thermoregulation process is chaotic. In a model of the oscillatory temperature-sensing ability of skunk cabbage, the temperature of the spadix is maintained at a certain level where heat production and loss, due to radiation, evaporation, conduction, and convection, are balanced. An as-yet unidentified thermal sensor detects changes in temperature, and if the change exceeds the threshold for over about 30 min, thermogenesis is modified. The temperature oscillator in skunk cabbage appears to be distinct from known circadian rhythms and other kinds of biological rhythms.

B. Internal energy

To see more clearly how the First Law operates, we need to add internal energy and work to our conceptual toolkit. As with heat, both internal energy and work are measured in units of joules (or

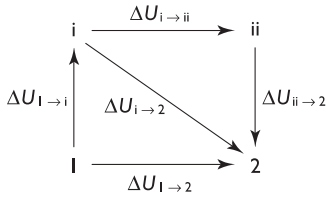

Fig. 2.3 Thermodynamic cycle. The difference in internal energy between state 2 and state I is $\Delta U_{I\to 2}$. Because U is a state function, the energy difference is independent of path; the internal energy of a system is determined by the specific physical properties of the state of the system and not on how the state was prepared. It follows that if the system begins in state I and is brought back to this state, $\Delta U = 0$. In symbols, $\sum_{loop} \Delta X = 0$. This holds not just for the internal energy but for any state function X.

calories). But not all heat is work, and internal energy will be heat or work only under certain circumstances. Say again? The internal energy is the energy *within* the system, U. For our purposes U will represent only those kinds of energy that can be modified by a chemical process – translational, vibrational, rotational, bonding, and non-bonding energies. A particle in a system may translate from A to B, a bond may rotate and vibrate, a bond may break and reform, and particles may interact non-covalently, for instance, by electrostatics. We'll leave out nuclear energy, even though it is always present and important in energy production and fusion reactions in the Sun. Nuclear energy simply does not play much of a role in the typical biochemical reaction, and when we think about a particular biochemical reaction, we take the atoms involved as given and we do not consider their history. So we can leave nuclear energy out of any calculation of the internal energy because the nuclear energy does not change in the typical biochemical reaction. Keeping track of quantities that do change is complicated enough!

The *internal* energy defines the energy of a substance in the absence of *external* effects, for instance, those owing to capillarity, electric fields, and magnetic fields. But U is an *extensive* property of a substance, meaning that its value depends on the size of the sample. For instance, the internal energy of 2 g of fat is twice as great as the internal energy of 1 g of fat under the same conditions. An intensive property; by contrast, for example, the concentration of sodium in a solution of sodium bicarbonate, is independent of the amount of the sample. U is a special kind of thermodynamic quantity called a *state function*. This means that U can be expressed in a certain mathematical form, and that the value of U depends only on the *current* state of the system (e.g. temperature, pressure and number of particles) and not at all on how the particles of the system came to be arranged in a particular way. An example will help to illustrate the point. The internal energy of an aqueous buffer solution depends only on its current state and not on whether it was made directly by mixing some chemicals with water or was prepared from a concentrated stock solution that had been frozen at $-20\,^{\circ}C$ for however long. Other general properties of state functions will be introduced as we go along.

The internal energy of a system cannot be measured *directly*; it is calculated from other measured properties. Moreover, it is not U that is measured but a change in U. But this presents no problems because normally we are interested in changes in U and not U itself. When a process brings about a change in a system from state 1 to state 2, the internal energy changes from U_1 to U_2, and the difference $\Delta U = U_2 - U_1$. For example, when salt is dissolved in water a large amount of heat is released, and solvation of the ions can be measured as a change in temperature. State 1 is the crystalline form of the salt and pure water, and state 2 is the salt when it is completely dissociated into ions and solvated. It does not matter whether we think of dissolution occurring in several steps (e.g. separation of ions in vacuum followed by solvation) or all in one go (Fig. 2.3); the

computed energy difference between states 1 and 2 is the same. This implies that ΔU for a complete cycle, say, a change from state 1 to state 2 and back again, will be 0, regardless of the *path* of the process – the succession of states through which the system passes. Many experiments corroborate the rule, and no exception is known. This is the experimental basis on which U is considered a state function. All state functions are path-independent.

The path-independence of U has the ring of the First Law about it. In fact, changes in U are what the First Law is about! In the money analogy of Chapter 1, the total amount at the end of the day did not depend at all on whether payment was made in coins and banknotes, nor on the order in which the customers made their purchases (and definitely not on the identity of the customers); it depended only on which purchases were made on a particular day. There are many, many ways in which money could change hands and still compute to a net change of Δm. The situation with ΔU is clearly very similar. Let's now set internal energy aside for a moment and have a look at work.

C. Work

Work, w, is a key physical quantity in thermodynamics; we had better know something about it. Adequate treatment of work requires the concept of force, F, so let's look at F and then w. A force is any influence that can cause an object to be accelerated, and it is usually measured in newtons, N (1 N = 1 kg- m- s^{-2}). There are as many kinds of force as there are kinds of energy. A familiar example of a force is the gravitational force of attraction of Earth for some object, e.g. the book you are reading. The force of gravity on an object is proportional to the quantity of matter (its mass), and for objects near the surface of Earth, the acceleration due to gravity, g, 9.8 m- s^{-2}. These concepts are combined in Newton's famous second law, $F = ma$, where m is the mass of the object and a is the acceleration. When the force is the gravitational force, $a = g$ and $F = mg$. The gravitational force on a 70 kg man, i.e. his weight, is 70 kg × 9.8 m- s^{-2} = 690 N. The ratio of weight (F) to mass (m), namely, the acceleration due to gravity (g), is the same for a large object and a small object. Similarly, the ratio of circumference to diameter is the same for a large circle and a small circle. Time to get to work!

Work is similar to heat. When heat is added to a system, the internal energy changes (it increases). When work is done on a system, for example by compressing the volume of a gas, the internal energy changes (it increases). Both heat and work are forms of energy transfer *across* the boundary of a system; q and w are "boundary phenomena" (Chapter 1). You may have noticed that, unlike the internal energy, both heat and work are represented by lower case symbols. This is because U is a state function, but neither q nor w is a state function. Instead, q and w are *path* functions. In practical terms

this means that both q and w are *transient* quantities, unlike U, which is stored in a system. So heat and work are similar. But they are also different, and that's why different names are needed. They differ in that work is the *equivalent* of a force (e.g. gravity) acting through the displacement of an object, while heat is the transfer of energy owing to a temperature difference. Work involves the *non-random* movement of particles, heat the *random* movement of particles.

There are many different kinds of work. Not many different possible jobs for readers of this book, but different kinds of w. Here are a few of the latter sort: lifting of a weight against the force of gravity, expansion of a gas against an external pressure, movement of a charge against an electric field gradient (voltage), rotation of a shaft driving a fan, driving of an electric generator, expansion of a liquid film against its surface tension. In each case a force acts through a displacement, resulting in work being done by or on the system. The system does work when it pushes on the surroundings, and work is done on the system when the surroundings push on the system. When a system consisting of gas in a syringe is compressed by pushing on the plunger, the surroundings, which may include your hand and arm, do work on the system, and the internal energy of the gas increases.

The technical definition of *work* is similar to the one we are familiar with from everyday life. If someone "works hard," they put a lot of effort into accomplishing a task or reaching a goal. Similarly, in physics work is done when an object is moved against an opposing force. For example, when a crate is lifted vertically against the opposing force of gravity, the atoms of the box are involved in an *organized* transfer of energy; all the atoms of the box move together in the same direction. The heavier the crate, the more work done. But there are also differences in meaning, so we need to clarify what is meant by *work*. Although it may be income-earning work (non-technical meaning) for a porter to tote baggage at some distance above the ground, if the distance above ground is fixed no work (technical meaning) is done. This is because the suitcase is not displaced against the opposing force of gravity, though it is maintained at a fixed height against the pull of gravity. We have assumed that the suitcase alone is the system. If the person carrying the suitcase is made part of the system – and we are free to define the system however we like – then a sort of work, "physiological" work, is done in holding the suitcase at a fixed distance off the floor. Physiological work is done to maintain the muscle tension needed to hold the suitcase in place. This work results from transformation of chemical energy into mechanical energy in striated muscle (red meat) – a process that involves a lot of nerve impulses, the energy molecule ATP (Chapter 5), and polymerized actin and myosin (Chapter 8).

Another example will help to illustrate how work is done in coordinated movement against an opposing force. Pumping air into a bicycle tire is easy if the tire is flat; there is not much stuff in the tube to resist the flow of air in. Once the tube starts to fill, however,

and the pressure begins to build, it gets harder and harder to force air in. Here air in the pump is being moved in an organized way against the opposing force of the compressed air in the tire. During inflation, the tire expands somewhat but not much. This is because tires are made to adopt a certain shape, one well-suited to their function. The volume of the tire is approximately constant. Why does the pressure increase as you pump more air in? More and more molecules get stuffed into more or less the same volume.

Similarly, you may have been to a nightclub or concert, or been wearing a headphone, and found the music painfully loud. This is not merely a psychological effect: it comes from huge waves of air pressure smashing into your eardrum! Even in the middle of a grassy plain on a still day, where there is not a sound to be heard anywhere, the eardrum is in contact with the air. Nitrogen, oxygen, and carbon dioxide bombard it continually. We usually take no notice of this, because the same types of molecules are bombarding the eardrum from the opposite side with *equal* force. We detect a disturbance of the air as sound only when there is a pressure *difference* across the eardrum (a type of membranous boundary) and the difference is large enough. When a sound is so loud that it makes your ears hurt, it's because the pressure on the outside of your eardrum is far greater than the pressure on the inside, and to save your hearing it would be advisable to plug your ears or leave!

When particles of a system have reached *thermal equilibrium*, all the particles will have the same (average) thermal energy. In a gas, as we said above, some particles will be moving faster than others, but we can think of the like particles as having the same average speed. By the Zeroth Law, the temperature of each object in the system will be the same at equilibrium. What we're getting at here is that the *thermal energy* of a collection of particles is proportional to T, the *absolute* temperature. Suppose our system is a gas at thermal equilibrium. The gas particles move about freely within the system in all directions; particle movement is *disorganized*, or *random*. From time to time (very often!) a gas particle will collide with a wall of the container, the system boundary. The impact of such collisions will give rise to a pressure exerted by the particles on the contained. If we keep the volume constant but increase the number of particles in the container, the number of collisions between particles and boundary rises and the pressure is increased. If the volume and number of particles is constant but heat is added, the speed of the particles goes up and so does the temperature. Faster particles strike the walls of the system more often, increasing pressure. This descriptive view of temperature and pressure fits the simple mathematical relationship called the ideal gas law: $pV = nRT$, the pressure times the volume equals the number of moles of gas times the gas constant times the absolute temperature. This law has a long empirical foundation, and it can be derived from more basic principles. The ideal gas law is too simplistic to provide an accurate description of most real gases, but like many of the examples of elementary physics, it is none the less useful for

making sensible qualitative predictions about the behavior of matter under most of the conditions that most of us are likely to care about. We can leave the ideal gas law for now, but there will be reasons to pay it another visit later on.

What if we have a mixture of two different kinds of gas particles, a "light" gas like hydrogen and a "heavy" one like nitrogen? At thermal equilibrium, all the particles will have the same thermal energy. But will the helium molecule and nitrogen molecules bombard the walls of the container with equal force? No! Why not? Their masses are different. From physics the energy of motion of a particle, its *kinetic* energy (K.E.), is proportional to its mass times its velocity *squared*: K.E. $\propto mv^2$. (The direction of motion is not important here, so we can think of velocity as speed.) K.E. is a *non-linear* function of v. If the velocity of an object doubles, say, from 1 to 2 m s^{-1}, its K.E. quadruples. We can make the proportionality into an equality by including a multiplicative factor, in this case 1/2, but it's not needed for the present discussion. The *momentum* of a particle $\mathbf{p} = mv$. It is a *linear* function of v. The momentum of an automobile traveling at velocity v_1 is clearly much greater than that of a bicycle traveling at v_1. Historically, it took a good long while for physicists and philosophers to clarify the difference between K.E. and momentum and the relation of these quantities to energy and force, but we now think this is understood pretty well. By simple substitution K.E. $\propto \mathbf{p}^2/m$. Momentum matters to our present study because a change in momentum per unit time is proportional to a pressure. A change in momentum per unit time is a *force*, and a force per unit area is a *pressure*. In symbols, $\Delta\mathbf{p}/\Delta t = F = p/A$. At thermal equilibrium, the (average) kinetic energy of a particle is equal to the (average) thermal energy, so $\mathbf{p}^2/m \propto T$ at equilibrium. Solving this relationship for $\mathbf{p}$ gives $\mathbf{p} \propto (Tm)^{1/2}$. Thus, in our mixture of gases a "heavy" nitrogen molecule will have a greater average momentum than a "light" hydrogen molecule. Such ideas underlie all of physical biochemistry, and it will help us to keep them running in the background of our thinking as we make our way through the many avenues of our subject.

Before concluding this section, we want to introduce the concept of a heat engine. We'll give the heat engine the once-over-lightly treatment here and come back to it with vigor in Chapter 3. As shown in Fig. 2.4A, a heat engine does work by transferring heat from a source (e.g. a radiator) to a sink (e.g. a cold room). Only some of the heat transferred can be used to do work, because there is a fundamental limit on engine efficiency. (This limitation is a statement of the Second Law of Thermodynamics, as we shall see in Chapter 3.) The heat energy that is *not* used to do work enters the heat sink as randomized molecular motion. Work is energy transfer by ordered motion, heat is energy transfer by random motion. Note here how the First Law applies: the energy lost by the heat source (ΔU) is either converted into work (w) or transferred to the heat sink (q), and w and q must sum to ΔU. Figure 2.4B shows a diagram of a cell, say, an epithelial cell; it could just as well represent a tissue, organ or entire organism. The

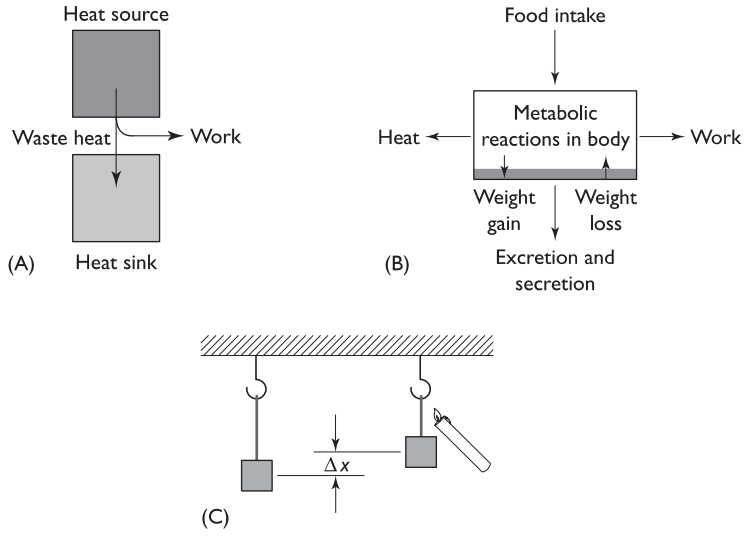

Fig. 2.4 Heat transfer. (A) Heat is transferred from a source (a warm body) to a sink (a cold body). Some of this heat can be used to do work, but certainly not all of it (Chapter 3). (B) Schematic representation of energy transformations within the body. The energy "input" is food. There are several "outputs." Aside from heat and work, which are mentioned explicitly in the First Law, there is excretion and change in body weight. In general, (food intake) – (waste excreted) = (change in body weight) + (heat) + (work), according to the First Law of Thermodynamics. (C) A very simple heat engine. The rubber band contracts when heated, lifting a weight. Because the weight is translated against the force of gravity, work is done. Thus, some portion of the heat is turned into work. The efficiency of this engine is low! Panel (C) is based on Fig. 44–1 of Feynman *et al.* (1963).

system of inputs and outputs resembles the situation in panel (A), but in panel (B) *everything is at the same temperature*. An organism is an isothermal system. Figure 2.4C shows how the heat energy of a candle can be used to do work. A rubber band dangles from a horizontal support, and attached to the rubber band is a weight (a mass accelerating under the force of gravity). When heat from the candle is absorbed by the molecules of the rubber band, the rubber band contracts. The attached weight is translated a distance Δx against the opposing force of gravity, and work w is done. Some of the heat of the candle will of course be lost to the surrounding air (this heat engine is rather inefficient), and only if adequate care is taken will the rubber not melt before our eyes, leaving no engine at all! Bearing all this in mind, let's take a closer look at how the First Law works.

D. The First Law in operation

By convention, the internal energy of a system will *increase* either by transferring heat *to* it or by doing work *on* it (Table 2.1). Knowing this, we can express the First Law of Thermodynamics as follows:

$$\Delta U = q + w. \tag{2.1}$$

Note that, in keeping with our earlier comments on measuring energy, the First Law defines only changes in ΔU. The conceptual background to Eqn. (2.1) was formulated in 1847 by the eminent German physicist and physiologist Hermann Ludwig Ferdinand von Helmholtz (1821–1894).[8] The idea of energy conservation had been

[8] See *Ueber die Erhalting der Kraft* (Berlin: Reimer, 1847). Helmholtz was son of a teacher of philosophy and literature at the Potsdam Gymnasium, a top-grade secondary school; his mother was descended from William Penn, a Quaker who

Table 2.1. *Sign conventions for heat and work*

Heat is transferred to the system	$q > 0$
Heat is transferred to the surroundings	$q < 0$
The system expands against an external pressure	$w < 0$
The system is compressed because of an external pressure	$w > 0$

proposed in 1842 by the German physiologist Julius Robert von Mayer (1814–1878).[9] It is interesting that a physiologist played such an important role in establishing one of the most important concepts of thermodynamics. When a system does work on its surroundings, w makes a negative contribution to ΔU because the system loses energy. Similarly, if heat is lost from the system, q makes a negative contribution to ΔU. In other words, ΔU measures the *net* amount of energy change in the system; it is the difference between the energy gained from the surroundings and the energy lost to the surroundings.

Let's look at some examples of Eqn. (2.1) in action. James Prescott Joule (1818–1889), son of a brewer in Manchester, England, is famous for his studies on the conservation of thermal energy understood as the mechanical equivalent of heat (1843). Perhaps the best-known experiment Joule did was to monitor the temperature of a vat of water during stirring. In this experiment, increases in water

was expelled from Oxford University for refusing to conform to Anglicanism and who later founded the American Commonwealth of Pennsylvania. Helmholtz's earned degree was in medicine; he would eventually receive an honorary doctorate in physics. Music and painting played a large part in his science. The present author, who was a Ph.D. student at Johns Hopkins University and a post-doctoral research fellow at University of Oxford, is connected to Helmholtz in various ways. Helmholtz's student Otto Richard Lummer (1860–1925) was awarded the doctorate in physics at the Humboldt University of Berlin. And Lummer was thesis adviser of George Ernest Gibson, a Scot, at University of Breslau, who was the adviser of Henry Eyring (see Chapter 8) in the Department of Chemistry at University of California at Berkeley, who was adviser of *I* in the Department of Chemistry at University of Utah, who was thesis adviser of *J* in the Department of Chemistry at University of Minnesota, who was thesis adviser of *K* in the Department of Pharmacology at University of Virginia, who was thesis adviser of the author. Johns Hopkins University was founded in 1876 by a wealthy Quaker merchant on the German model, which emphasized specialized training and research. The person who advised Helmholtz's medical thesis was Johannes Peter Müller, son of a shoemaker. Müller's academic degree was in medicine. His *Handbuch der Physiologie des Menschen für Vorlesungen* was recognized throughout the world, and it established a positive interchange between physiology and hospital practice in Germany, stimulated further basic research, and became a starting point for the mechanistic concept of life processes.

[9] The conservation of *mechanical* energy (kinetic energy + potential energy = constant) had in fact been proposed much earlier, by the German philosopher and mathematician Gottfried Wilhelm Leibniz (1646–1716), son of a professor of moral philosophy, and was an accepted principle of mechanics. Mayer's more general statement stemmed, curiously, from an analysis of the color of blood – a key means of distributing food energy throughout the body.

temperature represent positive increments in q, the heat transferred to the system. A motor turns a wheel in contact with water. The system is the water plus the wheel. As the wheel turns, mechanical energy is converted into increased motion of the water, and as we have seen, the motion of water is related to its temperature. Individual water molecules collide with the wheel and with each other. Vigorous and protracted stirring could eventually bring the vat of water to the boil. The *system* does no work; it does not expand against an opposing pressure or anything like that, so $w = 0$ and $\Delta U = q$. As a second example, suppose we have a motor. Suppose it has been determined that the motor generates 30 kJ of mechanical work per second, and that 9 kJ is lost to the surroundings as heat in the same amount of time. The change in internal energy of the motor per second is $-9\,\text{kJ} - 30\,\text{kJ} = -39\,\text{kJ}$. The energy produced by the motor is negative because work is done by the system on the surroundings and heat is lost to the surroundings. OK, but we also want to see how these ideas can be applied to something biological.

Above we saw that no work is done in holding up a heavy crate when the person is excluded from the system. And even when the person is included, no physical work is done, just physiological work. And if the energy expended in holding up the crate is not replenished by food, there will be a net decrease in the internal energy of the body. Our bodies do work even when we're sleeping! When you touch a metallic door handle on a wintry day, unless you have gloves on you can feel the heat being transferred from your flesh to the metal, and it might hurt! This heat transfer makes a negative contribution to the internal energy of the body, and the energy used to keep the body warm comes from food. When you walk up a flight of stairs, you do work against the force of gravity. If there are many steps to climb, as for instance on the way up to the cupola of Santa Maria del Fiore in Florence, or to Lady Liberty's torch in the Big Apple, by the time you've reached the top you may well be out of breath and dripping with perspiration. But if you're captivated by the view, you will not mind too much! Not only will you have lost energy to move your weight against Earth's gravitational pull on your body, you will be losing a lot of heat energy to the surroundings to keep your body cool. In any case, the energy used to climb stairs and the energy lost as heat comes from food.

We can be more quantitative about work with relatively little additional effort. From physics, the work done when an object is displaced a distance Δx ($x_{\text{final}} - x_{\text{initial}}$, where x refers to position) against an *opposing* force (hence the minus sign) of constant magnitude F is calculated as

$$w = -F\Delta x. \tag{2.2}$$

We see that work is the product of an "intensity factor" that is independent of the size of the system (the force) and a "capacity factor" (the change in the position of the object on which the force acts). For instance, the work done against gravity by a 50 kg woman in climbing

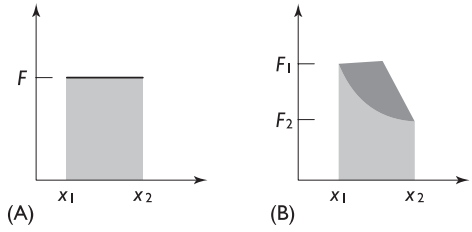

Fig. 2.5 Graphical representation of work: $|w| = F\Delta x$, where $|w|$ means "the absolute magnitude of w." For example, $|-3| = 3$. (A) For a constant force, the magnitude of work is the area $F \times \Delta x$. (B) If the force is variable, $|w|$ can no longer be calculated simply as $F \times \Delta x$. The figure illustrates why w cannot be considered a state function: its value depends on the path. See Fig. 2.3. The graphical method of calculating the work done by a system is said to have been introduced by James Watt.

to a point on a ladder 4 m above the ground is $-(50\,\text{kg} \times 9.8\,\text{m s}^{-2}) \times 4\,\text{m} = -1960\,\text{kg m}^2\,\text{s}^{-2} = -1.96\,\text{kJ}$. (Note: $1\,\text{J} = 1\,\text{kg m}^2\,\text{s}^{-2}$. Oddly, the dimensions of energy are [mass][length]^2[time]$^{-2}$.) The minus sign indicates that energy has been expended by the system, in this case, the woman. Diagrams help visualize the situation. The work done in Eqn. (2.2) can be represented graphically as an *area*, as shown in Fig. 2.5A. Figure 2.5B shows that the work done during a process depends on the path, because the shaded area need not be the same for all processes. This is a way of depicting that w is a path function; its magnitude depends on the path.

When a piston in a cylinder moves against a *constant* external pressure p_{ex} (as for instance in Fig. 2.6), the work done is

$$w = -p_{ex}\Delta V, \tag{2.3}$$

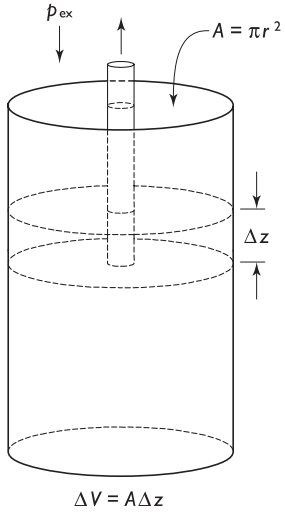

Fig. 2.6 Gas-filled cylinder. There is a constant external pressure, p_{ex}. For a change in position of the piston, Δz, there is a corresponding change in volume, ΔV. The work done *by* the system is $w = -p_{ex}\Delta V$. If ΔV is negative, if the gas in the cylinder is compressed, there is a positive contribution to ΔU.

where ΔV represents the change in the volume of the *system*; and $p_{ex} = nRT/V$, to the extent that whatever is pushing on the system can be modeled as an ideal gas. This type of work is called *pV*-work. Again, the work done is the product of an "intensity factor" (p_{ex}) and a "capacity factor" (ΔV). If the volume *of the system* increases, $\Delta V > 0$; the energy for expansion against an opposing force comes from the system itself, so the work done is negative. If there is no external pressure (if the surroundings are vacuum), then $p_{ex} = 0$; there is no force to oppose expansion of the system, and no work is done as V increases. Both p and V are state variables: they specify the state of the system.

E. Enthalpy

Another thermodynamic state function we need to know about is the enthalpy, H. It is covered in this book for several reasons, the most important one being that H is a component of the Gibbs free energy (Chapters 4 and 5). The term *enthalpy* is from the Greek *enthalpein*, to warm in, and it was coined around 1850 by the German physicist Rudolf Julius Emanuel Clausius (1822–1888), son of a pastor and schoolmaster. The enthalpy is the heat absorbed by a system at constant pressure (subscript "p"). Let's suppose we are working under constant pressure. Rewriting the First Law in terms of q, we have

$$q_p = \Delta U - w. \tag{2.4}$$

When the pressure is constant and the system expands from state 1 to state 2, the system does work on the surroundings. If the only type of work is pV-work, Eqn. (2.4) becomes

$$q_p = U_2 - U_1 + p\ (V_2 - V_1). \tag{2.5}$$

We can rearrange Eqn. (2.5) as

$$q_p = (U_2 + pV_2) - (U_1 + pV_1) = \Delta U + p\Delta V. \tag{2.6}$$

The complicated quantity $\Delta U + p\Delta V$ is equivalent to the heat exchanged at constant pressure. The right-hand side of Eqn. (2.6) is a state function, called the enthalpy, H:

$$H = U + pV. \tag{2.7}$$

Equation (2.7) can seem confusing and abstract; we need a way of making things fit better with what we have said already and our everyday experience of the world. We said above that $w = -p_{ex}\Delta V$ is a *path* function. But how can a state function plus a path function equal a state function? It is precisely because, although the product of p and V is a path function, p and V themselves specify the state of the system, and like U, they are independent of how that state was reached. H is therefore a state function, and it is no less or more of a state function than U is. And the development leading up to Eqn. (2.7), e.g. Eqn. (2.6), says that if the pressure is constant, the amount of heat exchanged during a reaction is independent of whether the product is formed directly or indirectly, in one or in a series of steps.[10] This statement, which is based on the results of experiments, says that state functions and state function differences (e.g. ΔU or ΔH) are additive (compare Fig. 2.3).

Looked at another way, the enthalpy can be thought of as the amount of energy *in* a thermodynamic system for *transfer* between itself and the environment. For example, in the calorimetry experiments in Chapter 1, the change in enthalpy was (very close to) the heat of oxidation, which was the energy transferred from the oxidized compounds to the calorimeter. When a system changes *phase*, for example, when a quantity of liquid water becomes solid, the change in enthalpy of the system is the "latent heat" of fusion, the heat given off to the environment in the freezing process. And in a temperature change, for example, the cooling off of a food item when placed in a fridge, the change in the enthalpy per unit temperature reflects a property of the material.

Let's see what happens when the enthalpy varies by a small but measurable amount. From Eqn. (2.7) we have

$$\Delta H = \Delta(U + pV) = \Delta U + \Delta(pV) = \Delta U + p\Delta V + V\Delta p. \tag{2.8}$$

[10] This is known as the "law of constant summation" of the Swiss–Russian chemist Germain Henri Hess (1802–1850), a physician and chemist. Hess's Law restates the First Law, though historically the former preceded the latter.

Note that p and V are assumed to vary independently. If the external pressure is constant, $\Delta p = 0$ and the last term vanishes. Substituting in Eqn. (2.1) and requiring pV-work only gives

$$\Delta H = q_p - p\Delta V + p\Delta V. \tag{2.9}$$

The last two terms on the right-hand side cancel, and we are left with

$$\Delta H = q_p. \tag{2.10}$$

Just as we said, the heat transferred to a system at constant pressure measures the change in the enthalpy of the system. Why the emphasis on heat transfer at constant pressure in a book on biological thermodynamics? Most of the reactions biochemists study are carried out at constant pressure (usually 1 atm), and as we shall see in Chapter 4, H is a component of a state function known as the Gibbs free energy, G, which predicts the direction of spontaneous change for a process at constant pressure and temperature, the biological scientist's favorite experimental constraints.

How can we *understand* the difference between ΔH and ΔU? Equations presented above make them out to be quite different, but the discussion about them sounds quite similar. In fact, the difference between ΔH and ΔU is often small enough to be neglected, but not always. If a reaction occurs in solution, and gas is neither produced nor consumed, $\Delta V \approx 0$. Under such circumstances $\Delta U \approx q_p$, as we can see from Eqn. (2.5), and so $\Delta U \approx \Delta H$. An example will help to illustrate that as a general rule it is a mistake not to take account of differences when there are reasons to suspect they might be significant. From Eqn. (2.7),

$$\Delta H = \Delta U + \Delta(pV). \tag{2.11}$$

The ideal gas law is $pV = nRT$, so assuming that the gas involved in our experiment can be modeled as an ideal gas, Eqn. (2.11) can be written as

$$\Delta H = \Delta U + \Delta(nRT). \tag{2.12}$$

If we now require constant temperature, $\Delta(nRT) = RT(\Delta n)$, where Δn represents the change in the number of moles of gas in the reaction; R, the universal gas constant, is 8.3145 J K^{-1} mol^{-1} in SI units (1.9872 cal K^{-1} mol^{-1} is also still in use); and T is the absolute temperature.

To illustrate, let's express the combustion of ethanol as:

$$C_2H_5OH(1) + 3O_2(g) \rightarrow 2CO_2(g) + 3H_2O(1). \tag{2.13}$$

From a bomb calorimetry experiment at 298 K and constant volume, 1368 kJ mol^{-1} of heat are released in the reaction. Now, $\Delta n = 2 - 3 = -1$. Therefore, by Eqn. (2.12), $\Delta H(298\,K) = \Delta U(298\,K) - RT = -1\,368\,000\,J\,mol^{-1} - 2480\,J\,mol^{-1} = -1370\,kJ\,mol^{-1}$. This is a small difference between ΔH and ΔU – less than 1%, i.e. ΔH is approximately equal to ΔU for beer, wine, and other such beverages – but it is a difference. We learn from this example that, although the

oxidation heats of Chapter 1 are changes in internal energy, they are very close to the corresponding changes in enthalpy. Check whether the combustion data you use for calculations do or do not take the pV term into account! A process for which the change in enthalpy is negative is called *exothermic*, as heat is let out of the system into the surroundings; a process for which the change in enthalpy is positive is called *endothermic*, as heat is let into the system from the surroundings.

Combustion of food in a bomb calorimeter tells us more than just how much heat is produced when food is completely burnt to a crisp. Indeed, tables of oxidation would be of little use to nutritionists if the numbers did not say something about the energetics of metabolism. Such tables are useful to the physicist, the biochemist, and the nutritionist because the laws of physics are assumed to be independent of time and location. In other words, the enthalpy of oxidation of glucose is not one value in a bomb calorimeter and some other value in the striated muscle connecting your big toe to the rest of your body. By Hess's Law, this enthalpy equivalence holds despite the fact glucose oxidation occurs in the body by a large number of sequential steps involving a large number of chemical intermediates. This discussion suggests that we can use machines like calorimeters to investigate the thermodynamic properties of the body and the molecules the body is made of. It also suggests that our bodies themselves are very much like machines.

Finally, suppose we have a system of pure water. We know from careful measurements that when ice melts at $+0.1\,^{\circ}\mathrm{C}$, barely above the melting temperature, $\Delta H = 1437.2\,\mathrm{cal\ mol^{-1}}$. When melting occurs at $-0.1\,^{\circ}\mathrm{C}$, just below the freezing point, $\Delta H = 1435.4\,\mathrm{cal\ mol^{-1}}$. The difference in enthalpy differences, $\Delta(\Delta H)$, is $1.8\,\mathrm{cal\ mol^{-1}}$. This is only about *half* the enthalpy change we would expect on changing the temperature of water by $0.2\,^{\circ}\mathrm{C}$ *in the absence of melting*. (See Section H below.) The difference arises from the change of phase that occurs between the initial state and final state, the melting of the solid into a liquid. It is necessary to account for the heat effect of any changes in the state of matter (solid, liquid or gas) when calculating ΔH.

F. Standard state

Changes in enthalpy (and other state functions) in tables of thermodynamic data are generally given for processes occurring under a standard set of conditions. The *standard state* is usually defined as one mole of a pure substance at 298.15 K (25.00 °C) and 1 bar ($1\ \mathrm{bar} = 10^5\,\mathrm{Pa} = 0.986\ 932\,\mathrm{atm}$). An example is the standard enthalpy change accompanying the conversion of pure solid water to pure liquid water at the melting temperature and a pressure of 1 bar:

$$\mathrm{H_2O(s)} \rightarrow \mathrm{H_2O(l)} \quad \Delta H^{\ominus}(273\,\mathrm{K}) = 6.01\ \mathrm{kJ\ mol^{-1}}. \tag{2.14}$$

Note that this enthalpy change is *positive*: heat must be added to ice at 0 °C in order to melt it. The standard enthalpy change used by the biochemist, ΔH°, is the change in enthalpy for a process in which the initial and final states of *one mole* of a substance in pure form are in their standard state: 25 °C and 1 atm pressure. The difference in enthalpy from the difference between 1 bar and 1 atm is almost always small enough to be neglected in biochemical reactions. But one should nevertheless be aware of the different ways in which thermodynamic data of use to the biochemist are presented in tables and be on the lookout for situations where the small differences cannot be neglected.

G. Some examples from biochemistry

Equation (2.10) is useful to the biochemist. As we have seen, it helps to make sense of the oxidation heats measured by bomb calorimetry. It can also be used in the study of protein stability, an important subject for several reasons. One is that about half of the dry mass of the human body is protein, and knowing how a polypeptide folds up into its native state would be of tremendous value in making good use of all the protein-encoding DNA sequence data that has been revealed by the Human Genome Project.

How can Eqn. (2.10) be used to study the thermodynamic properties of proteins (or of nucleic acids)? The native state of a protein is like an organic crystal. It is fairly rigid, and held together by a large number of different kinds of "weak" non-covalent interactions, including hydrogen bonds (largely electrostatic in character), van der Waals interactions[11] and "salt bridges" (electrostatic attractions between ionized amino acid side chains) (Tables 2.2 and 2.3). A native protein is "folded." In the "core" of a folded protein, apolar amino acid side chains interdigitate and are tightly packed, forming rigid and specific contacts. The rigidity of a folded protein is important to its biological function and, in favorable circumstances, permits determination of its structure at atomic resolution. This is not to say that a folded protein exhibits no fluctuations of structure or rotations of bonds. Native protein structure certainly does fluctuate, as we know for example by nuclear magnetic resonance studies, and such fluctuations can be important in the binding of small compounds to macromolecules (Chapter 7) and to enzyme function (Chapter 8). But the folded state of a typical protein is nevertheless quite rigid. In contrast, the unfolded state of a protein is more flexible and fluid-like. Bonds in amino acid side chains rotate relatively freely in an unfolded protein, and in the

[11] Named after the Dutch physicist Johannes Diderik van der Waals (1837–1923). Van der Waals was awarded the Nobel Prize in Physics in 1911.

Table 2.2. *Energetics of non-covalent interactions between molecules*

Type of interaction	Equation	Approximate magnitude (kcal mol^{-1})
Ion–ion	$E = q_1 q_2 / Dr$	14
Ion–dipole	$E = q\mu\theta / Dr^2$	−2 to +2
Dipole–dipole	$E = \mu_1 \mu_2 \theta' / Dr^3$	−0.5 to +0.5
Ion–induced dipole	$E = q^2\alpha / 2Dr^2 r^4$	0.06
Dispersion	$E = 3h\nu\alpha^2 / 4r^6$	0 to 10

[a] Charge q_1 interacts with charge q_2 at a distance r in medium of dielectric D.
[b] Charge q interacts with dipole μ at a distance r from the dipole in medium of dielectric D. θ and θ' are functions of the orientation of the dipoles.
[c] Dipole μ_1 interacts with dipole μ_2 at an angle q relative to the axis of dipole μ_2 and a distance r from the dipole in medium of dielectric D.
[d] Charge q interacts with molecule of polarizability at α distance r from the dipole in medium of dielectric D.
[e] Charge fluctuations of frequency ν occur in mutually polarizable molecules of polarizability α separated by a distance r.
The data are from Table 1.1 of van Holde (1985).

Table 2.3. *Characteristics of hydrogen bonds of biological importance*

Bond type	Mean bond distance (nm)	Bond energy (kJ mol^{-1})
O–H···O	0.270	−22
O–H···O^-	0.263	−15
O–H···N	0.288	−15 to −20
N^+–H···O	0.293	−25 to −30
N–H···O	0.304	−15 to −25
N–H···N	0.310	−17
HS–H···SH_2	—	−7

The data are from Watson (1965).

ideal case all amino acid side chains are completely exposed to solvent (Table 2.4).

The non-covalent interactions that stabilize folded protein structure (or double-stranded DNA or folded RNA structure) can be "broken" in a number of ways. One is by adding heat. If all the non-covalent bonds break simultaneously, in an all-or-none fashion ("cooperative" unfolding), then there are in essence just two states of the protein: the folded state and the unfolded state. The *transition* from the folded state to the unfolded state is like melting. So inducing the unfolding of protein by heat or some other means is something like melting a solid. This is true even if one is working not with a mass of freeze-dried protein but with folded proteins dissolved in aqueous solution. The *cooperativity* of the transition, the all-or-none character of going from being folded to being unfolded,

Table 2.4. *Principal features of protein structure*

Folded (native) state	Unfolded (denatured) state
Highly ordered polypeptide chain	Highly disordered chain – "random coil"
Intact elements of secondary structure, held together by hydrogen bonds	No secondary structure
Intact tertiary structure contacts, as in an organic crystal, held together by van der Waals interactions	No tertiary structure
Limited rotation of bonds in the protein core	Free rotation of bonds throughout polypeptide chain
Desolvated side chains in protein core	Solvated side chains
Compact volume	Greatly expanded volume

results from the concurrent breaking of a large number of weak interactions. In water, these interactions are hydrogen bonds; in proteins, they are the several kinds mentioned above. The melting of pure water or any other pure solid is a cooperative phenomenon. That is, melting occurs at a single or over a very narrow range of temperatures, not over a broad range. The same is true of cooperative protein unfolding or the melting of DNA.

A number of experimental studies have been carried out to measure the energy required to break a hydrogen bond at room temperature. This is pertinent not only to the unfolding of proteins but also to the "melting" of double-stranded DNA, which is held together by hydrogen bonds. Estimates of the bond energy vary, but a reasonable and generally agreed rough figure is 1 kcal mol^{-1}. Individual hydrogen bonds are weak; collections can be quite strong.

In terms of Eqn. (2.10), the enthalpy of the folded state of a protein is H°_F, the enthalpy of the unfolded state is H°_U, and the difference, $H^\circ_U - H^\circ_F$, is the enthalpy of denaturation or unfolding, ΔH°_d. In this case the folded state of the protein is the *reference state*, as the enthalpy of the unfolded state is measured with respect to it. What is this enthalpy difference? As discussed above, the enthalpy change for a process is equal to the heat absorbed by the system at constant pressure, and the rigid folded state of a protein can be pictured as a solid, and the flexible unfolded state as a liquid. So the enthalpy difference between the unfolded and folded states of a protein is the amount of heat needed to unfold the protein. As we shall see, the magnitude of that heat depends on the temperature.

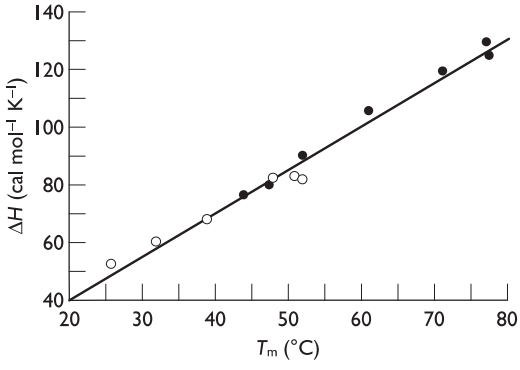

Fig. 2.7 Enthalpy of unfolding of hen egg white lysozyme as a function of transition temperature. Filled symbols: intact lysozyme. Open symbols: lysozyme in which one of the four native disulfide bonds has been removed. When folded, 3-SS lysozyme closely resembles the native state of intact lysozyme. Change in transition temperature was induced by a change of pH. Note that ΔH is approximately linear in T_m. The data are from Cooper *et al.* (1991).

The temperature at which a protein unfolds (or double-stranded DNA melts) is called the melting temperature, T_m. This temperature depends not only on the number and type of non-covalent bonds in the folded state but also on the pH and other solution conditions. T_m also depends on the pressure, but most biological science experiments are done at 1 atm pressure. In the case of proteins, changing the pH of solution changes the net charge on the protein surface. This can have a marked impact on T_m and ΔH°_d, as shown in Fig. 2.7 for the example of hen egg white lysozyme, a well-studied small globular protein. The figure also illustrates that the slope of ΔH° against T_m for this protein is more or less constant throughout the pH range shown.

Above we saw how a bomb calorimeter can be used to obtain thermodynamic information. Here we introduce isothermal titration calorimetry (ITC)[12] and explain how it can be used to measure the enthalpy of a biochemical process (Fig. 2.8). By Eqn. (2.10) the heat absorbed at constant pressure measures the enthalpy change. Suppose, for example, we are interested in the energetics of the binding of the F_c portion of immunoglobulin G (IgG), important in humoral immunity and biotechnology, to soluble protein A, a bacterial protein. We need not be concerned at the moment just which part of IgG the F_c portion of is: we just need to know that antibody molecules can be dissected into components and that the F_c portion is one of them. The thermodynamic states of interest here are the unbound state, where protein A is free in solution, and the bound state, where protein A is physically associated with F_c. The heat exchanged at constant pressure upon injection of protein A into a calorimetric cell containing the antibody can thus be used to determine ΔH°_b, the enthalpy of binding (under standard state conditions). The heat of injection will change as the number of vacant binding sites decreases.

[12] The isothermal titration calorimeter was first described in 1922 by Théophile de Donder, founder of the Brussels School of thermodynamics.

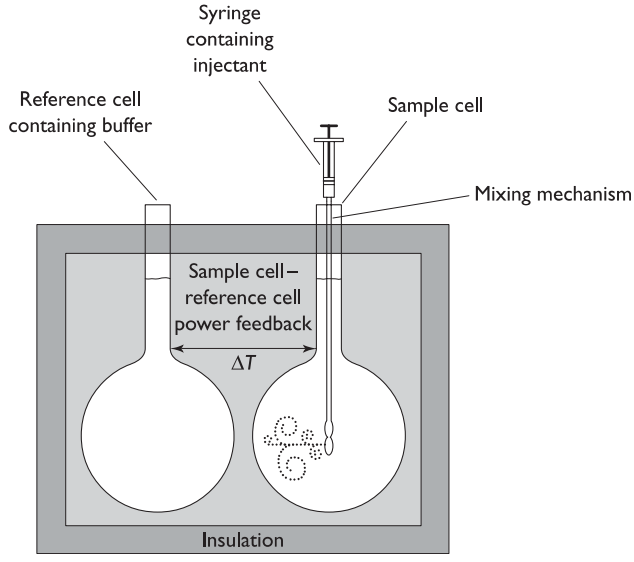

Fig. 2.8 Isothermal titration calorimeter. The temperature is constant. There are two identical chambers, the sample cell and the reference cell. In most cases, the sample cell will contain a macromolecule, and the syringe/stirrer is used to inject a ligand into the sample cell. The syringe is usually coupled to an injector system under software control and rotated at a constant speed. The reference cell is filled with buffer; no reaction occurs there. ΔT measures the temperature difference between cells, which are surrounded by insulation to minimize heat exchange with the surroundings. Electronic (power feedback) circuitry minimizes ΔT on a continuous basis. If injection of ligand results in binding, there will ordinarily be a change in the temperature of the sample. The sign of the change will depend on whether the reaction is exothermic or endothermic. An experiment consists of equal-volume injections from the syringe into the sample cell.

What if we're interested in the energetics of an enzyme binding to its substrate? This can be measured if a suitable substrate analog can be found or the enzyme can be modified. For instance, ITC has been used to measure the enthalpy of binding of a small compound called 2′-cytidine monophoshate (2′CMP) to ribonuclease A, which hydrolyzes RNA to its component nucleotides. 2′CMP binds to and inhibits the enzyme. If the enzyme of interest is, say, a protein phosphatase with a nucleophilic cysteine in the active site, mutation of the Cys to Ser or Asn will abolish catalytic activity, as in the N-terminal domain of the cytoskeleton-associated protein tensin, and the energetics of binding can be studied. A deeper understanding of binding will be sought in Chapters 5 and 7.

If you've spent any time in a biochemistry lab, you may have experienced the large heat given off by a salt solution as the salt dissolves. There are several contributions to the effect, but the main one is the enthalpy of hydration. This is the enthalpy change that occurs when an ion in vacuum is dropped into a sea of pure water. Water molecules form what is called a hydration shell around the ion, the number depending on the radius of the ion and its charge. Calorimetry can be used to measure the hydration enthalpy of biologically important ions. Values are given in Table 2.5. Why is this important? In one example, some of the water molecules hydrating an ion must be stripped away before the ion can pass through a selective ion channel in the plasma membrane, and this requires an input of energy. Complete dehydration of the ion would require a very large input of energy, so it is easy to imagine that a few water molecules remain associated with an ion as it passes through a pore. Ion channels that are specific for the passage of certain types of ion are part of the molecular machinery underlying the transmission of nerve impulses.

Table 2.5. *Standard ion hydration enthalpies*

H^+	−1090	Mg^{2+}	−1920
Li^+	−520	Ca^{2+}	−1650
Na^+	−405	Ba^{2+}	−1360
K^+	−321	Fe^{2+}	−1950
—	—	Zn^{2+}	−2050
NH^{4+}	−301	Fe^{3+}	−4430

The data refer to $X^+(g) \rightarrow X^+(aq)$ at 1 bar and are from Table 2.6c in Atkins (1998). 1 bar = 10^5 Pa = 10^5 N m^{-2} = 0.987 atm. 1 Pa = 1 pascal. Blaise Pascal (1623–1662) was a French scientist and religious philosopher.

H. Heat capacity

Above we noted that the heat taken up or released per unit change in temperature from a material at constant pressure is a property of that material. The name of this property is the heat capacity at constant pressure, C_p.[13] This quantity is readily measured, and it can be used to calculate changes in the enthalpy. The heat capacity per unit mass of Coke, for example, which is mostly water, differs from the heat capacity per unit mass of an egg, which contains a large amount of protein; the amount of heat energy that must be extracted from 1 g of each in order to lower the temperature by 1 degree K will not be the same in the two cases. The heat capacity tells us how much energy is in the system for transfer between itself and the environment *per degree*.

It is evident from Fig. 2.7 that the enthalpy of a protein rises as its temperature is increased. This is true of substances in general. The numerical relationship between H and T, however, depends on the conditions. We concern ourselves here with constant pressure only. The slope of a graph of H versus T at constant pressure is the heat capacity at constant pressure.

We are all familiar with heat capacity, even in the absence of a formal introduction. Returning to our water-in-a-saucepan example, if the water is heated at a high enough rate, it will eventually boil. The amount of heat that must be added to increase the temperature of 1 g of a substance by 1 degree K is the *specific heat capacity*. At 1 atm pressure, the heat capacity of liquid water varies only very slightly with temperature over the range 0–100 °C. This comes from the bonding structure of water (Fig. 2.9). Just as the extent of motion changes substantially when a quantity of water freezes or vaporizes, the heat capacity of water depends substantially on its state. This is true of substances in general. But the number of hydrogen bonds

[13] The heat capacity per unit mass of material, or specific heat, was first described in detail by Joseph Black.

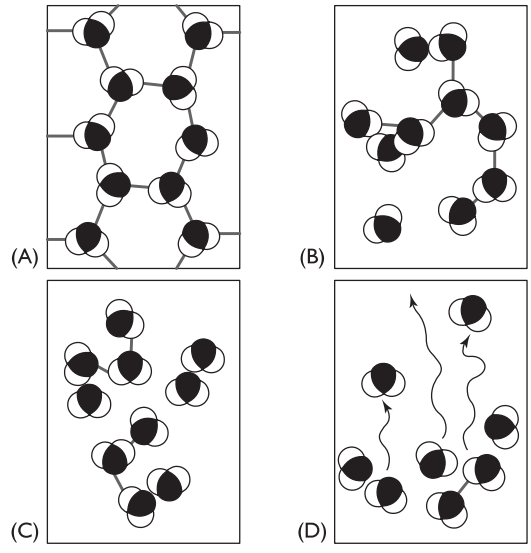

Fig. 2.9 Schematic diagram of the structure of water under different conditions: (A) solid state, (B) melting point, (C) liquid state, and (D) boiling point. Oxygen is shown in black, hydrogen in white. The black bars represent hydrogen bonds. Hydrogen bonds are relatively persistent in the solid state. The number of bonds decreases at the melting point, as molecules move out of the lattice. In the liquid state, hydrogen bonds are present, but they are formed only transiently. Boiling water has such a high thermal energy that persistent hydrogen bonds are rare. As the temperature increases, there are increases in translational, vibrational and rotational energy. The change in translational energy is not very difficult to detect; for example, when water is brought to the boil on the stove. Increases in vibrational and rotational motion of water cannot be seen with the naked eye. Based on Fig. 3.3 in Voet and Voet (1995).

formed by an individual water molecule is roughly constant throughout the temperature range 0–100 °C at 1 atm pressure. A quantity of water vapor can be much hotter than 100 °C, and water vapor must lose a good deal more energy than liquid water to fall in temperature by 1 degree K. This makes steam hazardous for people, but it reduces the time to cook the claw muscle of Chesapeake Bay blue crabs.

With regard to purified proteins in solution, just as the folded and unfolded states have different enthalpies, they also have different heat capacities. The heat capacity of the folded state is $C_{p,F}$, while that of the unfolded state is $C_{p,U}$. The heat capacity difference between states at constant pressure is $C_{p,U} - C_{p,F} = \Delta C_{p,d}$. In principle, $C_{p,F}$, $C_{p,U}$ and therefore $\Delta C_{p,d}$ are temperature-dependent. In practice, however, the variation with temperature can be and often is sufficiently small to be ignored. An example of a case where $\Delta C_{p,d}$ is only slightly dependent on temperature is shown in Fig. 2.7. That $\Delta C_{p,d}$ is positive is related to the increase in hydrophobic surface that is in contact with the solvent. The side chains of the hydrophobic core are largely sequestered from the solvent in the folded state.

Now we are in a position to write a general expression for the enthalpy of a substance as a function of temperature. It is

$$H(T_2) = H(T_1) + C_p(T_2 - T_1), \tag{2.15}$$

where T_1 is the temperature of the system in state 1 and $H(T_2)$ is the enthalpy of the system in state 2. Another way of writing Eqn. (2.15) is

$$\Delta H = C_p \Delta T, \tag{2.16}$$

where $\Delta H = H(T_2) - H(T_1)$ and $\Delta T = T_2 - T_1$. Note that ΔH would have the same magnitude but the opposite sign if the labels attached to the

states were reversed; the enthalpy is a state function. From a mathematical point of view Eqn. (2.16), which can be written $C_p = \Delta H/\Delta T$, tells us that the constant pressure heat capacity can be obtained from a plot of H versus T in the interval ΔT. When C_p is constant throughout the temperature range, H versus T will be constant. As we have said, C_p is effectively constant over small temperature ranges for many materials in the absence of a change of phase. But the unfolding of a protein can be described as a phase change (melting of a solid), and we should therefore expect that there will be a difference in heat capacity between the folded and unfolded states. The corresponding expression to Eqn. (2.8) for the enthalpy difference between the unfolded and folded states of a protein is

$$\Delta H_d^\circ(T_2) = \Delta H_d^\circ(T_1) + \Delta C_{p,d}(T_2 - T_1), \tag{2.17}$$

where the heat capacity change is independent of temperature. Equations (2.15) and (2.17) apply to many different situations (not just protein folding/unfolding!) and are known as Kirchhoff's enthalpy law, after the German physicist Gustav Robert Kirchhoff (1824–1887).

One way of determining $\Delta C_{p,d}$ for protein unfolding is to denature the protein under different conditions. A common method is to measure ΔH_d° and T_m for different values of pH, as shown in Fig. 2.7. This can be done with a technique called differential scanning calorimetry (DSC), which measures the heat absorbed as a function of temperature (Fig. 2.10). The experiment is repeated at a variety of pH values to generate a curve like that shown in Fig. 2.7. As we shall see in Chapter 5, the relatively large $\Delta C_{p,d}$ of protein unfolding has a big impact on how much work must be done to unfold a protein, and how this amount of work depends on temperature.

Box 2.2. A micromachined nanocalorimeter for life sciences research and diagnostics

Receptors are membrane-embedded protein molecules that recognize and respond to the body's own chemical messengers, for example, hormones or neurotransmitters. In pharmacology, receptor affinity and efficacy together determine the potency of a drug. Differences in efficacy make a drug an agonist or antagonist. A drug of sufficient efficacy and affinity for a receptor to affect cell function is an agonist. A drug that binds the receptor but does not elicit a response is an antagonist. Pre-clinical screening of agonists and antagonists and assessment of the toxicity of novel lead compounds is generally done with specialized cell-based assays. Often, a specific cell line is required. An understanding of the nature of the molecules and cellular pathways involved is generally needed to interpret the results of such assays, and therefore a substantial investment of time and money. Close monitoring of cell temperature could provide a means of detecting changes in cell metabolism that are not stimulus-specific, enabling simpler, less expensive and more general cell-based screening than with specialized cell-based assays. Researchers in the Department of Electronic and Electrical Engineering at University of Glasgow have developed a

Box 2.2. Cont.

micromachined nanocalorimeter which functions as a biosensor. A small number of living cells are present in a sub-nanoliter chamber. The small size of the chamber could be useful for rapid screening of small samples. The sensor comprises a 10-junction gold and nickel thermopile on a silicon chip. A thermopile is a number of thermocouples, 10 in this case, connected end on end, and a thermocouple is simply a temperature-measuring device consisting of two wires of different metals fused at each end. A temperature difference between the metals results in a difference in an electrical potential, which can be calibrated to a temperature. The nanocalorimeter of the Glasgow group can detect a mere 13 nW of power generated by the cells on exposure to a chemical stimulus, the temperature resolution is 0.125 mK, the heat capacity is 1.2 nJ mK^{-1}, and the response time is 12 ms. Primary cell lines or tissue biopsies can be analyzed.

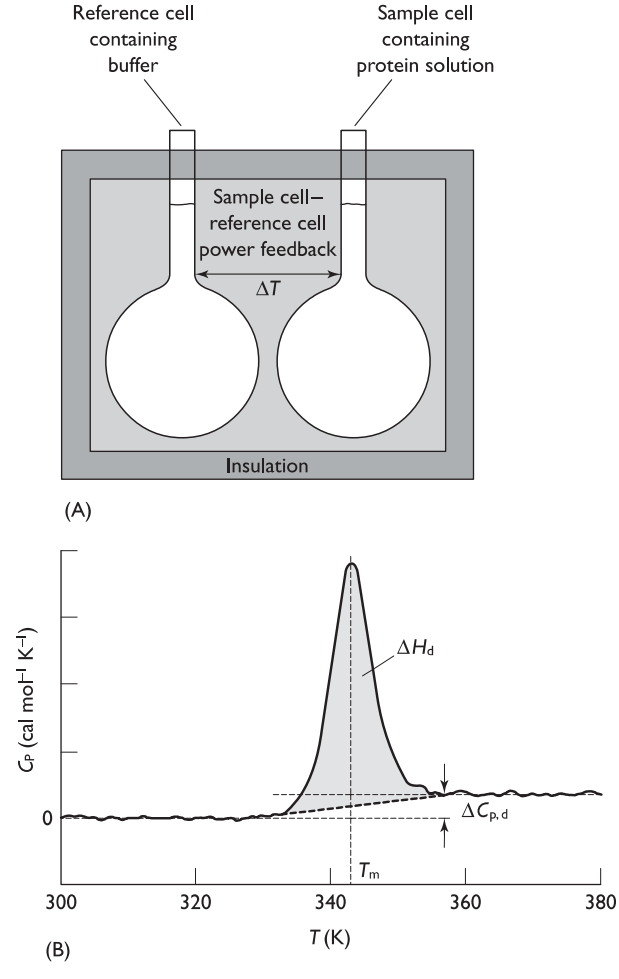

Fig. 2.10 Differential scanning calorimetry. (A) Schematic diagram of the instrument. In this case the reference cell contains buffer only, and the sample cell contains the macromolecule dissolved in buffer. Both cells are heated very slowly (e.g. 1 °C min^{-1}) in order to maintain equilibrium, and feedback electronic circuitry is used to add heat so that $\Delta T \approx 0$ throughout the experiment. Other types of DSC have been used for other purposes in biophysics, for example, to investigate the physiological limits of the freeze tolerance and freeze-avoidance strategies taken by different insect species to survive subzero temperatures. (B) Data. The heat added to keep $\Delta T \approx 0$ can be plotted as a function of temperature. The endothermic peak corresponds to heat absorbed, for example, on protein denaturation. The peak maximum corresponds roughly to the transition temperature, or melting temperature. The area below the peak is $\Delta H_d(T_m)$. The heat capacity of the unfolded state of a protein minus the heat capacity of the folded state is $\Delta C_{p,d}$. There is more about DSC in Chapter 5.

I. Energy conservation in the living organism

The First Law tells us that, if a system does work, w makes a negative contribution to ΔU; the system loses energy. This implies that not even the most sophisticated known "machine" – the human body, as far as we know – can do work without an energy source. And no matter how much the urge to eat might conflict with other ways we might rather spend time, there is no getting around having to eat – relatively often. But this does not necessarily mean that the First Law applies to living organisms.

In Chapter 1 we noted that calorimetry experiments on whole organisms were carried out as early as 1781 by Lavoisier and Laplace. They measured the heat given off by animals (and other objects) as the amount of water produced by melting ice, relative to a control in which no animal was present. The greater the volume of water at the end of the experiment, the greater the amount of heat given off during the experiment. Lavoisier and Laplace also collected and measured the gaseous "waste" from the animals used in their experiments. The quantity of heat and carbon dioxide produced by a guinea pig was compared with what was found for the combustion of carbon. Lavoisier later used the data from such experiments to establish that the combustion of food in animals leads to the production of heat, CO_2, and H_2O. About a century later, in 1904, a German physiologist named Max Rubner (1854–1932) reported on similar experiments with dogs. Rubner's work was effectively the final word on whether thermochemistry applied to physiology. For he was able to show that the heat production of a dog can be accounted for by the carbon and hydrogen balance of its respiration and the heats of combustion of fat, protein, and excrement. And on that cheerful note, we bring the text of this chapter to a close.

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K. Exercises

1. Invent three syllogisms. See Aristotle's *Topics* for ways of making use of syllogisms in formulating arguments.

2. Give the etymologies of *kinetic* and *potential*.

3. Give an example of a law of biology. What makes it a law?

4. Equation (2.1) involves a difference in internal energy. Differences in energy are much easier to measure than absolute magnitudes. Explain.

5. Figure 2.3 shows a thermodynamic cycle. The state function shown is U, though in principle a cycle of this sort could be given for any state function. Suppose that each arrow represents an experimental process, and that each internal energy represents an experimentally determined quantity. Give representative values for each energy change so that the condition $\sum_{\text{loop}} X = 0$ is satisfied.

6. The "Δ" in Eqn. (2.1) represents, effectively, a *measurable* change. What does this mean? Strictly speaking, the "Δ" should be used with state functions only; it should not be used to represent changes in q or w. Given this, and referring to Fig. 2.5, suggest a definition of *path function*. Does it follow that q (or w) can *never* be considered a state function? Why or why not?

7. Show that the right-hand sides of Eqns. (2.2) and (2.3) have the same dimensions.

8. We used Eqn. (2.2) to show that -1.96 kJ of work is done against gravity as a 50 kg woman climbs 4 m. Let the system be the woman. Evaluate ΔU? Explain how energy is conserved.

9. How many joules are expended by a 70 kg man climbing up 6 m of stairway? Does this quantity represent a maximum or minimum energy expenditure? Why? How much work is done if the climbing takes place on the surface of the moon? (Assume that the acceleration due to gravity on the moon's surface is 1.6 m s^{-2}.)

10. How many meters of stairway could a 70 kg man climb if all the energy available in metabolizing an 11 g spoonful of sugar to carbon dioxide and water could be converted to work?

11. A cylinder of compressed gas has a cross-sectional area of 50 cm^2. How much work is done by the system as the gas expands, moving the piston 15 cm against an external pressure of 121 kPa?

12. Indicate whether the temperature increases, decreases or remains the same in the following four situations: an endothermic/exothermic process in an adiabatic/non-adiabatic system. An adiabatic process is one in which no heat is exchanged with the surroundings.

13. A mathematical statement of the First Law of Thermodynamics is $\Delta U = q + w$. This holds for all processes. Assume that the only type of work done is *pV*-work. Show that $\Delta U = +w$ for an *adiabatic* process. Show that $\Delta U = 0$ for a process in an isolated system. Show that $\Delta U = q$ for a process that occurs at constant volume. Show that $\Delta H = 0$ for an adiabatic process at constant pressure.

14. When glucose is burned completely to carbon dioxide and water,

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O,$$

673 kcal are given off per mole of glucose oxidized at 25 °C. What is ΔU at this temperature? Why? What is ΔH at this temperature? Why? Suppose that glucose is fed to a culture of bacteria, and 400 kcal mol^{-1} of glucose is given off while the growing bacteria converted the glucose to CO_2 and H_2O. Why there is a discrepancy between the oxidation heats?

15. Conservation of energy is said to be implicit in the symmetrical relation of the laws of physics to time. Explain.

16. A person weighing 60 kg drinks 0.25 kg of water. The latter has a temperature of 62 °C. Assume that body tissues have a specific heat capacity of 0.8 kcal kg^{-1} K^{-1}. The specific heat of water is 1.0 kcal kg^{-1} K^{-1}. By how many degrees will the hot drink raise the person's body temperature from 37 °C? Explain how arriving at the answer involves the First Law of Thermodynamics.

17. Prove that Eqn. (2.14) follows from Eqn. (2.13).

18. Non-polar moieties in proteins make a positive contribution to $\Delta C_{p,d}$. This is known from measurements of the change in heat capacity of water on dissolution of non-polar compounds, e.g. cyclohexane. Is this true for polar moieties as well? What is the sign of their contribution to $\Delta C_{p,d}$? Explain your reasoning.

19. Early protein microcalorimetry studies were done by Peter Privalov, a Soviet biophysicist who emigrated to the United States in the early 1990s. One of the most thorough of all microcalorimetric studies of a protein is Privalov and Pfeil's work on hen egg white lysozyme, published in 1976. According to this work and later studies, $\Delta C_{p,d} = 1.5$ kcal mol^{-1} K^{-1}, and at pH 4.75, $\Delta H_d(25\,°C) = 52$ kcal mol^{-1}. Calculate the enthalpy difference between the unfolded and folded states of lysozyme at (a) 78 °C, the transition temperature, and (b) −10 °C. What is the physical meaning of ΔH in part (b)?

20. You have been asked to investigate the thermodynamic properties of a newly identified small globular protein by differential scanning calorimetry. The following results were obtained.

pH	T_m (°C)	$\Delta H_d(T_m)$ (kJ mol^{-1})
2.0	68.9	238
2.5	76.1	259
3.0	83.2	279
3.5	89.4	297
4.0	92.0	305
4.5	92.9	307
5.0	93.2	308
5.5	91.3	303
6.0	88.9	296
6.5	85.9	287
7.0	82.0	276
7.5	79.4	268
8.0	77.8	264

Plot $\Delta H_d(T_m)$ v. T_m. Describe the curve and rationalize its shape. Now plot $\Delta H_d(T_m)$ v. pH. What is happening?

21. ITC can be used to measure the enthalpy of protonation of amino acid side chains. Suppose three peptides were separately dissolved in weak phosphate buffer at pH 8 and injected into weak phosphate buffer at pH 2.5. There is a change in side chain ionization in going from one pH to the other. The peptides and the measured heats of reaction were Gly–Asp–Gly (-7.2 ± 0.8 μcal), Gly–Glu–Gly (-5.4 ± 0.8 μcal) and Gly–His–Gly (-5.5 ± 1.0 μcal). The data represent an average of 10 experimental data points, heat of injection minus background signal (injection of the pH 8 buffer into the pH 2 buffer in the absence of peptide). Gly = glycine, Asp = aspartate, Glu = glutamate, His = histidine. The peptide concentrations for the experiments were 0.64 mM, 0.57 mM and 0.080 mM, respectively. At pH 8, the side chains are approximately completely deprotonated, while at pH 2 they are approximately completely protonated. These solutions were injected into a sample cell in 10 μl aliquots. What is the physical basis of the background signal? What are the approximate protonation enthalpies of the Asp, Glu and His side chains? Suggest why tripeptides were used for these experiments rather than free amino acids. Would pentapeptides be any better? What could be done to account for the possible contribution of the terminal amino or carboxyl group?

22. Table C in Appendix C gives enthalpies of protonation for a number of popular biochemical buffers. Which five of these are likely to be best for thermodynamic measurements? Why?

23. The conditions of the standard state are chosen arbitrarily. What additional condition(s) might a biochemist add to those given in the text? Why?

24. Explain in structural and thermodynamic terms how the unfolding of a protein is like the melting of an organic crystal.

25. A protein called α-lactalbumin is a close homolog of hen egg white lysozyme. Unlike lysozyme, α-lactalbumin binds Ca^{2+} with high affinity. The measured enthalpy of binding, however, is much smaller in magnitude than the enthalpy of hydration. Explain.

26. Design a series of experiments to test whether the First Law of Thermodynamics applies to all living organisms.

27. Figure 2.7 shows that the enthalpy change on protein folding is large and positive. Suggest what gives rise to this.

28. Matter can neither be created nor destroyed, merely interconverted between forms. Discuss the statement in terms of the First Law of Thermodynamics.

29. Living organisms excrete the excess nitrogen from the metabolism of amino acids in one of the following ways: ammonia, urea, or uric acid. Urea is synthesized in the liver by enzymes of the urea cycle, excreted into the bloodstream, and accumulated by the kidneys for excretion in urine. The urea cycle – the first known metabolic cycle – was elucidated in outline by Hans Krebs and Kurt Henseleit in 1932. As we shall see in Chapter 5, urea is a strong chemical denaturant that is used to study the structural stability of proteins. Solid urea combusts to liquid water and gaseous carbon dioxide and nitrogen according to the following reaction scheme:

$$CO(NH_2)_{2(8)}(s) + 1.5O_2(g) \rightarrow CO_2(g) + N_2(g) + 2H_2O(l).$$

According to bomb calorimetry measurements, at 25 °C this reaction results in the release of 152.3 kcal mol^{-1}. Calculate ΔH for this reaction.

30. Giant sequoias, an indigenous species of California, are among the tallest trees on Earth. Some individuals live to be 3500 years old. Water entering at the roots must be transported up some 300 m of xylem in order to nourish cells at the top of the tree. Calculate the work done against gravity in transporting a single water molecule this distance.

31. Suggest three proofs that heat is not a fluid is the sense that liquid water is a fluid.

Chapter 3

The Second Law of Thermodynamics

A. Introduction

We have seen that a given amount of energy can be distributed in many different ways – something like how a certain volume of fluid can adopt many different shapes and adapt itself to its container. In this chapter we turn the spotlight on a thermodynamic function that enables us to *measure* how "widely" a quantity of energy is distributed.

The First Law of Thermodynamics relates heat, work and internal energy, and it tells us that energy is neither created nor destroyed in all its changes of form; the total energy of a reaction, and indeed of the universe, is *constant*. The First Law tells us with breathtaking generality that a boundary on the possible is a basic characteristic of our universe. It is not hard to see, though, that the First Law does not tell us some things we would like to know. For instance, if we put a "hot" system into contact with a "cold" one and allow them to come to thermal equilibrium, we find that the final temperature of the two objects, which will persist indefinitely if the combined system is isolated, is at some intermediate value. The value of ΔU for this reaction, however, which obviously proceeds spontaneously, is 0. Similarly, if we add a "concentrated" solution of substance A to a "dilute" solution of substance A, we find that the final concentration of the combined system, which will persist indefinitely if the system is isolated, is between the initial concentrations (Fig. 3.1). Again, $\Delta U = 0$, this time for spontaneous mixing. We see a similarity in behavior between heat energy and matter, and there is a correlation between $\Delta U = 0$ and spontaneity of reaction. Wait a minute. Won't $\Delta U = 0$ for a system that undergoes no change at all? In general the magnitude or sign of ΔU does not indicate the direction of spontaneous change!

Could we get our two objects in thermal equilibrium to return from being two warm objects to one that's hot and one that's cold? Could we get the solution at the intermediate concentration to spontaneously unmix and return to the concentrated solution and the dilute one? No! At least not spontaneously. For in both cases

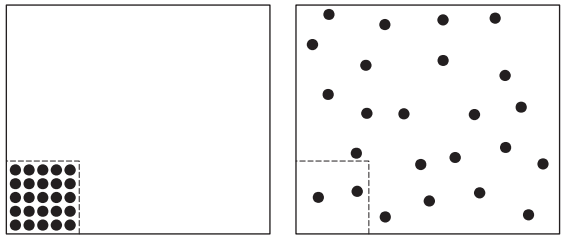

Fig. 3.1 Distribution of substance A before and after mixing. Panel (A) shows the situation before mixing, panel (B) when mixing is complete. Let substance A be perfume molecules on a heavily scented person who enters a small room, say, an elevator (lift). The molecules are initially very close together; the concentration of perfume is high. In such a high dose, it might be hard to distinguish perfume from insect repellent! Visual inspection of the wearer might not help much. After a while, maybe by the time you've reached the 57th storey, the perfume molecules are spread approximately randomly throughout the accessible space; the perfume is much more diffuse than at first; the concentration is uniform. This process is irreversible! The driving force for change is a movement of the system toward the most probable distribution of perfume molecules. Entropy, the key thermodynamic function of this chapter, measures the change in distribution. Further drops of understanding can be squeezed out of this figure. Imagine that the dots correspond not to molecules but to heat energy. Let the region where all the heat is collected in panel (A) be one object, and let the rest of the area be another object. The small object is hot, the big one is cold. Panel (B) shows the situation some time later, when the two objects have reached thermal equilibrium. (For example, 500 mL cans of Guinness Draught say that the contents should be chilled at 4 °C for 3 h, giving the gas plenty of time to dissolve in the liquid. The head is not quite so creamy on less cooling.) The heat energy has been redistributed throughout the matter, and both objects are at the same temperature. The driving force for change is a movement of the system toward the most probable distribution of heat energy. The comparison of perfume and heat suggests something exceptionally similar about matter and energy. Indeed, this relationship lies at the heart of $E = mc^2$.

something has been lost, *something* has changed – and the change is *irreversible*. The First Law, useful as it is, does not provide even the slightest clue about what that *something* is. Nor does it answer any of the following important questions: In which direction will a reaction proceed spontaneously? Is there a limit to how much work can be obtained from a reaction? If so, what is it? Why do highly purified enzymes degrade even when stored in the cold?

To be able to do more than just wave hands in positing answers to these queries, we must turn from the First to the Second Law of Thermodynamics. Like the First Law, the Second is an empirical result and a window on the relationship of heat to work. In still qualitative terms, the Second Law provides a way of describing the conversion of heat into work. It gives a precise definition of a thermodynamic state function called the entropy, and the sign of this function (plus or minus, not Leo or Libra!) tells us whether a process will occur spontaneously or not. This is something that no ΔU or w or q alone can do. Ice changes spontaneously into liquid water at 1 °C and 1 atm, despite the increase in translational motion (K.E.) of the molecules.

Our approach in this chapter is mainly that of "classical" thermodynamics. But we should be careful not to prejudge the discussion and think that its age makes it somehow less illuminating or useful. People who think that way are all too common, and few of them are good at thermodynamics. In learning a foreign language, one starts with relatively simple prose and not with poetry of the highest art! Moreover, this chapter is not an end in itself: it is a piece of the foundation for what comes next. In Chapters 4 and 5 we'll see the role played by the entropy in the Gibbs free energy, the biochemist's favorite thermodynamic function. And in Chapter 6 we'll turn our attention to statistical thermodynamics, and that will enable us to see how the statistical behavior of particles underlies the classical concept of entropy and other thermodynamic functions we'll have met by then.

We're all familiar with becoming aware of the cologne, aftershave, frou-frou juice, what-have-you someone nearby is wearing, or maybe the malodorous molecules underarm bacteria are pumping into the environment. The particles waft along an air current to the olfactory apparatus, gain entry, bind to receptors embedded in cell membranes, and thereby cause signals to be induced in brain-bound neurons. In some cases, for instance, when you walk by someone who is wearing a scent, it's your motion relative to that person that explains the phenomenon. But the relative motion of one person's neck to another's nose is not the essential ingredient of the present point. For on entering a room in which a heavily scented person has been present for a short while, you can smell the perfume immediately, even if that person's no longer present. What in the Milky Way does this have to do with thermodynamics?

The sweet-smelling volatile molecules in perfume may consist of various organic chemical components such as aromatic aldehydes. When heated by the body to 37 ^{o}C and exposed to the atmosphere, these molecules quickly become airborne. Then convection currents resulting from differences of temperature in patches of air play a role in spreading the cologne about. Finally, and most important here, the aromatic amines are bombarded constantly by the random motion of gaseous nitrogen and oxygen, and this moves them around a good deal – even in the absence of convection currents – by a process called *diffusion*. After some time, the concentration of perfume molecules will be approximately *uniform* throughout the room. Amazing! And no machine was required to achieve this end. Experience tells us that such behavior is the *only* thing that will happen (as long as there is no open window or something like that). That is, leaving a perfume-filled room and returning to find that all the aromatic aldehydes had, like some Arabian genie, somehow gone back into the bottle, seems extremely highly improbable. It *is* extremely highly improbable! As we shall see, diffusion is "governed" by the Second Law of Thermodynamics.

The Second Law is about the tendency of *particles* to go from being concentrated to being spread out in space – spontaneously. It is also about the tendency of *energy* to go from being "concentrated" to being

"spread out" – spontaneously. Consider a mass in a gravitational field, for example, a football that has been kicked high above the pitch. The driving force for change of the airborne ball is motion towards the most probable state, the state of lowest potential energy, the state of lowest energy of *position*. In this example, the state of lowest potential energy is just the ball at rest somewhere on the football field. The tendency of concentrated particles to become more uniformly dispersed is a reflection of the tendency of (chemical) energy to disperse itself into its most probable distribution, the state of lowest potential energy. We see this tendency in action on adding some cold cream to a cup of tea or coffee. Initially, the cream is seen to form distinct swirls, but before long the color and temperature of the liquid become more uniform. The Second Law, which helps to describe this process, is marvelously general: it applies not just to the mixing of cream and coffee but also (and equally well) to the spontaneous dissipation of aromatic aldehyde molecules from an open scent bottle, the spontaneous cooling of a hot saucepan when removed from the stove, the spontaneous folding of a polypeptide into a protein, the spontaneous movement of ions down their concentration gradient when a membrane channel opens.

Some students find the Second Law hard to grasp. One reason is that there are numerous formulations of the Law, and it's not always readily apparent that they are equivalent. It can be instructive to look at matter through the lenses of different observers, so to speak, as each sheds new light on the topic and helps to understand it in a different way. One of the earliest formulations of the Second Law from the historical point of view is of particular interest here because it helps to reveal the practical nature of the human activity out of which the science of thermodynamics developed. It is *impossible* for a system to turn a given amount of heat into an equivalent amount of work. In other words, if we put some quantity of heat q into the system, whatever work w is done by the system will be such that $w < q$. This comes to us from the work of the visionary French military engineer Nicolas Léonard Sadi Carnot (1796–1832). Publication of Carnot's *Réflexions sur la puissance motrice du feu et es machines propre à développer cette puissance*[1] (at the age of 28!) outlined a theory of the steam engine and inaugurated the science of thermodynamics. We shall encounter M. Carnot again shortly, but in a different chamber of the mansion of biological thermodynamics.

B. Entropy

The foregoing discussion brings us to the thermodynamic state function S, entropy (Greek, *en*, in + *trope*, transforming; also coined by Clausius). Being a state function, the entropy change for a process is independent of path, regardless of whether the change is

[1] *Report on the driving force of heat and the proper machines to develop this power.*

reversible or irreversible. The entropy is an index of the tendency of a system to undergo spontaneous change; it is a measure of the state of differentiation or distribution of the energy of the system. The entropy is the key to understanding energy transformation. As such, the entropy enables us to rationalize why solutes diffuse from a concentrated solution to a dilute one without exception, why smoke leaves a burning log and never returns, why wind-up clocks always run down, why magnets demagnetize spontaneously, why heat always flows from a hot body to a cold one. All this might suggest that entropy is something physical, as indeed many people have believed. It is important to realize, however, that entropy is not so much a "thing" as a highly useful mathematical object that provides insight to the nature of change in the material world.

As we have said, the entropy is a measure of the *order* of a system. For now, let a non-technical definition of *order* suffice; in Chapter 6, a more precise definition will be given. Entropy is less a "thing" than a way of describing how particles are *arranged* in space (e.g. perfume in the bottle or distributed throughout the room) and how particle arrangement changes as a system is subjected to changes of temperature, pressure, number of particles, volume, etc. The tendency toward a condition of *no further change* that we have seen in the examples above is a general property of thermodynamic systems. In fact, it is so fundamental to all of physics that most scientists consider the Second Law of Thermodynamics the most universal "governor" of natural activity that has ever been revealed by scientific study. Entropy measures how close a system is to the state corresponding to no further change, or equilibrium.

Let's avoid drifting into the ethereal world of abstractions and come back to Earth with another illustration, one that is closely related to the phenomenon of diffusion. Suppose we have two glass bulbs of equal volume connected by a stopcock, as shown in Fig. 3.2. Initially, the stopcock is closed. A gas occupies one bulb only; the other is evacuated. When the stopcock is opened, the gas molecules stampede into the evacuated bulb. There is a net flow of molecules into the formerly empty bulb until the concentration of molecules is identical (on the average) throughout the accessible volume. Such expansion of a gas is accompanied by an *irreversible* increase in entropy. The process is irreversible because a substantial amount of *work* would have to be done to herd all the gas molecules back into one bulb. The state in which all the molecules are distributed at random throughout the volume of the two bulbs – state 2 at equilibrium – is *less ordered* than the state in which all the molecules were randomly distributed throughout the volume of one bulb – state 1 at equilibrium. In this context, *equilibrium* just means a state of no further (net) change. Similarly, perfume is more ordered when it's still in the bottle than when it's been applied, volatilized by strenuous activity and increased body temperature, and scattered to the four corners of a room.

In Fig. 2.4C we saw how heat can be used to make a rubber band contract and lift a weight, to do work. Now let's see what insights

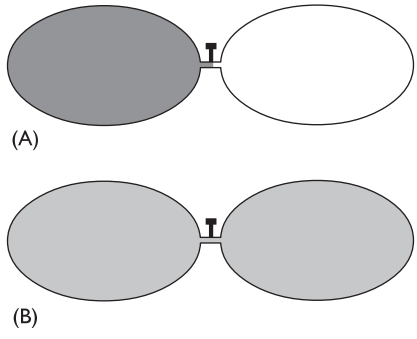

Fig. 3.2 Irreversible expansion of a gas. In panel (A), when the stopcock is closed, the gas is concentrated and confined to the left bulb. The right bulb is evacuated. When the stopcock is opened, as shown in panel (B), the gas flows rapidly from the bulb on the left to the bulb on the right. After a sufficiently long time, the condition known as equilibrium is reached; the concentration of gas is the same in both bulbs and the net flow of molecules between the bulbs is zero. This is just a more idealized view of the heavily scented person we met in the elevator.

can be gained by shrinking in size by several orders of magnitude and examining the mechanics of our rubber band machine. Rubber consists of long, chainlike molecules (Fig. 3.3). Stretching makes them align and become more orderly, decreasing the number of different spatial arrangements. Like forcing all the gas molecules back into one bulb, stretching a rubber band requires *work* to be done *on* the system, so $w > 0$. Your fingers, hands and arms together do the stretching; the energy comes from the cleavage of chemical bonds in your muscles. The contracting muscle is then used to stretch the rubber beyond its relaxed, equilibrium position, and there is a change in mechanical energy.

Box 3.1. The celebrated jumping insect of *Cantabrigiensis*

Insecta is the largest class of the phylum Arthropoda, which is itself the largest of the various phyla of the kingdom Animalia. Of all the animal species so far described by science, five of every six are insects. Beasts like bedbugs, beetles, and butterflies are a few of the more familiar members of the class. About 10^6 different insect species are known in total, and it is estimated that about as many more have yet to be described. Insects are poikilotherms: body temperature follows that of the surrounding environment within the range of tolerance for a species. Most of our knowledge of genetics has come not from humans but from experimental studies on the insect *Drosophila melanogaster*, one of about 10^3 different species of vinegar fly (more commonly but also misleadingly called fruit flies). Insects have jointed legs, and a segmented body covered by a hard exoskeleton (external skeleton) which is composed in part of the protein chitin. The exoskeleton serves as the point of attachment for muscles, which consist

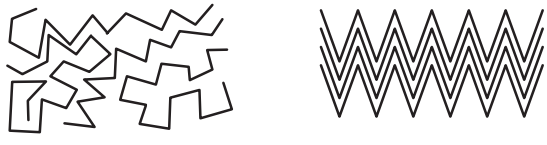

Fig. 3.3 Schematic diagram of a rubber band in the unstretched (equilibrium) state (left) and stretched state (right). In the unstretched state, the molecules are highly disordered. When the band is stretched, the molecules form a relatively orderly array. The entropy of the molecules is lower in the stretched state than in the unstretched state.

Box 3.1. Cont.

largely of the proteins actin and myosin. Locomotion is effected by muscles acting on the exoskeleton. In leaping insects (fleas, grasshoppers, locusts and the like) the force of muscle contraction compresses a "pad" of resilin. This protein is a member of a family of elastic proteins that includes elastin, gluten and spider silks. Resilin is also found in the sound-producing organs of cicadas. This long-chain molecule consists of many copies of a short elastic repeat sequence of amino acid residues joined in tandem. Chains are crosslinked between tyrosine residues, providing low stiffness, high strain, efficient energy storage, and a very high fatigue lifetime. The elasticity of resilin is thought to be the result of the extremely dynamic nature of amorphous hydrophobic regions which form a kinetically free, random-network polymer. At the microscopic level, rubbery materials like resilin become more ordered when extended, decreasing entropy, and they uncoil in elastic recovery. The energy stored in an elastic material can be transformed under stress into other forms of energy, for example, the K.E. of a projectile from a catapult. In the case of a cricket, the energy stored in coiled resilin is released by way of a catch mechanism that allows force generated before the jump to release rapidly; the projectile is the entire body of the bug. The current champion jumper of all species is the insect *Philaenus spumarius*, the froghopper (spittle bug), according to a recent report by a researcher at Cambridge University. The body of a froghopper accelerates at 2800–4000 m s^{-2} in a jump. The average jump requires about 50 μJ, which translates into a power output of 36 W g^{-1}. The force exerted in an average jump is 34 mN, about the same as the gravitational pull of the Earth on one-third of a gram of water at the planet's surface.

But how is work done by the *contraction* of rubber? Just as heating decreases the order of perfume molecules by bringing about a sort of thermodynamic diaspora, the addition of heat decreases the order of the rubber molecules and the rubber band contracts to its equilibrium position. Similar to muscle, the contraction of a rubber band permits work to be done. In this case, however, work is not done *on* the system, it is done *by* the system. We can *calculate* the magnitude of this work knowing gravitational acceleration, the mass of the object lifted by the rubber band,[2] and the distance the mass is moved against the force of gravity (Δx in Fig. 2.4C).

Stretching increases the order of the long, stringy molecules. In the taut state, the molecules make fewer random collisions with each other; the entropy of the system is reduced relative to the relaxed state. When the rubber band relaxes, the orderliness of the molecules is lost and the entropy increases. The closer the molecules are to being randomly ordered, the greater the entropy of the system. This study of rubber bands has helped to show how changes in entropy relate to transfers of heat, and heat is a form of energy related to molecular motion, as discussed in Chapter 2.

[2] By Newton's Second Law $\Delta \mathbf{p}/\Delta t = F = ma = mg$, where m is mass, a is acceleration, and g is the gravitational acceleration.

A related illustration. When a scented candle burns, the thermal energy of aromatic molecules embedded in the wax goes up. The greater the thermal energy of one of these molecules, the more easily it can overcome the attractive forces of other molecules in the melted wax, break forth from the surface, escape into the air, and after some time go spelunking in the cavernous recesses of your nasal passage. Blimey! Can this camp example bespeak aught of biology? The point is that the addition of heat increases the entropy of the system, the "breadth" of the distribution of the energy of the system. And in fact, both lords and ladies of the plant *Arum maculatum* attract pollinating insects to their flowers by heating and vaporizing aromatic molecules. At certain times of the day, the temperature of the part of the flower called the appendix increases rapidly, resulting in the evaporation of volatile compounds produced within. Insects "smell" the aromatic compounds, find them stimulating, and move up the concentration gradient until reaching the flower. Then, movement within the flower as the bug gathers up its prize leads to pollination.

Let's have another quick look at Fig. 2.4. Panel (A) shows heat being transferred from a hot system to a cold one. This is similar to Panel (C), where heat is being transferred from the candle to the rubber band. Back in Panel (A), kinetic energy is being shared as heat by means of multiple collisions between the molecules of the heat source and the molecules of the heat sink. A warm object is in contact with a cool one, and the warm molecules are banging on the cool ones harder than the cool ones are banging on the warm ones (or each other!). Heat transfer from the warm object to the cool one enables work to be done, as in the rubber band heat engine, and this situation continues as long as there is a temperature *difference* between the two systems.

When a thing is cold it moves relatively little. K.E. is proportional to thermal energy is proportional to absolute temperature. So addition of heat to something cold can disorder that thing more than the addition of the same amount of heat to the same thing at a higher temperature. On this basis we can guess that if a given amount of heat q is transferred to a system, it will increase the randomness of the system by an amount that is inversely proportional to the absolute temperature. Because we have described entropy as a measure of the breadth of a distribution of energy, we should expect $\Delta S \propto q/T$, though at this point we don't know if it's the first power of the temperature that's needed or something else.

Now let's become definite about the relationship between heat and entropy. The entropy change, ΔS, on heat transfer at *absolute* temperature T, is *defined* as

$$\Delta S \geq q/T, \tag{3.1}$$

where, by the convention we've adopted, $q > 0$ if heat is added *to* the system. Equation (3.1) is a fruit of labor on quantifying the maximum amount of work that can be obtained from an ideal reversible

engine. The equality holds only under an exceptional but none the less important constraint: when heat transfer is carried out *very* slowly and any change in the system is reversible, *i.e.*, when both the system *and* its surroundings can be returned to their *original* states. A reversible process is one that occurs through a succession of equilibrium or near-equilibrium states. The inequality corresponds to any other kind of process. No wonder we said that the expansion of a gas into vacuum is irreversible! Equation (3.1) tells us that although heat is a path function, the heat exchanged in a reversible *and* isothermal process is independent of path. So q can be independent of path but generally is not. S is always independent of path.

Equation (3.1) says that when a quantity of heat is transferred from a hot system to a cold one, $\Delta S_{\text{hot}} < 0$, $\Delta S_{\text{cold}} > 0$, $|\Delta S_{\text{hot}}| < |S_{\text{cold}}|$, and $\Delta S_{\text{total}} = \Delta S_{\text{hot}} + S_{\text{cold}} > 0$. Regardless of the magnitudes of q, T_{hot}, and T_{cold}, the *total* entropy change *must* be greater than zero. To make the discussion more concrete, let's look at an example. Suppose we wish to calculate the entropy change in the surroundings when 1.00 mol of $H_2O(l)$ is formed from H_2 and O_2 at 1 bar and 298 K. This is of interest here because liquid water is the only known matrix in which life occurs. We require the reaction to occur slowly. A table of standard thermodynamic quantities tells us that $\Delta H = -286$ kJ; the reaction is exothermic. Heat is transferred from the system to the surroundings, and $q_{\text{sur}} = +286$ kJ. Substituting this into Eqn. (3.1) and solving for ΔS_{sur} gives 286 kJ/298 K $= +959$ J K^{-1}. The entropy of the surroundings increases as heat is transferred *to* it. To put the numbers into perspective, 1 mol of water has a mass of 18 g and a volume of 18 ml, about the same as a large sip. Formation of a mole of water from hydrogen and oxygen at room temperature and ambient pressure increases the entropy of the universe by $\sim$1000 J K^{-1}.

C. Heat engines

This section describes how living organisms do *not* behave. You might guess therefore that this will be the usual sort of apparently useless, academic exercise that one is required to do to earn a degree. But in fact in trying to understand what something is, it can be instructive to seek to know *why* it is not what it is not. That's the spirit, anyhow, in which we discuss heat engines.

Let's suppose, as Carnot did, that heat q is transferred from a heat source to a heat sink (Fig. 2.4A). How much of this heat is available to do work? How much work can be done? No more than meets the requirement of the Second Law; the work done is usually much less than the Second Law allows! Let's calculate the limit. We set

$$\Delta S_{\text{hot}} + S_{\text{cold}} = 0. \tag{3.2}$$

Plugging in Eqn. (3.1), and calling the cold sink the place where the waste heat goes, we have

$$-q_{\text{transferred}}/T_{\text{hot}} + q_{\text{waste}}/T_{\text{cold}} = 0. \tag{3.3}$$

Rearranging,

$$q_{\text{waste}} = q_{\text{transferred}} T_{\text{cold}}/T_{\text{hot}}. \tag{3.4}$$

Here, q_{waste} is the *minimum* amount of heat transferred to sink; q_{waste} *cannot* be used to do work. In designing a heat engine, we would want to make q_{waste} as small as possible, making T_{cold} as small as possible and T_{hot} as large as possible, and remembering that energy must be "consumed" to make $T_{\text{cold}} < T_{\text{surroundings}}$ or $T_{\text{hot}} > T_{\text{surroundings}}$. The *maximum* work one can do is to use all the heat that remains, and that is $q_{\text{transferred}}$ less q_{waste}:

$$w_{\max} = q_{\text{transferred}} - q_{\text{transferred}} T_{\text{cold}}/T_{\text{hot}} = q_{\text{transferred}}(1 - T_{\text{cold}}/T_{\text{hot}}). \tag{3.5}$$

A simple numerical example is the following. If 30 J is transferred from a heat source at 300 K to a heat sink at 200 K (cold!), the maximum work that can be done is 30 J × [1 − (200 K / 300 K)] = 10 J. The efficiency of this process = $w_{\max}/q_{\text{transferred}}$ = 10/30 = 33%. We can see now that *an engine in which all the heat is converted to mechanical work cannot exist* – a suggestion that seems suspiciously like a limitation on what is possible in our universe. Which brings us to another way of formulating the Second Law, one due to Carnot himself: *heat of itself cannot pass from a colder body to a hotter one*; work is required. What does this have to do with biology? It helps us to realize that cells cannot do work by heat transfer because they are essentially isothermal systems (Fig. 2.4B). This applies not only to terrestrial mammals like armadillos, but also to the hyperthermophilic bacteria that live on the ocean floor in thermal vents, and presumably to any living thing anywhere in the universe.

We have seen that the transfer of heat can be used to do work but that the process generates waste heat, q_{waste}. By Eqn. (3.1), $S_{\text{irreversible}}$, the *minimum* irreversible entropy produced by heat transfer, is

$$S_{\text{irreversible}} = q_{\text{waste}}/T_{\text{cold}}. \tag{3.6}$$

Does this mean that an irreversible entropy increase must be written off as a pure loss? No! To see how a loss might be a gain of a sort, let's pay another visit to our friend the rubber band. When stretched, its entropy is low; the long rubber molecules are ordered. The release of tension results in decreased ordering of the molecules, so $\Delta S > 0$. We should therefore expect $q > 0$ on release of tension, and this is easily verified by experiment (try it!). The heat q_{waste} is lost to the surroundings. This heat is not completely useless, however, because the contraction of the rubber could be used to do something constructive, for example, lift a weight (Fig. 2.4C). An irreversible increase in entropy can be used to do work.

Having covered the necessary background, let's look at a biological example of an irreversible increase in entropy being used to do

work. Grasshoppers (and other hopping insects) store elastic energy in the compressed form of a protein called resilin, from *resilient*. This is something like a compressed spring, for instance, the spring in a loaded jack-in-the-box. When the insect leaps, elastic energy is released and the resilin becomes less ordered. ΔS for this process is large and positive. This form of energy release is just about as fast as the transmission of a nerve impulse and much faster than a typical metabolic reaction, enabling the grasshopper to make tracks if it senses danger from a predator. You will certainly know something about this if you have ever tried to catch one of these crispy critters while it was, so to speak, in the pink.

Now, before we wander too far away from equations, we ask: which is greater in magnitude, $q_{\text{transferred}}$ or q_{waste}? Or, suppose we have a process that can be carried out either reversibly or irreversibly. For which process will q be larger? Combining Eqn. (3.1) with the First Law gives

$$\Delta U \leq T\Delta S + w, \tag{3.7}$$

which, upon rearrangement, becomes

$$w \geq \Delta U - T\Delta S. \tag{3.8}$$

The most negative value of w that this expression can yield, and therefore the greatest amount of work that can be done *by* the system, is

$$w_{\max} = \Delta U - T\Delta S. \tag{3.9}$$

That is, the work done is maximal when the process is carried out reversibly. (Note that if $w_{\max} = 0$ and $\Delta U = 0$, then $\Delta S = 0$ at any T.) By the First Law,

$$\Delta U_{\text{rev}} = q_{\text{rev}} + w_{\text{rev}} \tag{3.10}$$

for a reversible process, and

$$\Delta U_{\text{irrev}} = q_{\text{irrev}} + w_{\text{irrev}} \tag{3.11}$$

for an irreversible one. But if the starting and ending points are the same, then $\Delta U_{\text{rev}} = \Delta U_{\text{irrev}} = \Delta U$. And if work is done by the system on the surroundings, then the sign of w is negative, and Eqns. (3.10) and (3.11) are, respectively,

$$\Delta U = q_{\text{rev}} - w_{\text{rev}} \tag{3.12}$$

and

$$\Delta U = q_{\text{irrev}} - w_{\text{irrev}}. \tag{3.13}$$

Combining these equations, which we can do because the change of state is identical in the two cases, gives

$$\Delta U = q_{\text{rev}} - w_{\text{rev}} = q_{\text{irrev}} - w_{\text{irrev}} \tag{3.14}$$

or, upon rearrangement,

$$q_{\text{rev}} - q_{\text{irrev}} = w_{\text{rev}} - w_{\text{irrev}}. \tag{3.15}$$

Above we found that $w_{rev} \geq w_{irrev}$, which means that both sides of Eqn. (3.15) must be positive. This implies that

$$q_{rev} \geq q_{irrev}. \tag{3.16}$$

And so we have answered the question we set ourselves. What does Eqn. (3.16) mean? In an endothermic process, the heat extracted from the surroundings will be greatest when the process is reversible. In an exothermic process, the heat released to the surroundings will be smallest when the process is reversible. So, living organisms would release the smallest possible amount of energy as heat if the processes going on inside them were reversible. And we do release a lot of heat to the surroundings. But many of the processes going on inside you are irreversible! To keep from cooling down to the temperature of the surroundings, we must consume energy. And it comes from food.

D. Entropy of the universe

As we have seen, the total entropy of an isolated system increases in the course of a spontaneous change. Put another way, the Second Law says that no natural process can occur unless it is accompanied by an increase in the entropy of the universe (Fig. 3.4 and Table 3.1). The upshot is that every process that occurs in nature is ultimately irreversible and unidirectional, the direction being dictated by the requirement of an overall increase in entropy. This can be symbolized in a compact mathematical form as $\Delta S_{total} = \Delta S_{hot} + \Delta S_{cold} > 0$. Rewriting this in a more general way, we have

$$\Delta S_{system} + \Delta S_{surroundings} = \Delta S_{universe} > 0. \tag{3.17}$$

In order for a physical change to occur spontaneously, the entropy of the universe *must* increase. Leafing back to a preceding page of this chapter, we see that ΔS in Eqn. (3.1) is we see that $\Delta S_{universe}$, and in the previous two sections the entire universe consisted of just a heat source and a heat sink, one being the system and the other being the surroundings.

It is important to realize that Eqn. (3.17) does *not* say that entropically "unfavorable" reactions (ones for which the entropy change is negative) *cannot* occur. Such reactions can and do occur, albeit *not spontaneously*. When an entropically unfavorable process is made to occur, the *overall* change in the entropy of the universe *will* be greater than zero, by the Second Law of Thermodynamics. Will this be true for a rather complicated and organized thing like an amoeba, an ant, or an aardvark? Yes! And what if we want to *measure* the entropy production of an organism? Can we do that and decide whether the change occurred spontaneously? No! Because the sign of ΔS for a system indicates whether a reaction will proceed spontaneously *only* if the system is isolated from its surroundings or both the entropy

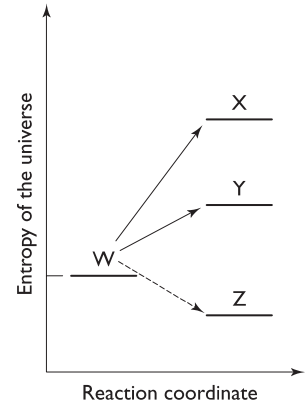

Fig. 3.4 The Second Law of Thermodynamics. No process will occur spontaneously unless it is accompanied by an increase in the entropy of the universe. This applies to an isolated system, a closed system, and an open system.

Table 3.1. | *Comparison of the "orderliness" of different types of energy*

Form of energy	Entropy per unit energy
Nuclear reactions	10^{-6}
Internal heat of stars	10^{-3}
Sunlight	1
Chemical reactions	1–10
Terrestrial waste heat	10–100

Note how the entropy of a given amount of energy increases as it is transformed from a nuclear reaction to the heat given off by biological organisms on the surface of Earth.

change of the system *and* the surroundings have been measured. These conditions are not easily met. As we have seen, $q_p = T\Delta S$ *only* if a reaction is reversible. The decomposition of an organism into its various molecular components is irreversible! Also, we must consider how one might go about measuring the entropy change of the surroundings. There is nothing to say such measurements are impossible as a matter of principle, but in many cases they cannot be made in practice! The next chapter will show us a way of circumventing this obstacle, enabling us to determine whether the change in a system has occurred spontaneously. We'll do that by employing a less general but in many respects more useful index of reaction spontaneity, the Gibbs free energy.

E. | Isothermal systems

Now we wish to look a little more closely at the specialized situation of constant temperature. Isothermal conditions are of great importance to the biochemist, because the temperature of a living organism is more or less uniform throughout its body, and it is practical for bench-top experiments to be done at constant temperature. In making a biochemical measurement, say of enzyme activity, one would not want to have to report that the temperature at which the reaction took place fluctuated over a range of a dozen degrees during the experiment.

Human beings and many other organisms can tolerate a change in body temperature of no more than a few degrees. We have terribly sophisticated negative feedback systems for thermoregulation and a circulatory system for distributing heat energy; when these systems are functioning normally they keep the entire body at about the same temperature. Now, consider a cell deep within your body to be a thermodynamic system. And suppose this cell undergoes a reversible change at constant pressure that transfers heat q to the surrounding cells of the tissue. Then

$$\Delta S_{\text{surroundings}} = -q/T, \tag{3.18}$$

where $T_{\text{surroundings}}$ is so close to T_{system} that both temperatures are T. Because $\Delta T \approx 0$, the pV-work that can be done from the heat transfer is practically negligible, and $q = \Delta H$. Substituting into Eqns. (3.17) and (3.18) gives

$$\Delta S_{\text{system}} - \Delta H/T > 0, \tag{3.19}$$

which, after rearrangement, can be written as

$$\Delta H - T\Delta S_{\text{system}} < 0. \tag{3.20}$$

The quantity $H - TS$ is a thermodynamic state function called the Gibbs free energy. ΔH measures the heat exchanged at constant pressure, as discussed in Chapter 2, and $T\Delta S$ can be thought of "isothermally unavailable" energy, as transfer of this energy out of the system would result in a temperature decrease, and we have said that the temperature is constant. The Gibbs energy enables prediction of the direction of spontaneous change of a system under the constraints of constant temperature and constant pressure.

We can approach the idea of a portion of heat released by a reaction being unavailable for work from another direction. First, let's see if we can convince ourselves (again) that some of the available energy cannot be used to do work. Suppose we have a system which undergoes a process that results in a decrease in the entropy of the system, for example, liquid water allowed to come to equilibrium in the solid state at -1 °C. If all the heat released by this process, which is about 6 kcal mol^{-1}, were exported to the surroundings as *work*, there would be *no* increase in the entropy of the surroundings, and the overall entropy for the process would be negative. But this contradicts Eqn. (3.17), the requirement of the Second Law that any real process will result in an increase in the entropy of the universe. So, at least some of the heat generated by the process must be unavailable to do work. But how much?

We can find the answer by continuing with a qualitative treatment. Let's suppose that the reaction reduces the entropy of the system by some amount, ΔS. In order for the reaction to be spontaneous, the entropy of the surroundings must increase by at least as much. By Eqn. (3.6), this is q_{waste}/T. Solving for the heat supplied, $q_{\text{waste}} = T\Delta S$, where ΔS is the *decrease* in the entropy of the system (q_{waste} is negative). This is the energy that *is not* available to do work. The energy that *is* available to do work is the difference between the total energy and the energy that is not available to do work. And this energy is the Gibbs free energy. Note the resemblance of this qualitative example to Eqn. (3.20). The Gibbs free energy is so important to the biological sciences that the next two chapters will be devoted to it.

Above we combined the First and Second Laws to arrive at the Gibbs free energy. Now we wish to combine these laws again, but in a somewhat different way. The result will be different, and it will provide new insight into the expansion of a gas into vacuum

discussed above and prepare the way for a discussion of osmosis in Chapter 5. Moreover, it will provide a good starting point for our coverage of statistical thermodynamics (Chapter 6). Let's require the system to be adiabatic; energy can neither enter nor leave, $\Delta U = 0$. By the First Law, $q = -w$. If we require pV-type work only, then $q = p\Delta V$; and if the system is an ideal gas, then $q = nRT\Delta V/V$. Suppose now that the process we'd like to carry out is reversible. Then, by the Second Law, $q = T\Delta S$. Combining the First and Second Laws gives

$$T\Delta S = nRT\Delta V/V. \tag{3.21}$$

Cancelling the Ts leaves

$$\Delta S = nR\Delta V/V. \tag{3.22}$$

This is the increment in entropy when an ideal gas is allowed to expand by a small but measurable volume, ΔV. If the expansion is carried out reversibly over a large change in volume, the total change in entropy is the sum of all the small changes, and the result, which can be found with a little calculus, is

$$\Delta S = nR\ln(V_f/V_i), \tag{3.23}$$

where V_f is the final volume and V_i is the initial volume. The entropy is a state function, so as long as the initial and final states are whatever they are, the entropy difference between states will be independent of how the change occurs. The entropy change on opening the stopcock of an adiabatic version of the system shown in Fig. 3.2 can be found by Eqn. (3.23). It is $\Delta S = nR\ln(2V_i/V_i) = nR\ln 2$. The entropy change of the universe for irreversible change must be greater than the entropy change of the universe of a reversible change.

F. Protein denaturation

Let's see how Eqn. (3.18) (or Eqn. (3.1)) can be used to describe the reversible isothermal entropy change of any biochemical system we like. Here, we apply it to protein denaturation, but it describes equally well the "melting" of DNA, the dissociation of the double-stranded helix into two single strands. In Chapter 4 we shall see how it is yet even more general.

Rewriting Eqn. (3.18) with symbols introduced in the previous chapter, we have

$$\Delta S_d = \Delta H_d/T_m. \tag{3.24}$$

The minus sign has vanished because heat is being transferred *to* the system and we are describing the entropy change of the protein system, not the surroundings. Suppose T_m is 342 K and we wish to

know ΔS_d at 25 °C. What can we do? If the heat transfer is carried out reversibly, then, by Eqns. (3.1) and (3.18), $\Delta S = -q/T$. The heat transferred, q, will increase the enthalpy of the system according to Eqn. (2.16), if the system is at constant pressure. Combining these equations gives

$$\Delta S = C_p \Delta T/T. \tag{3.25}$$

If we now sum up all these small contributions to find the entropy change over a measurable range of temperatures (T_1 – T_2) and use a wee bit of mathematical wizardry (the same sort used in the previous section), the result is

$$\Delta S(T_2) = \Delta S(T_1) + C_p \ln(T_2/T_1), \tag{3.26}$$

where $\Delta S(T_i)$ is the change in entropy evaluated at temperature T_i, not the mathematical product of ΔS and T_i, and we have assumed that C_p is constant throughout the temperature range. It can be shown that Eqn. (3.26) becomes

$$\Delta S(T_2) = \Delta S(T_1) + \Delta C_p \ln(T_2/T_1), \tag{3.27}$$

if state 1 and state 2 differ in heat capacity and ΔC_p is constant throughout the relevant temperature range. As an example, suppose that $\Delta S = 354$ cal mol^{-1} K^{-1} at 80 °C and $\Delta C_p = 1500$ cal mol^{-1} K^{-1}. Then $\Delta S(25\,°C) = 354$ cal mol^{-1} K^{-1} + (1500 cal mol^{-1} K^{-1}) × ln(298.16 K/353.16 K) = 100 cal mol^{-1} K^{-1}.

There is another way of thinking about proteins and entropy, one that does not involve a large change in heat capacity. As the pH decreases, acidic amino acid side chains become protonated. From an exercise in Chapter 2, the enthalpy change of amino acid side chain protonation is about 1 kcal mol^{-1}. This is so small as to be negligible in comparison with the enthalpy change of unfolding the protein in the absence of protonation effects. Changes in pH can nevertheless have a dramatic effect on protein stability; indeed, we have already seen how lowering the pH reduces the transition temperature of hen egg white lysozyme. It follows that protonation of Glu and Asp is mainly an *entropic* effect, with regard to the binding of the proton to the amino acid side chain *and* the effect on protein unfolding. The T_m of the protein decreases upon reduction of pH because the entropy difference between the folded and unfolded states decreases at a faster rate than the enthalpy difference, making $\Delta H_d/\Delta S_d$ progressively smaller. The ionization of food molecules in the low-pH environment of the gut denatures proteins, facilitating their breakdown into short peptides by digestive proteases.

How do we *interpret* the pH denaturation of proteins, and explain it on a more detailed level? As the pH goes *down* there is a change in the ionization state of the *acidic* side chains. This results in a net

increase in the surface charge of the protein. So at low pH, the positive charges repel each other by electrostatic interactions more than at high pH, destabilizing the folded conformation. The situation can be represented as follows:

$$P_{folded} + nH^{+} \rightarrow P^{*} \rightarrow P_{unfolded}, \tag{3.28}$$

where P* is an unstable folded conformation. The effect of charge on protein stability was first described mathematically in 1924 by the Danish physical biochemist Kaj Ulrik Linderstrøm-Lang (1896–1959). The earliest known experiments on the use of a strong acid to denature proteins were done about a century earlier, by a Dutch chemist named Gerardus Johannes Mulder (1802–1880), who is also said to have stimulated the eminent Swedish chemist Jöns Jacob Berzelius (1779–1848) to coin the word *protein* (Greek, of the highest importance). Linderstrøm-Lang's mentor at Carlsberg Laboratory, Copenhagen, Denmark, was the Danish biochemist Søren Peter Laurtiz Sørensen (1868–1939), who developed the now universally adopted pH scale for measuring the acidity of an aqueous solution and ushered in the modern era of protein chemistry. We shall meet Linderstrøm-Lang again in Chapters 6 and 8.

G. The Third Law and biology

Yet another way of stating the Second Law brings us to the Third Law. *Any system not at absolute zero has some minimum amount of energy that is a necessary property of that system at that temperature*. This energy, of magnitude *TS*, is the "isothermally unavailable" energy from above (Section E). Now, the Third Law of Thermodynamics states that the entropy of a *perfect* crystal is *zero* when the absolute temperature is *zero* (0 K = −273 °C = cold!). A perfect crystal is like the ideal diamond in which each atom is at its proper place in an orderly array. The reason why we care about the Third Law is that it implies that the rapid and complex changes exhibited by living organisms, for instance, in a eukaryotic cell migrating during embryogenesis, can only occur far from thermodynamic equilibrium.

There is a substantial scientific literature on the freezing of *living* organisms. Some creatures have been stuck in a rather sophisticated sort of meat locker, taken to an extremely low temperature (~4 K), and allowed to thaw again. It is remarkable that some relatively "simple" organisms, e.g. bacteria, some types of microscopic animals, and plant seeds, return to room temperature from the deadly cold and function normally. Even some nematodes, which are comparatively complex organisms, having on the order of 10^3 cells at adulthood, are known to be able to withstand this process (depending on how it is carried out).

As discussed in Chapter 2, temperature measures the average kinetic energy of a collection of molecules. So, when the temperature is made to approach 0 K, all molecular motion ceases (excluding that

required by the Heisenberg uncertainty principle[3]). Thus, close to absolute zero, the only "memory" a biological system has of its life-before-deep-freeze is the *information* contained in the structure and arrangement of its macromolecules. When the organism thaws out, no new information is added; in fact, information is removed, because heating is a disordering process, as we have seen throughout this chapter.

The foregoing discussion would suggest that "all" one would have to do to "create" a cell would be to construct a being of the appropriate configuration of atoms. If the configuration (structure) were "the right type," the cell would function "on its own." From this point of view, it seems that a cell of an organism or indeed an entire organism might not be qualitatively different from some other collection of organic molecules. But on another view, a cell is simultaneously the most highly organized and the most complex collection of organic molecules of its size that we can imagine! Will it be possible to make "artificial" cells? What will be their properties?

H. Irreversibility and life

In the first several chapters of this book we have looked at ways in which living organisms can be thought of as machines. (Note: this does not mean that living organisms *are* machines!) Back in Chapter 1, for example, we discussed energy "consumption" as though biological machines really do "consume" energy. We've covered the First and Second Laws in some degree of depth, and we want to take a more critical look at energy consumption and relate it to life. If food is potential energy and we consume food, we consume energy; a sound argument if we agree on the meaning of *consume*. There is, however, another way of looking at energy consumption, and that is what we want to do now.

We do of course consume food, but that should not be taken to mean that we *consume* energy. For all a living organism or any type of system whatsoever can do is *transform* energy from one form to another; the total energy of the universe remains the same throughout such transformations by the First Law. The amount of energy returned to the environment by an organism, for instance as excretory products or heat, is equivalent in magnitude to the energy taken in, assuming no change in the weight. In this sense, living things do not consume energy at all; energy simply *flows through* them.

Just as important, the energy an organism returns to the environment *must* be less *useful* than the energy it "consumed."

[3] Named after the German mathematical physicist and philosopher Werner Karl Heisenberg (1901–1976), son of a professor of ancient history. Heisenberg was awarded the Nobel Prize in Physics in 1932.

Sure, excretory products make great fertilizer, but there are several good reasons why certain animals would not want to feed on them! As we have seen, any real process *must* increase the entropy of the universe; any change in the universe must result in an overall decrease in order. And as we shall see in the next chapter, *biologically useful* energy, or free energy, is the energy that can be used to do work under isothermal conditions. It is the sort of energy that humans, sheep, goats, even sea slugs, need to live. As the example of the heat engine has shown, heat transfer cannot be used to perform a substantial amount of work in biological systems, because all parts of a cell and its surroundings are effectively at the same temperature (and pressure). We have thus eliminated a major class of ways in which cells could conceivably do work, at least within the constraints of the physical properties of our universe. And we have narrowed the path to understanding. Whew! In playing the game of twenty questions, the ideal strategy is to pose a query whose yes-or-no reply will eliminate the largest number of possible answers and enable you to close in on the right one. The direction of spontaneous change in an isothermal system from a non-equilibrium to an equilibrium state is determined by the requirement that the extent of change be a maximum at every point on the reaction pathway. The suggestion that, say, uniformly dispersed smoke particles could somehow move spontaneously from all corners of a room back into a burning cigar seems absurd – except in a videotape run backwards.

There are various causes of the irreversibility of real-world processes. These include friction between two objects during relative motion, unrestrained expansion of a gas or liquid without production of work, the mixing of different substances that would require the input of work to separate them – all common phenomena. Because all atoms interact with each other, even noble gases, it would appear that there must be at least a small amount of irreversibility in any actual process. The inexorable increase in the entropy of the universe resembles, broadly speaking, the unidirectional flow of time. For as far as anyone knows, time moves in one direction only: forward! Why should this be so remarkable?

Time moves forward, and the past is, well, the past. This apparent conjunction of an interpretation of a scientific theory (the Second Law of Thermodynamics) and our ordinary (psychological?) perception of time is all the more intriguing because *all* organisms come into being and pass out of being *in time* and *all* the fundamental laws of physics are *time-reversible*.[4] Newton's laws of motion work equally well in either direction of time; they are time symmetrical. Maxwell's equations of electromagnetism work equally

[4] The decay of kaons and other sub-nuclear particles violates time symmetry; these particles appear to possess an intrinsic "sense" of past-future. See Christenson *et al.* (1964).

well forwards and backwards.[5] The time-dependent Schrödinger[6] equation of quantum theory is equally happy whether time is positive or negative. Einstein's theory of relativity works just as well in either direction of time. The time-reversibility or time-symmetry of laws of physics is related to energy conservation (the First Law).

The widely accepted mathematical formulations of physical law help us to rationalize many aspects of the nature of the universe and, moreover, provide tools for the creation of technology. Because of this, we cannot but be convinced that physics gives us at least an approximately right sense of the nature of reality. Nevertheless, and regardless of one's familiarity with physics, time marches on. The only law of physics that jibes with this aspect of our everyday experience of the world is the Second Law of Thermodynamics. This is all the more noteworthy here in that life on Earth has grown increasingly complex since the advent of the first cell; humans, composed as they are of billions of cells, are a good deal more complex than one-celled beasts like bacteria! We'll come back to this point in Chapter 9.

One can think about the irreversibility of chemical processes and life on different levels. Just as the increase in complexity of life forms on Earth is irreversible, in that it cannot be undone (though we could, it seems, blast ourselves to oblivion by means of well-placed and sufficiently large nuclear bombs): so at certain points in the development of an organism "commitment" occurs. For instance, in higher eukaryotes, once embryonic cells have "differentiated" into mesoderm or ectoderm, they ordinarily do not and in many cases apparently cannot become endoderm. If you have had the extreme misfortune of losing a limb, you will be acutely aware of the fact that a new one won't grow in to take its place. Some researchers think that biological ageing can be described in terms of the Second Law. On this view, what we call ageing is the process whereby a biological system moves from a point far from equilibrium toward equilibrium, a state of no further change. Another way of stating this is that order is a basic property of a living organism, and disorder, a dead one. There is a great deal that could be said on this topic, and it is a pity that there is not enough space to do more with it here.

The concepts of entropy and irreversibility (and energy conservation) have had a huge impact on humankind's view of the universe. Indeed, the concept of entropy has thrown into high relief philosophies of progress and development. "How is it possible to understand life

[5] James Clerk Maxwell, a Scot, lived 1831–1879. He is regarded as the nineteenth-century scientist who had the greatest influence on twentieth-century physics and is ranked with Isaac Newton and Albert Einstein for the fundamental nature of his contributions. He did important work in thermodynamics and the kinetic theory of gases.

[6] The Austrian physicist Erwin Schrödinger (1887–1961) was awarded the Nobel Prize in Physics in 1933. His little book *What is Life?* had a significant impact on the early development of molecular biology.

when the entire world is ordered by a law such as the second principle of thermodynamics, which points to death and annihilation?"[7] It is hard to see how a definitive answer can be given. Again, this topic deserves far more attention than it can be given here.

Finally, we wish to touch on the origin of irreversibility in many-body systems like large collections of small interacting particles. The thermodynamic description of such systems is so useful precisely because, in the usual case, there is no detailed knowledge or *control* over the (microscopic) variables of position and momentum for each individual particle. If such control were possible, the dynamics of many-body systems would presumably be reversible. When the number of microscopic variables is large, the state of maximum entropy is *overwhelmingly* probable, and the only lack of *certainty* that the entropy is maximal is the requirement that statistical "fluctuations" be allowed to occur. Under given constraints, the maximum entropy (equilibrium) state is the macroscopic state that can be formed in the greatest number of microscopic ways. More on this in Chapter 6.

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J. Exercises

1. Is the word *entropy* a misnomer? Why or why not?

2. State whether the following phrases pertain to (A) the First Law of Thermodynamics, (B) the Second Law, (C) both the First and Second Law, or (D) neither of the Laws.

(1) Is concerned with the transfer of heat and the performance of work.
(2) Is sufficient to describe energy transfer in purely mechanical terms in the absence of heat transfer.
(3) Indicates whether a process will proceed quickly or slowly.
(4) Predicts the direction of a reaction.
(5) Is a statement of the conservation of energy.
(6) Says that the capacity to do work decreases as the organization of a system becomes more uniform.
(7) Is a statement of the conservation of matter.
(8) Says that a quantity of heat cannot be converted into an equivalent amount of work.
(9) Says that the capacity to do work decreases as objects come to the same temperature.

3. Examine Eqn. (3.1). What happens to ΔS as $T \rightarrow 0$? In order to ensure that this equation remains physically meaningful as

$T \to 0$, what must happen to ΔS? The answer to this question is a statement of the Third Law of Thermodynamics.

4. Consider a heat engine. Suppose 45 J is transferred from a heat source at 375 K to a heat sink at 25 °C. Calculate the maximum work that can be done and the efficiency of the process.

5. We said that heat engines do not tell us very much about how living organisms work. Show that if the human body depended on *thermal* energy to do work, it would cook before it could demonstrate its efficiency as a heat engine. Assume that the "engine" has an efficiency of 20%.

6. One calorie (1 cal) is produced for every 4.1840 J (Joules) of work done. If 1 cal of heat is available, can 4.1840 J of work be accomplished with it? Why or why not?

7. In Chapter 2 we learned about thermal equilibrium. In the approach to thermal equilibrium when two objects of differing initial temperature are brought into contact, although no energy is lost (by the First Law of Thermodynamics), *something* certainly is lost. What is it?

8. Entropy change of protein unfolding. Suppose that $\Delta H_d(25\ ^\circ C) = 10$ kcal mol^{-1}, $T_m = 68\ ^\circ C$ and $\Delta C_p = 1{,}650$ cal mol^{-1} K^{-1}. Calculate $\Delta S_d(T_m)$, $\Delta S_d(37\ ^\circ C)$ and $\Delta S_d(15\ ^\circ C)$. At what temperature is $\Delta S_d = 0$? Give the thermodynamic significance of ΔS_d in molecular terms at each temperature.

9. Recall Exercise 19 from Chapter 2. Use the same data to evaluate $\Delta S_d(T_m)$ at each pH value. Rationalize the entropy values.

10. For irreversible pathways, q/T is generally dependent on the path. How can one discover the entropy change between two states? Knowing that $q_{reversible} > q_{irreversible}$ (Eqn. (3.16)), use the First Law to write down a similar inequality for $w_{reversible}$ and $w_{irreversible}$.

11. Explain in thermodynamic terms why water freezes.

12. Suppose you have a cyclic process, as shown in Fig. 2.3. The entropy change for the system must be 0. Is there any inconsistency with the Second Law of Thermodynamics? Explain.

13. In his book *What is Life?*, Erwin Schrödinger says "an organism feeds with negative entropy." What does he mean? (Hint: consider an organism that is able to maintain its body temperature and weight in an isolated system.)

14. Consider a gas, a liquid and a crystal at the same temperature. Which system has the lowest entropy? Why?

15. Can a machine exist in which energy is continually drawn from a cold environment to do work in a hot environment at no cost? Explain.

16. There are a number of different causes of undernutrition. Some of these are: failure of the food supply; loss of appetite; fasting and anorexia nervosa; persistent vomiting or inability to swallow; incomplete absorption, comprising a group of diseases in which digestion and intestinal absorption are impaired and there is excess loss of nutrients in the feces; increased basal metabolic rate, as in prolonged fever, overactivity of the thyroid gland, or some cancers; and loss of calories from the body; *e.g.* glucose in the urine in diabetes. Rationalize each type of undernutrition in terms of the First and Second Laws of thermodynamics.

17. The macroscopic process of diffusion can be identified with microscopic Brownian motion,[8] which subjects molecules to repeated collisions with the atoms of their environment and results in their random rotation and translation. Some people say that the time-asymmetry in the inevitable increase of randomness of the universe is not strictly true as Brownian motion may contravene it. What is your view? Support it with well-reasoned arguments.

18. Consensus is a weak but nonetheless important criterion of truth, particularly in the scientific community. Doig and Williams[9] claim that disulfide bonds make a substantial contribution to the enthalpy change of protein unfolding. Their view is rejected by most researchers who study protein thermodynamics. In the light of the results of the study by Cooper *et al.*[10], and considering the structure of a disulfide bond, rationalize the long-standing view of the scientific community to the thermodynamic role of disulfide bonds in proteins.

19. The Gibbs paradox: consider two gas bulbs separated by a stopcock. The stopcock is closed. Both bulbs are filled with the same inert gas at the same concentration. What is the change in entropy when the stopcock is opened?

20. Is it possible for heat to be taken in to a system and converted into work with no other change in the system or surroundings? Explain.

21. Organisms are highly ordered, and they continually create highly ordered structures in cells from less-ordered nutrient

[8] Brownian motion is named after the Scottish botanist Robert Brown, who was the first to observe it, in 1827.

[9] See Doig, A.J. & Williams, D.H. (1991). Is the hydrophobic effect stabilizing or destabilizing in proteins – the contribution of disulfide bonds to protein stability. *J. Mol. Biol*, **217**, 389–98.

[10] See Cooper, A., Eyles, S.J., Radford, S.E. & Dobson, C.M. (1992). Thermodynamic consequences of the removal of a disulfide bridge from hen lysozyme, *J. Mol. Biol*, **225**, 939–43.

molecules. Does this mean that organisms violate the Second Law of Thermodynamics? Explain.

22. The process whereby the Earth was formed and living organisms grew increasingly complex with time is "essentially irreversible," says Thomas Huxley. It "gives rise to an increase of variety and an increasingly high level of organization." Thus, this process appears not to square with the Second Law of thermodynamics. Explain.

23. It would appear that all living organisms on Earth are, essentially, isothermal systems. Relatively few organisms live where the surroundings are at a higher temperature than they are. Rationalize this observation in thermodynamic terms.

24. Tube worms thrive at black smokers at the bottom of the ocean. These invertebrates live as long as 250 years, longer than any other known spineless animal. Tubeworms have no mouth, stomach, intestine, or way to eliminate waste. The part of the worm that produces new tube material and helps to anchor the worm in its protective tube, a chitin proteoglycan/protein complex, is often planted deep within the crevices of a black smoker. The soft, bright-red structure (made so by hemoglobin) at the other end of the worm serves the same purpose as a mouth and can be extended or retracted into the surrounding water. Giant tubeworms are over 1 m long, and they have to cope with a dramatic temperature gradient across their length. The temperature at a worm's plume is about 2 °C, just above the freezing point of pure water at 1 atm, while that at its base is about 30 °C! Can tube worms be modeled as isothermal systems? Why or why not?

25. Individual model hydrogen bond donors and acceptors do not often form hydrogen bonds in aqueous solution. Why not?

26. You may have noted that Carnot's formulation of the Second Law of Thermodynamics involves a very bold and unusually strong word: "impossible." Is this always true? Why or why not?

27. The contraction of rubber is largely an entropic phenomenon. What are the sources of the enthalpic component?

28. Recall the example used to illustrate the entropy change in the surroundings when a mole of liquid water is formed from molecular hydrogen and molecular oxygen at 298 K. Use the data given in the text to calculate the entropy change per water molecule formed.

29. Protonation of the side chains of Glu and Asp is mainly an entropic effect. Why is this not true of His as well?

30. Show that when a system gains heat reversibly from surroundings held at constant temperature, there is no change in entropy.

31. "The entropy change during an irreversible process is higher than the entropy change during a reversible process." Is the statement true? Under what conditions?

32. What bearing does the Second Law have on pollution? (See pp. 241, 247 of Peusner (1974).)

33. Discuss Fig. 1.5 in terms of the concepts of Chapter 3.

Chapter 4

Gibbs free energy – theory

A. Introduction

This chapter discusses a thermodynamic relationship that provides a basis for explaining spontaneous chemical reactivity, chemical equilibrium, and the phase behavior of chemical compounds. The relationship involves a thermodynamic state function that enables prediction of the direction of a chemical reaction *at constant temperature and pressure*. The constraints of fixed T and p might seem annoyingly restrictive, because they are less general than the requirements of the Second Law, but in fact the gains made on imposing the constraints will outweigh the losses. How is that? One reason is at any given time an individual organism is practically at uniform pressure and temperature (but be sure to see the Exercises at the end of the chapter). Another is that constant temperature and pressure are the very conditions under which nearly all bench-top biochemistry experiments are done. Yet another is that, although the total entropy of the universe must increase in order for a process to be spontaneous, evaluation of the *total* entropy change *requires* measurement of both the entropy change of the system *and* the entropy change of the surroundings. Whereas ΔS_{system} can often be found without too much difficulty, albeit only indirectly, $\Delta S_{\text{surroundings}}$ can be *hard* to measure! How could one measure the entropy change of the rest of the universe? The subject of the present chapter provides a way around the difficulty.

A particularly clear example of the inadequacy of ΔS_{system} to predict the direction of spontaneous change is given by the behavior of water at its freezing point. Table 4.1 shows the thermodynamic properties of water for the liquid → solid phase transition. The decrease in internal energy (which is practically identical to the enthalpy as long as the number of moles of gas doesn't change; see Chapter 2) would suggest that water freezes spontaneously in the range 263–283 K. Going on internal energy alone, spontaneous freezing would seem even more probable at $+10\ ^{\circ}$C than at $-10\ ^{\circ}$C,

Table 4.1. *Thermodynamics of the liquid → solid transition of water at 1 atm pressure*

Temperature (°C)	ΔU (J mol^{-1})	ΔH (J mol^{-1})	ΔS (J mol^{-1} K^{-1})	$-T\Delta S$ (J mol^{-1})	ΔG (J mol^{-1})
−10	−5619	−5619	−21	5406	−213
0	−6008	−6008	−22	6008	0
−10	−6397	−6397	−23	6623	−226

Table 4.2. *Sign of ΔG and direction of change*

Sign of ΔG	Direction of change
$\Delta G > 0$	The forward reaction is energetically unfavorable, the reverse reaction proceeds spontaneously
$\Delta G = 0$	The system is at equilibrium, there is no further change
$\Delta G < 0$	The forward reaction is energetically favorable, the forward reaction proceeds spontaneously

because ΔU for this system becomes increasingly negative, meaning that the internal energy of the system decreases, with increasing temperature. The entropy change too is negative at all three temperatures, consistent with the solid state being more ordered than the liquid one. So the sign and magnitude of the entropy of the *system* does not predict the direction of spontaneous change (unless the system is isolated).

In contrast to ΔU (which is hardly different from ΔH in this case) and ΔS, the last column, ΔG, matches what we know about the physical chemistry of water: below 0 °C, it freezes spontaneously ($\Delta G < 0$), at 0 °C solid water and liquid water coexist ($\Delta G = 0$), and above 0 °C, ice is unstable ($\Delta G > 0$). *ΔG is negative for what we know is a spontaneous process, and it is positive for the reverse process* (Table 4.2). As we know from experience, a stretched rubber band will contract when released. What is the sign of ΔG for this process? Negative! ΔS is large and positive, making $-T\Delta S$ negative, and ΔH is negative. More generally, *$\Delta G < 0$ is a basis for explaining chemical reactivity, equilibrium, and phase behavior*. Providing a good understanding of the Gibbs free energy and how the biochemist can use it is one of the most important purposes of this book.

The thermodynamic state function of chief interest in this chapter is G, the Gibbs free energy. This quantity is an eponym of Josiah Willard Gibbs (1839–1903),[1] the American theoretical physicist and

[1] Gibbs was the fourth child and only son of Josiah Willard Gibbs, Sr, professor of sacred literature at Yale University. In 1863 the younger Gibbs became the first person to receive the doctorate in engineering in the USA. Gibbs never married, lived with his sister, and spent all of his life in New Haven, Connecticut, apart from

chemist who was the first to describe it. Like its cousins U and H, which we met in Chapter 2, G is measured in units of joules. If all these state functions have the same units, what distinguishes G from U and H? What *sort* of energy is the Gibbs free energy?

Free energy is energy that is available in a form that can be used to do work. You should not find this statement terribly surprising. Remember from the last chapter that *some* energy is *not* free to do work; heat transfer *always* generates waste heat, and waste heat *cannot* be used to do work. The Gibbs free energy measures the maximum amount of work that can be done by a process going from non-equilibrium to equilibrium (at constant temperature and pressure). The First and Second Laws place boundaries on what is possible in extremely general terms. The Gibbs free energy tells us how much work can be done by a system under the constraints of the First Law, the Second Law, constant temperature, and constant pressure. But there is no law of free energy!

Like H and U, G is defined for *macroscopic* systems, ones that involve a very large number of particles. This implies that, although measurement of ΔG does tell us how much work must be done to convert one state of a system into the other, or how much work could be done by a process, it does not explain *why* that much work should done. Nevertheless, thermodynamics is often all the more useful for its very power in dealing with systems described in qualitative of terms. For instance, a microcalorimeter enables the enthalpy change of a process, say, the unfolding of a protein, to be measured directly and with extraordinary accuracy, regardless of how little or much one knows about the structure of the protein. And a bomb calorimeter can be used to measure the heat of combustion of a beignet from Café du Monde in New Orleans or a *crème brûlé* from the Latin Quarter in Paris without knowing the slightest thing about ingredients, shape, structure of the molecules involved, or the nature of the interactions in either case. Indeed, one need not have heard of beignets to record good combustion data! A biophysicist can measure the heat effects of pure protein or DNA molecules whose three-dimensional structures are not known in detail. When a molecular interpretation of thermodynamic quantities is needed, one turns to a branch of physical chemistry called statistical mechanics (Chapter 6).

G is a thermodynamic *potential function*. As such, it is analogous to the gravitational potential function of classical mechanics, which describes how the gravitational energy of an object varies with position in a gravitational field. If you take a coin out of your pocket and let it go, it will change position spontaneously and rapidly! The coin falls because its gravitational potential energy is greater in the air than on

three years in Europe shortly after the American Civil War. He was appointed professor of mathematical physics at Yale University in 1871. Gibbs is arguably the most famous American-born scientist to date, owing partly to the promotion of his work by Maxwell.

the ground, and air gives relatively little resistance to a change of position. The coin moves down its gradient of gravitational potential. A potential function, like G, permits prediction of whether a system will change or stay the same under given conditions.

Of course any change in a system will happen at some rate – some extent of change per unit time. As we shall see, though, you don't need to know the rate of a reaction in order to say whether it will occur spontaneously. And the rate of a reaction is no predictor of the energy difference between reactants and products. But let's keep things simple and focus on energy differences here and tackle reaction rates in Chapter 8.

Finally, the mathematics of this chapter will be a little rougher ride than earlier on, so fasten your seatbelt. Don't worry, though, because we'll find that we can make the complete journey without anything more perplexing than algebraic equations and the odd logarithm. The ideas themselves will always be considered more important than the specific mathematical tricks by which the results are obtained. This is not to imply that the mathematics is unimportant; it's to put our priorities in proper order. Mathematics may be Queen of the Sciences, but Applied Mathematics is definitely Handmaid of the Sciences. Those who are prepared for a more demanding mental journey and wish to explore an all-terrain-vehicle treatment of the subject might consider consulting the more advanced references at the end of the chapter.

B. Equilibrium

We turn now to the very important concept of chemical equilibrium. It was first proposed by the Norwegian chemists Cato Maximilian Guldberg (1836–1902) and Peter Waage (1833–1900) in the 1860s, in the form of the law of *mass action*: when a system is at equilibrium, an increase (decrease) in the amount of reactants (products) will result in an increase (decrease) in the amount of products (reactants). An equilibrium system responds to change by minimizing changes in the relative amounts of reactants and products. For example, suppose we have a solution of our favorite protein molecule. At equilibrium some molecules will be in the folded state (reactant), some in the unfolded state (product). Now add a dash of protease. In the usual case, unfolded proteins are much more susceptible to proteolytic attack than folded proteins. If a proteolyzed protein is unable to refold, proteolysis will change the balance of folded proteins and unfolded ones. By the law of mass action, the response of the system to the decrease in the number unfolded proteins will be for folded proteins to unfold, in order to minimize the change in the relative amounts of reactants and products. Equilibrium is such an important concept that the Swedish chemist Svante August Arrhenius (1859–1927) called it "the central problem of physical chemistry." That was in 1911, eight years after

he had won the Nobel Prize in Chemistry. There will be more on Arrhenius in Chapters 8 and 9.

A detailed discussion of equilibrium was avoided in previous chapters for the simple reason that neither the First Law nor the Second Law depends on it. Another reason is that no living organism functions and practically no real process occurs under such conditions! But this hardly implies that thinking about systems at equilibrium is unimportant. That's because abstractions and idealizations play an extremely important role in scientific study, serving as models of reality or simple generalizations of otherwise complex phenomena. A basic idea of thermodynamics is that *any* physical system will *inevitably* and *spontaneously* approach a stable condition called equilibrium. This concept is bound up in the Second Law; it resembles the proposed relationship between entropy and ageing that we touched at the close of the previous chapter.

A system will exhibit a net change in time if it is *not* at equilibrium, even if the rate of change is imperceptible. An example of a system that is not at equilibrium is a pet parrot. It's prudent to keep it caged to prevent it becoming predator Pussy's supper, but unless Polly's fed, its protected perch won't lengthen its lifespan. This is because the metabolic reactions of the body require a continual input of chemical energy, and when the energy requirements are not met, the body winds down and dies. All living organisms are highly non-equilibrium systems. All have a tendency to decay. The tendency cannot be overcome without energy. Input panels (A) and (B) of Fig. 4.1 are clearly not at equilibrium; the fluid height is not level. There will be a net flow of liquid from one side to the other. The flow rate in one direction will be greater than the flow rate in the opposite direction. The system is at equilibrium in panel (C). There is no net flow. The flow rates in opposite directions are equal.

A system will not show net change if it is *at equilibrium* and *left unperturbed*. For instance, a plugged test-tube filled with a

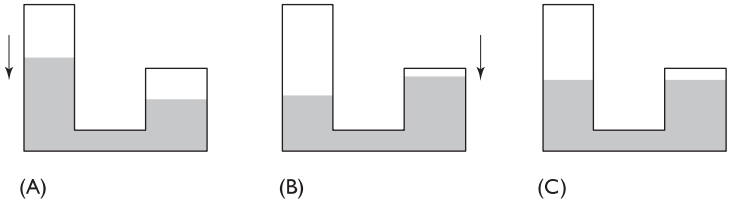

Fig. 4.1 Movement of a liquid system toward equilibrium. In panels (A) and (B) the system is not at equilibrium; we know from experience that change will occur. The driving force for change in this case is a difference in hydrostatic pressure, which is related to a difference in gravitational potential energy. The pressure difference is proportional to the difference in the height of the fluid in the two arms of the vessel. Water flows downhill! The rate of flow in one direction is greater than the rate of flow in the opposite direction. The system will continue to change until the fluid level is the same on both sides of the vessel. In panel (C), the flow rate is the same in both directions; the system is at equilibrium; no further change occurs.

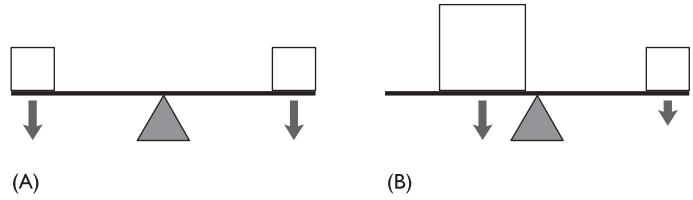

Fig. 4.2 Equilibrium. We know from experience that if the weights are the same, as in panel (A), the distance of each weight from the fulcrum must be the same. But if the weights are different, as in panel (B), the distance from the fulcrum cannot be the same. The lighter weight must be farther from the fulcrum than the heavier weight. By Newton's Second Law, at equilibrium the clockwise torque equals the counterclockwise torque, where torque = mass × gravitational acceleration × distance from fulcrum.

biochemical buffer and kept at constant temperature will not change (barring bacterial contamination and chemical degradation, of course). The system is in a rather stable equilibrium. A less stable equilibrium might be a heavy snow lying peacefully on a mountainside. Demonstration of the tenuous nature of this equilibrium could be regrettable for anyone or anything nearby: for a slight disturbance could turn a huge pile of resting snow into a raging avalanche! Another example of an unstable equilibrium is a balanced seesaw. The weights on either side of the fulcrum need not be equal, but if they are unequal, adjustments must be made in their distances from the fulcrum to achieve a balance, as in Newton's laws of mechanics (Fig. 4.2). What might be called a semi-stable equilibrium is one in which the energy of the system is at a minimum but not at the lowest possible minimum. For example, suppose a rock is resting at the bottom of a ravine between two hills. The rock will not leap up spontaneously and move elsewhere! If given a hard enough kick, though, it might roll up the side of the ravine, reach the crest of a hill, and tumble down the hill into the valley below. Various types of equilibrium are summarized in Fig. 4.3. The equilibrium state is one in which no further macroscopic change takes place because all forces acting on the system are balanced.

Let's look at the relationship of diffusion to equilibrium. Diffusion, a type of transport process, is important in many different biochemical reactions, for example, the chemical transmission of nerve impulses across synapses. Figure 4.4 shows one of many ways in which diffusion can be illustrated. We have two miscible liquids, and for the sake of argument we suppose that one can be layered aside the other, giving a sharp boundary, until we say "go." The top solution is colorless, like water, the bottom one opaque, like India ink. The densities are assumed to be about the same. Just after mixing has begun, which occurs spontaneously, there is a large concentration gradient across the boundary. But we know that this situation is unstable and will not persist. The result after a long time is that the dark liquid will be distributed uniformly throughout the combined volume.

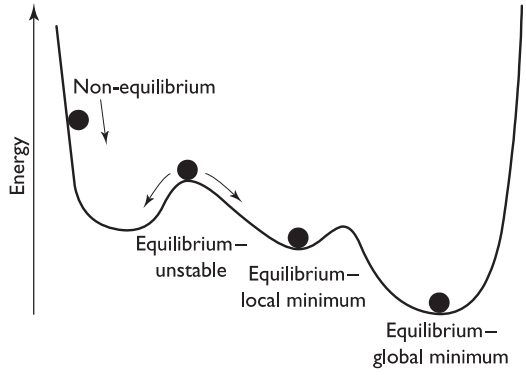

Fig. 4.3 Different types of equilibrium. The non-equilibrium situation will change immediately. The unstable equilibrium, like the snow at rest on a mountainside, will not change without the addition of energy. But the amount of energy needed to get over the barrier is very small. There are two main types of energy minimum: local minimum and global minimum. A local minimum has the lowest energy in the vicinity. The global minimum has the lowest free energy of all. An ongoing debate in protein folding research is whether the folded state of a protein corresponds to a local or a global free energy minimum. In some case, the functional state of a protein or enzyme might be a kinetically trapped conformation.

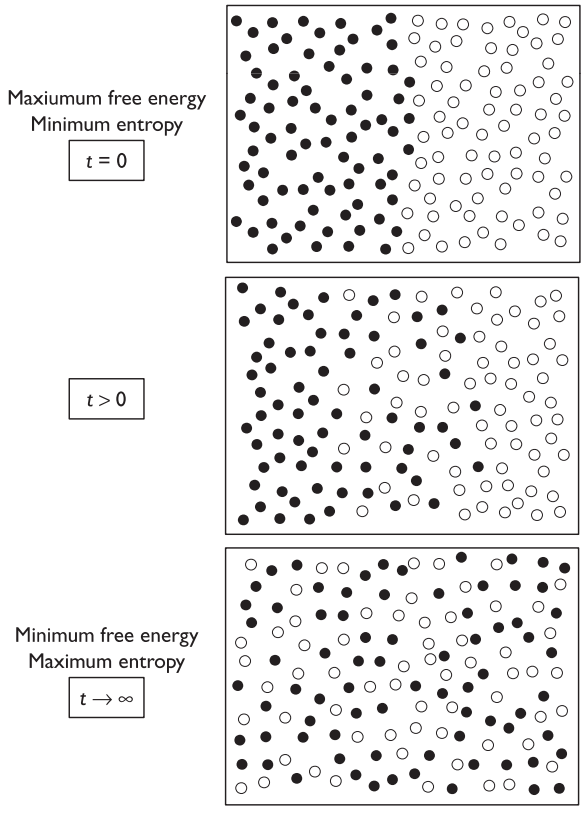

Fig. 4.4 Mixing. At time $t = 0$, when the partition is removed, the liquids are completely separate. The liquids are miscible so mixing occurs. After a sufficiently long time, the liquids are completely mixed. The unmixed state has a maximum of free energy for the system and a minimum of entropy. The completely mixed state has a minimum of free energy and a maximum of entropy. Based on Fig. 1.5 of van Holde (1985).

Figure 4.4A corresponds to the minimum entropy and maximum free energy of the system, while Fig. 4.4C, representing equilibrium, corresponds to the maximum entropy and minimum free energy of the system. Equilibrium will be achieved only if the temperature of the system is uniform throughout. For when the temperature is not uniform, convection currents and differences in particle concentration will be present, as for example in the swirls one sees shortly after pouring milk into a cup of coffee or tea. The convection currents and inhomogeneities will eventually go away, and when they have vanished, then the system will be at equilibrium. If we have two systems of identical molecules A and B, and the systems are in thermal equilibrium, then the distribution of the kinetic energy of the molecules of one system is identical to that of the other system. Some of the molecules will be moving very rapidly, others not so fast, but the distributions will be the same (Fig. 1.9). At equilibrium, the kinetic energy distribution of the molecules is one of maximum probability; the entropy, both of the individual systems and in combination, is a maximum; and the free energy, both of the individual systems and in combination, is a minimum.

An exercise from the previous chapter asked you to show that under the three constraints of *pV*-work only, constant internal energy, and reversibility, $\Delta S = 0$ at any temperature. We will now build on this foundation to develop the concept of equilibrium. Recall that when we required all change to a system to be reversible, we were saying that the system had to be at (or very close to) equilibrium throughout the process. In this context we interpret $\Delta S = 0$ to mean that the entropy must be at an extreme value when equilibrium is reached. By the Second Law, we know that the extremum is a maximum and not a minimum – the entropy of the universe always increases. In any non-equilibrium state of an isolated system, $S < S_{max}$, and the system will change *spontaneously* until $S = S_{max}$. Once equilibrium has been reached, there is no further increase in the entropy, $\Delta S = 0$. Somehow or other, a system "knows" when to stop "generating" entropy. This provides yet another way of stating the Second Law: an *isolated* system will change spontaneously until a maximum state of disorder is obtained.

Consider the reaction $Y \Leftrightarrow Z$. How far will the reaction go? Until equilibrium is reached, until there is no longer a tendency for a (macroscopic) change to occur spontaneously. At equilibrium, the average concentrations of Y and Z are constant in time; there is no further *macroscopic* change to the system. But this does not mean that the particles are no longer moving! For as long as the temperature is above 0 K, all the particles will be in motion, the amount depending on the temperature and whether the substance is in the solid, liquid or gas phase. If follows that at equilibrium Y and Z can and will interconvert, even if this happens only very slowly. Chemical equilibrium is a *dynamic equilibrium*. But the *concentrations* of Y and Z will not change – on the average.

If a process is made to occur through a succession of near-equilibrium states, the process must be slow, allowing the system to come to equilibrium after each small change. Real processes necessarily occur at a finite rate, so the best one can achieve in practice is a near-reversible process. Many biological macromolecules, for example small proteins, exhibit highly reversible order–disorder transitions ($> 95\%$) on thermal or chemical denaturation. It would appear in such cases that all the information required for the protein to fold to its native state is present in the matter it's made of, i.e. in the amino acid sequence. We'll return to protein folding in later chapters.

There are a number of other important features of the equilibrium state. One is that *for a system truly to be at equilibrium, it must be closed*. For example, if an unopened Pepsi can has reached thermal equilibrium, the contents will be at equilibrium. The amount of carbonic acid present and the amount of gaseous CO_2 will be constant, even if individual CO_2 molecules are constantly escaping from the liquid and returning thither. On opening the can, there will be a very rapid change in pressure and a jump to a non-equilibrium state. And a net loss of CO_2 will ensue for three main reasons. One, the system will no longer be closed, and gaseous CO_2 will escape immediately. Two, the decrease in abundance of CO_2 gas near the liquid–gas interface will promote the loss of CO_2 from the liquid. And three, if the can is held in your hand and not kept on ice, its contents will begin warming up, and this will drive off CO_2 because the solubility of the gas varies inversely with the temperature. We infer from all this that for a system to remain at equilibrium, variables such as T, p, V, and pH must be constant. For if any of them should change, or if the concentration of any component of the system should change, a non-equilibrium state would result, and the system as a whole would continue to change until equilibrium was reached.

C. Reversible processes

Now it's time for a mathematical statement of the Gibbs free energy:

$$G = H - TS. \tag{4.1}$$

We see that G is a sort of combination of the First and Second Laws, as it involves both enthalpy and entropy. We must bear in mind, though, that the temperature and pressure are constant for G to predict the direction of spontaneous change of a system.

What can be done with Eqn. (4.1)? For an incremental measurable change in G,

$$\Delta G = \Delta H - T\Delta S - S\Delta T. \tag{4.2}$$

If T is constant, the last term on the right-hand side vanishes, leaving $\Delta G = \Delta H - T\Delta S$. This tells us that the gain in *useful* work

from an isothermal system must be *less* than the gain in energy or enthalpy (ΔH). The difference is measured by the product of gain in entropy (ΔS) and the temperature at which the reaction occurs. $T\Delta S$ is "isothermally unavailable energy." ΔG is also the *minimum* work required to take a system from one equilibrium state to another.

Let's require that pV-work only be done in a reversible system. Then, because $\Delta U = T\Delta S - p\Delta V$, $H = U + pV$, and $\Delta H = \Delta U + p\Delta V + V\Delta p$, substitution into Eqn. (4.2) gives $\Delta G = [(T\Delta S - p\Delta V) + p\Delta V + V\Delta p] - T\Delta S - S\Delta T$, which simplifies to

$$\Delta G = V\Delta p - S\Delta T. \tag{4.3}$$

If we further require p and T to be constant, then $\Delta G = 0$. Just as with ΔS, we interpret this to mean that a reversible system has a maximal or minimal value of G when T and p are constant and the system is at equilibrium. In this case, and opposite to the entropy, the extremum is a minimum, just as with gravitational potential. In other words, the magnitude of ΔG measures the extent of displacement of the system from equilibrium, and $\Delta G = 0$ for a system at equilibrium.

Now we are positioned to see how the Gibbs free energy can be of great utility in predicting the direction of a process in a *closed* biochemical system. Have another look at Table 4.1, particularly the ΔG column. When ΔG is *positive* (if the energy change is *endergonic*), the process will *not* occur *spontaneously*. This is because the final state of the process has a higher free energy than the initial state, and this can be achieved only at the expense of the energy of the surroundings. When ΔG is *negative* for a process (if the energy change is *exergonic*), the reaction proceeds *spontaneously* in the direction of equilibrium, and when equilibrium is reached no further change will occur. *For any real process to occur spontaneously at constant temperature and pressure, the Gibbs free energy change must be negative*. Equation (4.1) shows that the lower the enthalpy (energy), the lower G, and the higher the entropy, the lower G. This tells us that spontaneity of a reaction is favored by a reduction of enthalpy (exothermic reactions) and by an increase of entropy (heat-releasing leading to increased disorder or increased energy that cannot be used to do work).

It must be emphasized that while the magnitude of ΔG tells us the size of the driving force in a spontaneous reaction, ΔG says nothing at all about the time required for the reaction to occur. Real physical, chemical, and biological processes occur at a finite rate, and all real chemical reactions are, to some extent, irreversible. Nevertheless, the basic principles of thermodynamics hold. Reversibility can be approached in the real world of biochemistry by having the process take place in a controlled manner, and that is what the biochemist often aims to do by studying reactions *in vitro*. The rate of a reaction will be developed in Chapter 8.

D. Phase transitions

We have already encountered phase transitions in two different contexts in this book: in likening the thermal denaturation of a protein molecule to the melting of an organic crystal, and in describing the physical properties of water. A *phase* is a system or part of a system that is homogeneous and has definite boundaries. A phase need not be a chemically pure substance. A phase transition is ordinarily caused by heat uptake or release, and when a phase change does occur it is at a definite temperature and involves a definite amount of heat. Phase changes are associated with a variety of fascinating and general aspects of biological thermodynamics. Let's take a few minutes to know more about them!

Phase transitions might look simple "from the outside;" in fact they are actually rather complex. No one really knows how to describe a phase change on the level of individual particles, though quantum mechanics must come into play at some level. Nevertheless, it is possible to give a description of a phase change on the macroscopic level in terms of classical thermodynamics. Some energetic quantities, for instance enthalpy and entropy, exhibit a *discontinuous* change at a phase boundary. What this means is that the enthalpy curve with temperature has the shape of a "step"; there is an abrupt change as the solid becomes a liquid or the liquid a solid, not a smooth change. Same for entropy. (See Fig. 4.5.) The amount of heat exchanged on melting or boiling is a heat capacity change, the latent heat of melting or vaporization, respectively. There is a relatively large change in heat capacity over an extremely small temperature range on a change of phase. Transitions of this type are "all-or-none" transitions; the material is completely in one phase on one side of the phase boundary and completely in another phase on the other side of the phase boundary. The two phases can coexist at the transition temperature. Such transitions are known as *first-order* phase transitions. These transitions resemble the "catastrophes" of an area of mathematics imaginatively called catastrophe theory. In many cases protein folding/unfolding closely resembles a first-order phase transition, for example, hen egg white lysozyme in Chapter 2. In more complicated situations, for example, gradual unfolding with heat increase, such a description is clearly inadequate (see Chapter 6).

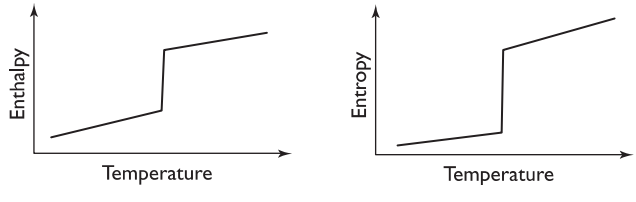

Fig. 4.5 First-order phase transition. The graphs show the behavior of the enthalpy and entropy functions versus temperature. Both of these thermodynamic quantities are *discontinuous* at the transition temperature, as is C_p.

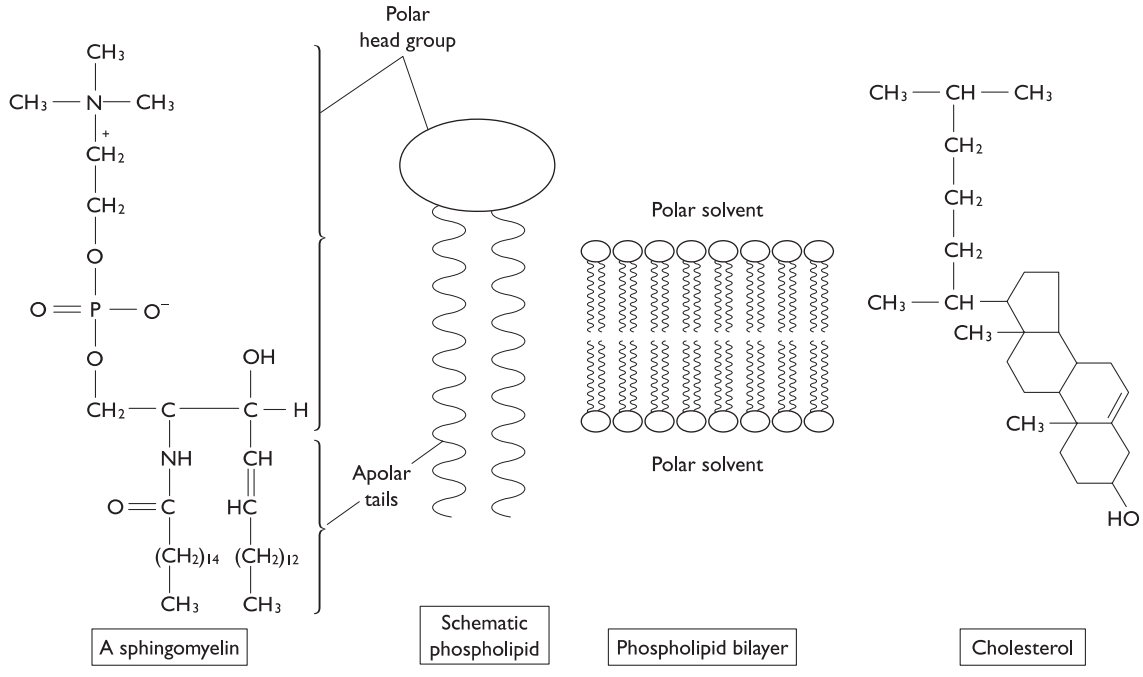

Fig. 4.6 Lipid bilayers. Lipids, or phospholipids, have two main regions, a polar "head" and aliphatic "tails." The head group is in contact with the solvent in a lipid bilayer, as shown. Sphingomyelins are the most common of the sphingolipids, which together form a major component of biological membranes. Sphingomyelins are just one type of phospholipid. The myelin sheath that surrounds and electrically insulates many nerve cell axons is rich in sphingomyelin. The most abundant steroid in animals is cholesterol. The metabolic precursor of steroid hormones, cholesterol is a major component of animal plasma membranes. In animals or plants, biological membranes are highly heterogeneous. They include not just several kinds of lipids and cholesterol, but membrane-spanning and membrane-associated proteins as well. At physiological temperatures, membranes are gel-like and allow lateral diffusion of their components.

The phase transition in water from the solid state to the liquid state is a first-order phase transition.

The liquid–solid phase boundary of water plays a key role in life on Earth – in more than one way. For instance, when the temperature drops, water begins to freeze on the surface of a pond, not at the bottom, and ice remains up top. Water on the surface loses its heat to the surroundings, the temperature of which can dip well below 0 °C. The density of solid water is lower than that of the liquid. Water is a peculiar substance! The physical properties of water also play a critical role in determining the level of the oceans and shaping the world's weather, determining what fraction of the oceans' water is liquid and how much is in the polar icecaps. In short, water determines the character of the biosphere.

Less well-known and obvious than the phase changes of water and proteins, perhaps, are those of lipids. These mainly water-insoluble molecules can undergo changes in state just as other compounds do. We say "mainly water-insoluble" because lipids are made of two parts, a small water-soluble "head" and a long water-insoluble "tail" (Fig. 4.6). In bilayers, which are surfaces of two layers of lipids in

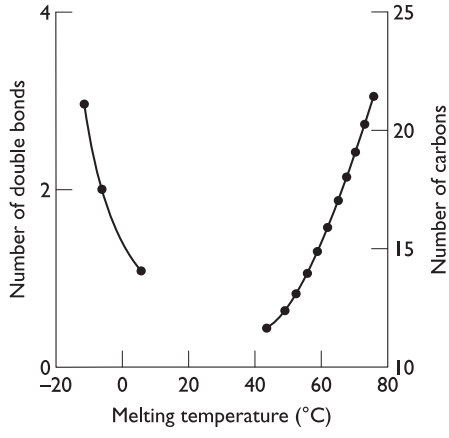

Fig. 4.7 Membrane melting temperature. In general, the melting temperature of a phospholipid bilayer decreases with increasing heterogeneity. An exception to the rule is cholesterol, which increases the melting temperature by increasing the rigidity of the bilayer. Increasing the number of double bonds in the aliphatic tail in lipids decreases the melting temperature by decreasing the ability of the molecules to pack against each other. A double bond introduces a kink in the tail. Increasing the length of the tail increases the melting temperature, because the aliphatic portions of lipids can interact favorably with each other by means of van der Waals forces. Redrawn from Fig. 7 of Bergethon (1998).

which the tails face each other, lipids can be in the liquid crystalline or gel state. This is an intermediate level of organization between the solid state, which is rigid, and the liquid state, which is fluid. The gel state is the one in which lipids are found in the membranes of the cells of living organisms. In pure lipid bilayers, there is a definite melting temperature between the solid state and the gel state, just as there is for water at the liquid–gas phase boundary and for a protein like lysozyme between its folded and unfolded states. The solid–gel transition of pure lipid bilayers is highly cooperative, also similar to the behavior of water and some proteins. Decreasing the purity of a bilayer, for instance by introducing a second type of lipid that is miscible with the first or by adding transmembrane proteins, the cooperativity of the solid–gel transition shows a corresponding decrease. Biological membranes in the living organism are highly heterogeneous: they are made of lipids in the fluid–lamellar phase, proteins, and some carbohydrate. Such membranes therefore do not exhibit very cooperative transitions, and melting occurs over a range of temperatures, usually 10–40 °C.

Other physical properties of lipids influence bilayer fluidity. One is length of the non-polar hydrocarbon tails (Fig. 4.7). The longer the chain, the higher the transition temperature. This is because the hydrophobic stuff of one lipid can interact fairly strongly with the hydrophobic stuff of another lipid. Another is the degree of saturation of the carbon–carbon bonds in the tails. Unsaturated bonds (double bonds) introduce kinks into the chain, making it more difficult for lipids in a bilayer to form an orderly array. Variation in the number and location of double bonds ensures that biological membranes do not become rigid. A contributor to membrane rigidity is lipid-soluble cholesterol, which decreases membrane fluidity by disrupting orderly interactions between fatty acid tails. Cholesterol itself is a rigid molecule (Fig. 4.6). The melting temperature also depends on solute and counter ion concentration! The physical properties of lipids, which are evidently complex, are of

vital important to the cell. Biological membranes permit membrane-bound proteins some degree of lateral movement, enable the cell to change shape and migrate, and make tissue formed by millions of cells relatively soft to the touch. Would a baby so enjoy nourishing itself at its mother's breast if her nipples were no more flexible than fingernails?

E. Chemical potential

Life as we know it could not exist without water. Small wonder that Thales of Miletus (fl. *c*. 500 BCE) considered water the ultimate substance, or *Urstoff*, the stuff of which all things are made! All physiological biochemical reactions take place in a largely aqueous environment, from enzyme catalysis to the folding of proteins to the binding of proteins to DNA to the assembly of large macromolecular complexes. It is probable that life on Earth began in a primordial sea of salt water (if it did not arrive from space). We had therefore better devote some energy to learning the basics of the thermodynamics of solutions. The path we shall take towards greater understanding will involve scaling a few jagged mathematical formulas, but the view we shall have at the end of the journey will make the effort worthwhile.

If you have doubted the usefulness of mathematics in previous study of biochemistry, it might help to bear in mind that mathematics is to biochemistry as a protocol is to producing a biochemical result. Mathematics is handmaiden, not master. Nevertheless, protocols themselves can be extremely useful, particularly when they are rather general in scope. One of the most highly cited scientific journal articles of all time is one of the most boring biochemistry articles ever published. This is because it has to do with a technique for separating proteins on the basis of size and says nothing specific about biology. But the paper in question is very important because the method it outlines (polyacrylamide gel electrophoresis) can be used in a very broad range of situations. In this sense, the protocol is even more important than any particular result it might be used to produce. In the same way, getting to know something of the mathematical background to a formula can be worth the time and effort, because the sort of thinking involved is in many cases of general utility.

Being quantitative about free energy changes is a matter of both being careful in making measurements and being clear about the conventions one has adopted. Teaching the first of these is beyond the scope of this book! To do the latter, we need to return to a topic introduced in Chapter 2: the standard state. Previously, we *defined* the standard enthalpy change, ΔH°, as the change in enthalpy for a process in which the initial and final states of one mole of a pure substance are at 298 K and 1 atm. Now we wish to define the *standard free energy change*, ΔG°. Here, the superscript indicates *unit activity* at standard temperature (298.15 K; three significant digits are usually

enough) and pressure (1 atm). The *activity* of a substance, a concept introduced by the American Gilbert Newton Lewis (1875–1946), is its concentration after correcting for non-ideal behavior, its effective concentration, its tendency to function as a reactant in a given chemical environment. There are many sources of non-ideality, an important one being the ability of a substance to interact with itself.

Ideal behavior of solute A is approached only in the limit of infinite dilution. That is, as $[A] \to 0$, $\gamma_A \to 1$. In the simplest case, the activity of substance A, a_A, is defined as

$$a_A = \gamma_A [A], \tag{4.4}$$

where γ_A is the *activity coefficient* of A on the *molarity* scale. When a different concentration scale is used, say the molality scale, a different activity coefficient is needed. The concept of activity is basically the same in both cases. According to Eqn. (4.4), $0 < a_A < [A]$ because $0 < \gamma_A < 1$. Activity is a *dimensionless* quantity; the units of the *molar* activity coefficient are $1\ mol^{-1}$.

Defining ΔG° at unit activity, while conceptually simple, is problematic for the biochemist. This is because free energy change depend on the concentrations of reactants and products, and the products and reactants are practically never maintained at molar concentrations throughout a reaction! Moreover, most reactions of interest do not occur at standard temperature. Furthermore, biochemistry presents many cases where the solvent itself is part of a reaction of interest. We need a way to take all these considerations into account when discussing free energy change.

The relationship between the concentration of a substance A and its free energy is *defined* as

$$\mu_A - \mu_A^\circ = RT \ln a_A, \tag{4.5}$$

where μ_A is the *partial molar free energy*, or *chemical potential*, of A, and μ_A° is the standard state chemical potential of A. The partial molar free energy of A is, in essence, just $\Delta G_A / \Delta n_A$, or how the free energy of A changes when the number of molecules of A in the system changes by one (Fig. 4.8). The chemical potential of A is a function of its chemical potential in the standard state and its concentration. Equation (4.5) could include a volume term and an electrical term (there are numerous other kinds of work, see Chapter 2), but let's assume for the moment that the system does not expand against a constant pressure and that no charged particles are moving in an electric field. It is appropriate to call μ the chemical potential because at constant T and p, G is a function of chemical composition alone.

Equation (4.5) tells us that when $a_A = 1$, $\mu_A - \mu_A^\circ = 0$. That is $\mu_A - \mu_A^\circ$ measures the chemical potential of A relative to the standard state conditions; the activity of a substance is 1 in the standard state. The chemical potential also depends on temperature as shown, and the gas constant puts things on a per-mole basis. Equation (4.5) also says that the chemical potential of a solvent

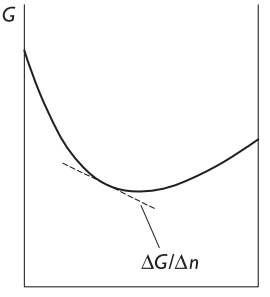

Fig. 4.8 Thermodynamic potential and solute concentration. The Gibbs free energy of a solute varies with concentration. The chemical potential measures the rate of change of G with n, or the slope of the curve at a given value of n ($\Delta G / \Delta n$). Note that G can decrease or increase on increases in concentration.

decreases as solute is added. The activity of a substance is always highest when that substance is pure. The reduction in chemical potential on mixing occurs even if the solution is ideal (enthalpy of mixing of zero),[2] as in the case where the solute is "inert" and does not interact with the solvent at all. This tells us that the decrease in chemical potential on mixing is fundamentally an entropic effect, even if for any real solvent and solute the change in chemical potential will contain an enthalpic component stemming from interparticle interactions.

Before getting too far down this road, let's look at an example that is aimed at helping to clarify the difference between ΔG° and ΔG (and thus $\Delta\mu^\circ$ and $\Delta\mu$). Suppose you're doing a study of the binding of a peptide hormone to a receptor situated in the plasma membrane of a cell. The cheapest way to obtain large quantities of pure hormone might be to synthesize it chemically and purify it using a type of liquid chromatography – quite possibly no problem at all if the peptide does not have to be glycosylated. Suppose that the sequence of the peptide you're studying is X–X–X–X–X–X–X–X–Ala–Gly, and because solid-phase chemical synthesis of peptides is done from the C-terminus to the N-terminus (the reverse of how it happens during translation of mRNA on a ribosome), the first coupling is of Ala to Gly attached to a resin. You'd like to know something about the energetics of formation of the peptide bond between these two amino acids. But when two free amino acids join to form a peptide bond, a water molecule is produced; the reaction is a type of dehydration synthesis. When the reaction occurs *in aqueous solution* under *standard state conditions* – that is, all products and reactants are at a concentration of 1 M *except water* (we'll say why later on) – the free energy change, ΔG°, is 4130 cal mol^{-1} – far from equilibrium! The driving force for change is in the direction of the reactants. When Ala and Gly are at 0.1 M, and Ala–Gly is at 12.5 μM, the reactants and products are no longer in their standard states, and the free energy difference is not ΔG° but ΔG. On doing an experiment to determine the energetics of peptide bond formation, one finds that the reaction is at equilibrium and that no change in the concentrations of reactants takes place unless the system is perturbed. In other words, $\Delta G = 0$. If the concentrations are again changed, so that Ala and Gly are at 1 M, as in the standard state reaction, but Ala–Gly is at 0.1 mM, you will find that the reaction proceeds in the direction of the products and $\Delta G = -1350$ cal mol^{-1}. ΔG on its own measures how far away a reaction is from equilibrium, and $\Delta G - \Delta G^\circ$ measures

[2] The given definition of an ideal solution is less general than it could be. We could say instead that an ideal solution is one for which the enthalpy of mixing is zero *at all temperatures*, implying that the solute always interacts with the solvent as it interacts with itself. An even more technical definition is that an ideal solution is a homogeneous mixture of substances whose physical properties are linearly proportional to the properties of the pure components, which holds in fact for many dilute solutions. This is known as Raoult's Law, after François-Marie Raoult (1830–1901), a French chemist.

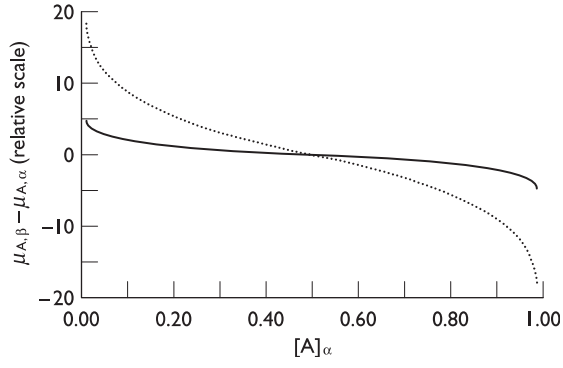

Fig. 4.9 Chemical potential difference as a function of concentration. The figure illustrates the behavior of the left-hand side of Eqn. (4.7) when the total concentration of A is fixed; $[A]_\alpha + [A]_\beta =$ constant. The dashed line represents a change in temperature.

how much the conditions differ from the standard state conditions. Yippee!

Let's have the discussion become a little more difficult. Suppose we have a two-component solution made of solvent and solute. The solute is some general substance A that is soluble in the solvent. The solvent could be water and the solute a metabolite. Assuming ideal behavior, $a_A = [A]$ and Eqn. (4.5) becomes

$$\mu_A - \mu_A^\circ = RT \ln[A]. \tag{4.6}$$

Let's now construct a *notional* partition between two regions of the solution and require that the system *not* be at equilibrium. Our mental boundary could be a liquid–gas interface or a membrane permeable to A. Substance A in any case can move across the boundary and back again by *random* motion. Calling the two regions of the solution α and β, the difference in chemical potential between them is

$$\Delta\mu_A = \mu_{A,\beta} - \mu_{A,\alpha} = RT \ln([A]_\beta / [A]_\alpha). \tag{4.7}$$

The standard state terms have vanished because the standard state free energy of A is the same in both regions. Intuitively, we would expect $\Delta\mu_A < 0$ when $[A]_\alpha > [A]_\beta$. When $[A]_\alpha > [A]_\beta$, the argument of the logarithm $([A]_\beta/[A]_\alpha)$ is less than one, and because $\ln x < 0$ for $x < 1$, our expectation is met (Fig. 4.9). When $\Delta\mu_A$ is negative, the solute particle will move spontaneously down its concentration gradient from α to β. If the concentration is greater in region α than region β, $\Delta\mu_A > 0$, and A will move spontaneously from β to α.

So far, so good. Backing up a couple of steps and combining Eqns. (4.4) and (4.5), we have

$$\mu_1 = \mu_1^\circ + RT \ln \gamma_1[1] \tag{4.8}$$

for component 1, the solvent. We can rewrite Eqn. (4.8) as

$$\mu_1 = \mu_1^\circ + RT \ln f_1 X_1, \tag{4.9}$$

where f_1 is the activity coefficient of component 1 on the *mole fraction*

scale. The mole fraction X_i is the *number of molecules* of i (i.e. n_i) expressed as a fraction of the total *number of molecules* in the system, n. In other words, $X_i = n_i/n$. We are still dealing with a two component system, so $X_1 = n_1/(n_1 + n_2) = 1 - X_2$, where X_2 is the mole fraction of solute A; the mole fractions of the individual components of solution must sum to 1. Writing $RT \ln f_1 X_1$ as $RT \ln f_1 + RT \ln X_1$ (which we can do because $\ln ab = \ln a + \ln b$), using $\ln(1 + x) = x - x^2/2 + x^3/3 \ldots$ (a relationship from mathematics which is valid for $-1 < x < 1$), and rearranging terms, Eqn. (4.7) becomes

$$\mu_1 - \mu_1^\circ = RT(-X_2 + \ldots) + RT \ln f_1. \tag{4.10}$$

We can simplify this beastly equation in two ways if the solution is dilute ($n_2 << n_1$). One is that the "higher order" terms in X_2 (namely, the square of X_2, the cube of X_2, etc.) are small because n_2 and therefore X_2 is small. So we consciously neglect them! The other assumption is that

$$X_2 \approx C_2 V_1^\circ / M_2, \tag{4.11}$$

where C_2 is the concentration of the solute in *molal* units (g l^{-1}), M_2 is the molecular weight of the solute, and V_1° is the molar volume of pure solvent. Equation (4.11) comes from $X_2 = n_2/(n_1 + n_2) \approx n_2/n_1$ when $n_2 << n_1$. Because $n_1 V_1^\circ = V$, the total volume of *solvent*, $X_2 \approx n_2 V_1^\circ / V = M_2 n_2 V_1^\circ / M_2 V = (M_2 n_2/V)(V_1^\circ/M_2) = C_2 V_1^\circ / M_2$. The dimensions of Eqn. (4.11) are (g l^{-1})×(l mol^{-1}) / (g mol^{-1}) = 1 (i.e., no units). That is, X_2 is dimensionless, as it must be! Substitution of Eqn. (4.11) into Eqn. (4.10) gives

$$\Delta\mu_1 = \mu_1 - \mu_1^\circ \approx -RTC_2 V_1^\circ / M_2 + RT \ln f_1 \tag{4.12}$$

for a dilute solution of component 2, the solute. Assuming that the second term on the right-hand side is small enough to be neglected (because $f_1 \approx 1$ and $\ln 1 \approx 0$), as is often approximately the case in biochemical experiments, we have a relatively simple and mathematical expression and can see that the chemical potential of a single-component solution relative to the pure solvent ($\Delta\mu_1$) is (approximately) directly proportional to the concentration of the solute and inversely proportional to solute mass. The higher the concentration of a solute, the higher its chemical potential. *A substance always diffuses from a region of higher concentration to a region of lower concentration*. The greater the mass of a particle, the lower its chemical potential. If we start to think about real biological macromolecules like proteins or DNA, things become more complex, because such molecules are almost always charged. Let's wait until Chapter 5 to see how to take charge into account.

F. Effect of solutes on boiling points and freezing points

Our comments on phase transitions have suggested that we should be aware of them in biochemistry. And the previous section

showed that the chemical potential of a substance depends on its concentration. Now we want to combine these areas of knowledge to obtain an expression for the change in boiling or freezing point of a solvent which results from the addition of a solute. We know that salt is used to keep roads clear of ice in the winter, and that salt is added to water to cook pasta, but why either is done may have been a mystery to you before now. Let's slog through a few more formulas and then look at examples of how to apply the gained knowledge to biochemistry.

From Eqn. (4.9) we have

$$\mu_1 - \mu_1^\circ = RT \ln f_1 X_1. \tag{4.13}$$

This can also be expressed as

$$(\mu_1 - \mu_1^\circ)/RT = \Delta G_{1,\mathrm{m}}/RT = (\Delta H_{1,\mathrm{m}} - T\Delta S_{1,\mathrm{m}})/RT = \ln f_1 X_1, \tag{4.14}$$

where, for instance, $\Delta G_{1,\mathrm{m}} = \Delta G_1/\Delta n_1$, and ΔG_1 represents the difference in the Gibbs free energy of component 1 between the one phase and the other. Because ΔH_m *and* ΔS_m *are relatively insensitive to temperature over short temperature ranges*, $\Delta H_\mathrm{m} = \Delta H_\mathrm{tr}$ and $\Delta S_\mathrm{m} = \Delta H_\mathrm{tr}/T_\mathrm{tr}$, where 'tr' stands for 'transition.' Substitution of these relations into Eqn. (4.14) gives

$$\Delta H_\mathrm{tr}/R \times (1/T - 1/T_\mathrm{tr}) = \ln f_1 X_1 \tag{4.15}$$

The difference $T - T_\mathrm{tr} = \Delta T$ is very small, so $1/T - 1/T_\mathrm{tr} \approx -\Delta T/T_\mathrm{tr}^2$. When this and Eqn. (4.10) are substituted in, we obtain

$$-\Delta H_\mathrm{tr}/R \times \Delta T/T_\mathrm{tr}^2 = -(X_2 + \cdots) + \ln f_1. \tag{4.16}$$

If the concentration of the solute, component 2, is small, then $\ln f_1 \approx 0$ and Eqn. (4.16) equation simplifies to

$$\Delta T \approx RX_2 T_\mathrm{tr}^2/\Delta H_\mathrm{tr}. \tag{4.17}$$

That's it! It is easy to see that ΔT varies proportionately with X_2. The greater the mole fraction of solute, the more the temperature of the phase transition will differ from that of the pure solvent. Because the effect depends on the mole fraction of the solute but not the solute's identity, it is called a colligative property, one that depends "on the collection" of solute molecules.

Are there practical applications in biological science of this hard-won knowledge of physical chemistry? Yes! In both cases, the addition of the "solute" changes the temperature of the phase change, in the one case lowering the freezing point and in the other raising the boiling point. There are also applications in the biochemistry lab. For instance, glycerol is often used to reduce the freezing point of aqueous protein solutions. The restriction enzymes so important for molecular biology and biotechnology are stored in *c.* 50% glycerol, which lowers the freezing point of water to below $-20\,^\circ$C! The enzymes are more thermostable at this temperature

than at 4 °C, and this preserves them for longer than if stored in the fridge by reducing the rate of spontaneous inactivation. Maintaining enzymes in the freezer comes with a cost – that of keeping the icebox running – so this is not a case of getting something for nothing! Then there are proteins that either raise or lower the freezing point of water with biological effect. The ones that lower it are known as anti-freeze proteins. Such proteins bind to and arrest the growth of ice crystals in the fish and thereby prevent it from freezing. By contrast, some bacterial proteins are known to *increase* the probability that supercooled water[3] will freeze.

Box 4.1 Puzzling proteins keep flounders from freezing

Pleuronectes americanus, the winter flounder, flourishes in the freezing waters of polar and subpolar oceans. Plasma proteins in the blood of this fish bind to ice crystals and stop them from growing. Type I anti-freeze protein, discovered in winter flounder 30 years ago, is a 3.3 kDa alanine-rich amphipathic molecule that forms a single α helix and binds to a pyramidal plane of ice. The concentration of this protein in fish blood plasma in winter is about 10–15 mg mℓ^{-1}, giving a non-colligative freezing-point depression, or thermal hysteresis, of 0.7 °C; far short of the −1.9 °C freezing point of sea water. The thermal hysteresis attributable to the colligative effects of other blood solutes adds another 0.8 °C to the total. But the 1.5 °C sum is not quite enough to keep the fish thawed. A piece of the jigsaw was missing. Recently, researchers in Ontario and Newfoundland, Canada, identified a previously unknown 16.7 kDa anti-freeze protein in the fish. This protein provides a thermal hysteresis of 1.1 °C at a concentration of just 0.1 mg mℓ^{-1}, about half the circulating concentration. It would appear that this new protein resolves the question of how the fish survives icy polar waters.

G. Ionic solutions

Ions in solution are called electrolytes. Charged particles get this name from their ability to conduct an electric current. Discussion about them takes up space in the present tome because water is found everywhere in the biological world, life as we know it could not exist without water, and most of the time plenty of ions are present in water. (An expensive water purification system is required to do work on ordinary water and separate ions from the solvent!) Moreover, many representatives of the three major classes of biological *macromolecule* – namely, proteins, nucleic acids, and polysaccharides – are charged at neutral pH, even if their net charge might be zero (though it usually isn't). And many species of lipid are charged. Charge properties help to give biomacromolecules important physical properties that are closely related to their physiological function.

[3] Liquid water cooled below its normal freezing point.

Box 4.2. Surviving a dip in the ice-cold ocean in a Speedo

In December 2005 British swimmer Mr. Lewis Pugh broke two records previously held by US swimmer Ms. Lynne Cox: the most southerly swim in the ocean, and the longest-duration swim near a Pole. For his first feat, Mr. Pugh swam 1 km under conditions that would kill a typical person within minutes. He spent 18 min 10 s in salt water just below 0 °C in the seas off the Antarctic Peninsula (latitude, 65° S). His second feat was a 1 mile swim for 30 min 30 s in mere 2–3 °C water. How was Mr. Pugh able to maintain body temperature and stave off the normally debilitating effects of the body's reaction to cold? In part by habituation, through increasingly frequent exposure to cold. The body can be trained not to shiver, allowing muscles to work more effectively in frigid surroundings. But when the surroundings are so cold, how is it possible to keep the temperature of the body above 35 °C, the cut-off point for hypothermia? Mental imagery plays a key role, stimulating the production of large amounts of heat by "anticipatory thermogenesis." Just as in sprinting and other sports, the release of stress hormones by the brain increases the metabolic rate of the body. Finally, there are the fitness and fatness factors. The latter is related to why women tend to be better than men at outdoor swimming: females tend to have more fat over their leg and arm muscles, which keeps them better insulated. Fitness favors the frantic movement of appendages, muscle activity, generation of heat, endurance, and speed. And men are generally ahead of women in that category. Mind over matter?

Ionic solutions tend to be dominated by electrical forces, which can, be very strong. Referring back to Table 2.2, you will see that the electrostatic energy of two electronic charges can be as great as 14 kcal mol^{-1}, an energy as large or larger than the free energy difference between the folded and unfolded states of a protein at room temperature. Often, though, electrostatic interactions are substantially weaker, as they depend not only on the distance between the interacting charges but also on the dielectric constant of the medium, D. In the middle of a protein, which is something like an oily solid, $D \approx 4$, and the electrostatic interaction is reduced to just 75 % from its vacuum value. In bulk aqueous solution, by contrast, $D \approx 80$! The polarity of water greatly reduces the distance over which the strength of the electric field created by a charge is significant. The strength of charge–charge interactions in water is often reduced even further by the orderly arrangement of a few water molecules in a "solvation shell" around an ion.

EDTA is a cation[4] chelator. It is a useful tool in the biochemist's kit because it can be used in a variety of practical situations. For instance, when preparing dialysis tubing, EDTA is used to "trap" divalent metal ions, "removing" them from solution, limiting their ability to bind to biomacromolecules one might want to prepare by

[4] Positive ions, e.g. Na^+, are cations. Cl^- and other negative ions are anions.

dialysis and proteases that might require them for activity. The activity of such proteases is greatly reduced in the presence of EDTA. ΔH for the binding of Mg^{2+} to EDTA is positive, but because ΔS is very negative, $\Delta G < 0$ and chelation occurs. Anyone who has ever tried to make an aqueous EDTA solution will know very well that the sodium salt does not dissolve very quickly at room temperature. If enough EDTA is present, when the solution comes to equilibrium only some of the salt will be dissolved. It might be possible to get more EDTA into solution by heating, which changes its solubility and the rate of dissolution.

What we want to do now is look at some general ways of thinking about thermodynamic properties of electrolytes. We'll do this by way of the example of EDTA. But let's bear in mind that the example will show how the approach is really much broader in scope. The solubility equilibrium of EDTA can be written as

$$Na_4EDTA(s) \Leftrightarrow 4Na^+ + EDTA^{4-}. \tag{4.18}$$

Based on this equation, at equilibrium,

$$\mu_{NaEDTA} = 4\mu_{Na^+} + \mu_{EDTA^-}. \tag{4.19}$$

The positive and negative ions appear as a pair because it is not possible to make separate measurements of the chemical potentials on the right hand side. To take this doubling effect into account, we define the *mean chemical potential*, $\mu_\pm$, which in this case is

$$\mu_\pm = \frac{4}{5}\mu_{Na^+} + \frac{1}{5}\mu_{EDTA^-}. \tag{4.20}$$

The coefficients account for the stoichiometry of dissociation of EDTA. Equation (4.19) can now be rewritten as

$$\mu_{NaEDTA} = 5\mu_\pm. \tag{4.21}$$

More generally,

$$W \Leftrightarrow \nu_+ A^{z+} + \nu_- B^{z-}. \tag{4.22}$$

In this equation W is a neutral compound, A and B are positive and negative ions with ion numbers z^+ and z^-, and ν_+ and ν_- are stoichiometric coefficients. The mean (not meaner!) chemical potential is

$$\mu_\pm = \frac{(\nu_+ A^{z+} + \nu_- B^{z-})}{V_+ + V_-} = \frac{\mu_{salt}}{\nu_+ + \nu_-}. \tag{4.23}$$

This is just W divided by the sum of the stoichiometric coefficients. Try working through the equations using EDTA as the example.

Substituting Eqn. (4.9) into Eqn. (4.20), we have

$$\mu_\pm = \frac{4}{5}(\mu^\circ_{Na^+} + RT\ln(f_{Na^+}X_{Na^+})) + \frac{1}{5}(\mu^\circ_{EDTA^-} + RT\ln(f_{EDTA^-}X_{EDTA^-})). \tag{4.24}$$

Making use of $x\ln a = \ln a^x$, a handy formula from mathematics, gives

$$= \mu_{\pm}^{\circ} + RT\ln\sqrt[5]{(f_{Na^+}X_{Na^+})^4 f_{EDTA^-}X_{EDTA^-}}, \tag{4.25}$$

where the standard state chemical potentials of Na^+ and pure $EDTA^-$ have been combined in the first term on the right-hand side of Eqn. (4.25).

Just as the chemical potentials of the ions cannot be measured separately, neither can one measure the activity coefficients separately. We therefore define a *mean ionic activity coefficient*, which for the present example is

$$f_{\pm} = {f_{Na^+}}^{4/5} {f_{EDTA^-}}^{1/5}. \tag{4.26}$$

This comes from $(ab)^{1/2}$, the *geometric mean* of a and b. In the more general case, Eqn. (4.26) looks like

$$f_{\pm} = (f_+^{\nu_+} f_-^{\nu_-})^{1/(\nu_+ + \nu_-)}, \tag{4.27}$$

where f_+ and f_- are the activity coefficients of the positive and negative ions on the mole fraction scale.

Knowing the mean activity coefficient of a salt can be important for interpreting the results of a biochemistry experiment. This will be especially true when the solution conditions are far from ideal, and particularly when the salt concentration is high. The bacteria that live in the Dead Sea, known as halophiles, thrive in a high salt environment. Somehow or other the molecular machinery of these bugs can cope with the high-salt surroundings. A high-salt solution is far from ideal, so the activity coefficients of ions in the surrounding environment of halobacteria deviate substantially from unity. In a similar example, the salt guanidinium chloride (GuHCl) is a strong protein denaturant. Most proteins are unfolded at a concentration of about 6 M GuHCl (a large concentration indeed, but one still many times smaller than the concentration of pure water, which is about ten-fold greater). Like HCl, GuHCl dissociates completely in aqueous solution to guanidinium ion and chloride ion (the solubility limit of GuHCl is well above 5 M at 25 °C). To explain in molecular terms what GuHCl does to protein structure, one needs to know its activity coefficient. We'll learn more about guanidinium chloride-induced unfolding of proteins in Chapter 5.

Now we want to think about electrolytes in a slightly different way. What follows is a simplified version of the theory of strong electrolytes developed by the Netherlander Petrus Josephus Wilhelmus Debye[5] (1884–1966) and the German Erich Hückel (1896–1980), published in 1923. The activity of an ion depends on a quantity known as the ionic strength, I, which is defined as

$$I = \frac{1}{2}\sum_i z_i^2 m_i. \tag{4.28}$$

[5] Debye was awarded the Nobel Prize in Chemistry in 1936.

Here m_i, the *molality*, is defined as X_i/M_s, the ratio of the mole fraction of solute i to the molecular mass of the solvent in *kilograms*, M_s. Note that if the salt you're working with is relatively simple, like NaCl, there is no problem in computing I: NaCl dissociates below its solubility limit, each particle carries just one charge, so a one molal solution of this salt has an ionic strength of $[(1^2\times1)+(1^2\times1)]/2=1$. $CaCl_2$ is somewhat more complicated, because the ions involved no longer carry the same charge.

Finally, it can be shown[6] that the activity coefficient of ion i on the molality scale is

$$\log \gamma_i = -Hz_i^2\sqrt{I}, \tag{4.29}$$

where γ_i is the activity coefficient on the *molality* scale. H is a complicated expression that depends the density of the solvent, the absolute temperature, the charge on an electron, the dielectric constant of the solvent ... For ions in water at 25 °C, $H\approx0.5$. And so there is a way to calculate γ_i. Things are much more complicated when dealing with polyvalent ions like proteins, because the degree of ionization is sensitive to pH. Polyvalent ions and ionization will be treated in greater depth below. Enough about ions for now!

H. Equilibrium constant

We have looked at the concept of equilibrium from various directions throughout the present chapter. But in fact, we have only scratched the surface of what could be known. Many things in life work like that. Here, we approach equilibrium in yet another way, one that is very useful to biological scientists, particularly biochemists.

Given a general reaction

$$a\mathrm{A} + b\mathrm{B} \Leftrightarrow c\mathrm{C} + d\mathrm{D} \tag{4.30}$$

the overall free energy change is

$$\Delta G = c\mu_\mathrm{C} + d\mu_\mathrm{D} - a\mu_\mathrm{A} - b\mu_\mathrm{B}. \tag{4.31}$$

Substituting Eqn. (4.31) into Eqn. (4.5), we have

$$\Delta G = \Delta G^\circ + RT\ln\left(\frac{[\mathrm{C}]^c\,[\mathrm{D}]^d}{[\mathrm{A}]^a\,[\mathrm{B}]^b}\right), \tag{4.32}$$

where $\Delta^\circ_G = c\mu^\circ_C + d\mu^\circ_D - a\mu^\circ_A - b\mu^\circ_B$ and we have not distinguished between activity and concentration, in case the reaction is not carried out at infinite dilution. The quantity $[\mathrm{C}]^c[\mathrm{D}]^d/[\mathrm{A}]^a[\mathrm{B}]^b$, called the *mass action ratio*, is based on Eqn. (4.30). Equation (4.32) indicates that the free energy change of a reaction has two parts: a constant term that depends only on the particular reaction taking place, and a

[6] See, for example, pp. 250–2 of Atkins (1998).

Table 4.3. *Relationship between ΔG° and K_{eq}*

Free energy change	Equilibrium constant
$\Delta G^\circ < 0$	$K_{eq} > 1$
$\Delta G^\circ = 0$	$K_{eq} = 1$
$\Delta G^\circ > 0$	$K_{eq} < 1$

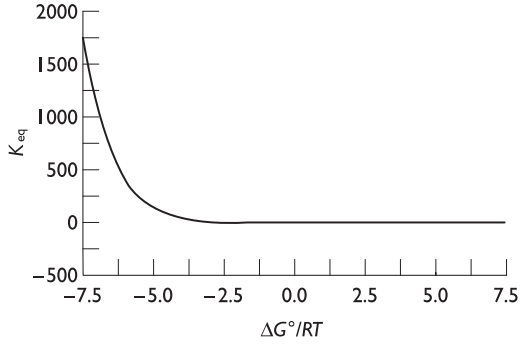

Fig. 4.10 Variation of K_{eq} with ΔG°. Note that K_{eq} is a function of the negative logarithm of ΔG°. When ΔG° is large and negative, K_{eq} is very large. When ΔG° is large and positive, K_{eq} is very small. In most biochemical reactions ΔG° will fall within the range shown here.

variable term that depends on temperature, concentrations of reactants and products, and stoichiometric relationships.

At equilibrium, the forward reaction balances the reverse reaction, and $\Delta G = 0$. So

$$\Delta G^\circ = -RT \ln K_{eq} = -RT \ln \left(\frac{[C]^c_{eq}\,[D]^d_{eq}}{[A]^a_{eq}\,[B]^b_{eq}} \right), \tag{4.33}$$

where the subscript "eq" signifies "equilibrium." The concentrations of reactants and products are the concentrations at equilibrium. The equilibrium constant of the reaction, K_{eq}, is *defined* as

$$K_{eq} = \left(\frac{[C]^c_{eq}\,[D]^d_{eq}}{[A]^a_{eq}\,[B]^b_{eq}} \right). \tag{4.34}$$

Note that K_{eq} will be unitless if $a + b = c + d$. Equation (4.33) indicates that K_{eq} can be calculated from standard state free energies. The form of relationship is illustrated in Fig. 4.10. You can get a feel for magnitudes by substituting in values (see Table 4.3). For instance, when $\Delta G^\circ = 0$, $K_{eq} = 1$. A 10-fold change in K_{eq} at 25 °C corresponds to $\Delta G^\circ = 5.7$ kJ mol^{-1}, an energy difference two to three times greater than thermal energy at the same temperature. Deviations from equilibrium will stimulate a change in the system towards the equilibrium concentrations of reactants and products. This is known as *Le Châtelier's principle*.[7] A reaction will go to completion for $K_{eq} \geq 10^4$.

[7] Named after the French chemist Henri Louis Le Châtelier (1850–1936). The principle was first enunciated in 1884, and it applies equally as well to reversible chemical

Comparison of Eqn. (4.32) with Eqn. (4.34) tells us that it will be hard to change the direction of a reaction with a very large or very small value of K_{eq} by changing the mass action ratio. In Chapter 5 we shall see how this difficulty is relevant in glycolysis, the process of glucose metabolism. Finally, Eqn. (4.34) also has a relationship to reaction rates, which was put forward by van't Hoff. We mention this here as a foretaste of the development in Chapter 8.

Here's a worked example of the relationship between Gibbs free energy change and equilibrium constant. Consider the glycolytic reaction in which a phosphoryl group is moved from carbon 1 of glucose to carbon 6:

$$\text{glucose-1-phosphate} \Leftrightarrow \text{glucose-6-phosphate}. \tag{4.35}$$

The equilibrium constant for this reaction, which is catalyzed by the enzyme phosphoglucomutase, is 19. How big is ΔG° at room temperature? By Eqn. (4.31), $\Delta G^\circ = -(8.314\ \text{J mol}^{-1}\ \text{K}^{-1}) \times 298\ \text{K} \times \ln(19) = -7.3\ \text{kJ mol}^{-1}$. The negative sign indicates that reaction proceeds to the right spontaneously *under standard conditions*. We need not have evaluated the magnitude of ΔG° to know this (Table 4.1), because when $K_{eq} > 1$, $\Delta G^\circ < 0$ and the forward reaction will be spontaneous. If $K_{eq} < 1$, the reaction will be energetically unfavorable. Let's see what happens when we alter the concentrations of reactants and products in Eqn. (4.35) and require the concentrations of reactants and products to be held at 10 mM and 1 mM, respectively. By Eqn. (4.32), $\Delta G = -7.3\ \text{kJ mol}^{-1} + (8.314\ \text{J mol}^{-1}\ \text{K}^{-1}) \times 298\ \text{K} \times \ln(1/10) = -13\ \text{kJ mol}^{-1}$. The reaction is considerably more exergonic than under standard state conditions. This tells us both that we are far from equilibrium, where $\Delta G = 0$, and the reaction will proceed to the right to reach equilibrium. Clearly, the magnitude of ΔG can depend significantly on the concentrations of reactants and products. In fact, the concentrations can be different enough in some cases (when $\Delta G \approx 0$) to reverse the direction of the reaction, a situation that is relatively common in metabolism and occurs in glycolysis. What is the position of the enzyme in this picture? Will it influence the rate of reaction? Yes! Will it change the free energy difference between products and reactants? No! Why not? The Gibbs free energy is a thermodynamic state function. As such, what it measures depends only on the state of the system and not on how the system was prepared.

I. Standard state in biochemistry

Most *in vitro* biochemical experiments are carried out at constant temperature and pressure in dilute *aqueous solution* near *neutral* pH. To be maximally useful, the *biochemist's definition of standard state*

reactions and reversible physical processes. The conclusions reached by Le Châtelier in his 1884 work had been anticipated in part by the American J. Willard Gibbs.

should take all these conditions into account. Doing this will seem to make things more complicated at first, but not ultimately.

We *define* the standard state of water to be that of the pure liquid. This means that the activity of water is *set* to 1, even though its concentration is 55.5 M. One justification for this is that if a reaction is carried out in dilute aqueous solution, the percentage change in concentration of water will ordinarily be negligible. Another is that it is pretty easy to multiply and divide by 1! You might agree that many *in vitro* experiments are carried out under relatively close-to-ideal conditions, but object that such conditions will often differ greatly from those in a cell. Which cell? Which organism? Where? We take 25 °C as the standard temperature because it is convenient for bench-top experiments. It is also close to the temperature of many organisms. The hydrogen ion activity is *defined* as unity at pH 7 (neutral solution), not pH 0 (highly acidic solution where the activity of H_3O^+ is 1).[8] Finally, no account is taken of the various ionization states of molecules that might be present at pH 7. This is particularly relevant to biological macromolecules, especially proteins, which can be ionized in a multitude of different ways. When all the stated conditions are accounted for, the standard state free energy change is symbolized as $\Delta G^{\circ\prime}$. The prime indicates pH 7. See Table 4.4.

To put the foregoing discussion in practice, consider the following chemical reaction, a sort of variation on Eqn. (4.30):

$$\mathrm{A} + \mathrm{B} \Leftrightarrow \mathrm{C} + \mathrm{D} + n\mathrm{H_2O}. \tag{4.36}$$

From Eqn. (4.34),

$$\begin{aligned}\Delta G^\circ &= RT \ln K_{\mathrm{eq}} \\ &= RT \ln([\mathrm{C}]_{\mathrm{eq}}[\mathrm{D}]_{\mathrm{eq}}[\mathrm{H_2O}]^n/[\mathrm{A}]_{\mathrm{eq}}[\mathrm{B}]_{\mathrm{eq}}) \\ &= RT \ln([\mathrm{C}]_{\mathrm{eq}}[\mathrm{D}]_{\mathrm{eq}}/[\mathrm{A}]_{\mathrm{eq}}[\mathrm{B}]_{\mathrm{eq}}) - nRT \ln[\mathrm{H_2O}],\end{aligned} \tag{4.37}$$

which, on invoking the biochemist's conventions, is

$$\Delta G^{\circ\prime} = RT \ln K_{\mathrm{eq}}{}' = RT \ln([\mathrm{C}]_{\mathrm{eq}}[\mathrm{D}]_{\mathrm{eq}}/[\mathrm{A}]_{\mathrm{eq}}[\mathrm{B}]_{\mathrm{eq}}). \tag{4.38}$$

Thus, the relationship between ΔG° and $\Delta G^{\circ\prime}$ is

$$\Delta G^{\circ\prime} = \Delta G^\circ + nRT \ln [\mathrm{H_2O}]. \tag{4.39}$$

What if protons are "consumed" in the reaction, taken up from the solvent by solute molecules? The situation can be modeled as

$$\mathrm{A} + \nu\mathrm{H}^+(\mathrm{aq}) \rightarrow \mathrm{P}. \tag{4.40}$$

By Eqn. (4.31),

$$\Delta G = \mu_{\mathrm{p}} - \mu_{\mathrm{A}} - \nu\mu_{\mathrm{H}^+}. \tag{4.41}$$

If A and P are in their standard states, then

$$\Delta G = \mu^\circ_{\mathrm{P}} - \mu^\circ_{\mathrm{A}} - \nu\mu_{\mathrm{H}^+}. \tag{4.42}$$

[8] H_3O^+ symbolizes the hydronium ion, the proton in aqueous solution.

Table 4.4. *Values of $\Delta G^{\circ\prime}$ for some important biochemical reactions*

Reaction	$\Delta G^{\circ\prime}$ (kcal mol^{-1})
HYDROLYSIS	
Acid anhydrides:	
Acetic anhydride + $H_2O \rightarrow$ 2 acetate	−21.8
$PP_i + H_2O \rightarrow 2P_i$	−8.0
$ATP + H_2O \rightarrow ADP + 2P_i$	−7.3
Esters:	
Ethylacetate + $H_2O \rightarrow$ ethanol + acetate	−4.7
Glucose-6-phosphate + $H_2O \rightarrow$ glucose + P_i	−3.3
Amides:	
Glutamine + $H_2O \rightarrow$ glutamate + NH_4^+	−3.4
Glycylglycine + $H_2O \rightarrow$ 2 glycine (a peptide bond)	−2.2
Glycosides:	
Sucrose + $H_2O \rightarrow$ glucose + fructose	−7.0
Maltose + $H_2O \rightarrow$ 2 glucose	−4.0
ESTERIFICATION	
Glucose + $P_i \rightarrow$ glucose-6-phosphate + H_2O	+3.3
REARRANGEMENT	
Glucose-1-phosphate $\rightarrow$ glucose-6-phosphate	−1.7
Fructose-6-phosphate $\rightarrow$ glucose-6-phosphate	−0.4
Glyceraldehyde-3-phosphate $\rightarrow$ dihydroxyacetone phosphate	−1.8
ELIMINATION	
Malate $\rightarrow$ fumarate + H_2O	+0.75
OXIDATION	
Glucose + $6O_2 \rightarrow 6CO_2 + 6H_2O$	−686
Palmitic acid + $23O_2 \rightarrow 16CO_2 + 16H_2O$	−2338
PHOTOSYNTHESIS	
$6CO_2 + 6H_2O \rightarrow$ six-carbon sugars + $6O_2$	+686

The data are from p. 397 of Lehninger, A. L. (1975) *Biochemistry*, 2nd edn. New York: Worth.

The chemical potential of H^+, from Eqn. (4.5), is

$$\mu_{H^+} = \mu^\circ_{H^+} + RT\ln a_{H^+} = \mu^\circ_{H^+} - 2.303RT(\mathrm{pH}), \tag{4.43}$$

where we have assumed ideal conditions and used the definition of pH ($\log[H^+] \approx -\mathrm{pH}$, see below). Combining Eqns. (4.42) and (4.43) gives

$$\Delta G = \mu^\circ_P = \mu^\circ_A - \nu(\mu^\circ_{H^+} - 2.303RT(\mathrm{pH})) = \Delta G^\circ + \nu 2.303\mathrm{RT}(\mathrm{pH}). \tag{4.44}$$

The biochemist's standard state, however, is defined for pH = 7. So,

$$\Delta G^{\circ\prime} = \Delta G^\circ - \nu 16.121RT \tag{4.45}$$

The free energy difference varies linearly with T and ν, the number of protons transferred in the reaction. Happily, if neither H_2O nor H^+ is involved in the reaction, then $n = 0$ and $\nu = 0$, and $\Delta G^{\circ\prime} = \Delta G^\circ$.

J. Effect of temperature on K_{eq}

The concentrations of reactants and products depend on the physical conditions, so too must the equilibrium constant. We can see that K_{eq} varies with temperature as follows:

$$\ln K_{eq} = -\Delta G^\circ/RT = -(\Delta H^\circ/R)(1/T) + \Delta S^\circ/R. \quad (4.46)$$

(See Fig. 4.11.) As before, the superscript indicates standard state, but no prime appears because we have not specified pH 7. In general, ΔH° and ΔS° depend on T. Often, though, the enthalpy and entropy changes will be relatively weak functions of temperature, and a plot $\ln K_{eq}$ versus $1/T$, called a *van't Hoff graph*, will be approximately linear. The slope and intercept of the line are $-\Delta H^\circ/R$ and $\Delta S^\circ/R$, respectively. The upshot is that if K_{eq} can be measured at several temperatures in the range, ΔH° and ΔS° can be *measured*, albeit indirectly. ΔH° determined from the behavior of K_{eq} is called the *van't Hoff enthalpy*, or ΔH_{vH}.

If ΔH° and ΔS° depend significantly on temperature, as with proteins (because of the large ΔC_p of unfolding), as long as folding/unfolding is cooperative, the phase transition will occur over a relatively narrow temperature range, as in the melting of a pure solid, and ΔH° and ΔS° will be *approximately* constant in the range. A van't Hoff plot can then be used to estimate these thermodynamic functions *at the transition temperature*. Such indirect measurements of protein energetics have been corroborated by direct calorimetric measurements. In scanning microcalorimetry (Fig. 2.10B), the heat measurement is made at constant pressure, so $q = \Delta H = \Delta H_{cal}$, the *calorimetric enthalpy*, the area below the heat absorption peak. If the folding/unfolding reaction is highly cooperative and involves effectively only two states, then the ratio $\Delta H_{vH}/\Delta H_{cal} = 1$. Deviations from such behavior, whether from oligomerization of the folded state or stabilization of partly folded species, have an enthalpy ratio greater than or less than one, respectively. We shall return to this subject in Chapter 5 and discuss it in considerable detail in Chapter 6.

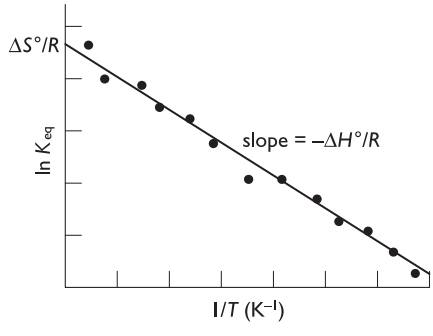

Fig. 4.11 A van't Hoff plot. This approach to data analysis will be useful when only two states are involved in the transition *and* if either of two conditions is met: ΔC_p is of negligible magnitude; or, if ΔC_p is large, the temperature range is small enough that ΔH° and ΔS° can be considered approximately independent of temperature. van't Hoff analysis is often used when data are collected by a technique that does not make a direct measurement of heat (e.g. any type of optical spectroscopy, NMR spectroscopy, viscometry, X-ray scattering, electrophoresis). See Chapter 7 for a discussion of multi-state equilibria.

How does a fluctuation in T translate into a fluctuation in $\ln K_{eq}$? In other words, how does $T \rightarrow (T + \Delta T)$ affect $\ln K_{eq}$? If the change brought about in $\ln K_{eq}$ by the temperature fluctuation is $\Delta \ln K_{eq}$, by Eqn. (4.44) we have

$$\ln K_{eq} + \Delta \ln K_{eq} = -(\Delta H^\circ/R)(1/(T + \Delta T)) + \Delta S^\circ/R. \tag{4.47}$$

To evaluate $1/(T + \Delta T) = (T + \Delta T)^{-1}$, we make use of a famous relationship in mathematics known as the binomial theorem, according to which $(x + y)^n = x^n + nx^{n-1}y + n(n-1)x^{n-2}y^2/2 + \ldots$ for $y^2 < x^2$. Our requirement is $\Delta T < T$, so the theorem can be applied! Making the substitution, we have

$$\begin{aligned} \ln K_{eq} + \ln K_{eq} &= -(\Delta H^\circ/R)[T^{-1} + (-1)T^{-2}\Delta T + (-1)(-2)T^{-3}(\Delta T)^2/2 + \cdots] \\ &\quad + \Delta S^\circ/R \ldots \\ &= -(\Delta H^\circ/R)(1/T) + (\Delta H^\circ/R)(T^{-2})(\Delta T) \\ &\quad - (\Delta H^\circ/R)(T^{-3})(\Delta T)^2/2 + \cdots + \Delta S^\circ/R. \end{aligned} \tag{4.48}$$

The first and last terms on the right-hand side of Eqn. (4.48) sum to $\ln K_{eq}$, so these terms can be dropped from both sides, leaving

$$\Delta \ln K_{eq} = (\Delta H^\circ/R)(T^{-2})(\Delta T) - (\Delta H^\circ/R)(T^{-3})(\Delta T)^2/2 + \cdots \tag{4.49}$$

If ΔT is small, then the second and following terms on the right-hand side will be much smaller than the first one. So we have

$$\Delta \ln K_{eq} \approx (\Delta H^\circ/R)(T^{-2})(\Delta T). \tag{4.50}$$

Does the approximation work? Let's test it! Suppose $\Delta H^\circ = 50$ kcal mol^{-1} at 300 K, a representative value for a small protein. If $\Delta T \sim 1$ K, then by Eqn. (4.50) $\Delta \ln K_{eq} = (50 \text{ kcal mol}^{-1}) \times (300 \text{ K})^{-2} \times (1 \text{ K}) / (1.9872 \text{ cal mol}^{-1} \text{ K}^{-1}) - (50 \text{ kcal mol}^{-1}) \times (300 \text{ K})^{-3} \times (1 \text{ K})^2 / (1.9872 \text{ cal mol}^{-1} \text{ K}^{-1}) = 0.2796 + 0.0009$. That is, in neglecting the second term, we incur an error of less than 1% – smaller than the usual uncertainty in measuring protein concentration! The error of the neglected terms would be greater if all of them were taken into account, but it can be shown that the total error would still be small. If, however, living organisms flourished at a temperature much closer to absolute zero, the relative magnitude of a 1 degree fluctuation would be much larger, the calculation we have just done would not involve division by such a large number, and the terms we have neglected would be much bigger. Life seems to depend on liquid water – the ability of water molecules to move around with a degree of independence – and on fluctuations in temperature and therefore thermal energy to be relatively small. The Antarctic bacteria mentioned in Chapter 1 are hardly "living" when frozen, even if they are not "dead." Finally, Eqn. (4.50) does provide a handy way of calculating the effect of temperature on $\ln K_{eq}$. You may have noticed, though, that we made a key simplifying assumption in the calculation – that the variation of ΔH° and ΔS° with temperature is small!

K. Acids and bases

A key application of the concept of equilibrium in biology concerns acids and bases in solution. According to the *Brønsted–Lowry definitions*, an *acid* is a proton donor and a *base* is a proton acceptor.[9] Biological situations usually concern water in some way or other. A measure of the acidity of water, due to the Danish biochemist Sørenson, is its pH, defined as

$$\mathrm{pH} = -\log a_{\mathrm{H_3O^+}}. \tag{4.51}$$

The pH of a solution determines the extent of proton dissociation from ionizable chemical groups in biological macromolecules and thus can have a profound effect on enzyme activity, protein–protein association, protein–DNA binding, and other types of biochemical reaction. We therefore had better put some effort into knowing this subject!

Suppose we have an acid HA. It participates in the following reaction in water:

$$\mathrm{HA(aq) + H_2O(l) \Leftrightarrow H_3O^+(aq) + A^-(aq)}. \tag{4.52}$$

The *acidity constant* for this reaction is *defined* as

$$K_\mathrm{a} = \left(\frac{a_{\mathrm{H_3O^+}} a_{\mathrm{A^-}}}{a_{\mathrm{HA}} a_{\mathrm{H_2O}}}\right) \approx \left(\frac{a_{\mathrm{H_3O^+}} a_{\mathrm{A^-}}}{a_{\mathrm{HA}}}\right). \tag{4.53}$$

The approximation is justified on the same grounds as before: if our concern is dilute aqueous solutions, the activity of water is close to 1 and essentially unchanging. At low concentrations of HA, the activity of hydronium ions is roughly equal to their molar concentration, and the acidity constant is usually written as

$$K_\mathrm{a} \approx [\mathrm{H_3O^+}][\mathrm{A^-}]/[\mathrm{HA}], \tag{4.54}$$

where the concentrations are in mol ℓ^{-1}. This approximation is valid only when *all* the ions in solution are present in low concentrations. Neglecting the limitations of the approximation can introduce complications. For instance, 1 mM imidazole[10] has an activity coefficient of about 0.95, and ignoring this can affect the equilibrium constant by as much as 10%! Acidity constant values are often tabulated in terms of their negative logarithm, pK_a:

$$\mathrm{p}K_\mathrm{a} = -\log K_\mathrm{a}. \tag{4.55}$$

The pK_a is related to the standard state free energy of ionization as

[9] Johannes Nicolaus Brønsted, a Danish physical chemist, lived 1879–1947. Thomas Martin Lowry was an Englishman. Brønsted and Lowry introduced their definitions simultaneously but independently in 1923.

[10] A common buffer in biochemistry. The structure of imidazole is the same as that of the side chain of histidine. The pK_a of imidazole is about 6.5, close to neutral pH.

Table 4.5. | pK_a' values of acidic and basic groups in proteins.

Group	Amino acid residue	pK_a'(25 °C)
α-Carboxyl		3.0–3.2
Carboxyl	Aspartic acid	3.0–4.7
	Glutamic acid	~4.5
Imidazolyl	Histidine	5.6–7.0
α-Amino		7.6–8.4
Sulfhydryl	Cysteine	9.1–10.8
Phenolic hydroxyl	Tyrosine	9.8–10.4
Guanidino	Arginine	11.6–12.6

Note that a range of values is given. The acidity constant will depend in part on the specific electronic environment of the acidic or basic group.

$$\mathrm{p}K_\mathrm{a} = \Delta \mathrm{G}^\circ/(2.303RT). \tag{4.56}$$

Taking the logarithm of both sides of Eqn. (4.54) yields the *Henderson–Hasselbalch equation*:

$$\mathrm{pH} = \mathrm{p}K_\mathrm{a} - \log([\mathrm{HA}]/[\mathrm{A}^-]). \tag{4.57}$$

The form of this relation is the same as that of Eqn. (4.7). Equation (4.57) tells us that the pK_a of an acid indicates the pH at which half of the protons are dissociated (when $[\mathrm{HA}] = [\mathrm{A}^-]$, $\log([\mathrm{HA}]/[\mathrm{A}^-]) = 0$). A buffer exhibits its greatest *buffering capacity* at this pH: a change in $[\mathrm{H_3O^+}]$ or $[\mathrm{OH}^-]$ has the smallest effect on the solution pH at the pK_a. The A^- ions at this pH can react with the largest amount hydronium ions produced on addition of strong acid, and the HA present can react with strong base added. The pK_a values of acidic and basic groups in proteins are given in Table 4.5. Proteins themselves are crucial for buffering fluids in living organisms. For example, about 80% of the buffering capacity of human blood comes from proteins, principally serum albumin and hemoglobin.

Let's use Eqn. (4.57) to calculate the pH of buffer solution containing 0.200 mol ℓ^{-1} $\mathrm{KH_2PO_4}$ (monobasic potassium phosphate) and 0.100 mol ℓ^{-1} $\mathrm{K_2HPO_4}$ (dibasic potassium phosphate). The equation describing the equilibrium between acid and base is as follows:

$$\mathrm{H_2PO_4^-(aq) + H_2O(1) \Leftrightarrow H_3O^+(ap) + HPO_4^{2-}(aq)}. \tag{4.58}$$

The pK_a for this reaction, which is based on measurement, can be found in standard tables and is 7.21. Plugging all the numbers into Eqn. (4.57) gives

$$\mathrm{pH} = 7.21 - \log(0.2/0.1) = 6.91. \tag{4.59}$$

It is clear why potassium phosphate is a favorite buffer of biochemists.

Despite the neat appearance of the calculation we've just done, the situation with phosphate buffer is rather complex. This is

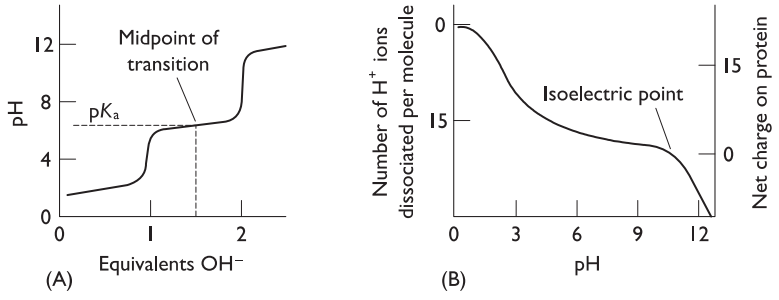

Fig. 4.12 Acid–base titration curves. Panel (A) shows titration of sodium phosphate at 25 °C. Phosphoric acid has three dissociable protons and therefore three pK_a values. The pK_as of phosphate are well-separated on the pH axis. This means that titration of the first site is effectively complete before titration of the second site begins, and so forth. Panel (B) shows the titration of ribonuclease at 25 °C. Ribonuclease is an extremely complicated molecule. Not only are there several different types of titratable group (the different types of ionizable amino acids), but the specific chemical environment of a dissociable proton can result in a substantial shift in its pK_a relative to the value for the free amino acid (in some cases by more than two pH units, or a 100-fold change in H_3O^+ concentration). This makes the titration curve of a protein very complicated. Panel (B) is based on a figure in Tanford & Hauenstein (1956).

because, unlike a simple acid like HCl, phosphoric acid is *polyprotic*. That is, phosphoric acid can donate more than one proton, so it has more than one pK_a. One of the other two pK_as is much higher than pH 7, the other is much lower. Fortunately, neither of the others makes a large contribution to the acid–base equilibrium at pH 7, and we can justify ignoring them in the neutral pH range. But don't neglect other contributions of a polyprotic acid if it has acidity constants that differ by less than about two pH units! Figure 4.12 shows how the net charge varies with pH for two polyprotic acids, phosphoric acid (panel (A)), in which there are three, well-separated ionization constants, and the protein ribonuclease (panel (B)), in which there are numerous ionizable groups of similar value. We'll have a further look at this topic in Chapter 6.

L. Chemical coupling

The Gibbs free energy is a state function. Individual contributions to the overall free energy change are therefore additive (see Fig. 2.3). Well, OK, but what does this say about biology? An endergonic reaction ($\Delta G > 0$) can be "powered" by an exergonic reaction ($\Delta G < 0$) if the two reactions are *chemically* "coupled" and the overall free energy change under the same conditions is negative. This is important! An analog will help to illustrate the situation. An example of *mechanical* coupling is the use of the downhill flow of a stream of water to turn a wheel to drive a system of gears to do something useful, for example, convert mechanical energy into electrical energy as at a hydroelectric dam or grind grain into flour.

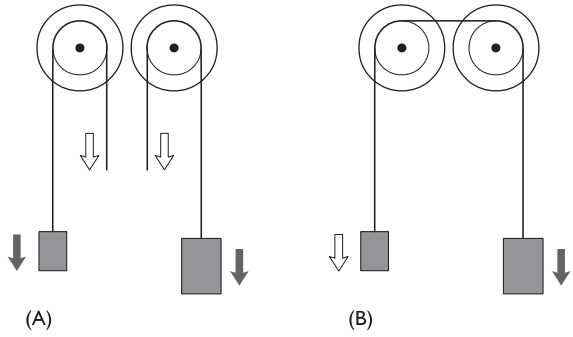

Fig. 4.13 Coupling. The figure shows a mechanical analog of chemical coupling, an extremely important means by which an endergonic reaction can occur. In panel (A), the two weights attached to strings are uncoupled. Both fall in accordance with Newton's law of gravitation. Neither weight does work on the other. In contrast, when the two weights are coupled, as in panel (B), one weight falls and the other one rises. The heavy weight does work on the light one. Note, however, that the net force acting on the "system" in panel (B) is less than that acting on the heavy weight in panel (A). The apparent mass of the heavy weight is the difference in mass between the coupled weights. Based on the figure on p. 167 of Atkins (1994).

Another type of mechanical coupling is depicted in Fig. 4.13. In panel (A), each weight represents an energetically favorable situation; both will fall to the ground spontaneously. The reverse reactions, which involve an increase in gravitational potential energy, will not occur spontaneously! But if the pulleys are coupled, as in panel (B), the more massive weight can be used to do work on the lighter one. Experimental studies have shown that coupling of biochemical reactions is an essential thermodynamic principle for the operation of metabolic pathways, including for instance the citric acid cycle (discussed in Chapter 5).

As an example of chemical coupling, consider the following two-step process:

$$A + B \Leftrightarrow C + D \qquad \Delta G_1, \tag{4.60}$$

$$D + E \Leftrightarrow F + G \qquad \Delta G_2, \tag{4.61}$$

Reaction (4.60) will occur spontaneously if $\Delta G_1 < 0$. But let's suppose $\Delta G_1 > 0$. Let's also suppose that the reaction of Eqn. (4.61) is spontaneous. Then the second reaction can be used to drive the first one if two conditions are met: the reactions involve a common compound (in this case, substance D), and the overall free energy change ($\Delta G_1 + \Delta G_2$) is negative. When both conditions are satisfied, the overall reaction proceeds spontaneously, even if the amount of compound D formed in Eqn. (4.60) is very small. The two reactions are said to be *coupled*, and D is called a *common intermediate* or *energy transducer*.

Let's look at a specific biochemical example. Transport of glucose into the cell is accompanied by phosphorylation. The negative charge on glucose prevents the diffusion of this valuable free energy molecule back out of the cell. The farmer will want to ensure that energy spent on harvesting wheat isn't wasted, so the grain is stored up a in building where the wind can't carry it off. The interior of the plasma membrane is made of hydrocarbon and, like the interior of a protein, is a region with a low dielectric. It is energetically unfavorable for something charged to pass into a membrane from bulk aqueous solution. Glucose is in the cell and phosphorylated. The overall coupled reaction here is one in which

a phosphoryl group is transferred from ATP to glucose, but it should be mentioned that neither of the relevant *half-reactions* (ATP hydrolysis or glucose phosphorylation) *obligatorily* drives the other reaction, as one might require in a more restricted definition of *coupled reaction*.

Let's look at some details of the energetics of glucose phosphorylation:

$$\begin{aligned} &\text{glucose} + P_i \Leftrightarrow \text{glucose-6-phosphate} + H_2O \\ &\Delta G^{\circ\prime} = 13.8\,\text{kJ mol}^{-1}. \end{aligned} \tag{4.62}$$

The blood concentration of glucose, the brain's primary fuel source, is ~5 mM. Given a cellular glucose concentration of about 300 μM, for the reaction to proceed to the right on the basis of concentration differences alone, the concentration of glucose-6-phosphate in the blood would have to be large – over 100 mM! (Can you prove it?) What actually happens in our bodies is that glucose phosphorylation is coupled to ATP hydrolysis. The overall reaction can be written as

$$\text{glucose} + \text{ATP} \Leftrightarrow \text{glucose-6-phosphate} + \text{ADP}. \tag{4.63}$$

This coupled reaction, for which $\Delta G^{\circ\prime} = -17.2$ kJ mol^{-1}, is clearly energetically favorable and will proceed to the right spontaneously – if the right "molecular hardware" is present.

Examination of Eqn. (4.63) can give a good sense of how this reaction coupling works on the molecular level. Minimizing the concentration of P_i would promote the forward reaction (by mass action), so an enzymatic nanomachine we might design to carry out the phosphorylation reaction should avoid the accumulation of P_i. Similarly, minimizing the concentration of H_2O in the vicinity of our molecule-sized "workbench" would minimize the probability of transfer of P_i from ATP to water; we want the phosphate group to be transferred to glucose! Crystallographic studies of the enzyme hexokinase, one of nature's own nanomachines, have revealed much about how the process actually occurs. Binding of glucose induces a conformational change in the enzyme that increases its affinity for ATP 50-fold and excludes water from the catalytic site. The functional groups of the amino acid side chains involved in catalysis move into proper alignment and a phosphoryl group is transferred from ATP to glucose. The low activity (concentration) of water in the active site of the enzyme is crucial for the reaction. Measurements have shown that the conformational change induced in the enzyme upon glucose binding results in the release of about 60 water molecules into bulk solution. This contributes a substantial increase in entropy to the overall energetics of the reaction, offsetting the unfavorable entropy change of bringing glucose and ATP simultaneously into an orientation that permits phosphoryl transfer to the appropriate hydroxyl group on glucose. Amazing!

M. Redox reactions

Utilization of the free energy of glucose and other nutrients consumed by organisms is controlled by means of *oxidation–reduction reactions*, or *redox reactions*. Some of these reactions occur in organelles called mitochondria, the "power-houses" of the cell. Redox reactions are of such great and general importance that much more than one section of one chapter of this book could be allocated to them. The present aim, however, is a brief introduction to the topic and not comprehensive treatment.

In redox reactions, electrons are transferred from one molecule to another. Electron transfer can be accompanied by the transfer of an atom or ion, but our main concern at the moment is electrons and changes in oxidation. The electron donor is called the *reductant* and the acceptor is the *oxidant*. Reductant and oxidant work in pairs, or couples.

Any redox reaction can be expressed as the difference of two reduction *half-reactions*. Conceptual in nature, half-reactions facilitate calculations by showing the electrons explicitly. An example of a half-reaction is the following:

$$\text{oxidized compound}(A_{ox}) + ne^- \rightarrow \text{reduced compound}(A_{red}), \tag{4.64}$$

where n electrons are transferred to the oxidized compound, giving the reduced compound. A more complicated redox reaction is

$$B_{ox} + ne^- + mH^+ \rightarrow B_{red}mH, \tag{4.65}$$

where n and m could be different. Note the proton (H^+) transfer. An example of a full redox reaction, where both redox pairs are given, is

$$A_{red} + B_{ox} \rightarrow A_{ox} + B_{red}. \tag{4.66}$$

A key physiological redox reaction is the reduction of oxygen to water by cytochrome *c*:

$$4\text{cyt}c^{2+} + 4H^+ + O_2 \rightarrow 2H_2O + 4\text{cyt}c^{3+}. \tag{4.67}$$

The transferred electrons are only implicit in this representation. Note that water is produced in this reaction.

The *standard redox potential*, $\Delta \boldsymbol{V}^{\circ\prime}$, represents the strength of a redox pair to exchange electrons, the "electron transfer potential." A redox potential is measured as an *electromotive force* (e.m.f.), or *voltage*,[11] of a half-cell consisting of both members of the redox couple (Fig. 4.14). Just as the Gibbs free energy (a *thermodynamic* potential) must be measured with respect to a chosen reference state, so the redox voltage measurement is made relative to a standard value. In most cases the standard is provided by the

[11] The volt, the SI unit of electrical potential, or voltage, gets its name from Count Alessandro Giuseppe Antonio Anastasio Volta (1745–1827), an Italian physicist.

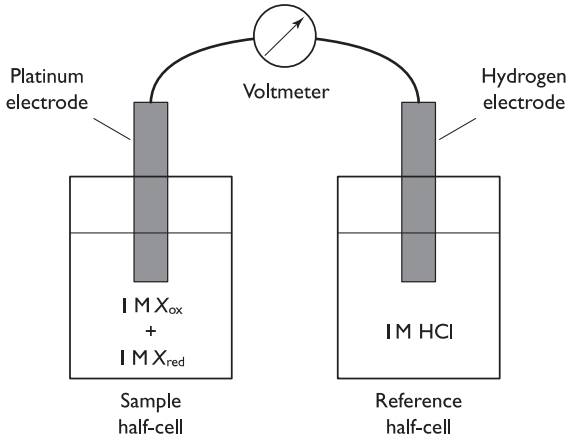

Fig. 4.14 Measurement of the standard redox potential. This device can be used to measure the standard redox potential of a compound. The hydrogen electrode in the reference cell contains 1 atm $H_2(g)$.

hydrogen electrode, the voltage of which is set to 0 V at pH 0. The redox potential is like the Gibbs free energy in that the magnitude depends on the concentrations of the reacting species.

To find the concentration-dependence of the redox potential, we note that work is done when a charge, $\boldsymbol{q}$, is moved through a difference in electrical potential, $\Delta \boldsymbol{V}$:

$$w' = -\boldsymbol{q}\Delta \boldsymbol{V}, \tag{4.68}$$

where the prime indicates *non-pV* work. This is the electrical work we mentioned in Chapter 2. As with *pV*-work, electrical work is the product of an "intensity factor" ($\boldsymbol{q}$) and a "capacity factor" ($\Delta \boldsymbol{V}$). If the amount of charge in Eqn. (4.68) is the same as that on 1 mole of protons, then $\boldsymbol{q} = F$, the Faraday constant (96.494 kJ V^{-1} mol^{-1}),[12] and the relationship is put on a molar basis. Recalling that the free energy change for a process is the maximum work that the system can do, and supposing that the stoichiometry of the reaction involves the transfer of n moles of *electrons*, we have

$$\Delta\mu = -nF\Delta \boldsymbol{V}. \tag{4.69}$$

The greater the number of positive charges transferred up a potential gradient, the greater the work that must be done by the system to bring about the change, and the less spontaneous the change. Rearranging Eqn. (4.69) in terms of $\Delta \boldsymbol{V}$ gives

$$\Delta \boldsymbol{V} = -\frac{\Delta\mu}{nF} \tag{4.70}$$

[12] The Faraday constant represents the electronic charge on 1 mole of electrons and is named after the renowned British physical chemist Michael Faraday (1791–1867), the first person to quantify the relationship between a chemical reaction and electric current. Faraday is a sort of anomaly in the history of science for his era: Most of the significant contributions to science were made by men of wealth or some form of high social status, and Faraday was from a poor family. Nevertheless, Faraday went on to become president of the Royal Institution, and the simple quantitative relationship he found between magnetic flux and induced e.m.f. was adopted by Maxwell as one of four elementary equations of electromagnetism.

Table 4.6. | *Standard redox potentials of some important biochemical substrates*

Electrode equation	n	$\Delta V^{\circ\prime}$ (V)
Acetate $+ 2H^+ + 2e^- \Leftrightarrow$ acetaldehyde	2	−0.58
$2H^+ + 2e^- \Leftrightarrow H_2$	2	−0.42
$NAD^+ + H^+ + 2e^- \Leftrightarrow NADH$	2	−0.32
Pyruvate $+ 2H^+\ 2e^- \Leftrightarrow$ lactate	2	−0.19
Cytochrome c $(Fe^{3+}) + e^- \Leftrightarrow$ cytochrome c (Fe^{2+})	1	+0.22
$\frac{1}{2}O_2 + 2H^+ + 2e^- \Leftrightarrow H_2O$	2	+0.82

$$\Delta \boldsymbol{V} = -\frac{\Delta\mu^{\circ\prime} + RT\ln\frac{[\text{products}]}{[\text{reactants}]}}{nF} = \Delta \boldsymbol{V}^{\circ\prime} - \frac{RT}{nF}\ln\frac{[\text{products}]}{[\text{reactants}]}, \tag{4.71}$$

where the standard state potential, $\Delta\mu^{\circ\prime}/nF$, is written as $\Delta \boldsymbol{V}^{\circ\prime}$. Standard state conditions are pH 7.0, 1 atm, and 25 °C. The right hand side of Eqn. (4.71) is known as the *Nernst equation*.[13] As we shall see below, it has many applications in biological systems. At equilibrium,

$$\Delta \mathrm{V}^{\circ\prime} = -\frac{RT}{nF}\ln K'_{\mathrm{eq}} \tag{4.72}$$

Now we have a way to relate the standard cell e.m.f. to the equilibrium constant. Some standard redox potentials are given in Table 4.6.

Equation (4.65) can be split into two parts,

$$\mathrm{A_{ox}} + n\mathrm{e}^- \rightarrow \mathrm{A_{red}} \tag{4.73}$$

and

$$\mathrm{B_{ox}} + n\mathrm{e}^- \rightarrow \mathrm{B_{red}}. \tag{4.74}$$

The half-cell reduction potentials here are

$$\Delta \boldsymbol{V}_{\mathrm{A}} = \Delta \boldsymbol{V}_{A}^{\circ\prime} - \frac{RT}{nF}\ln\frac{[\mathrm{A_{red}}]}{[\mathrm{A_{ox}^{n+}}]} \tag{4.75}$$

and

$$\Delta \boldsymbol{V}_{\mathrm{B}} = \Delta \boldsymbol{V}_{\mathrm{B}}^{\circ\prime} - \frac{RT}{nF}\ln\frac{[\mathrm{B_{red}}]}{[\mathrm{B_{ox}^{n+}}]}. \tag{4.76}$$

Note that the form of Eqns. (4.75) and (4.76) is exactly the same as that of Eqn. (4.7). The overall redox potential of any two half-reactions is

$$\Delta \boldsymbol{V} = \Delta \boldsymbol{V}_{\mathrm{e^-\,acceptor}} - \Delta \boldsymbol{V}_{\mathrm{e^-\,donor}} \tag{4.77}$$

A biochemical example will help to illustrate how these equations are put into practice. An important reaction of glycolysis is the reduction

[13] Named after Walther Hermann Nernst (1864–1941), the German physicist and chemist. Nernst is connected with the Third Law of Thermodynamics and was awarded the Nobel Prize in Chemistry in 1920.

of pyruvate to lactate by nicotinamide adenine dinucleotide (NADH, reduced form). The reaction is catalyzed with complete stereospecificity by the enzyme lactate dehydrogenase:

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \Leftrightarrow \text{lactate} + \text{NAD}^+. \tag{4.78}$$

The sum of the respective half-cell potentials, $\Delta \boldsymbol{V}^{\circ\prime}$, $+0.32$ V and -0.19 $\boldsymbol{V}$ (Table 4.6), exceeds 0. Therefore, by Eqn. (4.69) $\Delta\mu^{\circ\prime} = -2 \times 96.5$ kJ V^{-1} mol$^{-1} \times 0.13$ V $= -25.1$ kJ mol^{-1}, the forward reaction is the more probable one, and we should expect the spontaneous oxidation of NADH by pyruvate. We note that, although they do not appear explicitly in Eqn. (4.78), two electrons are transferred in this reaction. Nicotinamide adenine dinucleotide phosphate (NADPH, reduced form), a close relative of NADH, plays a key role in the cellular redox reactions that enable the synthesis of compounds that are thermodynamically less stable than glucose, a starting material.

What if we change the concentrations of reactants and products? What effect will this have on the spontaneity of electron transfer? As we have seen, the standard state redox potential of the NAD_{ox}/NAD_{red} couple is -0.32 V. Now we wish to calculate the potential of this half-cell reaction under non-standard state conditions. Let the couple be 75% reduced at $T = 70$ °C. By Eqn. (4.75),

$$\Delta \boldsymbol{V} = -0.32\,\text{V} - \frac{8.314\ \text{J mol}^{-1}\ \text{K}^{-1} \times 343\ \text{K}}{2 \times 96\,494\ \text{J V}^{-1}\,\text{mol}^{-1}} \ln\left(\frac{75}{25}\right) = -0.34\,\text{V} \tag{4.79}$$

There is relatively little deviation from the standard state potential at 75% reduction. At 50% reduction, $\Delta \boldsymbol{V} = \Delta \boldsymbol{V}^{\circ\prime}$. Marked deviations from the standard state potential occur only for extremes of temperature or extremes of the ratio of the concentration of oxidant to concentration of reductant.

Before bringing down the curtain on the text of this chapter, let us return the spotlight to photosynthesis. This biological process makes sugar, a reduced form of carbon, using water, a reducing agent. The overall chemical reaction can be expressed as

$$6\ \text{CO}_2 + 6\ \text{H}_2\text{O} + \text{light} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 + \text{energy}. \tag{4.80}$$

We covered some of the conceptual background to this equation in Chapter 1. Water is a relatively poor reductant, having a reduction potential of +820 mV; energy is required to separate electrons from water. This energy comes from the photons absorbed by chlorophylls *a* and *b* in photosystems I and II. Energy trapped by chlorophyll is transferred to the reaction center, whence electrons are transferred to pheophytin and plastoquinone. The reaction center is regenerated by "replacement" electrons from water, releasing the oxygen animals need to respire and generating a proton gradient across a lipid membrane. A more in-depth look at photosynthesis and glycolysis must wait until Chapter 5.

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O. Exercises

1. State whether the following phrases pertain to (A) the expansion of a gas into a vacuum, (B) two objects coming to thermal equilibrium, (C) both of these processes, or (D) neither of these processes.
(1) Involves a change in enthalpy.
(2) Involves an increase in entropy.
(3) Involves a decrease in Gibbs free energy.
(4) Can be made to proceed in the opposite direction.

2. State whether the following phrases pertain to (A) spontaneity, (B) reversibility, (C) both spontaneity and reversibility, or (D) neither spontaneity nor reversibility.

(1) Established for $\Delta G < 0$ at constant T.
(2) Established for $\Delta S < 0$.
(3) Established for a process in which the work done is a maximum.
(4) Illustrated by the migration of a solute from a region of high concentration to low concentration.
(5) Required for determination of ΔS by the heat transferred.
(6) Implies that the coupling of coupled reaction is very efficient.

3. State whether the following phrases pertain to (A) ΔU, (B) ΔG, (C) both ΔU and ΔG, or (D) neither ΔU nor ΔG.
(1) Does not depend on pathway during a change of state.
(2) Consists of the heat transferred and the work done.
(3) Must be negative if an isothermal and isobaric process is spontaneous.
(4) Measures the degree of disorder of a system.
(5) Is zero at equilibrium for an isothermal and isobaric process.
(6) Used to determine whether one reaction can drive another by coupling.
(7) Includes only energy that can do work, at constant temperature and pressure.

4. State whether the following phrases pertain to (A) ΔG, (B) ΔG°, (C) both ΔG and ΔG°, or (D) neither ΔG nor ΔG°.
(1) Equals $-RT \ln K_{eq}$.
(2) Equals $-nF\Delta V^{\circ\prime}$.
(3) Is zero if the change in the state of the system is spontaneous.
(4) Equals $\Delta H - T\Delta S$ at constant T.
(5) Is given for one mole of the reactants for a given reaction.
(6) Is equal to the sum of the chemical potentials of the products minus the chemical potentials of the reactants, with each chemical potential multiplied by the number of moles involved in the reaction.
(7) Is independent of the concentration of the components of a reaction.

5. State whether the following phrases pertain to (A) a, (B) μ, (C) both a and μ, or (D) neither a nor μ.
(1) Equals the concentration times the activity coefficient.
(2) Needed to calculate ΔG if ΔG° is known under specified conditions for all components of a reaction.
(3) Used to calculate ΔG for a process after multiplication by the number of moles of that component involved in the process.

6. In Chapter 1 we said that all living organisms depend on the Sun in order to meet the energy requirements of life. This is only partially true of the chemosynthetic bacteria that live at the bottom of the ocean. Explain the energy requirements for life in completely general terms. Although it may be that the Sun

played an indispensable role in the formation of life as we know it, is the Sun absolutely necessary for life? Why or why not?

7. What are the units of K_{eq}? Explain.

8. Calculate ΔG°(25 °C) for $K_{eq} = 0.001$, 0.01, 0.1, 1, 10, 100, and 1000.

9. The multi-component enzyme aspartate transcarbamoylase catalyzes the formation of *N*-carbamoylaspartate from carbamoyl phosphate and aspartate. Arthur Pardee has demonstrated that this reaction is the first step unique to the biosynthesis of pyrimidines, including cytosine, thymine and uracil, major components of nucleic acids. Aspartate transcarbamoylase has at least two stable folded conformations, known as R (high substrate affinity) and T (low substrate affinity). Interestingly, the relative stability of the T and R states is affected by the binding of ATP (a purine) to R and CTP (a pyrimidine) to T, a topic covered in Chapter 7. Measurement of the standard state free energy difference between R and T in the absence of ATP and CTP yielded the value 3.3 kcal mol^{-1}. Calorimetric determination of ΔH° for the transition was –6 kcal mol^{-1}. Calculate the standard state entropy change for the T → R transition.

10. When a photon in the visible range is absorbed in the retina by rhodopsin, the photoreceptor in rod cells, 11-*cis*-retinal is converted to the all-*trans* isomer. Light energy is transformed into molecular motion. The efficiency of photons to initiate the reaction is about 20% at 500 nm (57 kcal mol^{-1}). About 50% of the absorbed energy is available for the next signaling step. This process takes about 10 ms. In the absence of light, spontaneous isomerization of 11-*cis*-retinal is *very* slow, on the order of 0.001 yr^{-1}! Experimental studies have shown that the equilibrium energetics of retinal isomerization are $\Delta S^{\circ} = 4.4$ cal $mol^{-1}K^{-1}$ and $\Delta H^{\circ} = 150$ cal mol^{-1}. Calculate the equilibrium constant for the reaction.

11. Which one of the following equations is used to evaluate free energy changes in cells under physiological conditions? What makes it appropriate?
(a) $\Delta G = RT \ln K_{eq}'$.
(b) $\Delta G = \Delta G^{\circ\prime} + RT \ln$[products]/[reactants].
(c) $\Delta G = RT \ln$[products]/[reactants].
(d) $\Delta G = \Delta H - T\Delta S$.
(e) $\Delta G = \Delta G^{\circ\prime} + RT$ [products]/[reactants].

12. The direction of a reaction with a very large or very small value of K_{eq} is difficult, though not impossible, to alter by changing the mass action ratio. Explain.

13. Show that for a reaction at 25 °C which yields 1 mol of H_2O, $\Delta G^{\circ\prime} = \Delta G^{\circ} + 9.96$ kJ mol^{-1}.

14. Calculate K_{eq} for the hydrolysis of the following compounds at neutral pH and 25 °C: phosphoenolpyruvate ($\Delta G^{\circ\prime} = -61.9$ kJ mol^{-1}), pyrophosphate ($\Delta G^{\circ\prime} = -33.5$ kJ mol^{-1}), and glucose-1-phosphate ($\Delta G^{\circ\prime} = -20.9$ kJ mol^{-1}). Assume that the equilibrium constant includes water, accounting for the possibility that the water concentration is relatively low, as in the cell. These compounds are involved in the glycolytic pathway.

15. $\Delta G^{\circ\prime}$ for the conversion of fructose 1,6-bisphosphate (FBP) into glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) is +22.8 kJ mol^{-1}. This reaction is step four of the glycolytic pathway and is catalyzed by aldolase. In the cell at 37 °C the mass action [DHAP]/[GAP] = 5.5. What is the equilibrium ratio of [FBP]/[GAP] when [GAP] = 2×10^{-5} M? When [GAP] = 1×10^{-3} M?

16. Calculate ΔG when the concentrations of glucose-1-phosphate and glucose-6-phosphate are maintained at 0.01 mM and 1 mM, respectively. Compare the sign of ΔG with what was obtained in the worked example above. Suggest how this might be significant in metabolism.

17. Lactate dehydrogenase (LDH) catalyzes the oxidation of pyruvate to lactate and NADH to NAD$^+$ in glycolysis, the pathway by which glucose is converted to pyruvate with the generation of 2 mol of ATP mol^{-1} of glucose. The reaction is particularly important during strenuous activity, when the demand for ATP is high and oxygen is depleted. The relevant half-reactions and their standard reduction potentials are given in Table 4.6. Calculate ΔG for the reaction under the following conditions: [lactate]/[pyruvate] = [NAD$^+$]/[NADH] = 1; [lactate]/[pyruvate] = [NAD$^+$]/[NADH] = 160; [lactate]/[pyruvate] = [NAD$^+$]/[NADH] = 1000. What conditions are required for the reaction to spontaneously favor oxidation of NADH? [NAD$^+$]/[NADH] must be maintained close to 10^3 in order for the free energy change of the glyceraldehyde-3-phosphate reaction to favor glycolysis. This function is performed by LDH under anaerobic conditions. What is the largest [lactate]/[pyruvate] can be in order for the LDH reaction to favor the production of NAD$^+$ and maintain [NAD$^+$]/[NADH] = 10^3?

18. The citric acid cycle is the common mode of oxidative degradation in eukaryotes and prokaryotes (Chapter 5). Two components of the citric acid cycle are α-ketoglutarate and isocitrate. Let [NAD_{ox}]/[NAD_{red}] = 8; [α-ketoglutarate] = 0.1 mM; [isocitrate] = 0.02 mM. Assume 25 °C and pH 7.0. Calculate ΔG. Is this reaction a likely site for metabolic control? Explain.

19. Refer to Fig. 4.4. Mixing. In the text we noted that at first entropy is at a minimum and free energy is at a maximum.

Later, ... if the two liquids are the same, what are ΔS and ΔG of mixing?

20. Refer to Fig. 3.2. The stopcock is closed in panel (A). All of the inert gas is in the bulb on the left-hand side. In panel (B), the bulb on the left-hand side has inert gas at concentration x, and the bulb on the right has the same inert gas at concentration x. What are the entropy and the free energy differences between panels (A) and (B)?

21. Rationalize the change of sign in Eqn. (4.32).

22. Cytochromes are redox-active proteins that occur in all organisms except a few types of obligate anaerobes. These proteins contain heme groups, the iron atom of which reversibly alternates between the Fe(II) and Fe(III) oxidation states during electron transport. Consider the reaction

$$\text{cyt}c\ (\text{Fe}^{2+}) + \text{cyt}f\,(\text{Fe}^{3+}) \Leftrightarrow \text{cyt}c\ (\text{Fe}^{3+}) + \text{cyt}f\,(\text{Fe}^{2+})$$

involving cytochromes c and f. If $V^{\circ\prime} = 0.365$ V for electron transfer to cyt$f$ (Fe^{3+}), and $V^{\circ\prime} = 0.254$ V for electron transfer to cyt$c$ (Fe^{3+}), can ferrocytochrome c (2+ oxidation state) reduce ferricytochrome f (3+ oxidation state) spontaneously?

23. Calculate $\Delta \boldsymbol{V}$ in Eqn. 4.71 when the couple is 99% reduced and the temperature is 37 °C.

24. Table 4.1 presents the thermodynamic properties of water. On the basis of these data, rationalize the suitability, or lack thereof, of each thermodynamic function as an index of spontaneous change.

25. Cholesterol increases membrane rigidity. What effect will it have on the character of the lipid bilayer order–disorder transition? Why?

26. Some organisms are able to tolerate a wide range of ambient temperature, for instance bacteria and poikilothermic (cold-blooded) animals such as fish. The membrane viscosity of *E. coli* at its growth temperature is approximately constant over the range $15 - 43$ °C. Knowing aspects of the physical basis of the solid-gel transition in lipid bilayers, suggest how bacteria and fish might cope with changes of temperature.

27. Use your knowledge of the physical properties of lipids to outline several design characteristics of a liposome-based drug delivery system. A liposome is a bilayer structure that is self-enclosing and separates two aqueous phases.

28. The reversible deamination of aspartate yields ammonium and fumarate. Fumarate is a component of the citric acid cycle. Aspartate deamination is catalyzed by the enzyme aspartase.

Experimental studies on the deamination reaction have shown that

$$\log K_{eq} = 8.188 - (2315.5/T) - 0.010\,25T,$$

where T is in degrees kelvin (K). Note that the units of the coefficient of $1/T$ (i.e. 2315.5) must be K, while those of 0.01025 are K^{-1}. Calculate ΔG° at 25°C. Remember that $K = 10^{\log K}$ and $2.303\log x \approx \ln x$. Follow the development leading up to Eqn. (4.50) to show that $\Delta H^\circ = 2.303 \times R \times (2315.5 - 0.010\ 25T^2)$. Calculate ΔH° and ΔS° at 25 °C. From Chapter 2, $\Delta C_p = \Delta(\Delta H^\circ)/\Delta T$. Use this to show that $\Delta C_p = -2.303 \times R \times 0.0205T$. Evaluate ΔC_p at 25 °C.

29. State whether the following phrases pertain to (A) chemical potential of the solute, (B) chemical potential of the solvent, (C) both of these chemical potentials, or (D) neither chemical potential.
(1) Equals $RT \ln a$.
(2) Equals $\mu^\circ + RT \ln a$.
(3) At equilibrium, its value is the same on both sides of a membrane.
(4) Is proportional to the osmotic pressure (see Chapter 5).

30. Calculate the value of x for which the approximation $\ln(1 + x) \approx x$ gives an error of 5%.

31. State whether the following phrases pertain to (A) ΔG, (B) $\Delta \boldsymbol{V}$, (C) both ΔG and $\Delta \boldsymbol{V}$, or (D) neither ΔG nor $\Delta \boldsymbol{V}$.
(1) Indicates whether an oxidation-reduction reaction is spontaneous.
(2) Standard value for a reaction is determined with all components in their standard states.
(3) Is positive for a spontaneous reaction.
(4) Is called the standard electrode reduction potential.
(5) Can be used to calculate the equilibrium constant for a reaction for a known set of concentrations of all components of a reaction at a given temperature.

32. Chemical coupling. The equilibrium constant for $Glu^- + NH_4^+ \Leftrightarrow Gln + H_2O$ is 0.003 15 M^{-1} at pH 7 and 310 K; the reaction lies far to the left. The synthesis of Gln from Glu is made energetically favorable by coupling it to hydrolysis of the terminal phosphodiester bond of ATP. The products of ATP hydrolysis are ADP and P_i. The equilibrium constant for the coupled reaction, which is known from experiments with glutamine synthase, is 1200. Calculate the phosphate bond energy in ATP at pH 7 and 310 K.

33. What is the pH value of 0.001 M HCl solution?

34. Calculate the hydrogen ion concentration of solution of pH 6.0.

35. Calculate the ionic strength of a 0.35 molal aqueous solution of $MnCl_2$. Assume that dissociation of the salt into ions is complete at this concentration.

36. Calculate the ionic strength of 0.01 N acetic acid if the dissociation constant of the acid is 1.8×10^{-5}.

37. Calculate the activity coefficient and activities of the ions in aqueous solution of (a) 5 mM H_2SO_4 and (b) 2 mM NaCl.

38. The following data were obtained by German and Wyman (1937) for horse hemoglobin, an oxygen-binding blood protein, in 0.333 M NaCl.

Deoxygenated hemoglobin		Oxygenated hemoglobin	
Acid (−) or base (+) per gram Hb	pH	Acid (−) or base (+) per gram Hb	pH
−0.514	4.280	−0.514	4.280
−0.452	4.415	−0.453	4.410
−0.419	4.525	−0.420	4.525
−0.390	4.610	−0.392	4.618
−0.323	4.842	−0.324	4.860
−0.258	5.160	−0.259	5.188
−0.224	5.320	−0.225	5.430
−0.172	5.590	−0.173	5.800
−0.130	6.072	−0.130	6.055
−0.064	6.541	−0.063	6.430
0.0	6.910	+0.001	6.795
+0.070	7.295	+0.072	7.130
+0.131	7.660	+0.133	7.510
+0.171	7.860	+0.172	7.725
+0.208	8.140	+0.209	8.043
+0.254	8.545	+0.254	8.450
+0.288	8.910	+0.288	8.890
+0.311	9.130	+0.292	8.990
+0.331	9.350	+0.311	9.130
+0.350	9.410	+0.331	9.355
+0.357	9.465	+0.350	9.410
+0.407	9.800	+0.357	9.480
		+0.407	9.800

Plot the titration data to find which form of hemoglobin is the stronger acid. The stronger an acid, the more readily it gives up protons. We shall study hemoglobin in considerably greater depth in Chapters 5 and 7.

39. The history of science is full of "partly true" ideas pursued with vigor until they no longer became tenable. As we have

seen in Chapter 2, Galileo's assumption about the shapes of planetary orbits, which was based on the speculations of thinkers of classical antiquity, was eventually superseded by the very detailed measurements of the Danish astronomer Tycho Brahe (1546–1601) and analysis of Johannes Kepler. Similarly, Galileo's work on the relative motion of bodies was a great addition to the physics of his day (mostly that of Aristotle), and it prepared the way for Newton; but in the twentieth century, Galilean (Newtonian) relativity is seen to be a limiting case of the more general view proposed by Einstein. In the nineteenth century, research in thermochemistry was motivated in part by the belief that the heat of a reaction measured its "affinity": the greater the energy liberated, the greater the affinity of the reactants for each other. This view became untenable by the discovery of spontaneous endothermic reactions. Explain.

40. ΔG cannot generally be equated with ΔG°. To a very good first approximation ΔH can be equated with ΔH°. Explain.

41. Calculate the percentage non-ionized for an acid with a pK_a of 4 in an environment of pH 1.

42. Tris, a base, is a popular buffer for biochemical research. Its pK_a is strongly dependent on temperature. Would it make a very good buffer for a scanning calorimetry experiment? Why or why not? Assuming that $K_a = 8.3 \times 10^{-9}$ M, calculate the ratio of acid to base at pH 8.0. Let the total concentration of Tris be 150 mM, and divide the stock into to two parts. To one, add 10 mM HCl. How does the pH change? Hint: assume complete dissociation of HCl. To the other, reduce the concentration to 30 mM. How does the pH change?

43. The concentration of creatine in urine is *c.* 40-fold greater than in serum. Calculate the free energy change per molecule required for transfer of creatine from blood to urine at 37°C.

44. Which of the following redox pairs is the strongest reducing agent?

Redox pair	$V^{\circ\prime}$ in volts
Oxidized ferrodoxin/reduced ferrodoxin	−0.43
NADP/NADPH	−0.32
Oxidized glutathione/reduced glutathione	−0.23
Pyruvate/lactate	−0.19
Ubiquinone/hydroquinone	0.10

Chapter 5

Gibbs free energy – applications

A. Introduction

The Gibbs free energy is important in biology research because it enables one to predict the direction of spontaneous change for a system under the constraints of constant temperature and pressure. These constraints generally apply to all living organisms. In the previous chapter we discussed basic properties of the Gibbs free energy, showed how its changes underlie a number of aspects of physical biochemistry, and touched on what the biological scientist might do with such knowledge. Here, we build on the introductory material and explore how it can be applied to a wide variety of topics of interest to the biological scientist. A range of examples illustrate when, where, why, and how the Gibbs free energy is such a useful concept. We shall discuss the energetics of different types of biological structure, including small organic molecules, membranes, nucleic acids, and proteins. This will help to give a deeper sense of the relatedness of some seemingly very different topics one encounters in biological science.

B. Photosynthesis, glycolysis, and the citric acid cycle

This section presents a low-resolution view of the energetics of photosynthesis, glycolysis, and the citric acid cycle. There can be no doubt that the details we omit are important: entire books have been written on each subject! But our aim here is to consider biological energy in a global, qualitative way. We want to try to see "the big picture." So many of the protein, enzyme, chemical intermediate players do not have a speaking part in the present dramatic performance. Such details can be found in any good biochemistry textbook.

Over 99% of the free energy in our biosphere is from the Sun. Green plants, certain unicellular organisms like diatoms,

cyanophytes (blue-green algae), and various kinds of bacteria, collectively known as photoautotrophs, convert the light energy of the Sun and CO_2 into the chemical energy of bonding electrons in sugar molecules. The energy conversion process is called *photosynthesis*. The remaining less than 1% of our biosphere's free energy comes from the oxidation of inorganic matter, mainly hydrogen and sulfur, by microorganisms called chemolithotrophs. Whether photoautotrophs preceded or followed chemolithotrophs in the flowering of life on Earth is an intriguing open question (see Chapter 9).

The overall chemical reaction of photosynthesis is:

$$CO_2 + H_2O + \text{light} \rightarrow (CH_2O) + O_2. \quad (5.1)$$

CO_2 and H_2O are reduced to sugar and oxygen in this redox reaction. The process carried out in photosynthetic protists and cyanophytes resembles that in green plants, while compounds other than water serve as a reactant in photosynthetic bacteria and oxygen is not produced. All photosynthetic organisms[1] contain the light-absorbing pigment chlorophyll (Fig. 1.3). This molecule plays a key role in the transformation of light energy to chemical compounds. Chlorophyll, like the heme group (see below) of the vertebrate oxygen transport protein hemoglobin and the heme group of the electron transport protein cytochrome *c*, is derived from protoporphyrin IX, a complex ring structure synthesized from glycine and acetate (Fig. 5.1).

Figure 5.2 depicts the energetics of photosynthesis in schematic form. Absorption of photons ($h\nu$) results in the ejection of electrons from P680, the reaction center chlorophyll of photosystem II.[2] Each electron passes through a chain of electron carriers to plastoquinone, giving plastoquinol. By means of a series of redox reactions, the electrons are delivered to plastocyanin, which regenerates photooxidized P700, the reaction center chlorophyll of photosystem I. The electron ejected from P700 then passes through a chain of electron carriers to the oxidized form of nicotinamide adenine dinucleotide phosphate ($NADP^+$), an intracellular electron carrier. Photosynthetic electron transport drives the formation of a proton (pH) gradient, a difference in the concentration of protons on opposite sides of a membrane (in plants, the thylakoid membrane in chloroplasts). Movement of protons from a region of high chemical potential to low chemical potential powers the synthesis of ATP in manner that closely resembles oxidative phosphorylation, the endergonic synthesis of ATP from ADP and P_i in mitochondria in animal cells (see below). Plants also use light energy to make cellulose and other sugar molecules.

Glucose is the six-carbon sugar that is quantitatively the most important source of energy for cellular processes in all known

[1] Excluding halobacteria but including all other types of photosynthetic prokaryotes. Halobacteria thrive in the high salt environment of the Dead Sea.

[2] So called because 680 nm is the wavelength of the absorption maximum of the reaction center chlorophyll.

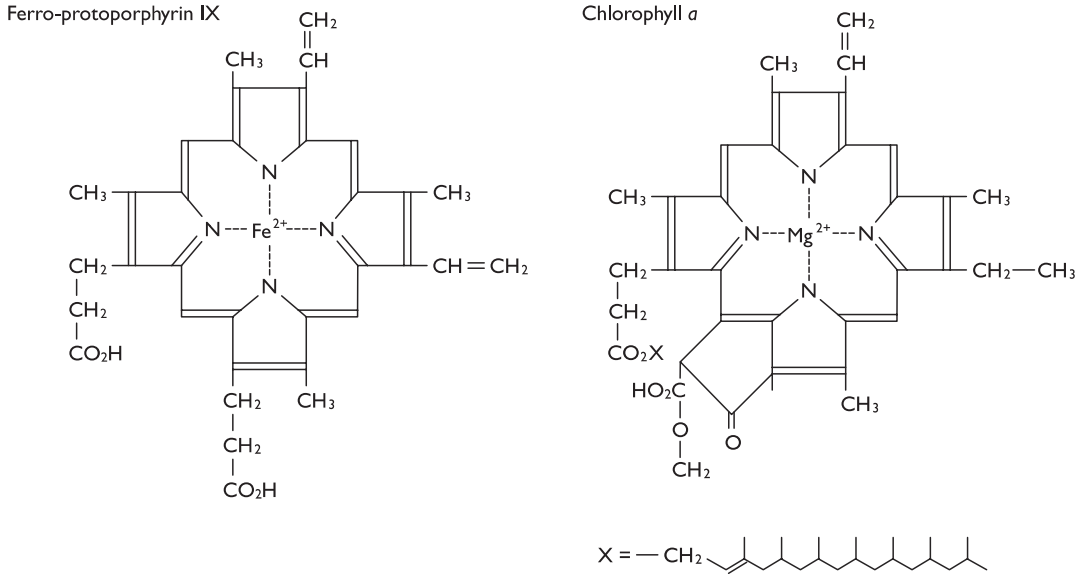


Fig. 5.1 Molecular formulas of the heterocyclic ring systems of ferro-protoporphyrin IX (heme) and chlorophyll *a*. The bound iron of heme is normally in the Fe(II) (ferrous, 2+) oxidation state regardless of whether oxygen is bound. The structure of chlorophyll *b* is nearly identical to that of chlorophyll *a*: a formyl group is found in place of a methyl group. Bacteriochlorophylls *a* and *b*, which are important in photon capture in photosynthetic bacteria, are very similar in structure to chlorophyll *a*. The long aliphatic tail of chlorophyll increases its solubility in a nonpolar environment. Note that in both heme and chlorophyll a divalent cation is bound. It is a remarkable indication of the unity of all known living things that such similar ring structures should play important roles in biological energetics in organisms as different as bacteria and humans.

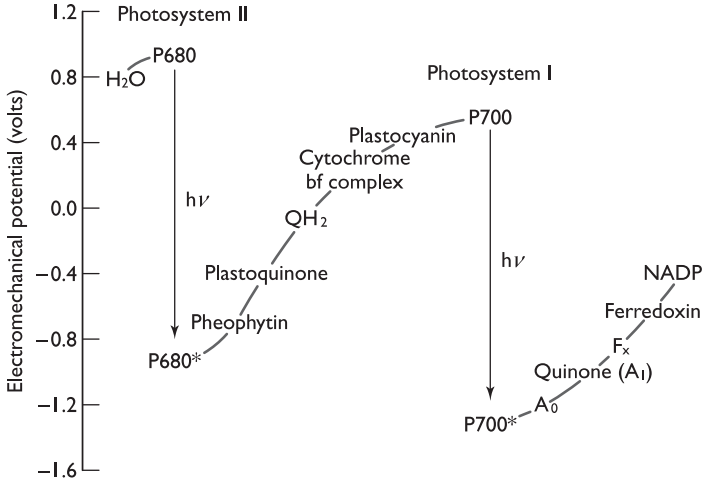


Fig. 5.2 Schematic diagram ("Z-scheme") of the energetics of electron transport in photosynthesis. The electrochemical potential (free energy) is measured in volts. Electrons tend to flow spontaneously from a state of higher to lower free energy. In terms of electrochemical potential, electrons migrate spontaneously from a more negative to a more positive reduction potential. PSII is coupled to PSI via the quinone Q and plastocyanin.

organisms. *Glycolysis*, the metabolism[3] of glucose, is a sequence of biochemical reactions by which one molecule of glucose is oxidized to two molecules of pyruvate, a three-carbon molecule (Fig. 5.3).

[3] Greek, *metabolikon*, disposed to cause or suffer change; coined by the German biologist Theodor Schwann (1810–1882).

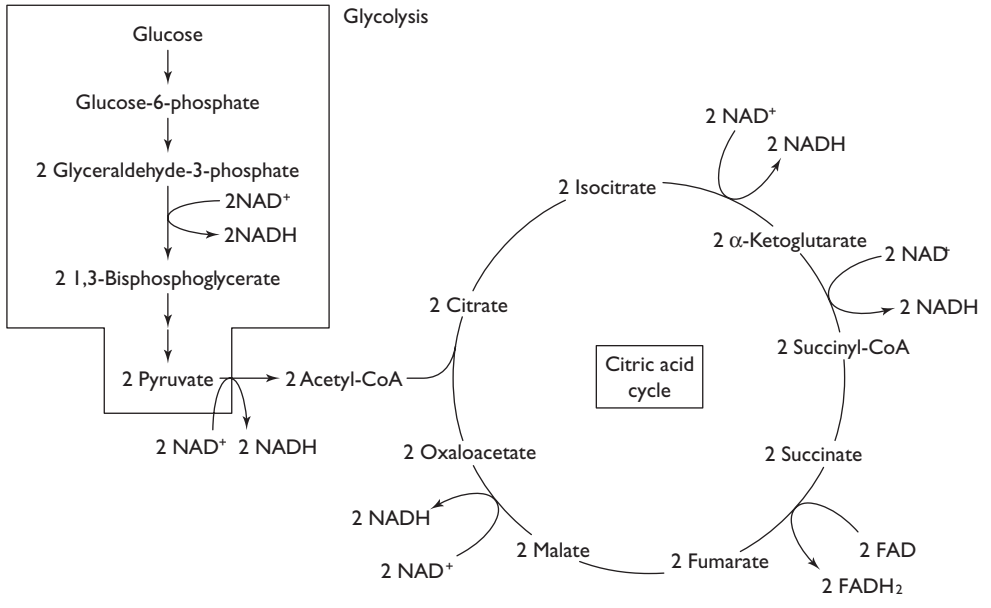


Fig. 5.3 Schematic diagram of glycolysis and the citric acid cycle. The figure shows the points at which the electron carriers NAD^+ and FAD are reduced by electron transfer to form NADH and $FADH_2$.

Pyruvate is then converted by a series of reactions to carbon dioxide and water. In combination with other aspects of oxidative carbohydrate metabolism, glycolysis is essentially the reverse process of photosynthesis. The *overall* chemical reaction for glucose metabolism is

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O. \qquad (5.2)$$

Compare Eqn. (5.2) with Eqn. (5.1). The free energy change for the *complete* redox reaction is $\Delta G^{\circ\prime} = -2823$ kJ mol^{-1}, and 24 electrons are transferred in the process. The standard state free energy change ($\Delta G^{\circ\prime}$) for glycolysis alone is -43.4 kJ mol^{-1}, while the physiological free energy change (ΔG) for glycolysis, which includes the synthesis of 2 moles of ATP, is -74 kJ mol^{-1}. Figure 5.4 depicts the physiological energetics of glycolysis in schematic form.

Glycolysis is similar in all organisms. Once a glucose molecule has entered the cell, it is immediately phosphorylated at the expense of one molecule of ATP. (It is interesting that ATP is expended in a process which, as we shall see, leads to ATP production.) Glucose phosphorylation is an essentially irreversible reaction because the free energy change of removal of the phosphoryl group from ATP is large and negative. Phosphorylation ensures that once it has entered the cell, the chemical energy of glucose is trapped there. The fate of pyruvate depends on the organism, tissue and conditions. In stressed, oxygen-depleted skeletal muscle, for instance, pyruvate is converted to lactate (the conjugate base of lactic acid) and one molecule of ATP is produced. Fermentation of yeast, a process integral to making bread, beer, and wine, involves the

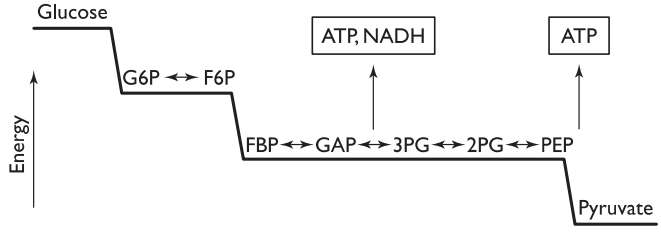
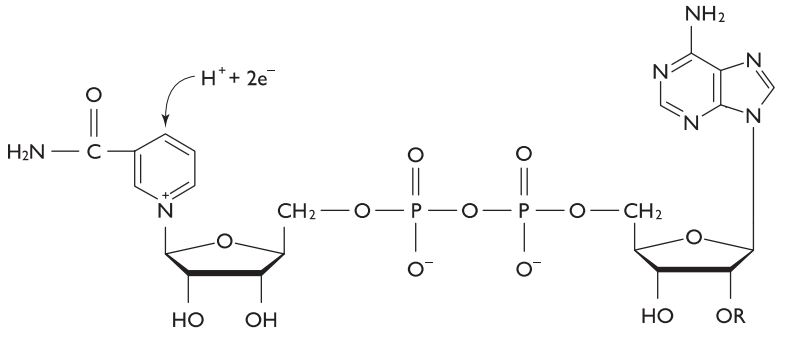


Fig. 5.4 Control by phosphofructokinase (PFK) of the flux of glycolysis breakdown products. Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose bisphosphate; GAP, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate. The physiological free energy changes (in kJ mol^{-1}) are: −27.2, − 1.4, −25.9, −5.9, +3.3, −0.6, −2.4, −13.9 (from Table 16–1 in Voet and Voet (1995)). There are three irreversible steps in the metabolism of glucose to pyruvate. These occur between glucose and G6P, F6P and FBP, and PEP and pyruvate. The irreversibility of these reactions is extremely important for cellular function. For only at an irreversible step of a process can control be exerted; irreversibility permits regulation of the speed of the reaction. The most important regulatory enzyme of glycolysis is PFK. This allosteric enzyme has four subunits and is controlled by several activators and inhibitors (see Chapters 7 and 8). PFK catalyzes the conversion of F6P to FBP. Because regulation of a pathway at a particular point affects all reactions that occur downstream, PFK controls the flux of glycolysis. Based on Fig. 1.3 of Harris (1995).

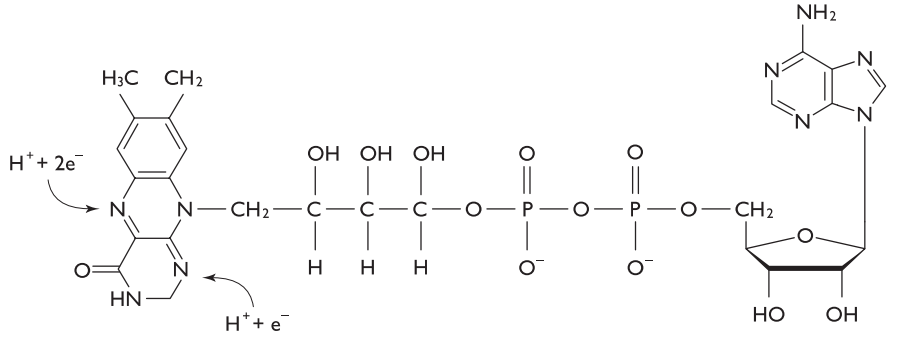
conversion of pyruvate to ethanol and CO_2. In the presence of oxygen, the three carbons of pyruvate are completely oxidized to CO_2.

In Chapter 4 we saw how an extreme value of K_{eq} corresponds to a mass action ratio that is difficult to shift by changes in the concentrations of reactants or products alone. Nevertheless, the thermodynamic unfavorability of a process can be overcome by the cell's maintaining concentrations that promote the reaction. One such reaction occurs in glycolysis. Fructose-1,6-bisphosphate (FBP) is cleaved by aldolase into two triose phosphates, dihydroxyacetone phosphate and glyceraldehyde phosphate (GAP). (Note that both trioses are phosphorylated, preventing escape from the cell!) Cleavage of the C–C bond is highly endergonic; ΔG° is large and positive. In order for the reaction to occur, $\ln([GAP]^2/[FBP])$ must be negative; the mass action ratio must be much less than 1. This step of glycolysis occurs only because the cellular concentrations of the products are kept below 1 μM; the mass action ratio is less than 1 for concentrations of FBP greater than 1 pM! There is a sense in which the cell is a sort of finely tuned machine.

The *citric acid cycle* (Fig. 5.3) is the terminal stage of the chemical processes by which the major portion of carbohydrates, fatty acids, and amino acids are converted into a form of chemical energy that is more useful to the cell. The cycle is the common mode of oxidative degradation in cells in animals, plants, microorganisms, and fungi; it is a main feature of cellular chemistry that is shared by all known forms of life. One complete cycle yields two molecules of carbon dioxide, one molecule of ATP, and numerous biosynthetic precursors. The cycle is entered twice in the oxidation of a single glucose molecule (one glucose gives two pyruvates), producing six molecules of nicotinamide adenine dinucleotide (NADH) and two molecules of flavin adenine dinucleotide ($FADH_2$) per glucose molecule by way of redox reactions (Fig. 5.5). The electron carriers NADH and $FADH_2$, which are synthesized from vitamin precursors, are of great importance to ATP production in oxidative phosphorylation (see below). The citric acid cycle was first proposed in 1937 by Sir Hans Adolf Krebs (1900–1981), a biochemist who emigrated from Germany to England in 1933. Krebs shared the 1953 Nobel Prize in Medicine or Physiology with the American Fritz Albert Lipmann (1899–1986).



(A) Nicotinamide adenine dinucleotide (oxidized).



(B) Flavin adenine dinucleotide (oxidized).

Fig. 5.5 Electron carriers in metabolism. NAD is a major soluble redox intermediate in metabolism. It is closely related to NADP, another redox intermediate. NAD and NADP differ in that the latter is phosphorylated on the adenylate ribose (R = phosphate in NADP, R = H in NAD). NADH shuttles electrons to electron transfer chains, NADPH provides electrons for biosynthesis. Neither NADH nor NADPH can form a stable one-electron intermediate, whereas FAD, a protein-bound cofactor, can form a one-electron semiquinone. Both NAD^+ and FAD comprise ADP and are synthesized from ATP (see Fig. 5.7). Energy and matter, matter and energy, energy and matter . . .

C. Oxidative phosphorylation and ATP hydrolysis

The NADH and $FADH_2$ molecules generated by the citric acid cycle play a central role in *oxidative phosphorylation*, the complex process whereby ADP and inorganic phosphate are combined to form ATP. From a quantitative point of view, oxidative phosphorylation is the most important means by which a cell generates ATP: complete metabolism of 1 mole of glucose by the citric acid cycle yields a maximum of 38 moles of ATP (2 from glycolysis, 2 from the citric acid cycle, and 34 from reoxidation of NADH and $FADH_2$). ATP is the most commonly utilized form of energy in a cell (Chapter 1).

The term *bioenergetics* usually refers to the way the in which cells generate energy from foodstuffs. The main concept of bioenergetics

is chemiosmotic theory, which states that energy stored as a proton gradient across a biological membrane (the so-called *proton motive force*) is converted to useful chemical energy in the form of ATP. One of the key contributors to the understanding of biological energy transfer has been the British biological chemist Peter Dennis Mitchell (1920–1992), who was awarded the Nobel Prize in Chemistry for his work in 1978.

The proton motive force is built up across the inner membrane of mitochondria in animals, the inner membrane of chloroplasts in plants, and the plasma membrane of aerobic bacteria (Fig. 5.6). Energy released from electron-transfer events in membrane-bound proteins is harnessed to generate the gradient. The chain of electron transfer reactions in mitrochondria terminates in the reduction of oxygen to water and the otherwise thermodynamically unfavorable pumping of protons across the membrane against the concentration gradient. The movement of protons down their gradient through the enzyme ATP synthase, the most complex structure in the inner mitochondrial membrane, results in the synthesis of ATP from ADP and inorganic phosphate. The difference in proton concentration across the membrane can be measured as a difference in pH. The role of mitochondria in coupling the phosphorylation of ADP to the electron transfer from reduced NAD to oxygen was shown by Albert Lester Lehninger (1917–1986) and associates at Johns Hopkins. Lest anyone think that the underlying electron-transfer reactions are unimportant to the end result, the mechanism underlying the toxicity of the highly poisonous cyanide ion involves binding to and inhibition of the cytochrome *a*-cytochrome a_3 complex (cytochrome oxidase) in mitochondria, and the poison sodium azide, which is added to protein solutions to inhibit the growth of bacteria, inhibits cytochrome *c* oxidase and thereby ATP synthase.

ATP, once synthesized, is put to use by the cell in many ways. For example, the free energy change of ATP hydrolysis is employed to power a tremendous variety of otherwise thermodynamically unfavorable biochemical reactions. In essence what ATP does in this context is provide free energy on the loss of its terminal phosphate group by hydrolysis of the *phosphoanhydride bond* (Fig. 5.7). Chemical coupling of ATP hydrolysis (Chapter 4) then "energizes" metabolic reactions which on their own cannot occur spontaneously. ATP is a common intermediate of energy transfer during anabolism, cellular processes by which energy is used to synthesize complex molecules from simpler ones.

In certain specialized cells or tissues, the chemical energy of ATP is used to do other kinds of chemical work, for example, the mechanical work of muscle contraction and cell movement (Chapter 8). ATP is required for osmotic work, the transport of ions other than H_3O^+ or metabolites through a membrane against a concentration gradient (below). ATP is also a major energy source in the synthesis of macromolecules from monomers, e.g. polysaccharides from individual sugar molecules and polypeptides from amino acids

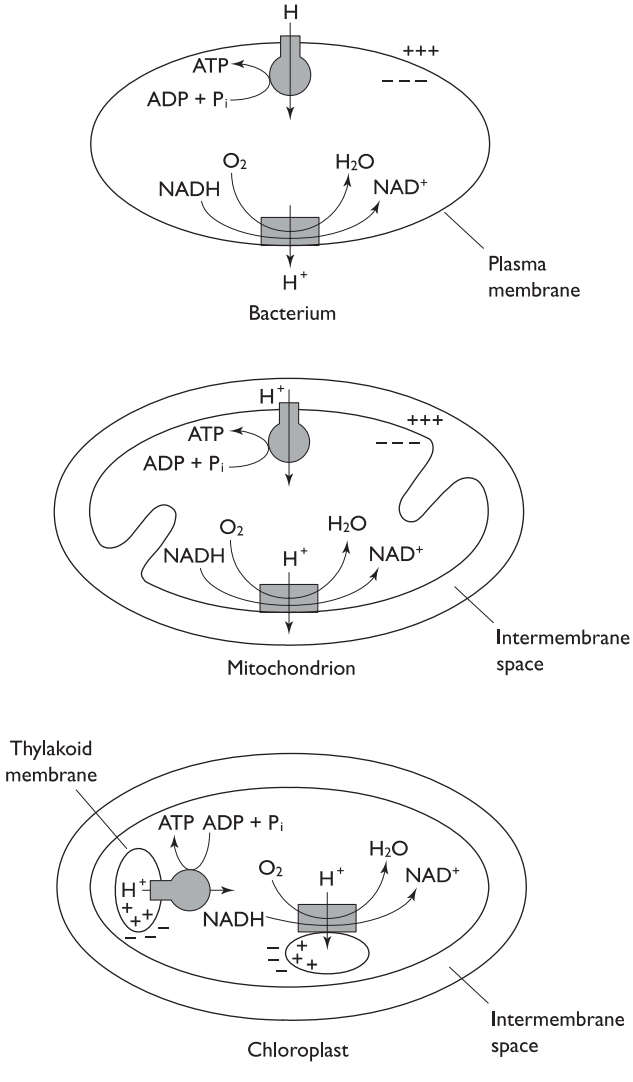


Fig. 5.6 Proton movement in bacteria, mitochondria, and chloroplasts. Note the similarities and differences in membrane orientation and direction of proton movement. In bacteria, mitochondria, and chloroplasts, the protein complex in which ATP is synthesized in situated on the cytosolic face of the membrane. Electron transport results in translocation of protons from the cytosolic side to the exoplasmic side of the membrane, creating a pH gradient. This is used to generate ATP as protons move down the pH gradient into cytoplasmic side. The similarities in ATP generation in bacteria, mitochondria, and chloroplasts point to the profound unity of all known living organisms. Adapted from Fig. 17–14 of Lodish *et al.* (1995).

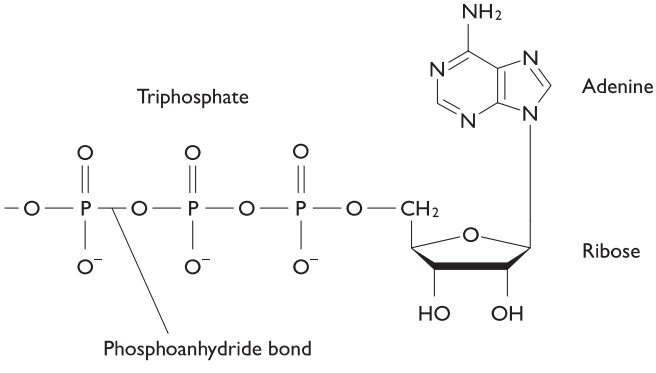


Fig. 5.7 The structure of adenosine triphosphate. There are three main components: adenine, a base found in RNA and DNA; ribose, a sugar; and triphosphate. In most biochemical reactions in which it is involved, ATP is hydrolyzed to ADP and inorganic phosphate. The bond broken in this reaction is a phosphoanhydride bond. The pK_as of the dissociable protons are different (see Chapter 4).

Table 5.1. *ATP requirements of macromolecule formation*

Macromolecule	Subunit type	ATP expenditure per monomer added (mol mol^{-1})
Polysaccharide	Sugar	2
Protein	Amino acid	4
Lipid	CH_2 unit from acetic acid	1
DNA/RNA polymerization	Nucleotide	2

(Table 5.1). In respect of all this, ATP is known as the "universal biochemical energy currency" (Chapter 1). We can see that there are many possible ways in which the free energy of a single glucose molecule can be distributed throughout a cell!

The vital importance of ATP in metabolism was first recognized by Fritz Lipmann and Herman Kalckar in 1941. Over 60 years on, the role of ATP in the cell is no less important than at any other point in the Earth's history (it seems). So we had better know something about it! The hydrolysis of ATP to ADP and P_i can be symbolized as

$$ATP + H_2O \Leftrightarrow ADP + P_i + H^+. \tag{5.3}$$

Using Eqns. (4.32) and (4.38), the free energy change for this reaction can be expressed as

$$\Delta G = \Delta G^{\circ\prime} + RT\ln[ADP][P_i]/[ATP]. \tag{5.4}$$

To keep things simple, we assume ideal behavior. Note that $[H^+]$ and $[H_2O]$, which are practically independent of the concentrations of the other species, are not included explicitly in Eqn. (5.4) (refer to the previous chapter if you are not sure why!). $\Delta G^{\circ\prime}$ for Eqn. (5.4) is about -7 kcal mol^{-1}. Does this hold for the cell, where conditions are of course very different from the standard state? Assuming that the cellular concentration of each species is 10 mM (a *very rough* estimate), Eqn. (5.4) says that $\Delta G = -7$ kcal $mol^{-1} + [1.987$ cal mol^{-1} $K^{-1} \times 298$ K $\times \ln(0.010)] = -7$ kcal $mol^{-1} - 2.7$ kcal $mol^{-1} \approx -10$ kcal $mol^{-1} \approx -42$ kJ mol^{-1}.[4] That's a 40% increase in the driving force for hydrolysis over standard state conditions! In other words, the equilibrium in Eqn. (5.3) makes a big shift towards the products when the solution is dilute. And according to the Second Law, if ATP hydrolysis releases about 10 kcal mol^{-1} at cellular concentrations, *at least* that much energy must have been consumed to synthesize ATP in the first place! Where does the energy come from?

The foregoing discussion increases our sense of awe of how the world is put together, but it also teaches some practical lessons.

[4] In skeletal muscle, [ATP] is $\sim 50 \times$[AMP] and $\sim 10 \times$[ADP]. Using these values, ΔG is even more exergonic, possibly as large as -60 kcal mol^{-1}.

Hydrolysis of ATP is clearly spontaneous in aqueous solution, and the reaction occurs relatively rapidly at 25 °C. (*In vitro*, the half-life of ATP is on the order of days at this temperature, and in the cell, where it is needed for metabolism, it is less than 1 s.) If the ratio of the *in vitro* to *in vivo* half-life were not large, ATP would be less a useful energy storage molecule than we know it to be. The hydrolysis rate of ATP and its dependence on concentration in the laboratory require that ATP-containing buffers be made up fresh and stored cold. For the same reason solutions of the free nucleotides used in the polymerase chain reaction (PCR, see below) are usually stored frozen at −20°C and thawed immediately before use.

Measurement of the enthalpy change of ATP hydrolysis shows that $\Delta H^\circ = -4$ kcal mol^{-1}. That is, hydrolysis of one mole of ATP at 25 °C results in about 4 kcal being transferred to the solution in the form of heat and about 3 kcal remaining with ADP and P_i in the form of increased random motion. We can combine our knowledge of the free energy and enthalpy changes to calculate the entropy change of ATP hydrolysis. Solving Eqn. (4.2) for ΔS when $\Delta T = 0$, we have $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$. At 310 K, $\Delta S^\circ = (-4 \text{ kcal mol}^{-1} - (-7 \text{ kcal mol}^{-1}))/(310 \text{ K}) = 10 \text{ cal mol}^{-1} \text{ K}^{-1}$. This is *roughly* the amount of entropy your body generates every time an ATP molecule is hydrolyzed. So, no matter how much you might feel your eyes glazing over at the sight of more Δs and cals, and no matter how much you might feel that biological thermodynamics is catalyzing the transition of your brain from a normal to a vegetative state, because you're simply alive you're doing a very fine job indeed of degrading the useful energy of the universe!

A couple of other pertinent points can be made here. One is that three of the four phosphate hydroxyl groups of ATP have pK_a values around 1.5. These are effectively completely ionized at neutral pH. In contrast, the fourth one has a pK_a of 6.5. This suggests that the net charge on any given ATP molecule might have a large impact on its cellular function. A second point is that the free energy difference between ATP and ADP + P_i is *not* the same as that between the plus-phosphate and minus-phosphate forms of other biomolecules. Glucose-6-phosphate, for instance, an important molecule in glycolysis, transfers its phosphate group to water with a standard state free energy change of about −3 kcal mol^{-1}. This is a substantially smaller energy change than for hydrolysis of ATP. The driving force for the chemical transfer of a phosphoryl group is known as *phosphoryl group-transfer potential*. ATP has the higher phosphoryl group-transfer potential of the two molecules. One might wonder whether ATP has the *highest* standard free energy of hydrolysis of all naturally occurring phosphates? No! ATP occupies a position about midway between extremes in tables of the standard free energy of hydrolysis of phosphate compounds (Table 5.2). ATP's being small and in the middle of the phosphate energy scale is likely an important determinant of its role in the cell.

Table 5.2. *Standard free energy changes of hydrolysis of some phosphorylated compounds*

Compound	$\Delta G^{\circ\prime}$ (kJ mol^{-1})
Glucose-1-phosphate	−20.9
Glucose-6-phosphate	−13.8
Fructose-6-phosphate	−13.8
ATP → ADP + P_i	**−30.5**
ATP → AMP + P_i	−32.5
Phosphocreatine	−43.1
Phosphoenolpyruvate	−61.9

Data are from Jencks, W. P., in Fasman, G. D. (ed.) (1976) *Handbook of Biochemistry and Molecular Biology*, 3rd edn, *Physical and Chemical Data, Vol. I*, pp. 296–304. Boca Raton: CRC Press.

Now let's look at a few other aspects of the cellular role of ATP: activity of glycogen synthase, synthesis of cyclic AMP, binding of ATP to hemoglobin, and inhibition of thermogenin in heat generation. Glycogen is a polymeric form of glucose that can be readily metabolized in times of need. Synthesis of glycogen involves the transfer of the glycosyl unit of uridine diphosphate glucose (UDPG) to an existing carbohydrate chain. UDPG is synthesized from glucose-6-phosphate and uridine triphosphate (UTP), a molecule involved in the synthesis of mRNA. Note the close "coupling" between energy storage and metabolism and information storage and expression. Marvellous efficiency! Replenishment of UTP occurs by means of a phosphoryl transfer reaction mediated by nucleotide diphosphate kinase. This enzyme catalyzes the transfer of a phosphoryl group from ATP to UDP, yielding ADP and UTP. Then replenishment of ATP occurs by means of a phosphoryl reaction mediated by ATP synthase and a proton gradient, and replenishment of the proton gradient occurs by means of oxidation of glucose ...

ATP is a precursor in the synthesis of 3′,5′-cyclic AMP (cAMP), an important intracellular signaling molecule known as a *second messenger* (Fig. 5.8).[5] The concentration of cAMP in the cell increases or decreases in response to the tight and specific binding of an extracellular molecule to a cell-surface receptor. For instance, [cAMP] goes up when a specific odorant receptor on a cell in the olfactory epithelium binds an odorant molecule, for instance, one of the aromatic ketones or amines mentioned in Chapter 3. Binding induces a conformational change in the receptor, and an intracellular protein that interacts with the cytoplasmic part of the receptor then activates adenylyl cyclase, the membrane-bound enzyme responsible for synthesis of cAMP from ATP. Once made, cAMP then moves throughout the cytoplasm, interacting with a wide range

[5] This term was introduced in 1964 by Earl Sutherland (1915–1974), an American, the discoverer of cAMP.

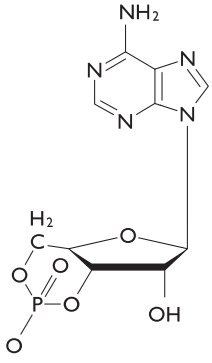


Fig. 5.8 Cyclic AMP. This molecule, which is synthesized from ATP, plays a key role in a variety of cellular processes. Principal among these is the control of glycogen metabolism in muscle. Glycogen is the highly branched high molecular mass glucose polysaccharide that higher animals synthesize to protect themselves from potential fuel shortage. The corresponding polymer in plants is starch (Fig. 1.1). Glycogen synthesis involves glycogen synthase. This enzyme catalyzes the transfer of the glucosyl unit of UDP-glucose (itself synthesized from glucose-1-phosphate and UTP, one of ATP's brother molecules) to glycogen. In glycogen breakdown, the enzyme glycogen phosphorylase cleaves the glycosidic bond linking glucose monomers by the substitution of a phosphoryl group. The products are a slightly smaller glycogen molecule and one molecule of glucose-1-phosphate (G1P), which is converted to glucose-6-phosphate by phosphoglucomutase. The nucleotides of information storage in genetic material play an important role in energy storage and utilization in all known living organisms. cAMP activates a protein kinase which activates phosphorylase kinase which, through phosphorylation, activates glycogen phosphorylase and inactivates glycogen synthase. The cellular concentration of cAMP is increased by adenylate cyclase, which is activated by the binding of glucagon or epinephrine to its receptor in the plasma membrane. When the hormone insulin binds to its receptor, glycogen phosphorylase is inactivated and glycogen synthase is activated.

of proteins. In this way, cAMP "mediates" the response of the cell to the ligand, be it an odorant molecule, hormone, or neurotransmitter. Again, there is a connection between energy and information, in that the small energy molecule ATP is involved in the communication throughout the cell of a signal received at the cell membrane. Later in this chapter we shall look at an example of the mechanics of binding interactions, and the subject will be covered in considerable detail in Chapter 7.

The physiological role of ATP does not always involve hydrolysis or chemical conversion into an electron carrier or second messenger. In fish and most amphibians, ATP binds tightly to deoxygenated hemoglobin but only weakly to oxygenated hemoglobin. The protein hemoglobin plays a crucial role in respiration by transporting oxygen to cells for oxidative phosphorylation. Binding to ATP regulates the function of hemoglobin by reducing its affinity for oxygen (see below and Chapter 7).

Box 5.1 Cool mice live longer

Obesity results when energy intake exceeds the energy expenditure. Experimental studies have shown that calorie restriction reduces core body temperature in rodents and primates. But is a lower core body temperature a simple consequence of calorie restriction, or is lower body temperature itself beneficial for health? Separate studies have found that lowering the core body temperature of poikilotherms like fish slows aging and prolongs life. But is this true of homeotherms like humans? To investigate the matter, Bruno Conti of the Scripps Research Institute in La Jolla, California and his colleagues created an engineered strain of laboratory mice. These animals have an overheated hypothalamus, the preoptic area of which is the brain's central thermostat. Heating up the hypothalamus dispatches "Chill out!" signals to the rest of the body and thus decreases the core temperature. Hypothalamic overheating in the engineered mice was achieved by over-expressing uncoupling protein 2 (UCP2). Found in the inner membrane of mitochondria, the powerhouses of the cell, UCP2 uncouples electron transport from ATP production and thus dissipates as heat the energy stored in the proton gradient across the mitochondrial membrane. UCP2 over-expression dissipated more proton gradient energy as heat than in normal mice, elevated the

Box 5.1. Cont.

temperature of the hypothalamus, and dropped the core body temperature 0.3–0.5 °C. Experimental female mice lived an average of 20% longer than controls; experimental males, 12% longer. Sex hormones might influence the rate at which some mice attain a lower core temperature. The experimental animals also appeared to show greater metabolic efficiency than controls, suggesting that fewer calories were needed to live. A take-home message for all you Ponce de Leon wannabes out there: the fountain of youth is within you.

Above we saw how the proton gradient in mitochondria can be coupled to the membrane protein ATP synthase and used to synthesize ATP. In brown adipose tissue, which contains large amounts of triacylglycerols (fatty acid triesters of glycerol, or fats, see Table 1.2) and many mitochondria, the proton gradient can be uncoupled from ATP synthesis by means of a channel protein called thermogenin. Dissipation of the proton gradient in the absence of ATP generation means that brown adipose tissue acts as a "built-in heating pad." Thermogenin is particularly plentiful in cold-adapted animals. The activity of thermogenin is under hormonal control. The adrenal hormone norepinephrine binds to its receptor and activates adenylate cyclase, which makes cAMP, which activates a kinase that phosphorylates a lipase, which hydrolyzes triacylglycerols to free fatty acids. When the concentration of free fatty acids is sufficiently high, thermogenesis is activated, and thermogenin changes the permeability of the inner mitochondrial membrane to protons and allows them back into the mitochondrial matrix without ATP production. Proton flow under the control of thermogenin is inhibited by ATP, GTP, and the diphosphate forms of these nucleotides.

D. Substrate cycling

The reaction catalyzed by the glycolytic enzyme phosphofructokinase is highly exergonic. Under physiological conditions,

$$\text{fructose-6-phosphate} + \text{ATP} \rightarrow \text{fructose-1,6-bisphosphate} + \text{ADP}, \tag{5.5}$$

with $\Delta G = -25.9$ kJ mol^{-1}. This reaction is so favorable that it is essentially irreversible. But the reverse reaction can occur! It just won't do so on its own. In fact, the enzyme fructose-1,6-bisphosphatase is present in many mammalian tissues, and it catalyzes the removal of a phosphate group from fructose-1,6-bisphosphate as follows:

$$\text{fructose-6-bisphosphate} + \text{H}_2\text{O} \rightarrow \text{fructose-6-phosphate} + \text{P}_\text{i}. \tag{5.6}$$

This also occurs spontaneously because $\Delta G = -8.6$ kJ mol^{-1}, but the reverse reaction is more probable than in Eqn. (5.5). The net reaction

is simply ATP hydrolysis, and $\Delta G = -34.5$ kJ mol^{-1}. Note that, although the overall free energy change is negative, this coupled reaction is less favorable than transfer of the terminal phosphoryl group of ATP to water. The opposing reactions of Eqns. (5.5) and (5.6) are called a *substrate cycle*.

Substrate cycles might seem to serve no useful purpose, since all they do is consume energy. But nature is a subtle lover, and she is more apt to reveal her charms to persistent humility than audacious presumption. The reverse reaction, far from being futile, constitutes a means of regulating the generation of product by the forward reaction, because enzyme activity itself is regulated. In cases where a substrate cycle is operative, metabolic flux is not simply a matter of the activity of an enzyme, but the combined activity of the enzymes working in opposite directions. There is thus exquisite regulation of a metabolic pathway, adjusting as needed to the cell's metabolic needs. The price paid for such control is the energy lost in the combined forward and reverse reactions.

Substrate cycles also function to produce heat, helping to maintain an organism's temperature.[6] So, although there is a high cost to control, it is clear that organisms make highly efficient use of the resource. It is also possible that heat production is important for controlling the rate of enzyme activity by controlling the temperature of the enzymatic reaction (Chapter 8). In bumblebees,[7] the presence of fructose-1,6-bisphosphatase in flight muscle is thought to enable these insects to fly at temperatures as low as 10 °C: honeybees, which do not have fructose-1,6-bisphosphatase, cannot fly when it's cold. Substrate cycling probably plays a key role in maintaining body heat in many animals, including humans. It is stimulated by thyroid hormones, which are activated upon exposure of the organism to cold. It's time for a change of topic.

E. Osmosis

We covered the concept of chemical potential in Chapter 4. Let's use it to develop a topic of general importance in biochemistry: osmosis (Greek, push). When mineral ions and charged molecules are absorbed by the small intestine, water follows *by osmosis*. We treat this subject in a fair amount depth for two reasons: *osmotic work* underlies many physiological functions – nerve conduction, secretion of hydrochloric acid in the stomach, and removal of water from the kidneys – and the subject involves a number of key subtleties of thermodynamics. Before looking at the mathematics of osmosis, let's first think about the situation qualitatively. This way, we'll be more certain to have a general sense of the matter before facing a

[6] Some organisms obtain a significant amount of heat from their surroundings, for instance basking lizards and snakes, which absorb heat from the Sun.

[7] Previously called humble-bees in the UK.

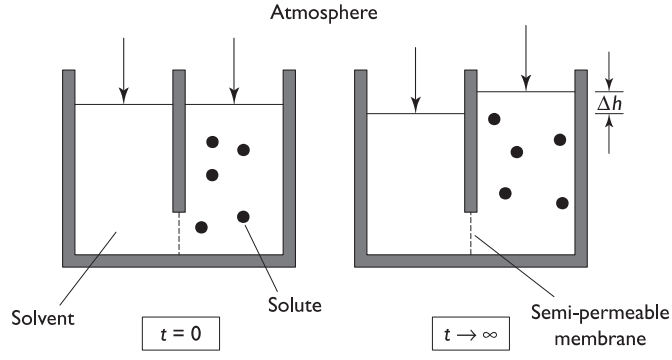


Fig. 5.9 A simple osmometer. A solute can move freely in a fraction of the total volume of solvent. The solution is separated from pure solvent by a membrane that is permeable to the solvent but not the solute. There is a net flow of solvent from the pure solvent to the solution, resulting in the development of a head of pressure. This pressure is the osmotic pressure, $\pi = \rho g \Delta h$, where ρ is density of the solvent, g is gravitational acceleration, and Δh is the difference in fluid levels. As described by van't Hoff, $\pi = CV_o RT/m$, where C is the mass of solute in the volume of solvent, V_o is the partial molar volume of the solvent, and m is the molecular mass of the membrane-impermeant solute. Note that π is an approximately linear function of C under some conditions. Osmotic pressure data can thus be used to measure the molecular mass of an osmotic particle.

page filled with equations. That *osmosis* is a pleasant-sounding word might help to move the discussion along ...

Osmosis is an equilibrium phenomenon that involves a semi-permeable membrane (not necessarily a biological membrane). *Semi-permeable* in this context means that there are pores in the membrane that allow small molecules like solvents, salts, and metabolites to pass through but prevent the passage of macromolecules like DNA, polysaccharides, and proteins. Biological membranes are semi-permeable: large solute molecules are *impermeant*. Like freezing point depression and boiling point elevation, osmosis is a colligative property.

Suppose we have an osmometer, also called a U-tube, with arms separated by a semi-permeable membrane (Fig. 5.9). Let the temperature be constant. If no solute is present the height of the solvent is the same on both sides, because the pressure of the external environment is the same on both sides. The situation changes on introduction of an impermeant solute to one side. Let the solute be a largish protein, say hemoglobin, and let it be freeze-dried before being added to the solvent. Freeze-dried protein occupies a relatively small volume. Initially, the height of the fluid is the same on both sides of the osmometer, just as when no solute was present. But whereas before the solute occupied a small volume on the bench-top, now it is able to move freely throughout one side of the osmometer. There has been a large increase in the entropy of the solute! (If you are not sure why, see the discussion on perfume in Chapter 3.) We require that the solute particles be free to roam about the entire volume on their side of the membrane, but that they not be able pass through the membrane. And just as a confined gas pushes against the walls of its container (Chapter 2), the solution pushes against the atmosphere and against the walls of the osmometer. What happens? There is a net transfer of solvent from the side where no solute is present to the other side. This decreases the volume of pure solvent and increases the volume of solution. How can we explain what has happened?

Addition of solute to solvent reduces the chemical potential of the solvent (Chapter 4). This creates a difference in the chemical potential of the solvent between the pure side and the impure side. The difference in chemical potential is thermodynamically

unstable; change must occur. The impure side has a lower solvent chemical potential, so water moves down its concentration gradient until equilibrium is reached. From an entropic point of view, the flow of water into the side with solute increases the entropy of the solute, making the situation more stable. How is entropy increased? Neglecting interactions between the solute and solvent, the greater the volume of solvent present, the larger the volume in which the solute can distribute itself. There is a resemblance to the ideal gas expansion discussed in Chapter 3 (see Eqn. (3.23)). In the context of the perfume example, if it is applied in the bathroom, the perfume molecules become distributed throughout the bathroom, but when the door is opened, the molecules begin to spread into the corridor. At equilibrium, the perfume molecules will occupy the bathroom and corridor, i.e. the entire accessible volume. The concentration is reduced in the process, entropy is increased, and more work would have to be done to gather all the molecules back together into the same place.

The flow of water from one side of the U-tube to the other must result in a change in the height of the water on the two sides. It becomes lower on the side of the pure solvent and higher on the side of the impure solvent. After enough time, the system comes to equilibrium, and the driving force for water to move through the membrane from the pure solvent to the solution will be equal in magnitude to the hydrostatic pressure arising from the difference in height of the water in the two arms ($p_{\text{hydrostatic}} = \rho g \Delta h$, where ρ is the density of the solution). The hydrostatic pressure is the same as the *additional* pressure one would have to apply to the side of the U-tube with solute in order to equalize the height on the two sides of the membrane. This pressure is called the *osmotic pressure*, and it was first studied in the 1870s by the German botanist and chemist Wilhelm Friedrich Philipp Pfeffer (1845–1920), the son of an apothecary.

Now let's take a more mathematical approach to osmosis. This way of thinking about the subject is not necessarily superior to the qualitative approach just because it involves more equations, but it will provide additional insight to our subject, and that's what we want. Mathematical or computational modeling of the physical world makes sense as long as it leads to insights that can be tested experimentally. In our approach to modeling, the system is regarded as consisting of two *phases*, x and y. In x, the impermeant molecules (component 2) are dissolved in the solvent (component 1). In y, only solvent molecules are present. Considering the *solvent* alone, the requirement for equilibrium between the two phases is

$$\Delta G = \mu_1^x \Delta n_1^x + \mu_1^y \Delta n_1^y = 0, \tag{5.7}$$

where Δn_1 stands for an incremental change in the number of moles of solvent. (See Eqn. (4.5).) Because $\Delta n_1^x = -\Delta n_1^y$ (because the gain of solvent molecules in one phase must come at the expense of the same number of molecules from the other phase),

$$\mu_1^x = \mu_1^y. \tag{5.8}$$

The ledger balances. But wait! Something funny's going on. For regardless of the amount of solvent transferred through the membrane, we can't avoid the requirement that $\mu_1 - \mu_1^\circ = \Delta\mu < 0$ (see Eqn. (4.10)). That is, the chemical potential of the solvent plus solute *must* be lower than that of the pure solvent. Nevertheless, Eqn. (5.8) does say that the chemical potentials of the solvent in the two phases must be equivalent. Where did we err?

We didn't! We conclude that there is a contradiction, that biological thermodynamics is illogical and therefore a waste of time, that it was a mistake to study biology or in any case to do it in a place where biological thermodynamics forms part of the curriculum, and that our best option would be to make our way to the college bar and drink away our misery. Right? No way! Things are just starting to get interesting! Let's see if we can't crack this nut now, and think about a celebratory night out later on. But what can we do?

Above we showed that $\Delta G = V\Delta p - S\Delta T$. Under isothermal conditions, $\Delta T = 0$ and the free energy change is proportional to Δp. To make the resulting expression tell us what will happen when the number of solvent molecules is changed, we divide both sides by Δn_1. This gives

$$\Delta G/\Delta n_1 = \mu_1 = V_{m,1}\Delta p, \tag{5.9}$$

where $V_{m,1}$ is the molar volume of component 1. We have found the "missing" term from our expression of the chemical potential earlier in this section! Taking into account the development leading up to Eqn. (4.12), where we saw how the chemical potential of a solvent changes when a solute is added, and adding in Eqn. (5.9), we have

$$\mu_1 - \mu_1^\circ \approx -RTC_2V_1^\circ/M_2 + RT\ln f_1 + V_1^\circ\pi, \tag{5.10}$$

where the pressure difference has been symbolized as π (this has nothing to do with the ratio of the circumference of a circle to its diameter; it is the symbol that is traditionally used to designate the osmotic pressure; π starts with the same sound as *pressure*). Adding in the extra term (Eqn. (5.9)) might strike you as a rather arbitrary way of doing math – one that would be fine for getting a correct result on an exam but maybe dubiously valid. But we need to remember that in order for the equilibrium condition to be met, we must have a balance of forces, and we can write down an equation – an expression of balance – only if we take into account *everything* that's relevant. There was indeed a contradiction earlier because we had assumed that the system was at equilibrium when in fact we hadn't taken the pressure term into account. Note that in Eqn. (5.10) we have assumed $V_{m,1} \approx V_1^\circ$, the molar volume of pure solvent, which is valid for dilute solutions. The pressure difference π is the pressure that must be applied to the solute side of the U-tube to make the fluid height the same on both sides.

We can simplify Eqn. (5.10) a bit. If the solution is ideal, $f_1 \approx 1$ and $RT\ln f_1 \approx 0$. At equilibrium, $\mu_1 - \mu_1^\circ = 0$. It follows that

$$\pi = RTC_2/M_2. \tag{5.11}$$

This is the van't Hoff law of osmotic pressure for ideal dilute solutions, named in honor of the scientist who gave Pfeffer's work a mathematical foundation.[8] Equation (5.11) can be used to measure the mass of an impermeant solute particle (though there are easier and more accurate ways to do it). Note how Eqn. (5.11) looks like Eqn. (4.12). You may already have noticed how closely Eqn. (5.11) resembles the ideal gas law ($pV = nRT$ or $p = nRT/V = CRT$, where n is number of particles and C is concentration). C_2, the concentration of solute, is the mass of solute particles added to a known volume of pure solvent. What van't Hoff found was that the measured osmotic pressure was basically the pressure of n solute particles moving around in volume V, the volume of the solvent through which the solute particles are free to move!

The degree to which Eqn. (5.11) matches experimental results varies with concentration and solute (Fig. 5.10). There are several different ways of trying to cope with the situation, but our concern will be with just one of them here. Time is spent on it at all because it's a generally useful method. We express the thermodynamic observable quantity (here, π) as a series of increasing powers of an independent variable, (here, C) and check that the dominant term is the same as we found before (Eqn. (5.10)) when the independent variable takes on an extreme value (low concentration limit, as we assumed above):

$$\pi = \frac{C_2 RT}{M_2}(1 + B_1(T)C_2 + B_2(T)C_2^2 + \ldots). \tag{5.12}$$

The $B_i(T)$ terms are *constant* coefficients whose values are solute- and temperature-dependent and must be determined *empirically*. If C_2 is small, only the first term makes a significant contribution to π (convince yourself of this!), just as in Eqn. (5.10). If only the first two

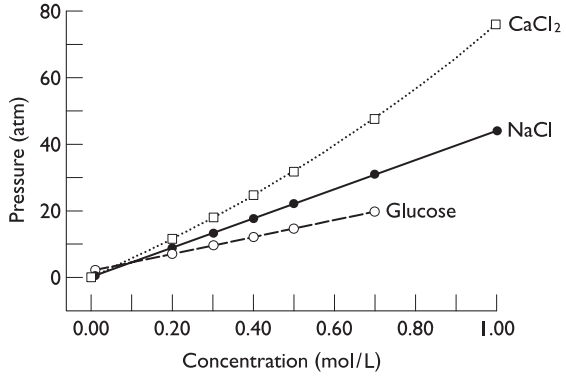


Fig. 5.10 Osmotic pressure measurements. Osmotic pressure increases with concentration of solute, as predicted by the van't Hoff law. The pressure at a given concentration of solute depends significantly on the solute. If the solute is a salt, dissociation in aqueous solution will result in a greater number of particles than calculated from the molecular mass of the salt. The van't Hoff law is exact for an ideal solution. At high solute concentrations, non-linear behavior can be detected. Such behavior can be accounted for by higher order terms in C. The data are from Table 6–5 of Peusner (1974).

[8] The Dutch physical chemist Jacobus Henricus van't Hoff (1852–1911) was the recipient of the Nobel Prize in Chemistry in 1901, the first year in which the prestigious awards were made.

terms make a significant contribution to π, a plot of π/C_2 will be linear in C_2 with slope $B_1(T)RT/M_2$ and intercept RT/M_2. This permits indirect measurement of M_2 and $B_1(T)$. Equation (5.12) can readily be generalized to include contributions from different species of osmotic particle:

$$\pi_{\text{total}} = \pi_1 + \pi_2 + \cdots + \pi_n = \Sigma\pi_i. \tag{5.13}$$

If a solute species is present on both sides of the membrane, and if this solute cannot pass through the membrane, it will make a contribution to the total osmotic pressure, but only if there is a concentration difference. In such cases, π_i is proportional not to C_i, as in Eqn. (5.12), but to ΔC_i, the concentration difference across the membrane.

Now let's leave the wispy world of mathematics and migrate over to a more material physical biochemistry. Osmosis can be a very strong effect. At 25°C, a 1 M solution of glucose, a relatively small "osmolyte," gives a pressure more than 25 times greater than that of the atmosphere; 1 M solutions of salts give even larger osmotic pressures (see Fig. 5.10), though the ions are smaller than glucose, even when hydration is taken into account. Osmotic forces are important in biology because they play a key role in membrane transport of water, in all kinds of situations. For example, red blood cells are full of impermeant solute particles, mainly hemoglobin; red cells have been called "bags of hemoglobin." M_2 is large, about 68 000, and C_2 is high, about 0.3 M or higher. When placed in pure water, there is initially a very large π across the membrane – about 8 atm or greater, the approximate pressure when scuba diving at a depth of 70 m! The plasma membrane cannot withstand an osmotic pressure of this magnitude and breaks, spewing its hemoglobin into the surrounding medium. Blood banks limit damage to red cells after separating them from plasma by centrifugation by resuspending the cells in a sucrose solution (sucrose is membrane impermeant) of approximately the same solute concentration as blood plasma (an *isotonic* solution).

Note the difference between the red blood cell in a solution of low solute concentration (hypotonic solution) and impermeant particles in an osmometer. In the osmometer, there is a real pressure difference. The presence of the impermeant particles results in the formation of a pressure head, and the solution pushes down harder than it would if the solute were not there. But what if the osmometer were configured as a capillary tube oriented horizontally with a semi-permeable membrane in the middle? Water would move through the membrane as before, and one could think of the effect as arising from the pressure of n solute particles confined to a volume V of solvent. The thought experiment suggests that red blood cells burst in hypotonic solution because the hemoglobin molecules inside the cell bang on the cell membrane much harder than the water molecules bang on the membrane from the outside. Is that right?

If a solution is at thermal equilibrium, then *all* the particles in the system have the same average thermal energy, irrespective of size. Big molecules like hemoglobin are relatively slow, little molecules like water are relatively fast (Chapter 1). But these molecules do not have the same *momentum* (Chapter 2). From physics, the K.E. of a particle is $\frac{1}{2}mv^2 = \mathbf{P}^2/m$, where m is the mass, v is the velocity and $\mathbf{P} = mv$ is the momentum. Thermal energy is proportional to T, and at thermal equilibrium the K.E. of a particle is equal to its thermal energy. So, $\mathbf{P} \propto (mT)^{\frac{1}{2}}$. In other words, the more massive the particle, the greater its momentum. Note that **P** has nothing to do with particle *volume*. So in a hypotonic solution, where there is nothing outside the cell that cannot get inside, hemoglobin molecules do bang into the membrane from all directions a lot harder than water molecules bang on the membrane from all directions. And the concentration of water molecules on the inside certainly is lower than the concentration on the outside, but the concentration of hemoglobin outside is 0. Therefore, it might be supposed, the banging of hemoglobin molecules on the cell membrane causes the cell to burst. But wait, is not water also rushing into the cell? Water is indeed pouring in, at a very high rate, seeking to destroy the gradient. The driving force for this flood, moreover, is very large. It is also driven almost entirely by an increase in entropy. Water could enter the cell before the surroundings become hypotonic, but the rate in and rate out were the same. Now, with a net flow of water inwards, the red blood cell swells, creating a larger and larger volume in which the massive hemoglobin molecules can diffuse, allowing them to display their Second Law tendency to disperse. It has been said that thermodynamics is a difficult subject because there are so many different ways of thinking about the same thing. Osmosis is a good example of the truth of that statement.

The actual situation with hemoglobin is more complicated than we've made it sound. This is because hemoglobin does not just float around in a sea of water in a red blood cell; the protein interacts with the solvent. When particle interactions are taken into account, the volume of the particle does matter, because the more space it takes up, the more surface it will expose to the solvent. This is the source of the higher-order terms in Eqn. (5.12).

In contrast to red cells, some bacteria do not burst when placed in a hypotonic solution. This is because these organisms (as well as plant cells and fungi) can withstand high osmotic pressure gradients by means of a rigid cell wall. When certain bacteria come into contact with lysozyme, an enzyme we have encountered already several times in this book, however, the bugs can be made to spill their guts. The biochemical activity of lysozyme is to cleave certain glycosidic bonds in the polysaccharides that give the bacterial cell wall its strength, weakening the cell wall. It is a rather good thing that our bodies station lysozyme molecules at common points of entry of foreign microbes, for example, the mucosal membrane in

the nasal passage. People with a mutant lysozyme gene have a tougher time than most in fighting off infection, and they tend to die relatively young. One might surmise that early death results from too little lysozyme being available to make the cell walls of bacteria more susceptible to osmotic stress. But the actual situation is more complex than that. That's because the immune system has a role to play in fighting off infection, few pathogenic bacteria are susceptible to lysozyme alone, and the mutant lysozyme proteins, which are less active than the wild-type enzyme, are also less thermostable than the wild-type enzyme and give rise to amyloid fibril formation. Not only is the amyloidogenic lysozyme less active and therefore less able to fight off infection, there is a net incorporation of the protein molecules into rigid fibril structures where they have effectively no enzymatic activity all. To make things worse, the body has a hard time ridding itself of the fibrils, and their continued increase in size can be pathological (Chapter 8).

F. Dialysis

This section is a close relative of the previous one. There are two basic forms of dialysis in biochemistry: non-equilibrium dialysis and equilibrium dialysis. We look at both here; the physics is basically the same in both cases. Dialysis is useful to the biochemist because it can be used to separate molecules according to size. It does this by means of a semi-permeable membrane, like the membrane in the section on osmosis (Fig. 5.11). Many semi-permeable membranes used for dialysis are made of cellophane (cellulose acetate).

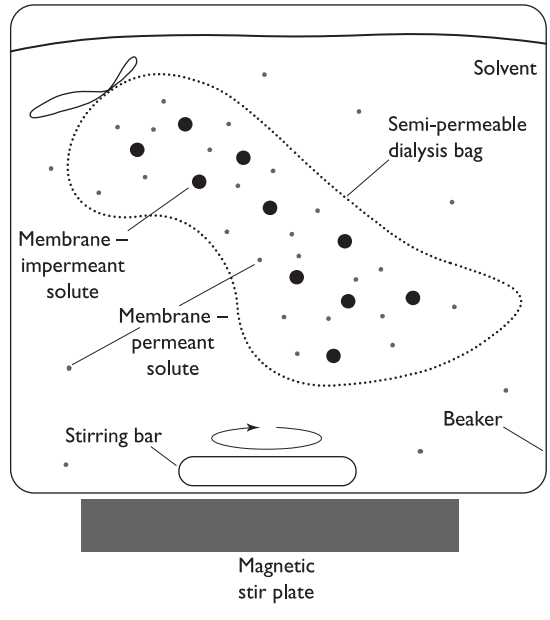


Fig. 5.11 Dialysis. A dialysis bag containing a membrane-impermeant solute is submerged in solvent in a beaker. Membrane-permeant solute appears on both sides of the membrane. The dialysis bag-solvent system is not at equilibrium. At equilibrium, the concentration of membrane-permeant solute will be the same on both sides of the membrane. A magnetic stir plate and stirring bar are used to accelerate the approach to equilibrium; the flow rate of membrane-permeant solute out of the dialysis bag is related to the concentration gradient of that solute.

Non-equilibrium dialysis is the use of a semi-permeable membrane to change the composition of the solution in which macromolecules are dissolved. For instance, one means of purifying recombinant proteins from *E. coli* host cells is to lyse the cells in 8 M urea, a small organic compound. Urea at a concentration of 8 M or higher will denature most proteins at room temperature. Once the recombinant proteins have been separated from the bacterial ones (often by a type of affinity chromatography), the recombinant protein solution is transferred to a dialysis bag. Then the bag is sealed and placed in a large volume of buffer containing no urea. When equilibrium is reached, several hours later, the concentration of urea inside the dialysis bag has decreased and the concentration outside has increased, and the concentration of urea is about the same on both sides of the membrane. What drives the change?

Initially, the concentration of solute particles is much higher within the dialysis bag than in the solution outside; some osmosis occurs. The chemical potential of urea is very much higher in the bag and very low outside, at least initially; there will be a net migration of urea out of the bag until the concentration gradient has vanished. The continuous change in concentration of urea both inside the bag and outside until equilibrium is reached and gives rise to a continuous change in osmotic pressure. The osmotic effect can be substantial when working with a high urea concentration, leading to a substantial increase in the volume of material inside the bag during dialysis. So, to avoid possible rupture of tubing and loss of a precious sample, leave room in the bag for an influx of water!

Is any work done in the net migration of urea out of the bag? No! Despite similarities to osmosis, the situation here is qualitatively different. True, urea moves down its concentration gradient, but there is no corresponding development of a pressure head as in osmosis. In other words, nothing retards the dilution of urea, just like nothing opposes the expansion of gas into vacuum, so no pV-work is done. The experiment can be carried out in isolation, so $q = 0$. Then by the First Law, $\Delta U = 0$. If the pressure is constant, then $\Delta H = 0$. And if the temperature is constant as well, G is a thermodynamic potential function. But the process we've outlined is irreversible! If we carry out the process very slowly, though, having the system pass through a series of near equilibrium states, we can evaluate ΔG. Recall that G is a state function, so its value depends only on the initial and final states of the system, and not on whether the process was reversible or irreversible. The expansion of the urea, just like the expansion of gas into vacuum, is spontaneous, so $\Delta G < 0$. This can only be true if $\Delta S > 0$, because $\Delta H = 0$. No violation the Second Law, no problems.

Another concern of non-equilibrium dialysis is charge. Typically, the macromolecule in the dialysis bag will be ionized, and this will affect osmosis by interactions with water. The solution being dialyzed out of the bag or into it will usually be a buffer of some sort, containing both charged and uncharged solute particles, and the

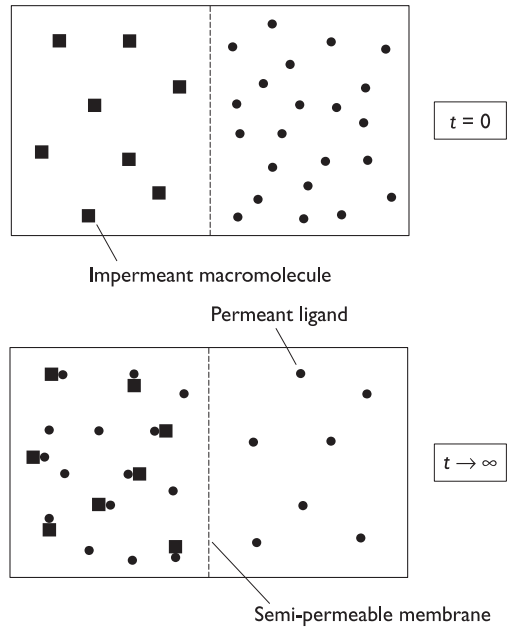


Fig. 5.12 Equilibrium dialysis. At the beginning of the experiment ($t = 0$), the membrane-impermeant macromolecule and membrane-permeant ligand are on opposite sides of a semi-permeable dialysis membrane. The two-chambered system is not at equilibrium. After a long time ($t \rightarrow \infty$), the concentration of *free* ligand is approximately the same on both sides of the membrane, in accordance with the Second Law of Thermodynamics. The number of ligand molecules is not the same on both sides of the membrane, however, as some ligands are bound to the membrane-impermeant macromolecules. The bound ligand molecules are nevertheless in equilibrium with the free ones. Measurement of the concentration of free ligand at equilibrium and the total concentration of ligand determines the amount of bound ligand at equilibrium.

ratio and relative abundance of these will have an impact on the migration of water through the membrane.

And equilibrium dialysis? In some respects it's rather similar to non-equilibrium dialysis. In others, it has a more specific meaning than *dialysis* and therefore deserves to be treated somewhat separately. Suppose you are interested in the binding of a macromolecule to a membrane-permeant ligand. This presents an opportunity for quantitative analysis of the binding interaction. To see how, suppose we have a two-chambered device like that shown in Fig. 5.12. In the left side, you introduce a known amount of macromolecule in your favorite buffer, and on the right side, a known amount of ligand dissolved in the same buffer. The ligand will diffuse in solution, and the net effect will be movement down its concentration gradient, through the membrane. By mass action the ligand will bind to the macromolecule. After a sufficiently long time, the two chambers will be at equilibrium; the concentration of free ligand will be the same on both sides of the membrane. The amount of ligand on the side of the macromolecule, however, will be higher by an amount depending on the strength of interaction between macromolecule and ligand. You can then use a suitable assay to measure the amount of ligand on both sides of the membrane, and the difference will be the amount bound to the macromolecule. You then compare the concentration of "bound" ligand to the concentration of macromolecule and to the concentration of "free" ligand, and use the results to calculate the binding constant and the number of ligand molecules bound per macromolecule. This is an important topic. See Chapter 7.

G. Donnan equilibrium

In our discussion of dialysis we barely mentioned charge effects. Here, we'll see just how much more complicated things are when charge is taken into account more formally. We need to engage with the subject with this added degree of complexity, because all major classes of biological macromolecule – proteins, nucleic acids, and some polysaccharides – are charged. Moreover, in the living organism these molecules are found not in pure water but in a saline solution.

Suppose we have a polyelectrolyte like DNA, and let it be dissolved in a solution containing a simple salt, say NaCl. Suppose further that there are two phases to our system, just as in our discussion of osmosis. Now, though, one phase consists of water, Na^+ and Cl^- (phase α), and the other consists of water, Na^+, Cl^- and DNA (phase β). The phases are separated by a semi-permeable membrane, and DNA alone is impermeant. At equilibrium, the concentration of ions will not be the same on the two sides of the membrane except in the limit that $[DNA] \to 0$. Why not? Because DNA is anionic, so we should expect the concentration of sodium to be higher on the side of the membrane with DNA than on the other side. In symbols, $[Na^{+\beta}] > [Na^{+\alpha}]$. Let's see if we can obtain a quantitative expression for the concentration of ions.

At equilibrium, even though the concentrations aren't equal, we must have

$$\mu^{\alpha}_{NaCl} = \mu^{\beta}_{NaCl}. \tag{5.14}$$

Let's keep things simple and assume that the solution is ideal. The chemical potential of the salt is

$$\mu_{NaCl} = \mu^{\circ}_{NaCl} + RT \ln[Na^+][Cl^-]. \tag{5.15}$$

At equilibrium, the standard state chemical potential must be the same in both phases, so

$$[Na^{+\alpha}][Cl^{-\alpha}] = [Na^{+\beta}][Cl^{-\beta}]. \tag{5.16}$$

And the net charge of each phase must be equal to zero, a condition known as *electroneutrality*, which is expressed mathematically as

$$[Cl^{-\alpha}] = [Na^{+\alpha}] \tag{5.17}$$

$$z[DNA^{\beta}] + [Cl^{-\beta}] = [Na^{+\beta}], \tag{5.18}$$

where z is the number of negative charges on the DNA. With a bit of algebra, these equations can be combined to give

$$[Na^{+\beta}] = [Na^{+\alpha}]\left(1 + \frac{z[DNA^{\beta}]}{[Cl^{-\beta}]}\right)^{1/2} \tag{5.19}$$

$$[Cl^{-\beta}] = [Cl^{-\alpha}]\left(1 - \frac{z[DNA^{\beta}]}{[Na^{+\beta}]}\right)^{1/2}. \tag{5.20}$$

As expected, $[Na^{+\beta}] > [Na^{+\alpha}]$, neutralizing the charge on DNA in phase β. Similarly, $[Cl^{-\beta}] > [Cl^{-\alpha}]$, though because of the minus sign in Eqn. (5.20) the difference between phases is not as great as for the DNA counterions (Na). In such situations, the observed osmotic pressure gradient is produced by both the impermeant macromolecule and the asymmetric distribution of small ions. This effect, called the *Donnan equilibrium*, was first described in 1911 by the physical chemist Frederick George Donnan (1870–1956), son of a Belfast merchant. The effect pertains not only to membrane equilibria but to any situation in which there is a tendency to produce a separation of ionic species. The asymmetric distribution of ions arises from the requirement of electroneutrality, and its magnitude decreases with increasing salt concentration and decreasing macromolecule concentration, as can be seen from Eqns. (5.19) and (5.20).

The Donnan effect is even more complicated for proteins than DNA. This is because the net charge on a protein, a sort of weak polyion, is highly dependent on pH, whereas DNA, a sort of strong polyion, has a net charge that varies relatively little with pH. The greater the net charge on a macromolecule, the greater the Donnan effect. For proteins, the Donnan effect is minimized at the isoelectric point, where the net charge on the molecule is zero. There are no conceptual difficulties here, but you might find it tricky to work with proteins at their isoelectric point in the laboratory. Protein solubility tends to be very low at the isoelectric point! A physiological example of where the Donnan effect is relevant is in the red blood cell (RBC). The effect is caused mainly by the huge concentration of hemoglobin inside the cell and the inability of hemoglobin to penetrate the membrane under isotonic conditions. Other ions present, for instance sodium and potassium, do not contribute to the Donnan effect because they are generally impermeant and their effects counterbalance ($[K^+]_{plasma} \approx [Na^+]_{cell}$ and ($[K^+]_{cell} \approx [Na^+]_{plasma}$). Chloride, bicarbonate, and hydroxyl ions, by contrast, can cross the membrane, and they contribute to the Donnan equilibrium. Experimental studies have shown that the cell-plasma ratios of these ions are 0.60, 0.685, and 0.63, respectively. The marked deviations from 0.5 arise from the confinement of hemoglobin within the cell. This has an impact on the pH of the blood since both bicarbonate and hydroxyl are bases.

H. Membrane transport

There is metabolic activity within cells, and an individual cell in a higher eukaryote is separated from its surroundings by its plasma membrane. The membrane enclosing the cell is about 10 nm thick. It comprises two layers of phospholipids, with the charged groups on the outside. The interior of a membrane is “oily” and thus generally impermeable to ions and polar compounds. Some charged

substances can pass through membranes, but most only by means of transport proteins embedded in the lipid bilayer.

Membrane transport is said to be *passive* if a solute moves down its concentration gradient, and *active* if it moves against it. An example of active transport in cells is the movement of Na^+ and K^+ across the cell membrane of red blood cells, nerves and muscle cells – against the concentration gradient. The concentration of K^+ in muscle is about 124 mM, some 60-fold greater than in serum. With Na^+ it's the other way around, the concentration being about 4 mM in muscle cells and 140 mM in serum. These ions will of course tend to move down their concentration gradients to minimize free energy. But the gradients, which are important to cell function, are maintained by a membrane-spanning enzyme called Na^+/K^+-transporting adenosine triphosphatase. In order to maintain the gradient, the cell must pay a cost – that of kicking out the unwanted ions that have come in and that of recovering the wanted ones that have left. Gradient maintenance requires moving ions from a region of low concentration to a region of high concentration and therefore the expenditure of energy. The Na^+/K^+-transporter acts as an ion pump and is powered by ATP hydrolysis. Another example of active transport is the secretion of HCl into the gut of mammals by parietal cells – the home of many mitochondria!

A numerical example will help to motivate the discussion that follows. The change in chemical potential of glucose when it is transported down a 1000-fold glucose concentration gradient at 37°C is given by Eqn. (4.32):

$$\Delta\mu = 8.314\ \mathrm{J\,mol^{-1}K^{-1}} \times 310\ \mathrm{K} \times \ln(1/1000) = -17.8\ \mathrm{kJ\,mol^{-1}}. \tag{5.21}$$

That is, if the glucose concentration in the blood is high, as after a meal, and the concentration in cells is low, the sugar molecules enter cells spontaneously. As we have seen, once the sugar gets in, it is "tagged" with a charged phosphoryl group, preventing its escape through the hydrocarbon membrane. And if the concentration of chloride in the blood is about 100 mM, whereas that in the urine is about 160 mM, work must be done to pump chloride out of the blood and into the urine. You can easily calculate the work done by the kidneys in this process: $\Delta\mu = 1.9872\ \mathrm{cal\,mol^{-1}\,K^{-1}} \times 310\ \mathrm{K} \times \ln(160/100) = 290\ \mathrm{cal\ mol^{-1}}$. Moreover, you can estimate the number of chloride ions transported per ATP molecule hydrolyzed: free energy change of ATP hydrolysis in the cell/energy required to transport $Cl^- = 10\,000\ \mathrm{cal\,mol^{-1}}/290\ \mathrm{cal\,mol^{-1}} \approx 34$. We have ignored charge effects in the second calculation, but the magnitude should be about right.

We know from our study of the Donnan equilibrium that if the solute particle is charged, as in the case of N^+ or K^+, the situation is more subtle. Equation (4.32) does still apply, but we also need to take into account the work done as the charged particle moves through the

electrical potential across the membrane, $\Delta \boldsymbol{V}$. The magnitude of $\Delta \boldsymbol{V}$ is 10−200 mV, depending on the cell type – giving an electric field strength of as much as 200 000 V cm^{-1} across a membrane about 100 Å thick. This is only one order of magnitude smaller than the field where dielectric breakdown of air occurs and lightning strikes! Across every membrane of every cell in your body! In Chapter 4 we saw that the magnitude of the free energy change for electrical work is $\Delta\mu = nF\Delta \boldsymbol{V}$ when the ionic charge is n. Adding this term to Eqn. (4.32), we obtain

$$\Delta\mu = RT \ln[I]_i/[I]_o + nF\Delta \boldsymbol{V}, \tag{5.22}$$

where I represents an ionic solute and $\Delta \boldsymbol{V} = \boldsymbol{V}_i - \boldsymbol{V}_o$. The reference state must be the same for both terms of the right-hand side of this equation; in this case it is the extracellular matrix. When there is no driving force to move an ion from one side of the membrane to the other, $\Delta G = 0$ and

$$nF\Delta \boldsymbol{V} = -RT \ln[I]_i/[I]_o \tag{5.23}$$

which, on rearrangement, becomes

$$\Delta \boldsymbol{V} = -\frac{RT}{nF} \ln \frac{[I]_i}{[I]_o}. \tag{5.24}$$

We can use Eqn. (5.24) and the measured potential across the membrane to determine the ratio of the concentrations of the ionic solute. Let's assume, for instance, that we are working with a monovalent cation ($n = +1$) at 300 K, and let $\Delta \boldsymbol{V} = 120$ mV. Solving for $[I]_i/[I]_o$, we have

$$\begin{aligned} [I]_i/[I]_o &= \exp(-nF\Delta \mathbf{V}/RT) \\ &= \exp[(-96.5\ \mathrm{kJ\,V^{-1}mol^{-1}} \times 0.12\ \mathrm{V})/(8.314\,\mathrm{J\,mol^{-1}\,K^{-1}} \times 300\,\mathrm{K})] \\ &= 0.01. \end{aligned} \tag{5.25}$$

$[I]_o$ is 100 times greater than $[I]_i$.

At a number of points in this book nerve impulses have cropped up, for example, in the context of olfaction, perfume, and the Second Law. Now we wish to expand on the underlying mechanisms, albeit in highly qualitative terms. The aim here is to show how the development of this section fits within the broader picture of how animals work. Neurons, like other cell types, have ion-specific "pumps" situated in the plasma membrane. These protein machines use the energy of ATP hydrolysis to generate ionic gradients across the membrane in a way that resembles how electron transport proteins use the energy of glucose metabolism to generate a proton gradient (below). When at rest, a neuron is not very permeable to Na^+ (which is concentrated outside the cell and dilute inside) or K^+ (concentrated inside, dilute outside). There is a voltage on the order of 60 mV across the "resting" membrane. Stimulation of a nerve

cell results in a "depolarization" of the membrane. In the process, voltage-sensitive channel proteins that are selective for specific ions are "activated" by the decrease in voltage, allowing Na^+ ions in and K^+ ions out. The combined effect of gain and loss of membrane permeability to these ions is a millisecond time scale spike in membrane potential, known as an *action potential* – the potential across the membrane is reflecting the action of nerve impulse transmission. Depolarization of one part of the membrane by an action potential triggers depolarization of the adjacent part of the membrane, thereby propagating the action potential down the axon of neuron. Nerve impulses travel in one direction only because a certain amount of time is required for the recently depolarized part of the cell to regenerate its ion gradient.

A protein that has been mentioned at several points above is ATP synthase, the most famous of all transport proteins. ATPase is a type of molecular motor that plays a vital role in bioenergetics. Equation (5.22) can be used to describe the energetics of the "energy-transducing" membranes involved in ATP synthesis. In this case, the membrane of interest is the inner membrane of mitochondria and the ion is hydronium. The term $\ln[I]_i/[I]_o$ becomes $\ln [H^+]_i/[H^+]_o$, which can be rewritten as $2.3\Delta pH$ ($pH = -\log[H^+]$, and $\ln x \approx 2.3\log x$). Substituting into Eqn. (5.22) and converting from units of energy to volts gives the *proton motive force* of chemiosmotic theory:

$$\Delta\mu_{H^+} = 2.3RT(pH_o - pH_i) + nF\Delta \boldsymbol{V}. \tag{5.26}$$

The measured membrane potential across the inner membrane of a liver mitochondrion is about -170 mV ($\boldsymbol{V}_i - \boldsymbol{V}_o$), and the pH of its matrix is about 0.75 units *higher* than that of its intermembrane space. Thus,

$$\begin{aligned}\Delta\mu = {} & [2.3 \times 8.314\,\mathrm{J\,mol^{-1}\,K^{-1}} \times 298\,\mathrm{K} \times (-0.75)] \\ & + [1 \times 96\,500\,\mathrm{J\,V^{-1}\,mol^{-1}} \times (-0.17\,\mathrm{V})],\end{aligned} \tag{5.27}$$

the sum total being about -21 kJ mol^{-1} for transport of a proton *into* the matrix.

The basic ideas discussed here apply not only to the synthesis of ATP from ADP and P_i and the accumulation of ions across a membrane, but also to a broad range of transport processes occurring across plasma membranes and neuronal synaptic vesicles. Before concluding this section, let's take the opportunity to see how ATP synthesis is a matter of energy coupling on a grand scale. This will help us to see how things tie together, how marvelously integrated the various aspects of the living cell are.

As we have seen, glucose oxidation in aerobic organisms is coupled to the reduction of oxygen to water. Electron transport proteins play a key role in the process. The overall redox reaction, which is energetically favorable, is used to pump protons *against* their concentration gradient to the opposite side of the membrane. In other words, the pH of solution on one side of the membrane is

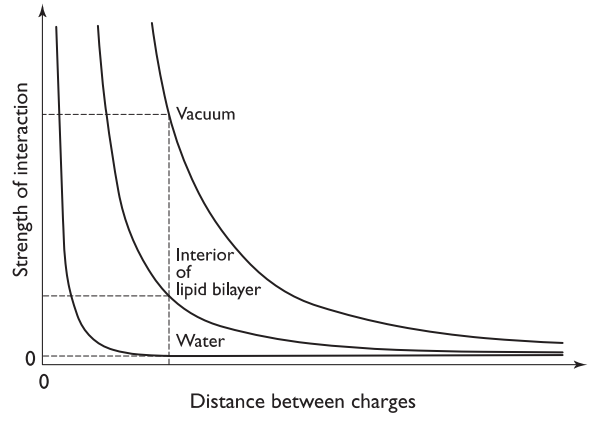


Fig. 5.13 Dependence of electrostatic energy on distance between charges and medium. The energy is inversely proportional to distance, so attraction or repulsion is greatest when the charges are close to each other. The energy also depends substantially on the stuff between the charges, varying inversely with the dielectric constant of the medium. The dielectric constant is constant for a given temperature and pressure, and it must be determined empirically. The interaction between charges is greatest in vacuum. In water, where the dielectric constant is very large, charges must be very close for the interaction between them to be significant. Charge–charge interactions are relatively large in the core of a protein or in the plasma membrane, because the dielectric constant of hydrocarbon is much lower than that of water.

different from that on the other side. And the voltage difference across the membrane, which is only about 10 nm thick, is about 200 mV, so the electric field strength in the middle of the membrane is *huge*! Protons migrate down their concentration gradient through a protein channel in the lipid membrane. Protons don't pass straight through the membrane because it is made of lipids. The channel is lined with polar chemical groups, making proton passage energetically favorable (Fig. 5.13). An amazing thing about this protein channel is that the energy change of proton translocation is coupled to an energetically unfavorable process – ATP synthesis. This is not a trivial chemical coupling; remember, the free energy change on hydrolyzing ATP to ADP is about 10 kcal mol^{-1} at cellular concentrations. For all practical purposes, hydrolysis of ATP is *irreversible*! The point of this discussion is that the cell (in fact, the mitochondria here, but the principles are the same for entire cells) must do work to generate the proton gradient. But there is a sort of purpose to the work. No wonder we need to eat from time to time! The cell is a sort of machine, and a very efficient one with regard to use of energy resources!

I. Enzyme–substrate interaction

In Chapter 2 we touched on the biological function of ribonuclease A (RNase A), a digestive enzyme that hydrolyzes RNA to its component nucleotides. We said that an inhibitor of the enzyme, 2′-cyclic monophosphate, can be used to study the enthalpy of nucleotide binding to RNase A. One aim of Chapter 5 is to illustrate the general utility of Eqn. (4.2). In the present section we focus on how Eqn. (4.2) applies to studies of the energetics of binding of small compounds to protein or DNA, taking RNase A as our example.

Figure 5.14 shows an outline of the reaction catalyzed by RNase A. The scheme is based on the isolation of 2′,3′-cyclic nucleotides from RNase A digests of RNA. There are four types of 2′,3′-cyclic

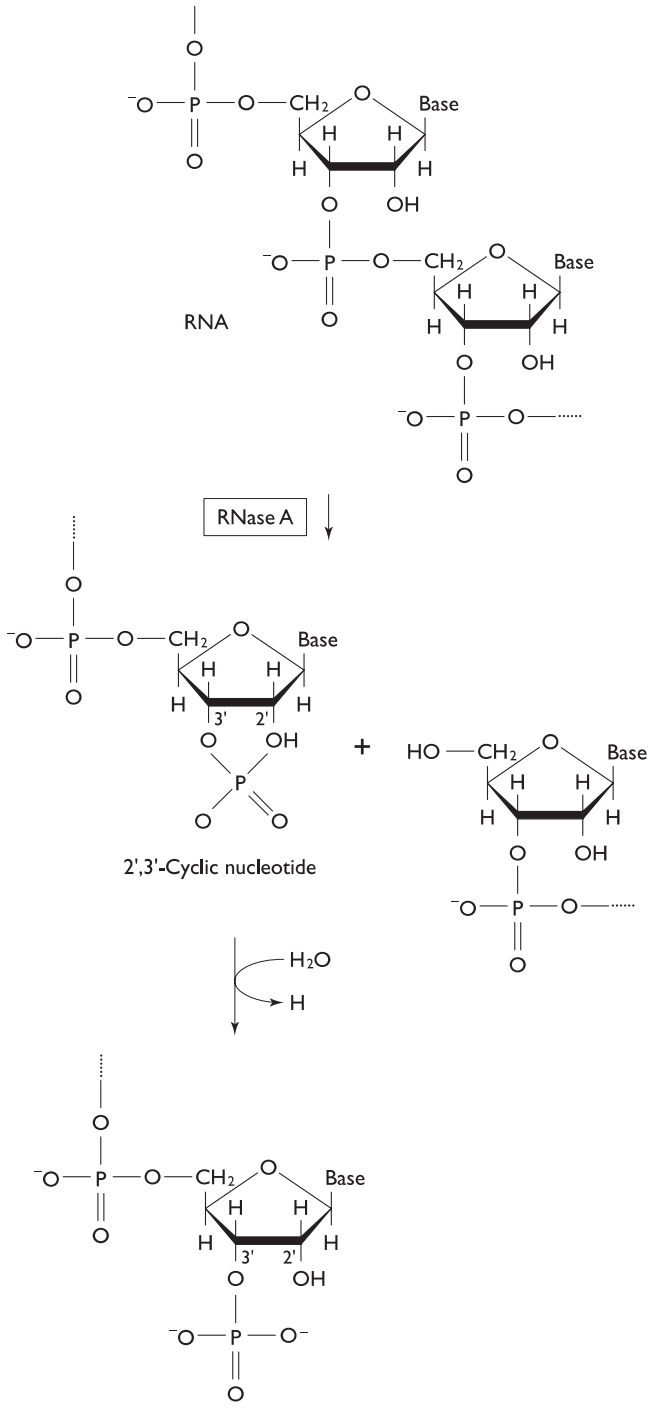


Fig. 5.14 Mechanism of RNase A activity. Bovine pancreatic ribonuclease A is an example of enzyme-mediated acid–base catalysis. The enzyme hydrolyzes RNA to its component nucleotides. The reaction scheme is based on the experimental finding that 2′,3′-cyclic nucleotides are present in RNase digests of RNA. RNase is inhibited by 2′-CMP. This binding interaction has been studied in considerable depth. See Chapter 8 for further information.

nucleotides. RNase A hydrolysis of one of them, cytidine 2′,3′-cyclic phosphate, has been studied extensively. The reaction is

$$\text{cytidine } 2',3'\text{-cyclic phosphate(aq)} + H_2O(l) \rightarrow \text{cytidine } 3'\text{-cyclic phosphate(aq)} \quad (5.28)$$

How might one determine ΔG° for this reaction?

From Eqn. (4.32),

$$\Delta G = \Delta H - T\Delta S. \quad (5.29)$$

If the products and reactants are in the standard state, the thermodynamic relationship is

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ. \quad (5.30)$$

We'll need to know ΔH° and ΔS° to calculate the energy. The enthalpy change of the reaction, which can be estimated by calorimetry, is -2.8 kcal mol^{-1}. But what is ΔS°? One approach would be to make use of the fact that S is a state function and combine measurements that, when summed, give ΔS° for Eqn. (5.28). The reaction scheme might look like this:

$$\text{cytidine } 2',3'\text{-cyclic phosphate(aq)} + H_2O(l) \rightarrow \text{cytidine } 2',3'\text{-cyclic phosphate(s)} + H_2O(l) \quad (5.31)$$

$$\text{cytidine } 2',3'\text{-cyclic phosphate(s)} + H_2O(l) \rightarrow \text{cytidine } 3'\text{-cyclic phosphate(s)} \quad (5.32)$$

$$\text{cytidine } 3'\text{- cyclic phosphate(s)} \rightarrow \text{cytidine } 3'\text{-cyclic phosphate(aq)}. \quad (5.33)$$

Equation (5.31) represents the dissolution of cytidine 2′,3′-cyclic phosphate, Eqn. 5.32 the conversion of cytidine 2′,3′-cyclic phosphate to cytidine 3′-cyclic phosphate in the solid state, and Eqn. (5.33) the dissolution of cytidine 3′-cyclic phosphate. The sum of these reactions is Eqn. (5.28), the conversion of cytidine 2′,3′-cyclic phosphate to cytidine 3′-cyclic phosphate in aqueous solution. If the entropy changes of these several reactions can be measured, ΔS° can be calculated for Eqn. (5.28). And combining ΔS° for Eqn. (5.28) with ΔH° for the overall reaction will give ΔG° for the overall reaction.

The entropy changes for Eqns. (5.31)–(5.33) have in fact been determined experimentally at 25°C. The values are: +8.22 cal mol^{-1} K^{-1}, −9.9 cal mol^{-1} K^{-1}, and +8.28 cal mol^{-1} K^{-1}, respectively. The overall ΔS° for these reactions is just the sum of the individual contributions, 6.6 cal mol^{-1} K^{-1}. Combining this entropy change with the calorimetric enthalpy change gives -2800 cal mol^{-1} $- 298\,\text{K} \times 6.6$ cal mol^{-1} K^{-1} $= -4800$ cal mol^{-1}. That ΔG° is negative suggests that cytidine 2′,3′-cyclic phosphate will hydrolyze *spontaneously* in aqueous solution, and this is confirmed by experiment. One could test the role that RNase A might play in this process by studying the effect of the wild-type enzyme and point mutants on the rate of reaction. See Chapter 8.

J. Molecular pharmacology

This is an important topic. The equations presented here are more general than the section title may suggest, as they can be applied not only to the interactions of drugs with membrane-bound receptor proteins but also to proteins that bind DNA, small molecules or ions. Binding interactions play a role in regulating enzyme activity and biosynthetic pathways, oxygen transport and regulation of blood pH, and many (!) other physiological processes. But for now, let's think of binding in the context of a single ligand–receptor interaction in pharmacology. This will help to motivate the mathematical development. A more thorough treatment of binding will be given in Chapter 7.

Equation (4.32) can be used to describe a chemical reaction in terms of reactants and products. But it could just as well represent the free energy difference between the "bound" and "free" states of a ligand, a small molecule or an ion. Under appropriate conditions, a ligand will interact with a macromolecule at a binding site. In some cases binding is highly specific; in other cases, not. In either case, ΔG° represents the driving force for binding under standard state conditions.

Here, we'll represent the binding reaction as

$$\mathrm{R} + \mathrm{L} \Leftrightarrow \mathrm{R} \bullet \mathrm{L}, \tag{5.34}$$

where R is the receptor, L signifies *free* ligand molecules, and R • L is the receptor–ligand complex. It is assumed that there is only one binding site for L per receptor molecule. The *association* constant is *defined* as

$$K_a = [\mathrm{R} \bullet \mathrm{L}]/([\mathrm{R}][\mathrm{L}]) \tag{5.35}$$

and the *dissociation* constant is

$$\mathrm{K_d} = \mathrm{K_a^{-1}} = [\mathrm{R}][\mathrm{L}]/[\mathrm{R}\bullet\mathrm{L}] = ([\mathrm{R}]_\mathrm{T} - [\mathrm{R}\bullet\mathrm{L}])[\mathrm{L}]/[\mathrm{R}\bullet\mathrm{L}], \tag{5.36}$$

where $[\mathrm{R}]_\mathrm{T} = [\mathrm{R} \bullet \mathrm{L}] + [\mathrm{R}]$ is the total receptor concentration. The fractional occupancy of ligand-binding sites, F_b, is

$$F_b = [\mathrm{R} \bullet \mathrm{L}]/[\mathrm{R}]_\mathrm{T} = [\mathrm{L}]/(\mathrm{K_d} + [\mathrm{L}]). \tag{5.37}$$

A plot of F_b against [L] is shown in Fig. 5.15A. The shape of the curve is a *rectangular hyperbola*. Equation (5.37) indicates that K_d corresponds to the concentration of L at which the occupancy of binding sites is half-maximal. Many physiological dissociation constants are on the order of μM−nM. A nM binding constant is considered "tight binding." When Eqn. (5.37) is plotted as percentage response against dose (for example, mg of drug per kg of body weight), it is called a *dose–response curve*. The dose is often plotted on a logarithmic scale, giving the curve a sigmoidal appearance (Fig. 5.15B), but the underlying relationship between dose and response is the same in both cases.

We can rearrange Eqn. (5.36) to obtain

$$[\mathrm{R} \bullet \mathrm{L}]/[\mathrm{L}] = ([\mathrm{R}]_\mathrm{T} - [\mathrm{R} \bullet \mathrm{L}])/K_d. \tag{5.38}$$

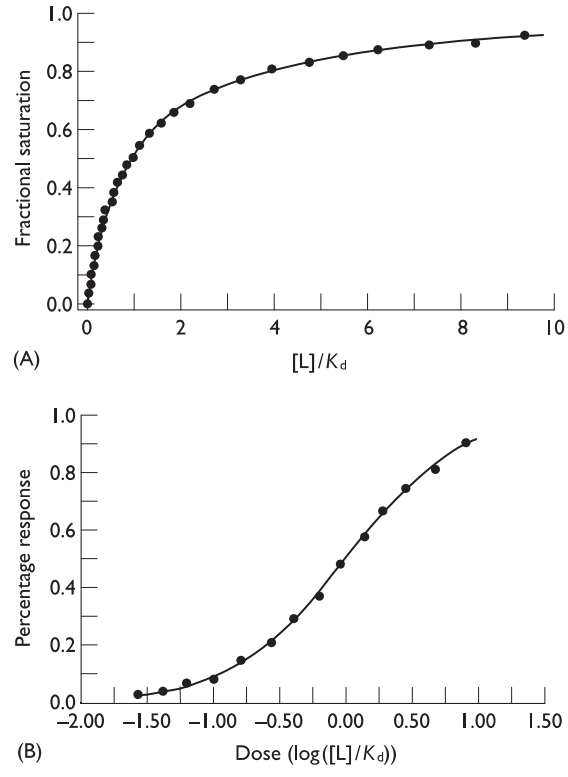


Fig. 5.15 Binding. In panel (A) the circles are experimental data points, the solid line is a theoretical description of binding. There is one ligand-binding site per macromolecule. In such cases, the mathematical relationship between the bound and free ligand concentrations is a rectangular hyperbola. Note that although half-saturation occurs when $[L] = K_d = 1/K_a$, $[L] = 9K_d$ gives only 0.9 saturation and $[L] = 99K_d$ but 0.99 saturation. In other words, most of the information about binding is in the free ligand concentration range $0–2K_d$. Experiments should be designed accordingly. Panel (B) shows a dose–response curve.

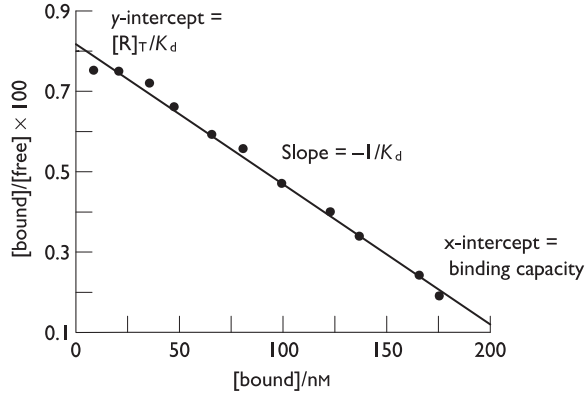


Fig. 5.16 Scatchard plot. The concentration of bound ligand divided by the concentration of free ligand is plotted against the concentration of bound ligand (nM). When binding data are presented in this way, the slope measures the negative inverse of the dissociation constant ($-1/K_d = -K_a$). The vertical axis-intercept is $[R]_T/K_d$, and the horizontal axis-intercept is the binding capacity (the concentration of binding sites).

In this representation [R•L]/[L], the concentration of bound ligand divided by the concentration of free ligand, is a *linear* function of [R•L]. The slope of the curve is $-1/K_d$ (see Fig. 5.16). The axis intercepts themselves represent interesting quantities: the intercept on the vertical axis is $[R_T]/K_d$, and the intercept on the horizontal axis is the "*binding capacity*," the "concentration" of ligand binding sites. A plot of bound/free ligand *versus* bound ligand is called a *Scatchard plot*, after the American physical chemist George Scatchard (1892–1973). Radioactive methods are one way that biological scientists measure the amounts of bound and free ligand.

Experiments can be done to determine the dissociation constant of other ligands that can compete for the same binding site as L. For instance, suppose you wish to test the effectiveness of a number of candidate drugs to compete directly with a physiological ligand L for a specific binding site on R. Let the candidate competitors be I_1, I_2, $I_3 \ldots$ According to this model,

$$K_{d,I_i} = [R][I_i]/[R \bullet I_i] \tag{5.39}$$

for a general inhibitor compound, I_i. It can be shown that in the presence of an inhibitor, the receptor–ligand complex, [R•L], is

$$[R \bullet L] = \frac{[R]_T[L]}{K_{d,L}\left(1 + \frac{[I_i]}{K_{d,I_i}}\right) + [L]}. \tag{5.40}$$

The relative affinity of a ligand in the presence of an inhibitor can be found by dividing Eqn. (5.40) by Eqn. (5.38). This gives

$$\frac{[R \bullet L]_{I_i}}{[R \bullet L]_0} = \frac{K_{d,L} + [L]}{K_{d,L}\left(1 + \frac{[I_i]}{K_{d,I_i}}\right) + [L]}. \tag{5.41}$$

Equation (5.41) is zero for all concentrations of I_i when there is no inhibition (compound I_i has no effect), and it is 1 at 100% inhibition. The concentration of competitor I_i that gives 50% inhibition is designated $[I_{i,50}]$. At this concentration,

$$K_{Ii} = \frac{[I_{i,50}]}{1 + \frac{[L]}{K_{d,L}}}. \tag{5.42}$$

Figure 5.17 shows the percentage inhibition for a number of different inhibitors. Note that the shape of the curves resembles that in Fig. 5.15a.

The above mathematical equations apply not only to natural ligands like the "fight-or-flight" hormone epinephrine and to competitive inhibitors like the "β-blocker" propranolol, which vies with epinephrine for binding sites on β-adrenergic receptors,[9] but also to noxious chemical substances like botulinum toxin. Moreover, the equations also apply to ligands of DNA, for example, repressor proteins that physically block the enzymatic transcription of mRNA by binding to an operator site, and to protein–protein interactions related to signal transduction. An example of ligand binding in a signal transduction cascade is the direct association of the SH2 domain[10] of the protein Grb2 to a specific phosphorylated tyrosine residue on a growth factor receptor (Fig. 5.18).

Phosphotyrosine-mediated binding is of particular interest in biological science for several reasons. One, it involves phosphorylated tyrosine, and the phosphoryl group is acquired via catalysis by a kinase from ATP, the energy molecule. Phosphorylation and

[9] The effects of β-blockers were first described by Sir James W. Black (1924–), a Scot. Sir James was awarded the Nobel Prize in Medicine or Physiology in 1988.

[10] SH2, Src homology 2.

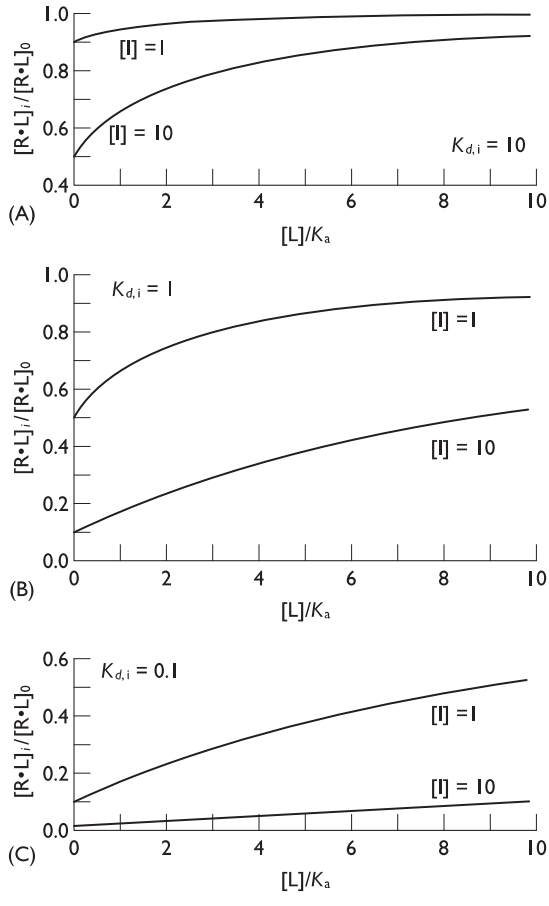


Fig. 5.17 Effect of inhibitor on ligand binding. When the concentration of inhibitor i is low and the inhibitor dissociation constant is high, as in panel (A), $[R\bullet L]_i/[R\bullet L]_0$ is nearly 1 even at low concentrations of ligand. Competition between ligand and inhibitor is more evident when the inhibitor concentration is increased by a factor of 10. In panel (B), the dissociation constant of the inhibitor is 10 times smaller than in panel (A). Note the marked impact this has on $[R\bullet L]_i/[R\bullet L]_0$. The effect of decreasing the dissociation constant by yet another factor of 10 is shown in panel (C). This is the sort of study a pharmaceutical company might do to characterize the properties of inhibitors that could be used as drugs. ITC can be used to screen different compounds. Analysis of such compounds will include not only *in vitro* binding experiments (high-affinity specific binding) but also assessment of side effects (low-affinity non-specific or high-affinity unwanted binding).

dephosphorylation of tyrosine is a type of dynamic molecular switch that regulates cellular activity by controlling which proteins can interact with each other. Phosphorylation also places severe restrictions on the relative orientation of interacting proteins. An important class of phosphotyrosine-mediated interactions is typified by phospholipase $C_{\gamma 1}$ (PLC), an enzyme that interacts with phosphorylated growth factor receptors by means of its two SH2 domains and is involved in lipid metabolism. Binding of Grb2 to a receptor resembles that of PLC, but Grb2 has no catalytic activity; Grb2 is a sort of "adaptor" protein. Two, there are several different types of phosphotyrosine recognition module, and they are found in many different proteins. Two of the best-known phosphotyrosine binding modules are the SH2 domain and the PTB (phosphotyrosine binding) domain. In some cases, both types are found in the same protein, for example, Shc[11] and tensin. Three, the breadth of the range of possible interactions of a given type of module is greatly increased by subtle differences in structure. As a general rule, the amino acid side

[11] Shc, Src homolog, collagen homolog.

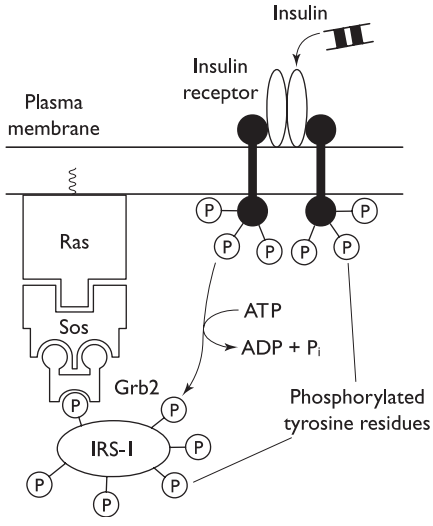


Fig. 5.18 Protein–protein interactions and phosphoryl transfer in signal transduction. The extracellular concentration of the hormone insulin, a peptide signal, is communicated across the plasma membrane by means of dimeric insulin-specific transmembrane receptor molecules. The binding of insulin to its receptor results in receptor autophosphorylation, the catalysis by one receptor molecule of the transfer of a phosphoryl group from ATP to a tyrosine side chain of the other receptor molecule. Phosphorylation of tyrosine acts as a molecular switch in the recruitment of proteins that recognize specific phosphorylated tyrosine residues. One consequence of the chain of events elicited by insulin binding is the phosphorylation of insulin-receptor substrate-1 (IRS-1). Again, ATP is involved. Once phosphorylated, IRS-1 can interact directly with the proteins Grb2, Sos, and Ras. The last of these plays a very important role in cellular signal transduction. The key point here is that several of the protein–protein interactions involved in this and many other signaling cascades are mediated by phosphorylated tyrosine, and phosphorylation of tyrosine depends on ATP. Signal transduction is a form of biological communication and information processing. We shall return to this point in Chapter 9. The figure is based on Fig. 20–48 of Lodish *et al.* (1995).

chains that interact directly with the ligand are conserved from module to module, but side chains nearby are specific to the protein in which the module occurs. Such peculiarities underlie the specificity of the interactions of an otherwise general protein structure. The general and specific interactions combine to give the overall binding free energy. We can see here a close connection between the existence and transmission of biological information and energy.

Finally, it should be mentioned that binding is a far more complicated phenomenon that we have made it seem. For instance, if a macromolecule can interact with more than one type of ligand at different binding sites, there is the possibility that one kind of metabolite can "sense" the concentration of another, despite the absence of a direct interaction between the metabolites. This aspect of the function of biological macromolecules, known as *allostery*, will be developed along with other aspects of binding in Chapter 7.

K. Hemoglobin

Most known organisms require oxygen for life; the only known exceptions are some types of bacteria and archaea. Reduction of molecular oxygen to water plays a key role in the generation of ATP. In every cell in an aerobic organism, oxygen is used as fuel in the combustion of glucose and production of ATP and carbon dioxide. Oxygen and glucose must be delivered to every cell in the body; carbon dioxide, a waste product, must be removed from every cell. Vertebrates carry out this food and gas transport by means of blood or blood-like fluid that moves through a closed system of tubes called the vasculature (Chapter 1). The vasculature makes contact with the lungs, gills or skin on the one hand, and the peripheral tissues on the other hand.

Molecular oxygen is transported throughout the blood by an allosteric transport protein called hemoglobin. In view of this, hemoglobin has been called the "molecular lung." Vertebrate hemoglobin is a tetrameric protein, $\alpha_2\beta_2$; it can be thought of as a dimer of $\alpha\beta$ heterodimers. In invertebrates, hemoglobins range from one to 144 subunits! Each subunit consists of a polypeptide chain called globin and a protoheme IX, a planar complex of an iron and ion protoporphyrin IX (Fig. 5.1). Iron plays a role in the coordination of bound dioxygen. The ability of hemoglobin to bind oxygen depends not only on the structure of the protein and oxygen but also on the partial pressure of oxygen.[12] In hemoglobin, the extent of oxygen loading into binding sites influences the affinity of the other binding sites to bind oxygen. The specific character of the amino acid chains near the protein–heme interface is essential for oxygen binding, as shown by amino acid replacement studies. Mutations in the region of the oxygen binding site can alter affinity for oxygen by over 30 000-fold! In the present section we introduce a number of aspects of hemoglobin thermodynamics. The treatment will be brief. A more in-depth look at oxygen binding is reserved for Chapter 7.

Now, if tetrameric hemoglobin is thermodynamically stable under normal physiological conditions, the tetramer must represent a minimum of free energy; the tetrameric state must be a lower free energy state than the other possible combinations of subunits, for example $\alpha\beta$ dimers. (There are other possibilities, for example, a kinetically trapped tetrameric state, but let's ignore them for now.) A number of natural variants of human hemoglobin are known. One of these is the famous sickle-cell variant. As shown in Table 5.3, the free energy difference between the tetrameric and dimeric states of

[12] The partial pressure of a gas is just the contribution that it makes to the overall gas pressure. By Dalton's Law, which is named after the John Dalton of the atomic hypothesis, the total pressure is just the sum of the partial pressures of the gases present. For example, if the air pressure is 1 atm, the partial pressures of nitrogen, oxygen, and carbon dioxide sum to 1 atm.

Table 5.3. *Thermodynamics of hemoglobin dissociation*

Hemoglobin	Substitution in mutant	ΔG° (kcal mol^{-1} of hemoglobin)
normal	—	8.2
"Kansas"	102β, Asn → Thr	5.1
"Georgia"	95α, Pro → Leu	3.6

The data are from Chapter 4 of Klotz.

hemoglobin can depend substantially on the primary structure, the sequence of amino acid residues. The free energy difference between normal hemoglobin and hemoglobin Kansas is "only" 3.1 kcal mol^{-1}, but the equilibrium constant differs by nearly 200-fold at 25 °C! Considerably less work must be done to dissociate tetrameric hemoglobin Kansas than wild-type hemoglobin into αβ dimers under the same conditions. For comparison, it is well known that inhalation of too much carbon monoxide will normally be fatal, even if exposure lasts just a few minutes. The spectrum of pathological effects of CO poisoning includes damage to the peripheral nervous system, brain damage, cell death in the heart, cell death in other muscles, and pathological accumulation of fluid in the lungs. All this results from the binding of CO to hemoglobin with an affinity constant "only" about 240 times greater than that of oxygen! Hemoglobin Georgia is even less stable than the Kansas variant, so its behavior as an oxygen carrier is very noticeably altered relative to the normal protein.

We have assumed that the tetrameric state of hemoglobin represents a lower free energy state than the dimer. And oxygen associates with hemoglobin. Binding occurs because the bound state is more thermodynamically favorable (has a lower Gibbs free energy) than the unbound state. Let's consider the oxygenation of hemoglobin in solution. For the moment, we'll take a rather simplistic view and assume that hemoglobin has just one binding site, or, more accurately, that each subunit binds O_2 with the same affinity. The reaction can be written as

$$\mathrm{Hb(aq)} + \mathrm{O_2(g)} \;\Leftrightarrow\; \mathrm{HbO_2(aq)}. \tag{5.43}$$

From experiments it is known that $K = 85.5$ atm^{-1} for the reaction as written. At 19 °C, $\Delta G = -2580$ cal mol^{-1}. What is the free energy change when the partial pressure of oxygen is 0.2 atm and oxygen is dissolved in solution with an activity of 1 (as in the standard state)?

The free energy difference between $p = 1$ atm and $p = 0.2$ atm is found using Eqn. (4.5):

$$\begin{aligned}\Delta G &= G(\mathrm{O_2}, 0.2\ \mathrm{atm}) - G^\circ(\mathrm{O_2}, 0.2\ \mathrm{atm}) - (G(\mathrm{O_2}, 1\ \mathrm{atm}) - G^\circ(\mathrm{O_2}, 1\ \mathrm{atm})) \\ &= RT\ln(\mathrm{O_2}, 0.2\ \mathrm{atm}) - RT\ln(\mathrm{O_2}, 1\ \mathrm{atm}) \\ &= RT\ln(0.2/1) \\ &= -930\ \mathrm{cal\ mol^{-1}}.\end{aligned} \tag{5.44}$$

That ΔG is negative is just what we should expect, since a substance will always move spontaneously from a region of higher concentration to a region of lower concentration.

At equilibrium, $\Delta G = 0$ between the oxygen vapor and the dissolved oxygen. To calculate the free energy difference between the concentration of dissolved oxygen in the standard state ($a = 1$) and the concentration at saturation, which is substantially lower, we need to account for the solubility of diatomic oxygen in water. This is 0.000 23 molal (kg l^{-1}) at 19°C. Thus, $\Delta G = RT\ln(1/0.000\ 23) = 4\,860$ cal mol^{-1}. Because the Gibbs free energy is a state function, the *net* free energy change on going from oxygen gas at 1 atm to dissolved oxygen at unit activity is just the sum of the individual contributions, or -930 cal $mol^{-1} + 0 + 4860$ cal $mol^{-1} = 3930$ cal mol^{-1}. The free energy change of the reverse reaction is, of course, -3930 cal mol^{-1}.

Now, the two reactions we're interested in are:

$$\mathrm{Hb(aq) + O_2(g) \Leftrightarrow HbO_2(aq)} \tag{5.45}$$

$$\mathrm{O_2(aq) \Leftrightarrow O_2(g)}, \tag{5.46}$$

which, when summed, give

$$\mathrm{Hb(aq) + O_2(aq) \Leftrightarrow HbO_2(aq)}. \tag{5.47}$$

ΔG for the overall reaction is -2580 cal mol^{-1} -3930 cal $mol^{-1} = -6510$ cal mol^{-1}. We can see that the driving force for oxygen association with hemoglobin is greater when the oxygen is solvated than when it is not solvated.

L. Enzyme-linked immunosorbent assay (ELISA)

Antibodies are protective proteins produced by the immune system in response to the presence of a foreign substance, called an antigen. Antibody recognition of an antigen is mainly a matter of shape complementarity and charge interactions in the antigen-binding site. The shape of the binding site must be a close match to a part of the surface of an antigen for specific binding to occur. Binding can be very tight indeed, with $K_{eq} \sim 10^9$ M^{-1} or greater, and highly specific. The following discussion, though it centers on ELISA, applies to a broad range of immuno-techniques, including for instance western blotting.

ELISA is a useful method for detecting small amounts of specific proteins and other biological substances ("antigens") in laboratory and clinical applications. For instance, it is used to detect the placental hormone chorionic gonadotropin in a commonly available pregnancy test. The assay is so useful because it is very general, antibody binding is very specific (K_{eq} is large), and the sensitivity of the binding "signal" can be increased in various ways, for instance, a covalent link between the antibodies used for detection and an enzyme (often horseradish peroxidase).

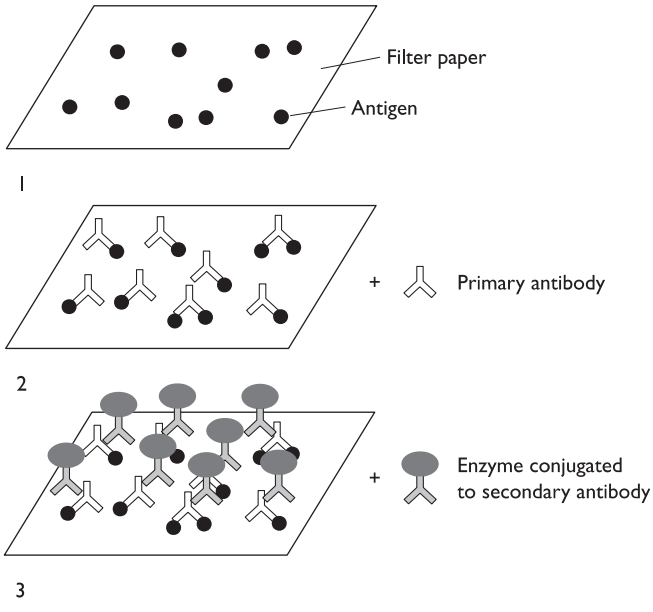


Fig. 5.19 ELISA. This very useful laboratory technique consists of three basic steps. First, the protein antigen is adhered to a solid support, often a nitrocellulose filter. This partially denatures the antigen. Next, (primary) antibodies are allowed to bind to the antigen. Finally, (secondary) antibodies that recognize the first antibody bind are allowed to bind the primary antibodies. Attached to each secondary antibody is an enzyme that is used to catalyze a reaction that facilitates detection. One assumes that detection of the enzyme linked to the secondary antibody implies detection of the antigen. This is often the case because antibody recognition of antigen is highly specific. But because nitrocellulose is very sticky, the milk protein casein is often used to bind sites not occupied by antigen in order to reduce the background signal arising from the non-specific adherence of primary antibodies. After step two, non-specifically adhered primary antibodies are rinsed off with buffer. Specifically bound antibodies are not lost in the rinsing procedure because the rate of dissociation of the antibody from the antigen is very low (Chapter 8).

The ELISA protocol involves adsorbing an "antigen" of interest to an "inert" solid support (usually a type of filter paper, Fig. 5.19). The binding of antigen to the solid support can be very strong indeed, though binding is usually relatively non-specific. The binding process usually results in partial denaturation of a protein antigen. After adsorption, the sample is screened with an antibody preparation (usually a rabbit antiserum) and "rinsed" to remove non-specifically bound antibody ($K_{eq} < 10^4$). The resulting protein–antibody complex on the solid support is reacted with an antibody-specific antibody to which the enzyme used for the detection assay is attached. This second antibody is often from goat. Why does the rinse step does not ruin the experiment?

As we shall see in Chapter 8, $K_{eq} = k_f/k_r$, where k represents reaction rate and "f" and "r" stand for "forward" and "reverse," respectively. When binding is specific, $k_f >> k_r$; the "on rate" (binding) is much greater than the "off rate" (release). So, even during rinsing tightly bound antibodies stay put, despite the requirement for mass action to release antigen to solution, where the antibody concentration is low. To put things into perspective, for $K_{eq} \sim 10^9$, the free energy change on binding is about -50 kJ mol^{-1}! We can get a sense of how big this free energy change is by considering the energy required to raise a 100 g apple a distance of 1 m. It is easy to show that this energy is about 1 J (Chapter 2). This tells us that 50 kJ could lift a 100 g mass about 50 km, over 5 times the height of Mt Everest! So $K_{eq} \sim 10^9$ M^{-1} is tight binding. This has been a very brief and consequently superficial treatment of ELISA. But it has been enough to illustrate yet another way the basic ideas of thermodynamics are useful for understanding biological science.

We'll come back to the important subjects of binding and chemical kinetics in Chapter 7 and Chapter 8, respectively.

M. DNA

Throughout this book we have put somewhat more emphasis on proteins than on DNA. In part this is a reflection of the expertise and interests of the author and not a conscious bias against nucleic acids or the people who study them! Besides, we shall see just how important DNA is to the entire story when we reach Chapter 9. Nevertheless, to redress lingering impressions of imbalance, this section looks at the thermostability of DNA and the next one discusses energetic aspects of the polymerase chain reaction.

The structure of the DNA double helix is illustrated schematically in Fig. 5.20. The types of interaction that stabilize the structure are hydrogen bonds and "stacking interactions." Three hydrogen bonds are formed between bases cytosine and guanine, two between adenine and thymine. These are the co-called Watson–Crick base pairs. The adenine of DNA (and RNA) is exactly the same as the adenine of ATP, cAMP, NADH and $FADH_2$. As shown in Fig. 5.14, however, only one of the phosphate groups of ATP actually becomes part of the polynucleotide. You might guess, then, that the stability of double-stranded DNA relative to single-stranded DNA will depend on the proportion of C–G pairs, because this will influence the average number of hydrogen bonds per base pair, and in fact that is correct (Fig. 5.21, Table 5.4). Analysis of structures of nucleic acids has revealed that the bases form extended stacks, interacting with each other by van der Waals forces. Both hydrogen bonds and van der Waals interactions contribute to the overall stability of the double helix.

The equilibrium between double- and single-stranded DNA can be symbolized as

$$D \Leftrightarrow S. \tag{5.48}$$

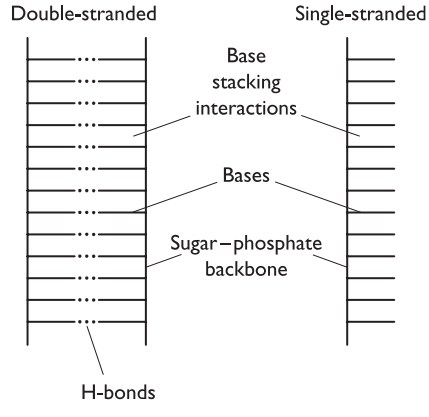


Fig. 5.20 Double-stranded and single-stranded DNA. DNA is composed of bases attached to a sugar–phosphate backbone. (See Fig. 5.14 for a higher resolution view of polynucleic acid.) There are two major types of interaction that stabilize double-stranded DNA: intermolecular hydrogen bonds (polar) and intramolecular base stacking interactions (non-polar). The number of hydrogen bonds depends on the bases involved: three are formed between cytosine (C) and guanine (G), and two between adenine (A) and thymine (T). Intermolecular hydrogen bonds are not present in single-stranded DNA. Based on Fig. 3.16 of van Holde (1985).

Table 5.4. *Association constants for base pair formation*

Base pair	$K(M^{-1})$
Self-association	
A•A	3.1
U•U	6.1
C•C	28
G•G	10^3–10^4
Watson–Crick base pairs	
A•U	100
G•C	10^4–10^5

The measurements were made in deuterochloroform at 25 °C. The data are from Kyoguko *et al.* (1969). Similar values have been obtained for T in place of U. Non-Watson–Crick base pairs are relatively unstable. The entropic component of K is roughly the same for each

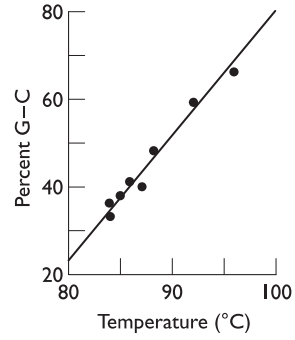


Fig. 5.21 Influence of G–C content on DNA melting temperature. As the percentage of G–C pairs increases, the number of intermolecular hydrogen bonds per base pair increases. The stabilizing effect on double-stranded DNA is reflected in the relationship between G–C content and melting temperature.

The equilibrium constant for this reaction is

$$K = [S]/[D]. \tag{5.49}$$

This equilibrium constant is the product of the Ks for the individual base pairings, as each base pair contributes to the overall stability of the double-stranded molecule.

Thermal denaturation of double-stranded DNA has been studied extensively. As mentioned above, C–G composition is an important determinant of stability of duplex DNA and therefore conditions under which $K = 1$. One means of promoting the dissociation of double-stranded DNA is to add heat. Just as with proteins, heat absorbed by DNA increases its thermal energy, fluctuations of structure become larger, and the disordered state becomes more probable than the ordered one. Measurement of the temperature at which double-stranded DNA is 50% "melted," the melting temperature, is one way of comparing the genetic material of one genome to another (Fig. 5.22). Research in this area has been used to work out empirical rules for the melting temperature of DNA as a function of C–G content, total number of base pairs, and concentration of ions, principally Mg^{2+}. Magnesium ions neutralize the electrostatic repulsion between the negatively charged phosphate groups in the sugar–phosphate backbone by decreasing the range and strength of the repulsive Coulombic interactions[13] between the phosphate groups on opposite strands of the double helix. Decreases in the concentration of such counterions increase the repulsion between strands and reduce the melting temperature of double-stranded DNA.

Figure 5.23 shows percentage of double-helix as a function of temperature for the forward and reverse reactions in Eqn. (5.48).

[13] Coulomb's law is an empirically derived mathematical description of the interaction between charged particles. It is named after the French physicist and military engineer Charles Augustin de Coulomb (1736–1806). The effect of Mg^{2+} counterions is explained by the Debye–Hückel theory of strong electrolytes.

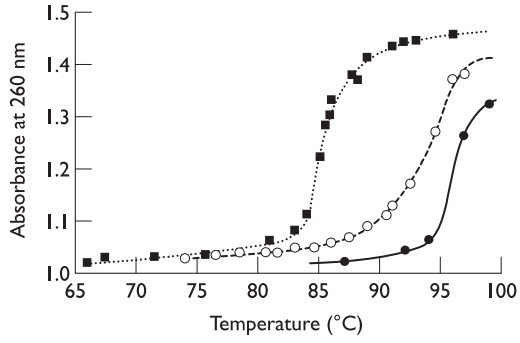


Fig. 5.22 DNA melting curves. The melting temperature varies not only with G–C content but also with size. In other words, a 100 base pair-long double-stranded DNA molecule will have a higher melting temperature than a 50 base pair-long double-stranded DNA for a given percentage and distribution of G–C pairs. Differences in G–C content and distribution and molecular size lead to differences in melting temperature.

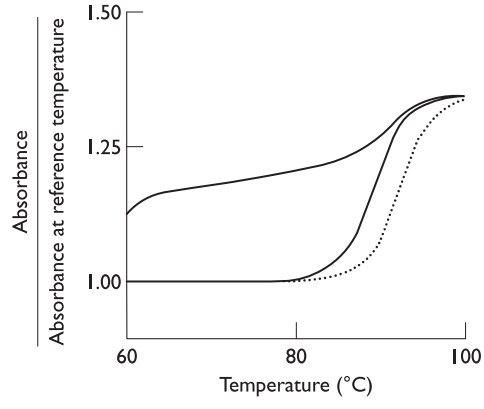


Fig. 5.23 Melting and cooling profile for double-stranded DNA. Solid line. The melting of double-stranded DNA is cooperative. Relatively few base pairs are broken below the melting temperature. Once melting has begun, however, relatively small increases in temperature result in the rupture of a relatively large number of hydrogen bonds. The melting profile differs greatly from the cooling profile. Unless cooling is carried out very slowly, the system will not be in a near-equilibrium state at every temperature value on the reaction pathway. Rapid cooling of melted DNA will not yield perfectly formed double-stranded DNA. Broken line. Melting temperature is influenced not only by G–C content and distribution and molecular size but also by ion concentration, particularly divalent cations. These ions interact favorably with the phosphate groups of the DNA backbone. The consequent reduction of electrostatic repulsion results in increased stability of the DNA duplex. Based on Fig. 3.15 of van Holde (1985) and Fig. 5.15 of Bergethon (1998).

The evident hysteresis (*Greek*, a coming late) in the reverse process arises from a difference in the rates of hydrogen bond breaking and specific annealing of the complementary strands, and the sample's not being at equilibrium throughout the experiment. The dissociation of strands is a much simpler reaction than the formation of perfectly matched double-stranded DNA. And as we have said, reversibility of a process depends on the system being taken through a series of equilibrium or near-equilibrium states. During the reverse reaction, if the system is not given sufficient time to come to equilibrium, some mismatching of bases is likely to occur, preventing or strongly inhibiting the return to the initial conditions.

Thus far we have described DNA in rather general terms. There are different types of DNA, however, not only differences in G–C content, and the different types have different thermodynamic properties. Genomic DNA of higher eukaryotes, for example, is linear: there is a distinct 3′-end and a distinct 5′-end. In plasmid DNA, by contrast, which is of great utility as a vector for carrying "foreign" genes into *E. coli* for production of "recombinant" proteins, there is no distinct 3′-end or 5′-end; this DNA is *circular*. Such DNA can exhibit a variety of conformations ranging from no

supercoiling, or no twisting, to tight supercoiling. This topological characteristic of circular DNA suggests that energetics of plasmid DNA melting will differ from that of linear DNA, even if the basic principles we have discussed thus far apply to both types.

A double-helical DNA molecule with covalently attached ends, as in a plasmid, will have a certain number of "coils." Such coils are analogous to the ones you can introduce in a belt before the buckle is fastened. It is easy to show that the number of coils cannot be changed after fastening the buckle without cutting the belt. In the same way, coils in circular DNA cannot be undone without cutting the polynucleotide strand. From a mathematical point of view, supercoiling can be expressed in terms of three variables as

$$L = T + W. \tag{5.50}$$

L, the *linking number*, is the integral number of times that one DNA strand winds around the other; it is the number of coils in our belt analogy. The *twist*, T, is the number of complete revolutions that one polynucleotide strand makes about the duplex axis (usually the number of base pairs divided by 10.6, the approximate number of base pairs per turn of DNA). T can be positive or negative, depending on the direction of the helix, and it can vary from one part of a molecule to another. W, the *writhe*, is the number of turns that the duplex axis makes about the superhelix axis. Like T, W can be positive or negative.

If the duplex axis of DNA is constrained to lie in a single plane, $W = 0$; there is coiling but no supercoiling, $L = T$, and the twist must be an integral number. From Eqn. (5.50) it is clear that different combinations of W and T are possible for a circular DNA molecule with L, which is a property of the molecule that is constant in the absence of a break in a polynucleotide strand. At equilibrium, one expects a given circular DNA molecule to fluctuate between a variety of conformations, each of which must have linking number L.

As a specific example of DNA supercoiling, consider the circular DNA molecule of the SV40 virus. This molecule is about 5300 base pairs long and is therefore expected to have $L = T \approx 500$ in the absence of supercoiling. The prediction is based on the most energetically favorable number of bases per turn. But in fact, the DNA isolated from SV40 is supercoiled.[14] This probably arises from an untwisted region being present at the end of DNA replication. Such "underwinding" is energetically unfavorable, because the average number of bases per turn is lower than optimal. The conformation of the molecule changes until the lowest free energy state is reached, but regardless of the conformation adopted L is constant (the DNA backbone is not severed). It has been found experimentally that $|W| \approx 25$, so by Eqn. (5.50), $W \approx -25$. The sign of W tells us that the supercoils are negative supercoils, which form to compensate

[14] Under normal salt conditions. The predominant conformation depends on salt concentration and temperature.

for the effects of helix underwinding. Because T is mainly a property of chain length, $T \approx 500$, and $L \approx 475$. Supercoiling increases the elastic strain in circular DNA, just as it does in any other circularized object, for instance, a rubber band! (See Chapters 2 and 3.)

N. Polymerase chain reaction (PCR)

PCR is an extremely useful laboratory process in which double-stranded DNA is replicated rapidly. Under favorable circumstances, a very small amount of starting material can yield a large, readily analyzed product. The technique was developed in the mid 1980s by Kary R. Mullis (1944–) and colleagues at the Cetus Corporation. Mullis, an American, was awarded the Nobel Prize in Chemistry for this work in 1993.

The procedure works as follows. DNA is dissolved in aqueous solution containing a DNA polymerase from a thermophilic bacterium (e.g. *Bacillus stearothermophilus*), polymerase buffer, free nucleotides (dATP, dCTP, dGTP and dTTP, where the "d" means "deoxy"), and oligonucleotide "primers". The primers are short sequences of single-stranded DNA that are designed to bind to either end of the DNA segment of interest. One primer binds one end of one of the complementary DNA strands, and the other primer binds the other end of the other complementary strand (Fig. 5.24).

In a typical PCR experiment, the solution described above is cycled repeatedly through three different temperatures. The first one is usually 95 °C. Thermal energy is used to break the hydrogen bonds and base stacking interactions that stabilize double-stranded DNA. The solution is then rapidly cooled to about 55 °C, at which temperature the primers bind to the complementary sites on the separated strands of the template DNA. The separated strands do not reanneal in the region of the primer binding site because the

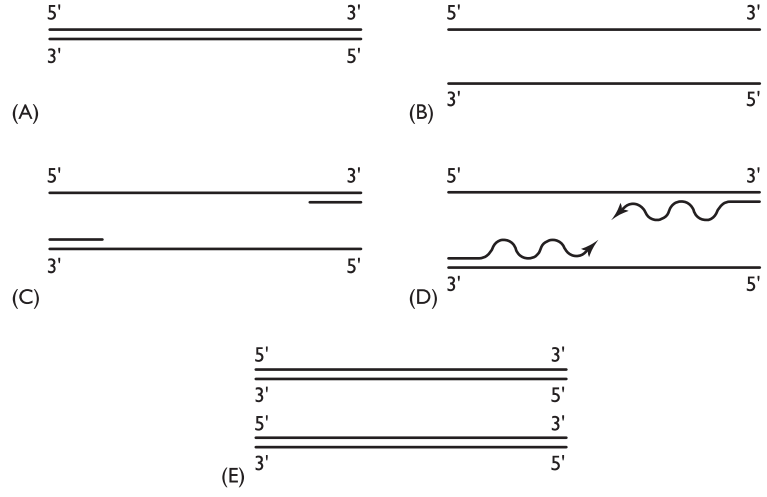


Fig. 5.24 Schematic diagram of PCR. When heated, a double-stranded template DNA (A) melts into two single-stranded molecules (B). If cooling is rapid, the template will not be able to reform. In the excess of complementary oligonucleotide primer, however, binding will occur at temperatures below the primer T_m (C). Increasing the temperature to the optimal value for polymerase activity results in extension of the primer (D). This completes the reaction cycle and yields two double-stranded DNA molecules from one (E). There is an approximately exponential increase in the amount of double-stranded DNA with each cycle.

primers, which are present in great excess, out-compete them. The third temperature of each cycle is usually about 72 °C. At this temperature DNA polymerase activity is high, and it catalyzes the synthesis of a new strand by joining free nucleotide bases to the 3′-end of the primers at a rate of several hundred bases per minute. Each time the thermal cycle is repeated, a strand that was formed with one primer is available to bind the complementary primer, the result being a new two-stranded molecule that is restricted solely to the desired segment of starting material; the region of DNA between the primers is selectively replicated. Further repetitions of the process can produce a billion identical copies of a small piece of DNA in 2–3 h. A well-designed PCR experiment yields the desired product with better than 99% purity.

A question of practical importance to the molecular biologist is: "How long should the oligonucleotide primer be?" There are two main considerations. One is cost: why spend more than is necessary? The other is that although specificity of an oligonucleotide increases with length, size is not necessarily an advantage. In order to answer these questions, let's think about how double-stranded DNA is held together.

Above we said that hydrogen bonds contribute to double-helix stability. How is that? Aren't the bases in single-stranded DNA able to form hydrogen bonds with water? Why should the hydrogen bonds in a double helix be any stronger? In the double helix, the inter-strand hydrogen bonds are not being made and broken constantly, as in single-stranded DNA interacting with water. The hydrogen bond donor or acceptor is, on the average, bonded more of the time in duplex DNA than in single-stranded DNA. Assuming that the enthalpy change of hydrogen bond formation is of roughly the same magnitude in both cases, we should therefore expect a difference in enthalpy between double-stranded DNA and its constituent strands when separated. If heat must be added to "melt" the double helix, then according to Eqn. (2.3) the single strands represent a higher enthalpy state than the double helix at a temperature favoring the double helix. This means that the longer the oligonucleotide primer, the larger ΔH_d, where the "d" stands for "denaturation of the double helix." Below T_m, base pair formation is energetically favorable with regard to enthalpy. What about entropy?

The double-stranded state has a much lower entropy than the single-stranded one with regard to DNA strands alone. Two particles sticking together is a more orderly situation than two particles floating freely in solution. Formation of a base pair *decreases* the entropy of a strand of DNA and is, therefore, energetically unfavorable. The unfavorability comes not only from requiring that two strands of DNA be in the same place in the sample volume, but also from the restrictions on the shape of both strands that are compatible with helix formation and on the orientation in space of individual bases.

As is often the case in science, thinking will get you only so far. At some stage it becomes necessary to do experiments to find out whether or not the world really is how you imagine it to be. There are two key experimental findings that will help us here. One is that G–C pairs contribute more to duplex-DNA stability than A–T pairs. This cannot be rationalized in terms of base stacking interactions alone, as the surface area of an A–T pair is not appreciably different from that of a G–C pair. The extra stability must come from the G–C pair's extra hydrogen bond. The other empirical finding is that oligonucleotide primers must be about 20 bases long in order for PCR to work well, the exact length depending on the G–C content of the oligo and the temperature at which annealing occurs (usually 55 °C). What this tells us is that we need to form about 50 hydrogen bonds for the favorable enthalpic contribution to the free energy change of double helix formation to exceed the unfavorable entropic contribution.

Now we can see why we will not want to make our oligonucleotide primers too short. We also know that we will not want to make them too long – every afghani, bhat, colon, dollar, euro ... yen, or zaire is dear. But there is another, thermodynamic reason why oligos should not be too long. Assuming a random base sequence in the template DNA strand, the absolute specificity of an oligonucleotide can only increase with length. But if the oligo is long, there will be many, many sites at which partial binding could occur on the template DNA. Moreover, the same oligonucleotide molecule may be able to bind not only to more than one place on the same template at the same time, but also to more than one template molecule! Such a situation, which can be energetically very favorable from an entropic point of view, will promote a huge number of side reactions and yield a very messy PCR product.

O. Free energy of transfer of amino acids

The free energy of transfer is the free energy change on transfer of a compound from one surrounding medium to another, usually, one solvent to another. Suppose we have two perfectly immiscible solvents (ones that do not mix) in the same beaker. There is an interface, with one solvent on one side and the other on the other. There is a large free energy barrier to mixing; this is what it means for the solvents to be immiscible. Now, if a solute is dissolved in one of the solvents, when the three-component system comes to equilibrium, solute will be found in the second solvent as well, if the solute is soluble in it. You will recognize this as a means of doing extraction of chemical compounds with organic solvents. This phenomenon is often grouped together with freezing point depression, boiling point elevation and osmosis, but strictly speaking it is *not* a colligative property. We are interested in it here because it will help us to have a better understanding of the solubility and thermodynamic

stability of biological macromolecules, not because of any particular relationship to colligative properties. A specific example will help to make headway in understanding.

The solubility of phenylalanine (Phe) in water at 25 °C on the molality scale is 0.170 mol (kg solvent)$^{-1}$; in 6 M urea it is 0.263 mol (kg solvent)$^{-1}$. Urea improves the solubility of hydrophobic side chains in aqueous solution, making urea a good chemical denaturant of proteins. Using the given information, one can calculate the standard state free energy of transfer of Phe from water to aqueous urea solution. To keep things simple, we'll assume that the activity coefficient of Phe is approximately the same in both media.

This situation can be pictured as follows (compare Fig. 2.3). In one process, Phe dissolved in water is in equilibrium with crystalline Phe; the solution is saturated. In another process, Phe dissolved in urea solution is in equilibrium with crystalline Phe. Both of these processes can be studied experimentally and the solubilities can be measured. In a third process, which is a sort of thought experiment, Phe in one solution is in equilibrium with Phe in the other solution. We construct a notional boundary between these solutions, and require that it be permeable to Phe but *not* to water or urea. There will be a net flow of Phe across the boundary until equilibrium is reached. The fourth "process" in the thermodynamic cycle is just solid Phe in equilibrium with solid Phe.

In mathematical terms,

$$\mu_{\text{water, sat.}} - \mu_{\text{solid}} = 0 \tag{5.51}$$

$$\mu_{\text{water}, a=1} - \mu_{\text{water, sat.}} = -RT \ln a_{\text{water, sat.}} = +1050 \text{ cal mol}^{-1} \tag{5.52}$$

$$\mu_{\text{urea, sat.}} - \mu_{\text{solid}} = 0 \tag{5.53}$$

$$\mu_{\text{urea}, a=1} - \mu_{\text{urea, sat.}} = -RT \ln a_{\text{water, sat.}} = +790 \text{ cal mol}^{-1} \tag{5.54}$$

$$\mu_{\text{solid}} - \mu_{\text{solid}} = 0. \tag{5.55}$$

The energy barrier between saturation and unit activity is greater in water than urea because the solubility of phenylalanine is lower in water than urea. The difference between Eqns. (5.52) and (5.54), which is what we set out to find, is $\mu_{\text{urea, } a=1} - \mu_{\text{urea, sat.}} - (\mu_{\text{water, } a=1} - \mu_{\text{water, sat.}}) = \mu_{\text{urea, } a=1} - \mu_{\text{solid}} - (\mu_{\text{water, } a=1} - \mu_{\text{solid}}) = \mu_{\text{urea, } a=1} - \mu_{\text{water, } a=1} = [\mu^{\circ}_{\text{urea, } a=1} + RT \ln(1)] - [(\mu^{\circ}_{\text{water, } a=1} + RT \ln(1)] = \mu^{\circ}_{\text{urea, } a=1} - \mu^{\circ}_{\text{water, } a=1} = 790 \text{ cal mol}^{-1} - 1050 \text{ cal mol}^{-1} = -260 \text{ cal mol}^{-1}$. This is the standard state driving force for transfer of Phe from saturated water to saturated 6 M urea. We can see from the sign of the chemical potential that the transfer is spontaneous; this is exactly what is expected from solubility data.

Though this example has involved a complete amino acid, there is in principle no reason why the experiment could not be done with

Table 5.5. *Thermodynamics of transfer at 25 °C from nonpolar solvent to water of various chemical groups*

Chemical group	ΔG_{tr} (cal mol^{-1} Å^{-2})	ΔH_{tr} (cal mol^{-1} Å^{-2})	ΔC_p (cal K^{-1} mol^{-1} Å^{-2})
Aliphatic: –CH_3, –CH_2–, CH	+8	−26	0.370
Aromatic	−8	−38	0.296
Hydroxyl	−172	−238	0.008
Amide & amino: –NH–, NH_2	−132	−192	−0.012
Carbonyl C: C=	+427	+413	0.613
Carbonyl O: =O	−38	−32	−0.228
Thiol and sulfur: –SH, –S–	−21	−31	−0.001

The data are from Ooi, T. and Oobataka, M. (1988) *J. Biochem.* 103, 114–120.

various small organic molecules "components" of the larger chemical. Comparison of the thermodynamic data with structural information would then provide clues to the thermodynamics of transfer of individual chemical groups. Table 5.5 gives thermodynamic values for the transfer of various chemical groups from nonpolar organic solvent to water.

There are at least two practical lessons we can draw from the above analysis. One is that the hydrophobic surface of phenylalanine, or indeed of any amino acid side chain which interacts with itself in the solid state by means of hydrophobic interactions, forms more favorable interactions with urea than water. Looked at another way, urea could be said to weaken hydrophobic interactions. Which leads to point number two. Empirical studies have shown that urea is a good chemical denaturant of proteins. We mentioned something about this in the context of dialysis but did not elaborate. The example of this section helps to rationalize the empirical finding. We know from X-ray analysis of the folded states of proteins that, although some hydrophobic side chains do appear on the protein surface, the core is mainly hydrophobic. In the presence of urea, where the solubility of the hydrophobic side chains is considerably increased relative to the absence of urea, the unfolded state of the protein is correspondingly more thermodynamically favorable. This fact can be used to investigate protein stability, as we shall see below and in Chapter 6.

P. Protein solubility

Here we are interested not so much in solubility of a substance *per se* but in solubility of proteins and protein–nucleic acid complexes. Our approach is qualitative and practical rather than quantitative and theoretical.

A protein molecule is a very complex polyion; there are numerous ionizable groups and a variety of pK_as. The solubility of a protein in aqueous solution will depend strongly on ionic strength and pH (Fig. 5.25). This is of the greatest practical significance for the choice of techniques that one might use to study a protein molecule. For instance, nuclear magnetic resonance (NMR) spectroscopy is a very high-resolution structural technique, making it valuable for protein structure determination and other aspects of biochemical research. But NMR is also an extremely insensitive technique, meaning that a very large concentration of protein is needed for a good signal-to-noise ratio, on the order of 1 mM or higher. At the same time, NMR structural studies generally require a relatively low rate of exchange of labile protons in tryptophan and tyrosine side chains, a pH-dependent phenomenon (see Chapter 8). In other words, the protein must not only be highly soluble, it must be soluble in a suitable pH range (near neutral pH). These requirements (and others!) restrict which protein structures can be studied by NMR.

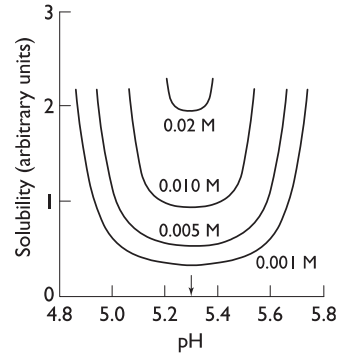


Fig. 5.25 Protein solubility. This depends not only on the net charge of the protein, which varies with pH, but also on ionic strength and temperature. The data shown are for a milk protein called β-lactoglobulin at different concentrations of NaCl. Solubility is very low at pH 5.3, the approximate isoelectric point of the protein. At this pH, the solubility of β-lactoglobulin increases exponentially with increasing ionic strength (solubility $\approx 0.255e^{10|pH}$). The data are from Fox and Foster (1957).

Here's an example of the effect of pH on protein solubility. The PTB domain of chicken tensin is highly soluble at pH 2, where its net charge is about +30. The net charge on each PTB molecule at acidic pH is so great that electrostatic repulsion inhibits the formation of protein aggregates, as long as the ionic strength is low. At pH 7, by contrast, the net charge on the PTB domain is 0 and it is not very soluble at all. This pH is the so-called *isoelectric* point, or p*I*, of the PTB domain. The isoelectric point of a protein depends primarily on *amino acid composition*; more specifically, the number of amino acids with ionizable side chains, and to some extent the location of each ionizable residue in the folded protein structure. If the number of basic side chains is relatively large and the number of acidic side chains relatively small, as with hen egg white lysozyme, the isoelectric point of the protein will be high, and the net charge is likely to be positive through most of the usual pH range (2–12). All that prevents a protein from aggregating at its p*I* is its solubility, which depends on the actual number of charged groups present, even if the sum of charges is 0. Charge properties can be used to purify a protein. For instance, recombinant PTB domain can be separated from some bacterial proteins by adjusting the cell lysate to pH 7. Separation of the precipitate effectively isolates recombinant PTB domain from the many bacterial proteins which remain soluble at neutral pH.

In general, the situation with protein solubility is more complex than we have made it seem thus far. At a given pH, a typical protein will have both positive and negative charges. Depending on the location of the charges on the protein surface, if the ionic strength is low, proteins can interact with each other by electrostatic attraction. It is often found therefore that *the solubility of a protein at low ionic strength increases with salt concentration*. This phenomenon is known as "*salting in*," and because it depends on the protein being charged, the effect is least pronounced at the isoelectric point. By contrast, at high ionic strength the protein charges are strongly shielded. Electrostatic

repulsion is negligible. Solubility is reduced. This effect is known as "*salting out*." The shape of a solubility curve with ionic strength is thus, roughly speaking, U-shaped (Fig. 5.25). Salting out is thought to arise from the screening of charges and from the numerous interactions between the salt ions and water resulting in a *decrease* in the water molecules available to solvate the protein.

Salting out is a useful means of purifying proteins. For instance, ammonium sulfate is often used to purify antibodies. Below a certain ionic strength, antibodies and some other proteins are soluble, but many other proteins are insoluble. The insoluble proteins can be removed from solution by centrifugation. Above a certain ion strength, the antibodies themselves precipitate. They can be separated from the rest of the solution and subjected to further purification. A similar procedure can be used to purify many different proteins. Once a protein is sufficiently pure, it is sometimes possible to crystallize it by dissolving it in a salt solution near the solubility limit of the protein. From a thermodynamic point of view, crystallization occurs because the crystalline state of the protein has a lower Gibbs free energy than the solution state.

Q. Protein stability

This section is on cooperative and reversible order–disorder transitions in proteins. It builds on several of the several previous sections, including those on DNA and PCR. A key difference between protein stability and duplex DNA stability is the size of ΔC_p between the ordered and disordered states: in proteins it is relatively large, in DNA relatively small. As we shall see, the magnitude of ΔC_p can have a marked effect on the thermostability of a protein.

When protein folding/unfolding is cooperative, effectively only two states are populated at equilibrium: the folded (native) state and the unfolded (denatured) state. The transition occurs over a relatively narrow range of the independent variable, be it temperature, pH or chemical denaturant concentration. In such cases, the equilibrium can be represented as

$$\mathrm{F} \Leftrightarrow \mathrm{U}. \tag{5.56}$$

The equilibrium constant (Eqn. (4.11)) is then

$$K_{eq} = [\mathrm{U}]/[\mathrm{F}]. \tag{5.57}$$

Note that Eqns. (5.56) and (5.57) are at least consistent with the idea that all the information required for a protein molecule to fold into its native form will be present in the amino acid sequence. The free energy difference between the folded state of a protein and its unfolded state is independent of the path! Regardless of the process by which a protein folds – in the cell or in a test tube – the free energy difference between folded and unfolded forms is the same (given the same temperature, ion concentrations, pH, etc.). But is a

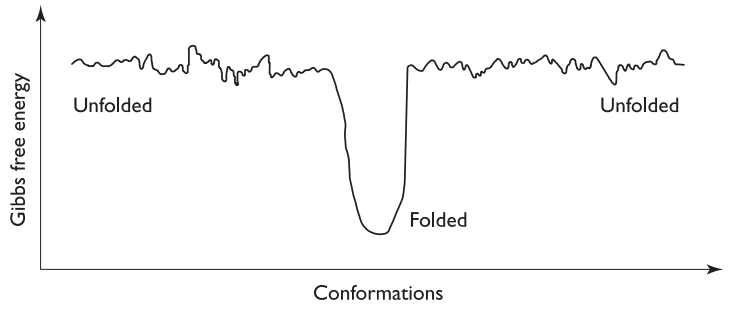


Fig. 5.26 Free energy profile of a "well-behaved" small protein. There are only two stable states: the folded state and the unfolded state. The number of unfolded conformations is vastly greater than the number of folded conformations. Although the unfolded conformations differ in energy, these differences are relatively small. Therefore the collection of unfolded conformations can be thought of as a single state. The energy difference between the unfolded state and the folded state is comparatively very large.

catalyst needed to get the reaction to proceed on a biologically relevant time scale?

Early attempts to give a thermodynamic description of reversible protein denaturation and coagulation appeared in the 1920s and 1930s in the work of American physical biochemists Alfred Ezra Mirsky (1900–1974), Mortimer Louis Anson (1901–1968), and Linus Carl Pauling.[15] In the 1950s, Rufus Lumry (1920-) and Henry Eyring (1901–1981), also both Americans, provided a more substantial mathematical analysis of reversible protein denaturation. This experimental and theoretical work modeled the folded state of a protein as corresponding to a (local) minimum of free energy, also known as an *energy well*, when conditions favor the folded state (Fig. 5.26). An important lingering difficulty, however, was the generality of the applicability of the thermodynamic description. Did it work for some proteins and not others? Did it apply to proteins with disulfide bonds? Christian Boehmer Anfinsen's investigations of the reversible denaturation of proteins showed conclusively that the native state of a protein with disulfide bonds could be recovered spontaneously, even when the disulfides were not formed in the denatured protein. This led to the general acceptance of the "thermodynamic hypothesis" for the folding of proteins, according to which attainment of the native structure rests solely upon the amino acid sequence. Anfinsen (1916–1995), an American, was awarded the Nobel Prize in Chemistry for this work in 1972. Since then, and particularly since the late 1980s, the goal of working out the structural basis of protein folding and thermostability has been pursued with considerable intensity throughout the world.

We have already discussed protein denaturation (in Chapters 2 and 3) in the context of illustrating the physical meaning of H and S and showing the utility of a van't Hoff analysis of equilibrium

[15] Pauling (1901–1994) was awarded the Nobel Prize in Chemistry in 1954 for his work on protein structure. His model of DNA structure, which had the bases pointing outwards, was no longer tenable after publication of the famous work of Watson and Crick. Something worth remembering: Nobel laureates are unusually accomplished rational animals, but they are capable of error. At the same time, though, it is fair to say that no one's discovered anything who hasn't also made a mistake.

constant data. Now let's consider protein denaturation in terms of free energy. At constant temperature, Eqn. (4.2) becomes

$$\Delta G^\circ_d = \Delta H^\circ_d - T\Delta S^\circ_d, \tag{5.58}$$

where as before the subscript signifies "denaturation." ΔG°_d is the difference in Gibbs free energy between the unfolded state and the folded state of the protein. In most cases, the energy of the unfolded state is measured relative to the energy of the folded state; i.e. the folded state is the reference state. There are two main reasons for the convention: the folded state has the least ambiguous conformation, and more often than not *equilibrium* studies investigate transitions *from* the folded state *to* the unfolded state; the folded state is often the starting state (not true of kinetic protein refolding experiments!). ΔG°_d alone tells us nothing about the relative magnitudes of ΔH°_d or ΔS°_d; an *infinite* number of combinations of these thermodynamic functions would be consistent with a given value of ΔG°_d. Of course, many of these combinations of ΔH°_d and ΔS°_d will have little or no physical meaning for the system under study, and only one combination will actually describe the system under study. In order to fix the values, we'll have to do at least one more experiment. It has been found that ΔH°_d and ΔS°_d for proteins can be very large in comparison with ΔG°_d. For instance, it is common for the maximum value of ΔG°_d for a protein in solution to be about 15 kcal mol^{-1}, and for ΔH°_d at the denaturation temperature to be more than an order of magnitude greater. ΔG°_d for proteins is thus a delicate balance of ΔH°_d and ΔS°_d.

At the *melting temperature*, also called the *heat-denaturation temperature*, the fraction of molecules in the folded state equals that in the unfolded state; the free energy difference between them, ΔG°_d, is 0. This leads to Eqn. (3.21) and enables you to calculate the entropy of unfolding from measurement of ΔH°_d. Including the temperature dependence of ΔH and ΔS explicitly, Eqn. (5.58) becomes

$$\Delta G^\circ_d(T) = \Delta H^\circ_d(T_r) + \Delta C_p(T - T_r) - T[\Delta S^\circ_d(T_r) + \Delta C_p \ln(T/T_r)], \tag{5.59}$$

where the subscript "r" means "reference." $\Delta G^\circ_d(T)$ is not $(\Delta G^\circ_d \times T)$ but ΔG°_d evaluated at temperature T. As an example, suppose that our reference temperature is 25 °C and that both ΔH°_d and ΔS°_d are known at this temperature. What is ΔG°_d at 35 °C? If ΔH°(25 °C) = 51 kcal mol^{-1}, ΔS°(25 °C) = 100 cal mol^{-1} K^{-1}, and $\Delta C_p = 1500$ cal mol^{-1} K^{-1}, then ΔG°(35 °C) = 51 kcal mol^{-1} + 1500 cal mol^{-1} K^{-1} × (308 K − 298 K) − 308 K + [100 cal mol^{-1} K^{-1} + 1500 cal mol^{-1} K^{-1} + ln(308 K/298 K)] = 20 kcal mol^{-1}. $\Delta G^\circ_d(T)$ is known as the *stability* of a protein. It tells you how much energy must be expended (more specifically, the minimum amount of work that must be done) to unfold the protein at a given temperature. A plot of $\Delta G^\circ_d(T)$ versus temperature (or any other independent variable, e.g. pH or concentration of chemical denaturant) is called a *stability curve*.

The stability curve as a function of temperature resembles a parabola and has a peak at which ΔG°_d is a maximum. It can be shown

(using Eqn. (5.58) and a little calculus) that at this temperature, called the *temperature of maximum stability*, $\Delta S^\circ_d = 0$ (compare Fig. 4.4). That is, the stability of the folded state of a protein is a maximum when the entropy of the folded state and surrounding solution is equal to the entropy of the unfolded state and the surrounding solution. At this temperature, which is often 40 or 50 K below the heat-denaturation temperature, enthalpic interactions alone hold the folded state together. Just *below* T_m (for heat denaturation), ΔG°_d is positive (if the folded state is the reference state). On the average, unfolding will not occur spontaneously, because $\Delta G^\circ_d > 0$.[16] To bring about unfolding by a further temperature increase, we expect ΔH°_d to be positive; this is roughly the energy required to disrupt non-covalent interactions in the folded state. We also expect ΔS°_d to be positive, as the polypeptide chain will be much more disordered in the unfolded state than in the folded one, and we don't expect the order of the surrounding solution to increase with temperature. But *on the balance*, $\Delta G^\circ_d > 0$ below T_m. Above T_m, the balance is shifted towards the entropy, $|T\Delta S^\circ_d| > |\Delta H^\circ_d|$, and there is net unfolding of the protein.

ΔC_p plays a key role in protein stability. Both the enthalpy change and the entropy change of denaturation depend on ΔC_p, so the free energy change depends on ΔC_p. Figure 5.27 shows how the Gibbs free energy difference between the unfolded and folded states changes as the magnitude of ΔC_p changes. If $H^\circ_d(T_r)$ and $S^\circ_d(T_r)$ are held constant, *decreasing* ΔC_p *increases* the breadth of the stability curve, and *increasing* ΔC_p *decreases* the breadth; all the curves intersect at the reference temperature. This tells us that if a protein has a small heat capacity change on unfolding, it is likely to have a relatively high transition temperature, and this is exactly what is observed experimentally. By contrast, when ΔC_p is relatively large, the stability curve becomes sharply peaked and can cross the temperature axis in more

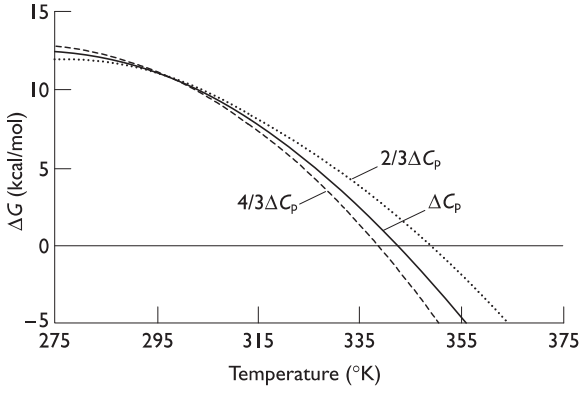


Fig. 5.27 Protein stability curves. Each one is a plot of ΔG versus T (or some other independent variable). This gives the free energy difference between the unfolded state and the folded state (the minimum amount of work that must be done to induce a transition from the folded state to the unfolded state). The curvature in ΔG v. T arises from the positive and relatively large ΔC_p of protein unfolding. The stability curve crosses the T-axis at two points, the heat- and cold-denaturation temperatures. In the figure only the heat denaturation temperatures are seen.

[16] This statement needs some qualification. In fact, unfolding *can and does* occur spontaneously when $\Delta G > 0$, but not in greater abundance than spontaneous refolding of unfolded protein (see Chapter 6). The more positive ΔG, the less probable spontaneous unfolding. The situation is just the opposite when $\Delta G < 0$.

than one place in the experimentally accessible range (when the solvent is in the liquid state).

The second intersection of ΔG°_d with the temperature axis, which occurs well below the heat-denaturation temperature, is known as the *cold-denaturation temperature*. The mathematical form of the stability curve, which is based on solid experimental evidence of *heat*-denaturation, suggests that protein unfolding can be induced by heating or, strange as it may seem, by cooling. This prediction has been confirmed by experimental studies in a number of cases, greatly underscoring the value of good mathematical modeling of experimental results for prediction of the behavior of biochemical systems. Cold denaturation seems rather counterintuitive. For in order to melt a crystal, one expects to have to *add* heat, in which case $\Delta H > 0$. The entropy change on protein unfolding, $\Delta S^\circ_d = \Delta H^\circ_d / T_m$, is therefore positive, in accord with intuition. By contrast, in cold denaturation $\Delta H^\circ_d < 0$! It follows that $\Delta S^\circ_d < 0$ for cold denaturation. Weird! Some peptide aggregates are known to exhibit cold denaturation on heating from room temperature. In other words, the peptides tend not to be aggregated in aqueous solution at 25°C, but heating the solution leads to spontaneous gelation, a type of peptide aggregation which resembles the condensation of hydrophobic residues in the folding of a small protein. In all these cases, ΔG°_d can pass through 0 more than once because both ΔH and ΔS depend on T.

So far we have been discussing ΔG as a function of temperature. There are other independent variables we could consider, for example, pH and chemical denaturant concentration. Let's look at the latter first. As chemical denaturant is added to a protein solution, the folded state becomes destabilized *relative to* the unfolded state, and the protein unfolds. At the so-called midpoint concentration of denaturant, $\Delta G = 0$, and the fraction of molecules in the folded state is equal to the fraction in the unfolded state. The superscript on G has disappeared because now we are considering the protein in solution in the presence of denaturant. Note how the midpoint concentration of chemical denaturation closely resembles the transition temperature of thermal denaturation. In pH denaturation, either acid or base is added to the protein solution to induce unfolding. At the midpoint pH, half of the molecules are in one state and half are in the other, and again, $\Delta G = 0$.

Chemical denaturation and pH denaturation are such common forms of studying protein stability that further details can be provided here. The stability of the folded state of a protein in the presence of a chemical denaturant is often modeled as

$$\Delta G_d = \Delta G^\circ_d - mc, \tag{5.60}$$

where c, the concentration of denaturant (usually in molar units), is the only independent variable, and m is a parameter that depends on temperature, pH, buffer, and – is it surprising? – the protein. Note that at the concentration midpoint, which is determined experimentally, $\Delta G^\circ_d = mc$. So if ΔG°_d is known independently, for

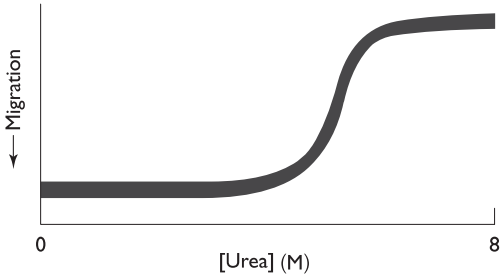


Fig. 5.28 Urea denaturation gel electrophoresis. Structural transitions in protein can be studied by polyacrylamide gel electrophoresis. The horizontal axis is a linear gradient in urea concentration. When the concentration is sufficiently high, the protein unfolds. This is detected as a change in the mobility of the protein in the gel matrix. Compact folded proteins generally migrate faster in the gel than extended unfolded proteins.

example, by calorimetry, m can be determined experimentally. Eqn. (5.60) "works"; it can be used to study protein denaturation. But it should be added that the physical meaning of m is not entirely clear, though it seems to correspond roughly to the increase in surface area of the protein in contact with the solvent upon unfolding. Figure 5.28 illustrates the effect on protein stability of changing the denaturant concentration. The denaturant is urea, and a change in the conformation of the protein is detected as a change in electrophoretic mobility. The more compact the protein, the higher the mobility in a network of non-migrating polymers. An unfolded protein migrates more slowly than a folded one. The model of protein stability outlined here is plausible for a number of reasons. At the same time, however, it says nothing at all about what the denaturant does to make the protein unfold. Moreover, experiments show that, although in many cases the dependence of ΔG_d on denaturant concentration is approximately linear, in some cases it is distinctly non-linear. The physical meaning of such non-linear behavior is usually uncertain.

As we have said, a change in pH can also cause a protein to unfold. Such denaturation usually occurs for two reasons. One is that a charged side chain can be partly buried, giving it an anomalous pK_a, and a change in the ionization state of the side chain can destabilize folded structure. Another is that at extremes of pH, the net charge on the protein can be very large, resulting in an unusually large destabilizing contribution to the overall thermostability. The change in stability of the folded state, $\Delta(\Delta G_d)$, varies with a change in pH as

$$\Delta(\Delta G_d)/\Delta(\mathrm{pH}) = 2.3RT\Delta Q, \tag{5.61}$$

where ΔQ is the difference in number of bound protons between the unfolded state and the folded state, Δ(pH) corresponds to a difference in $[H_3O^+]$, and 2.3 is a conversion factor related to logarithms. Using these hints, can you derive Eqn. (5.61)?

It has frequently been observed that point mutations in proteins lead to relatively large changes in ΔS° and ΔH° but a relatively small change in ΔG°. The changes in ΔS° and ΔH° are often difficult to rationalize in terms of changes in protein structure as assessed by NMR spectroscopy or X-ray crystallography. This "cancellation" of

changes in the enthalpy and entropy terms is known as *enthalpy–entropy compensation*. Apparently, subtle differences in structure and solvation can have significant thermodynamic consequences. This points up both the remarkable plasticity of the folded state of a protein and the still shallow understanding of the microscopic origins of macroscopic properties of materials and systems. We'll return to this topic in Chapter 9.

Now let's bring a bit of biology into the picture. We wish to cover two subtopics to round out this section: the engineering of enzymes to enhance their thermostability without altering specificity and the role of stability in protein degradation.

Protein engineering: enzymes are biological catalysts (Chapter 8). The ability to produce massive quantities of an enzyme by recombinant DNA technology has made it feasible to consider the use of enzymes in biomedical, chemical, and industrial applications. Often, though, the physical environment of the enzyme in a practical application will be different from the environment in which it is found in nature. An enzyme can be engineered by standard molecular biological techniques to tailor properties to specific applications. For instance, a way in which the stability of an enzyme can be increased is to decrease the disorder of its unfolded state. This will lead to a substantial decrease in ΔS_d but in some cases effectively no change in ΔH_d. The change can be brought about by replacing a Gly residue with any other residue type. Some Gly residues, for instance, ones in turns, won't work well for this purpose, because replacement has too large an impact on the structure of the folded state. In general, though, Gly residues make the polypeptide backbone very flexible, while the amino acids with side chains restrict bond rotations in the backbone. Pro residues allow practically no backbone flexibility, and these can be introduced into turns. Yet another approach is to add disulfide bonds. These link different parts of the polypeptide chain and reduce its mobility in the unfolded protein (see Chapter 6, Section C). A complementary approach to stabilization of a protein is to increase the enthalpy of the folded state. This can lead to a substantial increase in ΔH_d but effectively no change in ΔS_d. Unfavorable electrostatic interactions in the folded state can be replaced by favorable ones, and negatively charged side chains (particularly that of Asp) can be placed at the beginning of an α-helix to interact favorably with the helix dipole. Amino acid substitutions can be made within helices to increase their strength, and within the protein core to increase the hydrophobic surface area. All such changes can lead to a protein of increased stability.

Protein degradation: as we have said, protein stability can be measured by ΔG_d° or K_{eq}. Because K_{eq} measures the ratio of the forward and reverse rate constants (Chapter 8), when the folded state of a protein is stable (i.e. when ΔG_d° is large, if the folded state is the reference state), the rate of folding must be greater than the rate of unfolding. When the folded state is the more stable one, there will still be some molecules in the unfolded state, even though that

proportion will be small except in the region of the transition (see Chapter 6). When $K_{eq} = 1$, not only is there no free energy difference between the states, there is an equimolar mixture of folded state and unfolded state. In other words, the bottom of the energy well is at the same level as the ground surrounding the well! The proportion of molecules in one state or the other changes as the conditions are adjusted. Even when the stability of the folded state is relatively large, some unfolded proteins will be around, though the relative proportion of unfolded molecules could be 10^{-6} or less. A question we'd like to be able to answer is this: because the turnover rate of endogenous protein in an organism is high, i.e. because the body is constantly recycling its protein, (see Chapter 9), does the body clear protein by having a specialized degradation protein bind to and digest unfolded proteins? Does the degradation of an unfolded protein then diminish the population of folded proteins by mass action and thereby stimulate the synthesis of replacement protein? Or, does the body make "degradation proteins" that actively unfold proteins at random? If the former, it is thermal energy and the specific solution conditions of the body (salt concentration, pH, etc.) that play some role in clearing proteins. If the latter, then energy must be supplied to clear proteins, since work must be done to denature a stable folded protein. In fact, there appear to be proteins that can unfold and degrade stable, native proteins in the presence of ATP. One such protein in bacteria, ClpA, is a member of the Clp/Hsp100 "chaperone" family. Are there corresponding proteins in mammals? Another question we'd like to be able to answer is, if the body continually recycles protein, it must continually make it, and because proteins are synthesized on ribosomes from mRNA templates, genes must continually be transcribed, and if mutations in genes can lead to pathological proteins, and if mutations accumulate as the body ages, does the body somehow "program" its own death?

R. Protein dynamics

In an earlier chapter we described the folded state of a protein as an organic crystal. Indeed, if this were not so, it probably would not be possible to crystallize proteins! More importantly, without a relatively fixed geometry in the catalytic site, how could an enzyme carry out a specific function? Such considerations might give the false impression that the folded state of a protein has a rigidly fixed structure. Instead, folded states of proteins, though sturdy and crystal-like, are nevertheless flexible, and they exhibit many very rapid small-scale fluctuations. Evidence for dynamical behavior which arises from thermal motion tells us that the native structure of a protein is a large ensemble of similar and rapidly inter-converting conformations that have the same or nearly the same free energy. As we shall see, structural mobility in the native state has crucial functional significance.

First, let's see how this section links to the one on protein stability. The melting temperature of a protein showing two-state behavior depends on the balance of ΔH_d and ΔS_d. For given values of these thermodynamic functions, if the folded state of a protein were extremely rigid, then the change in entropy on unfolding would be large, and the protein would never fold; T_m would simply be too low (see Eqn. (3.21)). And if the entropy of the unfolded state of a protein were not very different from the entropy of the folded state, ΔS_d would be small, and $\Delta H_d/\Delta S_d$ would be so large that the protein would never unfold. This could be disastrous for the cell if a protein became modified in such a way as to be pathogenic.

Now, you might find it interesting that protein flexibility is demonstrated by the very fact that proteins can be crystallized! There are two points we can make. One is that high-resolution X-ray diffraction data provide valuable information on the motions of atoms more massive than hydrogen. Modeling of the protein structure is a matter of fitting a molecule with the known covalent constraints to an electron density map. The map does not reveal precisely where the center of mass of an atom will be, but only a volume of space where an atom is likely to be found. Analysis of such data shows that some atoms in a protein move very little while others move a great deal – when the protein is folded and in a crystal. There is another way in which protein crystals reveal that such motions exist, and it shows that the motions have physiological relevance. X-ray studies of the oxygen transport and storage proteins hemoglobin and myoglobin show that there is no obvious route for O_2 to move from the solvent to the binding site; oxygen takes up space. One concludes that O_2 (and CO_2) binding and release depend on fluctuations in structure known as "*breathing motions*."

The length and time scales of such motions depend on free energy differences relative to the minimum free energy structure. There are three basic types of dynamical motion: *atomic fluctuations*, *collective motions* and *triggered conformational changes*. Atomic fluctuations occur on a time scale on the order of picoseconds and are relatively small in magnitude, while conformational changes are typically much slower and larger. X-ray analysis of the active site cleft of hen lysozyme, for example, shows that some of its atoms move by $\sim$1 Å on substrate binding. Small but significant.

Other experimental methods that reveal the motions of folded proteins are NMR spectroscopy and hydrogen exchange. NMR can be used to measure the rate of 180°-flipping of the ring of a Phe or Tyr side chain about the C_β–C_α bond. The rate varies, but it generally falls in the μs–s range. Recent developments in NMR data collection and analysis permit a more general analysis of polypeptide backbone and amino acid side chain dynamics. NMR can also be coupled with the exchange of labile protons in the polypeptide backbone of a protein to gain information on protein dynamics. Exchange of such protons is temperature-dependent for two reasons: the stability of the native state of a protein varies with temperature, as we

saw above, and the exchange rate depends on temperature (as well as pH). These experiments involve D_2O, and deuterium is exchanged in for solvent hydrogen. The approach is particularly attractive from the point of view of experimental design and analysis of results, because hydrogen and deuterium are chemically identical but have completely different NMR characteristics (see Chapter 8).

S. Non-equilibrium thermodynamics and life

At some point in your study of thermodynamics you may have wondered: if the First Law requires the total energy of the universe to be constant, and the Second Law requires that every process be accompanied by an increase in the entropy of the universe, then how is life possible at all? Do the tremendously complex forms of matter we call living organisms violate the laws of thermodynamics? Clearly, the answer must be no, if the laws of thermodynamics as formulated actually do describe our universe.

In Chapters 4 and 5 we have used a combination of the First and Second Laws to look at a number of biological processes at equilibrium. We have seen how useful the Gibbs free energy function is for describing these processes. Although aspects of biochemistry can be described in this way, we should always remember that no living organism is at equilibrium! This holds not only for the organism as a whole but each of its cells. Moreover, it applies to every last bacterium inhabiting the cosy environment of your gut. Important for us, a non-equilibrium process is by definition irreversible (though possibly not completely irreversible)! Let's look at this topic somewhat more carefully.

An *in vitro* biochemical reaction is a closed system (Chapter 1). As such, it will change until equilibrium is reached. A living organism, on the other hand, be it an amoeba, a bombardier beetle, or a wildebeest, is an open system. An organism is therefore *never* at equilibrium. An organism takes in high-enthalpy and low-entropy compounds from its surroundings, transforms them into a more useful form of chemical energy, and returns low-enthalpy and high-entropy compounds to its surroundings. By means of such energy flows, living organisms degrade the quality of the energy of the universe. *Non-equilibrium systems "dissipate" the useful energy of the universe*.

Energy flow through an organism is like water flow through a channel. (But this does not mean that energy is a material particle or a collection of particles!). The rate of flow through an organism in adulthood is approximately constant, a situation known as *steady state*. A steady-state system changes continually, but there is no *net change* in the system – its physical makeup, the amount of matter present. (What changes occur in the brain when an adult learns something new and remembers it?) Steady state in an open system is the analog of equilibrium in a closed system. A steady inward flow of energy is the most stable state an open system can achieve. As

depicted in Fig. 2.5B, if the inward flow of energy differs from the rate at which energy is consumed, a change in weight occurs. A voracious reader is clearly something very different from a voracious eater.

Must a living organism be a non-equilibrium system? If it were not, it could not do useful work. An equilibrium system cannot do useful work. This is because at equilibrium, there is no free energy difference between reactants and products. An equilibrium process cannot be directed. It is "rudderless." The schematic diagrams of earlier chapters highlighted the machine-like qualities of living organisms; indeed, there are many similarities. For instance, both organisms and machines are made of matter, and the processes carried out by both are, at least to some extent, irreversible. Organisms and machines can do work. Because body temperature is not very different from and often greater than the temperature of the surroundings, an organism can do very little work by means of heat transfer, practically none. Instead, organisms do work by taking in free energy from their surroundings – food. Like machines, organisms "wear out" with use. Excessive physical activity can damage the body. Extensive bicycling on insufficient conditioning, for example, can result in a damaged nervous system, with neurons in the leg tingling constantly, possibly until death. Machines, by contrast, are basically "static" structures. The plastic in a computer keyboard, for example, is not changing dramatically from moment to moment. The keyboard molecules are not simultaneously being degraded by enzymes and replaced by newly synthesized ones. Living organisms are different. They need free energy because they must renew themselves to live. Their proteins are constantly being destroyed and new ones must be made to take their place. DNA is constantly being replicated. Certainly, organisms display machine-like qualities. But organisms are different from lifeless machines. A living organism cannot be at equilibrium.

Where does the thermodynamic irreversibility of a living organism come from? If many individual biochemical reactions are reversible, at what length scale does irreversibility set in? What is the microscopic origin of irreversibility in biological organisms? These are hard questions! We do not pretend to answer them fully here. There are numerous contributions to the overall fact of irreversibility, but a particularly important one is non-productive hydrolysis of ATP. As we have seen, ATP is hydrolyzed spontaneously in water. If hydrolysis is not coupled to a metabolic reaction, the energy released will go off as heat – irreversibly. There are also three "irreversible" steps in the metabolism of glucose to pyruvate. These occur between glucose and G6P, F6P and FDP and PEP and pyruvate. This turns out to be extremely important for cellular function, for it is really only at the irreversible steps of a process that "machine-like" control can be exerted: it permits regulation of the speed of the reaction. Such regulation is of considerable importance to reactions that occur downstream.

Lastly, non-equilibrium systems present a number of problems for the quantification of thermodynamic functions. The First Law has been verified experimentally and quantitatively for living organisms. It's harder to do this for the Second Law for reasons outlined above. Entropy, free energy and chemical potential cannot be measured for non-equilibrium systems. There is nevertheless a way of connecting a non-equilibrium system with something more amenable to study and analysis, and that is the internal energy, *U*. Suppose we wish to measure the internal energy of a non-equilibrium system. This can be done by isolating the system and waiting for it to come to equilibrium. Because the system is isolated, the internal energy will be the same at equilibrium as in any non-equilibrium state. If *U* of the equilibrium state is then measured with respect to some reference value, then *U* of the non-equilibrium state is known. Can you think of other ways of approaching the problem of quantifying thermodynamic functions and verifying the Laws for living organisms? Doing so might lead to new insights on what it is to be alive. Best wishes!

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U. Exercises

1. Speculate in broad terms on the effect on Earth of the cessation of photosynthesis.

2. The energy conversion process by which sunlight is converted into biomass is not completely efficient. What happens to the energy that does not become biomass? Rationalize your answer in terms of the First and Second Laws of thermodynamics.

3. Animal life as part-time plant? Sue Williams of the Department of Botany, University of Western Australia, says that the green-tinged sea slugs she studies "enslave" chloroplasts from the seaweed they ingest, and use them as a means of capturing up to 25% of their energy. Explain how this might work.

4. Use the following information to determine the standard free energy change of ATP hydrolysis.

$$\text{Glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP} \qquad \Delta G^{\circ\prime} = -16.7 \text{ kJ mol}^{-1}$$
$$\text{Glucose-6-phosphate} \rightarrow \text{glucose} + \text{P}_\text{i} \qquad \Delta G^{\circ\prime} = -13.8 \text{ kJ mol}^{-1}$$

Show all work.

5. Buffers containing ATP are ordinarily made up fresh and not stored as a stock solution. When a stock solution is made, it must usually be kept at 4°C (short term storage) or at −20°C (long term storage). Rationalize these practices. What bearing does this have on the necessary molecular machinery of a cell?

6. ATP is the energy currency of the cell. ATP is essential for life as we know it. Comment on the stability of ATP in aqueous solution and the constraints this may place on theories of the origin of life.

7. The free energy status of a cell can be described in various ways. One of these, called the *adenylate energy charge* (AEC), was first proposed by Daniel Edward Atkinson (1921–). The AEC is defined as

$$\mathrm{AEC} = ([\mathrm{ATP}] + 0.5[\mathrm{ADP}])/([\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}])$$

and it varies between 1.0, when all the adenine nucleotide is ATP, and 0, when all the β- and γ-phosphoanhydride bonds have been hydrolyzed. The relative amounts of ATP, ADP, and AMP can be determined by comparing the sizes of the respective peaks in a high-performance liquid chromatography (HPLC) profile. The AEC of a healthy cell is about 0.90–0.95. Malignant hypothermia is an inherited muscle disease in humans and pigs. Patients suffer rapid rises in body temperature, spasms in skeletal muscle, and increases in the rate of metabolism, which can be fatal if not treated with a suitable muscle relaxant. The following data were obtained before the onset of symptoms and just prior to the death of a pig afflicted with the disease.

	[ATP]	[ADP] μmol g^{-1} tissue	[AMP]
Before symptoms	4.2	0.37	0.029
Before death	2.1	0.66	0.19

Calculate the AEC before the symptoms began to occur and just before death. Comment on the magnitude of the values and what they indicate.

8. A 1 M solution of a glucose gives a pressure more than 25 times greater than that of the atmosphere. A 1 M solution of a salt gives an even larger osmotic pressure. Explain.

9. Suppose we have an osmometer that is constructed from a capillary tube with a membrane in the middle, and that the tube is oriented *horizontally* (why?). Now let some osmotic particles suddenly appear on one side of the tube only. Explain what happens.

10. You have a U-tube osmometer with cells of equal shape and volume. On one side, you place a sphere of volume V, and at the

same time and on the other side you place a cube of volume V. Neither particle is membrane-permeant. Suppose that these particles are able to interact with the solvent. Explain what will happen in the following situations: (a) the particles are so dense that they sit on the bottom of the cells of the osmometer; (b) the density of the particles is such that they are able to diffuse throughout the volume of their respective cells.

11. What causes the membrane of a red blood cell to burst when the cell is placed in hypotonic solution? Be as specific as possible.

12. Suppose you have an osmometer in which the solute particles are confined to a fixed volume, for instance an indestructible membrane of fixed volume. What happens? Why?

13. Suppose you have an osmometer with a membrane that is permeable to water but not to larger molecules. Add glucose to one side to a final concentration of 0.001 M and hemoglobin to the other side to a concentration of 0.001 M. Will a pressure head develop? If yes, on which side will the water level be higher? If no, why not?

14. Suppose you are involved in preparing recombinant protein for a physical biochemistry experiment. The approach involves 8 M urea, formation of which from urea crystals and water is highly endothermic. The bacteria are lysed in 8 M urea, a chemical denaturant, and the recombinant protein is separated from the bacterial proteins by column chromatography. Separation of the recombinant protein from urea is done by dialysis in two stages. In each, *c.* 100 ml of lysate is dialyzed against 5 l of water. The dialysis membrane allows the passage of water and urea but not protein. Will the volume of the protein preparation change in this procedure, and if so, how? Assuming that the volume of the protein solution at the end of dialysis is 100 ml, what is the final concentration of urea? Explain, in enthalpic and entropic terms, the driving force for the reduction in urea concentration in the first step of dialysis. Explain from a thermodynamic point of view what drives the further reduction in urea concentration in the second step of dialysis.

15. Recall what happens to a red blood cell when it's placed in a hypotonic solution. What must be done to ensure that dialysis tubing doesn't burst?

16. Prove that Eqns. (5.19) and (5.20) follow from the preceding equations.

17. Show that Eqn. (5.38) follows from Eqn. (5.36).

18. Derive Eqn. (5.40). (Hint: start with $R_T = R + R \bullet I + R \bullet L$, express R and $R \bullet I$ in terms of $R \bullet L$, and solve for $R \bullet L$.)

19. Equation (5.41) is 0 for all concentrations of I_i when there is no inhibition (compound I_i has no effect), and it is 1 at 100% inhibition. Explain.

20. Analysis of gene regulation involves study of structural and thermodynamic aspects of how proteins bind nucleic acid. One area of such research is the recognition of DNA operator sites by repressor molecules. Suppose protein P binds a single specific sequence on a molecule of DNA D. This is a common mechanism for the regulation of gene expression. At equilibrium, $P + D \Leftrightarrow P \bullet D$. A bacterial cell contains one molecule of DNA. Assume that cell is cylindrical, and that its diameter and length are 1 μm and 2 μm, respectively. Calculate the total concentration of D. Assume that $K_{eq} = 10^{-10}$ M. Calculate the $[P \bullet D]$, assuming that $[P] = [D]$. The concentration of *bound* D is just $[P \bullet D]$. Calculate the concentration of *unbound* D. Calculate $[P \bullet D]/[P]$. Give an interpretation of this quantity. The subject of binding will be discussed in detail in Chapter 7.

21. The previous problem involved the association and dissociation of two types of macromolecule, proteins and DNA. A basic feature of such situations is the dependence of the equilibrium on the total concentrations of the interacting species. The concept can be illustrated by means of the monomer–dimer equilibrium. Consider the equilibrium

$$2M \Leftrightarrow D. \tag{5.61}$$

The total concentration of monomer, $[M]_T$, is $[M] + 2[D]$, where the factor 2 accounts for there being two monomers in each dimer. This equation can be solved for [D]. Write down an expression for the equilibrium constant for the reaction in Eqn. (5.61). Combine this with your equation for [D] and solve the resulting quadratic equation for [M]. Show that $[M]/[M]_T \to 1$ as $[M]_T \to 0$, and that $[M]/[M]_T \to 0$ as $[M]_T \to \infty$. How does one interpret these limiting conditions?

22. What might be the structural basis for the low stability of Georgia hemoglobin relative to normal hemoglobin?

23. Hemocyanin is a Cu-containing oxygen-binding protein that is found in some invertebrates. In squid hemocyanin, when the partial pressure of oxygen gas is 0.13 atm at 25 °C, the oxygen binding sites are 33% saturated. Assuming that each hemocyanin molecule binds one molecule of oxygen gas, calculate the equilibrium constant. What are the units of the equilibrium constant? Calculate the standard state free energy change when hemocyanin interacts with O_2(aq). The solubility of pure oxygen in water at 1 atm and 25 °C is 0.001 17 mol $(\text{kg } H_2O)^{-1}$.

24. In ELISA, what type of interactions are likely to be most important for protein adsorption to the solid support? Why are antibodies able to bind to partially denatured protein?

25. Explain in thermodynamic terms how a single 30-cycle PCR experiment can yield billions of copies of double-stranded DNA.

26. Under normal conditions, complementary strands of DNA form a double helix. In the section on PCR we provided a way of rationalizing the stability of DNA. Compare and contrast our view with that put forward by Voet and Voet, authors of a popular biochemistry textbook (see pp. 866–70 of the second edition, published in 1995). Can the data in their Table 28–4 be trusted? Why or why not?

27. Equation (5.50) for DNA supercoiling resembles the First Law of Thermodynamics. List and explain the similarities and differences.

28. A certain machine of a biotechnology company provides a controlled environment for the automation of sequence-specific DNA analysis and performs all the reaction steps required for capture and detection of nucleic acids. A main feature of the product is its capture specificity. For instance, suppose a 300 bp PCR fragment derived from the filamentous bacteriophage M13 was specifically captured by using a series of complementary oligonucleotide probes 24 residues in length, and that the capture probes incorporated 0–6 mismatches with the target. Explain how optimizing the hybridization conditions (i.e. by adjusting the temperature) could distinguish sequences differing by a single base.

29. "Hot start." When plasmid DNA is used as the template in a PCR reaction, the enzyme buffer, plasmid, and oligonucleotide primers are often incubated at 95 °C for several minutes before starting thermal cycling. Why?

30. The release of insulin from pancreatic β-cells on uptake of glucose is a complex process. The steps of the process in rough outline are as follows. The resting membrane potential of a β-cell is determined by open ATP-sensitive K^+ channels in the plasma membrane. After a meal, glucose is taken into the cell and phosphorylated. Eventually, there is an increase in [ATP]/[ADP] ratio in the cell, and this closes the K^+ channels. The membrane depolarizes, stimulating the opening of Ca^{2+} channels. Calcium enters the cell, stimulating the release of insulin through exocytosis of secretory granules. Describe each step of this process in moderately detailed thermodynamic terms.

31. Isothermal titration calorimetry. The key condition underlying this technique is thermodynamic equilibrium. When an aliquot of titrant is injected, the Gibbs free energy of the system increases. A spontaneous chemical reaction occurs until G reaches a new

minimum and equilibrium is established once again. An ITC study of a ligand binding to a macromolecule was carried out at three temperatures, T_1, T_2 and T_3, where $T_1 < T_2 < T_3$. At T_1, $\Delta H_b > 0$; at T_2, $\Delta H_b = 0$; and at T_3, $\Delta H_b > 0$. The ligand is known to bind the macromolecule at all three temperatures by means of independent experiments. Explain what is happening in the reaction cell at each stage of a general ITC experiment, viz. before an injection and during an injection. Rationalize the results obtained.

32. Speculate on the possibility of observing the cold denaturation of DNA. What about tRNA?

33. The folded and unfolded states of a protein are in equilibrium as shown in Eqn. (5.57). Suppose that you are working with a solution of RNase A at a concentration of 2.0×10^{-3} M, and that fractions of protein in the *unfolded* state are as follows: 50 °C: 0.002 55; 100 °C: 0.14. In the thermal denaturation of this protein, there are essentially just two states, the folded one and the unfolded one, so the fraction of protein in the folded state is just one minus the fraction in the unfolded state. Calculate ΔH° and ΔS° for *unfolding* of RNase A. What key assumption must be made about temperature-dependence? Calculate ΔG° for *unfolding* of RNase A at 37 °C. Is this process spontaneous at this temperature? Determine the melting temperature of RNase A under standard state conditions (for a two-state reaction, at T_m half of the proteins are folded and half are unfolded).

34. The role of ΔC_p in protein stability and its molecular origin was discussed in publications by the American biochemist John Brandts as early as 1964. Use Eqn. (4.3) to investigate the role of ΔC_p in the thermostability of a protein. One relatively easy way to do this is to assume values for ΔH and ΔS at some reference temperature, say 298 K, and then to use a spreadsheet to calculate ΔG throughout a temperature range that includes 0–100 °C. Plot ΔG v. T for several different values of ΔC_p. Note that the curve crosses the T-axis at two points. What are the names of these intercepts? What if $\Delta C_p < 0$? Is this physically meaningful? Is it relevant to biological macromolecules?

35. Suppose you have designed a four-helix bundle. A four-helix bundle is just a polypeptide that folds into four helices of approximately equal length, and whose helices are bundled together. The helices interact with each other in the core of the protein. Various structural techniques show that at room temperature the structure is highly dynamic and not very much like an organic crystal, though all four helices are intact. Thermal denaturation studies, however, indicate that the unfolding temperature of your designed protein is over 100 °C! Explain. How could the design be modified to reduce the melting temperature and increase the specificity of interactions in the protein core?

36. Living organisms have been described as "relatively stable" systems that "show an organized collective behavior which cannot be described in terms of an obvious (static) spatial order" and are "not near thermal equilibrium." Explain.

37. The synthesis of ATP under standard conditions requires 7.7 kcal mol^{-1}, and this is coupled to the movement of $2H^+$ across a mitochondrial membrane. Calculate the pH difference across the inner mitochondrial membrane needed to drive ATP synthesis at 25 °C.

38. Oxidation–reduction reactions in *E. coli* generate a pH gradient of +1 (outside to inside) and a voltage gradient of –120 mV (outside to inside). What free energy is made available by this proton motive force? β-Galactosides are transported along with H + ions. Calculated the maximum concentration ratio of β-galactoside that can result from the coupling of its transport to the proton motive force.

39. An empirical expression for the melting temperature of double-stranded DNA in the presence of NaCl is

$$T_m = 41.1X_{G+C} + 16.6 \log[Na^+] + 81.5, \quad (5.62)$$

where X_{G+C} is the mole fraction of G–C pairs. Given a 1000 base pair gene with 293 Gs and 321 Cs, calculate the sodium ion concentration at which it will have a melting temperature of 65 °C.

40. Use the following osmotic pressure data for horse hemoglobin in 0.2 M phosphate and at 3 °C to determine the molecular mass of the protein.

Concentration of hemoglobin (g/100 ml)	Osmotic pressure (cm H_2O)
0.65	3.84
0.81	3.82
1.11	3.51
1.24	3.79
1.65	3.46
1.78	3.82
2.17	3.82
2.54	3.40
2.98	3.76
3.52	3.80
3.90	3.74
4.89	4.00
6.06	3.94
8.01	4.27
8.89	4.36

41. The effect of pH on the osmotic pressure of sheep hemoglobin was investigated by Gilbert Adair (Chapter 7). The following data were obtained.

pH	Osmotic pressure (mmHg/1 g protein/100 ml)*
5.0	21.5
5.4	13.4
6.5	3.2
6.7	2.4
6.8	2.4
6.8	3.5
6.8	4.5
7.2	5.0
9.6	15.6
10.2	21.4

*1 mmHg = 133.322 ... Pa.

Plot the data and use them to deduce the isoelectric point of sheep hemoglobin.

42. Why would it not be a good idea to water your houseplants with boiling water?

43. Suggestion biochemical means by which one might test the origin of the heat produced by *Arum maculatum* (see Chapter 3). (Hint: use tissue extracts of the spadix and appendix of the plant and consider poisons that block either electron transport or oxidative phosphorylation).

44. It is sometimes said that the two terminal phosphoanhydride bonds of ATP are "high-energy" bonds. This implies that the energy released as free energy when the bond is cleaved is stored within the bond itself. Why is the term *high-energy bond* misleading?

45. Mg^{2+} ions interact with ATP under physiological conditions. What is the likely effect of this on the free energy of hydrolysis of ATP? Why?

Chapter 6

Statistical thermodynamics

A. Introduction

Classical thermodynamics provides a phenomenological description of nature, and then only some aspects of nature. The mathematical relationships of thermodynamics are precise, but they do not tell us the molecular origin of the properties of matter. This chapter discusses a means of gaining a *molecular interpretation* of thermodynamic quantities. If you've spotted the trend we set from page one of this book, you will guess that mathematics will play an important role here. The required mathematical ability is greater than in pages past, but not greatly so. As before, all the main ideas can be expressed relatively clearly in figures or easily in words. And it is important to be able to *use* the mathematics, even if it is more important to the biochemist to have a good sense of *what* the mathematics says! We could say that this is what distinguishes the biochemist from the mathematician. It may be that *mathematics* applies to *everything*, and it may be that few things are as sublime or beautiful as Leonhard Euler's contributions to number theory, but with apologies to Thomas Alva Edison, it is the physical biochemist who hires the mathematician, not the mathematician who hires the physical biochemist.[1]

[1] Euler (1707–1783), a Swiss mathematician and physicist who spent much of his life in Russia and Germany, was son of a pastor and a pastor's daughter. He made numerous important discoveries in diverse fields of mathematics and introduced much of the modern mathematical terminology and notation. He was astonishingly prolific in mathematical output, rivalled perhaps only by the Hungarian Paul Erdös. Euler's eyesight was poor but he had a photographic memory. For instance, he could recite the entire Aeneid of Virgil. Edison (1847–1931) was an American inventor and businessman who developed many devices which have had a great impact on life worldwide into the twenty-first century. Edison had lost much of his hearing by early adolescence. He is considered one of the most prolific inventors in history, holding 1097 US patents as well as many others in the United Kingdom, France, and Germany. His inventions include telegraphic devices, the phonograph and the light bulb. Edison is credited with the creation of the first industrial research laboratory, set up for the specific purpose of continual technological innovation and improvement.

The need for working out a theory of thermodynamics grew increasingly evident as the work of British chemist and physicist John Dalton (1766–1844) and Russian chemist Dmitri Ivanovich Mendele'ev (1834–1907) on the atomic theory of matter became more generally accepted after the middle of the nineteenth century.[2] Classical thermodynamics is built on the tacit assumption that many particles are present, and it deals with *macroscopic properties* of such collections of particles. Thermodynamics itself does not give a molecular view of what it describes; it does not explain thermodynamic quantities in terms of the *microscopic properties* of individual particles.

An example will help to illustrate the difference between macroscopic and microscopic properties. As discussed in Chapter 2 (and Appendix B), DSC can be used to make good quantitative measurements of the heat absorbed by a solution of macromolecules as the molecules undergo an order–disorder transition. For instance, DSC has been employed in the study of the thermodynamics of tRNA folding/unfolding. The measured value of the heat capacity, however, says practically nothing about *why* a particular value should be observed. What kinds of bonds are broken as the temperature increases? Which bonds? How many bonds? How does the heat absorbed in an order–disorder transition correspond to the structure that is melted? The result of a DSC experiment does not answer these questions in the absence of additional information. Moreover, the mathematical relations of thermodynamics do not provide the answers.

Statistical mechanics, by contrast, provides a molecular theory or interpretation of thermodynamic properties of macroscopic systems. It does this by linking the behavior of individual particles or parts of macromolecules to classical thermodynamic quantities like work, heat, and entropy. Statistical mechanics can be thought of as a bridge between the macroscopic and the microscopic properties of systems. Using statistical mechanics, one can begin to rationalize,

[2] The belief that the world is made of atoms is in fact much older than this; it simply was not widely accepted until the nineteenth century. Its earliest known exponent is the Greek philosopher Democritus (*c.* 460–*c.* 370 BC), who held that the atoms of the heavy elements combined to form the Earth, while the atoms of the light ones formed the heavenly bodies (planets and stars). If you have ever doubted the significance of the idea that the world is made of atoms, consider the following quotation. In his *Lectures on Physics*, American Nobel Laureate in Physics Richard Phillips Feynman (1918–1988) says, "If, in some cataclysm, all of scientific knowledge were to be destroyed, and only one sentence passed on to the next generation of creatures, what statement would contain the most information in the fewest words? I believe it is the *atomic hypothesis* (or the atomic *fact*, or whatever you wish to call it) that *all things are made of atoms – little particles that move around in perpetual motion, attracting each other when they are a little distance apart, but repelling upon being squeezed into one another*. In that one sentence, you will see, there is an *enormous* amount of information about the world, if just a little imagination and thinking are applied." (Emphasis in original.) Feynman's statement should not be taken lightly.

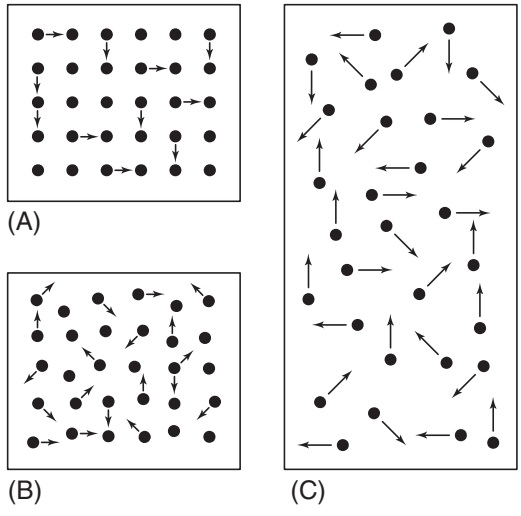


Fig. 6.1 Molecular interpretation of the three states of matter. In the solid phase (A), the molecules are in a regular array. Interactions between molecules occur, but overall there is practically no translational motion; each molecule stays put. Panel (B) depicts the liquid state. Molecules are free to translate in any direction. The volume, however, is not much different from in the solid state. In the gas phase (C), the volume occupied by the molecules is much larger than in the liquid phase or the solid phase. The average molecular speed is relatively high. After Figs. 4.1, 4.2, and 4.3 of Peusner (1974).

often in structural terms, how changes in a system connect up to the results of thermodynamic experiments.

You can easily see that the capture of light energy by plants is important for growth. The light energy provided and the change in mass of the plants can be quantified and analyzed in terms of classical thermodynamics. It is something different to describe energy capture in terms of the wavelength of photons involved and electronic bound states (Chapter 1). As discussed in Chapter 2, the inflation of a bicycle tire is a matter of stuffing a tube with air. The amount of air inside the tube can be measured as air pressure, but this says nothing at all about whether air is a continuous compressible fluid or a gaseous collection of particles which may or may not interact with each other. The physical properties of the different phases of water are of biological importance, and one can use classical thermodynamics to describe transitions between these phases. Such descriptions, however, say nothing at all about the structure of an individual water molecule or about how water molecules interact to give rise to the bulk properties of the solid phase, the liquid phase, or the gas phase (Fig. 6.1). Our interest in the present chapter is a molecular interpretation of macroscopic thermodynamic properties and the nature of connections between the world made of atoms and measurable thermodynamic quantities.

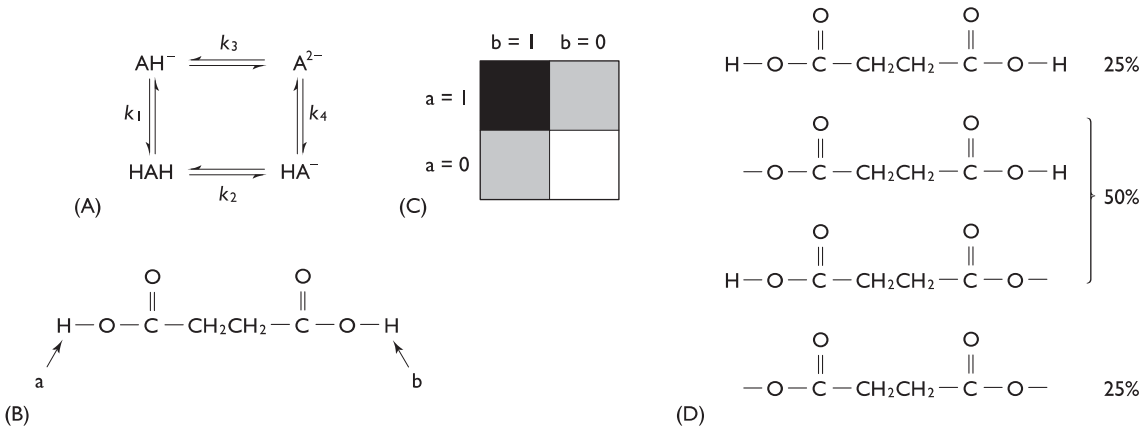
Fine. But *how* then does one go about providing a detailed description of molecular behavior? A macroscopic system might have on the order of 10^{23} particles, and on a practical level the complete description of each particle and each particle's motion seems *impossible*. Is there a way to keep track of what each and every particle is doing? On what level? How? The "problem" we face is not simply a matter of the number of particles involved. And that's because physics is good at describing interactions between two bodies, but not three or more! A middle way has to be found

between an explicit Newtonian (quantum mechanical, really) description of every last particle and a thermodynamic description of a system as a whole. The approach here is to use *statistical* methods and to assume that the average values of the *mechanical* variables of the molecules in a thermodynamic system (e.g. pressure and volume) are the same as the measurable quantities of classical thermodynamics, at least in the limit that the number of particles is very large ($>10^{10}$). The statistical treatment of the mechanical properties of molecules as they relate to thermodynamics is called statistical mechanics or statistical thermodynamics.

In Chapter 2 we described the Zeroth Law of Thermodynamics, showing how it justifies the concept of temperature. There, and in most sections of this book since then, heat has been treated as a sort of fluid that can pervade matter. In fact, people have historically treated heat transfer as the flow of a fluid from one body to another.[3] And extremely useful strides in mathematical physics have been made from treating heat as a fluid – one has only to think of Fourier analysis. But now we want to view heat more from a statistical perspective. Earlier we saw that *temperature measures the average kinetic energy of the molecules of a system*. The *average* value of some observable quantity is a *statistical* measure. Increases in temperature reflect average increases in translational motion – the motion of movement from one place to another. On this view, when two systems are in contact, energy is transferred between molecules as a result of *collisions*, not flow of a fluid-like heat from one system to the other. The transfer continues until a statistical *uniformity* is reached, which is thermal equilibrium.

Two chapters ago we discussed thermodynamic properties of acids and bases. We introduced the concept of pK_a, a macroscopic property, and learned something of its significance for biochemistry. That discussion required only a very qualitative idea of the structures of the acid and base involved. The model acid was HA, the conjugate base, A^-. Let's try to rationalize the measured macroscopic properties in terms of microscopic properties! Figure 6.2A shows a reaction scheme for a diprotic acid; there are two ionizable groups. The *k*s are *microscopic* equilibrium constants, each corresponding to the loss of a specific proton from a specific structure. In principle, either proton can be lost first in going from the fully protonated to the fully deprotonated state. If a diprotic acid is symmetrical, the two singly deprotonated states can be *indistinguishable*. Succinic acid, for example, an intermediate in the citric acid cycle, is a symmetrical molecule (Fig. 6.2B). The various states of the molecule can be represented diagrammatically as in Fig. 6.2C. The structures of the various species and the relative proportion of each possible type

[3] Count Rumford (Benjamin Thompson), founder of the Royal Institution and husband of the widow of the great French chemist Lavoisier, was born in America. Rumford showed that heat is not a fluid and proposed that it is a form of motion, or energy. He lived 1753–1814.



is given in panel (D). Thinking in such terms is part and parcel of giving a molecular interpretation of a thermodynamic phenomenon. Time to dig deeper.

Fig. 6.2 Molecular interpretation of acid–base equilibria. Panel (A) shows a thermodynamic cycle for a diprotic acid. The microscopic equilibrium constants contain information on the energetics of each step of the process. Panel (B) shows the molecular structure of succinic acid, an intermediate in the citric acid cycle. This molecule is symmetrical. The two dissociable protons are labeled "a" and "b." A schematic diagram of the possible states of a succinic acid molecule is given in panel (C). There are two possible ways in which the single-proton state can be formed from the two-proton state. Panel (D) shows the molecular structures of the various states of the molecule. Note that the two single-proton states are *indistinguishable*.

B. Diffusion

We have already encountered diffusion in this book on more than one occasion. In our discussion of gas pressure in Chapter 2, for example, the gas particles moved around the system in no preferred direction, i.e. at random. Then, in Chapter 3, perfume became distributed throughout a room as entropy increased. For a room at thermal equilibrium, the aromatic molecules will spread out on their own by diffusion. Diffusion also played a role in the sections on osmosis and dialysis in Chapter 5. So, you will have some idea of the character of diffusion now. This section develops a more quantitative and molecular understanding of diffusion. We are interested in diffusion because it is important in photosynthesis, respiration, molecular transport in cells, and the absorption of digested food from the gut into the bloodstream. So that's nearly everything, then.

Diffusion, unlike the thermodynamic concepts of energy, enthalpy, entropy, and all other strictly classical thermodynamic quantities, involves *time* explicitly. For this reason one might think the discussion belongs in a different place, perhaps in the chapter on kinetics. But it is included here because it helps to illustrate a very important aspect of statistical thermodynamics: that macroscopic properties of a system can be "built up" from the average behavior of its individual components. Moreover, the thinking involved here is essential to a mechanical view of the *dynamics* of equilibrium.

Suppose you have a system at equilibrium, for instance, a small volume of an aqueous solution of glucose. Some of the molecules will be moving rapidly, others slowly, but the average speed will relate to the kinetic energy that relates to the thermal energy, just

as before. Now, let the temperature of the system be constant. Any glucose molecule in the system will experience a sequence of collisions with solvent molecules and other glucose molecules. These collisions will involve molecules moving in no preferred direction, or *at random*. But the macroscopic level, there will be no net change; the system is at equilibrium.

We can describe the collisions made by a glucose molecule by using simple mathematics. We say "simple" because the equations *are* simple. Some students, though, find the thinking involved here more like trying to run a five-minute mile than walking from one class to another. So don't be surprised if you find that you want to read this section more than once.

In a unit of time **t**, our molecule will make an *average* of N collisions with other glucose molecules. If we increase the length of the observation time, say to 5**t**, the molecule will make and *average* of $5N$ such collisions. The *average* time between collisions, τ, a statistical quantity, is just

$$\tau = \mathbf{t}/N. \tag{6.1}$$

For comparison, suppose there are nine breaks during a two-hour TV program. Then the *average* time between breaks is 120 min/9 breaks = 13.3 min between breaks. With so many interruptions, it might be better to turn the tube off and find something more constructive to do!

What are the *odds* that one glucose molecule will collide with another glucose molecule during a small unit of time, Δt? This is just $\Delta t/\tau$. The larger the small unit of time, the larger the ratio, and the larger the *probability* of a collision. You can think about it another way. Instead of one molecule making N collisions, let's have N molecules make as many collisions as they will in a short period of time Δt. The number of collisions is then $N \times \Delta t/\tau$. Assuming that what is observed on one TV channel applies to them all, and using single-channel data for the calculation, we can evaluate the *odds* that a break will occur in a 1 min interval on any one of N channels. No surprise that the probability will rise with the number of channels!

How about the *probability* that the glucose molecule will *not* collide with another glucose molecule during an interval of time? You can compute it! Suppose there are N molecules in total, and suppose you watch the molecules for some amount of time, t. Suppose also that you find that the number of them that do *not* make a collision during this time is $N(t)$. As before, this notation means "the value of N is a function of time," not "$N \times t$." After an additional short period of time Δt, the number of molecules that still will *not* have made a collision is $N(t) + \Delta N(t)$. Don't be thrown off track by the "+"; this number must be *smaller* than $N(t)$! From above, the odds of a collision during a short period of time Δt among a group of N molecules is $N\Delta t/\tau$, so

$$N(t) + \Delta N(t) = N(t) - N(t)\Delta t/\tau. \tag{6.2}$$

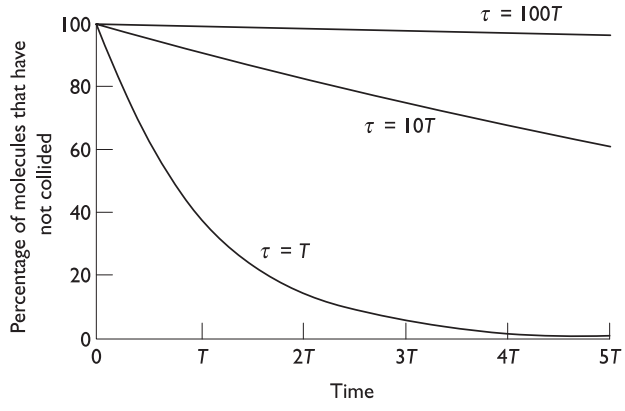


Fig. 6.3 Mathematical description of intermolecular collisions. The percentage of molecules that have not collided is plotted as a function of time. The shape of the curve in each case is a decaying exponential. Note how the rate of decay depends on τ, the average time between collisions. When τ is large, the percentage of molecules that have not collided declines relatively slowly.

Rearrangement gives

$$\Delta N(t)/\Delta t = -N(t)/\tau. \tag{6.3}$$

If the derivation is right, then the change per unit time in the number of molecules that have *not* collided is proportional to the ratio of the number of molecules that have *not* collided divided by the time between collisions. Just as the decrease with time in the number of TV channels on which an advertisement had *not* appeared after the start of observation would be proportional to the number of channels divided by the average time between breaks. A little calculus transforms Eqn. (6.3) into the following expression for *N*(t):

$$N(t) = N_0 \exp(-t/\tau), \tag{6.4}$$

where N_0 is the number of molecules being observed. A plot of $N(t)$ against time is shown in Fig. 6.3. $N(t)$ decreases exponentially with time, and the amount of time required for a decrease by a factor of e^{-1} is τ.

Now consider the distance a molecule will travel between two collisions. The *mean free path* of a particle, l, is defined as the average time between collisions, τ, times the *average* speed, v:

$$l = \tau v. \tag{6.5}$$

As required, this quantity has units of length, [s][m/s] = [m]. The greater the average speed of a molecule, the longer the mean free path for a given value of τ. If the concentration of a type of particle increases but the temperature is constant, the average speed of the particles does not change but τ decreases. Correspondingly, l decreases, and collisions are more frequent. If the average time between collisions between a bicyclist and flying insects is 6 s, and the average speed of the cyclist on her way to university is 15 m.p.h., the mean free path is just 1/40 of a mile. So it might be a good idea to wear goggles and nose and mouth protection (in addition to wet weather gear, especially in the UK)! The *odds* of a collision along a short stretch of cycle path will vary with the number of bugs per unit volume (distributed at random and flying in all directions), **N**; the size of the cyclist (actually, the

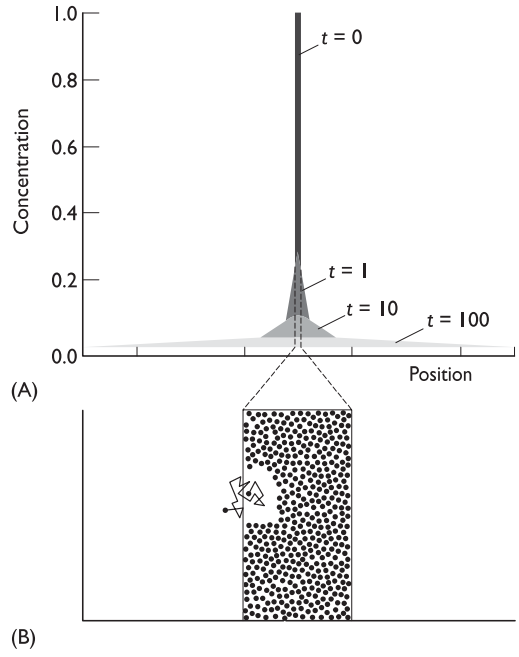


Fig. 6.4 Diffusion. Panel (A) shows how a concentrated substance spreads out in time. The area below the curve is the same in each case (the number of solute particles is constant). A magnified view of the concentrated solute is shown in panel (B). At this resolution it is possible to see individual solute particles. The trajectory of one particle is shown to the left, with the position indicated at the end of each time increment. The macroscopic result of all solute particles showing such microscopic behavior is shown in panel (A). The "random walk" of an individual particle is known as Brownian motion. Based on Figs. 4.7 and 4.8 of van Holde (1985).

frontal "cross-sectional" area of the cyclist on the bike – it would clearly be a good idea to be fit and trim in this example), A; and the length of the short stretch of cycle path, Δx:

$$\text{odds of cyclist–bug collision in } \Delta x = A\mathbf{N}\Delta x. \tag{6.6}$$

During weeks of the year when **N** is large, it might be a good idea to walk or use public transportation! And similarly for our glucose molecule.

Television involves programming, and cyclists decide which direction to ride in. But in diffusion, gas particles move around at *random*. Do the analogies we've used tell us anything? Yes! They help us to picture mechanical aspects of the mathematics used to describe diffusion. We also need randomness, though, as it is a basic assumption of Einstein's description of diffusion; the molecules have no preferred direction. The assumption is based on a key observation: Brownian motion, the microscopic and rapid motions that bits of particulate matter display when suspended in a solvent (Fig. 6.4).

Well, OK, we have acquired a deeper sense of collisions between molecules in solution. But how is the knowledge useful in the biological sciences? Suppose we introduce a small amount of concentrated glucose into a volume of water. What will happen? The glucose will distribute itself throughout the volume – by the Second Law of Thermodynamics! Diffusion tells us something about particle behavior as the system moves towards equilibrium. But it also tells us about particle behavior after the system has reached equilibrium. For in fact, from a qualitative point of view, there is no *essential* difference between the diffusion of non-interacting molecules in a volume in a non-equilibrium state and in an equilibrium state.

("Non-interacting" means that the molecules do not bind or repel each other any more than they bind or repel solvent molecules, not that they don't collide with each other.) The average length of time between solute–solute collisions will be smallest before the concentrated solution is diluted, and it will be largest at equilibrium.

Why else will you want to know about diffusion? Here's one reason of many: it's the "strategy" the body uses to take up nutrients from the gastrointestinal tract. Sucrose, lactose, maltose, and glucose chains, for example, are broken down into smaller units by enzymes on hair-like protrusions of the small intestine called microvilli. The sugars then cross the epithelium and enter the bloodstream by active transport or *facilitated diffusion*; they are too big for simple diffusion across the membrane! Active transport requires a membrane protein and additional energy; facilitated diffusion involves a channel through which sugar molecules can move down their concentration gradient.

Diffusion also helps to understand topics like enzyme function. Suppose you have a soluble substrate molecule in solution, e.g. glucose, and an enzyme that recognizes it, e.g. hexokinase. Suppose also that you can measure the rate of catalysis. What limits the rate? Is it the speed at which catalysis occurs? Maybe, and in that case the limitation would be a matter of the molecular mechanism of the reaction, or biochemistry. But it may be that the fundamental limitation on rate is the time required for the substrate to make a collision with the enzyme, how quickly the molecules are moving in solution (the temperature of the system) and concentration. There is an important difference between these possibilities. See Chapter 8.

C. Boltzmann distribution

Let's return to the subject of osmosis (Chapter 5). As we have seen, the underlying conditions for the phenomenon to occur under isothermal and isobaric conditions are solute particles that can move throughout a volume of solvent, the absence of a constraint on the volume of solution, and the separation of the solution from solvent by a semipermeable membrane. We stated with confidence that osmosis is an entropy-driven process, providing perhaps less evidence than you had liked. Let's use this opportunity to complete the discussion.

In Chapter 3 we derived the formula $\Delta S = nR\ln(V_f/V_i)$ for the reversible expansion of an ideal gas. Now divide up the volume V of gas into a bunch of notional volume elements. For simplicity, assume that each element is identical in size. When the overall volume of gas doubles, the number of volume elements will double. For even greater simplicity, let's think in just two dimensions, so that each volume element can be represented as an area. Our gas container can now be drawn on one side of a sheet of paper, as shown in Fig. 6.5. If the expansion corresponds to some factor of volume change, say, 272/25, the number of volume elements increases by the same ratio.

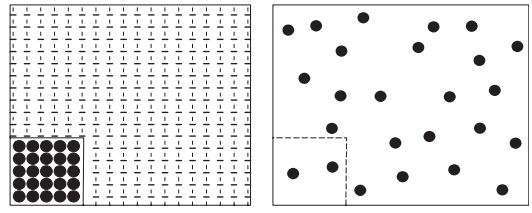


Fig. 6.5 Expansion of perfume throughout a room. In panel (A), the molecules are close together, as in a solid or a liquid. Only 25 of the $16 \times 17 = 272$ volume elements are occupied. In panel (B), the 25 molecules are spread throughout the volume. In other words, the volume accessible to the molecules is much greater in panel (B) than in panel (A), by a factor of 272/25. The entropy of the molecules is much greater in panel (B) than in panel (A). Based on Fig. 1.2 of Wrigglesworth (1997).

What role do the volume elements play in the present scene? They help to consider the number of ways of arranging particles. In Fig. 6.5A, for example, where all 25 gas molecules are stuffed into a tiny space, a mere 25 volume elements are occupied (1 molecule per volume element), and 247 elements are empty. The arrangement of molecules in panel (A) resembles the close packing of molecules in a crystal. But let's keep things simple and suppose we have a gas. The gas pressure is high! By contrast, in panel (B), 25 gas molecules are spread throughout the entire 272 volume elements. There are many more ways of placing 25 molecules in 272 elements than in 25 elements! As we shall see below, combining the division of space into volume elements with the $\Delta S = nR\ln(V_f/V_i)$ relationship from classical thermodynamics leads to the famous Boltzmann equation of statistical mechanics.

Energy "flows" from one part of the world to another, energy is transformed from one form to another, and in the midst of change the total amount of energy stays the same (Chapters 1 and 2). There are many ways in which a given quantity of energy can be distributed (Chapter 1). Here we are concerned not so much with the possibility of more than one distribution as with the relative *probability* of each possible distribution. And given a closed *macroscopic* system, we should guess that the *equilibrium* distribution will be the *most probable* distribution. There will be fluctuations of the system at equilibrium, but unless the system is particularly small, all probable fluctuations will be essentially negligible in magnitude. *If the number of molecules in a system is large, the behavior of the system will coincide with that predicted from a statistical consideration of the behavior of individual molecules of the system.*

As we saw in Chapter 1, photosystems are very good at absorbing light within a certain band of wavelengths. Indeed, this is why plants have a certain color: they absorb red and blue light but reflect green light. The absorption of a photon of a certain wavelength requires a suitable electronic structure. When the light-absorbing molecule adsorbs a photon of the "right" wavelength, the electron is elevated from its ground state to an excited state. The difference in energy between states is effectively *identical* to the energy of the photon. Energy is conserved! The important point here is that there are electronic energy *levels* in light-absorbing molecules: the electron will not be found at an intermediate energy; it is either in the

ground (low-energy) state or in some excited state, not in between (Fig. 6.6). More can be said about energy states and levels.

Let's choose as our system an ensemble of *indistinguishable* particles. The particles are indistinguishable because there is no way of telling them apart; maybe something akin to the sets of twins who drive the action in *The Comedy of Errors* – *indistinguishable* unless labels are attached. We assume that the system is such that its energy spectrum is discrete, as in Fig. 6.6. In other words, the particles are "bound," like an electron in a particular molecular orbital. Note that this situation differs from that modeled by Eqn. (1.1), in which the energy of a photon varies smoothly with wavelength. A photon travelling through space can have "any" energy, at least in principle. But if the photon will interact with matter, it needs to have a wavelength that corresponds to the energy difference between two electronic states of the matter.

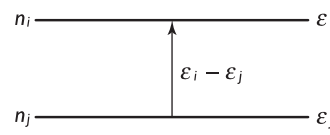


Fig. 6.6 Energy level diagram. There are n_i particles in energy level ε_i and n_j particles in energy level ε_j. The difference in energy between these states is $\varepsilon_i - \varepsilon_j$. When a photon is absorbed by chlorophyll or some other pigment molecule, the energy of the photon (hc/λ) is $\varepsilon_i - \varepsilon_j$, where i and j correspond respectively to the excited and ground states of the electron.

Note that an energy state of a single particle is *not* the same thing as a thermodynamic state of an entire system of particles. How can we distinguish between these cases? Suppose a house draws 3 A (amperes) of current, and suppose the only appliances on are three lights. Let each room of the house correspond to a particular energy state or level: 0 if the light is off, and 1 if the light is on. We could say that the state of the system, the house, is 3, a quantity that can be measured by putting a meter on the electric line connecting the house to the main electrical supply. This measured value, however, tells us nothing at all about whether a light is on in a particular room of the house. Are the three rooms the kitchen, sitting room, and bathroom, or the dining room, foyer, and a bedroom? There are clearly different combinations of lights that correspond to a specific current value, and if you are monitoring from outside the house, you can know the overall state of the system but not the state of each light. A light cannot be "half-way" on (this house has no dimmer switches!), so the state of the house must be an integral multiple of 1.

Now, suppose you have a definite number of particles, say seven, and let the measured total energy of the system be 15ε, where ε represents a unit of energy. You want to know *how many* different ways there are of arranging the particles so that two criteria are met. All the particles are accounted for ($\Sigma n_i = 7$; in words, the sum over all the individual energy levels (Σ) of the number of particles in energy level i, n_i, is 7), and the total energy is 15ε ($\Sigma n_i \varepsilon_i = 15\varepsilon$; in words, the sum over all the individual energy levels of the number of particles in energy level i times the energy of that level, ε_i, is 15ε). These two constraints make the system look the same from the outside, regardless of the specific arrangement of particles on the inside. There are only seven ways of arranging seven *indistinguishable* particles if six have energy ε: the seventh one *must* have energy 9ε. Figure 6.7 shows three other ways in which the particle and energy constraints can be met simultaneously. All these configurations are *possible*, but are they equally *probable*?

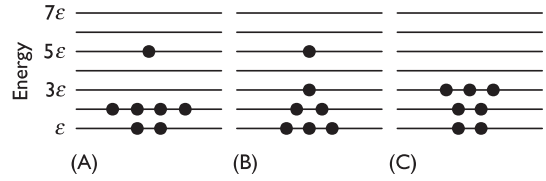


Fig. 6.7 Different ways of arranging seven indistinguishable particles under the constraints of constant total energy and constant number of particles. Arrangement (B) appears to be the most stable arrangement of the three. Based on Fig. 1.2 of van Holde (1985).

To answer the question, let's *assume* that *the most probable distribution is the one that corresponds to the largest number of ways of arranging particles in a given configuration*. It is easy to show that configuration (B) is more probable than (A) or (C). (This does not mean that *configuration* (B) is necessarily the *most probable* distribution of all possible distributions which satisfying both constraints.) Suppose we have three identical pea seeds, and let the seeds be indistinguishable. The seeds can be arranged into two "piles" of *configuration* {1,2}; two seeds are congregating in a single pile and one is a Lone Ranger. How many different ways can the seeds be arranged in two piles? Three! The lone seed can be any one of them, as shown in Fig. 6.8, and the other two form the second pile. If there are six seeds distributed into three piles with configuration {1,2,3}, the number of arrangements is 60 (prove it!).

In general the *number of ways*, Ω, of arranging N identical particles in configuration $\{n_1, n_2, \ldots\}$, with n_1 in one group, n_2 in another, n_3 in another, and so on, is

$$\Omega = N!/(n_1!n_2!n_3!...n_i!...), \tag{6.7}$$

where $x!$ ("x factorial") denotes $x(x-1)(x-2)\ \ldots\ 1$. A key assumption here is that any single particle of the system can occupy any state of the system with equally probability: the *postulate of equal a priori probability* requires that nothing predispose a particle to be in a particular state. And the two constraints of particle number and total energy must still be satisfied. Ω is called the *statistical weight* of the configuration. An example of Eqn. (6.7) in action is as follows. Suppose we have 20 identical objects ($N = 20$) with configuration {1, 0, 3, 5, 10, 1}; the objects are gathered into six different piles. In this case $\Omega = 20!\ /\ (1! \times 0! \times 3! \times 5! \times 10! \times 1!) = 9.31 \times 10^8$, a large number! ($0! = 1$ for reasons we cannot go into here.) Note that *the statistical weight depends only on the distribution of particles*, and not on whether there is one particle in energy state 1, three in state 3, five in state 4, and so on.

On the assumption that the most probable distribution is the one having the largest number of arrangements of particles, identifying the most probable distribution is the same as maximizing Ω under

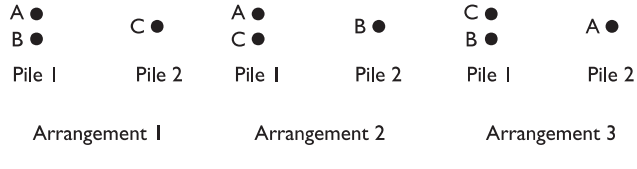


Fig. 6.8 Three possible ways of arranging three indistinguishable particles of configuration {1,2}. Labels have been attached to the particles to distinguish one from another.

the constraint that the number of particles and the total energy are constant. *Maximizing* the number of ways of arranging particles? That sounds curiously like the entropy being maximal at equilibrium; Ω does indeed resemble the entropy function discussed in Chapter 3, particularly in the context of $S_f - S_i = \Delta S = nR\ln(V_f/V_i) = nR\ln V_f - nR\ln V_i$. In fact, the connection between S and Ω, known as the *Boltzmann equation*, is

$$S = N_A k_B \ln\Omega = R\ln\Omega, \tag{6.8}$$

where k_B, the Boltzmann constant (1.381×10^{-23} J K^{-1}), is a fundamental constant of physics, and N_A is Avogadro's number.[4] If the system changes from state 1 to state 2, the molar entropy change is $S_2 - S_1 = N_A k_B(\ln\Omega_2 - \ln\Omega_1) = R\ln(\Omega_2/\Omega_1)$.

Note that by Eqn. (6.8) S is an *extensive* thermodynamic quantity; if our system consists of two parts, A and B, then the total entropy is $S_A + S_B$. If Ω_A is the number of ways of arranging particles in part A, and Ω_B is the number of ways of arranging particles in part B, and S is the total entropy, then

$$\begin{aligned} S &= S_A + S_B = N_A k_B \ln\Omega_A + N_A k_B \ln\Omega_B = N_A k_B(\ln\Omega_A + \ln\Omega_B) \\ &= N_A k_B \ln(\Omega_A\Omega_B) = N_A k_B \ln\Omega, \end{aligned} \tag{6.9}$$

where $\Omega = \Omega_A\Omega_B$. Relationships (6.8) and (6.9) were put forward in the late nineteenth century by Ludwig Boltzmann (1844–1906), an Austrian theoretical physicist. They are derived from $U = \Sigma n_i\varepsilon_i$, the same U we saw back in Chapter 3. This tells us that Boltzmann's discovery was not amazingly fortuitous; he did not just "happen" upon a *mathematical* relationship (Eqn. (6.8)) that works so well. The fact is that he had a good rough idea of what he was looking for. He started from the *physical* point of view, and he made a number of clever guesses and approximations. For instance, he assumed not only that all gas particles of an ideal gas move in all possible directions, but also that all the particles move with the same speed. A simplifying assumption, to be sure, but one that goes a long way toward understanding how things work. Remember from Chapter 1 that particle speeds won't all be the same; there will be some form of *distribution*. Boltzmann's particle-speed approximation works because thermal energy is proportional to the average kinetic energy, and the average energy is consistent with many possible distributions, including the one where all particles move at the same speed! Boltzmann also built on the work of Carnot and Clausius and went far beyond what they had done. This is not to diminish the work of Boltzmann, for there is no doubt that he made an extremely important contribution to physics! It is, rather, to put Boltzmann's work in perspective, and to provide clues as to why his mathematical results have proved so valuable.

[4] Named after the Italian physicist Amadeo conte di Quaregna Avogadro (1776–1856).

Boltzmann did not stop with Eqn. (6.9). He was able to show by means of a few mathematical tricks that need not concern us here, that when $N = \Sigma n_i$ is very large, say on the order of N_A,

$$n_i = n_1 \exp[-\alpha(\varepsilon_i - \varepsilon_1)], \tag{6.10}$$

where n_i is the number of particles with energy ε_i, n_1 is the number of particles with the lowest energy, ε_1, and α is a constant. This equation is called *the Boltzmann energy distribution* or ordering principle. *The Boltzmann distribution is the most probable distribution for a large system at or very near equilibrium.*

If energy level i of a single particle corresponds to ω_i arrangements, then

$$n_i = n_1 \exp[-\alpha(\varepsilon_i - \varepsilon_1)](\omega_i/\omega_1). \tag{6.11}$$

State 1 is the *reference state*, and measurement of the energy of state i is made relative to it. The constant term in the argument of the exponential, α, is $1/k_B T$, where T is the absolute temperature; ω_i is known as the *degeneracy* of state i. Note that we are now using the lower case of Ω. This is because here we are interested in the possible ways of ordering the atoms of a single molecule, not the number of ways of arranging a given number of molecules in space. In other words, the entropy of a system refers not only to the different ways of arranging particles in space but also to the ways in which the atoms of an individual molecule can be arranged. This way, the entropy term includes the various possible arrangements of a chemical group owing to bond rotation and the like.

To illustrate the usefulness of Eqn. (6.11) by way of example, we turn to the subject of disulfide bonds. These covalent links form between cysteine residues, usually of the same polypeptide. One of our favorite proteins, hen egg white lysozyme, has four intramolecular disulfide bonds. One of these bonds joins residues 6 and 127. There are only 129 amino acids in hen lysozyme, so disulfide bond 6,127 connects the N- and C-termini of the protein (Fig. 6.9). It so happens that 6,127 is mostly solvent-exposed (this can be seen by examination of the crystallographic structure), while the other disulfides are solvent-inaccessible. This makes 6,127 susceptible to selective reduction and chemical modification. One can carry out a certain redox reaction and obtain a three-disulfide derivative of hen lysozyme in which 6,127 alone is modified (3SS-HEWL).

Now, the thermodynamic properties of 3SS-HEWL have been investigated by scanning microcalorimetry. Modified lysozyme exhibits approximately "two-state" behavior throughout the acidic pH range and in the temperature range 0–100 °C, and ΔH_d is essentially the same for 3SS-HEWL as the wild-type (WT) enzyme at the same temperature. Thus, the main thermodynamic consequence of removal of disulfide 6,127 is to increase the *entropy* difference between the folded and unfolded states. Because the enthalpy of unfolding is about the same for both WT-HEWL and 3SS-HEWL, one

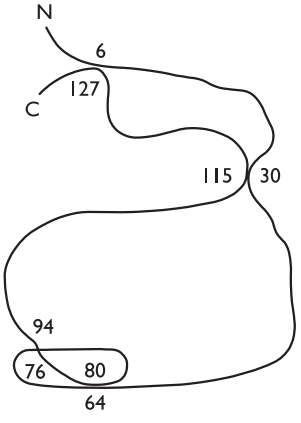


Fig. 6.9 Schematic diagram of the topology of hen egg white lysozyme (and α-lactalbumin). The N-terminus and C-terminus are marked, as are the residue numbers of the cysteine residues, all of which are involved in disulfide bonds. Note that disulfide 6,127 joins the chain termini together, forming a loop with disulfide bridge 30,115. Breaking disulfide 6,127 opens this loop, increasing the mobility at equilibrium of all the encompassed amino acid residues but especially those at the chain termini.

might expect that the folded forms of both proteins have about the same structure, about the same number of hydrogen bonds, about the same number and type of van der Waals interactions, and so on. This guess has been confirmed by determination of the structure of 3SS-HEWL by X-ray crystallography.

Assuming a limited difference in entropy between the folded state of the wild-type enzyme and the folded state of the derivative (an approximation, of course), the difference in entropy of unfolding between the modified and unmodified proteins, $\Delta\Delta S$, is interpreted as an increase in *conformational entropy*. This term is defined as the number of different ways that the covalent structure of a protein can be arranged in space at a given energy. From experiments, $\Delta\Delta S \approx 25$ cal mol^{-1} K^{-1} at 25 °C for 3SS-HEWL; the entropy of the unfolded derivative is considerably greater than the entropy of the unfolded WT protein. It makes sense.

Equation (6.8) can be used to calculate the increase in the number of conformations of the unfolded state (U) as follows.

$$\begin{aligned}\Delta\Delta S_{\text{conf,4SS}\rightarrow\text{3SS}} &= \Delta S_{\text{d,3SS}} - \Delta S_{\text{d,WT}} \\ &= N_A k_B(\ln\omega_{\text{U,3SS}}/\omega_{\text{F,3SS}} - \ln\omega_{\text{U,WT}}/\omega_{\text{F,WT}}).\end{aligned} \quad (6.12)$$

Assuming that the entropy of the folded state is the same in both forms of the enzyme, the ω_F terms cancel out, leaving

$$\Delta\Delta S_{\text{conf,4SS}\rightarrow\text{3SS}} = N_A k_B \ln(\omega_{\text{U,3SS}}/\omega_{\text{U,WT}}) = N_A k_B(\ln\omega_{\text{U,3SS}} - ln\omega_{\text{U,WT}}). \quad (6.13)$$

Substituting in known values,

$$\begin{aligned}25 \text{ cal mol}^{-1}\text{K}^{-1} = &(6.02\times10^{23} \text{ mol}^{-1}) \\ &\times(1.381\times10^{-23}\text{J K}^{-1})\times0.239 \text{ cal J}^{-1} \\ &\times\ln(\omega_{\text{U,3SS}}/\omega_{\text{U,WT}})\end{aligned} \quad (6.14)$$

from which

$$\omega_{\text{U,3SS}}/\omega_{\text{U,WT}} \approx 3\times10^5. \quad (6.15)$$

That is, breaking disulfide bond 6,127 of hen lysozyme results in a *c.* 300 000-fold increase in the number of conformations of the unfolded state, assuming that the entire magnitude of ΔS can be attributed to a redistribution of the preferred conformations of the unfolded state – an average increase of over 2000 conformations per residue on the loss a single disulfide bond! A more realistic interpretation would be that if the folded state is uniformly rigid, there is an even larger increase in the number of conformations per amino residue at the N- and C-termini and a smaller increase elsewhere.

Let's revisit the assumption that the folded-state entropies of the two forms are the same. According to Eqn. (6.11),

$$\omega_{\text{U,WT}}/\omega_{\text{U,3SS}} \propto n_{\text{U,WT}}/n_{\text{U,3SS}}. \quad (6.16)$$

This says that the distribution of conformations in the unfolded state of the three-disulfide derivative is much more probable than

the distribution of conformations in the unfolded state of the wild-type protein when disulfide bond 6,127 is severed. That is, given a sample of about 300 000 three-disulfide derivative lysozyme molecules, we should expect only about one of them on the average to have both Cys residues as close in space as in the WT enzyme. In short, *disulfide bonds play a key role in stabilizing proteins*.

D. Partition function

Equation (6.11) says that as $T \to 0$, the argument of the exponential becomes very large and negative, and $n_i \to 0$ for all $i > 1$. As $T \to \infty$, the denominator in the exponential term becomes so large that the magnitude of the numerator becomes irrelevant, and all energy levels are equally populated. Between these extremes, the probability that a particle is in state j is

$$P_j = \frac{n_j}{N} = \frac{n_j}{\sum n_i} = \left(\frac{n_j}{n_1}\right) \Big/ \sum_i \left(\frac{\omega_i}{\omega_1}\right)\left(\frac{-(\varepsilon_i - \varepsilon_1)}{k_B T}\right) \tag{6.17}$$

$$= \frac{\omega_j \exp\left(\frac{-(\varepsilon_j - \varepsilon_1)}{k_B T}\right)}{\sum_i \omega_i \exp\left(\frac{-(\varepsilon_i - \varepsilon_1)}{k_B T}\right)}. \tag{6.18}$$

So $\Sigma P_j = 1$. It is clear from Eqn. (6.17) that P_j increases with increasing n_j and decreasing $\Delta\varepsilon$. Thus, the closer energy state j is to the lowest energy state (the ground state), the more likely it is to be occupied. If we now show ω_1 in the numerator and denominator of Eqn. (6.18) and rearrange, we obtain

$$P_j = \frac{\frac{\omega_j}{\omega_1} \exp\left(\frac{-(\varepsilon_j - \varepsilon_1)}{k_B T}\right)}{\sum_i \frac{\omega_i}{\omega_1} \exp\left(\frac{-(\varepsilon_i - \varepsilon_1)}{k_B T}\right)} \tag{6.19}$$

$$= \frac{\exp[\ln\omega_j - \ln\omega_1] \exp\left(\frac{-(\varepsilon_j - \varepsilon_1)}{k_B T}\right)}{\sum_i \exp[\ln\omega_i - \ln\omega_1] \exp\left(\frac{-(\varepsilon_i - \varepsilon_1)}{k_B T}\right)} \tag{6.20}$$

$$= \frac{\exp\left(\frac{-[(\varepsilon_j - \varepsilon_i) - k_B(\ln\omega_j - \ln\omega_1)T]}{k_B T}\right)}{\sum_i \exp\left(\frac{-[(\varepsilon_i - \varepsilon_1) - k_B(\ln\omega_i - \ln\omega_1)T]}{k_B T}\right)}. \tag{6.21}$$

Putting the arguments of the exponential terms on a molar basis (by multiplying numerator and denominator by N_A), we have

$$P_j = \frac{\exp\left(\frac{-[(H_j - H_1) - T(S_j - S_1)]}{RT}\right)}{\sum_i \exp\left(\frac{-[H_i - H_1) - T(S_i - S_1)]}{RT}\right)} = \frac{K_j}{\sum_i K_j} = \frac{K_j}{Q}, \tag{6.22}$$

where $N_A\varepsilon_i$ and $N_A k \ln\omega_i$ have been *interpreted*, respectively, as the thermodynamic functions H_i (see Eqn. (2.7)) and S_i (see Eqn. (6.7) and Chapter 3). Note that $\exp(-\Delta G/RT)$ has been written as K, as though K were an equilibrium constant. K is an equilibrium constant! Each exponential term of the form $\exp(-\Delta G/RT)$ is a *Boltzmann factor*, and there is one of these for each accessible state of the system (the reference state has a Boltzmann factor of 1, $\Delta G = 0$ and $e^0 = 1$). Note also that if all the probabilities in Eqn. (6.22) are summed, the result is 1, as necessary. The sum in the denominator, the sum of all the Boltzmann factors, is called the (canonical) *partition function*. It is often symbolized as Q. Equation (6.22) tells us that, given a large collection of molecules, the fraction of them that will be in state j at any given time is given by the ratio of the Boltzmann factor for state j divided by the sum of all the Boltzmann factors, Q.

The various terms in the partition function reflect how energy is distributed in the system under specified conditions. The partition function contains *all* the thermodynamic information available on the system; it is a key concept in statistical thermodynamics. As useful as the partition function is, however, one's being able to write it down for a given situation and manipulate it flawlessly does not necessarily imply a good understanding of the physics of a situation, even more the biophysics of a situation. It is an idea worth pondering. One might be tempted to think that if statistical mechanics is useful and provides a molecular interpretation of thermodynamics, then learning classical thermodynamics is a waste of time. No! For it often turns out that a classical description of a situation is more intuitive and just plain simpler than a full-blown statistical treatment. Living is difficult enough without making things harder than they need to be. There are times, however, when statistical mechanics does not only what classical thermodynamics cannot do but also just what is needed. So, if you had to make a choice, it would be a good idea to prefer to know both classical thermodynamics and statistical mechanics.

E. Analysis of thermodynamic data

We now wish to make a step towards connecting Eqn. (6.22) to measurements that can be made in the laboratory. P_j stands for the population of state j, or the fraction of molecules in state j. As we shall see below, this is important for rationalizing the measured value of an observable property of a system, whether the observable quantity is the ellipticity of a sample of macromolecules at a given wavelength, the intrinsic fluorescence emission at a given wavelength, the partial heat capacity at a given temperature, or the intrinsic viscosity of sample at a given pH. The idea is a rather general one. Moreover, Eqn. (6.22) applies whether the macromolecules are proteins, strands of DNA, lipids, carbohydrates, or different combinations of these types of macromolecule. There is something amazing in being able to

use the relatively simple mathematical theory outlined above to analyze the results of such a wide variety of methods and samples. It is, perhaps, what Hungarian-American Physicist and Nobel laureate Eugene P. Wigner (1902–1995) meant by "the unreasonable effectiveness of mathematics in the natural sciences."

We saw in Chapter 2 that the thermal denaturation of hen lysozyme in aqueous solvent is a two-state, first-order phase transition. That is, the observed folding/unfolding properties of the protein can be modeled as arising from a folded state and an unfolded state only. No partly folded states are present? Some such species are present, but the fraction of them is so small in this case that deviation from two-state behavior is within the error of other aspects of the experiment (e.g. determination of protein concentration). Protein folding/unfolding, for which the free energy difference between states corresponds to a relatively intuitive change in structure, isn't the only process that can be modeled as a two-state process, but *any* system in which there are two main states which differ by some amount of energy. For example, in the binding of a ligand to a single binding site on a macromolecule, the two states are the unbound and the bound, and the energy difference between states is the binding free energy. Like its cousins in the thermodynamic family of functions, binding energy is a sort of all-encompassing quantity that can and usually does include various other contributions, for example, from structural rearrangement. One can easily imagine, though, a situation where the structure of the macromolecule does not change substantially on binding, say, when binding is mainly electrostatic in origin and the geometry of the binding site does not change on binding. Such situations can be rationalized in terms of the so-called *two-state approximation*.

In doing a biochemistry experiment, you can reduce the susceptibility of a measurement to ever-whimsical thermal fluctuations and increase the signal-to-noise ratio by increasing the number of molecules present. There might be other very practical reasons why your experiment will be done at a certain concentration. For instance, to do a protein NMR experiment, you might need as much as 1 ml of a 1 mM sample to get a decent signal. Unfortunately, though, many proteins are not soluble at such a high concentration (See Chapter 5)! And before the development of recombinant DNA technology and production of recombinant protein, very few proteins could be obtained in such large quantities, providing a historical dimension as to why the structures of myoglobin, hemoglobin, and lysozyme were the first ones to be determined at high resolution. Fluorescence emission, by contrast, is so sensitive that a quantity orders of magnitude smaller is needed for an experiment. In fact, it can be a liability to have too high a sample concentration in a fluorimetry experiment, even if solubility is not a concern. But fluorimetry can hardly provide the level of structural information obtainable by NMR! Regardless of the technique, in such experiments implicit use is made of the *ergodic hypothesis*. According to this

idea, *the average short-duration behavior of a large collection of identical particles is assumed to be identical to the average long-duration behavior of a single particle under given conditions.* In other words, the system under study is a collection of molecules which, at any given moment, represent what would be observed for a single molecule over a long period of time. An *observable quantity* is "normalized" by the concentration of the molecule of interest, and the average value is *assumed* to represent the average properties of a single molecule.

Let O represent an observable quantity of interest – heat capacity, intrinsic fluorescence, resonance, what-you-like. The measured value is the sum of the fractional contributions of the accessible states:

$$\langle O \rangle = \Sigma P_i o_i \tag{6.23}$$

where $\langle O \rangle$ is the average value of O and o_i is the contribution of state i to O, that is the characteristic value of the observable quantity for this state. Regardless of the number of states, the set of numbers $\{P_i\}$ defines the *distribution* of states. The distribution tells us the fraction of particles in state 1, the fraction in state 2, the fraction in state 3, and so on, just as before.

For the simple case of a two-state system (A $\Leftrightarrow$ B), Eqn. (6.23) becomes

$$\langle O \rangle = P_A o_A + P_B o_B = \frac{1}{1+K} o_A + \frac{K}{1+K} o_B, \tag{6.24}$$

where $0 < P_i < 1$, $i = $ A, B. For example, suppose you are using a fluorimeter to study the reversible binding of a protein to DNA. Assume that the protein and protein–DNA complex have the necessary fluorescence emission properties to distinguish the bound state from the unbound state of the protein. Assume also that the ligand exhibits a negligible contribution to the fluorescence emission in the unbound state at the wavelength of interest, or that any contribution it does make can be accounted for. Assuming that there has been no problem in data collection, it is not necessary to know anything about how a fluorimeter works or the physical basis of fluorescence emission to analyze the data resulting from the experiment. In fact, it is not even necessary to know that there are just two states! We're going to treat the situation rather mathematically. And for the sake of simplicity we'll assume that there are two and only two states, and that there is a difference in the observed value of fluorescence when binding occurs. Real life is generally more complicated, but some simple examples, like toys, can reveal deep and useful truths.

Now, suppose your experiment shows that the fluorescence intensity of your folded but unbound protein is 225 (in relative fluorescence units), and that the intensity of the same concentration of protein at the same temperature but in the presence of a sufficient amount of DNA ligand to saturate the binding sites is 735. Then $o_A = 225$, $o_B = 735$. In the absence of ligand, $P_A = 1$ and $O = 225$; in the presence of large quantities of ligand, $P_B = 1$ and $O = 735$. Intermediate concentrations of DNA ligand will give a fluorescence

intensity between these extremes. The measured value O is an average of o_A and o_B that is weighted by P_A and P_B, the proportion of molecules in the bound state and unbound state, respectively.

It is possible to calculate the population of a state, say A. To do this, we rewrite P_B in Eqn. (6.24) as $1 - P_A$ and solve for P_A. The result is

$$P_A = \frac{\langle O \rangle - o_B}{o_A - o_B} \tag{6.25}$$

In words, the population of state A (or state B) can be found from the baseline measurements (225 and 735 in the example above), and the measured value will correspond to an intermediate degree of saturation of binding sites.

Qualitatively speaking, the analysis would proceed in basically the same way if your observable were an NMR chemical shift instead of fluorescence intensity. Suppose you are using NMR to measure the pK_a of a titratable side chain. Changes in the protonation state of a side chain can have a marked effect on nearby protons, and this can be detected as a change in chemical shift. With NMR the chemical shift changes can be detected not only in one dimension, as in the fluorescence experiment described above (where fluorescence intensity was the sole dependent variable), but in two dimensions, using one of the two-dimensional techniques of NMR structure determination.

In a real situation, things are not likely to be as simple as they have been made to seem above. The baseline values of fluorescence, resonance frequency, heat capacity or whatever might not be constant (Fig. 6.10). The baselines might suffer from a drift related to

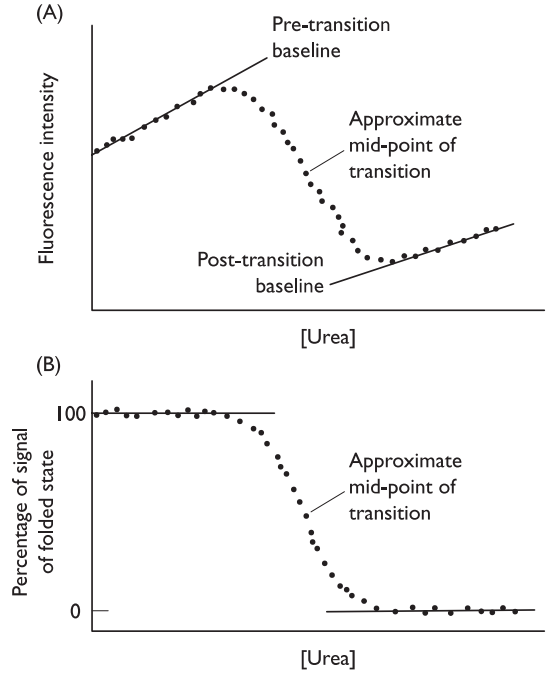


Fig. 6.10 Cooperative unfolding of a protein at equilibrium. The protein contains at least one partially buried tryptophan side chain. Panel (A) shows the fluorescence intensity as a function of urea concentration. Note that although the baselines are linear, neither is constant. Panel (B) shows the result of a mathematical transformation of the data of panel (A). The baselines have been accounted for, the fluorescence signal has been converted to a percentage change.

electronic properties of the measuring tool. The baselines might not be linear. The baseline properties might change from experiment to experiment. Often enough, though, it will be possible to model the baselines as straight lines or to transform the baselines into straight lines. In general, the slope and *y*-intercept of the pre-transition baseline will differ from the slope and intercept of the post-transition baseline. The procedure one uses for calculating the population of states is the same as before, except that a linear function is used in place of a constant value for the baseline.

Suppose you have linear baselines. There are different ways of determining slope and intercept in each case. One method is the following. Guess on the basis of experience which data points represent the baseline, and use linear least-squares fitting to find the slope and intercept of the best fit line to these points. The parameters define the baseline. Use the same procedure to find the other baseline. Then, use automated non-linear least-squares regression to fit an assumed model to the remaining data points. The fitting procedure will adjust the values of the parameters to optimize the fit of the model to the data, determining the energetics of the transition in the process. For example, if the object of study is protein stability and the independent variable is urea concentration, as in Fig. 5.28, you could use $\Delta G = \Delta G^\circ - m[\text{urea}]$ to model the relationship between denaturant concentration and free energy difference between states. You then plug this free energy formula into the expression for K in Eqn. (6.23) and fit the model to the data by wiggling the adjustable parameters (not variables!),[5] in this case ΔG° and m, until the model fits the data "well enough."

To continue with the example, once the non-linear least-squares procedure has yielded sensible values of ΔG° and m, one might consider making the baseline slopes and intercepts adjustable parameters as well. Doing this, however, will increase the number of adjustable parameters to be determined *simultaneously*: ΔG°, m, *slope1*, *intercept1*, *slope2* and *intercept2*. This is just the same number of adjustable parameters we've had to determine from the beginning, but now we're making no assumption about which data points represent the baseline, though we are still assuming that the baselines are linear. In practice, you might find that this approach does not lead to a sensible outcome unless you make "good" guesses as to what the final parameter values will be. In any event, this basic approach to analysis can be taken even further. For instance, if one of the baselines is not very straight, you could substitute a second-order polynomial function for the baseline in place of the straight

[5] An adjustable parameter is *not* a variable, and a variable is not an adjustable parameter! A variable represents a condition of the experiment, e.g. T or [urea]. It is called a variable because you can set it to a desired value. A parameter has a value that is determined by fitting a model to experimental data that were obtained at known values of the relevant variables.

line (first-order polynomial). What if temperature is an independent variable, as in a DSC experiment? Because ΔC_p of protein unfolding is often relatively large, ΔH_d and ΔS_d are sensitive functions of temperature, then there are not two but three adjustable parameters, unless there are grounds on which some of the parameters can be fixed or ignored. And if the two-state approximation should seem inadequate, because there are good reasons to believe that more than two states are thermodynamically relevant, even more fitting parameters must be included. And so on.

But wait! It is rightly said that given enough adjustable parameters it would be possible to fit an elephant. For increasing the number of parameters will *always* improve the appearance of the fit and certain quantitative gauges of its quality. One should therefore never forget that *convention* might not support the determination of so many adjustable parameters, even if there are reasons why some of them might be included. One should be particularly cautious if the number of data points is not very large or the data are especially noisy. Such conventions are ignored at the risk of credibility. It also should be mentioned that expertise in data analysis is no substitute for creativity or native ability in science. Entire books have been written on data analysis. *Basta*!

F. Multi-state equilibria

Systems of three or more states both do and do not differ in kind from a two-state system. A two-state transition is a first-order phase transition. If three states are present, the order–disorder transition as a whole is not all-or-none, but it might still be possible to model the multi-state transition as a sum of two-state transitions (Fig. 6.11). For instance, suppose one has a protein molecule in which several α-helices but no β-sheets are present in the folded state. In the first approximation, because the helices in proteins tend to be short and unstable in the absence of the rest of the protein, the folding/unfolding transition of each helix can be modeled as a two-state transition; each helix is either completely folded or completely unfolded. In a four-helix bundle there are four helices, and each can be in one of two states, so the total number of states is $2 \times 2 \times 2 \times 2 = 2^4$. Some (often all) of the partly folded forms of the protein, however, might be so unstable at equilibrium as to be but negligibly populated and below the level of detection. A multi-state transition, by contrast, might involve a continuum of partly folded conformations (Fig. 6.11). In practice it could be hard to distinguish between these two types of multi-state equilibrium.

Even when it is known that more than two states are present at equilibrium, it can be difficult to provide convincing evidence of the fact. And it can be even harder to say just how many states of any significance are present. In part this is because thermodynamic data

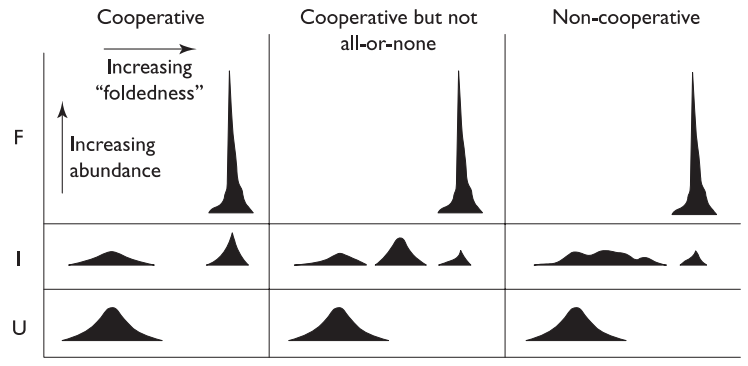


Fig. 6.11 Cooperative versus non-cooperative folding/unfolding. When folding/unfolding is highly cooperative, only two states are present at equilibrium. Each of these states is a distribution of conformation, but the distributions are relatively sharply peaked. Moreover, there is a large gap between the two distributions. Cooperative denaturation need not involve just two states, but the distributions of the various states must be distinct. In a non-cooperative transition there is no clear distinction between states. The folded state is likely to be unambiguous, but there may be a continuum of denatured states. The population of a denatured conformation will depend on the conditions (pH, salt concentration, temperature, etc.).

alone do not unambiguously establish a reaction mechanism. So the first and most important step in dealing with a suspected or known multi-state situation is to show that at least one property of the system simply cannot be explained by a two-state model. For instance, in optical spectroscopy, a necessary but insufficient condition for a two-state transition is an *isosbestic point*, a wavelength at which a family of curves has the same molar absorptivity. Each curve of the family might correspond to a different concentration of denaturant.

Thermodynamic techniques are useful for assessing the cooperativity of a process. In a previous chapter, we said that one criterion of two-state behavior in the absence of oligomerization and aggregation was the equivalence of the van't Hoff enthalpy and the calorimetric enthalpy. The van't Hoff enthalpy change for a process can be determined from the data of any technique that allows you to follow the relative concentrations of reactant and product; this enthalpy is a measure of the rate of change of the ratio of the concentration of production to the concentration of reactant. To calculate the van't Hoff enthalpy change, you have to assume that two and only two states are substantially populated throughout the process. The calorimetric enthalpy change, by contrast, is a measure of the heat absorbed during a reaction. This quantity does not depend at all on the model you might try to use to rationalize the data. So, under normal circumstances, comparing the van't Hoff enthalpy and the calorimetric enthalpy provides a means of assessing the number of states populated during a process.

Box 6.1 Stayin' Alive during Saturday Night Fever

Japanese bees are known to form a compact ball around a giant hornet when it attempts to invade the hive, and then raise their body temperature a whopping 12–13 °C from the normal 35 °C. The higher temperature kills the hornet but not the bees. Of course, the bees will "burn" a large amount of chemical energy per unit time in the process, so there is a cost to protection. What if humans could somehow turn up their temperature at will? A key step in a notional protein design project to keep blood functional at the boiling point of water might be to find a way to prevent hemoglobin, albumin, immunoglobulins, and other proteins from denaturing, losing functionality, precipitating, and creating a general mess at the higher temperature. Perhaps some lessons could be learned from structural comparisons of hyperthermophilic proteins and their mesophilic counterparts? In fact, such comparisons have suggested that large ion-pair networks are crucial to the protein hyperthermostability. Glutamate dehydrogenase from *Pyrococcus furiosus*, for instance, has about the same amount of secondary structure as the corresponding *Clostridium symbiosum* enzyme, and there is no significant difference in the accessible surface area or packing density of the indole-3-glycerophosphate synthases from *Sulfolobus solfataricus* and *Escherichia coli*. But large networks of ion-pairs are present in the hyperthermophilic protein but not in the mesophilic one in both cases. In separate work, replacement of the buried Arg–Glu–Arg ion triplet in the Arc repressor with Met–Tyr–Leu retains activity and increases thermostability relative to the wild-type protein. And comparison of the Klenow fragment of *E. coli* DNA polymerase I with *Taq* polymerase, an important enzyme for polymerase chain reaction, has revealed the absence of some unfavorable electrostatic interactions, an increased hydrophobic core, and an increased interdomain interface area in the hyperthermophilic enzyme. The jury is still very much out on the matter, but the most important factor for protein thermostability might be minimization of the surface-to-volume ratio by tight packing of intedigitating moieties.

There are many specific examples one could present to describe multi-state behavior. Let's focus on a well-studied case, that of α-lactalbumin and hen lysozyme. We have already said a good deal about lysozyme, and this should make the present ground seem less like *terra incognita*. This example is a nice one because lysozyme and α-lactalbumin have *practically identical* three-dimensional structures when folded. Both proteins have a relatively large helical "domain" comprising the same number of α-helices, and a relatively small "domain" where several β-strands are found. There are four disulfide bonds in both proteins – in just the same places. It is very likely that the lysozyme gene and the α-lactalbumin gene descend from a common ancestral proto-gene. So many similarities make the differences all the more interesting. Hen lysozyme displays a cooperative unfolding transition under a wide range of conditions (Chapter 2), whereas α-lactalbumin shows marked deviations from two-state behavior, especially in the absence of calcium. The similarities and

differences between these proteins were first studied systematically in the 1970s.

Now if these two proteins are so similar, *why* do they display such different properties? The overall structures are all but indistinguishable to the untrained eye, but the amino acid sequences are less than 50% identical. The percentage identity of amino acids is, however, not likely to be the main cause of the differences in folding characteristics. Instead, α-lactalbumin but not lysozyme binds a divalent metal ion with high affinity. Because selective binding to the folded state stabilizes the folded state relative to all other accessible states (Chapter 7), removal of the cation by EDTA reduces the stability of the folded state, probably because the aspartate side chains in the binding pocket repel each other. When the folded state is destabilized, the relative stability of partly folded states of α-lactalbumin is increased to a detectable level. An equilibrium partly folded state of a protein is called a *molten globule* when fixed tertiary structure is absent but elements of secondary structure are present and the protein is compact. In some cases molten globule species seem to correspond to partly ordered conformations on the protein folding pathway.

Suppose that three states of a small globular protein are present at equilibrium. The observable quantity, whatever it is, can be expressed in terms of the populations of states as

$$\begin{aligned}\langle O\rangle &= P_A o_A + P_B o_B + P_C o_C \\ &= \frac{1}{1+K_B+K_C}o_A + \frac{K_B}{1+K_B+K_C}o_B + \frac{K_C}{1+K_B+K_C}o_C,\end{aligned} \tag{6.26}$$

where the variables have the same meaning as before. K_B, for instance, measures the free energy difference between the intermediate state and the folded state at the temperature of the experiment. Fitting such a model to chemical denaturant data would require a minimum of four adjustable parameters (if the baselines were known): ΔG_A, ΔG_B, m_A, and m_B. Another approach to measurement of populations would be to plot the values of specific probes of the folded and unfolded states as a function of denaturant concentration. If circular dichroism spectroscopy, for instance, is used to monitor the unfolding of your protein, ellipticity in the "far-UV" (190–260 nm) can be used as a probe of secondary structure content, while ellipticity in the "near-UV" (260–310 nm) will provide information on tertiary structure. When the change in ellipticity with independent variable in the far-UV coincides with that in the near-UV, unfolding is "cooperative". But if the curves are not coincident, as in Fig. 6.12, unfolding is "complicated." The difference between the probes of the folded and unfolded states measures the fraction of molecules in intermediate states. Figure 6.13 shows the value of an observable quantity for two different independent variables, pH and [urea]. Note that unfolding involves three states along one axis and only two along the other. That is, the cooperativity of the

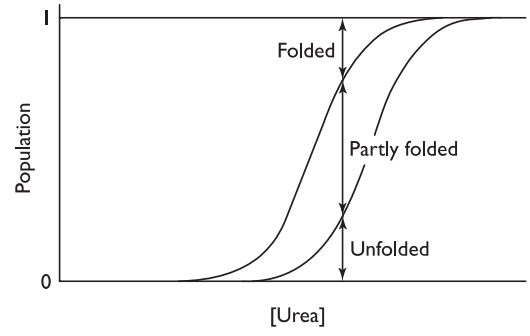


Fig. 6.12 Populations of states. Depending on the experimental approach, it might be possible to make independent measurements of the populations of the folded state and the unfolded state under the same conditions. This can be done by using circular dichroism spectroscopy. If the curves are not coincident, as shown here, at least one partly folded state must be present. Note that the population of the partly folded state first increases and then decreases with increasing denaturant concentration. At low concentrations of denaturant, the partly folded state is less stable than the folded state, and at high concentrations of denaturant, the partly folded state is less stable than the unfolded state. Somewhere between these extremes, the population of the partly folded state will be maximal. (Compare Fig. 6.15.)

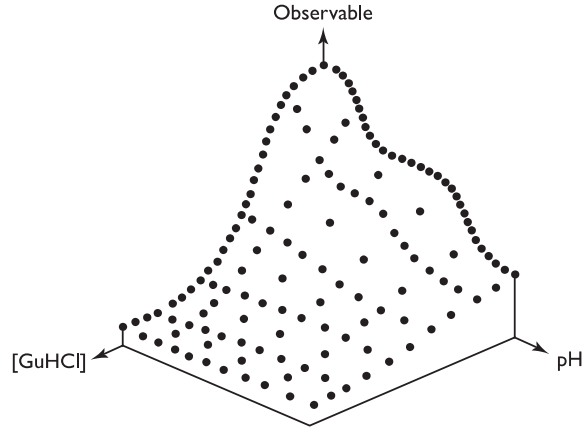


Fig. 6.13 Stability surface. The figure shows how an observable quantity like fluorescence or ellipticity or chemical shift or heat capacity might depend on two independent variables, in this case pH and urea. There are at least three accessible states, as one can see from the pH axis. The set of ordered triples (pH, urea, observable) define a surface. Further analysis would take into account baseline values, as in Fig. 6.9, and enable determination of the free energy difference between states under any combination of pH and urea at the temperature of the experiments. A plot of ΔG against two independent variables is called a stability surface. (Compare Fig. 5.28.)

Table 6.1. *Summary of the energetics of two-domain protein*

State	Free energy change	Boltzmann factor
A_FB_F	0, reference state	1
A_UB_F	$\Delta G_A + \Delta g_B$	$K_A\Phi_A$
A_FB_U	$\Delta G_B + \Delta g_A$	$K_B\Phi_B$
A_UB_U	$\Delta G_A + \Delta G_B + \Delta g_{AB}$	$K_AK_B\Phi_{AB}$

A sum of free energy terms translates into a product of Boltzmann factors by way of a basic property of exponential functions: $e^{(x\,+\,y)} = e^x e^y$, where $x = -\Delta G/RT$.

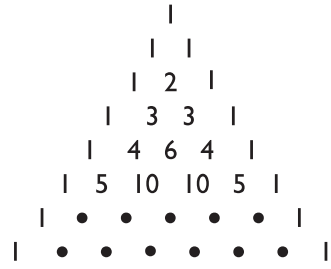


Fig. 6.14 Pascal's triangle. This triangular array of binomial coefficients (we saw the binomial theorem in Chapter 4) was taught as early as the thirteenth century by Persian philosopher Nasir al-Din al-Tusi. The triangle's discovery several centuries later by Pascal was apparently independent of earlier work.

unfolding transition depends on the medium in which unfolding occurs in this not-so-uncommon case.

What if we have a protein with multiple subunits? Suppose there are four identical subunits, and let each one have two accessible states, x and y. The enthalpy of any one subunit is H_x or H_y. There is only one way in which all subunits can be in enthalpy state H_x, and the enthalpy of the reference state, H_0, is $4H_x$. There are four ways in which one subunit can have enthalpy state H_y ($H_1 = 3H_x + H_y$), six ways of having two subunits in enthalpy state H_y ($H_2 = 2H_x + 2H_y$), four ways that three subunits can be in state H_y ($H_3 = H_x + 3H_y$), and only one way of having all four subunits in state H_y ($H_4 = 4H_y$). The actual number of states is $1 + 4 + 6 + 4 + 1 = 16$, but the enthalpic properties of some cannot be distinguished from others. The maximum number of *experimentally distinguishable* enthalpy states is 5, and they are H_0, H_1, H_2, H_3, and H_4. The various states and their features are presented in Table 6.1. The degeneracy of each enthalpy state is given by Pascal's triangle, from probability theory (Fig. 6.14). If there were three identical subunits, each in one of two possible states, then according to Pascal's triangle there would be a total of four enthalpy states, and the degeneracies would be 1, 3, 3 and 1.

What does the partition function look like for the multi-subunit protein? By Eqn. (6.17) and the definition of the partition function,

$$Q = \sum_{i=1}^{16} \exp\left(\frac{-(\varepsilon_i - \varepsilon_1)}{k_B T}\right). \tag{6.27}$$

The subscript runs from 1 to 16, the total number of states. When this equation is rewritten in terms of the five enthalpy states, we have

$$Q = \sum_{i=0}^{4} \omega_i \exp\left(\frac{-(H_i - H_0)}{RT}\right) = \sum_{i=0}^{4} \exp\left(\frac{-(G_i - G_0)}{RT}\right) = \sum_{i=0}^{4} \exp\left(\frac{-\Delta G_i}{RT}\right). \tag{6.28}$$

As always, the partition function is the sum of the Boltzmann factors. Don't worry that the range of the index has changed on going from Eqn. (6.27) to Eqn. (6.28). The index is just a "dummy" variable, a handy device that helps to distinguish one state from another.

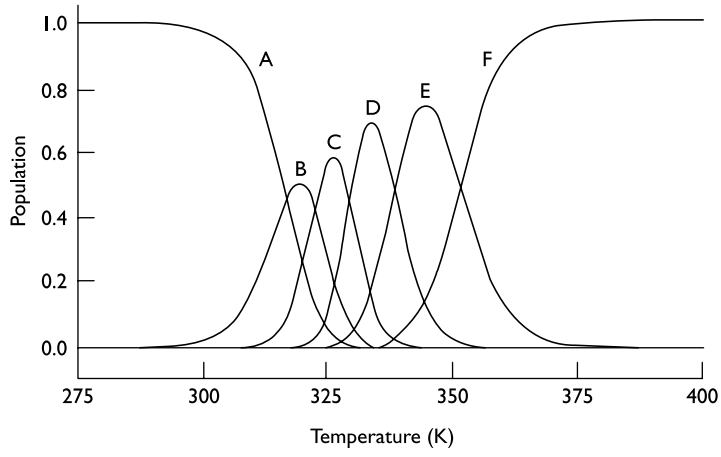


Fig. 6.15 Population of states as a function of temperature. The population of an accessible state is never zero. Depending on conditions, however, the population of some states could be practically negligible. For example, whenever states A, B, and C are stable (populated), the population of state F is small. Despite the jump in energy between states, the populations are smooth functions of temperature, as long as the number of molecules in the system is so large that fluctuations are extremely small.

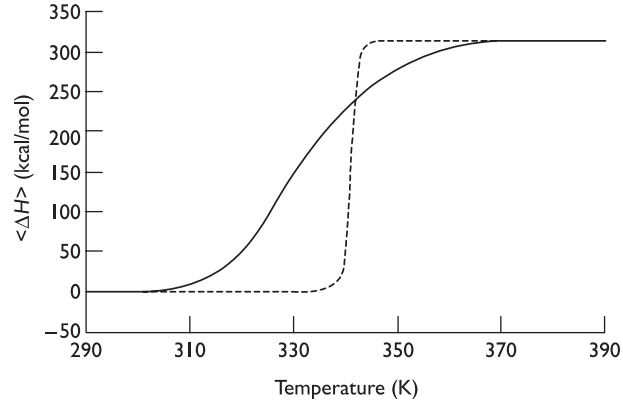


Fig. 6.16 Average enthalpy of a system. The solid line is a plot of Eqn. (6.30) for the system depicted in Fig. 6.12. The broken line illustrates how the average enthalpy would change with temperature if the transition did not involve intermediate states; i.e. if the transition were completely cooperative.

And now for this section's finale. The thermal unfolding of $tRNA^{phe}$ has been studied by DSC, which measures *the heat capacity function*, the heat absorbed as a function of temperature. Analysis of the heat capacity function of this tRNA has suggested that six states are populated during thermal denaturation. A plot of the population of states as a function of temperature is given in Fig. 6.15. In the six-state case, Eqn. (6.26) is written

$$\langle O \rangle = \sum_{i=1}^{6} P_i o_i \tag{6.29}$$

where the sum is over all the accessible states. If our experimental observable is enthalpy, Eqn. (6.29) has the following appearance

$$\langle \Delta H \rangle = \sum_{i=1}^{6} P_i \Delta H_i = \sum_{i=1}^{6} \frac{\exp(-\Delta G_i / RT)}{Q} \Delta H_i. \tag{6.30}$$

$\langle \Delta H \rangle$ is called the average enthalpy. A plot of Eqn. (6.30) as a function of temperature for the populations of states given in Fig. 6.15 is shown in Fig. 6.16. The heat capacity function is the variation in $\langle \Delta H \rangle$ with temperature.

G. Protein heat capacity functions

Recall from Chapter 2 that $\Delta C_p = \Delta\Delta H/\Delta T$. When ΔH is your observable quantity – when you measure the average heat absorbed or evolved at a given temperature, i.e., $\langle \Delta H \rangle$ – and if you make the measurement as a function of temperature, you measure $\Delta\langle \Delta H \rangle/\Delta T = \langle \Delta C_p \rangle$. This is what a scanning calorimeter does. The rest of the name of the instrument, "differential," refers to how the measurement is made (Fig. 2.10A, Appendix B). More precisely, a *differential* scanning calorimeter measures the *partial* heat capacity of a sample. The heat capacity of the solution of macromolecules is measured relative to the heat capacity of buffer in the absence of macromolecules, so only *part* of what could be measured (the *difference* between sample and reference) is actually measured. What follows is a qualitative description of how to rationalize DSC data.

In principle, it does not matter whether the sample is a protein, tRNA, a protein–DNA complex, a protein–lipid complex, or something else. The approach to analysis is basically the same in each case, though some tricks might apply more in one case than another. After accounting for buffer baseline effects, which can be done by measuring the heat capacity of the buffer with respect to itself, you analyze DSC data using the temperature derivative of Eqn. (6.30):

$$\langle \Delta C_p \rangle = \sum_i \frac{\Delta H_i^2 K_i}{RT^2(1+K_i)^2} + \sum_i \Delta C_{p,i} \frac{K_i}{1+K_i}. \tag{6.31}$$

This relationship can be derived from Eqn. (6.30) with a little calculus. The sums are over all states. The left-hand side of Eqn. (6.31) is what is measured in a DSC experiment, and what is measured is then interpreted in terms of the quantities on the right-hand side. The first term on the right-hand side is the "bell-shaped" heat absorption peak (see Fig. 2.10B). The second term gives the change in "baseline" heat capacity, which arises from the order–disorder transition. In the context of proteins, the second term measures the increase in exposure to solvent of hydrophobic residues as the protein denatures. As discussed in Chapter 2, ΔC_p can be large for large proteins, as they have a large solvent-inaccessible surface in the folded state and a small solvent-inaccessible surface in the unfolded state. For nucleic acids this term is generally small, because the heat capacity difference between the folded and unfolded forms of DNA and RNA is small.

When only two states are present, Eqn. (6.31) reduces to

$$\langle \Delta C_p \rangle = \frac{\Delta H^2 K}{RT^2(1+K)^2} + \Delta C_p \frac{K}{1+K}. \tag{6.32}$$

As before, the first term represents the heat absorbed during the transition, the second the shift in baseline heat capacity. Ignoring

the baseline shift, the area below the curve is the "calorimetric enthalpy." We have supposed that each macromolecule of the sample has two accessible microscopic states. The sharp heat-absorption peak occurs where the two microscopic states are equally populated and transitions between them – i.e. fluctuations – are a maximum. There is no free energy barrier between the states at the transition temperature.

The probability that any given molecule will be in the unfolded state is just the statistical weight of that state divided by the partition function, or $K/(1+K)$. The second term on the right-hand side of Eqn. (6.32) therefore makes good intuitive sense. When the population of the unfolded state is small, the contribution of this term to the change in baseline is small, practically zero. And when the population of the unfolded state is large, the contribution of the term approaches ΔC_p, the heat capacity difference between the folded and unfolded states (Fig. 2.10B).

Scanning calorimetry is useful for studying the thermodynamics of order–disorder transitions in macromolecules when the enthalpy change between states is "large." DSC is, however, a relatively blunt instrument, as it provides no direct or detailed information about the conformations adopted by the molecule of interest, even when statistical mechanics is used to analyze the data. The molecular interpretation of a DSC signal will therefore often depend on other techniques, e.g., fluorescence, CD, or NMR. In your own work, you will ideally use a combination of techniques to characterize a system.

H. Cooperative transitions

Suppose you have a dimeric protein in which both subunits unfold simultaneously by heat denaturation. Will the van't Hoff enthalpy change for denaturation be the same as the calorimetric enthalpy change? No! Why not? The van't Hoff enthalpy measures the rate of change with temperature of the population of the folded dimeric state with respect to the unfolded monomeric state, and the calorimetric enthalpy measures the heat absorbed on unfolding subunits. (We are neglecting interaction terms between subunits, which will be dealt with in the next section.) Each time a dimer unfolds, the population of folded state will decrease by one while the population of unfolded state will increase by two. But the heat absorbed during thermal denaturation will be the same whether the protein is one large monomer or a dimer of two half-sized monomers.

Defining cooperativity, c, as the ratio of the van't Hoff enthalpy change to the calorimetric enthalpy change, we have

$$c = \Delta H_{vH}/\Delta H_{cal}. \tag{6.33}$$

In the case of a *monomeric* protein that shows cooperative unfolding, $\Delta H_{vH} = \Delta H_{cal}$ and $c = 1$; there is but one cooperative unit. A

monomeric protein that exhibits multi-state unfolding has $c < 1$. Systems composed of a very large number of identical subunits, for example polynucleotides and phospholipid membranes, have $10 < c < 500$.

To see the matter more clearly and to give the discussion an experimental context, suppose you have a multimeric protein that exhibits the following equilibrium:

$$c[U] \Longleftrightarrow [F]_c. \tag{6.34}$$

The folded state is composed of c identical copies of a polypeptide. The overall equilibrium constant for the folding/unfolding reaction is

$$K = \exp(-c\Delta G/RT). \tag{6.35}$$

Note the factor c in Eqn. (6.35). ΔG is the free energy change of folding one subunit; $c\Delta G$ is for c subunits. The average enthalpy is

$$\langle \Delta H \rangle = \Delta H P_F = K/(1+K), \tag{6.36}$$

where ΔH is the enthalpy difference between the folded and unfolded states of a subunit and P_F is the population of the folded state. It can be shown with a little calculus and Eqn. (6.33) that

$$\langle \Delta C_p \rangle = \frac{K}{(1+K)^2}\frac{c\Delta H^2}{RT^2} = \frac{K}{(1+K)^2}\frac{\Delta H_{vH}\Delta H_{cal}}{RT^2}. \tag{6.37}$$

The effect of changing c on $\langle \Delta C_p \rangle$ is illustrated in Fig. 6.17. As the cooperativity c increases, the curve becomes more and more sharply peaked, but the area below the curve (ΔH_{cal}) remains the same.

At the midpoint of the transition, where $\Delta G = 0$ and $K = 1$,

$$\langle \Delta C_P \rangle_{T_m} = \frac{\Delta H_{vH}\Delta H_{cal}}{4RT_m^2}. \tag{6.38}$$

It can be shown that when the transition occurs far from absolute zero, the temperature at which the heat absorption peak has a maximum is practically indistinguishable from T_m, the temperature at which $K = 1$. The only unknown in Eqn. (6.38) is ΔH_{vH}. The van't Hoff enthalpy can therefore be calculated from the result of a DSC experiment. The cooperativity of the transition is assessed by comparing ΔH_{vH} to ΔH_{cal}, as in Eqn. (6.33).

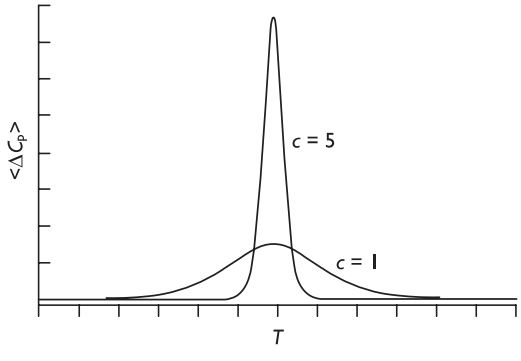


Fig. 6.17 Cooperativity and the heat capacity function. Heat capacity functions are shown for $c = 1$ and $c = 5$. The calorimetric enthalpy and melting temperature are the same in both cases.

I. "Interaction" free energy

What if your protein has more than one domain or subunit? There are numerous examples of multi-domain proteins and multi-subunit proteins in nature. For example, the extracellular matrix protein fibronectin consists of a number of repeats of a small unit called a fibronectin domain, and each domain comprises about 100 amino acids and is globular. The domains are joined together like beads on a string. In many cases but not in all, the folded states of the individual domains are stable at room temperature in the absence of the rest of the protein. This is possible only if the stability of a domain does not depend substantially on interactions with other parts of the protein. We have already encountered the multi-subunit protein hemoglobin, and we shall study it in greater depth in the next chapter. Whether the interactions in your protein are between domains or between subunits, they involve specific chemical groups in a particular spatial arrangement. In the best case, detailed structural information will be available to you from an X-ray study or NMR.

Now, suppose you are interested in a two-domain protein, for example, yeast phosphoglycerate kinase (PGK). Our object of study need not be a protein, but it will simplify things in accounting for all the contributions to the thermodynamics to let the system be a single covalent structure. (Why is that?) The individual domains of phosphoglycerate kinase are not very stable; they are denatured at *room temperature*, even when combined in a 1:1 stoichiometric ratio. This implies that the combined thermodynamics of the continuous polypeptide chain and the domain–domain interactions are responsible for the stability of the intact protein.

The situation can be modeled as follows. Let the two domains be called A and B. The free energy difference between the folded and unfolded states *of domain* A *alone* is ΔG_A. This energy term does *not* include contributions from *interactions* with domain B (Fig. 6.18.). The free energy change of exposing the surface of domain B that is

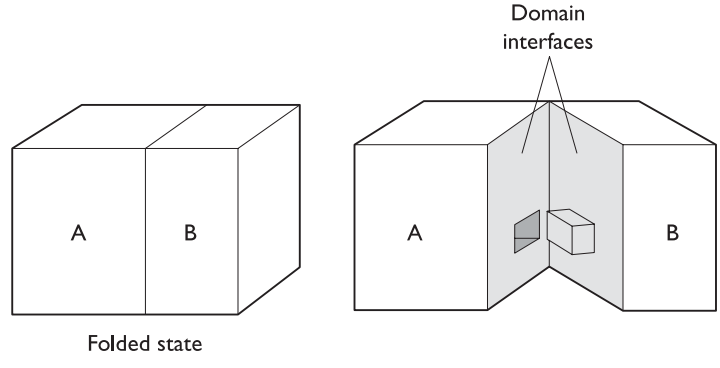


Fig. 6.18 Domain interface of a two-domain protein. In the folded state, interactions between the domains are inaccessible to the solvent. There is a free energy cost to exposing the domain interface to solvent, even if the domains themselves remain folded. The domain interface consists of two geometrically complementary surfaces.

solvent inaccessible when domain A is present, is Δg_B. And vice versa. The states, free energy changes, and Boltzmann factors are displayed in Table 6.1. Note that in general, $\Delta g_A \neq \Delta g_B$, though in practice the values might be very similar. $\Delta g_{AB} = \Delta g_A + \Delta g_B$. Table 6.1 is completely general, for if there is no interaction between domains, $\Delta g_A = \Delta g_B = 0$, and the corresponding Boltzmann factors are simply equal to 1.

In the presence of the chemical denaturant guanidine hydrochloride, PGK unfolds reversibly on heating or cooling; it exhibits cold denaturation. The character of the transition is, however, different in the two cases. In heat unfolding the transition is cooperative, both domains unfold simultaneously, and the individual domains are apparently too unstable to be found in appreciable abundance. In the low temperature transition, by contrast, unfolding is non-cooperative; the domains denature independently. This suggests that the cooperativity of the folding/unfolding transition depends on the thermodynamic properties of the domain interface. A similar phenomenon is observed when a multi-subunit protein dissociates into its individual subunits at temperatures around 0 °C.

One approach to rationalizing the thermodynamic data is to examine available structural information, to see if it might provide clues as to the molecular origin of the macroscopic behavior. Analysis of the crystal structure of PGK reveals that each domain has a certain amount of solvent inaccessible surface which becomes solvated when the protein unfolds. On heat denaturation, both domains unfold simultaneously, and one assumes that if domain A has, say, 65% of the total buried surface, it accounts for *c.* 65% of the heat absorbed and *c.* 65% of the heat capacity change. The same sort of reasoning can be applied to estimating the enthalpy of unfolding of domain B and the domain interfaces at the transition temperature.

Further analysis of the PGK structure shows that each domain contributes to the interface somewhat less than 500 Å^2 of hydrophobic surface and nine hydrogen bonds. This information can be coupled with the thermodynamics of solvation of small model compounds and used to evaluate Δg_A and Δg_B. The missing terms are reference values for Δs_A and Δs_B, and there is unfortunately no reliable way of predicting them by crystal structure analysis. To get round this obstacle, one can simply substitute in different values, simulate the heat capacity of the entire two-domain protein as a function of temperature using Eqn. (6.31), and compare the results of the simulation with the observed experimental data. When the simulation matches the observed behavior, one can assume that the values of the thermodynamic quantities used in the simulation are at least close to what one would find if experiments to measure the thermodynamics of exposing the domain interface to solvent could actually be carried out.

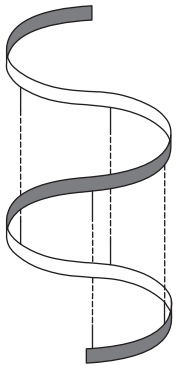


Fig. 6.19 The α-helix. Helices in proteins are right-handed. That is, the thumb of the right hand points from N to C if the fingers of the right hand curl around the helix in the direction of N to C. This very low resolution diagram is intended to give a sense of how hydrogen bonds stabilize this element of secondary structure. Hydrogen bonds are formed between N–H groups and C=O groups along the polypeptide backbone. There are 3.6 peptide units per turn in the α-helix. In other words, each peptide unit corresponds to a 100 ° rotation about the helix axis.

J. Helix–coil transition theory

The α-helix is a very basic structural motif in proteins; most known folded proteins contain at least one α-helix (Fig. 6.19). It is generally believed that α-helix formation in a protein occurs as a very early event on the folding pathway. On this view, the coil–helix transition plays a key role in the mechanism of protein folding. A qualitative description of helix–coil theory is included here for two reasons: it is of basic interest to people studying protein folding, and it illustrates how statistical thermodynamics can be used in biological thermodynamics research. Theoretical descriptions of the other major type of secondary structure element, the β-sheet, are not as advanced as for the α-helix and will not be discussed here.[6]

Helical peptides show heat-induced unfolding. This probably indicates that helix formation is enthalpically driven and that the sign of the heat capacity difference between the coil and helix states is positive, as in protein denaturation. ΔC_p for the helix–coil transition, though unknown, is likely to be small. In any case, the enthalpy change on helix formation will have two major components: the difference in enthalpy between (a) hydrogen bonds formed between water and polypeptide backbone donors (amide hydrogen atoms) or acceptors (carbonyl oxygen atoms) and (b) hydrogen bonds formed between backbone donors and acceptors themselves; and the enthalpic effect of changes in the solvation of other parts of the peptide. It is possible but unlikely that formation of a hydrogen bond in an α-helix results in the same change in enthalpy as formation of a hydrogen bond between two water molecules. Irrespective of the precise partitioning of the enthalpy into its various components, experiments suggest that ΔH° for helix formation is $\sim$1 kcal mol^{-1} residue^{-1}. Hydrogen bond strength is likely to depend on the donor and acceptor involved, as well as the distance between donor and acceptor and the electronic properties of the medium in which the bond forms. But all such tiny details are ignored in the present treatment in order to keep things simple.

In the *Zimm–Bragg model* of helix–coil transition theory,[7] helix formation is considered to involve two steps: *nucleation* and *propagation*. Nucleation is the process of forming one hydrogen bond characteristic of an α-helix between two amino acid residues, when no other such bonds are present. Propagation of the helix, or elongation, depends entirely on nucleation already having occurred. Although nucleation can occur randomly at multiple locations along the polypeptide chain, each nucleation event is relatively improbable; nucleation is energetically unfavorable. This is because

[6] The four major levels of protein structure – primary structure, secondary structure, etc. – were first described by Linderstrøm–Lang in 1952, several years before the first protein structure was visualized at atomic resolution.

[7] B. H. Zimm and J. K. Bragg.

it involves a substantial decrease in entropy, the cost of fixing the orientation of residues in space so that the first helix hydrogen bond can be formed. Once a helix has been nucleated, however, the additional decrease in entropy on fixing the geometry of the polypeptide chain is more than offset by the energetically favorable formation of hydrogen bonds.

The thermostability of existing helical structure is explained as follows. After initiation, a helix would extend indefinitely and encompass all amino acids in a polypeptide chain if it were not entropically favorable for there to be several helical regions instead of one. In view of this, the helical content of a homopolypeptide depends primarily on length; nucleation is unfavorable, so more than one nucleation site in a short polypeptide is improbable. Given an ensemble of identical polypeptides, the specific location of helices and overall helix content will vary from molecule to molecule. This is because from an entropic point of view it is favorable for molecules with the same percentage helix to differ somehow or another. It follows that one cannot use a two-state model to describe the helix–coil transition, unless the polypeptides involved are as short as the helices found in proteins.

Measurement of helix content of an ensemble of identical polypeptides estimates not the percentage helix in each polypeptide, but the average helix content in all the polypeptides in the ensemble. There is a distribution of helix content in a collection of identical polypeptides. The stability of helical structure in any particular case will depend on the character of the amino acids involved, as some amino acids have a higher *helix propensity* than others. The helix-forming tendency of an amino acid type can be rationalized in terms of its structure (see below).

In Zimm–Bragg theory, then, two parameters give a quantitative account of the helical content of a polypeptide. These are σ, the helix nucleation parameter, and s, the helix propagation parameter. Note that σ and s are parameters, not variables, because their values are determined by optimizing the "fit" of the model to experimental data. The cooperativity of the helix–coil transition will depend on the value of s. When s is large ($\sim$1), cooperativity is low; when s is small ($\sim 10^{-4}$), cooperativity is high. For real peptides, $\sigma \sim 10^{-3}$, $s \sim 1$.

The first helical residue (i) in a Zimm–Bragg helix has a Boltzmann factor of $\sigma s = \exp(-\Delta G_{\text{initiation}}/RT)$, while that of the second helical residue ($i + 1$) is $s = \exp(-\Delta G_{\text{propagation}}/RT)$. The Boltzmann factor is 1 for both a coil residue following a helical one and a coil residue following a coil. Note that because the Boltzmann factors refer to α-helical hydrogen bonds formed between peptide groups, σ is a property of more than one peptide group and several residues. The equilibrium constant between the helical state (H_n) and coil state (C) for n peptide groups in a homopolymer is $K_n = [H_n]/[C] = \sigma s^n$. In the first approximation for heteropolymers neither σ nor s depends on residue type, excluding proline and glycine, which have very different polypeptide backbone characteristics from the

other amino acids. This is easily seen by comparing the respective Ramachandran plots.

Another approach to analysis of the helix–coil transition is that of Lifson and Roig. In the *Lifson–Roig model*, the Boltzmann factors correspond to amino acid residues, not peptide groups. This model facilitates accounting for effects at the ends of helices, which do contribute to helix stability but not to the same extent as residues in the middle of a helix. The ends of helices are "frayed," and the percentage of time that helical hydrogen bonds are formed at the end of a helix is less than in the middle. In the Lifson–Roig model, the equilibrium constant of the conformation ccccchhhhhccchhcc is *uuuuuvwwwvuuuvvuu*. The coil–helix junction (ch) is represented by a Boltzmann factor of *v*, hh by *w*, and cc by *u*.

The propensity of a given amino acid type to form helical structure has been measured by so-called host–guest experiments. A water-soluble polypeptide serves as the host, and amino acid replacement is carried out at a specific site in the polypeptide chain. One can generate a set of twenty different peptides by chemical synthesis, measure helix content, and rank the various amino acids according to their ability to promote helix formation. Helix content is usually assessed by circular dichroism spectroscopy, which measures the difference in absorption of right- and left-circularly polarized light. The technique is particularly sensitive to helical structure in the so-called far-UV region of the spectrum.

A helix propensity scale is shown in Table 6.2. The experimental data have been normalized to the result for Gly. This amino acid has no chiral center, and because it has no side chain, the bonds of the polypeptide backbone on either side of the α carbon are able to rotate freely. There is therefore a large entropic cost to placing severe restrictions on the motion of these atoms, as would be necessary to form an α-helix. By contrast, the next most complicated amino acid, Ala, stabilizes helical structure more than any other amino acid type. How is that? Ala has a very small side chain, just a methyl group. Formation of helix from the coil state results in a relatively small decrease in the motions of the Ala side chain (rotations of the methyl group about the $C_\alpha - C_\beta$ bond and of the $C_\beta - H$ bonds; Ala has no γ substituent); there is a smaller decrease in the entropy of the side chain of Ala than for other amino acid residues. This is the physical basis of the high helix propensity of Ala. Pro has a very low helix propensity because of the restrictions it places on polypeptide backbone geometry. A question you should be asking yourself is whether study of helical peptides alone will say anything definitive about helices in proteins or protein folding. Probably not.

Another approach to sorting out the role of individual amino acids in stabilizing α-helix structure is to make mutations at a solvent-exposed site in the middle of a helix in a well-studied protein. In many cases, mutations of this sort cause no significant distortion of helix geometry relative to the wild-type protein, effectively ruling out one possible origin of helix stability. Instead,

Table 6.2. *Helix propensity scale*

Amino acid residue	Relative contribution to stability of α-helical conformation (kcal mol^{-1})
Ala	−0.77
Arg	−0.68
Lys	−0.65
Leu	−0.62
Met	−0.50
Trp	−0.45
Phe	−0.41
Ser	−0.35
Gln	−0.33
Glu	−0.27
Cys	−0.23
Ile	−0.23
Tyr	−0.17
Asp	−0.15
Val	−0.14
Thr	−0.11
Asn	−0.07
His	−0.06
Gly	0
Pro	≈3

Data are from O'Neil and DeGrado (1990). Compare these values with Table B in Appendix C.

the effect of a mutation on overall protein stability correlates with the change in the difference in hydrophobic surface area exposed to solvent of the mutated residue in the folded state of the protein (crystal structure) and in the unfolded state (fully extended chain). In symbols, $\Delta T_m = T_{m,\ mutant} - T_{m,\ wild\text{-}type} \propto \Delta(A_{unfolded} - A_{folded})$, where A is solvent-exposed hydrophobic surface area. In general, there is relatively good agreement between various helix propensity scales. Differences are likely to be attributable more to structural changes that are propagated throughout the host than to experimental error. This suggests all the more that analysis of the experimental thermodynamic properties of model helices will probably not be able to say substantially more about what stabilizes proteins than what is known already. Unfortunately, this will not stop some people from spending their entire lives learning more and more about less and less.

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L. Exercises

1. Explain in statistical mechanical terms why it is easier to remove a book from a specific place on a bookshelf than it is to put the book in a specific place on a bookshelf.

2. Suppose you have two glass bulbs of equal volume that are connected by a stopcock. Initially, one bulb contains N identical molecules of an inert gas, and the other bulb is evacuated. When the stopcock is open, there is an equal probability that a given molecule will occupy either bulb. How many equally probable ways are there that the N molecules can be distributed among the two bulbs? The gas molecules are, of course, indistinguishable from each other. How many different states of the system are there? Write down a formula for W_L, the number of (indistinguishable) ways of placing L of the N molecules in the left bulb. The probability of such a state occurring is its fraction of the total

number of states. What is that probability? What is the value of W_L for the most probable state? Calculate $W_{N-2\pm1}/2^N$ for $N = 10^1, 10^3, 10^5, 10^{10}$ and 10^{23}. Explain the significance of W as a function of N.

3. Given a system with configuration {0, 1, 5, 0, 8, 0, 3, 2, 0, 1}, calculate Ω.

4. Given a system in which $N = 20$, give the configuration that maximizes Ω. Show how you arrived at your answer.

5. The 17th June 1999 issue of *Nature*, the international weekly journal of science, published a research on chimpanzee "cultures." There is a famous joke attributed to renowned British physicist Sir James Hopwood Jeans (1877–1946) that goes something like this. A population of chimps typing randomly but diligently at a computer keyboard would eventually produce Shakespeare's greatest work, "Hamlet." Calculate how long, on the average, it would take 107 chimps to type the phrase "to be or not to be, that is the question"? Assume that each chimp has a 45-key keyboard plus a space bar (assume no shift key). How long, on the average, would it take one chimp to type this phrase if its computer was programmed to shift to the right after each correct keyboard entry? Compare these numbers and comment on their possible significance for theories of the origin of life and chimp cultures.

6. The protein folding/unfolding example described in Sections E and F involves changes in the urea concentration. Is the system open or closed? Why? Can the results be analyzed using equilibrium thermodynamics? Why or why not? Give a protocol for carrying out such an experiment that is independent of the instrument used and the observable monitored.

7. Polyacrylamide gel electrophoresis is used to separate proteins on the basis of size. Figure 2.28 shows the pattern one finds when a monomeric protein that unfolds cooperatively in the presence of denaturants is studied by urea gradient polyacrylamide gel electrophoresis. The gel has been stained with a protein-sensitive dye. Rationalize the result in terms of urea concentration and migration behavior of proteins in a polyacrylamide gel. Assuming that there is no geometrical distortion in the sigmoidal curve, describe a method of determining the free energy of unfolding in the absence of denaturant and the urea concentration at the midpoint of the transition. Would you expect this method to give very accurate results in practice? Why or why not?

8. Explain how disulfide bonds stabilize the folded states of proteins.

9. Sickle-cell hemoglobin differs from normal wild-type hemoglobin by one amino acid change. This results in aggregation of hemoglobin molecules under certain conditions. The hemoglobin

filaments formed at 37 °C can be disaggregated by cooling to 0 °C. Rationalize this behavior.

10. Write down the partition function for the three-state unfolding of a protein in a chemical denaturant.

11. Note that the phase transition shown in Fig. 2.10B is not very sharp. The transition occurs over a range of temperatures, not at a single temperature, as one might expect for a pure substance. We have claimed, however, that this transition is essentially no different from what finds for the cooperative thermal denaturation of a protein. What is the origin of the behavior shown in Fig. 2.10B?

12. Suppose you are using differential scanning calorimetry to study a two-domain protein. Suppose also that thermal denaturation shows a heat absorption peak for which $\Delta H_{vH}/\Delta H_{cal} = 1$. Does it necessarily follow that thermal denaturation is cooperative? Why or why not?

13. In Eqn. (6.6), why is the speed of the cyclist irrelevant to the number of collisions?

14. At 310 K, ΔG° for ATP hydrolysis is -30.5 kJ mol^{-1}, and $\Delta H^\circ = -20.1$ kJ mol^{-1}. Calculate ΔS° for this process and explain the result in molecular terms.

15. How many possible different tetrapeptides can be made using the twenty standard amino acids? How many possible different tetrapeptides could be made from a pool of twenty different amino acids? How many possible different tetrapeptides of the same composition could be made from a pool of twenty amino acids. How many possible different tetranucleotides are there?

16. Pascal's triangle. Consider Fig. 6.14. Give the next three rows of Pascal's triangle.

17. Given a two-domain protein with a binding site in one of the domains, enumerate and describe the various accessible states.

18. Devise a test for assessing whether or not the inclusion of additional fitting parameters actually improves the goodness-of-fit of a model to experimental data.

19. Given a three-state system, describe the population of the intermediate state relative to the other two states for an independent variable that results in a transition from the completely ordered state to the completely disordered state.

20. Consider a molecule of ethane. How many different ways are there of arranging the position of one methyl group relative to the other, accounting only for rotations about the sigma bond between the carbon atoms? What is the entropy of this bond rotation?

21. The thermodynamics of hen egg white lysozyme at pH 4.5 and 25 °C are as follows: $\Delta H = 50\ 000$ cal mol^{-1}, $\Delta S = 132$ cal mol-K^{-1}, $\Delta C_p = 1500$ cal mol-K^{-1}. Calculate ΔG at 60 °C. Evaluate the partition function, P_F, and P_U at this temperature. The thermodynamics of *apo* α-lactalbumin at pH 8.0 and 25 °C are $\Delta H = 30\ 000$ cal mol^{-1}, $\Delta S = 94$ cal mol-K^{-1}. Calculate ΔG at 60 °C. Assume that the protein exhibits a two-state transition under the stated conditions. Evaluate the partition function, P_F, and P_U at this temperature.

22. Helix–coil transition theory. Give the statistical weight of cchhhhhhhhcchhchhhccc.

Chapter 7

Binding equilibria

A. Introduction

We have seen how the First and Second Laws are combined in a thermodynamic potential function called the Gibbs free energy. We have also seen how the Gibbs energy can be used to predict the direction of spontaneous change in a wide variety of systems under the constraints of constant temperature and pressure. One type of application of the Gibbs energy, which we studied in Chapter 5, is the binding of a ligand to a macromolecule. And in Chapter 6 we became introduced to statistical thermodynamics, a mathematical formalism that permits a molecular interpretation of thermodynamic quantities. The present chapter combines and extends all these ideas. *Binding* is a ubiquitous and immensely important general topic in biochemistry. While binding can be considered just one of many different types of equilibrium process, the key role it plays in the physiology of biological macromolecules makes the present chapter one of the most important of the book.

Let's do a brief survey of areas in biology where binding plays a role – before plunging into a sea of mathematical equations. In our discussion of the First Law (Chapter 2) we encountered RNase A, a digestive enzyme that can bind tightly to a nucleotide inhibitor. Then in Chapter 5, we looked at receptor–ligand interactions and an introductory analysis of oxygen binding to hemoglobin. And an exercise in Chapter 5 involved the binding of a regulatory protein to DNA (Fig. 7.1). All these intermolecular interactions are from different biological processes, and all of them have quite different biological effects. From the perspective of physical chemistry, however, they all bear a distinctly similar mark. In every case, binding is made specific by steric complementarity between ligand and macromolecule (hand fits in glove), complementary charges on macromolecule and ligand (opposites attract), complementary polar and non-polar surfaces on macromolecule and ligand (likes attract), and so on. In a word, complementarity! In the present chapter, we'd

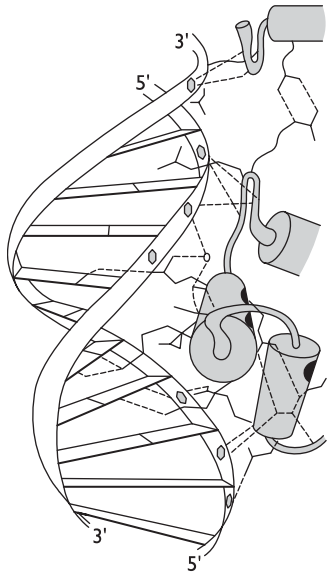

Fig. 7.1 Binding. There are many different kinds of biomolecular interaction (see text). Protein–DNA binding is one type of binding. The figure shows one helix–turn–helix motif of the 434 repressor protein interacting with its DNA binding site. Binding of the repressor to the operator site in DNA inhibits transcription of the gene regulated by the operator; 434 repressor is a dimeric protein, and it associates with DNA with two-fold symmetry. The conformation of the protein in the protein–DNA interface closely resembles that of DNA itself. The protein interacts with both paired bases and the sugar–phosphate backbone through a complex network of hydrogen bonds, salt bridges, and van der Waals contacts. The DNA bends around the protein in the complex. The energetic cost of the strain induced by bending must be offset by the other interactions made between the DNA and protein. *All* binding interactions are essentially electrical in nature. After A. K. Aggarwal *et al.* (1988).

like to see how all these similarities translate into a general mathematical theory of binding.

Let's look at an example. Binding occurs when the hormone insulin interacts physically with the extracellular portion of its membrane-spanning receptor protein. The interaction elicits a cascade of events inside the cell where the receptor is located, the ultimate result depending on cell type. In muscle cells, for instance, insulin binding leads to an increase in glucose metabolism. In fibroblasts, insulin acts as a growth factor. And in liver cells, insulin stimulates the activity of enzymes that synthesize glycogen, a polymeric form of the energy molecule glucose. In this chapter you will learn ways to quantify the interaction between ligand and receptor.

Many enzymes require a bound ion or multi-atom cofactor in order to carry out their catalytic function. The milk protein α-lactalbumin, for example, is stabilized in the native state by calcium (Chapter 6), and the *holo* (ligand-bound) protein binds to galactosyltransferase to form galactose synthetase, a heterodimeric enzyme. As we saw in Chapter 5, the DNA polymerases from hyperthermophilic

bacteria that are so important in PCR require divalent cations for activity. And several of the enzymes involved in the citric acid cycle (Chapter 5) bind a flavin (FAD^+) for use in redox reactions (Chapter 4). These are just a few examples of the many that illustrate the importance of binding in the living organism.

Now let's zoom in on the direct interaction of molecular oxygen with the blood protein hemoglobin (Hb) and the muscle protein myoglobin (Mb), two closely related proteins. In humans, Hb plays a vitally important role in the transfer of oxygen from the lungs to cells situated throughout the body. Hb does this by being confined to red blood cells, a.k.a. "bags of hemoglobin." Oxygen is loaded onto Hb in the lungs, where the partial pressure of oxygen is high, and unloaded in the extremities of the vasculature, where the partial pressure of oxygen is low. Oxygen simply moves down its concentration gradient in both locations. In striated muscle, the offloaded oxygen is picked up by the protein Mb and stored until needed for respiration. This "macroscopic" description, while true, does not provide any sense of the marvelous complexity of the proteins involved, nor how their complex structure enables them to do their jobs.

Research in this area had begun decades earlier, but it was not until the 1920s that Hb, the "molecular lung," was found to be a tetramer with an average of about one oxygen binding site per subunit. Eventually, the structure of Hb would be solved at low resolution by Max Ferdinand Perutz (1914–2002) and co-workers at Cambridge University in 1959.[1] This work showed that the tetramer is held together by ion pairs, hydrogen bonds, and hydrophobic interactions. Detailed analysis of binding data implies that the binding equilibrium involves not just H_2O, O_2, Hb, and $Hb(O_2)_4$, but several other molecular species as well, namely, $Hb(O_2)$, $Hb(O_2)_2$, $Hb(O_2)_3$. Important for the present discussion, there are multiple forms of these partially ligated species, since there are multiple permutations of the ligands bound. To make things even more interesting, each intact species of Hb is in equilibrium with subunit dimers and monomers! And to go to an even higher level of complexity, the binding affinity of oxygen for a subunit depends not only on whether it is in the monomeric, dimeric, or tetrameric state, but also on whether other oxygen molecules are bound! Hb is a complex molecular machine.[2] Determination of the structure of Hb was aided by the existing structure of Mb, which has been solved at low resolution in 1957 by John Cowdery Kendrew (1917–1997) and colleagues. This was possible because there are clear structural similarities between the α-chain of Hb and Mb; a reflection of their similar functions. It is probable that hemoglobin

[1] The British crystallographers M. F. Perutz and J. C. Kendrew were awarded the Nobel Prize in Chemistry in 1962.

[2] Richard Feynman's famous 1959 talk, "Plenty of Room at the Bottom," is often taken as the starting point of nanotechnology. There is compelling anecdotal evidence that some of Feynman's remarks were inspired by hemoglobin. The hypothesis is elaborated in Haynie *et al.* (2006) *Nanomedicine: Nanotechnology, Biology, and Medicine*, **2**, 150–7. The title of Feynman's talk comes from an Academy Award winner.

and myoglobin genes originate from the same proto-gene, which is certainly very ancient since myoglobin-like heme-containing proteins are also found not only in vertebrates but in plants, eubacteria, and archaea. And yet, as we shall see, Hb and Mb are also very different. An aim of this chapter is to provide a means of discussing such differences in terms of relatively straightforward mathematics.

As we shall see, one of the happier aspects of using mathematics to describe binding is that a relatively small number of equations can be applied very effectively to a tremendous variety of situations. Some of the questions we shall consider in the present chapter are: How many ligand-binding sites are there per macromolecule? How strongly does a ligand bind? If there is more than one binding sites per macromolecule, is the binding of a ligand to one site independent of binding to another site on the same macromolecule?

B. Single-site model

We have already discussed the single-site binding model in moderate detail in Chapter 5, in the section on molecular pharmacology. (Before getting into the thick of things here, you might find it helpful to return to Chapter 5 for a quick review of basic concepts.) Equation (5.39) says that the *average* number of moles of ligand bound per mole of macromolecule (the fraction of sites occupied or *fractional saturation*), ϕ, is

$$\phi = [\mathrm{M} \bullet \mathrm{L}]/[\mathrm{M}]_\mathrm{T} = [\mathrm{L}]/(K_\mathrm{d} + [\mathrm{L}]), \tag{7.1}$$

where M is the macromolecule (e.g. DNA or protein), $[\mathrm{M}]_\mathrm{T}$ is the total concentration of macromolecule, [L] is the concentration of *free* ligand, and K_d is the *dissociation* constant. Note that when $[\mathrm{L}] = K_\mathrm{d}$, $\phi = 1/2$. That is, the dissociation constant measures the concentration of free ligand at which the binding sites are half-saturated. In terms of the *association* constant, $K_\mathrm{a} = {K_\mathrm{d}}^{-1}$, the fraction of sites bound is

$$\phi = K_\mathrm{a}[\mathrm{L}]/(1 + K_\mathrm{a}[\mathrm{L}]), \tag{7.2}$$

where $K_\mathrm{a} = \exp(-\Delta G_\mathrm{b}/RT)$, and ΔG_b, the free energy of binding, is the free energy difference between the bound state and the unbound state. We can see from Eqn. (7.2) that if K_a is large (if binding is "tight"), even small concentrations of free ligand will give $\phi \sim 1$. Rearrangement of Eqn. (7.2) in terms of $K_\mathrm{a}[\mathrm{L}]$ gives

$$K_a[L] = \phi/(1 - \phi). \tag{7.3}$$

This is the *Langmuir adsorption isotherm* (Fig. 7.2).[3] "Adsorption" because bound ligand molecules can be pictured as being adsorbed onto macromolecules, and "isotherm" because the temperature is

[3] The isotherm is an eponym of Irving Langmuir, the American physical chemist who lived 1881–1957. His research program combined interests in basic and applied science ("Pasteur's quadrant"). He was awarded the Nobel Prize in Chemistry in 1932.

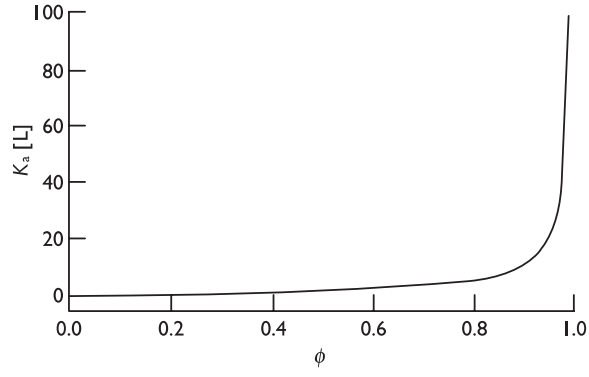

Fig. 7.2 Langmuir isotherm: K_a[L] is plotted against ϕ. As K_a becomes infinitely large, K_a[L] / (1 + K_a[L]) approaches 1.

constant. But in fact, earliest work on the adsorption isotherm had nothing to do with biological macromolecules! A biomolecule is a chemical.

Box 7.1. You give me fever . . .

Immunity in humans consists of all the physiological mechanisms which enable the body to detect "foreign" or "abnormal" materials and to neutralize or eliminate them. There are two types of immune response: specific and nonspecific. The entry of a "foreign invader" into the body triggers the nonspecific immune response called inflammation. Some aspects of the inflammatory response are local, for example, dilation of vessels of the microcirculation nearby the invader; others are systemic, involving tissues or organs far from the site of infection. The most common and striking sign of infection or some other sort of injury is fever, which results from a disturbance in the regulation of body temperature by the central nervous system. The body's thermoregulatory system resembles an engineering control system, monitoring "inputs" by sensing body temperature at various locations and controlling "outputs" by maintaining body temperature near 37 °C. The "thermostat" is located in the hypothalamus, a 5–6 cm^3 region of the brain. A rise in body temperature above 42–44 °C will result in death. The substance which is most responsible for resetting the body's internal temperature in the wake of infection is a protein known as endogenous pyrogen, which is secreted at the site of infection by the white blood cells known as monocytes and macrophages. Endogenous pyrogen is transported by the bloodstream to the brain, where it binds its receptor. If fever is such a consistent indicator of infection, one wonders whether fever might also serve a protective function? Recent evidence suggests that such is the case. The use of aspirin and other drugs to suppress fever might therefore not always be advantageous for health. You give me fever . . .

Equations (7.1) and (7.2), simple though they are, can nevertheless be extremely useful to the biochemist; they describe an amazing variety of biochemical situations. Consider, for instance, the role of phosphorylated tyrosine residues in signal transduction: if a certain phosphotyrosine recognition module interacts with a target molecule at just one site, then these equations apply. It just so

happens that this is indeed the case with a large number of known phosphotyrosine recognition motifs, including the SH2 domain and the PTB (phosphotyrosine binding) domain. PTB domains interact with phosphorylated tyrosine residues in the cytoplasmic domain of growth factor receptors with $K_d \sim 1\ \mu M$. Some proteins, for instance the large cytoskeleton-associated signaling protein tensin, have multiple phosphotyrosine recognition domains, enabling them to interact with several proteins at once. Tensin has an SH2 domain and a PTB domain.

C. Multiple independent sites

The situation here is obviously more complicated than the one-site model! Suppose there are n binding sites per macromolecule. The concentration of *bound* ligand is

$$[L]_b = [M \bullet L] + 2[M \bullet L_2] + 3[M \bullet L_3] + \cdots = \sum_{i=1}^{n} i[M \bullet L_i]. \tag{7.4}$$

The integral coefficients on the right-hand side {1, 2, 3, ...} tell us how many ligand molecules are bound to the n binding sites per macromolecule. The average fraction of sites bound is

$$\phi = \frac{\sum_{i=1}^{n} i[M \bullet L_i]}{\sum_{i=0}^{n} [M \bullet L_i]}. \tag{7.5}$$

Note the differences between numerator and denominator of Eqn. (7.5). The terms of the series in the denominator have no coefficient (there is no weighting of a term's contribution to the sum), and the sum starts at zero (to include macromolecules with no ligands bound).

Now let's think about the various ligand-bound states in terms of equilibrium constants. The relevant equilibria, which represent the time-average situation, look like this:

$$\begin{aligned} M + L &\Leftrightarrow M \bullet L & K_1 &= [M \bullet L]/[M][L] \\ M + 2L &\Leftrightarrow M \bullet L_2 & K_2 &= [M \bullet L_2]/[M][L]_2 \\ M + 3L &\Leftrightarrow M \bullet L_3 & K_3 &= [M \bullet L_3]/[M][L]_3 \end{aligned} \tag{7.6}$$

and so forth, where each K is a *macroscopic association constant*, or apparent association constant. K represents an all-or-none association of ligands and macromolecule in each case, not the sequential addition of a single ligand. This is a convenient mathematical device, not an accurate model of the physical process! Because the free energy of a bound state must be lower than the free energy of an unbound state, the more ligands bound, the more stabilized the complex. Binding stabilizes – always.

Substitution of the relationships in the right-hand column into Eqn. (7.5) gives

$$\phi = \frac{\sum_{i=1}^{n} iK_i[\mathrm{M}][\mathrm{L}]^i}{\sum_{i=0}^{n} K_i[\mathrm{M}][\mathrm{L}]^i} = \frac{\sum_{i=1}^{n} iK_i[\mathrm{L}]^i}{\sum_{i=0}^{n} K_i[\mathrm{L}]^i}. \tag{7.7}$$

This is the famous *Adair equation*, first described in 1924 by the British physiologist Gilbert Smithson Adair (1896–1979). Though complex, the equation is nice and compact and does not depend on assumptions on the specific character of binding. And when there is just one binding site, the Adair equation reduces to $\phi = K_1[\mathrm{L}]/(K_0[\mathrm{L}]^0 + K_1[\mathrm{L}]) = K_1[\mathrm{L}]/(1 + K_1[\mathrm{L}])$, which is Eqn. (7.2). (Good!) $K_0 = 1$ because the unbound macromolecule is the reference state, i.e. $\Delta G_0 = 0$.

Note that Eqn. (7.7) makes no distinction between binding to the folded state and binding to the unfolded state. Generally speaking, though, the geometry of the binding- site will be suitable for specific binding in the folded or native state only. The constellation of chemical groups that coordinate the ligand in the native state will rarely be suitable for binding a ligand specifically when the macromolecule is denatured. The situation is different, however, if a ligand binds non-specifically.

Consider, for example, the chemical denaturants urea and guanidine hydrochloride. The precise mode of binding of these small molecules to proteins is not entirely clear, and they probably alter the structure of solvent as much as or more than they bind to proteins at the concentrations required for denaturation. But it is a fact that many proteins unfold cooperatively as the chemical denaturant concentration goes up. Regardless of whether or not denaturant molecule–protein interactions are the principal cause of denaturation when a chemical denaturant is present, the situation can be *modeled* as though binding were the cause of unfolding. In this context, the *difference* in the nominal number of denaturant binding sites between the folded and unfolded state is a quantity of interest. This number is purely phenomenological, not only because it is a thermodynamic quantity and all thermodynamic quantities are phenomenological in the absence of a plausible structural interpretation, but also because the number of binding sites determined experimentally is an effective number and not the number actually believed to bind. In short, if you plug the phenomenological quantity into the right equation, your theoretical curve will closely resemble the data, but you do not therefore believe that the phenomenological quantity gives an accurate description of the situation on the physical level. But if the binding of a chemical denaturant to a macromolecule is non-specific, there *must* be more binding sites in the unfolded state than in the folded one. As the denaturant concentration increases, the folded state becomes progressively *stabilized* through interacting non-specifically with ligand molecules, but the loosely organized

unfolded state becomes stabilized even more, because it has many more non-specific "binding sites" than the compact folded state. To a first approximation, then, the phenomenological *m*-values discussed in the sections on protein denaturation in Chapters 5 and 6 are proportional to the solvent-*inaccessible* surface area of the protein in the *folded* state. The solvent-accessible surface area in the unfolded state is, roughly, the total surface area of the extended polypeptide chain: the solvent-accessible surface plus the solvent-inaccessible surface in the folded state. An *m*-value will also be roughly proportional to the difference in number of denaturant binding sites between the unfolded state and folded state of a protein. In the usual case it will be hard to interpret an *m*-value beyond what is said here.

Equation (7.7) may seem rather frightening in appearance, sending chills up and down your spine and causing you to break out in a cold sweat. Take heart: the good news is that we can simplify things a bit, not merely because this is an introductory text but because that's what's usually done in practice! We want to be able to see, for example, how ϕ has a maximum value of n when we impose the condition that the n binding sites per macromolecule be *independent* and *identical*. If we require the binding of one ligand *not* to affect the affinity of ligands at the other binding sites, and if each site binds the same type of ligand with the same affinity, there is indeed a simpler way of writing down Eqn. (7.7). By way of a few mathematical tricks (some "hairy" algebra), you can show that

$$\phi = nk[\mathrm{L}]/(1 + k[\mathrm{L}]), \tag{7.8}$$

where k is the so-called *microscopic association constant*, or intrinsic association constant. This binding constant is defined as $k_i = [\mathrm{ML}_i]/[\mathrm{ML}_{i-1}][\mathrm{L}]$, and it represents the free energy change on binding of the ith ligand to a macromolecule with $i-1$ ligands already bound. It is assumed that $k_i = k$ for all i; in other words, every ligand binds with the same affinity. Note that k differs from K, the equilibrium constant for the free energy difference between the completely unbound state of the macromolecule and the state in which all i ligands are bound. It is important to say that *the microscopic and macroscopic approaches, although they might seem quite different, describe binding equally well*. The approach you choose to describe a particular situation will be governed by personal preference, the research situation, simplicity.

Now let's look at the mathematics of ligand binding in the context of *titration*, the gradual filling up of sites. Such experiments are very common in biochemistry, so the odds that this knowledge will be of *practical* use to you at some point in your life in science are fairly good. You might even find yourself analyzing isothermal titration calorimetry (ITC) data someday. We are interested in the general reaction

$$\mathrm{M} + n\mathrm{L} \Leftrightarrow \mathrm{M} \bullet \mathrm{L}_n, \tag{7.9}$$

where the symbols have the same meaning as before. The total ligand concentration is

$$[L]_T = [L] + [L]_b \tag{7.10}$$

$$= [L] + [M]\phi \tag{7.11}$$

$$= [L] + [M]nk[L]/(1 + k[L]), \tag{7.12}$$

where we have used Eqn. (7.8) to get from Eqn. (7.11) to Eqn. (7.12). Multiplying both sides of Eqn. (7.12) by $(1 + k[L])$ gives

$$(1 + k[L])[L]_T = (1 + k[L])[L] + [M]nk[L], \tag{7.13}$$

which can be expanded to

$$[L]_T + k[L][L]_T = [L] + k[L]^2 + nk[M][L] \tag{7.14}$$

and rearranged as

$$k[L]^2 + (nk[M] - k[L]_T + 1)[L] - [L]_T = 0. \tag{7.15}$$

This equation is *quadratic* in [L] (the highest power of the ligand concentration is 2), so you can simply plug the coefficients of [L] into the quadratic formula and turn the crank to solve for [L].[4] The result is

$$[L] = \frac{-(1 + nk[M] - k[L]_T) \pm \sqrt{(1 + nk[M] - k[L]_T)^2 + 4k[L]_T}}{2k}. \tag{7.16}$$

This is the *free* ligand concentration (Fig. 7.3). Beware: only positive values of [L] are physically meaningful.

We can connect the foregoing theory with concrete measurements made by ITC (Fig. 7.4). What we'd like to know is how to relate the

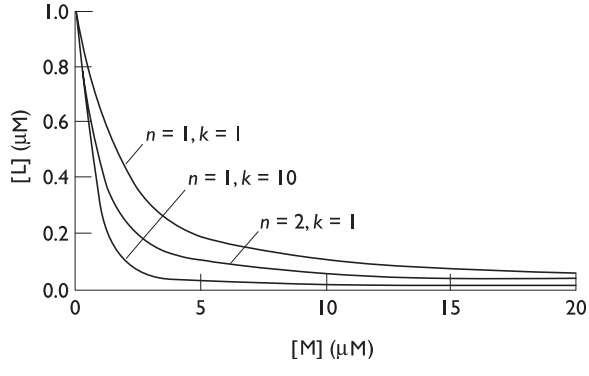

Fig. 7.3 Variation of free ligand concentration. The curves show the effect on the variation of [L] with [M] of changing the number of binding sites (n) or the microscopic binding constant (k). As expected, as [M] goes up, [L] falls; the macromolecule "mops up" the ligand, reducing the free concentration for a fixed quantity of ligand. Note that k is in units of $(\mu M)^{-1}$.

[4] The general form of a quadratic equation is $ax^2 + bx + c = 0$. There two solutions to this equation: $x = (-b \pm [b^2 - 4ac]^{\frac{1}{2}})/2a$.

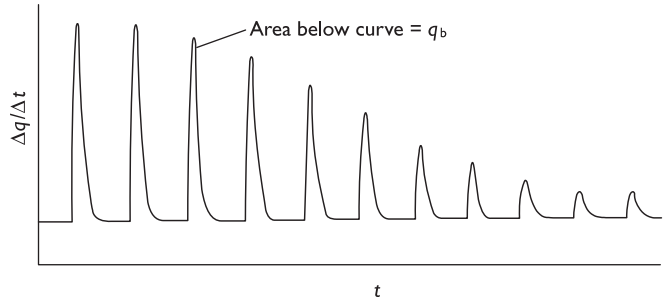

Fig. 7.4 Isothermal titration calorimetry. An experiment consists of equal-volume injections of ligand solution into the macromolecule solution. Each peak results from a single injection and in this case corresponds to an exothermic process. The first few peaks are similar in size, indicating that nearly all of the injected ligand is binding. The injection peaks become smaller as binding sites become occupied and the fraction of injected ligand decreases. The last few peaks are about the same size: binding is effectively complete at the end of the experiment, but there is a small heat of injection. Figure 2.8 shows a schematic diagram of an ITC instrument.

heat released upon binding, q_b, a macroscopic quantity, to molecular interactions. At constant pressure, we can write down q_b as:

$$q_b = \phi \Delta H_b \tag{7.17}$$

$$= V_{cell}[L]_b \Delta H_b \tag{7.18}$$

where V_{cell} is the volume of the reaction cell (Chapter 2). Note that $V_{cell} \times [L]_b = \phi$ has dimensions [volume][moles/volume] = [moles] (compare Eqn. (7.8)). Substituting Eqns. (7.10) and (7.16) into Eqn. (7.18) gives

$$q_b = V_{cell}\left([L]_T + \frac{(1 + nk[M] - k[L]) - \sqrt{(1 + nk[M] - k[L]_T)^2 + 4k[L]_T}}{2k}\right)\Delta H_b. \tag{7.19}$$

When the macromolecule is saturated with ligand, the concentration of bound ligand is equal to the concentration of macromolecule times the number of binding sites per macromolecule:

$$q_{b,sat} = V_{cell} n[M] \Delta H_b \tag{7.20}$$

which, when solved for the binding enthalpy, is

$$\Delta H_b = \frac{q_{b,sat}}{V_{cell} n[M]}. \tag{7.21}$$

If the number of binding sites per macromolecule and the cell volume are known, and if the macromolecule concentration and heat of binding can be measured, the enthalpy of binding can be calculated as shown.

What if there is more than one class of binding site *for the same ligand*? That is, what if all the ligands are the same but the *k*s are different? A more general form of Eqn. (7.8) is needed, and it is

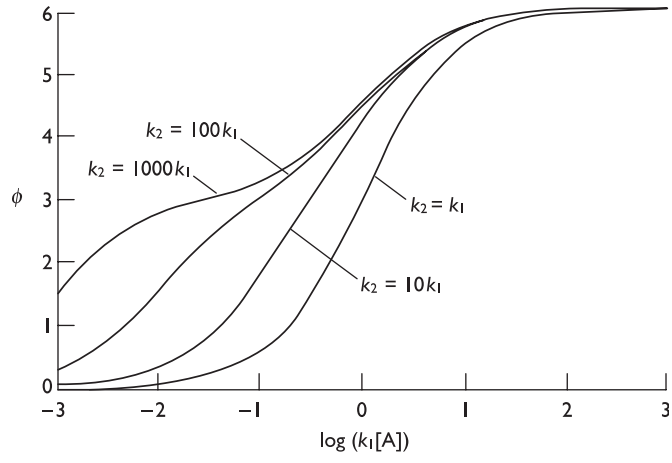

Fig. 7.5 Multiple classes of binding site. Here there are two different types of site, one with k_1 and another with k_2. Note that $n_1 = n_2 = 3$. When $k_1 = k_2$, the curve is indistinguishable from that for six identical sites. As k_2 becomes increasingly different from k_1, the curve becomes increasingly less sigmoidal. We could replace ϕ/n, which varies between 0 and 1 and $n = n_1 + n_2$, by the percentage change in another observable quantity.

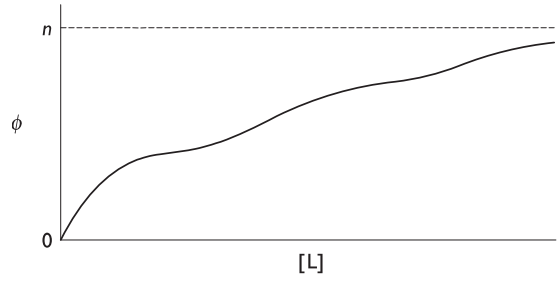

Fig. 7.6 Complex binding process. It would be very difficult to say in such cases how many different classes of binding site are present, but there is no doubt that *something* is happening. What?

$$\phi = \Sigma n_i k_i[\mathrm{L}]/(1 + k_i[\mathrm{L}]), \tag{7.22}$$

where the sum is over the various different kinds of binding site. For example, if there are two types of binding site, Eqn. (7.22) becomes

$$\phi = \frac{n_1 k_1[\mathrm{L}]}{1 + k_1[\mathrm{L}]} + \frac{n_2 k_2[\mathrm{L}]}{1 + k_2[\mathrm{L}]}. \tag{7.23}$$

There are n_1 sites with binding constant k_1 and n_2 sites with constant k_2. Figure 7.5 shows how ϕ varies with k_1[L] for different values of k_2/k_1. As you might expect, when $k_2 = k_1$ the curve is indistinguishable from Eqn. (7.8) with $n = n_1 + n_2$. Marked deviations from Eqn. (7.8) occur if k_2/k_1 is large (>50) or small (<0.02). When k_2/k_1 lies between these values, if you neglect to take great care in collecting and handling experimental data it might be difficult to distinguish between a large number of identical sites with $k_2 < k < k_1$ and two smaller numbers of two classes of site. Such difficulties in data analysis are common in biochemistry, and a lot of clever thinking might be required to make a convincing case for a certain number of binding sites. A plot of ϕ versus [L] for a complex binding process is shown in Fig. 7.6.

D. Oxygen transport

In Chapter 5 and in the introduction to the present chapter we saw how Hb plays a key role in oxygen transport. Here we build on the previous discussions and make a more detailed comparison of Hb and Mb in the context of binding. The approach is abstract and need not concern the biological *origin* of the proteins for the moment; let's think about them as mere objects of physical chemistry. Then, we'll see what additional insight accounting for biological function can add to the mathematical development. Our overall aim is to see how the physicochemical properties of these proteins underlie their biological functions.

As discussed above, Hb and Mb are similar and different. They are similar in that the three-dimensional shape of native Mb, known as the myoglobin fold, closely resembles the native structure of the α-subunits of Hb. Both proteins bind *heme* (Greek: *hyma*, blood) in much the same way, and the bound iron atom (Fig. 5.1) helps to coordinate diatomic oxygen (or carbon monoxide!). Hb and Mb differ in that the former is tetrameric (two α-chains and two β-chains) while the latter is monomeric under physiological conditions. As we shall see, this structural difference has profound consequences for regulation of protein function. Indeed, the macroscopic oxygen-binding properties of these two proteins are very different (Fig. 7.7). Oxygen binding to Hb can be regulated by more than just the partial pressure of oxygen: it is also sensitive to Cl^- concentration, pH, and a small ionic compound called diphosphoglycerate (DPG).

Analysis of the structure and thermodynamic properties of Hb has shed much light on how protein assemblies "self-regulate" in response to environmental "signals." The "signals" could be changes in the concentration of certain solutes or temperature, and "self-regulation" is the effect of such changes on protein conformation and ligand binding. The structure of Hb shifts subtly with changes in

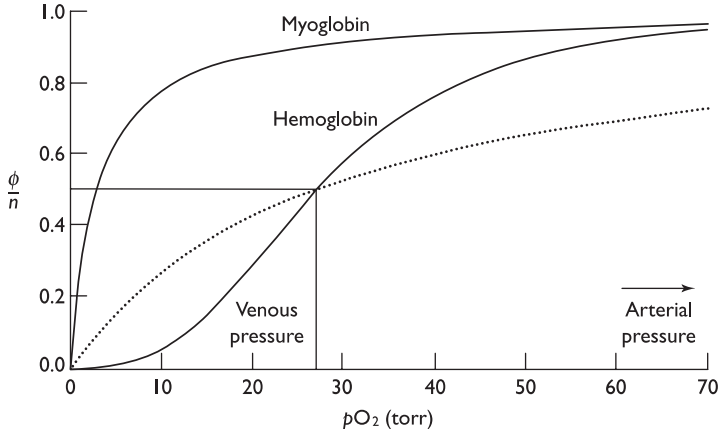

Fig. 7.7 Oxygen-binding properties of myoglobin and hemoglobin. The myoglobin oxygen-binding curve is a rectangular hyperbola. The hemoglobin curve is sigmoidal. The shape of the hemoglobin curve arises from interactions between subunits. The broken line represents the percentage saturation of hemoglobin in the absence of inter-subunit cooperativity, assuming that the partial pressure of oxygen required for 50% binding (p_{50}) is the same as when cooperative interactions are present. At low partial pressures of oxygen, the affinity of oxygen is much higher for myoglobin than for hemoglobin. This explains how oxygen is unloaded from hemoglobin in capillaries.

the chemical environment, in order to minimize free energy. The energy changes can be large, and although the associated shifts in structure are small, they can have dramatic effects on protein biochemistry. In short, Hb is a sophisticated transport system. In-depth analysis of Hb and comparison with other biological macromolecules shows that Hb is a useful model for the development of general theories of regulation of biochemical processes at the molecular level. So Hb can be thought of as a type of *molecular switch*. Its switch-like character "arises" from the *interactions* between subunits.

And now for a more mathematical look at the oxygen binding capabilities of Hb and Mb. The binding of oxygen to Mb can be described as

$$\mathrm{Mb} + \mathrm{O_2} \;\Leftrightarrow\; \mathrm{Mb} \bullet \mathrm{O_2}. \tag{7.24}$$

The dissociation constant for this reaction is

$$K_\mathrm{d} = [\mathrm{Mb}][\mathrm{O_2}]/[\mathrm{Mb} \bullet \mathrm{O_2}] \tag{7.25}$$

and, by Eqn. (7.1), the fraction bound is

$$\phi = [\mathrm{Mb} \bullet \mathrm{O_2}]/[\mathrm{Mb}]_\mathrm{T} = [\mathrm{O_2}]/(K_\mathrm{d} + [\mathrm{O_2}]). \tag{7.26}$$

When this relationship is written in terms of the partial pressure of oxygen ($p\mathrm{O_2}$) and the pressure at which half of the binding sites are occupied (p_{50}), it looks like this:

$$\phi = p\mathrm{O_2}/(K_\mathrm{d} + p\mathrm{O_2}) = p\mathrm{O_2}/(p_{50} + p\mathrm{O_2}). \tag{7.27}$$

The shape of Eqn. (7.27) is a rectangular hyperbola (Fig. 7.7). In humans, $p_{50} \approx 2.8$ torr.[5] Because this is well below venous pressure, it is thermodynamically unfavorable for $\mathrm{O_2}$ to be released back into the blood once it has become bound to Mb. And Hb?

Experimental studies have shown that the variation in fractional saturation as a function of $p\mathrm{O_2}$ is rather different for Hb. The binding curve of Hb is *not* a rectangular hyperbola. An early attempt to use mathematics to rationalize the oxygen-binding characteristics of Hb was advanced by Archibald Vivian Hill in 1911.[6] According to his work, for a ligand L and macromolecule M with n binding sites,

$$\mathrm{M} + n\mathrm{L} \;\Leftrightarrow\; \mathrm{M} \bullet \mathrm{L}n. \tag{7.28}$$

This is just Eqn. (7.9). If the ligand molecules are assumed to bind in an all-or-none fashion (an oversimplification that will be dealt with shortly), there is but one (macroscopic) *dissociation* constant:

$$K_\mathrm{d} = [\mathrm{M}][\mathrm{L}]^n/[\mathrm{M} \bullet \mathrm{L}_n]. \tag{7.29}$$

[5] 1 torr = 1 mm Hg at 0 °C = 0.133 kPa; 760 torr = 1 atm. The torr is named after Evangelista Torricelli (1608–1647), the Italian physicist and mathematician who is credited with inventing the barometer.

[6] A.V. Hill (1886–1977), a Briton, was awarded the Nobel Prize in Physiology or Medicine in 1922, for his discovery relating to the production of heat in muscle.

The degree of saturation, defined as before, is

$$\phi = \frac{n[\mathrm{M} \bullet \mathrm{L}_n]}{[\mathrm{M}] + [\mathrm{M} \bullet \mathrm{L}_n]}. \tag{7.30}$$

The factor n appears in the numerator on the right-hand side because there are n ligands bound to each macromolecule at saturation; ϕ is the average number of ligands bound per macromolecule. Rearrangement of Eqn. (7.30) gives

$$\frac{\phi}{n} = \frac{[\mathrm{M} \bullet \mathrm{L}_n]}{[\mathrm{M}] + [\mathrm{M} \bullet \mathrm{L}_n]}. \tag{7.31}$$

which could be called the fractional saturation, since it varies between 0 and 1. On substituting in Eqn. (7.29),

$$\frac{\phi}{n} = \frac{[\mathrm{L}]^n}{K_\mathrm{d} + [\mathrm{L}]^n}. \tag{7.32}$$

This is the famous *Hill equation*. Figure 7.8 shows plots of the Hill equation for different values of n. Note that although one would not ordinarily think of a non-integral number of ligands bound, n can in principle take on any real value. In other words, n is a *phenomenological representation* of the *average* number of ligands bound, or the *effective* number of ligands bound. Unfortunately, making use of statistical mechanics to analyze binding data does not necessarily get around the phenomenological quality of binding data, particularly when the situation is the slightest bit ambiguous, as it is with the interaction of chemical denaturants with proteins. But when binding is very specific, the measured number of binding sites will ordinarily be close to the number one would "see" on visualizing the structure of the macromolecule at atomic resolution. Note that n can be negative as well as positive. When it is positive, the binding cooperativity is positive; and vice versa. "Negative binding" (of water) occurs in

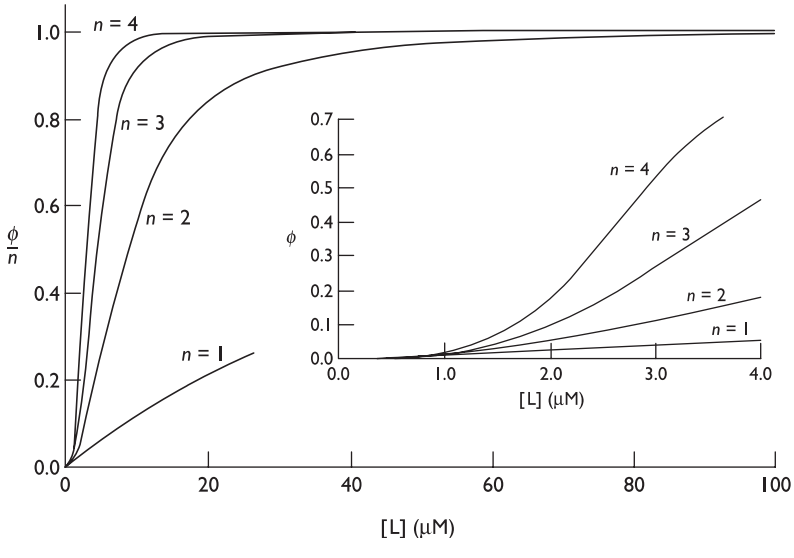

Fig. 7.8 The Hill equation. Fractional saturation is plotted as a function of ligand concentration and n. As n increases, the concentration of ligand required for saturation decreases if binding affinity is constant. The inset helps to reveal the increasingly sigmoidal shape of the curve as n increases from 1. $K_\mathrm{d} = 1\ \mu\mathrm{M}$.

the context of exclusion of the solvent from the surface of a macromolecule in the presence of an "inert" co-solvent like sucrose, which do not bind the protein but nevertheless influence protein stability.

Now let's dial in a higher-powered objective lens and look at oxygen binding to Hb at increased magnification. The various binding equilibria to consider are:

$$\begin{array}{lll} \mathrm{M}+\mathrm{L} \Leftrightarrow \mathrm{M}\bullet\mathrm{L} & K_{a,1}=4k_{a,1} & k_{d,1}=4K_{d,1} \\ \mathrm{M}\bullet\mathrm{L}+\mathrm{L} \Leftrightarrow \mathrm{M}\bullet\mathrm{L}2 & K_{a,2}=3k_{a,2}/2 & k_{d,2}=3K_{d,2}/2 \\ \mathrm{M}\bullet\mathrm{L}2+\mathrm{L} \Leftrightarrow \mathrm{M}\bullet\mathrm{L}3 & K_{a,3}=2k_{a,3}/3 & k_{d,3}=2K_{d,3}/3 \\ \mathrm{M}\bullet\mathrm{L}3+\mathrm{L} \Leftrightarrow \mathrm{M}\bullet\mathrm{L}4 & K_{a,4}=k_{a,3}/4 & k_{d,4}=K_{d,3}/4. \end{array} \tag{7.33}$$

Note the relationships between the microscopic *association* constants (the $k_{a,i}$s) and the macroscopic dissociation constants (the $K_{d,i}$s). The coefficients of the microscopic binding constants, called *statistical factors* (Chapter 6), arise as follows. There is only one way in which the ligand can dissociate from a singly ligated Hb molecule, but four possible ways in which it can bind in the first place; hence $K_{a,1}=4k_{a,1}$. When one site is occupied, there are three possible ways in which a second ligand can bind, and two ways in which a doubly ligated molecule can lose ligands, so $K_{a,2}=3k_{a,2}/2$. And so forth.

The concentrations of the various bound states are:

$$\begin{aligned} [\mathrm{M}\bullet\mathrm{L}] &= [\mathrm{M}][\mathrm{L}]/k_{d,1} \\ [\mathrm{M}\bullet\mathrm{L}_2] = [\mathrm{M}\bullet\mathrm{L}][\mathrm{L}]/k_{d,2} &= [\mathrm{M}][\mathrm{L}]^2/k_{d,1}k_{d,2} \\ [\mathrm{M}\bullet\mathrm{L}_3] = [\mathrm{M}\bullet\mathrm{L}_2][\mathrm{L}]/k_{d,3} &= [\mathrm{M}][\mathrm{L}]^3/k_{d,1}k_{d,2}k_{d,3} \\ [\mathrm{M}\bullet\mathrm{L}_4] = [\mathrm{M}\bullet\mathrm{L}_3][\mathrm{L}]/k_{d,4} &= [\mathrm{M}][\mathrm{L}]^3/k_{d,1}k_{d,2}k_{d,3}k_{d,4}, \end{aligned} \tag{7.34}$$

where dissociation constants have been used. The fractional saturation of the macromolecule is

$$\frac{\phi}{4}=\frac{[\mathrm{M}\bullet\mathrm{L}]+2[\mathrm{M}\bullet\mathrm{L}_2]+3[\mathrm{M}\bullet\mathrm{L}_3]+4[\mathrm{M}\bullet\mathrm{L}_4]}{4([\mathrm{M}]+[\mathrm{M}\bullet\mathrm{L}]+[\mathrm{M}\bullet\mathrm{L}_2]+[\mathrm{M}\bullet\mathrm{L}_3]+[\mathrm{M}\bullet\mathrm{L}_4])}. \tag{7.35}$$

The coefficients in the numerator refer to the number of ligands bound to each species. Substituting in Eqns. (7.34) gives

$$\frac{\phi}{4}=\frac{\begin{array}{l}[\mathrm{M}][\mathrm{L}]/k_{d,1}+2[\mathrm{M}][\mathrm{L}]^2/k_{d,1}k_{d,2}+ \\ 3[\mathrm{M}][\mathrm{L}]^3/k_{d,1}k_{d,2}k_{d,3}+4[\mathrm{M}][\mathrm{L}]^4/k_{d,1}k_{d,2}k_{d,3}k_{d,4}\end{array}}{\begin{array}{l}4([\mathrm{M}]+[\mathrm{M}][\mathrm{L}]/k_{d,1}+[\mathrm{M}][\mathrm{L}]^2/k_{d,1}k_{d,2}+ \\ [\mathrm{M}][\mathrm{L}]^3/k_{d,1}k_{d,2}k_{d,3}+[\mathrm{M}][\mathrm{L}]^4/k_{d,1}k_{d,2}k_{d,3}k_{d,4})\end{array}} \tag{7.36}$$

$$\frac{\phi}{4}=\frac{[\mathrm{L}]/k_{d,1}+2[\mathrm{L}]^2/k_{d,1}k_{d,2}+3[\mathrm{L}]^3/k_{d,1}k_{d,2}k_{d,3}+4[\mathrm{L}]^4/k_{d,1}k_{d,2}k_{d,3}k_{d,4}}{4(1+[\mathrm{L}]/k_{d,1}+[\mathrm{L}]^2/k_{d,1}k_{d,2}+[\mathrm{L}]^3/k_{d,1}k_{d,2}k_{d,3}+[\mathrm{L}]^4/k_{d,1}k_{d,2}k_{d,3}k_{d,4})}. \tag{7.37}$$

In terms of the macroscopic dissociation constants, Eqn. (7.37) is

$$\frac{\phi}{4}=\frac{\begin{array}{l}[\mathrm{L}]/K_{d,1}+3[\mathrm{L}]^2/K_{d,1}K_{d,2}+ \\ 3[\mathrm{L}]^3/K_{d,1}K_{d,2}K_{d,3}+[\mathrm{L}]^4/K_{d,1}K_{d,2}K_{d,3}K_{d,4}\end{array}}{\begin{array}{l}1+4[\mathrm{L}]/K_{d,1}+6[\mathrm{L}]^2/K_{d,1}K_{d,2}+ \\ 4[\mathrm{L}]^3/4K_{d,1}K_{d,2}K_{d,3}+[\mathrm{L}]^4/K_{d,1}K_{d,2}K_{d,3}K_{d,4}\end{array}} \tag{7.38}$$

Note that the statistical factors in the denominator are the same as line five of Pascal's triangle (Fig. 6.13), reflecting their origin in combinatorics. We shall return to this development in the second part of the next section.

E. Scatchard plots and Hill plots

Now we turn our attention to useful ways of graphing binding data. The discussion follows on from the previous discussion. At the close of this section we'll note strengths and weaknesses of the Scatchard plot and Hill plot, and comment on the more general utility and greater value of non-linear least-squares regression methods in data analysis.[7]

Rearrangement of Eqn. (7.1) gives

$$[\mathrm{M} \bullet \mathrm{L}] = [\mathrm{M}]_\mathrm{T} - K_\mathrm{d}[\mathrm{M} \bullet \mathrm{L}]/[\mathrm{L}]. \tag{7.39}$$

This is a variant of the *Scatchard equation* (see Eqn. (5.38) and Fig. 5.16). The bound ligand concentration ([M•L]) is linear in [M•L]/[L] with slope $-K_\mathrm{d}$; a *Scatchard plot* can be used to obtain K_d graphically. Note that Eqn. (7.39) assumes that there is just one binding site per macromolecule. A more useful equation would be one that could be used to analyze data directly, whether the instrument used for experiments was a fluorimeter, circular dichroism spectrometer, NMR spectrometer, calorimeter, or whatever. Assuming that the change in the observable quantity, ΔO, is directly proportional to [M•L], the bound concentration, as is often the case, then $\Delta O = [\mathrm{M}\bullet\mathrm{L}]$. And if ΔO_max is the change in observable on saturation of the binding sites, then $\mathbf{c}\Delta O_\mathrm{max} = [\mathrm{M_T}\bullet\mathrm{L}]$. Note that **c** is a proportionality constant whose value we do not know *a priori* (in advance of doing the analysis); the value will depend on the protein–ligand system being studied, the solution conditions, and of course the technique used for measurements. Making the appropriate substitutions into Eqn. (7.39), we have

$$\mathbf{c}\Delta O = \mathbf{c}\Delta O_\mathrm{max} - K_\mathrm{d}\mathbf{c}\Delta O/[\mathrm{L}] \tag{7.40}$$

or

$$\Delta O = \Delta O_\mathrm{max} - K_\mathrm{d}\Delta O/[\mathrm{L}]. \tag{7.41}$$

ΔO simply measures the change in signal (fluorescence intensity, ellipticity, resonant frequency, heat uptake, ...) when [L] is added, and K_d measures how much O will change for a given change in [L].

[7] Least-squares methods were developed about 1794 by Carl Friedrich Gauss (1777–1855), who with Archimedes and Newton ranks as one of the greatest mathematicians of all time. Gauss also made important contributions to astronomy, geodesy, and electromagnetism, and his treatment of capillary action contributed to the development of the principle of the conservation of energy. In least-squares analysis, the best-estimated value is based on the minimum sum of squared differences between the "best-fit" curve and experimental data points.

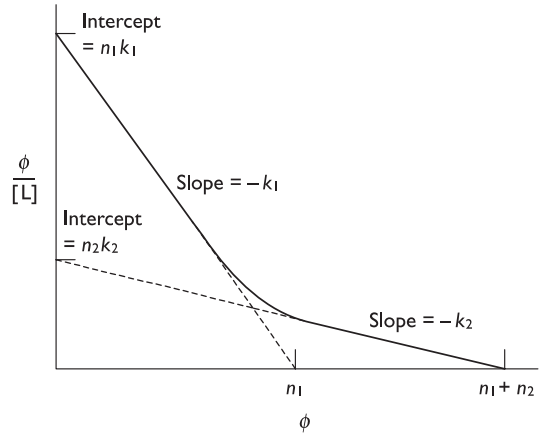

Fig. 7.9 Scatchard plot. There are two types of ligands, $k_1 > k_2$, and $n_1 \neq n_2$. The ks are microscopic association constants. The horizontal axis intercepts are particularly informative here: they provide information on the number of ligand binding sites.

Making use of $K_d = 1/K_a$, Eqn. (7.41) can also be written as $\Delta O/[L] = K_a \Delta O_{max} - K_a \Delta O$. How does this relationship compare with others we've seen?

From Eqns. (7.1) and (7.2),

$$\phi = [M \bullet L]/[M]_T = K_a[L]/(1 + K_a[L]). \tag{7.42}$$

Multiplication of both sides by $(1 + K_a[L])$ gives

$$\phi(1+K_a[L]) \; = \phi + \phi K_a[L] \; = K_a[L], \tag{7.43}$$

which, when solved for ϕ and divided by [L], gives

$$\phi/[L] \; = K_a - \phi K_a, \tag{7.44}$$

yet another form of the Scatchard equation. A plot of $\phi/[L]$ against ϕ gives a line of slope $-K_a = -1/K_d$. The curve intersects the vertical axis at K_a, the association constant, and the horizontal axis at 1, the number of binding sites. This approach to analysis can also be used in more complicated cases, for instance, when there are two classes of binding site and both n_1 and n_2 are greater than 1. From Eqn. (7.23)

$$\phi/[L] \; = n_1k_1/(1+k_1[L]) + n_2k_2/(1+k_2[L]). \tag{7.45}$$

Figure 7.9 shows the appearance of $\phi/[L]$ versus ϕ when $k_1 > k_2$. Here the ks are microscopic association constants.

A simple rearrangement of the Hill equation (Eqn. 7.32) leads to the *Hill plot* – not a scheme hatched in the Capitol Building, not a garden on a mound, but a useful way of graphing binding data. The Hill plot is obtained by multiplying both sides of Eqn. (7.32) by the denominator on the right-hand side, $(K_d + [L]^n)$, grouping terms in $[L]^n$, and solving for $[L]^n/K_d$. The result is

$$\frac{\frac{\phi}{n}}{1 - \frac{\phi}{n}} = \frac{[L]^n}{K_d}. \tag{7.46}$$

This relationship, which is linear in $1/K_d$, can be made somewhat more useful by taking the logarithm of both sides. This gives

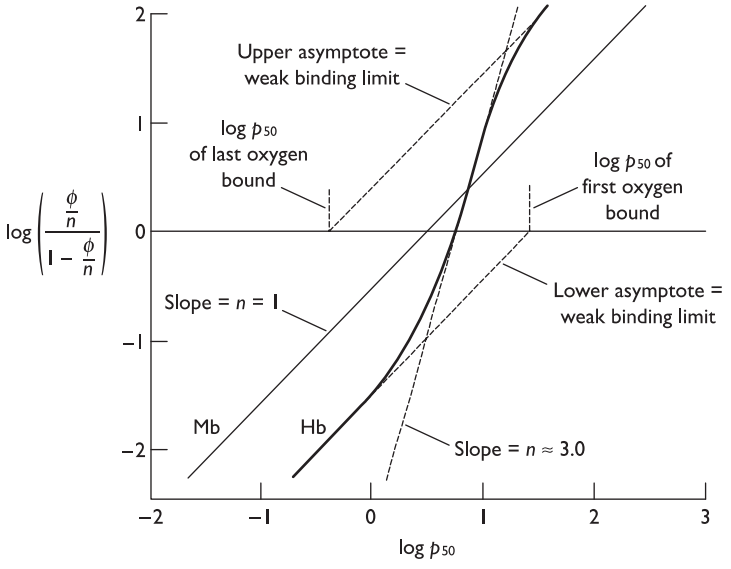

Fig. 7.10 Hill plot. When the data are of good quality, this type of plot can be very informative. The slopes of the upper and lower asymptotes are 1 in cooperative binding. Between the onset of binding and saturation, the slope changes dramatically. The slope at the point where the data curve crosses the line $n = 1$ gives the *apparent* number of ligand binding sites. In this case it is about 3.0, as in hemoglobin. The actual number of binding sites differs from the apparent number because the binding process is not perfectly cooperative. The apparent number of sites is a phenomenological parameter; it does not provide an atomic resolution view of the binding process. It will go without saying that the Hill coefficient and p_{50} depend on conditions, e.g. temperature, ionic strength, *etc.*

$$\log\frac{\frac{\phi}{n}}{1-\frac{\phi}{n}} = \log[\mathrm{L}]^n - \log K_\mathrm{d} = n\log[\mathrm{L}] - \log K_\mathrm{d}. \tag{7.47}$$

The left-hand side of this expression, which is admittedly complex, is nevertheless a *linear* function of log[L] with slope n and ordinate-intercept $-\log K_\mathrm{d}$ and therefore convenient for graphical analysis.

The utility of Eqn. (7.47) can be illustrated as follows. If the ligand is oxygen, as in hemoglobin, Eqn. (7.47) becomes

$$\log\frac{\frac{\phi}{n}}{1-\frac{\phi}{n}} = n\log pO_2 - \log K_\mathrm{d} \tag{7.48}$$

where, as before, pO_2 is the partial pressure of diatomic oxygen. What is K_d? It can be found from Eqn. (7.32). At half saturation $\phi/n = 0.5$, so $K_\mathrm{d} = (p50)^n$. A comparison of the form of Eqn. (7.48) for $n = 1$ (Mb) and $n \approx 3$ (Hb) is shown in Fig. 7.10.

If only things were *always* so simple! Before the advent of inexpensive desktop computers, the Scatchard plot and Hill plot were very sensible graphical approaches to the routine analysis of binding data, and they were used all the time. This is because linearized versions of the formulas made determining parameters and estimating

Table 7.1. | *Outline of non-linear regression analysis*

- Collect data and enter values into a spreadsheet
- Choose a model
- Guess the best-fit values of the fitting parameters
- After fitting, question whether the parameter values make good sense
- Compare the results of fitting different models to the data set
- Use various criteria to decide which model is the best one

errors a cinch using linear regression, which can be carried out relatively painlessly with a no-frills hand-held calculator, or as in ancient days, by hand. Despite the apparent simplicity and neat appearance of linear equations, linearizations of more complicated mathematical relationships *should not* normally be used for data analysis. Why not? Linearization (or any type of mathematical transformation for that matter) *distorts* the experimental error. Two assumptions of basic linear regression are that the errors in the experimental data points follow a *Gaussian* distribution,[8] that all errors are exclusively random, and that the variation in the error of the independent variable (pO_2 in the last example) is very small and constant throughout its range. These conditions, which can be difficult to realize in practice in the best of circumstances, are not likely to hold after data have been transformed mathematically. Moreover, mathematical transforms can change the form of the relationship between the independent variable and the dependent variable. For instance, in a Scatchard plot the dependent variable ([bound]/[free]) not only varies with the independent variable ([free]), as is generally the case, but it is also multiplied by its inverse! To summarize, the algebra involved in deriving the Scatchard equation and the Hill equation is sound, but the gains in using these equations may be more than offset by the losses, at least in serious data analysis. Good experimental work requires a good deal of care, effort, and time, so you should look for the most useful and sound technique for analyzing data and not just one that happens to appear in just about every biochemistry book ever published. What should you do? Non-linear regression analysis is in many cases the best way forward.

A non-linear function is just that: any (continuous) function that is not a straight line. It can be a polynomial of order two or higher ($y = a + bx + cx^2 + \ldots$; the order of a polynomial is given by the highest power of the independent variable; a first-order polynomial is a straight line) or any other type of relationship that is not a straight line (e.g. $y = \sin x$, $y = e^x$, etc.). Let's take this opportunity to cover in simple terms what non-linear regression can and cannot do. An outline of regression analysis is given in Table 7.1. You start with a spreadsheet (e.g. Excel, Origin, ...) into which your experimental

[8] A "bell-shaped" curve, named after C. F. Gauss.

data are entered, a mathematical model that *might* describe the physical or chemical process which gave rise to the data, and some sensible guesses as to the likely final values of the fitting parameters (adjustable quantities that are determined by the outcome of experiments under specific conditions). Guessing well as to final parameter values can be a very important aspect of fitting when a model is complex. This is because the usual algorithms for regression analysis "search" for the best-fit parameters by trying to minimize the deviations of the model from the experimental data, just as in linear regression. And when the model is complex, many possible combinations of parameter values can give a fit that appeals to the eye but is not physically meaningful. The calculated deviations resulting from the initial guesses can sometimes be so large that the program will not be able to adjust the parameters in such a way as to home in on the "correct" values.

That what's regression analysis does for you. What it does not do is choose your model! And for that reason regression analysis can be a non-trivial task. It is not simply a matter of finding any old mathematical function that gives a good fit, which would be fine for data presentation but of no help at all for understanding how your "system" works. Instead, you want to look for an equation that fits the data well and that is *physically meaningful*. For when the best-fit parameters have been found, they will satisfy the mathematical criterion of minimizing the deviations of the model from the data, but it will still be necessary to question whether the values (and the model) make sense! You would like to be able to stand back and say, "These results and analysis suggest that the underlying mechanism is *x*, *y* or *z*, and we can test this hypothesis by doing a certain kind of experiment." In the best case, all the tools needed to do that experiment will be available to you where you are! But be careful, for there may well be more than one physically plausible model that will fit your data well! You may well need more kinds of information to choose one model over another. Indeed, modeling can be more a matter of ruling out what seems not to work instead of choosing what is *known* to be "right."

A great many books have been written on data analysis, and data analysis is hardly the main subject of the present one, so we'll end this digression presently. The important thing to remember is that knowing the ins and outs of non-linear regression is important and useful in modern biochemistry. Regression analysis, like all areas of scientific study beyond textbook-level knowledge, is something of an art. At the same time, though, expertise in data analysis (or information technology) will not necessarily make you a good physical biochemist.

F. Allosteric regulation

The metabolic and physiological processes of living cells and tissues are regulated in a plethora of ways. In general, regulatory biochemical

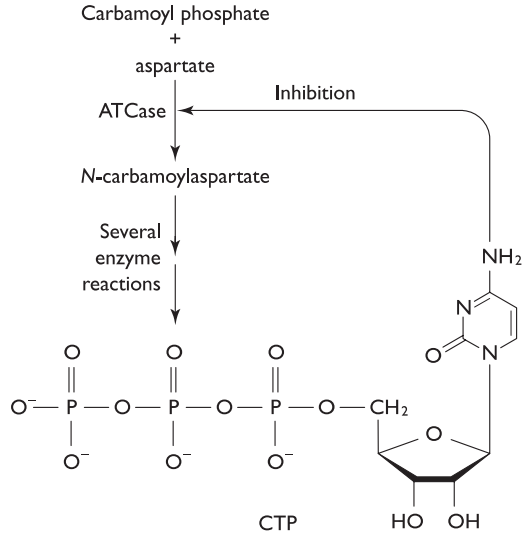

Fig. 7.11 Feedback inhibition. CTP closely resembles ATP. But ATP is the universal energy currency of the cell, not CTP. When the concentration of CTP is low, it does not inhibit the enzyme ATCase because there are not enough CTP molecules to bind. ATCase and the other enzymes of the pathway produce CTP. When [CTP] is high, it binds ATCase and inhibits production of CTP. Why should the energy resources of the cell be spent unnecessarily?

mechanisms respond to change by *damping* the effect (though there are, of course, situations were *amplification* occurs). A general type of molecular-scale metabolic control that is common to all living things is *feedback inhibition*, wherein an increase in the product of a metabolic pathway regulates an enzyme upstream in the cascade by binding to it and decreasing enzyme activity. Many enzymes regulated in this way are ones that catalyze the first step in a synthetic pathway (Fig. 7.11). This gives the cell very great control over metabolism and utilization of energy resources. Although feedback inhibition can be brought about in a variety of ways, all share the property that when the concentration of metabolite is low its production proceeds, and when it is high, production is inhibited. The cell's energy resources are spent on an as-needed basis, not willy-nilly.

The synthetic pathway of the amino acid histidine provides a specific example of feedback inhibition. There are about ten different enzymatic "nanomachines" on the molecular "assembly line" in this case. If sufficient histidine for protein synthesis and other purposes is present in the cell, the "nanomanufacture" of histidine slows and the "burn rate" of energy resources and the generation of heat and entropy are minimized. On a mechanistic level, inhibition of a key nanomachine on the assembly line results from the physical association of "end-product" histidine with the first enzyme in its "fabrication process." The regulation of gene expression by DNA-binding proteins provides another class of examples. Certain proteins called repressors limit the synthesis of unneeded enzymes by binding to specific locations on chromosomal DNA, preventing synthesis of the corresponding mRNA. If the substrates of the encoded enzymes are present, however, enzyme synthesis proceeds; synthesis is *induced* by the presence of the substrate. For example, addition of galactose to a growth medium containing *E. coli* will

induce the synthesis of β-galactosidase. In this way, synthesis of the galactose-metabolizing enzyme is regulated and the energy of the cell is not spent unnecessarily. (See Chapters 8 and 9.)

The regulation of ligand binding to a macromolecule is called *allosteric regulation* (Greek, *allos*, other + *stereos*, space), and it is one of the most interesting of all aspects of function of biological macromolecules. The term *allosteric* gets its name from the influence that binding at one site has on binding at a remote location of the same protein molecule, possibly the active site in the case of an enzyme. Note the qualitative difference between allosteric regulation and the independent binding of different types of ligand to different sites on the same enzyme with no effect on structure or function. The molecule that brings about allosteric regulation of the binding of another ligand is called an *effector* or *modulator*. The ligands involved in allostery can be identical, as in the case of oxygen binding to hemoglobin, or different, as in aspartate transcarbamoylase (ATCase). The binding of the effector can either increase or decrease the affinity of the protein for another ligand. For instance, the binding of the first oxygen molecule to hemoglobin increases the affinity of the other sites for oxygen; this is positive allostery. And the binding of ATP to ATCase increases enzymatic activity, while binding of CTP decreases activity. CTP is a negative allosteric effector of ATCase.

Let's take a closer look at ATCase. This oligomeric enzyme of identical subunits catalyzes the formation of *N*-carbamoylaspartate from carbamoyl phosphate and aspartate (Fig. 7.11). Synthesis of *N*-carbamoylaspartate is the first step in the biosynthesis of pyrimidines, including cytosine, thymine, and uracil, molecules important to the biosynthesis of polymers involved in the transmission of genetic information. ATCase has at least two stable folded conformations, known as R (high substrate affinity) and T (low substrate affinity). The relative stability of these states is affected by the binding of ATP (a purine) to R and CTP (a pyrimidine) to T. Note, however, that although different nucleotides bind to different conformations, they bind to the same site on the enzyme! That is, ligand binding is competitive. Both ATP binding and CTP binding to ATCase are examples of *homoallostery*. That's because the binding sites in ATCase are intrinsically identical.

There are two basic models of allosteric regulation in proteins: the *sequential model* and the *concerted model* (Fig. 7.12). In the sequential model (*KNF model*), proposed by Daniel Edward Koshland, Jr. (1920–2007), G. Némethy, and D. Filmer, the folded structure of a macromolecule is assumed to be sufficiently plastic for the binding of a ligand at one site to directly alter the conformation of the macromolecule at another site and thereby affect the affinity of the second site for its ligand. When cooperativity is positive, binding of the first ligand results in a conformational change that increases the affinity for a ligand at the second site, and so on. Binding affinities in allosteric systems usually vary within a range of a few kilocalories per mole. The binding of different ligands to the same site could

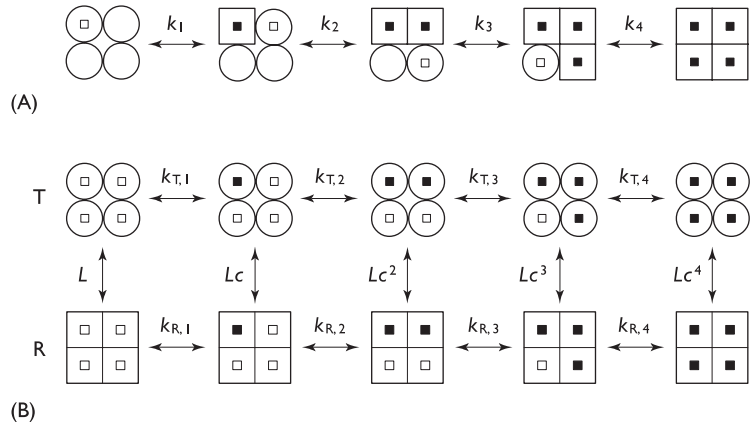

Fig. 7.12 Models of ligand binding. The ligand is not shown for the sake of clarity. (A) The KNF or "sequential" model. The binding affinity increases as the number of ligands bound increases. (B) The MWC or "concerted" model. An equilibrium constant L describes the structural change between the T state and the R state in the absence of ligand. The R conformation has a higher affinity for ligand than the T conformation by the factor c, the ratio of the microscopic binding affinities ($k_{R,i}/k_{T,i}$). Conceptually, L and c are completely separable, but in the case of Hb they are linked, complicating independent evaluation.

have different effects on enzyme conformation at remote locations. In the concerted model (*MWC model*), proposed by Jacques Lucien Monod (1910–1976) and Jean-Pierre Changeux (1936–), both Frenchmen, and Jeffries Wyman (1901–1995), an American, each subunit of a multi-component macromolecule has two folded conformations (T and R), the conformations are in equilibrium regardless of the presence of a ligand, the binding of a ligand to one site has no direct influence on the binding of a ligand to another site, the affinity of a ligand for a subunit depends only on the conformation of the subunit and not on the number of ligands bound, and all subunits are either in one conformation or the other. Instead of altering the conformation of the enzyme, ligand binding shifts the equilibrium between the two conformations of the occupied subunit. The association constants include statistical factors that account for the number of ligands bound (see above). In any particular situation, it might be difficult if not impossible to say which of these two models better represents the physical situation.

G. Proton binding

Hydrogen is the smallest atom. Nevertheless, proton binding can have a dramatic impact on protein structure and function. We saw in Chapter 2 how changes in pH can affect protein thermostability. Even when it brings about no large change in structure, proton binding or release can have marked physiological consequences. Hb provides a convenient and important example.

A 1904 research paper on the effect of pH on the affinity of oxygen for Hb describes what is known as the *Bohr effect*[9]: the release of oxygen when Hb binds protons (Fig. 7.13). In the reverse process, roughly two protons are released when Hb binds three oxygen

[9] The Bohr effect gets its name from the Danish physician Christian Bohr, father of the Nobel laureate in physics, Neils Bohr.

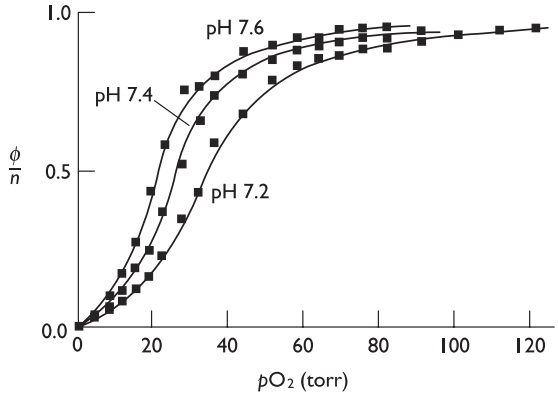

Fig. 7.13 Bohr effect. The degree of saturation of Hb depends not only on pO_2 but also on pH, as shown. The Bohr effect describes the effect of changes in pH on the number of oxygen molecules bound. The binding of oxygen is *linked* to the binding of protons. In general a thermodynamic change of the state of Hb will involve contributions from oxygen binding, proton binding, ion binding, and structural rearrangement.

molecules. These protons add up, and together they can result in a significant change in the pH of a microenvironment. We can see how the Bohr effect fits into the broader scheme of things by recalling the beginning of Chapter 5, where we discussed some aspects of respiration on a detailed level. For every mole of O_2 consumed by humans in respiration, approximately 0.8 moles of CO_2 are formed. Carbonic anhydrase, a red blood cell enzyme, catalyzes the conversion of CO_2 to carbonate and H^+, keeping the partial pressure of CO_2 low in the capillaries. As a result, the pH of the capillaries is acidic. Combination of low pH and low partial pressure of O_2 in the capillaries results in release of oxygen from Hb. The Bohr effect is particularly important in very active muscle, where the demand for O_2 is high. The role of specific inter-subunit interactions in bringing about such properties is highlighted by the finding that tetramers of the β-chain, which occur in some types of thalassemia (an inherited anemia resulting from the impaired synthesis of a hemoglobin subunit), exhibit neither cooperativity nor the Bohr effect. The unfortunate individuals who suffer from some types of thalassemia require regular blood transfusions – inconvenient and, in many countries, rather risky.

How can proton binding be fit into the formalism developed above? Returning to Eqn. (7.8), and letting the ligands be protons, we have

$$\phi = nk[H^+]/(1 + k[H^+]), \tag{7.49}$$

where n is the number of dissociable protons that bind with microscopic *association* constant k. In terms of the microscopic dissociation constant, Eqn. (7.49) is

$$\phi = \frac{n[H^+]/k_d}{1 + [H^+]/k_d}. \tag{7.50}$$

Defining ρ as the average number of protons dissociated from the fully protonated macromolecule,

$$\rho = n - \phi \tag{7.51}$$

because ϕ is the average number of protons bound. Substituting in Eqn. (7.50) gives

$$\rho = n - \phi = n - \frac{n[H^+]/k_d}{1 + [H^+]/k_d} = n\left(1 - \frac{[H^+]/k_d}{1 + [H^+]/k_d}\right) = \frac{nk_d/[H^+]}{1 + k_d/[H^+]}. \quad (7.52)$$

Rearranging Eqn. (7.51) and substituting in Eqn. (7.52) for ρ, yields

$$n - \rho = n - \frac{nk_d/[H^+]}{1 + k_d/[H^+]} = \frac{n}{1 + k/[H^+]} \quad (7.53)$$

Combining Eqns. (7.52) and (7.53) gives

$$\frac{\rho}{\phi} = \frac{[A^-]}{[HA]} = \frac{\rho}{n - \rho} = \frac{\frac{nk_d/[H^+]}{1+k_d/[H^+]}}{\frac{n}{1+k_d/[H^+]}} = \frac{k_d}{[H^+]}. \quad (7.54)$$

Dividing the numerator and denominator of the third term in Eqn. (7.54) by n yields

$$\rho/(n - \rho) = \psi/(1 - \psi) = k_d/[H^+], \quad (7.55)$$

where ψ is the fraction of protons *dissociated*. Taking the logarithm of both sides of Eqn. (7.55) gives

$$\log[\psi/(1 - \psi)] = \log k_d - \log[H^+] = -pk_a + pH. \quad (7.56)$$

Note how closely Eqn. (7.56), a form of the Hill equation, resembles the Henderson–Hasselbalch equation (Eqn. 4.57)!

The derivation just done made two implicit assumptions: one type of ionizable chemical group only, and all protons bind with the same affinity. Real proteins are more complicated, of course, not only because different kinds of ionizable side chain are present, but also because the specific electronic environment of a particular type of side chain can have a profound effect on pK_a. Glutamic acid 35 of hen lysozyme, for example, has a pK_a of about 6 in the folded state, more than two pH units above the pK_a of glutamic acid side chains in other parts of the folded state of the protein! And several anomalously low pK_as of histidine residues in myoglobin play a role in the stability of the molten globule state of apomyoglobin[10] at acidic pH. Numerous other examples could be given. In view of this, Eqn. (7.52) is more realistically rewritten as

$$\rho = \frac{n_1 k_{d,1}/[H^+]}{1 + k_{d,1}/[H^+]} + \frac{n_1 k_{d,2}/[H^+]}{1 + k_{d,2}/[H^+]} + \ldots, \quad (7.57)$$

where there are n_1 protons with dissociation constant $k_{d,1}$, n_2 with $k_{d,2}$, and so on. In the absence of detailed information on a particular ionizable group, each dissociation constant can be assumed to be the same as the "intrinsic k_d," the value for the free amino acid. This is the same as assuming that no titratable site is influenced by the electric field of another titratable site. This of course is an

[10] Myoglobin from which heme has been removed. From the Greek, *apo*, meaning from.

oversimplification, as the example of Glu 35 proves, but it is a way of getting started with analysis, and the approximation does in fact work relatively well in many cases.

Proteins can be denatured at extremes of pH (Chapter 2). Let's study how to model the situation in terms of free energy contributions! One way is to write the total free energy difference between the unbound and bound states of each proton as the sum of the intrinsic free energy of binding ($\Delta G^\circ_{\text{in}}$) plus the free energy of electrical effects ($\Delta G^\circ_{\text{e}}$):

$$\Delta G^\circ = \Delta G^\circ_{\text{in}} + \Delta G^\circ_{\text{e}}. \tag{7.58}$$

In terms of dissociation constants,

$$-RT \ln k = -RT \ln k_{\text{in}} + \Delta G^\circ_{\text{e}} \tag{7.59}$$

$$-RT \log k = -RT \log k_{\text{in}} + \Delta G^\circ_{\text{e}}/2.303 \tag{7.60}$$

$$\text{p}k_{\text{a}} = \text{p}k_{\text{a,in}} + \Delta G^\circ_{\text{e}}/(2.303RT). \tag{7.61}$$

If $\text{p}k_{\text{a,in}}$ is known and $\text{p}k_{\text{a}}$ can be determined experimentally, for example, by NMR spectrometry, then $\Delta G^\circ_{\text{e}}$, the work that arises from electrical effects, can be measured.

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I. Exercises

1. There are four fundamental physical forces: gravity, electromagnetism, the weak nuclear force, and the strong nuclear force. Electromagnetism and the weak nuclear force have recently been shown to be aspects of the same force, the electroweak force. Which of these forces mediates interactions between a ligand and a macromolecule to which it binds?

2. Find a mathematical expression for the binding free energy in terms of the dissociation constant.

3. When $[L] = K_d$, $F_b = 0.5$. Calculate the concentration of ligand required for 90% saturation and 99% saturation.

4. Describe protein folding/unfolding in terms of binding of heat.

5. Once O_2 has become bound to Mb it is not released back into the blood, because p_{50} is below venous pressure, and venous pressure is lower than arterial pressure. Describe the association of oxygen with Mb in terms of the chemical potential of O_2.

6. Write down a general equation relating k_i to K_i.

7. The following data were collected in a binding experiment.

[L]	ϕ	[L]	ϕ
0	0.0	55	0.85
5	0.33	60	0.86
10	0.50	65	0.87
15	0.60	70	0.875
20	0.67	75	0.88
25	0.71	80	0.89
30	0.75	85	0.89
35	0.78	90	0.90
40	0.80	95	0.90
45	0.82	100	0.91
50	0.83	—	—

Plot the data by the Scatchard method to evaluate the dissociation constant. Use a spreadsheet and the relevant equations from the text to determine the binding constant by non-linear least-squares regression.

8. Show that $\phi/[\mathrm{L}] = nK_\mathrm{a} - \phi K_\mathrm{a}$ for n identical binding sites.

9. Binding of a ligand stabilizes the folded conformation of macromolecule. Explain in thermodynamic terms why this must be so.

10. Given the definition of *homoallostery*, define *heteroallostery*.

11. Show that

$$\frac{\phi}{n} = \frac{x(1+x)^{n-1} + Lcx(1+cx)^{n-1}}{(1+x)^n + L(1+cx)^n}$$

for homotropic allosteric interactions. Write down an expression for the fractional saturation for ligand binding. Define $x = [\mathrm{L}]/k_\mathrm{R}$ and $c = k_\mathrm{R}/k_\mathrm{T}$. Use $k_\mathrm{R} = \{(n-i+1)/i\}[\mathrm{R}_{i-1}][\mathrm{L}]/[\mathrm{R}_i]$, $i = 1, 2, 3, \ldots, n$ to show that

$$([\mathrm{R}_1] + 2[\mathrm{R}_2] + \cdots + n[\mathrm{R}_n]) = [\mathrm{R}_0]\{n\alpha + 2n(n-1)\alpha^2/2 + \cdots + nn!\alpha^n/n!\} = [\mathrm{R}_0]\alpha n(1+\alpha)^{n-1}.$$

Show also that

$$([\mathrm{R}_0] + [\mathrm{R}_1] + \cdots + [\mathrm{R}_n]) = [\mathrm{R}_0]\{1 + n\alpha + \cdots + n!\alpha^n/n!) = [\mathrm{R}_0]\alpha n(1+\alpha)^{n-1},$$

that

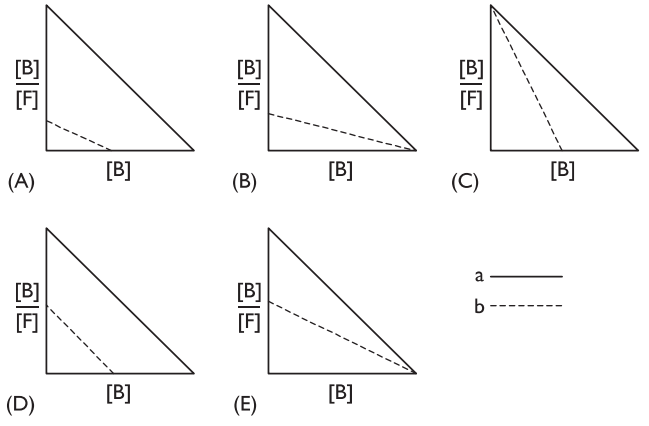
$$([\mathrm{T}_1] + 2[\mathrm{T}_2] + \cdots + n[\mathrm{T}_n]) = [\mathrm{T}_0]([\mathrm{L}]/k_\mathrm{T})n(1 + [\mathrm{L}]/k_\mathrm{T})^{n-1} = \mathrm{L}[\mathrm{R}_0]c\alpha n(1+c\alpha)^{n-1},$$

and that

$$([\mathrm{T}_0] + [\mathrm{T}_1] + \cdots + [\mathrm{T}_n]) = [\mathrm{T}_0](1 + [\mathrm{L}]/k_\mathrm{T})^n = \mathrm{L}[\mathrm{R}_0](1+cx)^n.$$

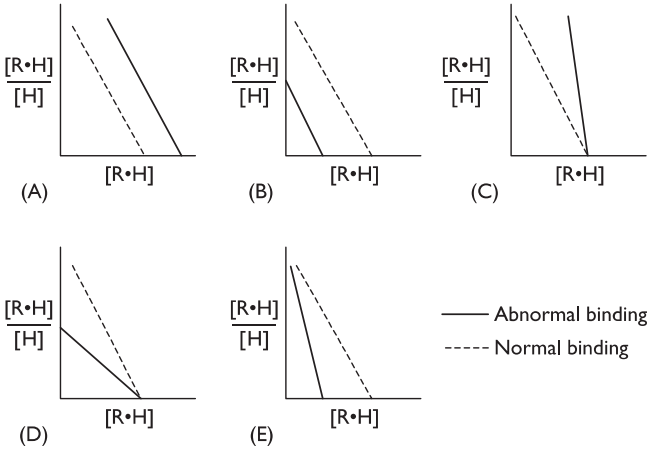
Note that $L \neq [L]$. (See Fig. 7.12.) Combine these terms to give the general result for homotropic allosteric interactions.

12. Which of the Scatchard plots shown below indicates that compound *a* binds with half the affinity as compound *b* but to twice the number of sites?



13. The Scatchard plots below compare data on the binding of a hormone (H) to the receptors (R) of five abnormal persons (solid line in plots (A)–(E)) and a normal person (broken line in each plot). [R•H] = concentration of bound hormone, [H] = concentration of free hormone. Which patient shows a decreased number of binding sites but the same receptor affinity as the normal person?

14. Consider a homotrimeric protein with three identical and independent binding sites and microscopic association constants of 10^6. Plot the fractional saturation of the protein against the free ligand concentration. Write down equations describing the macroscopic binding constants (K_1, K_2, K_3) in terms of the microscopic binding constant.



15. Suppose you are studying the binding of heme to myoglobin using equilibrium dialysis (Chapter 5). The total concentration of myoglobin is 10 μM. The following data were obtained at equilibrium.

Experiment	[heme] in chamber without myoglobin (μM)	[heme] in chamber with myoglobin (μM)
1	3	5.3
2	30	37.5

Calculate the concentration of bound and free myoglobin in the chamber where myoglobin is present. Use these values to calculate the fractional occupancy of myoglobin at the two ligand concentrations. Determine the affinity of heme for myoglobin using your favorite method. Can these data be used assess binding cooperativity? Explain.

16. Give three biological examples of negative feedback. Give three biological examples of positive feedback.

Chapter 8

Reaction kinetics

A. Introduction

The foregoing chapters focused on practical and applied aspects of thermodynamics and statistical mechanics. These subjects provide ways of thinking about energy transformation, methods for determining the direction of spontaneous change, the magnitude of thermodynamic quantities when a system passes from one state to another, and the molecular origin of change. Useful as they are, however, thermodynamics and statistical mechanics do not tell us everything we'd like to know: they give no direct indication of the rate at which a chemical change will occur nor how the rate of change will vary with conditions.

The present chapter seeks to bridge some of the gaps which remain from a strictly thermodynamic treatment of topics of interest in biochemistry. It might seem counter-intuitive for this chapter to appear next to last instead of first, since one of the most basic aspects of our experience of the world is change. Plants grow, go to seed, and die, while animals move, eat, reproduce, and die. And the molecules bacteria, plants, and animals are made of are *always* moving. But the title of this book is *Biological Thermodynamics*, not *Biological Kinetics*!

As we have seen, analysis of free energy changes provides a way of answering such questions as "Why is most of the energy of glucose harvested in the citric acid cycle and not in glycolysis?". But in order to respond well to "Does H_2CO_3 break down fast enough on its own to permit metabolic CO_2 to be excreted into the atmosphere?" we must turn to reaction kinetics. In a previous chapter we met the allosteric enzyme ATCase. This enzyme is inhibited by CTP and activated by ATP, though neither of these nucleotide triphosphates binds in the active site. ATCase provides an excellent example of how the rate of enzymatic activity can be regulated through allostery; you might like to investigate the matter in your further study.

Chapter 4 suggested that chemical equilibrium can be described as a condition of balanced forward and reverse rates of reaction.

When these rates are identical, their ratio is 1, the corresponding equilibrium constant is 1, $\ln(1) = 0$, and so $\Delta G = 0$, the condition for equilibrium at constant T and p. Awareness of the relative rates of the forward and reverse reactions can be useful in a biochemistry laboratory in many ways. For example, the ELISA assay discussed in Chapter 5 is based on the reverse ("off") rate of antibody–antigen binding being tiny in comparison with the forward ("on") rate, even though dissociation of antibody from antigen is predicted by mass action. Rates are also important to the molecular collisions that give rise to diffusion (Chapter 6). A more in-depth treatment of association and collision rates was postponed until now because we wanted to keep equilibrium thermodynamics as our principal focus. This chapter provides an introduction to the measurement of kinetic properties of macromolecules, include enzyme activity.

Our general approach will be to start with the phenomenological perspective. Once we've ascertained the general topography of the field, we'll pose questions about the more detailed mechanisms that have shaped it. We're not going to start from highly polished theoretical concepts and work our way down to approximations that are actually useful. Development in most areas of science has not followed a clean-cut, top-down approach. Instead, in most cases the creation of new knowledge is more organic, with the most significant breakthroughs being completely unplanned. Nevertheless, most of this chapter will deal with modeling kinetic behavior, and that is necessarily a matter of mathematics. Importantly, the equations we shall derive are simple enough to be useful in practice, predictions of the models have been tested, and in many cases the predictions correspond well enough to measured values. We also aim to have shown by the chapter's end how the kinetics of certain phenomena – protein folding kinetics, hydrogen exchange kinetics, muscle contraction – link to the thermodynamic quantities that have been such a large concern in previous chapters.

Figure 8.1 shows an energy profile for a generic chemical reaction. As we have seen, in order for the reaction to proceed spontaneously at constant temperature and pressure, the Gibbs free energy of the products must be lower than the Gibbs energy of the reactants. The reaction wants to proceed, but an energy "hill" or "barrier" separates reactants from products, preventing immediate conversion of the one into the other. The height of the barrier is the *activation energy*, E_a. (The relationship between E_a and G is discussed below.) This is the (average) energy required to convert reactants to products. Note that the height of the barrier depends on the direction of the reaction. On a hike in the countryside, a steep hill raises questions about whether and how to proceed: in the absence of climbing gear, a sheer cliff is practically an absolute barrier, while a gentle grade might do little more than retard progress temporarily. Similarly, if a reaction pathway is relatively slow, E_a is large, perhaps so large that its value will be hard to determine accurately. When the rate of a reaction is relatively high, E_a can often be

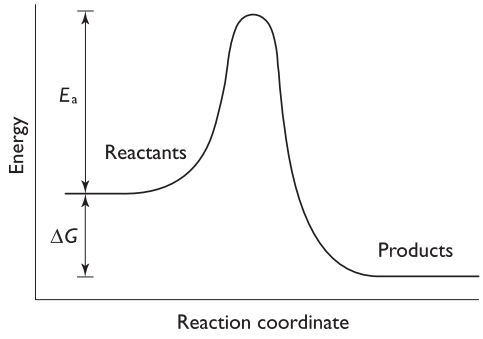


Fig. 8.1 Reaction profile. Gibbs free energy is plotted against the reaction coordinate, a pseudo-coordinate that represents the progress of the reaction. The Gibbs free energy of the products is lower than the energy of the reactants; the products will form spontaneously. But in order for the reactants to form products, a substantial energy barrier must be traversed. The height of this barrier, the activation energy (E_a), determines the kinetics of the process: the reaction is slow when the barrier is high and fast when it is low. The relationship between the activation energy and free energy is discussed in the text.

measured with a small error term. In the context of metabolic reactions, off-pathway reactions *always* occur, despite the exquisite specificity of enzymes, but such processes are often so slow (the energy barrier is so high) that they can often be assumed not to occur. But not always! Indeed, some cancers are characterized by a change in the proportion of certain types of off-pathway reactions, leading to abnormal cell properties. The top of the energy hill represents the free energy of the *transition state*, a transient chemical species that is unstable at equilibrium (see below).

A couple of examples will help to illustrate how the concept of activation energy sheds light on how the world works. Some years ago a big fire broke out in Malibu, California. To the amazement of many observers, a few houses began blazing away without having been touched by the flames. It was later determined that the fire had generated such an intense heat that infrared radiation passed through the glass of closed windows. The drapes hanging inside heated up and eventually ignited; the heat energy was so intense that it had exceeded the activation energy for oxidation of the curtains. Other houses nearby did not go up in flames. These dwellings were apparently protected by aluminum[1] blinds hanging in the windows, which deflected enough of the infrared radiation to keep the temperature inside below the activation energy for oxidation of curtains.

Nitroglycerin is a liquid explosive. It was invented in 1846. Unlike curtains, which are usually made of cellulose or a synthetic material and take a lot of heat to combust, nitroglycerin has a low activation

[1] Aluminium.

energy. Relatively slight agitation of the liquid is sufficient to set off very rapid formation of hot gas, despite a small free energy difference between reactants and products. Oxidation occurs rapidly because nitroglycerin is made of oxygen. Combustion releases as well as consumes oxygen, so the overall rate of reaction is not limited by access to atmospheric oxygen, as is the case for combustion of a hydrocarbon like octane. Alfred Nobel, the man after whom the annual prizes in physics, chemistry, medicine or physiology, economics, literature, and peace are named, was driven to invent a safer explosive near the end of the nineteenth century when his brother and four workers were killed in the family nitroglycerin plant. Nobel made what he called dynamite[2] by mixing nitroglycerin, which is oily, with powdery silica. The mixture is a dry solid that is much more stable (has a higher activation energy) than nitroglycerin alone and can also be molded into shapes that stack compactly and can be carrier easily – cylindrical "sticks."

You might suppose from these examples that chemical change in the universe is "controlled" not so much by the laws of thermodynamics as by the laws of chemical change, and that there is not really any point to knowing thermodynamics. But the First and Second Laws are by all appearances "boundary conditions" on the physically possible: all known chemical changes are consistent with these laws, but the actual rate of change will depend on the chemistry involved and the physical conditions. This chapter provides insight into how reaction rates relate to the First and Second Laws.

Finally, you might find it interesting to know something about the breadth of the range of rates of biochemical processes. Some processes are exceedingly fast, others slow. For instance, the lifetime of the excited state of chlorophyll in the capture of electromagnetic energy is about 10^{-10} s. This is so fast that chlorophyll would be practically useless in a typical biochemical reaction. By contrast, the reduced organic molecules into which the Sun's energy is transformed have a lifetime on the order of months or years. The fastest enzymes do their work at a rate that is diffusion controlled; they are limited only by the time required for a substrate molecule to move into the active site or for the product to move out. Some polypeptides are stable in pure water for tens of thousands of years; protein turnover in cells is obviously much faster.

B. Rate of reaction

A rate is of course a measure of how quickly something happens. For example, the rate of food intake in adult humans is about 3 meals per day; it's higher for babies! The rate of inflation is a measure of the change in value with time of a monetary unit, for example the yen or the peso. In chemistry, the *rate of reaction*, J, is simply a

[2] Note similarity to *thermodynamics*.

measure of how rapidly the concentration of a product or reactant changes in time. One should in general expect J itself to vary with time, as it clearly does when the amount of a reactant is limited and consumed by a process.

Recall ATP hydrolysis, which we discussed at some length in Chapter 5. The nucleotide hydrolyzes spontaneously because there is a Gibbs free energy decrease on going to ADP and P_i; hydrolysis is thermodynamically favorable. This reaction is so favorable that it is essentially irreversible at room temperature; hydrolysis results in the increasing loss of ATP. The smaller the number of ATP molecules per unit volume, the smaller the number of reactions per unit volume and the lower the rate of reaction. We can express this in symbols as follows:

$$\mathrm{ATP} \rightarrow \mathrm{ADP} + \mathrm{P_i}. \tag{8.1}$$

In terms of the depletion of ATP, the rate of reaction, J, is

$$J = -\Delta[\mathrm{ATP}]/\Delta t, \tag{8.2}$$

where t is time. We can see that J has dimensions [concentration] [time]$^{-1}$. The rate of reaction can also be expressed in terms of the products, as

$$J = +\Delta[\mathrm{ADP}]/\Delta t = +[\mathrm{P_i}]/\Delta t \tag{8.3}$$

based on the stoichiometry of the reaction. Figure 8.2 shows the concentration versus time for ATP and ADP.

For a more general reaction, say,

$$a\mathrm{A} + b\mathrm{B} \rightarrow c\mathrm{C} + d\mathrm{D} \tag{8.4}$$

the rate is given by

$$J = -\frac{1}{a}\frac{\Delta[\mathrm{A}]}{\Delta t} = -\frac{1}{b}\frac{\Delta[\mathrm{B}]}{\Delta t} = +\frac{1}{c}\frac{\Delta[\mathrm{C}]}{\Delta t} = +\frac{1}{d}\frac{\Delta[\mathrm{D}]}{\Delta t}. \tag{8.5}$$

The reason why the rate appears as $-(\Delta[\mathrm{B}]/\Delta t)/b$ and not $-\Delta[\mathrm{B}]/\Delta t$ will be clear from another example. Suppose

$$\mathrm{A} + 2\mathrm{B} \rightarrow \mathrm{C}. \tag{8.6}$$

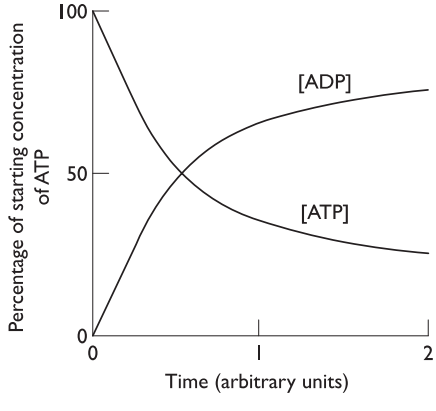


Fig. 8.2 Kinetics of nucleotide hydrolysis *in vitro*. ATP hydrolyzes spontaneously at room temperature, and the reaction is essentially irreversible. The rate of ATP depletion is equal to the rate of ADP accumulation. At some stage during the reaction the concentration of ATP will be half the starting concentration. At this time, [ATP] = [ADP].

For every mole of A converted into C, two moles of B are consumed. So the rate of consumption of B is twice as great as that of A. Taking this into account, $-\Delta[B]/\Delta t$ must be divided by 2 in order to equal $-\Delta[C]/\Delta t$ or $-\Delta[A]/\Delta t$. It is important to bear in mind that J measures the rate of reaction as a whole, not the rate of consumption or production of one component of the reaction, which is given by $-\Delta[Z]/\Delta t$ for chemical species Z.

To summarize, we know from experience that change is a basic aspect of the world. We know that a chemical reaction will occur at a certain rate. And we have seen a way of describing the rate of reaction in simple mathematical terms. Time to move on to deeper things!

C. Rate constant and order of reaction

We also know from experience that the shape of a kinetic trace for a given chemical is not the same for every reaction. In other words, in one reaction you might find a curve of a certain shape for the rate of change of a particular chemical species, but in a different reaction you might get a curve that is clearly different from the first one for the same chemical species. Why? One possibility might be that the stoichiometry is different in the two situations. Another might be that the mechanism is different. To describe the various possible types of chemical behavior, we need to make our mathematical description of things a bit more sophisticated.

It has been found *experimentally* that, in general, the rate of a reaction is related to the concentration of a reactant as

$$J \propto [A]^n, \tag{8.7}$$

where n is called the *order of reaction*. Experiments have shown that *the order of reaction for a component is often but not always identical to its stoichiometry; the order of a reaction must be determined empirically*. We can upgrade Eqn. (8.7) to the status of an equality by adding a constant of proportionality:

$$J = k[A]^n. \tag{8.8}$$

The *rate constant*, k, a phenomenological parameter, is the inverse of the time constant, τ (compare the average time between collisions in Chapter 6). (NB: this k is not the microscopic binding constant of the previous chapter! We are free to use symbols however we like, but be sure it's clear enough what the symbol represents!) The value of k depends entirely on the reaction of interest and it can only be determined experimentally; the theory of reaction rates is still at too primitive a stage of development to predict k outside of specialized situations. The rate constant is a measure of the activation energy, and it is expressed in terms of the activation energy as

$$k = \mathbf{A}\exp(-E_a/RT), \tag{8.9}$$

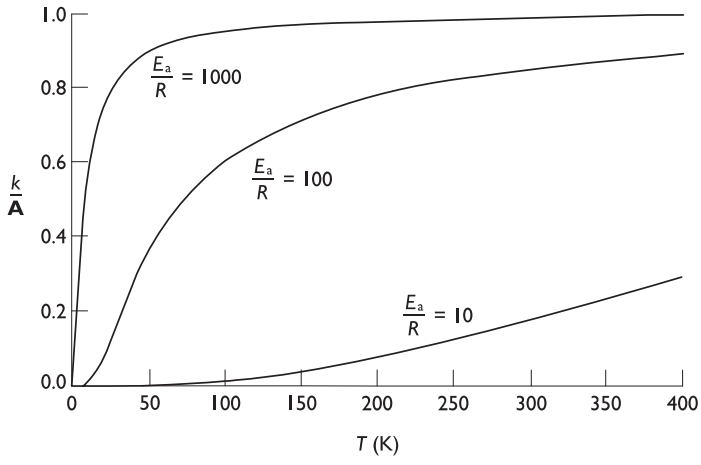


Fig. 8.3 Temperature dependence of $k/\mathbf{A}$ for different values of the activation energy. Note E_a is in units of K^{-1}. The lower the activation energy, the higher the temperature at which $k/\mathbf{A}$ attains its maximum value, 1.

where **A**, another empirical quantity, is known as the *frequency factor* (Fig. 8.3). Note the resemblance of this equation to $K = \exp(-\Delta G/RT)$. Equation (8.9) tells us that if k can be measured and T is known, then E_a can be determined. For instance, if $k = 10^{-3}\ s^{-1}$ at 300 K and $\mathbf{A} = 1$, then $E_a = -RT\ln k = -8.314\ J\ mol^{-1}\ K^{-1} \times 300\ K \times \ln 10^{-3} = 17\ kJ\ mol^{-1}$. The exponential function is always positive, k is always positive. We do not think of rates of chemical reactions as being negative; they go forward or reverse.

In the general situation,

$$A + B + C + \cdots \longrightarrow \text{products}. \tag{8.10}$$

The overall rate of reaction can be written as

$$J = k[A]^{n_A}[B]^{n_B}[C]^{n_C}\cdots, \tag{8.11}$$

where n_A is the order of reaction with respect to component A, *etc.* The overall order of reaction is $n_A + n_B + n_C + \ldots$ Often, the exponents in Eqn. (8.11) will reflect the stoichiometry of the reaction, but that is certainly not always the case; the magnitudes of the orders of reaction as well as k must be determined experimentally. Because the form of the mathematical expression for J depends on the reaction, so do the units of a rate constant. For instance, in a *first-order reaction*, the unit of concentration is raised to the first power and k has units of inverse time. In a *second-order reaction*, the dimensions of k are $[\text{concentration}]^{-1}[\text{time}]^{-1}$. If a reaction rate is independent of the concentration of a reactant, the rate is zeroth-order with respect to that reactant.

D. First-order and second-order reactions

We want to focus on first- and second-order reactions for a couple of reasons. One is that it is often difficult to obtain data of sufficient quality that would support a more complex model (a higher-order

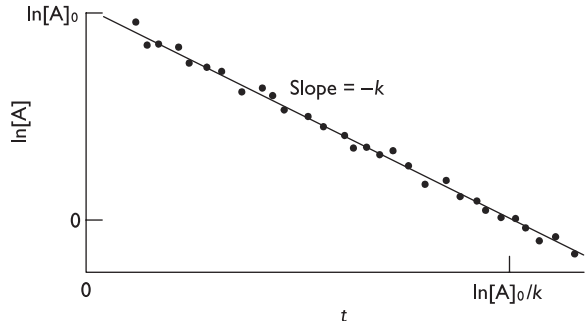


Fig. 8.4 First-order process. A plot of ln[A] against time is linear. The slope is negative, and its magnitude is the rate constant for the reaction. The curve intercepts the axes at two points: at $t = 0$, ln [A] = ln[A]$_0$, and at $t = \ln[A]_0/k$, ln [A] = 0.

reaction). Another is that a very large number of reactions of interest to the biochemist can be described well enough as first-order or second-order, making it hard to justify a more complex one.

Suppose you are interested in the first-order reaction A → P. Combining Eqn. (8.2) and Eqn. (8.8) gives

$$-\Delta[A]/\Delta t = k[A], \tag{8.12}$$

which can rearranged into

$$\Delta[A]/[A] = -k\Delta t. \tag{8.13}$$

Using a bit of calculus and algebra, it can be shown that

$$\ln[A] = \ln[A]_0 - kt, \tag{8.14}$$

where $[A]_0$ is the concentration of A when $t = 0$ (the beginning of the experiment). An important feature of Eqn. (8.14) is that it is *linear* in t with slope $-k$ (units of [time]$^{-1}$) and vertical axis-intercept ln[A]$_0$ (Fig. 8.4). So if you collect experimental rate data, plot the logarithm of the concentration of a reactant, and find that the curve is a straight line, you can conclude that the reaction was (approximately) first-order in that reactant. You should take care in doing the analysis, as it can be difficult to distinguish between a reaction of one order and another order; see below.

Exponentiation of both sides of Eqn. (8.14) gives

$$[A] = \exp(\ln[A]_0 - kt) = e^{\ln[A]_0} e^{-kt} = [A]_0 e^{-kt}, \tag{8.15}$$

which on rearrangement is

$$[A]/[A]_0 = e^{-kt}. \tag{8.16}$$

This says that in a first-order reaction, the ratio of the concentration of the reactant to the starting concentration is a negative exponential function of time. When $t = 0$, the right-hand side of Eqn. (8.16) is 1, and $[A] = [A]_0$, just as expected. As $t \to \infty$, the right-hand side becomes very small, and $[A]/[A]_0 \to 0$. The rate at which the left-hand side approaches 0 depends on the size of k. When k is very large, $[A]/[A]_0 \to 0$ rapidly!

How long does it take for [A] to decrease to half its starting value? The so-called *half-time* or *half-life* ($t_{1/2}$) of a first-order reaction can be

found by setting $[A]/[A]_0 = 0.5$ in Eqn. (8.16). Solving for t gives

$$t_{1/2} = -\ln(1/2)/k = \ln 2/k. \tag{8.17}$$

You can describe a process in terms of its half-life whether the process of interest is a biochemical reaction or nuclear decay. A mathematical formula, model, description can be very general indeed. Equation (8.17) shows that if k is large, as in the case of fast photosynthetic reaction mentioned in the first section of this chapter, $t_{1/2}$ is small; the reaction could go to completion in a fraction of a second. When $t_{1/2}$ is large, by contrast, as for instance with a relatively slowly decaying radioactive isotope like ^{14}C, a useful one in biochemical research, only a small fraction of a given amount of isotope will decompose in the course of a human lifetime. It is therefore a good idea to avoid eating ^{14}C!

The simplest type of second-order reaction is $2A \rightarrow P$, where two molecules of A react with each other to form P. The analog of Eqn. (8.12) for this case is

$$-\Delta[A]/\Delta t = k[A]^2. \tag{8.18}$$

Rearrangement gives

$$\Delta[A]/[A]^2 = -k\Delta t, \tag{8.19}$$

which, by a little jiggery-pokery (some elementary calculus), yields

$$\frac{1}{[A]} = \frac{1}{[A]_0} + kt. \tag{8.20}$$

As in the first-order case, we have a function of the concentration of reactant that is linear in time. Now, though, instead of the natural logarithm of [A], 1/[A] has appeared, the slope of the line is $+k$ (with units of $[\text{concentration}]^{-1}[\text{time}]^{-1}$), and the intercept is $1/[A]_0$ (Fig. 8.5). Multiplying both sides of Eqn. (8.20) by $[A]_0$ and taking the inverse gives

$$\frac{[A]}{[A]_0} = \frac{1}{1+kt}. \tag{8.21}$$

Above we said that it can be difficult in practice to distinguish between a reaction of one order and another. Figure 8.6 helps to illustrate the idea. A plot of $[A]/[A]_0$ *versus* time is shown for a first-order model and a second-order model when $[A]/[A]_0 = 0.5$ at the

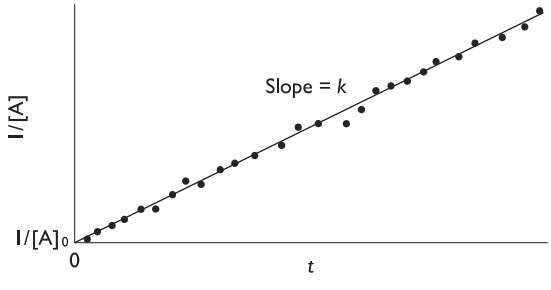


Fig. 8.5 Second-order process. The inverse of the concentration of reactant is linear with time. The slope is positive with magnitude k. The curve crosses the t-axis at $1/[A]_0$.

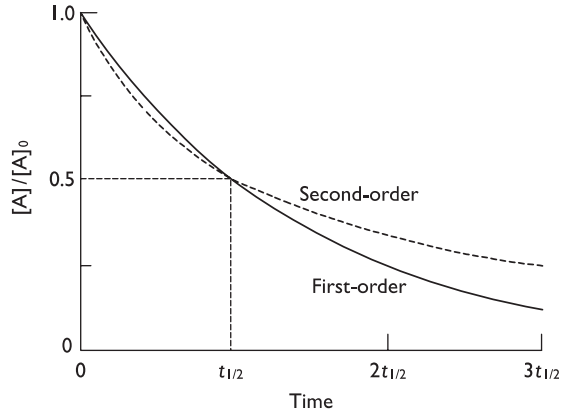


Fig. 8.6 Comparison of a first-order process and a second-order process. The fraction of reactant remaining is plotted against time. The second-order rate constant has been chosen so that $[A] = [A]_0/2$ after one time constant in the first-order reaction. Note how similar the two curves look. Reproducible data with a good signal-to-noise ratio would be needed to be able to judge whether the process was first-order or second-order.

half-time of the first-order model. Notice how similar the curves appear to the eye. If the data you've collected are somewhat noisy, as all real data are, it might be very difficult to form a clear sense of the order of the reaction without further analysis. What you could do in this case is *assume* that the reaction is first-order and then *calculate how well* the model fits the data, taking into account any uncertainty in the measured values. Then do further fitting on the assumption of a second-order reaction. Then compare quantitative measures of goodness-of-fit or, even better, a more useful criterion for model selection. And finally, decide which model provides the better description of the chemical process. But don't be surprised if the best thing you can do to elucidate the reaction is obtain more data.

E. Temperature effects

Reaction rates depend on temperature! For example, your curtains won't ordinarily combust spontaneously at room temperature, so k for this reaction must be fairly large when T is about 300 K. So the rate of reaction for the combustion of curtains will increase with temperature. The growth rate of *E. coli* depends significantly on temperature. At −80 °C, water is frozen, even in the presence of about 50% glycerol, and the growth rate of bacteria is nil. This is one reason why glycerol stocks of bugs are maintained at such a low temperature. At 37 °C, by contrast, where the doubling time is maximal for this species of bacteria, cells divide every 30 min or so on the average. The human gut is a bacterium's paradise: plenty of food, protection from predators, and no need to pay for the heat needed to drive metabolic reactions forward! The rapid growth rate of bacteria at room temperature is just one of the reasons why it's a good idea to refrigerate certain foods – something cooks discovered a very long time before anyone knew microorganisms existed.

The rate of reaction and therefore the rate constant increase with temperature. We can see how the rate of reaction depends on temperature by manipulating Eqn. (8.9):

$$\ln \ell k(T) = \ln \mathbf{A} - E_a/(RT), \tag{8.22}$$

where ln*k* is a linear function of the inverse of *T*. Experiments have shown that *in many cases the rate of reaction doubles or triples for a 10 degree increase in temperature*. Equation (8.22) is known as the Arrhenius equation, and it gets its name from the Swedish Nobel laureate in chemistry (1903). Arrhenius is also known for promoting the view that life on Earth arose from "panspermia," according to which micro-organisms or spores drifted through space by radiation pressure until finally landing on Earth. We'll consider the extremely interesting question of how life got going on our planet in the next chapter.

For now, suppose you know from experiments that the rate of the biochemical reaction $\mathrm{A} + \mathrm{B} \rightarrow \mathrm{P}$ doubles on raising the temperature from 25 °C to 35 °C. What is E_a? Assuming that E_a is approximately independent of temperature in this range, we have

$$\ln k(T_1) = \ln \mathbf{A} - E_a/(RT_1) \tag{8.23}$$

$$\ln k(T_2) = \ln \mathbf{A} - E_a/(RT_2). \tag{8.24}$$

Subtraction of Eqn. (8.24) from Eqn. (8.23) gives

$$\ln k(T_1) - \ln k(T_2) = -E_a/(RT_1) + E_a/(RT_2) = E_a\{-1/RT_1 + 1/RT_2\} \tag{8.25}$$

which, when solved for E_a, is

$$E_a = \frac{\ln\{k(T_1)/k(T_2)\}}{\frac{T_1 - T_2}{RT_1T_2}}. \tag{8.26}$$

Plugging in the given values and turning the computational crank yields

$$E_a = 12.6 \text{ kcal mol}^{-1}. \tag{8.27}$$

Here is a rule of thumb: *doubling the rate of reaction on a temperature increase of 10 degrees corresponds to change in activation energy a tad under 13 kcal mol*$^{-1}$.

F. Collision theory

This section and the next discuss two theories of reaction rates, collision theory and transition state theory. Each theory attempts to rationalize the results of experiments. We have already encountered two basic aspects of collision theory earlier on. In Chapter 2, in the discussion of air molecules, pressure and a bicycle tire, we saw that the pressure of a system is related to the number of collisions that the particles within make with the system boundary, and that the pressure can be increased either by raising the temperature or by

pumping more particles into the system. That discussion was linked to the kinetic theory of gases, which is based on the assumption that gas particles are constantly banging into the walls of their container. And in Chapter 6, while looking more deeply at the molecular interpretation of thermodynamic quantities, we saw how the Brownian motion and diffusion can be explained in terms of particle collisions. Bearing these *physical* changes in mind, we now turn our attention to chemical changes that might depend on particles colliding.

Particle collisions *must* have *something* to do with temperature, particularly in a gas or a liquid, because the speed at which the particles move will depend very much on the temperature. As noted in a previous chapter, you can get a sense of particles in motion by watching what happens to water when it is heated on the stove, particular when it starts to boil. Now, if a certain chemical reaction requires two particles to collide, as when ADP and P_i meet to form ATP, the rate of reaction will also depend on the concentration of reactants. This is just as we saw above in Eqn. (8.11). In other words, [ADP] and [P_i] measure the likelihood of an encounter between them. As we saw in Chapter 6, if the concentration of bugs in the cycling lane is high, a biker will collide with them relatively frequently. But there must be more to a chemical reaction than collisions per se, because experimental studies show that in a typical reaction only about one in 10^{14} collisions leads to the formation of products! Taking the exquisite stereospecificity of enzymes and enzyme-catalyzed reactions into account helps to throw some light on the subject. It's not a mere collision between enzyme and substrate that brings about an enzymatic reaction, it's a collision that brings the substrate into the active site of the enzyme in an orientation that will be productive for the catalytic reaction. For instance, a hand can "collide" with a glove in a huge variety of ways, but only a relatively small number of collisions will result in a glove going on the correct hand. Things are usually less specific with non-biological molecules, but the principles involved are similar.

Box 8.1. Tongue of the chicken and nose of the cat

Birds normally maintain body temperature within a range of less than 1°C; they are homoiotherms. Direct electrophysiological evidence has revealed that the skin of pigeons contains both cold and warm thermoreceptors. In chicken tongues, individual nerve fibers serving cold receptors display a high level of static nervous activity, increasing 4–7-fold on an initial temperature decrease from 44°C to 9°C. The mammalian sensory system can discriminate thermal stimuli ranging from the painfully cold to the painfully hot. The nose of a cat, for example, contains numerous receptors that are highly specific for excitation by cold and warm stimuli; these receptors do not respond to mechanical deformation of the skin; each thermoreceptor is connected to a single nerve fiber. The main temperature sensors

in mammals belong to the transient receptor potential family of cation channels. When open, such channels allow cations to pass through the plasma membrane of the cell. The marked temperature sensitivity to the opening and closing ("gating") of some of these channels is tightly linked to membrane electrical potential. The channels become "activated" during plasma membrane depolarization, the process whereby different ions cascade down their respective concentration gradients through pores in the plasma membrane, leading to a loss of the physiological voltage across the cell membrane. Changes in temperature give graded shifts in the voltage-dependent channel activation curves. Recent analysis by researchers located in Leuven, Belgium, and Homburg, Germany, has found that the temperature sensitivity of channel gating stems from a tenfold difference in the activation energies associated with voltage-dependent gating. For the cold-sensitive channel TRPM8, the opening rate has an activation energy of 16 kcal mol^{-1}; temperature dependence is weak. The closing rate of the channel, by contrast, has an activation energy of 170 kcal mol^{-1} and is strongly temperature-dependent. The temperature dependencies of the gating of TRPV1, a heat-sensitive channel, are just the opposite: $E_{a,open}$ = 210 kcal mol^{-1}, and $E_{a,close}$ = 23 kcal mol^{-1}. Mechanisms of thermoreception in cats, chickens, pigeons and people are likely to be similar in many respects.

Another requirement for a reaction in collision theory is that the total relative kinetic energy of the colliding reactants *must* be greater than a certain amount. At a given temperature, some molecules are moving rapidly, others slowly, according to the kinetic theory of gases. So, in the reaction $A + B \rightarrow P$, not only do A and B have to collide to form P, their relative orientation must be right and they must collide with sufficient energy. This energy is none other than E_a.

The Maxwell law says that the distribution of the relative kinetic energy of particles is

$$\frac{\Delta n}{n} = \frac{\Delta E}{RT}\exp(-E/RT), \tag{8.28}$$

where n is the number of molecules of a particular type and E is the energy. It can be shown by way of some calculus that $n(E_a)$, the fraction of molecules with $E > E_a$, is

$$n(E_a) = n\ \exp(-E_a/RT) \tag{8.29}$$

an expression that looks a lot like the Boltzmann law (Chapter 6). In other words, the fraction of molecules with $E > E_a$ is $n(E_a)/n$, and these are the molecules that have enough energy to react (Fig. 8.7). We can now try to tie things together by writing

$$J \propto \text{collision rate} \times \exp(-E_a/RT). \tag{8.30}$$

Notice how similar Eqn. (8.30) is to the rate of a first-order reaction, $J = k[A] = [A]\mathbf{A}\exp(-E_a/RT)$. The comparison suggests that the collision rate, the number of collisions per unit volume per unit time, is $[A] \times \mathbf{A} \times$(a scaling factor). If E_a is large, $n(E_a)/n$ and $\exp(-E_a/RT)$ are small, and the rate of reaction will be low. This makes sense, but productive collisions are not the only way to think about reactions.

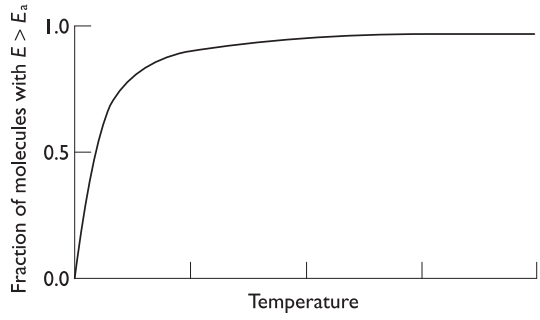


Fig. 8.7 Fraction of reactant molecules with sufficient energy to jump over the activation energy barrier. There is a strong temperature dependence to this quantity. At 0 K, no molecules can cross; at very high temperatures, effectively all the molecules are energetic enough to get over. Reaction rates increase with temperature.

G. Transition state theory

As intuitive and straight-forward as collision theory is, it suffers from a number of defects. The most important of these is that its predictions do not always match up with the results of experiments! This must be considered a significant flaw of the theory, for *the ultimate test of any scientific theory is how well it predicts the outcome of experiments that can actually be done*. Transition state theory was invented in the 1930s by Henry Eyring (see footnote 8 in Chapter 2) and others to improve on some of the shortcomings of the collision approach. The main conceptual device of Eyring's theory is the *transition state*. The rate of formation or breakdown of the transition state determines the rate of reaction. Let's look at an example.

Suppose we have the bimolecular reaction

$$A - B + C \rightarrow A + B - C. \tag{8.31}$$

At some point on the reaction pathway, a high-energy "intermediate" complex must be formed. This complex is unstable and transient in nature. Its lifetime is so short that no one can observe it (under ordinary conditions, except perhaps by computational modeling). You might guess, though, that the transition state will look something like $A \cdots B \cdots C$, where the dashed lines are bond-like connections. Whatever the transition state looks like, we can call this chemical species whatever we like, so let's name it the transition state or *activated complex*, X.

Now consider the reaction

$$A \Leftrightarrow X \rightarrow P. \tag{8.32}$$

And assume that X is in *rapid equilibrium* with A and B, and that the rate of formation of X from A and B–C is so small as to be negligible. We also assume that the reaction takes place at constant temperature and pressure, for reasons that will become clear momentarily. We know from above that

$$\Delta[P]/\Delta t = k'[X]. \tag{8.33}$$

We can also write down an equilibrium constant, $\boldsymbol{K}$, for the formation of the transition state:

$$\boldsymbol{K} = [\mathrm{X}]/[\mathrm{A}]. \tag{8.34}$$

From Chapter 4, K can be written as

$$\Delta G_\mathrm{a} = -RT \ln \boldsymbol{K}, \tag{8.35}$$

where the subscript "a" refers to activation, just as in E_a. Combining this equation with Eqn. (8.33) gives

$$\Delta[\mathrm{P}]/\Delta t = k'[\mathrm{X}] = k'\boldsymbol{K}[\mathrm{A}] = k' \exp(-\Delta G_\mathrm{a}/RT)[\mathrm{A}]. \tag{8.36}$$

This equation makes good intuitive sense: the more energy required to form the transition state from the reactants, the smaller the exponential term on the right-hand side and the smaller the overall rate of reaction. This energy, ΔG_a, corresponds to the activation energy, E_a, under the constraints of constant temperature and pressure, and the chemical species it represents, $\mathrm{X_a}$, is formed at the crest of the *activation barrier* (or *kinetic barrier*) in the energy profile in Fig. 8.1. Another symbol for ΔG_a is $\Delta G^{\ddagger}$.

How about k', the rate of formation of P from $\mathrm{X_a}$? Can a mechanistic interpretation of this rate constant be found? Yes! Suppose that k' is proportional to a vibrational frequency, ν, and the probability that $\mathrm{X_a}$ will decompose to form P. Then

$$k' = \nu\kappa. \tag{8.37}$$

The probability κ is known as a *transmission coefficient*. This quantity is something like the odds that a salmon will be adequately fit to jump several feet in the air on its journey upstream to the spawning area. It will often take a salmon several tries to clear a given waterfall, and it might not ever make it up and over. The vibrational frequency takes us very far back upstream in the course of this book to Planck's law, Eqn. (1.1). From statistical mechanics, the energy of an oscillator is

$$E = hc/\lambda = h\nu = k_\mathrm{B}T. \tag{8.38}$$

Combining Eqns. (8.37) and (8.38) gives

$$k' = \kappa E/h = \kappa k_\mathrm{B}T/h \tag{8.39}$$

and inserting this result into Eqn. (8.36) gives

$$J = \Delta[\mathrm{P}]/\Delta t = \kappa k_\mathrm{B}T \exp(-\Delta G_\mathrm{a}/RT)[\mathrm{A}]/h. \tag{8.40}$$

We can see from comparing Eqn. (8.40) with Eqn. (8.10) that the rate constant of the forward reaction is

$$k = \kappa k_\mathrm{B}T \exp(-\Delta G_\mathrm{a}/RT)/h. \tag{8.41}$$

When $\kappa = 1$, as in many reactions, the rate is $k_\mathrm{B}T \exp(-\Delta G_\mathrm{a}/RT)/h$. Now we have a direct connection between reaction rate, something that can often be measured relatively easily, and the free energy of the transition state. If addition of a catalyst brings about a ten-fold

increase in the rate of reaction, the catalyst must *reduce* the free energy barrier by $RT\ln 10 = 8.31\ \text{J mol}^{-1}\ \text{K}^{-1} \times 298\ \text{K} \times \ln 10 = 5.70\ \text{kJ mol}^{-1}$ at 25°C. To put things in perspective, the magnitude of this energy change is roughly the same as the *enthalpy* change on forming a single hydrogen bond.

We can take the analysis one step further by returning to Eqn. (8.35) and writing the activation free energy in terms of enthalpy and entropy:

$$\Delta G_a = \Delta H_a - T\Delta S_a. \tag{8.42}$$

Inserting this relationship into Eqn. (8.41) and setting $\kappa = 1$ gives

$$k = \frac{k_B T}{h} \exp(-\Delta H_a/RT)\exp(\Delta S_a/RT). \tag{8.43}$$

Here, ΔH_a and ΔS_a are the enthalpy and entropy of activation. Equation (8.43) says that for a given activation enthalpy, the higher the activation entropy, the faster the reaction. The entropy of the activated complex, however, will generally be substantially lower than the entropy of the reactants, making $\Delta S_a < 0$. But if the formation of the activated complex can be coupled to the release of water molecules, as in the case of hexokinase (Chapter 4), the total entropy change might make the activation energy sufficiently low for the reaction to occur. By means of a little calculus, it can be shown that

$$\frac{\Delta \ln k}{\Delta T} = \frac{\Delta H_a}{RT^2} + \frac{1}{T}, \tag{8.44}$$

which ties together the rate for the forward reaction and the enthalpy of activation in a tidy equation. It can be shown that for a *unimolecular* reaction in the *gas* phase, $E_a = \Delta H_a + RT$, which when substituted into Eqn. (8.43) gives

$$k = \frac{kT}{h} \exp(-E_a/RT)\exp(\Delta S_a/RT)\exp(1). \tag{8.45}$$

Comparison of Eqn. (8.45) with Eqn. (8.9) indicates that

$$A = \frac{kT}{h} \exp(\Delta S_a/RT)\exp(1). \tag{8.46}$$

In other words, the phenomenological frequency factor measures the activation entropy.

The preceding discussion pertained to the relatively simple case of $A \rightarrow P$. If a stable intermediate, I, is involved, the reaction scheme looks like this:

$$A \rightarrow I \rightarrow P. \tag{8.47}$$

Now there are two activated complexes, one for each step of the reaction. The rate constants for the first step, k_1, can be either greater or less than the rate constant for the second step, k_2, depending on the reaction. The smaller one corresponds to the higher activation energy. The step of the overall reaction with the highest free energy barrier is the *rate-determining step* of the reaction

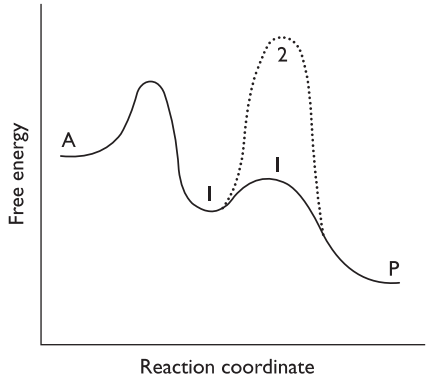


Fig. 8.8 Rate limiting step of a reaction. In reaction 1, the first step in the formation of P from A is rate-limiting; the energy barrier of this step is higher than for the second step. Consequently, the first step determines the overall rate of this reaction. In reaction 2, the second step is the rate-determining one. The rate of reaction is independent of the Gibbs free energy difference between P and A.

(Fig. 8.8); something like a narrow passageway through the Alps on a road or rail trip from Florence to Frankfurt. As we shall see below in the context of enzyme kinetics, catalysts speed up a reaction by reducing the energy barrier of the rate-determining step.

H. Electron transfer kinetics

In Chapter 4 we looked at electron transfer reactions in the context of redox couples. And in Chapter 5 we touched on the role of redox reactions in the all-important processes of photosynthesis, glycolysis, and the citric acid cycle. Now let's take a slightly deeper look at electron transfer and consider the kinetics of the process.

In 1992 the Nobel Prize in Chemistry was awarded to Rudolph A. Marcus (1923–), an American, for his work on electron transfer. The *Marcus theory* relates the rate of electron transfer, k_{et}, to properties of the redox system involved. Specifically,

$$k_{et}=k_0\exp(-\beta d), \tag{8.48}$$

where d is the distance between electron donor and acceptor and the coefficient β, which falls between 0.7 $Å^{-1}$ (van der Waals contact) and 4 $Å^{-1}$ (vacuum), depends on the intervening medium. The maximum rate of electron transfer is about 10^{13} s^{-1}. That's very fast; dinosaurs roamed Earth 10^{13} min ago. The intervening medium between donor and acceptor is not the only determinant of the transfer rate. It is also affected by ΔG, the driving force of the reaction. Notice how this situation is qualitatively different from what we saw above, where the free energy difference between products and reactants had little or no effect on the rate of reaction; what mattered most was the relative free energy of the transition state.

The dependence of rate of electron transfer on ΔG is complex. How could it not be when it must account for the size of ions involved, the spatial orientation of neighboring electronic charges, and the number and orientation of solvent molecules? One particular arrangement of all these contributors will give the minimum

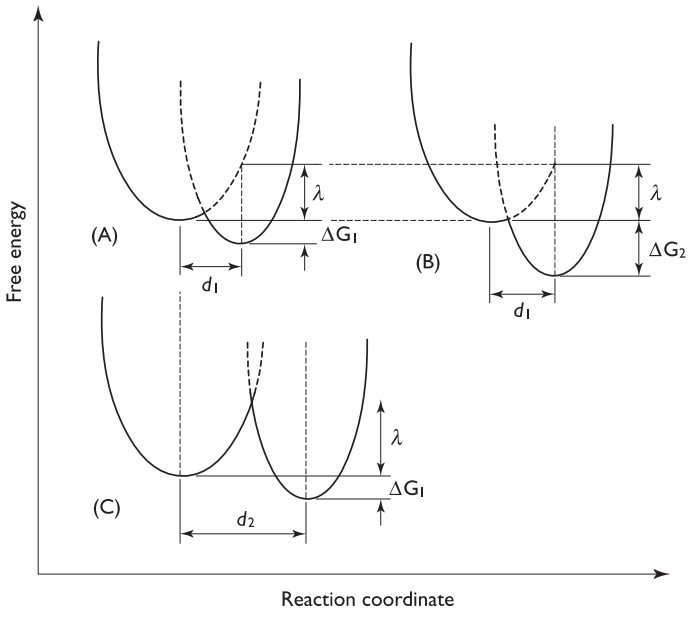


Fig. 8.9 Electron-transfer kinetics. The kinetics vary with free energy. Electronic motion is very fast, much faster than nuclear motion. This allows separation of the energy into electronic and nuclear components. The parabolas represent the energy of the nuclear component. When the distance between donor and acceptor is constant, as in plots (A) and (B), varying the free energy difference between electron donor and acceptor affects the height of the energy barrier (the energy of the transition state). In panel (B), where $\Delta G_2 = \lambda$, the reorganization energy, the rate of transfer is larger than when $\Delta G \neq \lambda$. Panel (C) shows that, although the free energy difference between donor and acceptor is the same as in panel (A), the rate is different, because a change in distance separating the donor–acceptor pair has resulted in a large increase in the energy of the transition state.

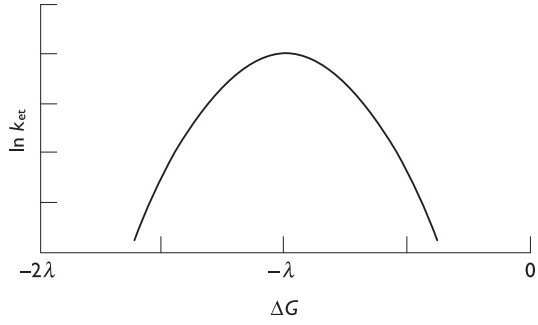


Fig. 8.10 Electron transfer rate as a function of λ. The rate is maximal when $|\Delta G| = \lambda$.

free energy for transfer; all other arrangements will have a higher energy. Figure 8.9 illustrates how the Gibbs free energy varies with the reaction coordinate. In each of the three cases, two overlapping energy wells are shown. These represent the electron donor and electron acceptor. The electron is in a bound state. Electrons move on a much faster time scale than do the comparatively heavy nuclei, so in the course of electron transfer there is effectively no nuclear motion. In order for transfer to occur, the energy of the acceptor must be the same as that of the donor – the point where the energy wells overlap.

According to the Marcus theory, k_{et} varies with the free energy difference between donor and acceptor (ΔG°) and the energy required to reorient the nuclei so that the energy state of the electron will be the same in both donor and acceptor (λ). (Note that the meaning of "λ" here is not the same as in Eqn. (1.1).) In polar

solvents like water, the major contribution to λ is reorientation of solvent molecules resulting from the change in the charge distribution of the reactant. The magnitude of λ is reduced if electron transfer occurs in a medium of low dielectric, for example, a lipid bilayer or the interior of a protein. Note that λ also depends on changes in the shape and charge distribution of the electron donor as the reaction proceeds. A prediction of the theory is that the rate of electron transfer is a maximum when $\Delta G^\circ = -\lambda$ (panel (B) of Fig. 8.9). As ΔG° becomes more negative but the distance between nuclei remains the same, as in panel (A), the rate of electron transfer decreases, because $\Delta G^\circ \neq -\lambda$. Note that changes in the distance between nuclei (d) result in changes in the height of the energy barrier, the energy at which the curves cross. Figure 8.10 shows how k_{et} varies with $\Delta G^\circ/\lambda$. This sections helps to deepen the earlier discussion of redox reactions, and it goes some distance towards tying together energy, thermodynamics, and kinetics. Many enzyme mechanisms involve electron transfer.

I. Enzyme kinetics

Unlike "mere" electron transfer, the complicated phenomenon of enzyme[3] catalysis has no explicit dependence on the free energy difference between reactants and products. Instead, it depends on the free energy of the transition state as outlined above. An entire section of the book is devoted to this subject because many of the chemical reactions of life are mediated and catalyzed by enzymes! Enzymes not only promote biochemical reactions: they also effectively ensure that the reactions will proceed rapidly enough for the cell metabolism to be maintained. All catalysts speed up a reaction by reducing the free energy of the transition state (Fig. 8.11), but enzymes are extraordinary catalysts. A great deal could be said about how extraordinary enzymes are, but let it suffice to mention just a few of their defining features. Then we'll devote the rest of the section to mathematical relationships useful for describing their behavior.

Enzymes are of course no less subject to the laws of physics than anything else made of matter. But they do nevertheless have a number of properties that set them well apart from ordinary catalysts. Here are just a few such properties: high rate of reaction, effectiveness under mild reaction conditions, astonishing specificity, and capacity to be regulated. The rate of a typical enzyme-catalyzed reaction is several orders of magnitude greater than a reaction catalyzed by a non-biological catalyst, and 10^6–10^{12} times greater than

[3] Greek, *en*, in + *zyme*, leaven. Technical use of this word is at least as ancient as the Middle Ages, when it played a part in theological discussions about whether the Eucharist should be celebrated with leavened bread (enzyme) or unleavened bread (azyme). See Fruton (1999), p. 148.

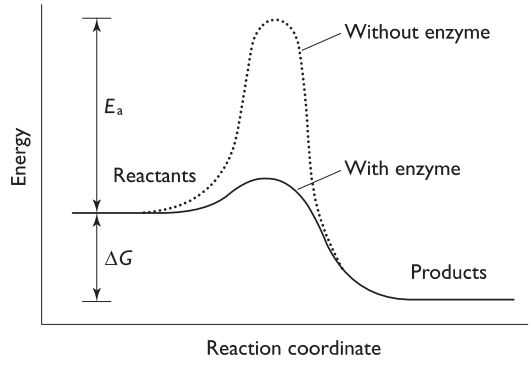


Fig. 8.11 Reaction profile for an enzyme-catalyzed reaction. The precise geometrical arrangement of atoms in the active site stabilizes the transition state of a biochemical reaction, lowering the free energy barrier between reactants and products. Because the rate of a reaction scales with E_a, a catalyzed reaction is faster than an uncatalyzed one.

the uncatalyzed reaction. In other words, if a biochemical reaction occurs at a rate of about 1 day^{-1} in the absence of enzyme, when the enzyme is present it is likely to occur at a rate of 1 s^{-1} to 1 000 000 s^{-1}. Amazing! The astonishing specificity of enzymes probably depends in general on the induced fit of the macromolecule to the substrate, as we saw with hexokinase (Chapter 4). What's more, enzyme-catalyzed reactions work best under remarkably mild conditions: temperatures at which water is in the liquid state, near-neutral pH values, near-atmospheric pressure. Most industrial catalytic processes, by contrast, require extremes of the same variables for commercially viable efficiency. Being made out of chiral subunits, enzymes are themselves chiral. The site where the enzymatic reaction occurs is chiral. This means that enzymes can discriminate between molecular "sheep" and "goats," gating who can and cannot enter into the active site, though some enzymes are more specific than others. Hexokinase, for example, phosphorylates not just glucose but other hexoses as well, while transfer of the phosphoryl group to glucose-6-phosphate by phosphoglucomutase is 10^{10} times more probable than transfer to water. Side reactions are the bane of anyone who has tried to synthesize a polypeptide by chemical methods, and yet this is done by cellular enzymes with near total fidelity. And enzymes can be regulated in a huge variety of ways, depending on the enzyme. The activity of ATCase, for instance, depends on the concentrations of ATP and CTP, even though neither molecule is directly involved in the reaction catalyzed by the enzyme.

The first general theory of chemical catalysis was formulated by Berzelius in 1835, and Hermann Emil Fischer (1852–1919),[4] a German, discovered that glycolytic enzymes can distinguish between stereoisomeric sugars in 1894. Adrian John Brown (1852–1919), a Briton, reported on the rate of hydrolysis of sucrose by β-fructofuranosidase in 1902. About three decades later, the American John Howard Northrop (1891–1987)[5] crystallized pepsin and

[4] Fischer was awarded the Nobel Prize in Chemistry in 1902.
[5] Northrop was awarded the Nobel Prize in Chemistry in 1946.

demonstrated conclusively that its catalytic activity was a property of the protein. The first enzyme structure (hen lysozyme, see below) was visualized at atomic resolution in Oxford about another three decades later, in the mid 1960s. These landmark accomplishments give a very rough idea of the pace of change in the first half of the twentieth century. The rate of discovery in this field today is a whole lot greater than when "Yesterday" was a new hit tune.[6]

Formation of a complex between enzyme (E) and substrate (S) and decomposition of the complex (E•S) into product (P) and enzyme can be symbolized as follows:

$$\mathrm{E} + \mathrm{S} \Leftrightarrow \mathrm{E} \bullet \mathrm{S} \rightarrow \mathrm{P} + \mathrm{E}. \tag{8.49}$$

Note that the complex is *assumed* to be *in equilibrium* with free enzyme and substrate, an important simplification that will crop up below. Note also that when the substrate concentration is high and all available enzyme is in the E•S form, the overall rate of reaction will be independent of [S]. The situation resembles the saturation of binding sites in Chapter 7. The rate or "*velocity*" of this reaction is

$$J_{\mathrm{P}} = \Delta[\mathrm{P}]/\Delta t = k_2[\mathrm{E} \bullet \mathrm{S}]. \tag{8.50}$$

Here, k_2, the rate constant of formation of P from E•S, is also known as k_{cat} and the *turnover number*, the number of substrate molecules converted to product per unit time (when all enzyme active sites are filled with substrate). The time rate of change in [E•S] is

$$J_{[E \bullet S]} = k_1[E][S] - k_{-1}[E \bullet S] - k_2[E \bullet S], \tag{8.51}$$

where k_1 is the rate constant of formation of E•S from E and S and k_{-1} is the rate constant of decomposition of the complex back into E and S. Under *steady-state* conditions, the rate of formation of the complex is equal to the rate of its decomposition, [E•S] is constant, and $J_{[\mathrm{E} \bullet \mathrm{S}]} = 0$. The *steady-state assumption* of enzyme catalysis was first put forth by the Englishmen John Burdon Sanderson Haldane (1892–1964) and George Edward Briggs (1893–1985) in 1925.

Let's make this exercise in formula derivation seem more like it's actually getting us somewhere we'd like to go by reformulating relationships in terms of things that can be *measured*. Such things include the *total* enzyme concentration, $[\mathrm{E}]_{\mathrm{T}}$, and the concentration of substrate at the beginning of the reaction. We have assumed that the enzyme binding site can be in one of two states, occupied or free, so

$$[\mathrm{E}]_{\mathrm{T}} = [\mathrm{E}] + [\mathrm{E} \bullet \mathrm{S}]. \tag{8.52}$$

Substitution of this equation into Eqn. (8.51) under steady-state conditions gives

$$k_1([\mathrm{E}]_{\mathrm{T}} - [\mathrm{E} \bullet \mathrm{S}])[\mathrm{S}] - (k_{-1} + k_2)[\mathrm{E} \bullet \mathrm{S}] = 0 \tag{8.53}$$

[6] This Beatles ballad is one of the most famous pieces of popular music of all time. The composer, Paul McCartney, was knighted by Queen Elizabeth II in 1997.

which, when solved for [E•S] is

$$[\mathrm{E{\bullet}S}] = \frac{[\mathrm{E}]_\mathrm{T}[\mathrm{S}]}{K_\mathrm{M} + \mathrm{S}}, \tag{8.54}$$

where $K_\mathrm{M} = (k_{-1} + k_2)/k_1$. The quantity K_M is called the Michaelis constant, after the German enzymologist Leonor Michaelis (1875–1949). Note that K_M is the ratio of the sum of the rates of depletion of the enzyme–substrate complex to the rate of formation of the complex. When $k_{-1} \gg k_2$, $K_\mathrm{M} \approx k_{-1}/k_1$, and K_M is *like* a dissociation constant. Values of K_M range between about 10^{-8} M and 10^{-2} M, and the value for a given enzyme will depend on the conditions under which measurements are made. When K_M is small, k_1 is relatively large, the free energy barrier to complex formation is relatively small, binding of substrate to enzyme is tight, and the enzyme will catalyze formation of product at very low concentrations of substrate. K_M thus reflects an enzyme's ability to bind substrate and carry out catalysis; K_M is not a true binding constant.

Substituting Eqn. (8.54) into Eqn. (8.50) gives

$$J_\mathrm{P} = \Delta[\mathrm{P}]/\Delta t = k_2[\mathrm{E{\bullet}S}] = k_2[\mathrm{E}]_\mathrm{T}[\mathrm{S}]/(K_\mathrm{M}+[\mathrm{S}]), \tag{8.55}$$

the rate of formation of product at any time during the experiment. The relationship can be used to determine K_M if $[\mathrm{E}]_\mathrm{T}$ and [S] are known, as at $t = 0$, enabling J_P to be measured. This $J_\mathrm{P} \approx J_\mathrm{P}(0)$. Later in an experiment, although $[\mathrm{E}]_\mathrm{T}$ will be known because it will not have changed [S] will not be known because some of the substrate will have been consumed, complicating determination of K_M.

The velocity of the reaction we are studying cannot be any faster than when every enzyme molecule present is in a complex with a substrate molecule at all times, when $[\mathrm{E{\bullet}S}] = [\mathrm{E}]_\mathrm{T}$. So,

$$J_\mathrm{P,max} = k_2[\mathrm{E}]_\mathrm{T}. \tag{8.56}$$

This equation reveals that the turnover rate, k_2, is the ratio of the maximum rate of reaction to enzyme concentration. The turnover rate usually varies from 10^5 to 10^9 molecules of product formed per enzyme molecule per second. Substituting this relationship into Eqn. (8.55) gives

$$J_\mathrm{P} = J_\mathrm{P,max}[\mathrm{S}]/(K_\mathrm{M}+[\mathrm{S}]). \tag{8.57}$$

This is the famous *Michaelis–Menten equation*, a basic equation of enzyme kinetics (Fig. 8.12). It was developed in 1913 by Michaelis and Maud Leonora Menten (1879–1960). The Michaelis–Menten equation says that when $[\mathrm{S}] = K_\mathrm{M}$, the velocity of the reaction is half-maximal. When K_M is relatively small, the rate of enzyme catalysis is maximal at a relatively low substrate concentration. And when K_M is relatively large, the concentration of substrate must be very large for the rate of enzyme catalysis to be a maximum. The fraction of active sites occupied can be found from the Michaelis–Menten equation by dividing both sides by $J_\mathrm{P,max}$:

$$J_\mathrm{P}/J_\mathrm{P,max} = [\mathrm{S}]/(K_\mathrm{M}+[\mathrm{S}]). \tag{8.58}$$

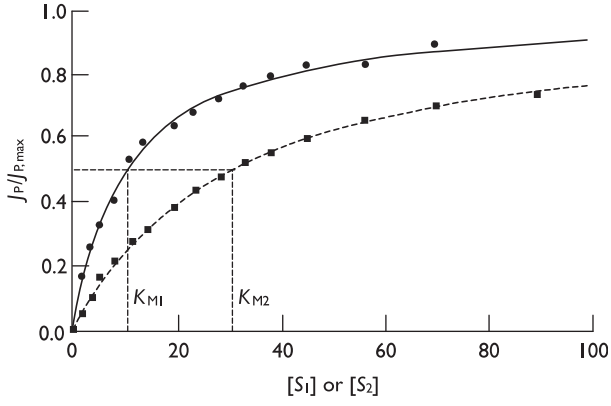


Fig. 8.12 Relative rate of reaction as a function of substrate concentration. Note that the rate of reaction here is the *initial* rate, i.e. the rate before the concentration of substrate has changed substantially, or the rate one would measure if the substrate concentration were held constant throughout the measurement. Data are shown for substrates with different values of K_M. The larger K_M, the greater the substrate required to give a half-maximal rate.

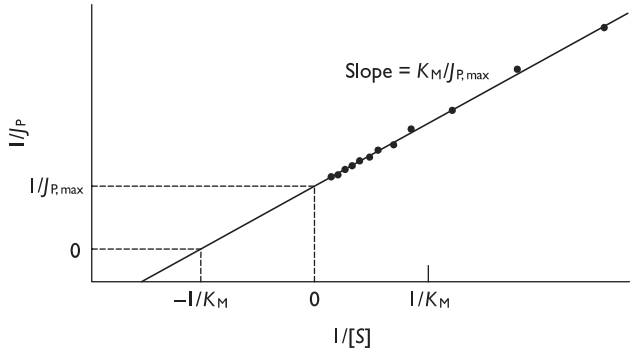


Fig. 8.13 A linearized version of the rate of reaction *versus* substrate concentration. Note how the data are clustered together, an effect of the mathematical transformation. Two points of interest are: $1/J_P = 1/J_{P,max}$, which occurs where $[S] \to \infty$, and $1/J_P = 0$, which will be the case when $[S] = -K_M$. A negative concentration is a mathematical fiction, but it useful for evaluating K_M. Unfortunately, linearization of kinetics data is often less helpful than one would like; reasons are given in the text. The type of plot shown here is a Lineweaver–Burk plot.

When [S] is small, $J_P/J_{P,max}$ is small, and as $[S] \to \infty$, $J_P/J_{P,max} \to 1$, as required.

Taking the reciprocal of Eqn. (8.57) yields one way of plotting kinetic data:

$$1/J_P = (K_M + [S])/(J_{P,max}[S]) = (K_M/J_{P,max})(1/[S]) + 1/J_{P,max}. \quad (8.59)$$

This equation is conveniently linear in 1/[S] with slope $K_M/J_{P,max}$, vertical axis-intercept $1/J_{P,max}$, and horizontal axis-intercept $-1/K_M$ (Fig. 8.13). But beware: use of the linearized form of the Michaelis–Menten relationship is beset by the same difficulties that we saw in Section E of the previous chapter. Moreover, in cases where the concentration of substrate is relatively high, there will be a tendency for experimental data to cluster together in a fairly narrow range of 1/[S]. In this way small errors in [S] can lead to large errors in K_M and $J_{P,max}$.

Equation (8.55) can be used to determine the efficiency of an enzyme-catalyzed reaction. Let $J_P = J_P(0)$. When [S] is very small in comparison with K_M, $[E]_T \approx [E]$, because very little E•S can form, and Eqn. (8.55) reduces to

$$J_P(0) \approx k_2[E]_T[S]/K_M \approx k_{cat}[E][S]/K_M, \quad (8.60)$$

Table 8.1. | The kinetic properties of some enzymes and substrates

Enzyme	Substrate	K_M(m)	$k_{cat}(s^{-1})$	k_{cat}/K_M $(M^{-1}s^{-1})$
Acetylcholinesterase	Acetylcholine	9.5×10^{-5}	1.4×10^{4}	1.5×10^{8}
Carbonic anhydrase	CO_2	1.2×10^{-2}	1.0×10^{6}	8.3×10^{7}
	HCO_3^-	2.6×10^{-2}	4.0×10^{5}	1.5×10^{7}
Catalase	H_2O_2	2.5×10^{-2}	1.0×10^{7}	4.0×10^{8}
Chymotrypsin	*N*-Acetylglycine ethyl ester	4.4×10^{-1}	5.1×10^{-2}	1.2×10^{-1}
	N-Acetylvaline ethyl ester	8.8×10^{-2}	1.7×10^{-1}	1.9
	N-Acetyltyrosine ethyl ester	6.6×10^{-4}	1.9×10^{2}	2.9×10^{5}
Fumarase	Fumarate	5.0×10^{-6}	8.0×10^{2}	1.6×10^{8}
	Malate	2.5×10^{-5}	9.0×10^{2}	3.6×10^{7}
Urease	Urea	2.5×10^{-2}	1.0×10^{4}	4.0×10^{5}

Data from Table 13–1 of Voet and Voet (1995). Note that k_{cat} is very high for catalase. Chymotrypsin is much more active against tyrosine residues than glycine or valine. Although acetylcholinesterase and fumarase have lower k_{cat} values than does catalase, all three of these enzymes have k_{cat}/K_M ratios of the same order of magnitude.

a second-order rate equation with rate constant k_{cat}/K_M. Here k_{cat} is defined as $J_{P,max}/[E]_T$; k_{cat} is identical to k_2 in the model discussed above. Note that k_{cat}/K_M measures the *catalytic efficiency* of an enzyme. When k_{cat}/K_M is large, on the order of 10^8–10^9 $M^{-1}s^{-1}$, enzyme activity is effectively limited solely by diffusion of the substrate into the binding site. In the case of catalase, for example, an enzyme that catalyzes the degradation of hydrogen peroxide to water and oxygen, k_{cat}/K_M is about 10^7. This enzyme operates very close to the diffusion-controlled limit! In other words, the catalytic efficiency of this enzyme is limited by physics and not by chemistry; no further change in the enzyme could increase its catalytic activity. It is k_{cat}/K_M, not K_M, that is the generally accepted parameter for characterizing enzyme catalysis (under given conditions). See Table 8.1.

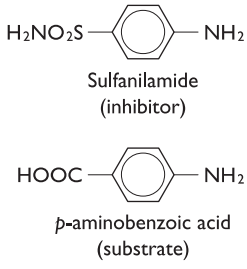


Fig. 8.14 A substrate and an inhibitor. The figure shows how similar the structure of an inhibitor and the substrate can be. In the pharmaceutical industry, medicinal chemists look for ways of synthesizing compounds similar in structure to known substrates. The potential inhibitors are then tested in a variety of ways. If a candidate inhibitor is able to inhibit an enzyme, and if it is not too toxic to cells, it might become a marketable drug.

J. | Inhibition

Enzyme activity can be inhibited in various ways. In *competitive inhibition*, molecules similar to the substrate bind to the active site and prevent entry of the usual substrate. The antibiotic penicillin, for example, serves as a competitive inhibitor by blocking the active site of an enzyme that many bacteria use to construct their cell walls. In *non-competitive inhibition*, by contrast, an inhibitor binds the enzyme at a location other than the active site. This can come about in several ways, including deformation of the specific geometry of the active site (*allosteric inhibition*, see Chapter 7).

An example of competitive inhibition is the action of sulfanilamide (Fig. 8.14) on an enzyme involved in the metabolism of folic acid,

a vitamin that is a coenzyme precursor. Sulfanilamide is sufficiently similar to the substrate, *p*-aminobenzoic acid, that it binds to the enzyme and inhibits *p*-aminobenzoic acid from reaching the active site. The enzyme in question is essential in certain disease-causing bacteria but not in humans, and this allows the chemotherapeutic use of sulfanilamide as a type of antibiotic called an anti-metabolite.

Note that both competitive inhibition and non-competitive inhibition are usually thought of as involving non-covalent interactions between inhibitor and enzyme. Other types of inhibitor, however, form covalent bonds with enzymes. For instance, the nerve gas diisopropyl fluorophosphate, forms a covalent bond with an amino acid residue side chain in the active site of acetylcholinesterase and thereby prevents binding of the neurotransmitter acetylcholine and blocks nerve action. Various types of protease inhibitor are added to protein preparations to prevent digestion of the sample. Some protease inhibitors bind irreversibly to proteases by forming a covalent bond with amino acid side chains in the active site.

Having looked at qualitative aspects of inhibition, let's see if we can model it mathematically. In competitive inhibition the enzyme interacts with the substrate, S, or the inhibitor, I, so the total concentration of enzyme is

$$[\mathrm{E}]_\mathrm{T} = [\mathrm{E}] + [\mathrm{E}\bullet\mathrm{I}] + [\mathrm{E}\bullet\mathrm{S}]. \tag{8.61}$$

As before,

$$K_\mathrm{M} \approx [\mathrm{E}][\mathrm{S}]/[\mathrm{ES}]. \tag{8.62}$$

The inhibitor is in equilibrium with the enzyme and E•I, so

$$[\mathrm{E}][\mathrm{I}]/[\mathrm{E}\bullet\mathrm{I}] = K_\mathrm{I}. \tag{8.63}$$

Solving this equation for [E•I] and substituting Eqn. (8.62) in for [E] gives

$$[\mathrm{E}\bullet\mathrm{I}] = [\mathrm{E}][\mathrm{I}]/K_\mathrm{I} = (\mathrm{K_M}[\mathrm{E}\bullet\mathrm{S}]/[\mathrm{S}])[\mathrm{I}]/\mathrm{K_I}, \tag{8.64}$$

and substituting Eqn. (8.64) into Eqn. (8.61) and solving for [E•S] gives

$$[\mathrm{E}\bullet\mathrm{S}] = \frac{[\mathrm{E}]_\mathrm{T}[\mathrm{S}]}{K_\mathrm{M}\left(1 + \frac{[\mathrm{I}]}{K_\mathrm{I}}\right) + [\mathrm{S}]}. \tag{8.65}$$

As before, $J_\mathrm{P} = k_2[\mathrm{E}\bullet\mathrm{S}]$, so

$$\mathrm{J_P} = \frac{K_2[\mathrm{E}]_\mathrm{T}[\mathrm{S}]}{K_\mathrm{M}\left(\frac{1+[\mathrm{I}]}{K_\mathrm{I}}\right) + [\mathrm{S}]}. \tag{8.66}$$

Comparison of this relationship with Eqn. (8.55) shows that the effect of increasing the concentration of competitive inhibitor is to increase the apparent magnitude of K_M. This is why the quasi-equilibrium character of the Michaelis constant must be treated with caution. Equation (8.66) is plotted for different values of $[\mathrm{I}]/K_\mathrm{I}$ in Fig. 8.15. $[\mathrm{I}]/K_\mathrm{I} = [\mathrm{E}\bullet\mathrm{I}]/[\mathrm{E}]$, approximately the proportion of the total amount of enzyme that has formed a complex with the inhibitor.

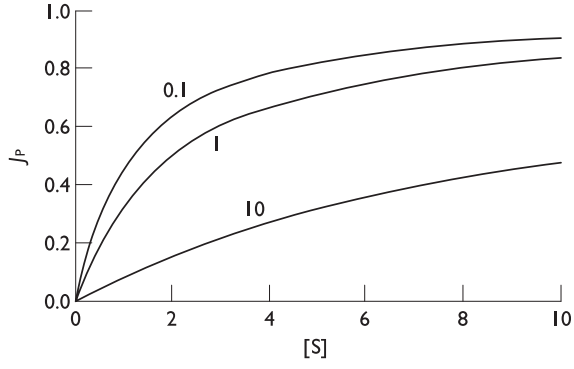


Fig. 8.15 The effect of an inhibitor on the rate of enzyme activity. The three curves represent different values of the ratio [I]/K_I. For a given K_I, the plots show the effect of increasing inhibitor concentration. As expected, increases in [I] result in decreases in J_P for a given substrate concentration. The middle curve could also represent [I]/K_I = 10 and a 10-fold decrease in K_M relative to the lowest curve. In other words the smaller K_M, the larger the rate of catalysis for a given concentration of substrate.

The derivation for the case of mixed inhibition is very similar. It is left as an exercise to show that

$$J_P = \frac{k_2[E]_T[S]}{K_M\left(1+\frac{[I]}{K_I}\right)+[S]\left(1+\frac{[I]}{K_I'}\right)}, \tag{8.67}$$

where K_I is defined as before and $K_I' = [E\bullet S][I]/[E\bullet S\bullet I]$. $K_I = K_I'$ in non-competitive inhibition.

K. Reaction mechanism of lysozyme

As discussed in Chapter 5, lysozymes hydrolyze the β(1→4) glycosidic linkage of oligosaccharides in the cell wall of bacteria. This weakens the cell wall and leads to osmotic lysis. Lysozyme also digests chitin, a polysaccharide which strengthens the cell wall of most fungi. Our present interest is the mechanism of lysozyme activity.

Much of what is known about the mechanism of lysozyme is based at least in part on the structure of the enzyme at atomic resolution, which was first obtained by David Chilton Phillips (1924–1999) at Oxford in 1965. A prominent structural feature of the folded enzyme is its large active site cleft, which binds up to six residues of polysaccharide. The rate of enzyme activity depends on the length of the sugar. Enzyme-catalyzed hydrolysis is up to 100 million times faster than the uncatalyzed reaction. When six residues are bound in the active site, hydrolysis occurs between residues four and five. The location of hydrolysis is somehow related to the experimental finding that the free energy of binding of the fourth residue to the enzyme is the weakest of the six (Table 8.2). This weakness in binding is thought to reflect the distortion of the sugar at residue four that is required for tight binding of the polysaccharide to occur. The most favorable binding of the enzyme is to the third residue of the six-residue polysaccharide. How does lysozyme go about its business?

Table 8.2. *Binding free energies of polysaccharide subunits in the active site of hen lysozyme*

Site	Bound saccharide	Binding free energy (kJ mol^{-1})
1	NAG	–7.5
2	NAM	–12.3
3	NAG	–23.8
4	NAM	+12.1
5	NAG	–7.1
6	NAM	–7.1

[a] *N*-acetylglucosamine.
[b] *N*-acetylmuramic acid.
Lysozyme hydrolyzes the $\beta(1\rightarrow4)$ glycosidic linkages from NAM and NAG in the alternating NAM-NAG polysaccharide component of bacterial cell wall peptidoglycans. Data are from Chipman & Sharon (1969).

There are two acidic groups in the active site cleft, Glu 35 and Asp 52, and they are close to the bond of the polysaccharide that is cleaved during catalysis. Replacement of Asp 52 by Ser leads to virtually complete loss of enzyme activity. There is, however, nothing particularly unusual about the pK_a of this side chain (it is *c*. 3.5), as one might expect for a side chain involved in catalysis. The carboxyl group of Asp 52 is in a polar environment. Glu 35, by contrast, has an anomalous pK_a in the folded state of the enzyme (it is ~6.3!), and this must result from the specific electronic environment of the side chain. Indeed, the carboxyl group of Glu 35 is situated in the relatively low dielectric environment of a mostly hydrophobic pocket. This shift in pK_a has important consequences for the mechanism of enzyme action, as experimental studies have shown that activity is *maximal* at pH 5, well *below* the pK_a of Glu 35. In other words, enzyme activity is less than it could be if the Glu 35 side chain were ionized. Therefore, a plausible mechanism of lysozyme activity is that the carboxyl group of Glu 35 transfers its proton to the bond between polysaccharide units four and five. It seems that the negative charge on Asp 52 must play a role in the enzyme mechanism, but it is still not entirely clear how. This is just one of biochemistry's many, many unanswered questions.

L. Hydrogen exchange

Biological macromolecules are full of covalently bound but labile hydrogen atoms, many of which can exchange readily with hydrogen atoms in the solvent. What we have in mind here are not the ionizable protons of Glu and Asp but the amide protons of the polypeptide backbone. Because these protons undergo exchange with the solvent on a convenient time scale, hydrogen isotopes can be used to "label" solvent-exposed parts of a macromolecule (Fig. 8.16). The use of such labeling techniques in biochemistry,

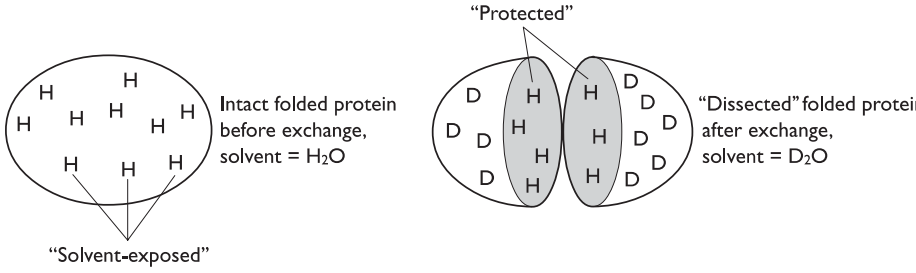


Fig. 8.16 H/D exchange labeling. Proteins and other biological macromolecules have hydrogen atoms that can readily exchange with protons in the solvent. In proteins, such protons are found in the amide groups of the polypeptide backbone in certain amino acid side chains; aliphatic protons do not exchange with solvent on a time scale that is suitable for protein structure studies. In general, the more exposed to solvent a labile proton, the more rapidly it exchanges. One can determine the rate of exchange at different sites in a protein by exchange labeling, in which deuterons are exchanged for protons, or vice versa. This works because on the chemical level deuterium is identical to hydrogen: there is just a single electron and a single proton. The difference lies in the number of nucleons: hydrogen has no neutrons and deuterium has one. The change in nucleus changes not only the mass of the atom but also its magnetic properties, rendering deuterium "invisible" to an NMR spectrometer set up to acquire data on proton resonances. The effect of exchange on the NMR spectrum is that proton peaks diminish in size as deuterium gets exchanged in for hydrogen, since only the remaining hydrogen at a particular site will contribute to the proton spectrum.

which was pioneered by Linderstrøm-Lang in the 1950s, has been extremely useful in studies of the structure, stability, dynamics, and folding properties of biological macromolecules. For instance, the rate of hydrogen exchange is a key consideration in NMR structure determination, because if the side chain protons of aromatic residues in the core of the protein exchange too rapidly, it will be hard to obtain definite information on how they interact with other protons. Such information is needed to determine the 3-D structure of a protein by usual methods of NMR. In this section we'll look at hydrogen exchange kinetics in two contexts: protein stability at equilibrium and acquisition of native-like structure on the protein folding pathway. The outlined approaches can also be used to study protein–ligand interactions, whether the ligand is an ion, a small organic molecule, a peptide, or a nucleic acid.

Not all hydrogen atoms exchange rapidly enough to be useful for physical biochemistry experiments. Aliphatic hydrogen atoms, for example, exchange extremely slowly, so these hydrogens are essentially fixed on the time scale of an experiment you might do. Polypeptide backbone amide hydrogens (protons), however, undergo exchange on a more convenient time scale, making them particularly valuable for the experiments described here. Other exchangeable protons are indicated in Fig. 8.17. The dependence of rate of exchange on pH arises from the reaction's being both acid-catalyzed and base-catalyzed (though by different mechanisms). The rate of exchange also depends on temperature, tripling every 10 °C or so. Together, pH and temperature determine the *intrinsic rate of exchange*, the rate in an unstructured polypeptide (see Appendix D). Other influences on exchange rate include electronic properties of the local environment and the exchange medium (whether the

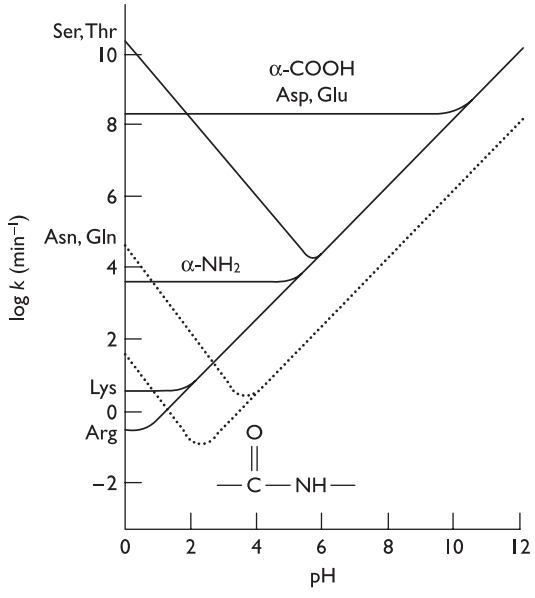


Fig. 8.17 The pH-dependence of the hydrogen exchange rate for different types of chemical group. There is a 10-fold change in reaction rate for a change in pH of 1 unit; exchange is both acid- and base-catalyzed, giving v-shaped curves. Peptide bond amino protons show a minimum rate of exchange at acidic pH. The rate of exchange of these protons depends only on whether the amide group is buried or solvent exposed and on the type of secondary and tertiary structure in which it is involved, but also to some extent on the flanking residue. (See Appendix D.)

solvent water contains hydrogen or deuterium, the ionic strength, and so on). Important for studies of protein folding, the rate also varies substantially with how often the exchangeable hydrogen comes into contact with the solvent. Backbone amide protons deep in the hydrophobic core are not likely to exchange rapidly, because they rarely come into contact with the solvent when the protein is in the folded state. But interior labile protons *can* exchange! This phenomenon is usually described in terms of *local unfolding* of the protein, or "*breathing*," an imaginative image provided by Linderstrøm-Lang.

There are two basic types of exchange, and both come from thinking of exchange as a race between the protein and the exchange mechanism. If the rate of exchange of a solvent-exposed labile hydrogen is higher than the rate at which this hydrogen ceases to be solvent-exposed (usually because of reorganization of molecular structure), exchange is said to follow the *EX_1 mechanism*. But if the rate of exchange of a solvent-exposed labile hydrogen is lower than the rate at which this hydrogen becomes "protected," exchange follows the *EX_2 mechanism*. The second of these, in which the rate is determined by the kinetics of the exchange reaction because exchange is slower than refolding, is the type displayed by proteins under most conditions. There are many ways in which you could make use of protein hydrogen exchange data!

Consider the relationship between hydrogen exchange and NMR spectrometry and mass spectrometry. There are many variations on the theme in NMR studies of proteins, but there are two basic ways of doing hydrogen exchange (HDX) experiments: *equilibrium exchange* of protons for deuterons (or vice versa) and *quenched-flow pulse labeling* of deuterated protein with protons. In either case, one uses a non-exchangeable proton as an intrinsic probe, typically, a tryptophan

side-chain proton, which allows normalization of the proton resonance magnitude from spectrum to spectrum. In equilibrium HDX, the protein sample is dissolved in a buffer made with D_2O instead of H_2O. As discussed in Chapter 5, even at equilibrium structure of a protein will fluctuate, and at any given time some fraction of the molecules will be partly folded or unfolded. These fluctuations and folding/unfolding transitions can be sufficient for the solvent to penetrate to the core of the protein, though the time-average duration of contact of protein surface with the solvent will obviously be much lower for core residues than surface ones. Exchange is generally very slow under conditions favoring the folded protein, so the height of a ^1H NMR resonance, which is proportional to the number of hydrogen atoms at a given location in the protein, is measured over a course of up to several months. Between measurements, the protein sample is kept at constant temperature (and pressure).

After hydrogen peak heights have been measured and normalized by reference to the height of a non-exchangeable hydrogen resonance, the data for each exchangeable proton is plotted as a function of time. A fitting procedure can then be used to determine the rate of exchange of hydrogen on a case by case basis. Typically, exchange at a particular site will be dominated by a single rate. The rates of exchange for different protons in the same protein under the same conditions are then compared with each other, to discern similarities and differences. When exchange of core protons occurs via global unfolding of the protein, as in an all-or-none transition (Chapter 4), all such protons exchange with about the same rate. The process can be more complex, however, as for example if one part of the protein is particularly flexible and another is especially rigid. In such cases the rate data for individual protons might cluster into two or more groups. So HDX data can be used to build models of the mechanism of protein folding/unfolding.

Equilibrium HDX data can be used in other ways. For example, the measured rate of exchange can be compared with the calculated intrinsic rate of exchange under the same conditions. The ratio of rates measures the extent of "protection" against exchange of a particular proton under the conditions of the experiment. This ratio, called a *protection factor*, resembles an equilibrium constant (Chapter 4), and it can therefore be used to calculate the free energy difference between the folded state of a protein (for which the rate of exchange is measured) and the unfolded state (the intrinsic rate of exchange) by Eqn. (4.38). The free energy so calculated should be compared with one obtained by some other method, for example, calorimetry. Comparable values are consistent with a two-state process; different values suggest something more complex.

Many single-domain proteins follow a two-state folding/unfolding mechanism under usual conditions; essentially only two states are relevant to the process. Some techniques used to monitor structural transitions in proteins are differential scanning calorimetry and spectroscopic methods like fluorescence (Chapters 2, 5,

and 6). These methods measure bulk properties of a sample and do not distinguish explicitly between different states. Mass spectrometry, by contrast, which can determine the mass of a particle to a resolution of 1Da (dalton), allows not only the identification of different co-existing conformations but also determination of their exchange behavior. In the case of two-state behavior, only two peaks will be seen in the mass spectrum at any point in the folding/unfolding reaction: one corresponding to the folded state and the other to the unfolded state. Depending on how the data are processed, the mass spectrometry peak heights will reflect the relative abundance of the two states. When the folding/unfolding mechanism is more complicated, for instance, when three states are present, in principle it will be possible to identify all three states as separate peaks in the mass spectrum.

A quenched-flow pulse labeling machine can be used to label proteins during the folding process. In a typical experimental set-up, the protein is deuterated and dissolved in deuterated chemical denaturant. The experiment is begun by rapid mixing of the protein sample with a non-deuterated buffer that favors protein refolding, which is initiated on diluting the concentration of denaturant. Some time later, usually between just a few milliseconds and one second, a high-pH pulse is added to the refolding protein solution. The driving force for hydrogen exchange is very high under these conditions, and deuterons exposed to the solvent are replaced by protons. The number of deuterons that can exchange will clearly decrease as the length of time between initiation of folding and the high-pH pulse increases. The duration of the high-pH pulse is constant. To inhibit further exchange, the high-pH pulse is followed immediately by a change back to the refolding buffer. Data collection at different time points during refolding yields a series of snapshots of the protein, revealing which parts are solvent-exposed and how long they are that way. By this approach, it has been possible to show that one part of a protein folds more rapidly than another.

M. Protein folding and pathological misfolding

Protein folding is a large topic, one that has continued to develop rapidly since the early 1980s, though groundbreaking studies were done long before then (see Chapter 5). Justice could not possibly be done to the subject in the span of a few pages, so our aim is not to attempt an exhaustive treatment but to give a sense of the place of protein folding in the more general subject of reaction kinetics. The section closes with comments on protein misfolding and disease.

One of the first aims of a protein folding study might be to measure the overall rate of attainment of native structure from an adequately defined denatured state. We say “adequately” and not

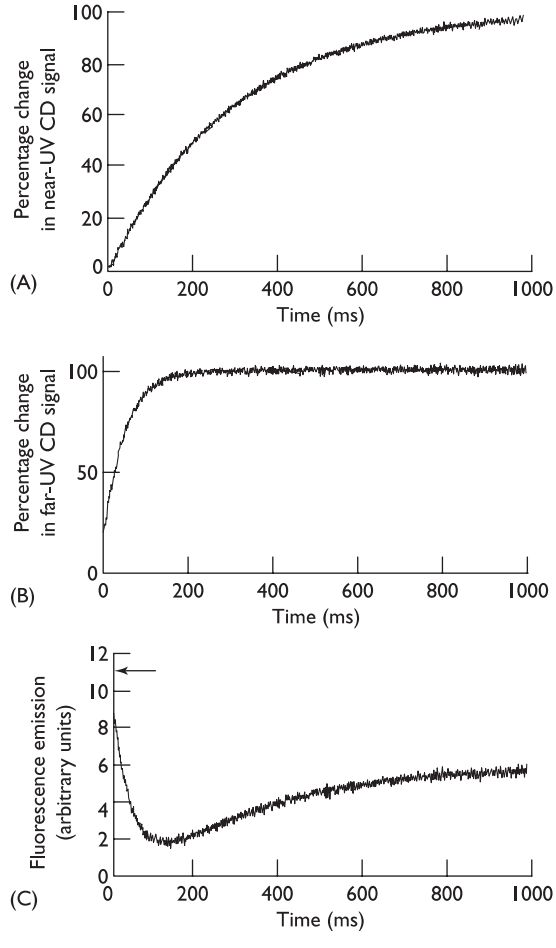


Fig. 8.18 Kinetic protein folding data. (A) Far-UV CD. The signal reaches the equilibrium value relatively rapidly. (B) Near-UV CD. Compared with the far-UV data, the signal reaches its maximum value relatively slowly. (C) Intrinsic fluorescence emission. This kinetic trace is more complex than either of the CD curves. The process is biphasic, the first phase being relatively fast and the second one slow. The data were acquired with instruments outfitted with stopped-flow modules, which enable rapid mixing of solutions.

"completely" because it can be very difficult to say just how unfolded a denatured state is. Suffice it to say here that the heat-denatured state will not always be very close in structure to the pH-denatured state or some other type of denatured form, because the way amino acids interact with each other will depend on solution conditions. In general, the form of the denatured state matters more for folding studies of big proteins (greater than about 130 residues) than small ones. A popular way of studying folding is to denature the protein in 6 M GuHCl and to initiate refolding by diluting one volume of denatured protein with 10 volumes of refolding buffer.

Figure 8.18 shows what you might find by optical spectroscopy in analyzing the refolding of a small globular protein from a denatured state. The data were obtained by circular dichroism spectroscopy (panels (A) and (B)) and fluorescence emission spectroscopy (panel (C)). The near-UV CD signal monitors organization of specific interdigitation of side chains in the native state. We see from panel (A) that this occurs with relatively slow kinetics, and is not complete

until about 1 s after initiation of folding. But according to panel (B), which shows the far-UV CD signal, the secondary structure of the protein forms rather more quickly: by 200 ms into the reaction apparently all secondary structure is as formed as under native conditions. Comparison of panels (A) and (B) would suggest that there are at least two "phases" in the folding reaction. Moreover, about one-fifth of the native far-UV signal is present by the time the first reliable signal can be acquired (about 3 ms into the experiment). This so-called burst phase of protein folding is a bit tricky to study because of its short time scale.

Fluorescence emission can be used to follow changes in the solvent accessibility of tryptophan side chains. When tryptophan is exposed to the highly polar solvent, emission intensity is low; when it is buried in the hydrophobic core of a folded protein, emission intensity is high. The curve in panel (C) is biphasic, distinguishing it from panels (A) and (B). The apparent rate of refolding found by fluorescence is, however, about the same as that of near-UV CD (panel (A)). Moreover, the first phase has kinetics that closely resemble the view obtained by far-UV CD (panel (B)). Thus, in a somewhat non-obvious way the fluorescence data corroborate the data in panels (A) and (B).

Denatured polypeptides do not always fold into native proteins under conditions favoring the folded state. In some case, misfolding *in vivo* can be "corrected" by other proteins, the so-called chaperones, whose general function appears to be to assist folding. This is particularly important to the life of the cell, tissue, and organism, because under some conditions misfolding can result in pathological protein aggregation.

Generally speaking, misfolding yields a partly folded structure, one that is more compact than a fully extended polypeptide chain and may contain native-like elements of secondary structure but less thermostable and usually more hydrophobic than the native state. The increased solvent exposure of apolar side chains in misfolded proteins relative to folded proteins makes misfolded proteins "sticky," giving them a tendency to aggregate with kinetics that are governed by protein concentration, temperature, and solution conditions.

Why does aggregation occur when the folded state is likely to have a lower free energy than a partly folded state? Just as a large number of relatively weak individual hydrogen bonds can stabilize a folded protein, a large number of weak interactions between misfolded proteins can stabilize aggregates. Experimental work has shown that protein aggregation is often mediated by intermolecular β-strands, the strands of a β-sheet being contributed by different protein molecules. Individual partly folded states are in a shallower energy well than the native state, but aggregates probably have a lower free energy than the same number of fully folded proteins, making them stable and particularly difficult for the body to clear.

Protein misfolding appears to be the common cause of the various amyloid diseases,[7] which are characterized by abnormal extracellular accumulations of protein called amyloid plaques. Clumps of degenerating neurons surrounding deposits of protein, for example, are called neuritic plaques; twisted protein fibers in nerve cells are known as neurofibrillary tangles. If these descriptions conjure up images of senility, then you are right on target: the occurrence of these structures in the brain correlates with symptoms of Alzheimer's disease.[8] Plaques and tangles may actually cause the disease. Neuritic plaques form around aggregates of amyloid β-protein, a proteolytic fragment of a larger molecule called amyloid precursor protein, a normal component of nerve cells. When separated from the rest of the precursor protein, amyloid β sticks to itself like glue, forming large deposits that probably interfere somehow with normal cellular activity and lead to impaired brain function. The fibers of neurofibrillary tangles consist of a different protein, called tau, a normal protein in neurons. Tau molecules clump together and form tangles when protein processing goes awry.

Something similar happens with amyloidogenic lysozyme. In this case, however, the aggregate-forming molecules are intact; they are not the result of proteolysis or incorrect processing. The tendency of human lysozyme to aggregate in some people (an extremely small percentage of the population in Britain and probably elsewhere) comes not from misfolding or from incorrect disulfide bond connections but from native state instability brought about by a single point mutation. Several amyloidogenic variants of lysozyme are known, and in the most pathological one the relative instability of the native state leads to non-cooperative protein denaturation at room temperature.

N. Polymerization

Polymerization has to do with the formation of linear polymers from subunits. Here, though, our concern is polymers in which the subunit interactions are non-covalent ones, not the covalent bonds of amino acid polymers (polypeptides) or nucleic acid polymers (polynucleotides). The most famous non-covalent biological polymer is perhaps the actin filament, but in fact many biomacromolecules can polymerize. Indeed, lysozyme amyloid fibril formation comes from mutations that lead the partly folded enzyme to polymerize – spontaneously. Non-covalent polymerization is thought to be an excluded-volume effect, one that favors compact conformations. In

[7] Greek, *amylon*, starch + *eidos*, form, coined by Rudolf Carl Virchow (1821–1902), a German pathologist. Most of the mass of an amyloid plaque is protein, not starch.

[8] Named after the German psychiatrist and neuropathologist Alois Alzheimer (1864–1915).

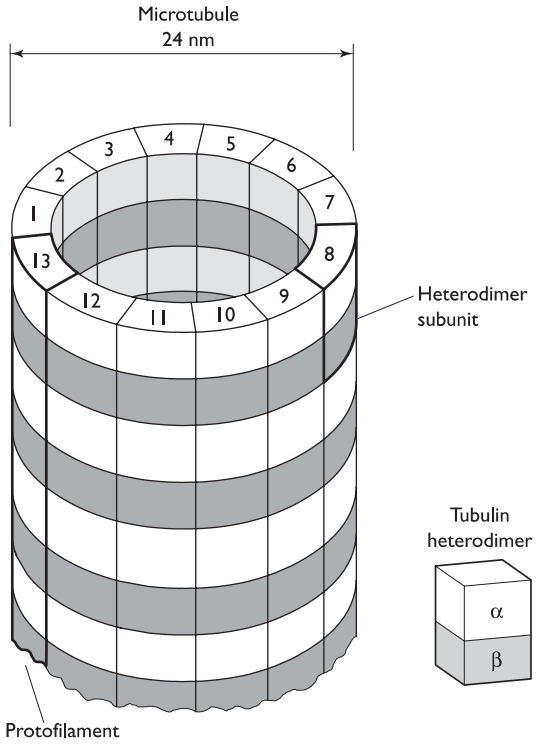


Fig. 8.19 Microtubule structure. Microtubules are composed of tubulin, of which there are two types of subunit, designated α and β. A single tubule consists of thirteen protofilaments made of alternating α and β subunits. Note that an intact tubule, which is 24 nm wide, is helical and hollow. Microtubules have received a fair amount of popular press in recent years, as the noted British mathematical physicist and science popularizer Sir Roger Penrose (1931–) has suggested that microtubules and a reworking of the foundations of physics might be keys to understanding consciousness. Is it not amazing that a human brain, which on one level can be thought of as just a highly organized collection of atoms, can investigate how those atoms are organized and indeed the structure of the atoms themselves? But why microtubules and not some other type of filamentous structure, say actin? After all, actin has more than one biochemically relevant conformational state, uses ATP for polymerization, plays an indispensable role in contractility, and serves as a conveyor for biomolecules in the cytosol. There is a great deal of disagreement among scientists, philosophers and theologians as to the origin and material requirements of human consciousness! Because no one has all the answers on such matters, it is probably best to avoid being too presumptuous about which viewpoints are worthy of further investigation or debate.

this sense, polymerization of proteins is similar to the folding of a single protein but on a larger scale. Highly elongated protein polymers (e.g. actin microfilaments) tend to form higher-order phases in which the elongated protein polymers associate with each other to form even more compact structures. The actin cytoskeleton consists of bundles of actin filaments that are highly "cross-linked" by actin-binding proteins. Our main interest in the present section is actin, the polymerization of which involves hydrolysis of ATP. Before focusing our attention on microfilament assembly, let's look briefly at a related example of polymerization in the living organism.

Microtubules are cylindrical tubes with a diameter of 24 nm (Fig. 8.19), and they play a big part in filling the cytosol of a cell from the nucleus to the plasma membrane. These polymeric filaments are crucial for cell movement, from the beating of cilia, to the transport of vesicles from the cell membrane to places within the cell, to the separation of chromosomes during cell division, to the extension of the neuronal growth cone. Microtubules also play a structural role, helping to give a cell its shape. Microtubules are composed of globular α- and β-tubulin subunits. The inherent asymmetry of the subunit gives rise to an asymmetric structure. At low temperatures (and also in the presence of calcium), microtubules dissociate into tubulin protomers (heterodimers). *Both types of tubulin subunit interact with GTP*. The α-subunit binds the nucleotide irreversibly; the β-subunit is a GTPase. *The energy of GTP hydrolysis in the β-subunit drives addition of tubulin at the end of a growing microtubule*. A single tube comprises 13

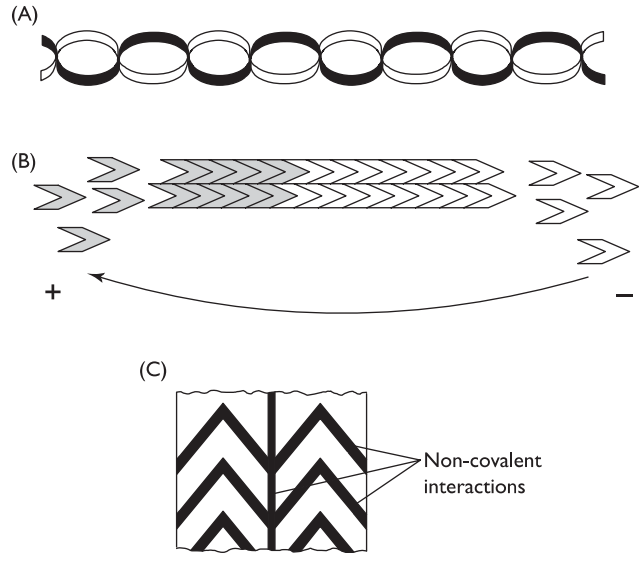


Fig. 8.20 Actin filament structure. (A) A single filament, also known as a microfilament, is a double helix, each strand of which is a string of actin monomers held together by *non-covalent* interactions. (B) Actin filaments are polar: the ends differ because subunits are asymmetrical. Preferential ATP-bound monomer association occurs at the barbed end of the filament, preferential ADP-bound monomer dissociation occurs at the point end. ATP is clearly important for actin polymerization, but actin itself is not known to be an ATPase. (C). The situation is much more complicated than panels (A) and (B) would suggest. For not only is there physical contact between the heads and tails of monomers, subunits interact non-covalently with each other across the helix axis.

protofilaments. Several tubes can join together to form larger diameter and stronger structures. Now on to microfilaments.

Actin is ubiquitous and usually the most abundant cytoplasmic protein in eukaryotic cells. A major component of the cytoskeleton, actin forms microfilaments *in vivo* and *in vitro*. The monomeric form predominates at low temperature, low salt concentration, and alkaline pH. Monomers can associate and dissociate from both ends of the filament (Fig. 8.20). The kinetics of association and dissociation, however, differ at the two ends. The "*plus end*" of the filament, or "*barbed end*," is where *ATP-bound actin monomers associate*; the "*minus end*" of the filament, or "*pointed end*," is where *ADP-bound actin monomers dissociate*. The arrow-like monikers come from electron microscopic studies of actin filaments after "decoration" with the actin-binding portion of myosin (next section). The complex has a chevron-like appearance, reflecting the underlying asymmetry of each actin monomer. At some point between association of an actin monomer at the barbed end and dissociation from the pointed end, ATP hydrolysis occurs. The precise role of ATP hydrolysis in actin polymerization is unknown, but it is known that ADP-actin polymerizes much less well than ATP-actin.

Both monomer addition at the pointed end and monomer dissociation from the barbed end occur with low probability. Under appropriate conditions, actin filaments *in vitro* will exhibit a phenomenon called "*treadmilling*"; the rate of monomer addition at the barbed end being about the same as the rate of monomer dissociation from the pointed end; the average length of the filaments is constant. The ability of actin to polymerize and depolymerize readily is probably very important to cell function. For when filaments are stabilized by the binding of phalloidin, a toxic

component of certain poisonous mushrooms, the cell displays a highly abnormal appearance. Before filament elongation can occur, a "nucleus" of monomers must form. Nucleation involves the thermostable association of at least three actin monomers and is therefore improbable (compare nucleation of α-helix formation in Chapter 6). But in the presence of well-formed nuclei, when the concentration of actin monomers exceeds the "*critical concentration*" for polymerization, elongation occurs rapidly.

The kinetics of polymerization can be determined by fluorescence spectroscopy if the actin monomers have been labeled with a fluorescent dye. For example, it is possible to conjugate pyrene to actin. There is an increase in fluorescence intensity when polymerization occurs, because the polarity (dielectric constant) of the environment of the dye molecules is lower in the filament than in the monomer, and the intensity of fluorescence emission of a fluorophore varies inversely with the polarity of its surrounding environment. Experiments involving analysis of the kinetics of actin polymerization are called *polymerization assays*.

Polymerization assays are a useful tool for studying the association of actin with actin-binding proteins. For instance, a protein that associates with the barbed end of actin will decrease the rate of actin polymerization under given conditions. Such experiments can be used to determine an association constant for the actin-binding protein. Binding of a barbed end-binding protein to actin can also result in net depolymerization of actin filaments. This arises from the dissociation of monomers from the pointed end and the inhibition of monomer addition at the barbed end.

There are other types of actin-binding protein. Profilin, for example, forms a 1:1 complex with actin monomers called profilactin and thereby prevents actin polymerization. The importance of this interaction can be seen exceptionally clearly in the fertilization of a sea urchin egg. Each sperm head is loaded with a bag full of profilactin called the acrosome. Contact between sperm head and the jelly surrounding the egg sets off a reaction that increases the pH of the acrosome. This change in the net charge of actin and profilin results in dissociation of profilactin, and actin nuclei begin to form. A couple of seconds later, a thin bundle of actin filaments called the acrosomal process begins to protrude from the sperm head. The process penetrates the jelly, joining sperm and ovum. Once activation of the formation of the acrosomal process has occurred, elongation is rapid and long; the rate is greater than 10 $\mu M\ s^{-1}$ for *c.* 6 s.

O. Muscle contraction and molecular motors

About 2 of every 5 g of the weight of a healthy adult human is muscle. The mechanical and contractile properties of non-muscle cells, erectile tissue, and other types of muscle depend in part on the actin filaments described above. In skeletal muscle, for example,

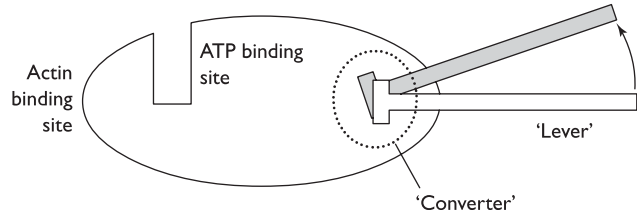


Fig. 8.21 Structure of the head group of myosin. A catalytic "domain" binds actin and ATP, and an elongated, α-helical carboxyl-terminal "domain" contains a variable number of proteins called calmodulin-like light chains. A plausible model of myosin function is that ATP hydrolysis leads to small intramolecular movement in the catalytic domain that is converted into a large rotation of the light-chain domain. This then acts like a lever arm in the motor mechanism. The pivot, called the "converter," fixes the point on which the lever rotates. The process results in the movement of the end of the lever by several nanometers.

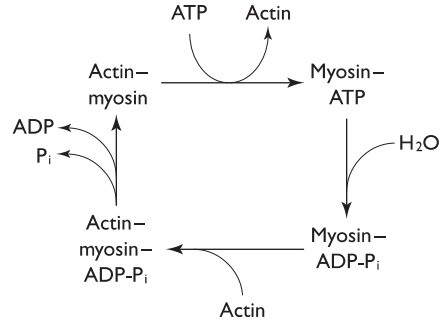


Fig. 8.22 The actomyosin ATPase reaction cycle. Compare Fig. 2.3.

which allows the skeleton to operate as a system of levers, actin filaments form regular arrays with filaments of *myosin*. This gives muscle its shape as well as its ability to contract. The action of muscles enables animals to move wings, legs, or fins, digest food, focus eyes, circulate blood, maintain body warmth, and perform a variety of other physiological functions. Our concerns in this section are kinetic and mechanistic aspects of muscle contraction.

Experimental data on the interaction of actin and myosin have provided a basis for molecular models of "vectorial" force generation and contraction in living muscle. On the mechanistic view, muscle contraction results from the relative motion of actin ("thin") filaments and myosin ("thick") filaments (collectively, *actomyosin*), which are oriented parallel to each other and to the long axis of the muscle. Such organization enables muscle to contract and stretch while maintaining structural integrity. The *sliding-filament model* was proposed in 1954 by the British molecular biologists Hugh Esmor Huxley (1924–) and Emmeline Jean Hanson (1919–1973). Later, analysis of the work of Albert Szent-Györgi (1893–1986), a Hungarian biochemist, and the results of kinetics studies of the biochemical properties of myosin and actin led others to propose models of actomyosin-mediated ATP hydrolysis.

Details of myosin function are complex. In resting muscle, the head group of a myosin subunit (Fig. 8.21) is bound to an actin filament. Release occurs on a conformational change in the head group, which is itself induced by the binding of ATP (Fig. 8.22). Hydrolysis of bound nucleotide by the ATPase activity of myosin results in repositioning of the head group about 6 nm down the fibril. The head group associates with the filament again, dislodging P_i. On releasing ADP, the head group pulls the actin filament about 5–6 nm, ending the cycle about 200 ms after it began. In short, the energy of ATP hydrolysis is used to do the mechanical work of muscle contraction. The $\sim$0.05 s^{-1} rate of hydrolysis in isolated myosin is far lower than in contracting muscle, where the rate is $\sim$10 s^{-1}. The rate of hydrolysis is greater in the presence than absence of actin, because interaction between myosin and actin stimulates the release of P_i (and ADP), enabling ATP to bind.

A mechanical motor is designed to perform a given function in a periodic fashion. A gasoline[9]-powered, self-driven lawnmower, for example, uses combustion to turn both a blade for cutting grass and wheels for moving forward. An example of a macroscopic biological motor is the mammalian heart. Comprising several chambers and valves, the heart is a motorized muscle that pumps blood throughout the body, delivering oxygen and food to all cells and returning carbon dioxide to the lungs. And from what we've seen in this section, myosin is a molecular biological machine; it is an example of a class of biomolecular machines known as protein motors. Cardiac tissue is a motor made of a lot of little motors. As discussed above, the head group is the location of myosin's motor function.

Like the engines that propel cars and planes, protein motors convert chemical energy into mechanical energy. Protein motors also control the viscoelasticity of the cell cortex and undergird the transport of cell components along polymer tracks made of actin filaments, microtubules, or DNA. Some protein motors operate as individual molecules; others cooperate in large ensembles. But common to all these motors is energy consumption in the form of ATP hydrolysis and an associated small conformational change. "Linear motor" molecules, for example, move along a track in nanometer-sized steps, each step corresponding to the hydrolysis of single ATP molecules. Myosin is one of the best-studied linear protein motors. All myosins move along an actin filament track by hydrolysis of ATP, but at least one type of myosin can move in the opposite direction from skeletal muscle myosin. Amazing! Analysis of the physico-chemical properties of different motors is expected to shed light on general mechanisms of energy transduction in motor proteins. It is time to close for now, but we shall revisit molecular motors in Chapter 9.

[9] Petrol.

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Q. Exercises

1. How is E_a overcome in the oxidation of materials in a bomb calorimeter in Chapter 1? (See Fig. 1.11.)

2. The following statements pertain to energy transfer within a cell and between a cell and its surroundings.

(a) A cell can convert energy into a useful form by allowing carbon and hydrogen to combine with oxygen.

(b) Chemical energy is converted by a cell to heat, where the energy is transferred into a more ordered form.

(c) A cell obeys the Second Law of Thermodynamics by acting like a closed system.
(d) Enzymes are important for cell catabolism because they lower the change in free energy of the reaction.

Which of these statements are true? Which are false? Explain.

3. Which of the following are true?
(a) Resting cells do not produce any heat.
(b) Growing cells release less heat to the environment than do resting cells because they use more energy.
(c) Life is a thermodynamically spontaneous process.
(d) Enzymes that couple unfavorable reactions to favorable reactions cause a decrease in total entropy.
Explain.

4. What are the units of k in a fourth-order reaction?

5. Does the Arrhenius equation hold for enzymes? If yes, under what conditions? If no, why not?

6. Prove that if k_2/k_1 is small in comparison with the dissociation constant of substrate binding (which relates to the quantities of chemical species as [E][S]/[E•S]), K_M is a measure of the affinity of an enzyme for a substrate.

7. Referring to panel (B) of Fig. 8.9, describe the effect on the rate of electron transfer of increasing ΔG°.

8. The rate of ATP hydrolysis to ADP and P_i is influenced by the muscle protein myosin. The following data are tabulated at 25 °C and pH 7.0.

Velocity of reaction in μmoles inorganic phosphate produced l^{-1} s^{-1}	[ATP] in μM
0.067	7.5
0.095	12.5
0.119	20.0
0.149	32.5
0.185	62.5
0.191	155.0
0.195	320.0

Find the Michaelis constant of myosin.

9. Show that

$$J = \frac{\dfrac{J^{f}_{max}[S]}{K^{S}_{M}} - \dfrac{J^{r}_{max}[P]}{K^{P}_{M}}}{1 + \dfrac{[S]}{K^{S}_{M}} + \dfrac{[P]}{K^{P}_{M}}} \qquad (8.68)$$

for a reversible enzymatic reaction. The reaction scheme might look like this

$$E + S \Leftrightarrow ES \Leftrightarrow P + E$$

and

$$J^{f}_{max} = k_2[E]_T \qquad J^{r}_{max} = k_{-1}[E]_T$$
$$K^{S}_{M} = \frac{k_{-1} + k_2}{k_1} \qquad K^{S}_{M} = \frac{k_{-1} + k_2}{k_{-2}}.$$

10. The rate of hydrogen exchange is a function of temperature. Assuming that the rate increases threefold for every increase in temperature of 10 °C, calculate the activation energy for exchange.

11. Suppose you have a bimolecular reaction in which $2A \rightarrow P$. Using standard methods of calculus, it can be shown that $[P(t)] = [A]0kt/(1 + 2[A]_0kt)$, where $[A]_o = [A(t = 0)]$ and k is the rate constant. Compare $[P(t)]$ for a unimolecular reaction and a bimolecular reaction in the form of a graph.

12. Skeletal muscle is involved in maintaining body warmth. Explain how this might occur.

13. At low temperatures, addition of heat increases enzyme activity. The trend usually begins to reverse at about 55–60 °C. Why? (Hint: see Chapter 5.)

14. Would life be possible if the rates of biochemical reactions were not determined by activation energies? Why or why not?

15. Why does plant life tend to be more robust in tropical climates than closer to the poles?

16. Cellular respiration involves the oxidation of glucose to gluconic acid. The reaction is catalyzed by glucose oxidase. Suggest a means of measuring the rate of reaction.

17. List four variables that can affect the rate of a reaction.

18. Urea is converted into ammonia and carbon dioxide by the enzyme urease. An increase in the concentration of urea increases the rate of reaction. Explain. (Hint: see Chapter 6 Section B.)

19. Suggest several ways in which an enzyme inhibitor might be used therapeutically.

20. Outline an experimental program by which site-directed mutagenesis could be used to study properties of the transition state of a protein folding reaction.

21. Equine lysozyme is an unusual lysozyme in that it has a calcium-binding site. The location of the ion-binding site is identical to

that in α-lactalbumin (see Chapter 6). Unlike the α-lactalbumins, equine lysozyme has Glu35 and Asp53 (Asp52 in hen lysozyme), important for lysozyme activity. Morozova-Roche *et al.* have measured rates of exchange of polypeptide backbone amide protons in equine lysozyme under different conditions. Determine the protection factors of the residues shown in the table below. Helix B encompasses residues 24–36, and residues 40–60 form an anti-parallel β-sheet in the native protein. Comment on the calculated protection factors in the light of this structural information.

	pH 4.5, 25°C	pH 4.5, 25°C	pH 2.0, 25°C	pH 2.0, 25°C
Residue	k_{ex}	k_{in}	k_{ex}	k_{in}
Asn27	1.7×10^{-7}	9.5×10^{-7}	3.6×10^{-4}	1.5×10^{-3}
Trp28	2.0×10^{-7}	1.0×10^{-6}	8.4×10^{-4}	8.3×10^{-3}
Val29	5.6×10^{-8}	2.7×10^{-7}	5.3×10^{-5}	3.0×10^{-4}
Met31	1.4×10^{-7}	8.0×10^{-7}	2.2×10^{-4}	6.4×10^{-4}
Ala32	1.3×10^{-8}	8.2×10^{-8}	2.1×10^{-4}	2.3×10^{-3}
Glu33	6.1×10^{-8}	2.5×10^{-7}	—	—
Tyr34	1.5×10^{-7}	7.2×10^{-7}	2.7×10^{-4}	1.0×10^{-3}
Glu35	6.1×10^{-7}	2.5×10^{-6}	9.9×10^{-5}	7.1×10^{-4}
Ser36	7.5×10^{-7}	3.5×10^{-6}	1.1×10^{-3}	1.5×10^{-3}
Thr40	2.6×10^{-7}	1.1×10^{-6}	–	–
Ala42	1.6×10^{-6}	7.0×10^{-6}	3.7×10^{-3}	2.2×10^{-3}
Lys46	4.7×10^{-6}	1.8×10^{-5}	1.2×10^{-3}	2.2×10^{-3}
Ser52	5.2×10^{-5}	1.7×10^{-4}	–	–
Asp53	1.2×10^{-5}	4.7×10^{-5}	–	–
Tyr54	3.5×10^{-7}	1.5×10^{-6}	1.3×10^{-3}	1.6×10^{-3}
Phe57	4.3×10^{-7}	1.8×10^{-6}	1.4×10^{-3}	1.1×10^{-3}
Gln58	3.7×10^{-6}	1.5×10^{-5}	3.3×10^{-3}	6.6×10^{-4}
Leu59	6.3×10^{-7}	2.6×10^{-6}	1.2×10^{-3}	7.2×10^{-4}

22. Derive Eqn. (8.67).

Chapter 9

The frontier of biological thermodynamics

A. Introduction

Thus far our principal concern has been fairly well established aspects of energy transformation in living organisms, the macromolecules they're made of, and the environments in which living things flourish. There has been a decidedly practical slant to much of the discussion to show how concepts from thermodynamics are useful in today's biochemistry laboratory. In the present chapter, we'll change tack and set sail for waters less well charted. The exploration will aim to locate the material covered thus far in the broader scheme of things, and also to see how the development of topics of considerable current interest must conform somehow, probably, to laws of thermodynamics. Our course might be somewhat off the mark, as the questions we wrestle with here are more speculative than above; often, no right answer is known. But the journey will not be any less worth the effort, as it will help to reveal how lively a subject biological thermodynamics is today and draw attention to a few of the areas where there is still much work to be done. An undercurrent of the discussion is a research program proposed over a century ago by the great British physicist Lord Kelvin: (1824–1907) to explain *all* phenomena of the world, both natural and manmade, in terms of energy transformations. The absolute temperature scale we have used throughout this book to quantify thermal energy is named in Kelvin's honor.

B. What *is* energy?

Many students find the concept of energy difficult to grasp. Definitions tend to be abstract, and they are often framed in mathematical terms that may seem far-removed from your everyday experience of the world. Physics textbooks don't always help much here, as *energy* is

usually defined as "the capacity to do work." Even when interpreted strictly mechanically, such definitions are more open-ended than one might expect of the most basic concept in science. After all, *science* comes to us from the Latin verb *scire*, meaning "to know." Shouldn't you expect to know something if you know its definition? Digging deeper doesn't always help either, because in some cases knowing more can make the basic outline of the object of study seem all the more enigmatic or obscure. When turning for guidance, for instance, to that august and scholarly compendium of knowledge, *Encyclopædia Britannica*, you will find, "*the term energy is difficult to define precisely, but one possible definition might be the capacity to produce an effect*." How's that for scientific clarity, precision, and certainty!

Nevertheless, headway can be made, and the temptation to make the plainly obvious seem privileged knowledge or the mysterious seem mundane should be avoided. There are similarities, of course, between scientific circles and Gnostic cults, but there is no particular initiation rite in the present case besides an inner desire to understand the world and life (but odds are high that you would benefit much from the help of a mentor – choose wisely). From exercises in Chapter 1, Einstein's famous formula, $E = mc^2$, says that the energy of a thing is not some arbitrary quantity but the amount of matter in that thing times the speed of light in vacuum squared. In other words, *energy is a property of matter*. Moreover, energy is a *universal* property of matter, because *all material things somehow possess energy*. Grand statements of this sort do not provide a very specific idea of what energy is, but they do at least give a sense of its nature. The Einstein energy relation says less than you might like it to say. That's because, as written, it is not at all clear how it will apply to photons, the massless particle-like entities that are the main source of free energy in photosynthesis. *Matter considered generally is not the same as mass*. To describe the energy of a photon we need Eqn. (1.1). This relationship describes photon energies very well over an extremely wide range of frequencies. The form of the equation, however, suggests that there is no upper bound on the energy of a photon. What might that mean? Does Eqn. (1.1) make sense if the universe is finite, as suggested by Big Bang cosmology? For if the universe is finite, then surely the energy of a photon cannot be arbitrarily large. Or is Eqn. (1.1) just a convenient model for photons of energies that are neither smaller nor larger than we have encountered thus far in our investigation of the universe? And if Eqn. (1.1) can be joined to the Einstein equation, what sort of matter is the energy of photons equivalent to? Sub-atomic particles? Hydrogen? Dark matter? Can these questions be answered?

As we have seen throughout this book, energy can be described qualitatively and mathematically in seemingly very different ways. This can make the abstract general concept of energy that much more difficult to make concrete. For example, we know from an earlier chapter that heat energy has liquid-like properties, and

advanced analysis shows that energy of this sort can be modeled by mathematics developed to describe fluid flow. And yet, it was demonstrated experimentally hundreds of years ago that heat energy is not a fluid in the sense that liquid water is a fluid. Is the mathematics in this case nothing more than a convenient fiction? Is the mathematics of elementary particles (and many other areas of physics), which involves $\sqrt{-1}$, any less a fiction, even if some of the relationships have been tested experimentally and "verified"? And we have mentioned that energy conservation, a basic principle with a simple mathematical expression, is related to the time-symmetry of physical law, a very basic principle. But it is not at all clear how the concepts of fluid-likeness and time-symmetry link up. Maybe the universe is just a very strange place, and maybe it seems even stranger when a person perceives how very great and how very limited our knowledge of it is.

In the first few chapters of the book our discussion of energy concentrated mainly on such questions as "*How* can a biological process be described in energetic terms?" and "*What* can be done with energy?" The harder question, "What *is* energy?", was raised but not answered explicitly. This approach was adopted to take account of both the puzzling and difficult nature of energy and *the marked tendency of modern science to be more concerned with mechanism and utility than with being as such*. In a fast-pace world where what counts is "results," it can be difficult to see the importance of pondering the very foundations of a science, of going beyond what can obviously be applied in a real situation. But it is important to question and probe and reflect; indeed, it is *necessary* for the development of science. Recalling the first few chapters, we say – with a good deal of confidence! – that *energy is conserved*, citing the First Law of Thermodynamics. We say – with even greater confidence! – that the Second Law tells us that the extent to which the energy of the universe is organized can only decrease. We can *define* energy as the capacity to do work, and write down precise mathematical equations for energy transformation. We can solve mathematical equations that give accurate predictions of what can be measured. We begin to think that we know something. And indeed, *something* is known. But one should not lose sight of the fact that such knowledge does not tell us what energy *is*.

You should not feel too bad about this, though, because *no one* can say what energy *is*. "Ah," you say, "but don't we *know* that the kinetic energy of a body like a comet is its mass times its velocity squared divided by two, and hasn't this relationship been tested many, many times with objects under direct control?" "Indeed, but do we *know* what *mass* is? Einstein tells us that mass and energy are, in some sense, equivalent, and we find our reasoning quickly becoming circular. We know that energy exists, and we know that the total amount of it doesn't change, but we cannot say what energy is." The suggestion is that *although we certainly can know about the world and describe its properties both qualitatively and quantitatively, it*

is by no means certain that we can know or describe its most basic aspects. The situation resembles numbers and the concept of infinity. We accept that if we add or subtract a finite quantity from infinity we get infinity back again. Is infinity a number? Maybe, but we do not know whether it is even or odd. No one can say what infinity *is*. In other words, we can *know* that the infinite exists, though perhaps only as a concept, even though we cannot define it conclusively or comprehend the fullness of its nature. It seems that the concept of energy is something like that.

There does seem to be a mysterious quality to energy. *Why* is that? Is it a pesky relic of a pre-modern age in dire want of further scientific research and clarification? Or is it a deep insight into the basic nature of the universe? Whatever one thinks about this, however one responds, it is clear that *it is not necessary to be able to say what energy is for the concept to be useful* in science, technology, or everyday life. This should not come as such a great surprise. Because, for example, for most purposes for example, it is hardly necessary to *know* technical details of how a computer works in order to make practical use of one, or what paper is made of in order to press it into service in the form of a book, a cereal box, or money. Neither should the difficulty we have in "understanding" energy be seen necessarily as a failure of scientific research. We can at least admit the possibility that what is known now might actually be a glimpse of the basic character of reality, and that it might not be possible to go any deeper in the sense of being able to say with greater specificity and clarity what energy is. But any lack of certainty we might have about such things should not lead us into cozy contentment but rather spur us on to test anew whether ideas inherited from previous generations are incomplete or possibly wrong (including basic thermodynamic relationships), to seek a more definite or complete awareness of the *nature* of the world, and to consider what it all means.

C. The laws of thermodynamics and our universe

Einstein is cited in the Preface as having said that of all the laws of physics the First and Second Laws of thermodynamics are the ones least likely to be overturned or superseded. Was he right? What makes these laws so special? We have mentioned that they are *extremely* general, and anything purporting to be a law should be general. The First Law meets this criterion. Indeed, there are compelling reasons to believe that it applies just as well to the entire universe as to a purified solution of DNA in a test tube. Similarly, the Second Law pertains not only to the entropy of a system of interest but indeed the entire universe. (Doesn't it?)

Perhaps just as important as the *universality* of these Laws is their *simplicity*. From a mathematical point of view, the First Law has the form $a + b = c$, and the Second Law, $x \,/\, y \leq z$. Summing two numbers

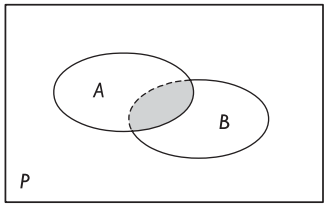

Fig. 9.1 Possible universes and the laws of thermodynamics. The Venn diagram shows all possible universes in which energy transformation can occur (*P*), those in which the First Law holds (*A*), those in which the Second Law holds (*B*), and the intersection of *A* and *B*, where our universe is a mere dimensionless point. Even under the constraints of the First and Second Laws, there is no limit on the number of different universes that are possible; not different successive states of the same universe, but different universes. Ours is but one possible universe.

and comparing the magnitudes of two numbers are among the most basic mathematical operations conceivable. The First Law says that for any chemical reaction the sum of the energy changes must not result in a change in the total energy, while the Second Law says that for there to be a net change in a system the ratio of the heat exchanged to the temperature of the system must increase. That's it. *The principles of thermodynamics are profound not merely because they work and are universal, but because they are so simple. And there is a sort of truth in simplicity*.

The simplicity of thermodynamic relationships helps to make them of great practical importance in science and engineering. Their utility also stems from the severe restrictions they place on what is possible in principle. The limitations imply that a large class of conceivable machines, for example, machines that produce more energy than they consume, cannot exist (in our universe). So let's suppose that the First and Second Laws provide a fundamentally correct description of our universe, as Einstein, Eddington, and many others have *believed*. We can conceive of the set of all possible universes in which energy can be transformed from one type to another (*P*), and represent this in the form of a Venn diagram[1] (Fig. 9.1). Subsets of *P* are the set of all universes in which the First Law holds (*A*) and the set of all universes in which the Second Law holds (*B*). The intersection of *A* and *B*, which includes all universes in which both the First Law and the Second Law apply, is where our universe is found. Twenty billion years old and vast in extent, our universe occupies but a single dimensionless point in the diagram. This helps to begin putting things in perspective, including our very lives! And any theory claiming to describe how living organisms originated by natural causes and continue to exist must be compatible with the First and Second Laws of thermodynamics.

The Second Law tells us that as time goes on the entropy of the universe will increase indefinitely. A rather curious aspect of this is that although the Law demands irreversible change, the Law itself does not change. That is, the Law seems not to apply to itself. All the ways in which we have thought about the Second Law thus far have concerned the behavior of *particles and energy*, and while the Law describes what particles must do, at least in general terms, the Law itself is not made of matter; it is a *relationship*.

Some people say that the universality of the relationships of thermodynamics eliminates an "artificial" distinction between the animate and the inanimate, and thereby enables one to see the world as a single thing. A piece of granite, however, is no more a willow tree than a plant is a kangaroo. *That the world is one does not necessarily imply that qualitative differences of universal proportion will not be found in it*. For this reason (and others, see below) a cell is considered the smallest structure that can be considered alive, at least at this time.

[1] The British logician John Venn lived 1834–1923.

D. Thermodynamics of (very) small systems

The relationships of classical thermodynamics describe the properties of *macroscopic* systems – systems of a large number of particles. In a typical differential scanning calorimetry experiment, for example, the protein concentration might be 1 mg ml^{-1}. Given a cell volume on the order of 1 ml and a protein molecular mass of 20 kDa, the number of molecules in an experiment is of the order 10^{16}. This number is big, about 1% of the age of the universe in seconds! By contrast, a bacterial cell might contain only a small number of copies of a given macromolecule, rarely more than 1000 (excluding strains engineered for constitutive expression of recombinant protein). So what?

Average values of thermodynamic quantities of small systems must be the same as for large systems. But, just as the uncertainty of a measured value is inversely related to the number of measurements made, deviations from the average value are large when the concentration is low or the number of particles is small, because each molecule plays a relatively important role in determining the properties of the system. The Boltzmann distribution says that the population of an energy level scales as the negative exponential of the energy (Chapter 6). *The Boltzmann distribution is the most probable distribution in the limit that the system consists of a large number of particles; it is not necessarily the most probable distribution in a small system*. In other words, the lower the concentration of molecules, the less well the Boltzmann distribution will describe how the particle energies are distributed.

A single molecule can play no greater role in determining the thermodynamics of a system than when it is the *only* molecule of that type in the system. This is pertinent here because much remains to be known about biological processes at the mechanistic level, and there is rapidly growing interest in techniques that can be used to interrogate single complex biomolecular machines and the roles they play in organisms. Consider, for example, DNA replication in the cell, which occurs when regions of single-stranded DNA become available for binding by DNA polymerase, a type of molecular machine that replicates DNA for transmission to daughter cells. Polymerase catalyzes DNA synthesis. The enzyme is believed to move itself along the template DNA by making use of the favorable free energy change of incorporating nucleotides into the growing nucleotide strand, which involves hydrolysis of phosphodiester bonds. It has been found by single-molecule experiments that the catalytic activity of polymerase depends on the tension in the DNA molecule. Enzyme activity is highest when the tension is about 6 pN;[2] below or above this tension, activity is less than maximal. This suggests that entropic properties

[2] 1 N = 1 kg m s^{-2}. This unit of force is named after Isaac Newton.

of single-stranded DNA play a role in determining how quickly polymerase does its job. If the chain is not flexible enough, polymerase doesn't work too well; if the chain is too flexible, polymerase is also less effective than it could be.

Single-biomolecule systems are interesting and important in other ways as well. There are certain biological machines known as molecular switches, whose movement is driven by chemical, electrochemical, or photochemical forces. As we have seen, an example of a molecular switch is hemoglobin. Molecular switches are of great interest now because of the hope that their properties can be exploited in molecular-scale information processing. One can envision, for example, protein- or nucleic acid-based computers, and interesting work in this area is underway.

Experiments involving single molecules raise questions of fairly basic importance. How does one cope with thermal fluctuations on the level of a single molecule? How meaningful are the results of single-molecule experiments for describing the properties of molecules generally? A basic condition of credibility in experimental science is repeatability. This is obviously more easily achieved when one is investigating properties of a large collection of molecules than a single molecule. Will analysis of the fluctuations exhibited by single molecules provide insight into how macroscopic properties develop out of them? How do thermodynamic relationships apply to macromolecules when quantities are limited? Can the behavior of single molecules tell us anything about how life got going?

E. Formation of the first biological macromolecules

Microfossils in ancient rocks in Australia and South Africa strongly suggest that the first organisms flourished on Earth as early as 3.5 billion years ago. Even older rocks in Greenland contain features that seem to have resulted from living organisms. So, about 100 million years after the earliest time when Earth could safely have supported life, living organisms had probably already established a toehold. (To put this in perspective, the dinosaurs met their demise a mere 65 million years ago.) The timing of life's earliest appearance on Earth suggests that there may have been "help" from space. It is hardly overstating the matter to say that going from a bacterium to a baboon is easier than going from a mixture of amino acids to a bacterium. Ignoring the odd meteorite, Earth is a closed system (Chapter 1), and it is by no means certain that the mixture of chemicals present early on in the history of our planet was sufficiently complex to permit the spontaneous organization of the first cell. Although such reasoning might "explain" the origin of life on Earth, it obviously would not explain how life began in the first place. In this section we consider

several aspects of the origin of life as we know it, all of which may pertain regardless of where life began.

Abiotic synthesis of biopolymers

Could bacteria have formed from non-living chemicals in a single step? Most scientists think not. It is hard to imagine how there could not have been "intermediate forms," perhaps aggregates of organic polymers. Candidates are the various classes of biological macromolecule: proteins, nucleic acids, and polysaccharides (and lipids). Such molecules constitute the bulk of tissues and cellular components. Knowing something about the biosynthetic pathways and physical properties of these macromolecules might provide clues as to which ones were important in "pre-biotic life," so let's take a look at some of the more important features.

Cell component biosynthesis occurs in two main stages. In the first, intermediate chemical compounds of the main thoroughfares of metabolism are shunted to pathways that lead to the formation of the building blocks, or *precursors*, of macromolecules. These reactions are wholly dependent on the extraordinary functional specificity and speed of a broad range of enzymes and other proteins. In the second stage of biosynthesis, precursors are joined to form a protein, nucleic acid, polysaccharide, or lipid, as the case may be. Through biosynthesis of certain macromolecules, principally chromosomal DNA and DNA-binding proteins, the *biological information* specifying the identity of the cell, tissue, and entire organism is both expressed and maintained.

The cell makes biological polymers with relative ease – consider how readily bacteria are nowadays engineered to produce huge quantities of recombinant polypeptide. The *abiotic synthesis* of specific biological polymers from precursors is rather more difficult (for us)! From the physical point of view, abiotic synthesis is hard because two monomers must be in the right spatial orientation in the same place at the same time, and there is no enzyme binding pocket present to facilitate specific interaction. Synthesis is difficult from the chemical point of view as well, because the collision between precursor and growing polymer must be sufficiently energetic to overcome the activation energy barrier, and the chemical groups of an enzyme catalyst are absent. Moreover, the underlying mechanism of synthesis, known as dehydration (a molecule of water is lost in joining two subunits), requires the presence of dehydrating agents or condensing agents (for example, cyanamide). In other words, there are rather severe restrictions on the conditions that could have supported abiotic synthesis of the polymers of life. This is particularly true of enzymes, which have distinct catalytic properties; the polyamino acids of living things simply cannot be random polymers. In view of this, it is most unclear how the first biopolymers came into being billions of years ago.

The British X-ray crystallographer John Desmond Bernal (1901–1971) has suggested that pre-biotic aqueous solutions of molecular intermediates of biopolymers were perhaps concentrated and protected from degradation by adsorption onto clay or some mineral. For instance, one possibility is that *phosphates* were involved, which would help explain the apparently preferential incorporation of phosphorous into organic molecules (nucleotides) at a time when the extremely complex biological concentration mechanisms we know about today – for example, membranes and ion pumps – did not exist. The primitive oceans in which life is commonly thought to have begun may have contained high concentrations of organic molecules, and evaporation or freezing of pools may also have helped to concentrate precursors of biopolymers. In any case, *the means by which the first biological polymers were formed is still very much an open question*.

Proteins

One proposal regarding Earth's first biopolymers is that they were not "home made" but "delivered" – by *meteorites*. These bits of debris from space are made mostly of metal and rock, but some of them contain complex organic compounds like nucleobases, ketones, quinones, carboxylic acids, amines, and amides. Of all these molecules, the amino acids command the most attention. Why? Several reasons. One is that all proteins are made of them. The genetic code, which is nearly identical in all forms of life, specifies the 20 "usual" amino acids employed by cells to build proteins. Eight of these amino acids have been found in meteorites. And although nucleic acids are the molecules of genetic inheritance, it would appear that proteins are necessary for (almost) all of the specific chemical reactions in cells. Another reason is that amino acids (and other biological molecules, e.g. sugars) exist in mirror-image pairs, a molecular quality called *chirality*, or handedness, and ribosomes in all known organisms "manufacture" proteins with just one of them. More specifically, individual amino acids are either left-handed (L-form) or right-handed (D-form), depending on the placement of atoms that are singly bonded to the centrally located α-carbon. The energies of formation of the two forms are identical, so there is no apparent thermodynamic advantage in making and using one type instead of the other. *Despite the absence of a (known) thermodynamic criterion, all the amino acids in proteins are all left-handed*.[3]

Some people believe that the peculiar handedness of proteins is a matter of chance, the result of an unpredictable combination of

[3] More accurately, D-amino acid residues are found in some short bacterial polypeptides that are synthesized enzymatically instead of on ribosomes. Such peptides are found in the bacterial cell wall, and this may help to protect the bugs against proteolytic attack. D-amino acid peptides are also found in bacterially produced antibiotics. It is currently believed that D-amino acids are synthesized enzymatically from L-amino acid precursors.

"blind" pre-biotic processes that may have involved polarized light. Others think that primitive life forms may have incorporated both L- and D-amino acids in polypeptides, but that the mechanisms for D-amino acid synthesis were lost long ago, again by chance as to whether L- or D- would persist, in order to conserve the energy resources of the cell. If extraterrestrial starting ingredients were involved in the origin of life on Earth, they could be responsible for the chiral bias. Convincing evidence of this based on the composition of comets or meteorites, however, has not yet been found. And, as stated above, *although sufficient proof of an extraterrestrial origin would answer an important question about life on Earth, it would not tell us how the biased distribution of amino acids types arose in the first place*. Related questions on this topic are: What would be the effect of changing the handedness of a protein but keeping its chemical make-up the same? Would the information content of the folded state of the protein change? Is it important for life that water is a non-chiral solvent? Does the universe as a whole have a hand? If so, what is its origin? Is there a thermodynamic reason for it?

The energies of formation of D- and L-amino acids are the same, but could thermodynamics nevertheless have played a role in the asymmetry of their roles in life on Earth? Research has shown that some far-from-equilibrium chemical systems can both generate and maintain chiral asymmetry *spontaneously*. The dominance of L-amino acids in living organisms might then be the result of small but significant chiral asymmetry that has its origin in the *electroweak interactions* of electromagnetism and the weak nuclear force. Such interactions give rise to effects at the atomic and molecular levels, spin-polarized electrons in radioactive decay, and polarized radiation emitted by certain stars. It has been estimated that the chiral asymmetry of the electroweak interaction could result in a difference in the concentration of chemical mirror pairs (enantiomers) on the order of one part in 10^{17} – a tiny difference! However, calculations suggest that if the production of chiral molecules were fast enough and maintained long enough, the enantiomer favored by the electroweak force could dominate – after a very long time. *As of now, however, convincing experimental support for the hypothesis that electroweak interactions in far-from-equilibrium systems underpin the origin of biomolecular chiral asymmetry is decidedly lacking*.

Nucleic acids

Proteins were not necessarily required to catalyze all the biochemical reactions that have been important to life since its advent. Indeed, nucleic acids and not proteins may have been the first biopolymers. In support of this, some RNAs go beyond their protein-encoding function and exhibit enzymatic properties. Moreover, RNA can serve as a template for DNA synthesis, the reverse of the normal transcription process, as in retroviruses like HIV. Taken together, these facts suggest that RNA could have been the type of macromolecule that got things going.

On this view, polymeric RNA molecules came into being spontaneously in a "nucleotide soup," assembling themselves into something "proto-biological." Importantly, RNA can self-replicate, and just as in DNA incorrect base incorporation leads to new sequences and possibly new enzymatic properties, tRNA-like adaptor molecules must eventually have appeared, and these would have associated directly with amino acids, which must have been available by this time, and ribosomal RNA would have self-assembled with proteins into ribosomes, on which others proteins were synthesized. Polypeptides thus made would eventually "acquire" the enzymatic properties needed to synthesize the necessary components of a membrane, metabolizing foodstuffs, and sustaining growth. And DNA, which is more stable chemically than RNA and thus better for storing genetic information, would eventually replace RNA as the storage molecule of genetic information.

In support of this "*RNA world*,"[4] it has been found that highly active RNA ligases – enzymes that link RNA monomers together – can be derived from *random* RNA sequences. This suggests that biomolecular functionality *can* arise out of randomness. And recently a polymeric RNA lacking cytidine was found to exhibit enzymatic activity, implying that no more than three subunit types were necessary for catalytic activity in "pre-biotic life." Though these findings are perfectly credible, it must be appreciated that they depend on the design of the experiments and the functional selection processes involved. A *person* designed an assay to *select* molecules exhibiting a certain property. Moreover, it is hardly irrelevant that outside the controlled environment of the laboratory one would be extremely hard-pressed to find biologically meaningful RNA strands *of any size*. This is because in addition to being difficult to synthesize abiotically, RNA is chemically unstable. Just as important, the known range of catalytic activities exhibited by RNA is rather narrow. In other words, the RNA hypothesis must be taken seriously, but *we are far from having proof that the spontaneous appearance of RNA catalysts was the means by which life originated on Earth*. This view is corroborated by that of British Nobel Laureate Sir Francis Harry Compton Crick (1916–2004), who has said that "the gap from the primal 'soup' to the first RNA system capable of natural selection looks forbiddingly wide." Another Nobel Laureate, Ilya Prigogine (1917–2003),[5] has expressed a similar view, though one not necessarily intended as a comment on the RNA world: "The probability that at ordinary temperatures a macroscopic number of molecules is assembled to give rise to the highly ordered structures and to the co-ordinated functions characterizing living organisms is

[4] The term *RNA world* was apparently coined in 1986 by Walter Gilbert (1932–), an American molecular biologist and Nobel Laureate in Chemistry (1980).

[5] Prigogine, son of a chemical engineer, was born in Russia but spent nearly all of his life in Belgium. He was awarded the Nobel Prize in Chemistry in 1977 for his contributions to non-equilibrium thermodynamics (see Chapter 5).

vanishingly small. The idea of spontaneous genesis of life in its present form is therefore highly improbable, even on the scale of billions of years during which prebiotic evolution occurred." In view of this, *the idea that life on Earth originated from an extra-planetary source looks very attractive indeed. But should the panspermia hypothesis continue to prove the most plausible one, we still would not necessarily know when, where, or how life began.*

F. Bacteria

Leaving aside the really hard questions, let's just take the existence of bacteria for granted and move on to other topics. We begin with bacteria because they are the simplest living things known to humankind. *All known types of bacteria exhibit all the essential features of a living organism: the ability to capture, transform, and store energy of various kinds in accordance with information encoded in their genetic material. Viruses* and *prions* (infectious proteins), by contrast, which exist on the fringe of the fabric of life, are themselves not alive. True, viruses (but not prions) contain genetic information in the form of DNA or RNA. Viruses, like cats, are open systems. Viruses, like cats, reproduce. And both viruses and cats change from generation to generation by way of alterations to genetic material. What viruses cannot do, however, is self-subsist: they require the metabolic machinery of a host like a cat to produce the energy molecules required for their replication. And although some enzymes are known to be encoded in viral genomes, all viral enzymes appear to be mutated forms of normal cellular enzymes produced by the host, as in the case of Src, source of the SH_2 domain discussed above. And after their constituent molecules have been synthesized, viruses *assemble spontaneously* into highly symmetrical structures which resemble inorganic crystals and represent a minimum of free energy or a kinetically trapped state; viruses do not *develop*.

Most known species of bacteria, by contrast, like cats, require oxygen to synthesize the energy molecules required for replicating DNA, making proteins, growing, and reproducing. Other types of bacteria, however, for instance sulfate-reducing ones, are strict anaerobes, and culturing them in the presence of oxygen is a surefire way of putting them to death. *Sulfate-reducing bacteria* use sulfate, not oxygen, as the terminal electron acceptor in respiration, and they "generate" energy from a variety of simple organic molecules and molecular hydrogen. Sulfate is reduced to hydrogen sulfide, a substance that smells like rotten eggs. Both mesophilic and thermophilic species of sulfate-reducing eubacteria (non-archaebacteria) are known. At hyperthermophilic temperatures (85–110 °C), however, only certain archaebacteria are known to thrive by sulfate-reduction. These bugs love X-treme conditions and are therefore called "*extremophiles*."

Box 9.1 World's top thermophile is from sea's bottom

In 2006 it was reported in *Science*, the weekly journal of the American Association for the Advancement of Science, that biological nitrogen fixation had been found to occur in hot hydrothermal vents on the ocean's floor. All organisms need nitrogen for synthesis of nucleic acids and proteins. And N_2 accounts for over two-thirds of the dissolved gas in seawater. But for most aquatic microbes, N_2 is a useless resource. The archaebacterium FS406-22, however, which flourishes 1500 m below sea level in the Juan de Fuca Ridge in the Pacific Ocean, not only converts N_2 to ammonia, it does it at a steamy 92 °C, a gold-medal temperature by nearly 30 degrees. As shown by researchers in the USA, FS406-22 achieves this feat with a nitrogenase enzyme. A sulfur-rich metal cluster in the active site enables N_2 to be converted into NH_3. The identity of the bound metals is not known, but likely candidates are molybdenum and tungsten – elements more commonly thought of as the stuff of spacecraft parts and light bulbs than protein molecules. If life on Earth originated here, a strong candidate location for the earliest forms must be deep-sea vents that cycled hot fluid in a reducing environment. Most known organisms thrive at a temperature well below 92 °C; most proteins studied to date are not only aggregated at this temperature but also "denatured" and therefore unable to carry out a specific function. Analysis of the structural basis of the high heat stability of FS406-22's proteins could be useful for engineering proteins for industrial applications. The lead author of the ***Science*** study did the work as part of her doctoral research. It took some 600 attempts to get the microbe to grow in the laboratory. Persistence will not guarantee to pay off, but it will make it considerably more probable.

Another kind of extremophilic archaebacteria are the *methanogens*. Enclaves of these organisms have been detected *thousands* of meters below the surface of Earth, in crystalline rock aquifers within the Columbia River basalt group in North America. Archaebacteria are the only organisms known to live under such "harsh" conditions. Methanogens are strict *anaerobic autotrophs*, meaning that they can synthesize *all* of their cellular constituents from "simple" molecules like H_2O, CO_2, NH_3, and H_2S – all in the absence of oxygen. Methanogens appear not to depend on the Sun's energy one whit; they use *chemosynthesis* to produce methane and water from carbon dioxide and hydrogen gas.

To come to the point. Although some species of archaebacteria live under extreme conditions, their basic metabolic machinery nevertheless closely resembles that of eubacteria and eukaryotes. This strongly suggests, though by no means requires, that archaebacteria, eubacteria, and eukaryotes have a common origin. Further, *some people speculate that archaebacteria, being able to exist under conditions more closely resembling the early Earth than the atmosphere of today, are the probable precursors of eubacteria and eukaryotes*. The properties of archaebacteria have also been interpreted as suggesting that life on Earth may have begun deep within instead of on the stormy, oxygen-deficient surface. On this view, the heat of Earth's core, not the

Sun, would have provided the free energy needed to sustain the first-formed living organisms and bring about their origin in the first place. An argument in support of the hypothesis is the following. In general, the less energy required to carry out a process, the more probable it will be, as for instance when an enzyme lowers the activation energy of a reaction and thereby speeds it up. The chemical equations for photosynthetic fixation of carbon dioxide are:

$$6CO_2 + 12H_2S \rightarrow C_6H_{12}O_6 + 6H_2O + 12S \quad \Delta G^{\circ\prime} = +406 \text{ kJ mol}^{-1}, \tag{9.1}$$

$$6CO_2 + 12H_2O \rightarrow C_6H_{12}O_6 + 6H_2O + 6O_2 \quad \Delta G^{\circ\prime} = +469 \text{ kJ mol}^{-1}. \tag{9.2}$$

These equations show that anaerobic synthesis of sugar from carbon dioxide, in which hydrogen sulfide is the terminal electron acceptor, has a smaller energy demand than the corresponding aerobic process. *Ergo*, ...

At this stage, however, too little is known to say whether archaebacteria gave rise to life that depends on the Sun or they branched off from earlier photosynthetic bacteria and then adapted to a variety of more extreme environments. For one can easily imagine that the widespread and frequent volcanic eruptions characteristic of earlier stages of Earth's history may have led to the isolation of a population of photosynthesizing bacteria that became able to make good use of other sources of free energy. And if an isolated population did not have access to sunlight, it is possible that with time the genes encoding proteins required for photosynthesis were lost, transformed by mutations, or simply expressed with increasing improbability, as they were no longer required for continued existence. *No one knows for sure how life began on Earth or how it developed.*

G. Energy, information, and life

Regardless of when, where, and how life began and has developed, it is clear that good internal energy resource management is important to the life of any organism. As we have seen in a previous chapter, energy management on the level of the entire organism determines whether weight is lost or gained. *Perhaps the most "successful" organisms (ants, bacteria, cockroaches, ...) are the ones that utilize their energy resources the most efficiently on the level of individual cells and overall*. Consider a metabolic pathway. In the usual case it will involve a number of different enzymes, each encoded by a separate gene. Functional genome analysis has shown, however, that the genes encoding enzymes of a particular pathway are often adjacent to each other in chromosomal DNA. Moreover, such genes are often turned on or off by the same molecular switch – not the same *type* of switch but the *same* switch. Such switches often come in the form of a repressor protein binding to a particular site on DNA, inhibiting gene

transcription. Repressor, mRNA and protein production are energy consuming, so "successful" organisms might be ones in which these processes are very efficient; or, perhaps, as efficient as possible. From an entropic point of view, it would seem most probable for the genes of a pathway to be distributed randomly in the genome. As this is often not the case, an organism will presumably expend energy to prevent it from happening. Are the demands of the Second Law met by the entropy increase resulting from maintaining the structural integrity of a genome being even greater than the entropy increase that would result from locating all genes in a genome at random locations? Could this possibility be tested by experiments with bacteria or yeast?

Maintenance of cell structure and growth require a huge number of metabolic and synthetic reactions: a range of complex mechanisms is needed to regulate the highly heterogeneous distributions and flows of matter and energy within the cell. In general, the biological macromolecules that carry out the biochemical reactions of metabolism, synthesis, and active (energy-consuming) transport are proteins. This class of nanomachine-like biomolecules is tremendously diverse, not only in terms of monomer composition but also with regard to biochemical activity. It is not much of an exaggeration to say that proteins are what really matter to the physicochemical properties and existence of a cell because proteins do all the work. In addition to catalyzing a plethora of biochemical reactions, proteins also give a cell its shape and determine its mechanical properties.

Proteins are similar to books. A simple calculation shows that there are 720 (=6!) different ways of arranging six books on a shelf in the side-by-side and upright position. And yet, only a few of these will be *meaningful* to the person doing the arranging (alphabetical by author, subject, or title, size, and so on). The mind selects these possibilities intuitively or by force of habit and does not consider explicitly the remaining universe of arrangements (of which there are about 700). Small proteins, for example hen lysozyme, are about 100 amino acids long. Given 20 different usual amino acids, there are $20^{100} \approx 10^{130}$ different amino acid sequences of this size. Even if a protein is required to have a sequence composition that matches the *average* protein, there are still some 10^{113} *possible* different sequences of this size. In other words, *effectively unlimited variety is possible at the level of the primary structure of a small protein. The total number of 100 residue-long sequences that encode functional proteins is, however, extremely small in comparison,* as we shall see presently. Is the relatively small number of sequences one finds in nature the result of a sort of thermodynamic optimization process? Do the constraints imposed by the First and Second Laws somehow drive this optimization?

Thermodynamic optimization and biology

Another question you might ask yourself is whether any given protein sequence in nature is optimized in some way, for example, for certain thermodynamic properties? Is the sequence of an enzyme as random

as possible without loss of enzymatic activity? As we have seen, thermodynamic stability is a measurable property of a protein (Chapter 5). Moreover, in some cases, notably relatively small proteins, all the information required for folding into something biologically functional is encoded in the amino acid sequence. The native states of such proteins represent either global free energy minima (under conditions favoring the native state) or energy wells that are sufficiently deep not to allow structure attainment to proceed to beyond the "kinetically trapped" native state.

Protein thermostability is related to structure, which is encoded in DNA, structure is related to function, and function is related to information and the ability to process information. A particularly clear example of this is *arc* repressor of bacteriophage P22. The wild-type repressor protein interacts with DNA and regulates the expression of genes, and the native protein contains a stabilizing electrostatic interaction involving amino acid residues Arg31, Glu36, and Arg40. In folded repressor, Glu36 is inaccessible to the solvent. Site-directed mutagenesis has been used to make all 8000 combinations of the 20 amino acids at positions 31, 36, and 40 ($20^3 = 2^3 \times 10^3 = 8000$), and all mutants have been tested for *arc* repressor activity in P22.

Remarkably, only four of the mutants (0.05%) are as active as the wild-type enzyme: Met–Tyr–Leu, Ile–Tyr–Leu, Val–Tyr–Ile, and Val–Tyr–Val. Another sixteen (0.2%) are partially active. Six of the twenty active mutants are more thermostable than the wild-type protein, by as much as 20 kJ mol^{-1}. These are Met–Tyr–Leu, Val–Tyr–Ile, Ile–Tyr–Val, Met–Trp–Leu, Leu–Met–Ile, and Gln–Tyr–Val; all hydrophobic combinations. Analysis of the crystal structure of the Met–Tyr–Leu mutant shows that it is practically identical to the wild-type protein, excluding the replaced side chains, which pack against each as well as might be expected in the core of a wild-type protein. Mutant Ala–Ala–Ala, by contrast, is about 16 kJ mol^{-1} less stable than the wild-type under usual conditions, though it does fold.

What do the data tell us? There would appear to be no loss of biological information in an amino acid replacement that does not impair biological activity. In all of the stabilizing mutants the favorable electrostatic interaction, which is fairly specific (low entropy), is absent. Hence, that interaction cannot be the main source of native stability in the wild-type protein. The mutated side chains in the stable mutants have large hydrophobic surfaces in close contact, so such interactions, which are relatively non-specific (high entropy), must help to stabilize folded structure. It is likely that some of the inactive mutants have folded states that are more stable than the wild-type protein, but there is no experimental proof of this because active mutants have been studied in any depth. And, importantly, the data show only that the thermostability of the biologically functional form of a wild-type protein can be increased without impairing functionality. That is, *the native states of natural proteins are not necessarily optimized for thermostability*.

The apparent lack of a requirement for thermodynamic optimization is intriguing. For *most natural processes occur in such a way that some physical quantity is "extremized."* Water flows downhill, and in doing so its gravitational potential energy is minimized. A closed system tends to equilibrium, and in doing so its free energy (or entropy) is minimized (maximized). It is likely that there is a thermodynamic explanation for the "minimum" size for a protein: if the polypeptide chain is not long enough, the stabilizing interactions between residues will not be great enough to overcome the energetic cost of restricting side chain motion on folding.[6] In much more general terms, the dynamical behavior of objects can be formulated in several logically equivalent ways, and one of these involves the minimization of a quantity called the *action*, the sum over time of the *difference* between the kinetic energy and the potential energy. There is nothing obviously similar to minimized action in proteins, which as we have seen are not necessarily extremized for thermostability. In other words, if existing biological macromolecules are extremized for anything, that something need not be free energy. Are biological macromolecules optimized instead for something *biological*? *The catalytic rate of some protein enzymes is as large as it possibly could be, since its rate is limited by the rate of diffusion of the substrate*. Most enzymes, however, operate with sub-maximal efficiency in simple, *in vitro* experiments which consist of enzyme, substrate, solvent, and perhaps some salt but none of the molecules of the cell that might have some impact on the optimal functionality of a particular enzyme.

Is there a biological *advantage* to a protein's not being optimized for stability? If a protein were extremely thermostable when it did not need to be, protein metabolism or programmed protein degradation might severely tax the energy resources of the cell. An extremely thermostable viral protein might be particularly harmful to an organism! Less-than-maximal thermostability allows molecular chaperones and other proteins to assist in folding, facilitate protein translocation across membranes, and eliminate incorrectly folded proteins. In view of this, if proteins are optimized for anything, perhaps it is *compatibility* with all aspects of their existence as a biological entity, from the availability of amino acids to folding on the ribosome, to transport, biological function, and degradation. *In view of what has been said about proteins, if the distinction between complex, highly organized living things and less complex, less organized inanimate things is artificial, it is at least unclear how it is artificial*.

Information theory and biology

Having come this far, we find ourselves not at the end of a quest to understanding the origin of cells and their ability to transform

[6] Proteins are generally no smaller than about 50 amino acids long. This is not to say that smaller peptides cannot be bioactive. Indeed, some peptide hormones are but a few amino acids long.

energy, but at the beginning. For in organisms as we know them, and despite all the functions they carry out, proteins are not the most basic repository of biological information: the instructions for making a protein are stored in DNA. At the current stage of development of molecular biology it is clear enough how the structure of DNA translates into the amino acid sequence of a protein, but there is no known way in which the sequence of a protein could be used as a template for the synthesis of a corresponding DNA sequence.

The biological information stored in genes can be analyzed in a variety of ways. One we'll look at here is *information theory*, a subject that developed out of the work in the communications industry by an American named Claude Elwood Shannon (1916–2001). Information theory has found its main applications in electrical engineering, in the form of optimizing the information communicated per unit time or energy. But because it is so general, the theory has proved valuable in the analysis of phenomena in other areas of inquiry. As we shall see below, information theory is closely akin to statistical thermodynamics and therefore to physical properties of biological macromolecules, and it gives insight into information storage in DNA and the conversion of instructions embedded in a genome into functional proteins.

According to information theory, *the essential aspects of communication are a message encoded by a set of symbols, a transmitter, a medium through which the information is transmitted, a receiver, and noise*. *Information* stands for messages occurring in any of the standard communications media, such as radio or television, and the electrical signals in computers, servomechanisms, and data-processing devices. But it can also be used to describe signals in the nervous systems of animals. Indeed, a sense organ can be said to gather information from the organism and its environment: the central nervous system integrates this information and translates it into a response involving the whole organism, and the brain can store information previously received and initiate action without obvious external stimulation. Ideas about how the mammalian central nervous system might work were the basis on which early electronic computer development proceeded.[7] In the biological expression of genetic information, the transmitter is the genome, the message is messenger RNA, and the receiver is the cell cytoplasm and its constituents (e.g. ribosomes).

The information content of a message has a more precise *meaning* in information theory than in "ordinary" human communication. Information theory aims to be quantitative and unambiguous. The remarkable subtleties of human communication no

[7] A key person in the development was John von Neumann (1903–1954), a Hungarian–American mathematician who made important contributions in quantum physics, logic, meteorology, and computer science. His theory of games had a significant impact on economics. In the early 1930s he gave precise formulation and proof of the ergodic hypothesis (Chapter 6). In computer theory, von Neumann was a pioneer in logical design, "memory," imitation of "randomness," and the construction of cellular automata that can reproduce their own kind.

longer exist; there is no such thing as the *inherent meaning* of a message in information theory, unless "inherent meaning" means nothing more than a semi-objective quantitative measure of the degree of order, or non-randomness, of information. In so far as it can be treated mathematically, information is similar to energy, mass, and other physical quantities. Nevertheless, information is not a physical quantity like inertia or electrical current; information concerns arrangements of *symbols*, which of themselves need not having any particular meaning – consider, for example, the individual letters by which the word *symbol* is symbolized. Any "meaning" said to be encoded by a specific combination of symbols can be conferred only by an intelligent *observer*.

Information theory resembles thermodynamics in a number of ways. As we saw in Chapter 6, in statistical mechanics state variables define the *macroscopic* state of a system. The "external," macroscopic view places constraints on what is happening "inside" the system, but it does not determine the state of the system. In other words, in general many different microscopic states correspond to the same macroscopic state. For example, a fixed current drawn by a house can correspond to a variety of different combinations of lights on inside, as we saw earlier. A biological parallel was encountered in our discussion of the Adair equation in Chapter 7. To be sure, the average number of ligand molecules bound is a measurable quantity, but in the absence of additional information the average number bound does not tell us which sites are occupied in a given macromolecule; many possible combinations of occupied sites would give rise to the same measured value.

The connection between information theory and thermodynamics can be further elaborated as follows. When the entropy of a system is low, as for instance in the crystalline state, the information content[8] is high, because it is *possible* to provide a very accurate description of the system on the microscopic level. One could say, for example, that all the atoms form a regular array with specific geometrical properties, a unit cell with precise dimensions. When the entropy of a system is high, by contrast, as it is in a gas, it is impossible to describe the arrangement of particles on the microscopic level. *The information content of a system is inversely related to the uncertainty of the microscopic state of the system*. There is, moreover, a relationship between information and work. A liquid can be converted into a solid by doing work on it, for instance, by using a motor to extract heat in order to lower the temperature. So the expenditure of work can result in an increase in information, albeit at the expense of an increase in the entropy of the universe. And as discussed in Chapter 4, a system at equilibrium undergoes no net change. In the context of the present discussion, this means that *an equilibrium system can neither gather information nor respond to it*.

[8] Or determinacy content.

Now let's take a word in English as the system of interest. The macroscopic state could be the number of letters of each kind and the microscopic state their specific arrangement. In some cases, knowledge of the composition of a word will be enough to determine arrangement, since only one of the many possible strings will be intelligible in English. An illustrative example is *example*, for which there is no anagram. In other cases, multiple arrangements of letters could make sense, and the "correct" arrangement would have to be inferred from analysis of a "higher" *semantic level* of the message. For instance, the two very different words *state* and *teats* have the same macroscopic state, and the one that is an actual constituent of a message could only be known by considering the broader context of the word. In the case of *state*, there would be the additional difficulty of deciding whether it was employed as a noun (Plato's *Republic* discusses construction of the ideal state, one for which the paramount concern is justice), verb (Aristotle, who surpassed his mentor Plato in many respects, would state that justice benefits the man who is just), or an adjective (were he alive today, Plato's mentor Socrates might well agree that state-supported higher education and graduate study was a worthwhile investment – if it could be freed from the political agenda of university administrators and government bureaucrats). This shows how the observer or experimenter plays a key role in determining the information content, or meaning, of a message or data; the observer *decides* how the message will be evaluated. Owing to such subjectivity, *there is no absolute value that can be placed on the information content of a message*.

There is, however, a difference between the *actual information* and *potential information* of a message, the extent depending on the macroscopic state and microscopic state in any particular case. Consider, for example, the relatively simple binary alphabet (*a*, *b*) and the set of all possible "words" that are 12 letters long. There are $2^{12} = 4096$ different words of this size. In the simplest case, where the message is a string of identical letters, e.g. *aaaaaaaaaaaa*, the actual information is maximal, because determination of the microscopic state adds nothing to what is known at the macroscopic level. The message *aabbaabbaabb*, by contrast, contains considerably more information than the macroscopic state, which of itself tells us only that the "word" consists of an identical number of letters *a* and *b*.[9] An intermediate semantic level might characterize the message as "*aab* followed by its mirror image, and the resultant followed by its inverse." The greatest possible potential information is encoded by words in which the number of letters *a* equals the

[9] The significance of this choice of message is as follows. If the message represents the order of bases in DNA, the sequence of the complementary strand is identical to the message itself (the coding strand and its complement read in opposite directions). Messages that read the same way forward and backward are called palindromes, and many of the known DNA sequences to which proteins bind to regulate gene expression are palindromic. Regulatory DNA-binding proteins are typically dimeric, for obvious reasons.

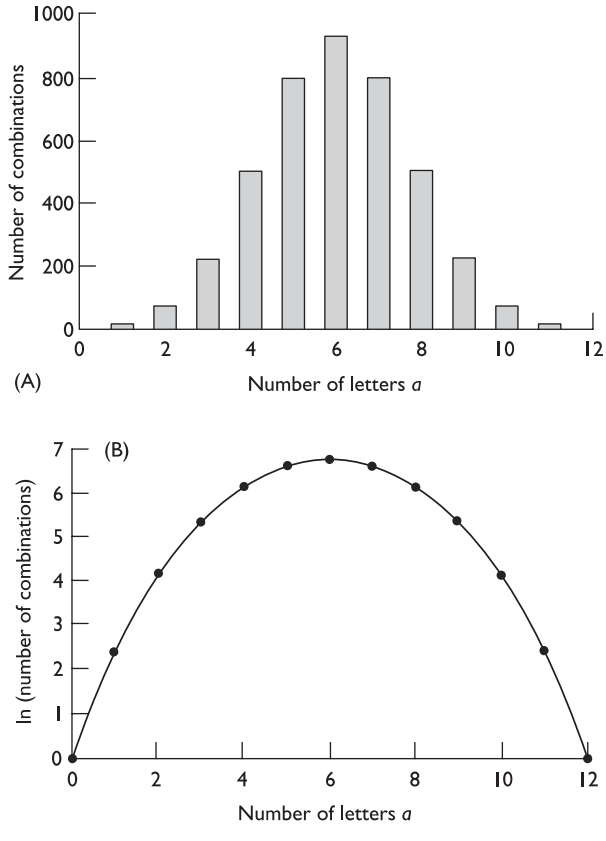

Fig. 9.2 Genome combinatorics. The alphabet considered here has but two letters, a and b, two fewer than DNA. Panel (A) shows the number of distinguishable combinations of letters in a 12-letter word as a function of the number of letters a. The curve is the famous bell-shaped one of probability and statistics. Panel (B) shows the logarithm of the number of distinguishable combinations. This number is related to both the information content of the word and its entropy. Note that the information content and entropy are greatest when the number of letters a equals the number of letters b, and that when the word consists of just one type of letter the information content is 0.

number of letters b, as this allows for the greatest *number of ways* of arranging the available symbols. As we saw in Chapter 6, the number of ways of arranging the particles of a system is related to the entropy function of classical thermodynamics, and in the case of *aabbaabbaabb* that number is $\omega = 12!/(6!6!) = 924$ (Fig. 9.2). In view of this it is said that *entropy and information are "isomorphous"* (Greek, *iso*, same + *morphe*, form).

We can be a wee bit more biological in our discussion of information theory. The complete DNA sequence is known for a rapidly increasing number of genomes, including that of *Haemophilus influenzae*. In the genome of this protist, which has 1727 coding regions, only 15% of the genomic DNA does not encode a protein, and over half of the genes have known counterparts in other organisms. Assuming that each nucleotide can occur at any position with the same probability, each nucleotide in the *Haemophilus* genome (or any polynucleotide for that matter) contains 2 *"bits" of information*. This is because at least two binary "decisions" are made each time a nucleotide is added: "Is this nucleotide a purine or a pyrimadine?" and either "Is this nucleotide a cytosine or a thymine?" or "Is this nucleotide a guanine or an adenine?" This intuitive view is supported by calculation as follows. The probability, **p**, that the base at

a given position is one type and not another is 1/4. This can be written in terms of powers of 2 as

$$\mathbf{p} = 1/4 = 1/2^I = 2^{-I}. \tag{9.3}$$

We are interested in powers of two because we wish to relate **p** to some number of binary decisions. The information content in terms of binary decisions, *I*, is found by taking the base 2 logarithm of both sides of the equation. The result is

$$I = -\log_2 \mathbf{p} = -\log_2(0.25) = -\log_2(2^{-2}) = 2\log_2(2) = 2 \text{ bits per nucleotide.} \tag{9.4}$$

Thus, a polynucleotide chain n nucleotides long has a sequence information content of $2n$ bits. Similarly, the information content of an arbitrary message in the English language is

$$I = -\log_2(1/27) = 4.76 \text{ bits per character} \tag{9.5}$$

assuming that 26 letters and a space are the only characters allowed and that the probability of occurrence is the same for each type of character – an obvious oversimplification – but one that will do as a first approximation.

The simplest is not so simple

Let's consider *Escherichia coli*. The genome of this "simple" beast is only about 4×10^6 bases long; its genetic information content is 8×10^6 bits. The total number of sequences of this length is not large. Rather, at $4^{4\,000\,000} \approx 10^{2\,400\,000}$ it is absolutely astonishingly mind-bogglingly gigantic. For comparison, the temperature at the core of the Sun is a mere 2×10^7 °C, the human genome comprises some 2.9×10^9 base pairs, Earth is about 10^{26} *nanoseconds* old, and the universe itself only ten-fold older. But only a tiny fraction of all the possible sequences ($\ll$ 1%) will encode anything *biologically meaningful – correspond to an organism, and enable it to maintain its cellular structure by transforming energy from one form to another, grow, adapt, and reproduce*. The conclusion is no different even if we exclude all sequences with a base composition different from that of the *E. coli* genome.

This reasoning can applied to more general questions. The *minimum* number of DNA sequences that are biologically meaningful is the number of different species (with a genome at least as big as that of *E. coli*) and all sequence variations within the species that have ever existed. The *maximum* number of sequences includes all those that have existed plus all others that *could* satisfy all the necessary requirements. All these sequences have the same information content. We have now entered a realm where information theory seems less helpful than we might like it to be, for as yet *we* have no way of making an absolute distinction between sequences that are biologically meaningful and those that are not on the basis of sequence information alone.

Given that some sequences are not biologically meaningful, how do the physical, chemical, and biological properties of living organisms

select sequences that are biologically meaningful from ones that are not? The emphasis on meaning is crucial, as a simple example helps to show. There is often little or no real knowledge communicated in information. Consider, for example, a telephone book. Though it may contain a great amount of information and be *useful* in a variety of ways, a *telephone book* is but a *directory*. A telephone book does not impart knowledge in the way, say, that Shakespeare does, and just as important, it is difficult to imagine how dramatization of a telephone book could be the slightest bit *interesting*. Where do interesting DNA sequences – ones that encode the genomes of living organisms – come from?

Molecular communication

The discussion of biological information can be taken a step further. Nucleic acids are not the only kind of molecular message important to the life of a cell. Another sort is typified by cAMP, which as discussed in a previous chapter is a "second messenger," a kind of intracellular signal. cAMP is generated by the cell upon receipt of an appropriate molecular *signal*, for example, the binding of the hormone insulin to the extracellular portion of its transmembrane receptor. There are important bioenergetic connections here: insulin plays a key role in regulating the concentration of glucose in the blood, blood delivers the sugar molecules to every cell in the body, and cells use the glucose as the main energy source for production of ATP. In addition, cAMP is the primary intracellular signal in the glycogen phosphorylase kinase and glycogen synthase cascades, important in the metabolism of the glucose-storage polymers called glycogen, and cAMP plays a role in the phosphotransferase system and smooth muscle contraction. And cAMP-dependent kinase controls the enzyme acetyl-CoA carboxylase, which catalyzes the first committed step of fatty acid biosynthesis and is one of its rate-controlling steps. It is clear that biological information comes in a number of forms.

In *E. coli*, transcription of the β-galactosidase gene occurs when the cell enters an environment in which the glucose level is relatively low and the lactose level relatively high; i.e. when a certain type of signal is received and other conditions are met. The binding of lactose (or, possibly, one of its metabolic products) to the *lac* repressor results in dissociation of the repressor from the operator site, allowing transcription of the β-galactosidase gene to proceed. Once the enzyme β-galactosidase has been synthesized, lactose can be metabolized as an energy source. Transcription of the β-galactosidase gene is inhibited in the absence of lactose to conserve the energy resources of the cell.

We can be somewhat more quantitative about the relationship between biological information and thermodynamics. The gain of one bit of information by a cell requires the expenditure of at least $k_B T \ln 2$ units of work: $S = k_B \ln \omega$, and the entropic term of the Gibbs free energy is TS. At 27 °C, one bit of information "costs" 3×10^{-21} J.

The energy required to generate the biological information of an entire cell at this temperature might be 3×10^{-12} J, assuming that the information content of the cell is roughly comparable to that of *Encyclopædia Britannica*. Because "informational" macromolecules like RNA, DNA, proteins, and polysaccharides constitute at least half of the mass of a bacterial cell, you will have a hard time saying how the energetics of information processing can be separated from other aspects of cellular activity. It is also clear that information processing on the cellular level must be a major impetus for the energy consumption of an organism, regardless of its size. Your body, then, is processing tremendous quantities of information while you're alive, whether you are studying biological thermodynamics, designing biological chemistry experiments, attempting to outfox opponents during a hand of contract bridge, or slumbering away. A nervous system is clearly not necessary for information processing – think of bacteria and yeast. Are humans at all different from other organisms with regard to information processing on a basic level? What does seem extraordinary about us is that *whereas plants (and digital computers) only carry out the information processing instructions in the program they are running (the plant genome in the one case, a specific piece of software in the other case), human beings can process information, be aware that they are doing it, build models in order to try to understand information/energy flow, search for practical ways of utilizing any new-found knowledge as a means towards a needed or desired end, and consider the possible meaning of what they are doing.*

To summarize this section. Information theory provides a semi-objective means of saying what information is and quantifying it. Information theory does not help us to distinguish between biologically meaningful and meaningless DNA sequences. Moreover, information theory does not tell us *where* biologically meaningful information comes from; much less *how* it came to be. (*Why* it came to be is a different matter altogether.) Information about a system is similar to the entropy of a system. These measures differ in that the latter relates to the system itself while the former is related to the observer. The distinction, however, must be considered somewhat artificial, since as we have seen thermodynamic quantities are defined only under rather arbitrary circumstances chosen by the observer (experimenter). Information theory in biology is a very large topic, and we have barely scratched the surface. Nevertheless, having compared DNA and human language in the context of information theory, we can clearly see that there is something rather earthy about human language, or something unmistakably ethereal about the organization of living matter, or both.

H. Biology and complexity

The situation in the living cell is still far more complex than our mostly qualitative discussion of information theory and biology has

so far suggested. For in living things there is not only highly specific encoding of biological information in genetic material, but also continual *interplay* between such information and biological macromolecules, not to say the surrounding environment. The process of protein biosynthesis is not simply a linear flow of matter, energy, or information, but a highly organized feedback circuit in which proteins and nucleic acids control *each other*. Regulatory proteins bind to DNA and thereby enable or disable the synthesis of mRNA required for making proteins on ribosomes. In most eukaryotes, the nucleic acid message must be spliced in order to remove the non-protein encoding regions called introns. This process, which is carried out by a marvelously complex macromolecular machine, can in some cases lead to a variety of spliced versions of the same protomessage. The result is that the same gene can be and often is used to produce different forms of the same protein in different tissues.

Each living cell comprises thousands upon thousands of proteins. Each bacterium is made of thousands of different proteins, each encoded by a specific gene and required in a relatively specific amount. The regulation of gene expression and protein metabolism on which the living cell depends is extremely complex. And yet, *all the proteins of the cell work together in a coordinated way to sustain the highly ordered living state of the cell.*[10] *Proteins do this by following an extremely well organized program encoded in the organism's DNA*. The inner workings of a cell are like the automated processes of a factory, only unimaginably more complex.

The immune system of vertebrates operates with an astonishing degree of complexity. This wonder of the living world can generate a virtually unlimited variety of antigen-binding sites; it can produce antibodies against almost any antigen it encounters. How is the body able to produce literally *billions* of different antibody structures? There are two basic mechanisms, and both contribute to *antibody diversity*: *somatic recombination* and *somatic mutation*. Recombination involves just a few gene segments that encode the so-called variable region of the immunoglobulin chain. A limited number of segments can combine in many different ways, much as a limited number of letters of the Roman alphabet can be joined together into different meaningful combinations. Even greater diversity is produced by mutations in the immunoglobulin gene that arise during the differentiation of B cells, the antibody-secreting cells of the immune system. Antibody diversity is an essential means of protection against foreign invaders.

The complexity of living things can make it hard to see how they are similar, but there are some common themes of basic importance. A few aspects of the common denominator have been touched on above. Here are a few more. The complexity of a biological organism

[10] On a higher hierarchical level, all cells of the body work together in a coordinated way to sustain the highly ordered living state of the healthy organism.

correlates with its being a far-from-equilibrium open system. It is difficult to imagine that this correlation does not point to something deep, something significant. The signal processing operations carried out by a cell must consume a lot of energy, whether by dissipating an electrochemical gradient or by the essentially irreversible breaking of chemical bonds, notably, the phosphodiester bonds of ATP. The molecules cells use to energize the biochemical reactions necessary to their existence *require* that cells be open thermodynamic systems. But it does not follow that some aspects of cellular activity must therefore violate either the First or Second Law of Thermodynamics. Every day in living cells everywhere on Earth, thousands of purines in genomic DNA hydrolyze spontaneously. To maintain the integrity of encoded information, damaged purines must be replaced, and the process consumes the enerby resources of the cell. The chemical structure of DNA itself plays a key role in the process: the redundancy of genetic information in the form of the complementary strands of the double helix greatly reduces the odds that spontaneous hydrolysis will lead to permanent change. Other types of mutation can occur in genes, and elaborate DNA repair mechanisms are present to reverse the changes in all organisms. Perhaps most importantly, the organizational plan of *all* organisms is encoded in DNA, and nearly all organisms use the same code for message transmission[11] and synthesize proteins of the same chirality. *The common features of known living organisms strongly suggest that all of them have a common origin*.

Parts of organisms and indeed entire organisms can usefully be thought of as machines, albeit complex ones, whether the organism has one cell or a lot of them. How far can this analogy be taken? Despite similarities, living organisms and machines are in fact fundamentally different, and as we shall see the difference has to do with complexity. *The physicochemical basis of order in biological organisms remains a major unsolved puzzle*, not least because the Second Law of Thermodynamics requires an increase in the entropy of the universe for any real process. To get a better understanding of the machine-like character of organisms, it might help to consider briefly the historical dimension of the concept.

In the "dualist" philosophy of the French mathematician and philosopher René Descartes (1596–1650), the "body," as distinct from the "mind," is assigned the properties of a "machine." By the middle of the eighteenth century, this extreme mechanistic philosophy had come

[11] The known exceptions are commonly accepted as slight variations on the central theme, not as very distinct competing alternatives. Though it is common knowledge that experimental proof of the triplet nature and degeneracy of the genetic code was worked out by Francis Crick and colleagues, it is less well-known that the triplet code was first proposed by the Ukrainian-American theoretical physicist Georgy Antonovich (George) Gamow (1904–1968), who is also known for developing the Big Bang theory of the origin of the universe with Ralph Alpher and Hans Bethe, popularizing science by means of his highly amusing and helpful Mr Tompkins science books, and collaborating in the design of the hydrogen bomb.

to be rejected by a number of leading biologists, including George Louis Leclerc de Buffon (1707–1788) and Pierre Louis Maupertuis (1698–1759), both Frenchmen, and Albrecht von Haller (1708–1777), a German. These researchers stressed the complexity of life over mechanism, and held that the animate was distinguished from the inanimate by such attributes as "sensibility," "irritability," and "formative drive."

To develop the organism–machine analogy, let the archetypal machine be a personal computer. A PC is made of matter, needs electrical energy to run, and has parts that can wear out. But a computer is a relatively static structure, even if disk drives can fail, chips and wireless network can be moved about with relative ease, and virus scan updates can be installed automatically over the Web. That's because the matter a computer is made of does not change all that much once the machine and its parts have been produced. A living organism is similar to a PC, but it is also very different. For an organism, be it a bacterium or a bat, utilizes the free energy it has acquired from its environment to carry out a continual process of *self-renewal*, and this is something no machine can do. Each and every cell of an organism simultaneously metabolizes proteins, many of which will have no actual defect, and cells synthesize new proteins as replacements. The process requires not merely the expenditure of free energy but a *huge amount* of free energy – on one level to produce the necessary precursors of macromolecules, on another to stitch them together, on another to get the molecule folded up in a biological meaningful way, and on another to get the protein sent to a location where it can do its job. If the necessary energy requirements are not met, the organism perishes. We'll return to death in a moment.

For now, let's try being quantitative about self-renewal in life. The half-life of a highly purified polypeptide in very pure aqueous solution is on the order of thousands of years. Protein turnover in the living organism is comparatively rapid. For example, a 70 kg man (or woman) synthesizes and degrades about 70 g of protein nitrogen – *per day*! The total nitrogen content of a 70 kg man is about 900 g, so the protein turnover rate is roughly 8% – *per day*! The free energy of formation of a peptide bond is about $+6$ kcal mol^{-1}, not very different in magnitude from the free energy of hydrolysis of ATP, so protein renewal requires a large fraction of an organism's daily energy intake (Chapter 1). We conclude that *although organisms have machine-like qualities, they are certainly not machines in the way that computers are machines, if organisms can be considered machines at all.*

Living biological systems are open systems, exchanging matter and energy with their surroundings. Living biological systems are most definitely not at equilibrium. In order to maintain themselves, they must have virtually constant access to a suitable form of free energy-rich matter. A great deal of research has been done in recent decades on relatively simple non-equilibrium systems and highly ordered structures that can arise within them. An example is the swirls that appear in a cup of tea immediately after a spot of milk has

been added. The patterns are clearly more ordered than when the milk has been completely stirred in. This sort of order is rather short-lived; it does not persist because maintaining it against dissipative forces would require energy input. Many other examples would fit here. Continuous heating of a fluid can result in the appearance of ordered structures called convection cells and turbulent flows. Under such conditions the entropy of the fluid is not maximal, as it would be if the density were uniform and the same in every direction. A large temperature gradient across a fluid can lead to the formation of Benard cells, highly ordered hexagonal structures, but a temperature gradient must be maintained in order for the Benard cells to persist. And complex weather patterns can result from the combination of solar heating and water vapor. *While these non-biological complex systems resemble life in certain ways, there is little doubt that they are a far cry from something as "simple" as a bacterium*.

The foregoing discussion raises the question of a possible relationship between biological complexity and the ability to survive. Bacteria, by far the simplest of living things known, have been around a very long time, and they are found nearly everywhere on the surface of Earth and many places below. Whatever it is that enables them to adapt to such a broad range of conditions works very well. In view of this, it seems probable that the ability of bacteria to survive all-out nuclear war or some other great calamity would be much greater than for humans. Assuming that humans are the product of a long evolutionary process, and that evolution is driven by the survival value of genetic changes, how is it that such complex but relatively "unstable" creatures as humans were able to evolve? Are humans really more fit for survival than bacteria? How so? If humans are eventually able to devise ways of leaving Earth permanently, for example, in order for a remnant to save themselves from destruction of the planet or simply to go where no human being has gone before, would they be able to depart without taking with them at least a few of the 100 trillion bacteria that inhabit each human gut? Can evolution be explained in terms of survival value or increasing complexity? Or is the possible or actual long-term survival value of a genetic change not always a relevant question in evolution?

The number of different eukaryotic species in existence today is in the millions, of which the animals, from ants to aardvarks, constitute a relatively large proportion. The diverse appearance of animals is largely superficial: the bewildering array of known forms, some of which are downright bizarre, can be sorted into a mere half-dozen or so body plans. Established during embryonic development, a *body plan* limits the size and complexity of an animal. Despite being so similar, different organisms differ strikingly in their ability to adapt to different conditions, e.g. temperature. Simple eukaryotes do not adapt to temperatures above about 60 °C. The upper limit for plants and animals is below 50 °C, and the majority of eukaryotes, including humans, are restricted to considerably lower temperatures. Above

about 60 °C, the only organisms that have any chance of survival are prokaryotes, unicellular organisms.

How do the complex interactions between organisms known as ecosystems form from individual organisms? was natural selection's acting on random mutations sufficient to generate not only the first living cell but also the totality of the tremendous variety of interacting life forms that have existed on Earth? Does the mutation–selection model predict that an ecosystem will have a *hierarchical* structure, with many interactions between organisms occurring at several different scales of size and complexity? If the employees of a particular company can communicate with each other, and if the communication of one company with another can lead to increased "productivity," can such a model help to explain the origin or the character of the interactions between different organisms in an ecosystem?

How about death? Aging and death may be related to changes in the information processing capability of a cell. One theory posits that life span is determined by a "program" encoded in DNA, just as eye color is determined genetically. Indeed, long life often runs in families, and short-lived strains of organisms like flies, rats, and mice can be produced by selective breeding. Nevertheless, there must be more to long life than the basic genetic program of aging, as decades of improvement in human nutrition attest. Another aging theory posits that cell death results from "errors" in the synthesis of key proteins like enzymes stemming from faulty messages. Errors in duplication of DNA, incorporation of errors into mRNA, pathological post-transcriptional control, or aberrant post-translational modification are several possible means by which "impaired" enzymes could be produced in the cell. The "somatic mutation" theory of aging says that aging results from the gradual increase of cells whose DNA has accumulated a significant number of mutations and which no longer function normally. It does seem that in some way or other the biologically meaningful information content of a cell declines toward the end of its life.

The Sun is a massive object, and we know from experience that the Sun's rays, having penetrated air, can be used to heat a pool of water on the surface of Earth or, when focused, to start a fire. The Sun transfers heat energy to its surroundings in accordance with the Second Law, and it does so by the transformation of its mass into electromagnetic radiation (at a rate of $\sim$100 million tons per minute). Something similar happens in our bodies. The biochemical reactions going on within lead to the dissipation of free energy as low frequency electromagnetic radiation, i.e. heat. Because of the various ways in which we use energy to make new proteins or other molecules and dissipate energy as heat, every few hours we have the urge to eat. From the point of view of thermodynamics, we satisfy this urge not to spend time with family or friends, nor to enjoy fine cuisine, but to supply our bodies with a source of free energy so that our bodies will not eat themselves! We need the energy input to maintain body weight and temperature. Without this energy, we will die

within a couple of months. And even if you are able to eat well throughout life, you will die after just a few score years. In this sense living organisms are like the Sun, which will eventually exhaust its energy resources.

I. The Second Law and evolution

When the biological world is viewed through a narrow window of time, what one sees is not how much organisms change from one generation to the next, but rather how much they maintain their exquisite order as a species. No wonder species were considered fixed when people thought Earth was relatively young! Nowadays there is no doubt that the order presently exhibited by a particular organism is the result of changes that have occurred over a very long period of time. Moreover, it is clear that in general terms the earliest organisms on Earth were much simpler than the ones in existence today. Exactly how the complex came from the simple is obscure, though in some way or other it must have involved changes in genetic material.

We might ask how there could be such a thing as life at all when the Second Law points to death, annihilation? How can there be a process whereby life forms become increasingly complex where the Second Law operates – everywhere? Has the chain of processes whereby the first cell on Earth became the many cells of all the organisms that have ever existed violated the Second Law of Thermodynamics? Some people think so, saying for example that zebras are clearly much more complex and highly ordered organisms than zebra fish and protozoa, protozoa have been around a lot longer than zebra fish and zebras, and that the Second Law demands ever-increasing disorder. This view, however, stems from a misunderstanding of what is possible in the context of the First and Second Laws. The Second Law says that the entropy of the universe must increase for any real process, not that order cannot increase anywhere. If order could not increase anywhere, how could rocks be transformed into a pile of cut stones, and stones turned into a majestic feat of architecture like an aqueduct or a cathedral? Or, leaving humans out of the argument, how could diamond form from a less ordered or less compact array of carbon atoms? The Second Law requires only that any process resulting in a decrease in entropy on a local level must be accompanied by an even larger increase in entropy of the surroundings.

If the degree of order exhibited by an open system increases *spontaneously*, the system is said to be "*self-organizing*"; the environment must exert no control over the system. Under some circumstances, such order can be retained spontaneously, albeit at some energetic cost. Self-organization, which is entirely consistent with the laws of thermodynamics, is usually initiated by "internal" processes, called "fluctuations" or "noise," the kind of particle motion one finds even in systems at equilibrium. Earlier we looked briefly at

processes that may have been involved in the abiotic synthesis of biological macromolecules. Such formation of macromolecular complexes, assuming it occurred, was presumably a matter of self-organization. Eventually, it was necessary for biological macromolecular complexes to catalyze the synthesis of macromolecules capable of storing information. How this could have occurred and where the information stored came from are unknown.

Remarkably, the "collapse" from order into disorder can be *constructive*, at least under some circumstances. For example, when concentrated guanidine hydrochloride is diluted out of a sample of chemically denatured hen lysozyme, the protein refolds – spontaneously. In other words, the free energy of the disordered state is energetically unfavorable in the absence of chemical denaturant (and at a temperature that favors the native state; see Chapter 2). The situation in which the guanidine concentration is low but the protein is unfolded must be far from equilibrium, and the *folding* of the protein must correspond to an *increase* in entropy, since by definition the equilibrium state is the one in which the entropy is a maximum. Because the protein itself is obviously more highly ordered when folded than when unfolded, the entropy increase must come from the dehydration of the protein surface on structure attainment. The released water molecules become part of the bulk solvent, where they have more degrees of freedom than when "bound" to the protein surface. *In general, an event like the folding of a protein does not occur in isolation from the rest of the world, and it is important to be aware of the possible consequences of how things do or do not interact with the rest of the world*. Certain biochemical reactions, for instance, can take place in cells only because they are chemically coupled to energetically favorable reactions (Chapter 4). The *interaction* between reactions may be crucial to the life of an organism. The Second Law requires that the overall entropy change in the universe be positive for any real process, but just how that entropy increase is achieved is quite another matter.

According to the Second Law, the entropy of the universe must increase for *any* real process, regardless of the involvement of non-equilibrium states. Non-equilibrium thermodynamics is helpful for thinking about biological processes because it provides a way of rationalizing the local decreases in entropy that are necessary for the formation of such extraordinarily ordered entities as living organisms. There is no violation of the Second Law since the entropy of the universe increases in any non-equilibrium thermodynamic processes. But non-equilibrium thermodynamics does not explain *how* the first living organism came into existence, *why* it came into being, or why, over time, there has been a marked increase in biological complexity. The First and Second Laws provide restrictions on *possible* processes, but we could not say, for example, that the laws of thermodynamics have required that a certain biophysicist will choose to investigate the thermodynamic properties of proteins! Nor is there any apparent way in which we could say

that thermodynamics *requires* that the enzyme catalase must have a catalytic efficiency that is limited by physics and, for example, not by chemistry.

How *purposeful* is the coupling of processes that bring about or underpin the order we see in living organisms? This would appear to be a question that thermodynamics cannot answer. While the great local decrease in entropy required for biological reproduction seems very purposeful, the accompanying increase in the entropy of the universe seems to lack purpose, excluding the possibility that its purpose is to enable local increases in order. The individual biochemical events on which brain processes and actions depend may seem purposeless on the level of individual atoms and molecules, but can this be said of the same processes when considered at a less detailed level? A book may make sense on the level of individual words used, sentences, paragraphs, sections, chapters, and indeed the entire book. But would there be any sense in thinking about the purpose of a book on the level of the molecules it's made of? As far as humans are concerned, what could be more purposeful than trying to make sense of the universe and what goes on inside it, being willing to learn, having a keen awareness of the essential and an overall sense of purpose, and testing ideas to see how right they might be? A philosophical scientist might argue that there is no particular meaning or purpose in life, and yet he will contradict himself in the very act.

In thinking about how the Second Law constrains biological processes, a *distinction* must be made between processes that express preexisting genetic information and ones that involve the appearance of "new" genetic information. In human growth and differentiation, for example, all the information required to construct the adult body is present in the fertilized ovum. The development of the conceptus into an adult involves the conversion of free energy and matter into increased material order and complexity as prescribed by genetics. In the usual case, the "potential complexity" of the fertilized egg is the same as that of the adult. The "expressed complexity" of the organism, however, increases dramatically throughout gestation, birth, adolescence, and early adulthood. *Despite similarities, then, animal development must differ considerably from speciation, for it is difficult to imagine how the first cell contained the genetic information required for the development not only of itself but also of amoebas, hyacinths, nematodes, koala bears, and people*.

Again assuming the existence of the first cell, it is not hard to see that "evolution" might not only be compatible with the Second Law of Thermodynamics but indeed driven by it. For replication of the bacterial genome, while good, is not perfect. Moreover, it may be that the fidelity of genome replication has "improved" with time, not become worse. In other words, given some starting material, the Second Law would seem to demand the generation of new varieties, particularly if the energetic cost of maintaining the genome is high, as it undoubtedly is.

Most mutations resulting from incorrect base pairing, however, are "corrected" by an elaborate system of repair mechanisms that are found in all organisms. Other mutations are "tolerated" because they result in no dramatic change in the organism. *Biological "stasis" on its own is energy consuming*. Does a model in which random mutation and natural selection drive evolution square with the inclusion in the genetic material of genes that encode enzymes that function to maintain the status quo? The rate of mutation is relatively low, much lower than one would expect if repair enzymes did not exist. Is there a selective advantage to having the repair enzymes? Such proteins would appear to slow down evolution. Do only exceptional cases of mutation and natural selection lead to new species? If the basic pattern exhibited by a species is to resist change, how have so many different species arisen in the short time that Earth has existed? And though many different, seemingly unrelated species have existed, because organisms from bacteria to humans have remarkably similar metabolic pathways, it is hard to see how all living (and extinct) organisms could not have a common origin.

Evolution might be slowed in more ways than one. *C. elegans* is a nematode worm that lives in the soil and feeds on detritus, decaying organic matter. Yum! The adult organism is just under 1 mm long and consists of fewer than 1000 cells. The generation time is a mere 3 days, and progeny are numerous. Studies of this organism have provided clues about many aspects of eukaryotic life, including for instance the importance of sexual reproduction in higher organisms. It is probable that reproduction of this kind enables an organism to cope with mutations in its genome and thereby to put the brakes on the rate of divergence of the genome. Most mutations have no detectable effect on the phenotype of offspring of sexually reproducing organisms. According to the "engine and gearbox" view, if you have two knackered cars, one lacking a gearbox and the other missing a driveshaft, you can combine parts and produce a single working vehicle. Something similar appears to happen during sexual reproduction when genetic material provided by the male is combined with the DNA of the female. This is particularly important if either dad or mom, but not both, carries an undesirable genetic trait, for example, sickle-cell anemia. In this way, sexual reproduction helps to keep the rate of change of a population down. Organisms that reproduce sexually "dilute out" the effect of mutations that would reduce either reproductive fitness or number of offspring.

Assuming that mutations and natural selection play an essential role in speciation, it may be that what might be called "persistent forms" (e.g. the species that exist) are something like local minima in a free energy landscape. On this view, once a species gets going it will not change very much; it is "stuck" in a "well" in the "genetic landscape" (Fig. 9.3). Divergence can still occur by genetic change, but this will entail a comparatively large-scale increase or decrease in organization of genetic information, and most instances of such

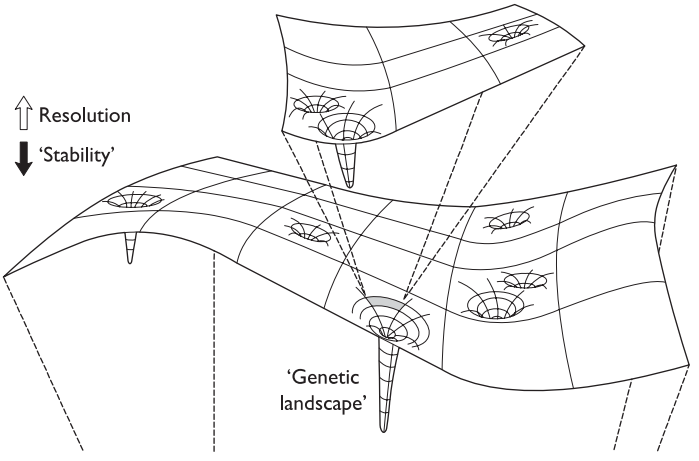

Fig. 9.3 Genetic "landscape" representing all possible sequences for DNA of a given number of base pairs. "Pockets" of genomic stability ("wells") correspond to actual species. Though each pocket is but a relatively small region of the total genetic landscape, it represents substantial genetic "stability" and allows for considerable sequence variation. There can be a large number of combinations of sequence in different ways: the larger the genome, the greater the number of possible sequences; and the more an organism reproduces itself, the higher the likelihood of variation. Bacteria have small genomes, but there are many of them, on the order of trillions in each human gut. Humans have large genomes, but there have been comparatively few of us ($<$ 50 billion). Does this imply that given enough time one could go from a dog, all breeds of which have come about through the selective mating of wolves over a time span of about 100 000 years, to a different *species*, *X*, which, unlike a dog, could not mate with a wolf?

reorganization will yield offspring that will not survive long enough to meet another organism with which it could produce viable next-generation offspring. In some cases, however, successful reproduction of offspring will be possible, and this can be represented diagrammatically as a move from one minimum in the genetic landscape to another. Organisms corresponding to one minimum will be sexually incompatible with organisms of another minimum (the definition of species), if not immediately then after some number of generations. Such a process will not violate the Second Law of Thermodynamics.

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K. Exercises

1. In order for a physical change to occur spontaneously, the entropy of the universe must increase. Can the increase of the entropy of the universe continue indefinitely? Why or why not? Relate your answer to the constraints on the energy in Eqn. (1.1).

2. Kurt Vonnegut's science fiction novel *The Sirens of Titan* tells the story of a man who has to cope with being converted into pure energy and knowing everything that has already happened or ever will. Is this possible in our universe? Why or why not?

3. If Earth was poor in biological information before life began and is now "biological information-rich," we should like to know what was the origin of the input of information. Is biological information inherent in the chemical composition of Earth? How did biologically meaningful information come into existence? Does the origin of information reside in the Big Bang?

4. Is a "DNA world" plausible? Why or why not.

5. Hen egg white lysozyme is 129 amino acid residues long. Calculate the information content of lysozyme on the level of the

amino acid sequence. Calculate the information content of the shortest piece of DNA that could encode this protein. Compare the values and comment.

6. According to Eqn. (9.4) each nucleotide in a strand has an information content of 2 bits. Thus a poly-G oligonucleotide 12 bases long has an information content of 24 bits. The logarithm of the number of distinct ways of arranging the 12 nucleotides, however, is 0. If entropy and information are isomorphous, why are these numbers not identical? (Hint: see Chapter 2.)

7. The human language–DNA analogy must break down at some point. Consider the three basic types of sentence – declarative, imperative, and interrogative – and discuss how they resemble and differ from gene expression.

8. Calculate the information content of the human genome. How many possible different DNA sequences are there with a size identical to that of the human genome?

9. The table below shows the probability of occurrence of letters in English. Note that, unlike the example presented in the text, the probability varies from character to character. When this is the case, the information content of a message is defined as

$$I = -\Sigma p_i^* \log_2 p_i.$$

Calculate the information content of this sentence.

Symbol	Probability, $\mathbf{p}$	Symbol	Probability, $\mathbf{p}$
space	0.2	C	0.023
E	0.105	F, U	0.0225
T	0.072	M	0.021
O	0.0654	P	0.0175
A	0.063	Y, W	0.0175
N	0.059	G	0.012
I	0.055	B	0.011
R	0.054	V	0.0105
S	0.052	K	0.008
H	0.047	X	0.002
D	0.035	J, Q, Z	0.001
L	0.029	–	–

Probability of occurrence of letters in English. From Brillouin (1962).

10. Is the information content of the sickle cell variant of hemoglobin different from or the same as that of the wild-type protein? Is one variant more biologically meaningful than the other? Justify your answer.

11. From the point of view of information theory, entropy measures the observer's lack of knowledge of the microscopic state of a

system. Because the information content of a message is only semi-objective, the observer can be said to be a part of any system being studied. Can this subjectivity be circumvented? Why or why not? If yes, how?

12. π, the value of which is 3.141 592 6 . . . , is a rather remarkable number. Not only does it crop up in areas of mathematics like number theory, but it plays a key role in theoretical physics; π is used to describe the geometry of space (the volume of a sphere is proportional to π). Moreover, π is a transcendental number[12] and therefore an "irrational" one, meaning that it cannot be expressed as the ratio of two *integers*; the trail of digits after the decimal place is *infinitely* long. As of later 1999, the value of π had been computed up to over 100 000 million decimal places. Analysis very strongly suggests that there is no organization to this string of numbers. In other words, no distinction can be made between the successive digits of π and numbers between 0 and 9 chosen *at random*. A consequence of this for computing is that there is no way of "compressing" the digits of π into a shorter string, as one can easily do with a number like 0.321 321 321 . . . Comment on the relationship between the compressibility of information and entropy. Relate this to the encoding of information in DNA. Speculate on what this might mean for the origin and propagation of life.

13. Mathematical transforms and thermodynamics. By means of a Fourier transform,[13] a signal $h(t)$ in the time domain, for instance a chord played on a piano, may be represented by its spectrum $H(f)$ in the frequency domain, the fundamental vibrational frequencies of the strings corresponding to the keys involved, and vice versa. In essence, the Fourier transform decomposes or separates a waveform into sinusoids of different frequency and amplitude, and when these are summed, they give the original waveform. Dr Sylvan Bloch, a professor of physics at the University of South Florida, has shown how π can be used to generate what is called a spread-spectrum wavelet by the "direct sequence method." The digits of π are then encoded on a noise-like wavelet, compressed in time (with a concomitant expansion in frequency), decompressed, and demodulated to recover the information. Explain the similarity between this situation and the First Law of Thermodynamics. What are the similarities and differences between this problem and question 4 of this chapter?

[12] The original proof of the irrationality of π was given by Lindemann in 1882. See, for example, Kasner & Newman (1943).

[13] Named after Baron Jean Baptiste Joseph Fourier (1768–1830), a French mathematician and physicist noted for his research on heat diffusion and numerical equations.

Appendix A

General references

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Appendix B

Biocalorimetry

A. Introduction

Calorimetry is the only means by which one can make direct, model-independent measurements of thermodynamic quantities. Spectroscopic techniques, though in many cases extremely sensitive or useful for obtaining high-resolution structure information, can give but an indirect, model-dependent determination of thermodynamic quantities. Calorimetric analysis therefore complements spectroscopic studies, giving a more complete description of the biological system of interest. Modern microcalorimeters are both accurate and sensitive, so that measurements require relatively small amounts of material (as little as 1 nmol) and can yield data of relatively low uncertainty.

Diffuse heat effects are associated with almost all physicochemical processes. Microcalorimetry provides a way of studying the energetics of biomolecular processes at the cellular and molecular level, and it can be used to determine thermodynamic quantities of conformational change in a biological macromolecule, ligand binding, ion binding, protonation, protein–DNA interaction, protein–lipid interaction, protein–protein interaction, protein–carbohydrate interaction, enzyme–substrate interaction, enzyme–drug interaction, receptor–hormone interaction, and macromolecular assembly. Microcalorimetry is also useful in the analysis of thermodynamics of very complex processes, for example, enzyme kinetics and cell growth and metabolism. Calorimetry is not narrowly applicable to processes occurring at equilibrium.

There are three broad classes of biological calorimetry: bomb calorimetry, differential scanning calorimetry (DSC), and isothermal titration calorimetry (ITC). Other biocalorimeters are usually derivatives of these types. The choice of instrument will ordinarily depend on the process of interest. Bomb calorimetry is used to measure the energy content of foods and other materials; discussion of the technique can be found in Chapters 1 and 2. This appendix focuses on DSC and ITC. An appealing and important feature of DSC and ITC is

that, because they are used to study the energetics of non-covalent or reversible interactions, they are at least in principle non-destructive. In fact, in practice most of a sample can often be recovered from a DSC or ITC experiment and put to further use.

B. Differential scanning calorimetry

DSC measures the heat capacity of a sample undergoing a process over a range of temperatures. The technique has been employed in the thermodynamic characterization of different kinds of biopolymers, but it has primarily been used to study the heat-induced unfolding of proteins and polynucleotides. Other applications of DSC include measurement of the stability of protein–nucleic complexes (e.g. ribosomes) and determination of the melting temperature of the gel-liquid crystal phase transition of lipid vesicles (Chapter 4).

Temperature is the principal independent variable in a DSC experiment. Cosolvent concentration, pH, or ion concentration can be a useful second independent variable, as for instance in studies of protein thermostability (Chapter 5). DSC experiments are carried out at constant pressure, so the heat effect corresponds to the enthalpy of the reaction (Chapter 2). The shape of the heat capacity function ($<C_p>$ versus T) provides information on the thermodynamics of the order–disorder transition. DSC is the only model-independent means of determining ΔH, ΔC_p, T_m, and cooperativity of a structural change in a biological macromolecule. Measurement of these thermodynamic quantities provides a complete *thermodynamic* description of an order–disorder transition, because these quantities are sufficient to simulate a heat capacity function ($<C_p>$ versus T). (See Chapter 6 for further details.) Determination of ΔH and T_m permits evaluation of ΔS (Chapter 2).

To rationalize the results of a DSC experiment, one often needs information on the thermodynamics of interactions between specific chemical groups. That is, although a single DSC experiment can in principle provide a "complete" description of the thermodynamics of an order–disorder transition, such a description is consistent with many possible molecular mechanisms, and information obtained from an independent source is needed to exclude some of the various possibilities.

The usual scanning rate in a DSC experiment is 1 °C per minute. This rate will be appropriate, however, only if the system will come to equilibrium relatively quickly throughout the temperature range of the experiment. A process is reversible if it proceeds through a succession of equilibrium or near-equilibrium states (Chapter 2), and if the reverse process yields the starting material *as it was before heating*. Some proteins exhibit nearly complete reversibility after unfolding. More generally, the degree of reversibility will depend on

solution conditions and the duration of heating in the unfolded state. When equilibrium is approached on a longer time scale than the scan rate, kinetic effects must be taken into account in interpreting the results of an experiment. Any change in protein association must also be accounted for, as for instance when the degree of oligomerization changes with temperature.

Analysis of the properties of point mutants has revealed that proteins are extremely complicated thermodynamic systems. Single amino acid replacement probably causes numerous small effects, and these are likely to be distributed throughout the molecule. This makes an observed thermodynamic quantity difficult to rationalize in terms of molecular structure. One of the most interesting findings of such studies, which is often though not always observed, is "enthalpy–entropy" compensation. This describes the situation where a point mutation alters the enthalpy of unfolding but the calculated free energy at, say, 25°C, is relatively unchanged. See Chapter 5 and cited references for further information.

DSC can also be used to study interactions between molecules. For instance, if a protein has a high-affinity binding site for an ion, say calcium, the concentration of calcium will have a marked effect on protein stability. The transition temperature will increase with ion concentration until all the sites remain filled as long as the protein is folded. DSC can thus be used to determine affinity constants of ligands (Chapter 7). The same approach can be used to measure the thermostability of polynucleotides, and how thermostability varies with concentration of ions or DNA-binding proteins.

C. Isothermal titration calorimetry

ITC is used to characterize the binding of a macromolecule to a ligand at constant temperature. The ligand can be another macromolecule, a peptide, an oligonucleotide, a small chemical compound, an ion or even just a proton. (See Chapter 7.) ITC permits analysis of the binding properties of native molecules without modification or immobilization. In a well-designed experiment, an ITC instrument can be used to acquire a complete profile of the binding interaction and to measure the binding constant, stoichiometry, and other thermodynamic functions. Modern instruments are accurate and easy to use. Specific applications of ITC include disaggregation of cationic micelles, enzyme–substrate interactions, antibody–antigen recognition, peptide–antibiotic interactions, and protein–DNA binding. The approach can also be used to study whole organism metabolism. ITC is a versatile technique.

ITC measures the heat of a reaction. The experiment takes place at constant pressure, in addition to constant temperature, so the heat absorbed or evolved is the enthalpy of reaction (Chapter 2). In an ITC experiment, small aliquots of a titrant solution containing ligand are added sequentially to a macromolecule in a reaction cell,

and the instrument records changes in enthalpy as the binding sites become saturated. The shape of the titration curve provides information on the strength of the binding interaction and the number of ligands recognized by the macromolecule. Determination of ΔH, K_{eq}, and n, the number of ligands, permits evaluation of ΔS (Chapter 4). ITC can also be used to measure ΔC_p if the titration experiment is carried out at several different temperatures ($\Delta C_p = \Delta\Delta H/\Delta T$, Chapter 2).

Results from ITC studies can provide insights into the nature of macromolecular interactions on the molecular level, particularly if high-resolution structural information on the macromolecule or ligand is available. Knowledge of the binding thermodynamics and structural details of the macromolecule in the free and liganded states can lead to insights on the rules governing such interactions and enable the manipulation of biomolecular recognition processes at the molecular level.

In antibody binding, for example, antibody in solution is titrated with antigen added in small aliquots, and the heat evolved in the formation of the antigen–antibody complex is measured with a sensitivity as high as 0.1 cal. The free energy of binding, the binding enthalpy, and the binding entropy can often be measured in a single experiment. Moreover, no spectroscopic or radioactive label must be attached to the antigen or antibody, simplifying both experimental design and interpretation of results. The change in heat capacity accompanying the formation of the complex can be determined by measuring the binding enthalpy over a range of temperatures. The binding heat capacity measured in this way is often found to be large and negative.

Unlike monomeric protein unfolding, a binding experiment gives several different types of thermodynamic data: protein–ligand interaction in the binding pocket, structural changes that might occur on binding, reduction of the translational degrees of freedom of the antigen. Clever experimental design can enable measurement of individual contributions to an overall heat effect. Suppose, for example, that the hydroxyl group of a Tyr residue of a macromolecule forms a hydrogen bond with a hydrogen bond acceptor in a ligand. Replacement of the Tyr by Phe could permit determination of the contribution of a single hydrogen bond to the binding thermodynamics, as long as no other changes to structure occurred. In practice, however, even such small changes as Tyr→Phe are often accompanied by the rearrangement of water molecules in the binding site, making it hard to be certain of the proportion of the measured effect that is attributable to the chemical modification.

ITC can be useful for drug discovery in the pharmaceutical industry. The data provided by the technique complement rational drug design. ITC can reduce the time or money required to take a lead compound to the marketplace, because sample through-put for the instrument is high. For instance, ITC can be used to screen potential inhibitors of an enzyme, taking the heat effect as an

indicator of binding. Additional experiments could then probe whether the ligands interfered with enzymatic activity. Direct measurement of the heat of reaction is one of the best ways to characterize the thermodynamics of binding, and ITC enables rapid determination of binding affinity.

High-affinity equilibrium binding constants are inherently difficult to measure. Biological processes in this category include cell surface receptor binding and protein–DNA interactions. Technique-independent difficulties stem from loss of signal intensity at low concentrations and from slow off-rate kinetics. The largest binding constant that can be measured reliably by titration microcalorimetry is about 10^9 M^{-1}. This poses a problem if the binding constant is large under physiological conditions. One way of dealing with this situation is to try to determine the binding constant under other conditions, and then to make necessary corrections for any changes in conditions. This can be done because the Gibbs free energy is a state function (see Chapter 2).

D. The role of biological techniques

Certain biological techniques play an important role in present-day biological calorimetry. For instance, site-directed mutagenesis enables study of the thermodynamic consequences of changing single amino acids. Large-scale production of recombinant proteins is also extremely useful. Although DSC and ITC are non-destructive techniques, protein folding/unfolding can be considerably less reversible than one might like, and recovery of a macromolecule or ligand from an ITC experiment might not be worth the effort if large quantities of pure material are relatively easy to prepare.

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Appendix C

Useful tables

A. Energy value and nutrient content of foods

	Energy (kJ)	Water (g)	Carbohydrate (g)	Protein (g)	Fat (g)	Alcohol (g)
Whole wheat flour	1318	14	63.9	12.7	2.2	–
White bread	1002	37.3	49.3	8.4	1.9	–
White rice, boiled	587	68	30.9	2.6	1.3	–
Milk, fresh, whole	275	87.8	4.8	3.2	3.9	–
Butter, salted	3031	15.6	trace	0.5	81.7	–
Cheese, cheddar	1708	36	0.1	25.5	34.4	–
Steak, grilled	912	59.3	0	27.3	12.1	–
Tuna, canned in oil, drained	794	63.3	0	27.1	9	–
New potatoes, boiled in unsalted water	321	80.5	17.8	1.5	0.3	–
Peas, frozen, boiled in unsalted water	291	78.3	9.7	6	0.9	–
Cabbage, boiled in salted water	75	92.5	2.5	0.8	0.6	–
Orange	158	86.1	8.5	1.1	0.1	–
Apple, raw	199	84.5	11.8	0.4	0.1	–
White sugar	1680	trace	105	trace	0	–
Beer*, canned	132	–	2.3	0.3	trace	3.1
Spirits* (brandy, gin, rum, whiskey)	919	–	trace	trace	0	31.7

All values per 100 g edible portion, except those indicated with an asterisk.
* Values per 100 ml. Data are from *The Composition of Foods*, 5th edn. (1991), The Royal Society of Chemistry and the Controller of Her Majesty's Stationery Office.

B. Physical properties of amino acids

Residue	Volume properties of amino acid residues			Accessible surface areas of amino acids in a Gly–X–Gly tripeptide in an extended conformation					Packing of residues in the interior of proteins	
						Side-chain atoms				
	Van der Waals volume (Å^3)	Partial volume in solution (Å^3)	Partial specific volume (cm^3 g^{-1})	Total (Å^2)	Main-chain atoms (Å^2)	Total (Å^2)	Non-polar (Å^2)	Polar (Å^2)	Fraction of residues at least 95% buried	Relative free energy of residue in interior relative to surface (kcal mol^{-1})[a]
Alanine, Ala (A)	67	86.4	0.732	113	46	67	67		0.38	−0.14
Arginine, Arg (R)	148	197.4	0.756	241	45	196	89	107	0.01	1.40
Asparagine, Asn (N)	96	115.6	0.610	158	45	113	44	69	0.12	0.75
Aspartic acid, Asp (D)	91	108.6	0.573	151	45	106	48	58	0.15	0.78
Cysteine, Cys (C)	86	107.9	0.630	140	36	104	35	69	0.40[a] 0.50[b]	−0.61[b, c]
Glutamine, Gln (Q)	114	142.0	0.667	189	45	144	53	91	0.07	0.80
Glutamic Acid, Glu (E)	109	128.7	0.605	183	45	138	61	77	0.18	1.15
Glycine, Gly (G)	48	57.8	0.610	85	85				0.36	0
Histidine, His (H)	118	150.1	0.659	194	43	151	102	49	0.17	0.02
Isoleucine, Ile (I)	124	164.6	0.876	182	42	140	140		0.60	−0.68
Leucine, Leu (L)	124	164.6	0.876	180	43	137	137		0.45	−0.59
Lysine, Lys (K)	135	166.2	0.775	211	44	167	119	48	0.03	2.06
	124	160.9	0.739	204	44	160	117	43	0.40	−0.65

B. (Cont.)

Residue	Volume properties of amino acid residues			Accessible surface areas of amino acids in a Gly–X–Gly tripeptide in an extended conformation					Packing of residues in the interior of proteins	
						Side-chain atoms				
	Van der Waals volume ($Å^3$)	Partial volume in solution ($Å^3$)	Partial specific volume ($cm^3\ g^{-1}$)	Total ($Å^2$)	Main-chain atoms ($Å^2$)	Total ($Å^2$)	Non-polar ($Å^2$)	Polar ($Å^2$)	Fraction of residues at least 95% buried	Relative free energy of residue in interior relative to surface (kcal mol^{-1})[a]
Methionine, Met (M)	135	187.3	0.766	218	43	175	175		0.50	−0.61
Phenylalanine, Phe (F)	90	120.6	0.748	143	38	105	105		0.18	0.50
Proline, Pro (P)	73	86.2	0.596	122	42	80	44	36	0.22	0.40
Serine, Ser (S)	93	113.6	0.676	146	44	102	74	28	0.23	0.32
Threonine, Thr (T)	163	225.0	0.728	259	42	217	190	27	0.27	−0.39
Tryptophan, Trp (W)	141	190.5	0.703	229	42	187	144	43	−0.15	0.28
Tyrosine, Tyr (Y)	105	136.8	0.831	160	43	117	117		0.54	−0.55
Valine, Val (V)			0.703							
weighted average										

[a] Calculated as $-RT\ln$(fraction in interior/fraction on surface), with the relative free energy of Gly set to zero.
[b] When in disulfide form.
[c] When in thiol form. Data from Tables 4.3, 4.4 and 6.3 of Creighton and references therein.

C. Protonation energetics at 298 K

Buffer	ΔH (kJ mol^{-1})	ΔC_p (J K^{-1}mol^{-1})	Buffer	ΔH (kJ mol^{-1})	ΔC_p (J K^{-1}mol^{-1})
Acetate	0.49	−128	Imidazole	36.59	−16
Cacodylate	−1.96	−78	TES	32.74	−33
MES	15.53	16	HEPES	21.01	49
Glycerol-2-phosphate	−0.72	−179	EPPS	21.55	56
ACES	31.41	−27	Triethanolamine	33.59	48
PIPES	11.45	19	Tricine	31.97	−45
Phosphate	5.12	−187	Tris	47.77	−73
BES	25.17	2	TAPS	41.49	−23
MOPS	21.82	39	CAPS	48.54	33

Data were prepared and revised by H. Fukuda and K. Takahashi, Laboratory of Biophysical Chemistry, College of Agriculture, University of Osaka Prefecture, Sakai, Osaka 591, Japan.

D. Buffer ionization constants

Acid	pK
Oxalic acid	1.27 (pK_1)
H_3PO_4	2.15 (pK_1)
Citric acid	3.13 (pK_1)
Formic acid	3.75
Succinic acid	4.21
Oxalate$^-$	4.27 (pK_2)
Acetic acid	4.76
Citrate$^-$	4.76 (pK_2)
Succinate	5.64 (pK_2)
MES[a]	6.09
Cacodylic acid	6.27
H_2CO_3	6.35 (pK_1)
Citrate^{2-}	6.40 (pK_3)
ADA[b]	6.57
PIPES	6.76
ACES	6.80
H_2PO^{4-}	6.82 (pK_2)
MOPS[c]	7.15
HEPES[d]	7.47
HEPPS[e]	7.96
Tricine[f]	8.05
TRIS[g]	8.08
Glycylglycine	8.25
Bicine[h]	8.26
Boric acid	9.24
Glycine	9.78
HCO^{3-}	10.33 (pK_2)
Piperidine	11.12
HPO_4^{2-}	12.38 (pK_3)

Data from Dawson *et al.*, *Data for Biochemical Research*, 3rd edn (Oxford: Clarendon, 1986) or Good *et al.* (1966) *Biochemistry*, **5**, 467.
Abbreviations:
[a] morpholinoethanesulfonic acid, [b] acetamidoiminodiacetic acid, [c] morpholinopropanesulfonic acid, [d] hydroxyethylpiperazine-ethanesulfonic acid, [e] hydroxyethylpiperazone-propanesulfonic acid, [f] trishydroxymethylglycine, [g] trishydroxymethylaminomethane, [h] bishydroxymethylglycine.

E. Energetics of the reactions of the citric acid cycle

Reaction	Enzyme	Comments	$\Delta G^{\circ\prime}$ (kJ mol^{-1})	ΔG (kJ mol^{-1})
Acetyl-CoA + H_2O oxaloacetate → citrate + CoASH	Citrate synthetase	An acetyl group is added to oxaloacetate, changing the carbonyl carbon at C-3 from a + 2 to a + 1 oxidation state. Water is used for release of free CoA. This favorable energy change makes the first step in the cycle essentially irreversible.	−31.5	<0
Citrate → *cis*-aconitate + H_2O → isocitrate	Aconitase	A hydroxyl group is transferred from C-3 to C-2 on citrate by successive dehydration and hydration reactions.	~5	~0
Isocitrate + NAD_{ox} → NAD_{red} + CO_2 + 2-oxoglutarate (α-ketoglutarate)	Isocitrate dehydrogenase	Isocitrate is decarboxylated via NAD-linked oxidation	−21	<0
2-oxoglutarate + CoASH + NAD_{ox} → NAD_{red} + CO_2 + succinyl-CoA	2-Oxoglutarate dehydrogenase multienzyme complex	2-Oxoglutarate is decarboxylated via NAD-linked oxidation. Succinyl is attached to CoA, forming succinyl-CoA.	−33	<0
Succinyl-CoA + P_i + GDP → succinate + GTP + CoASH	Succinyl-CoA synthase	Substrate-level phosphorylation is driven by the redox reaction in the previous step. The terminal phosphate group of GTP can be transferred to	−2.1	~0

E. (Cont.)

Reaction	Enzyme	Comments	$\Delta G^{\circ\prime}$ (kJ mol^{-1})	ΔG (kJ mol^{-1})
		ATP by nucleoside diphosphate kinase.		
Succinate + FAD_{ox} → fumarate + FAD_{red}	Succinate dehydrogenase	The FAD is covalently linked to the enzyme, which is bound to the inner mitochondrial membrane.	+6	~0
Fumarate + H2O → malate	Fumarase	Water is added to fumarate to form malate.	−3.4	~0
L-malate + NAD_{ox} → oxaloacetate + NAD_{red}	Malate dehydrogenase	This energy barrier is overcome by maintaining a low concentration of oxaloacetate in the mitochondrial matrix.	+29.7	~0

Data are from Table 19-2 of Voet and Voet (1996).

Appendix D

BASIC program for computing the intrinsic rate of amide hydrogen exchange from the backbone of a polypeptide

The computer program IRATE calculates intrinsic rates of hydrogen exchange for all backbone amide hydrogens in a given amino acid sequence, using the method of Bai *et al.* (1993) *Proteins*, **17**, 75–86. The input file must be an ASCII file and the amino acid sequence must be in single letter code. The program accepts both upper case or lower case letters, but the input file must not contain spaces.

```
   '
'    ***********************************************
'    *     Program: IRATE (Intrinsic RATe of Exchange)  *
'    *    Author: Donald T. Haynie, Ph.D.               *
'    *     Date of Original Program: May 1996           *
'    *     Previous Modification:    Th.23.v.96         *
'    *     Most recent Modification: Th.23.ix.99        *
'    *     Acknowledgement: Dr Christina Redfield       *
'    ***********************************************
'
CLS
PRINT "  "
'
' Read input file containing amino acid sequence.
'
INPUT "Name of ASCII file that contains amino acid sequence"; infile$
OPEN infile$ FOR INPUT AS #1
INPUT #1, sequence$
CLOSE #1
'
'  Determine length of the sequence.
'
length = LEN(sequence$)
'
'  Create an array of the length of the sequence.
'
DIM seq(length - 1) AS INTEGER
PRINT "The length of the sequence is:"; length
PRINT "  "
INPUT "Name of protein/peptide"; protein$
PRINT "  "
'
'  Create an output file.
'
INPUT "Output filename"; outfile$
```

```
OPEN outfile$ FOR OUTPUT AS #1
'
'  Convert the characters of amino acid sequence input string to integers and store
'  in sequential elements of array "seq." The input string must be in single letter
'  code.
'
position = 0
DO WHILE position < length
           aa$ = MID$(sequence$, position + 1, 1)
        IF ASC(aa$) > 96 THEN
                seq(position) = ASC(aa$) - 97
        ELSE
                seq(position) = ASC(aa$) - 65
        END IF
        position = position + 1
LOOP
'
'  Prompt for temperature and pD
'
PRINT "  "
INPUT "Temperature (deg. C)"; temp
PRINT "  "
INPUT "Exchange medium: H2O (0) or D2O (1)"; solvent
PRINT "  "
INPUT "Is protein/peptide deuterated (y/n)"; deuterated$
'
'  Set the reference exchange rates in accordance with experimental conditions.
'
flag = 0
DO WHILE flag < 1
           IF LEFT$(deuterated$, 1) = "y" OR LEFT$(deuterated$, 1) = "Y" THEN
                    IF solvent = 0 THEN
'
'  See Connelly et al. (1993) Proteins 17:87-92.
'
```

```
                        arefrate = 1.4
                       brefrate = 9.87
                        wrefrate = −1.6
                        flag = 1
               ELSE
                    PRINT "IRATE does NOT calculate rates of exchange of deuterons into D2O"
               END IF
        ELSE
               SELECT CASE solvent
                        CASE 0
'
'  See Connelly et al. (1993) Proteins 17:87-92.
'
                                arefrate = 1.39
                                  brefrate = 9.95
                                  wrefrate = 0!
                        CASE 1
'
'  See Bai et al. (1993) Proteins 17:75-86.
'
                                  arefrate = 1.62
                                  brefrate = 10.05
                                  wrefrate = −1.5
               END SELECT
               flag = 1
        END IF
LOOP
PRINT "  "
PRINT arefrate, brefrate, wrefrate
PRINT "  "
INPUT "Measured pH or pD (uncorrected) at 20 deg. C"; pd
'
'  If solvent is D20, a correction must be made to electrode reading.
'  Ionization constants are for 20 deg. C and are from p. D-166 of CRC
'
```

```
IF solvent = 1 THEN
        pd=pd+.4
       pod=pd-15.049
ELSE
        pod=pd-14.1669
END IF
'
'  Convert temperature to degrees K
'
temp=temp+273.15
PRINT "  "
'
'  Create table of the measured effects of amino acid side chains on HX rates of
'  neighboring peptides. See Bai et al. (1993).
'
DIM table(27, 3) AS SINGLE
table(0, 0)=0!              'Ala acid catalysis left
table(0, 1)=0!              'Ala acid catalysis right
table(0, 2)=0!              'Ala base catalysis left
table(0, 3)=0!              'Ala base catalysis right
table(17, 0)=-.59           'Arg acid left
table(17, 1)=-.32           'Arg acid right
table(17, 2)=.08            'Arg base left
table(17, 3)=.22            'Arg base right
table(13, 0)=-.58           'Asn
table(13, 1)=-.13
table(13, 2)=.49
table(13, 3)=.32
table(3, 0)=.9              'Asp base
table(3, 1)=.58
table(3, 2)=-.3
table(3, 3)=.18
table(1, 0)=-.9             'Asp acid
table(1, 1)=-.12
table(1, 2)=.69
```

```
table(1, 3)=.6
table(2, 0)=-.54          /Cysteine
table(2, 1)=-.46
table(2, 2)=.62
table(2, 3)=.55
table(9, 0)=-.74          /Cystine
table(9, 1)=-.58
table(9, 2)=.55
table(9, 3)=.46
table(6, 0)=-.22          /Gly
table(6, 1)=.22
table(6, 2)=.27
table(6, 3)=.17
table(16, 0)=-.47         /Gln
table(16, 1)=-.27
table(16, 2)=.06
table(16, 3)=.2
table(4, 0)=-.9           /Glu base
table(4, 1)=.31
table(4, 2)=-.51
table(4, 3)=-.15
table(14, 0)=-.6          /Glu acid
table(14, 1)=-.27
table(14, 2)=.24
table(14, 3)=.39
table(7, 0)=0!              /His base
table(7, 1)=0!
table(7, 2)=-.1
table(7, 3)=.14
table(20, 0)=-.8              /His acid
table(20, 1)=-.51
table(20, 2)=.8
table(20, 3)=.83
table(8, 0)=-.91              /Ile
table(8, 1)=-.59
```

```
table(8, 2)=-.73
table(8, 3)=-.23
table(11, 0)=-.57            /Leu
table(11, 1)=-.13
table(11, 2)=-.58
table(11, 3)=-.21
table(10, 0)=-.56             /Lys
table(10, 1)=-.29
table(10, 2)=-.04
table(10, 3)=.12
table(12, 0)=-.64             /Met
table(12, 1)=-.28
table(12, 2)=-.01
table(12, 3)=.11
table(5, 0)=-.52              /Phe
table(5, 1)=-.43
table(5, 2)=-.24
table(5, 3)=.06
table(15, 0)=0!               /Pro (trans)
table(15, 1)=-.19
table(15, 2)=0!
table(15, 3)=-.24
table(23, 0)=0!               /Pro (cis)
table(23, 1)=-.85
table(23, 2)=0!
table(23, 3)=.6
table(18, 0)=-.44             /Ser
table(18, 1)=-.39
table(18, 2)=.37
table(18, 3)=.3
table(19, 0)=-.79             /Thr
table(19, 1)=-.47
table(19, 2)=-.07
table(19, 3)=.2
table(22, 0)=-.4                /Trp
```

```
table(22, 1) = -.44
table(22, 2) = -.41
table(22, 3) = -.11
table(24, 0) = -.41                 'Tyr
table(24, 1) = -.37
table(24, 2) = -.27
table(24, 3) = .05
table(21, 0) = -.74                 'Val
table(21, 1) = -.3
table(21, 2) = -.7
table(21, 3) = -.14
table(25, 0) = 0!                    'N-term
table(25, 1) = -1.32
table(25, 2) = 0!
table(25, 3) = 1.62
table(26, 0) = .96                    'C-term base
table(26, 1) = 0!
table(26, 2) = -1.8
table(26, 3) = 0!
table(27, 0) = .05                     'C-term acid
table(27, 1) = 0!
table(27, 2) = 0!
table(27, 3) = 0!
'
'  Write information about protein and HDX conditions to output file.
'
PRINT #1, "Calculated intrinsic amide exchange rates for ** "; protein$; " **"
PRINT #1, "at ** pD (corrected)"; pd; "** and **"; temp; "K **"
PRINT #1, "  "
IF solvent = 1 THEN
        PRINT #1, "Solvent is D2O"
ELSE
        PRINT #1, "Solvent is H2O"
END IF
PRINT #1, "  "
```

```
PRINT #1, "All rates are in units of inverse minutes"
PRINT #1, "  "
PRINT #1, "res R L"; TAB(12); "fracar"; TAB(40); "fracal"; TAB(70); "acid rate"; TAB(90); "base rate"; TAB(110);
"water rate"; TAB(130); "intrinsic rate"; TAB(160); "dG++"
PRINT #1, "  "
'
'  Set the activation energies.
'
PRINT "  "
'
'  Acid
'
aae = 14000!
'
'  Base
'
bae = 17000!
'
'  Water
'
wae = 19000!
PRINT "  "
CLS
DEFDBL C, F, H, K, Z
'
'  Factor for converting log to ln
'
constant = LOG(10)
'
'  See Eq. (3) of Bai et al. (1993).
'
corr = (1 / temp - 1 / 293!) / 1.9872
'
'  Fraction protonated to the right
'
```

```
fracar = 0
'
'  Fraction protonated to the left
'
fracal = 0
position = 0
'
'  Calculate percentage of protons bound for acidic side chains.
'
DO WHILE position < length
'
'    What is this residue?
'
         SELECT CASE seq(position)
'
'    If this is a Cys, is it in an S-S bridge?
'
           CASE 2
                    PRINT "Residue"; position + 1; "is a Cys"
                    INPUT "Is it involved in an S-S bridge"; reply$
                    PRINT "   "
                    IF LEFT$(reply$, 1) = "y" OR LEFT$(reply$, 1) = "Y" THEN
'
'  If yes, change the array.
'
                             seq(position) = 9
                    END IF
                    logarl = table(9, 0)
                    logbrl = table(9, 2)
'
'  If this residue is an Asp, do this:
'
           CASE 3
                    PRINT "The default pKa of Asp"; position + 1; "is 3.95"
                    INPUT "Do you wish to change this value (y/n)"; reply$
```

```
                    PRINT "  "
                    IF LEFT$(reply$, 1) = "y" OR LEFT$(reply$, 1) = "Y" THEN
                            INPUT "What is the pKa for this residue"; pKa
                    ELSE
                            pKa = 3.95
                    END IF
                    PRINT "pKa is "; pKa
                    PRINT "  "
                    fracal = (10^(pKa - pd)) / (1 + 10^(pKa - pd))
                    logarl = fracal * table(1, 0) + (1 - fracal) * table(3, 0)
                    logbrl = fracal * table(1, 2) + (1 - fracal) * table(3, 2)
'
'  If this residue is a Glu, do this:
'
            CASE 4
                    PRINT "The default pKa of Glu"; position + 1; "is 4.4"
                    INPUT "Do you wish to change this value (y/n)"; reply$
                    PRINT "  "
                    IF LEFT$(reply$, 1) = "y" OR LEFT$(reply$, 1) = "Y" THEN
                            INPUT "What is the pKa for this residue"; pKa
                    ELSE
                            pKa = 4.4
                    END IF
                    PRINT "pKa is "; pKa
                    PRINT "  "
                    fracal = (10^(pKa - pd)) / (1 + 10^(pKa - pd))
                    logarl = fracal * table(14, 0) + (1 - fracal) * table(4, 0)
                    logbrl = fracal * table(14, 2) + (1 - fracal) * table(4, 2)
'
'  If this residue is a His, do this:
'
            CASE 7
                    PRINT "The default pKa of His"; position + 1; "is 6.5"
                    INPUT "Do you wish to change this value (y/n)"; reply$
                    PRINT "  "
```

```
                IF LEFT$(reply$, 1) = "y" OR LEFT$(reply$, 1) = "Y" THEN
                        INPUT "What is the pka for this residue"; pKa
                ELSE
                        pKa = 6.5
                END IF
                PRINT "pKa is "; pKa
                PRINT "  "
                fracal = (10^(pKa - pd)) / (1 + 10^(pKa - pd))
                logarl = fracal * table(20, 0) + (1 - fracal) * table(7, 0)
                logbrl = fracal * table(20, 2) + (1 - fracal) * table(7, 2)
'
' If this residue is none of the above...
'
        CASE IS < 2
                logarl = table(seq(position), 0)
                logbrl = table(seq(position), 2)
'
'  then just read values straight...
'
        CASE 5 TO 6
                logarl = table(seq(position), 0)
                logbrl = table(seq(position), 2)
'
'  from the array "table"...
'
        CASE IS > 7
                logarl = table(seq(position), 0)
                logbrl = table(seq(position), 2)
    END SELECT
'
'  If the second residue has been reached:
'
    IF position > 0 THEN
'
'  Check the identity of the previous residue.
```

```
'
          SELECT CASE seq(position - 1)
                    CASE 3
'
'  Compute weighted average rate if this is an Asp:
'
                              logar = fracar * table(1, 1) + (1 - fracar) * table(3, 1) + logarl
                              logbr = fracar * table(1, 3) + (1 - fracar) * table(3, 3) + logbrl
                    CASE 4
'
'  Compute weighted average rate if this is a Glu:
'
                              logar = fracar * table(14, 1) + (1 - fracar) * table(4, 1) + logarl
                              logbr = fracar * table(14, 3) + (1 - fracar) * table(4, 3) + logbrl
                    CASE 7
'
'  Compute weighted average rate if this is a His:
'
                              logar = fracar * table(20, 1) + (1 - fracar) * table(7, 1) + logarl
                              logbr = fracar * table(20, 3) + (1 - fracar) * table(7, 3) + logbrl
                    CASE IS < 3
'
'  Or just read the values...
                              logar = table(seq(position - 1), 1) + logarl
                              logbr = table(seq(position - 1), 3) + logbrl
                    CASE 5 TO 6
'
'  ...from the array "table"...
'
                              logar = table(seq(position - 1), 1) + logarl
                              logbr = table(seq(position - 1), 3) + logbrl
                    CASE IS > 7
'
'  ...if this is neither Asp nor Glu nor His.
'
```

```
                        logar=table(seq(position-1), 1)+logarl
                        logbr=table(seq(position-1), 3)+logbrl
        END SELECT
'
'  Take polypeptide chain end effects into account as follows:
'
        IF position=1 THEN
                logar=logar+(10^(7.4-pd)) / (1+10^(7.4-pd)) * table(25, 1)
                logbr=logbr=(10^(7.4-pd)) / (1+10^(7.4-pd)) * table(25, 3)
        END IF
        IF position=length-1 THEN
                 logar=logar+(10^(3.9-pd)) / (1+10^(3.9-pd)) * table(27, 0)+(1-(10^(3.9-pd)) /
(1+10^(3.9-pd))) * table(26, 0)
                logbr=logbr+(1-(10^(3.9-pd)) / (1+10^(3.9-pd))) * table(26, 2)
        END IF
'
'  Compute rates:
'
'  Acid rate:
'
        acidrate=EXP((arefrate+logar-pd) * constant-aae * corr)
'
'  Base rate:
'
        baserate=EXP((brefrate+logbr+pod) * constant-bae * corr)
'
'  Water rate:
'
        waterrate=EXP((wrefrate+logbr) * constant-wae * corr)
'
'  Sum rates to give total rate:
'
        k=acidrate+baserate+waterrate
         PRINT #1, position+1; TAB(6); CHR$(seq(position-1)+65); SPC(1); CHR$(seq(position)+65); TAB
(12); fracar; TAB(40); fracal; TAB(70); acidrate; TAB(90); baserate; TAB(110); waterrate; TAB(130); k; TAB
```

```
(160); USING "##.###"; -1.9872 * temp * LOG(6.6254E-27 * k / 1.38046E-16 / temp / 60!) / 1000!
      END IF
'
'  Switch left to right, etc., and go to the next residue:
'
      fracar = fracal
      fracal = 0
      position = position + 1
LOOP
CLOSE
```

Glossary

abiotic synthesis – non-biological synthesis of a biochemical, often a macromolecule.

acid – proton donor. Compare *base*.

acidity constant – pH at which dissociation of protons from a specific titratable site is half complete; a measure of the *free energy* of protonation.

activated complex – structure of enzyme–substrate complex in the transition state.

activation barrier – schematic representation of the *energy* that must be added to reactants to convert them to products.

activation energy – minimum *energy* input required to initiate a chemical reaction under given conditions.

active site – region on the surface of an enzyme where the *substrate* binds and catalysis occurs. See *catalyst*.

active transport – transport of ions or metabolites across a biological membrane against a concentration gradient at the expense of *energy* resources of the cell (*ATP* hydrolysis). Compare *diffusion* and *facilitated diffusion*.

activity – effective concentration of a chemical species.

activity coefficient – factor by which the concentration of a chemical species is multiplied to give the *activity* under specific conditions.

actual information – calculated information content of a message. Compare *potential information*.

Adair equation – general ligand binding equation first proposed by Gilbert Adair.

adjustable parameter – component of a mathematical model, the value of which is determined by fitting the model to experimental data.

anaerobic autotrophs – organisms that synthesize all their cellular constituents from simple molecules, some inorganic, in the absence of oxygen.

allosteric regulation – modulation of enzyme function through the binding of small molecules or ions to sites on the enzyme other than where catalysis occurs.

amino acid composition – percentage of each amino acid type for a given polypeptide.

antibody diversity – vast repertoire of antibodies produced in individual mammals by means of genetic recombination (combinatorics) and mutation.

association constant – binding constant for association of ligand and macromolecule. Compare *dissociation constant*.

ATP – small molecule compound that is the main *energy* "currency" of all known organisms. *ATP* is also utilized in the communication of biological information: it is directly involved in the synthesis of *second messengers*, *mRNA*, and *DNA*, and in the propagation of chemical signals by *phosphorylation* of amino acid side chains.

barbed end – the end of an actin filament where *ATP*-bound actin monomers associate preferentially. So named from appearance of myosin S1 fragment-bound actin filaments by scanning electron microscopy. Synonym of *plus end*.

base – proton acceptor. Compare *acid*. *Base* is also used to describe a hydrogen bond-forming information storage unit in DNA or RNA.
Big Bang – cataclysmic explosion about 20 billion years ago by which the universe is thought to have come into existence.
binding capacity – number of binding sites per macromolecule.
binding site – precise location on a macromolecule where a ligand binds.
biochemist's standard state – defined reference state of greatest use to biochemists, as it accounts for pH and assumes that reactions occur in aqueous solvent.
bioenergetics – the study of *energy* changes in living organisms, particularly as these concern glucose metabolism and *ATP* production.
biological information – the one-dimensional information content of genetic material and the three-dimensional information content of proteins and other biological macromolecules.
bit of information – information content of one binary decision.
body plan – one of but several different basic organization schemes into which all known organisms can be classified.
Bohr effect – effect of pH on oxygen-binding properties of hemoglobin, first described by Christian Bohr, father of Neils Bohr.
Boltzmann distribution – the most probable distribution of a system at equilibrium if the system contains a large number of molecules; first described by Ludwig Boltzmann.
Boltzmann factor – relative contribution of a *state* to the magnitude of the *partition function*; named after Ludwig Boltzmann. Synonym of *statistical weight*.
boundary – notional barrier where the system meets the surroundings which may or may not be permeable to heat or matter.
breathing motions – stochastic fluctuations in the structure of proteins and other biological macromolecules. See *Le Châtelier's principle*.
Brønsted–Lowry definitions – see *acid* and *base*.
buffering capacity – quantitative ability of a buffered solution to resist changes in pH upon addition of *acid* or *base*.
calorimetric enthalpy – heat absorbed or evolved during a *process*, usually occurring at constant pressure.
calorimetry – science of measuring heat transfer from system to surroundings and vice versa.
carbon – extraordinary element whose ability to form up to four relatively stable covalent bonds per atom is essential for life as we know it.
catalyst – substance whose presence increases the rate of a chemical reaction but is not consumed by the reaction.
chemical potential – how the Gibbs energy of a system changes as a substance is added to it. For a pure substance, the chemical potential is the same as the molar Gibbs energy.
chemosynthesis – biochemical *process* by which *ATP* is synthesized by reduction of inorganic compounds and not by absorption of photons. Compare *photosynthesis*.
chirality – molecular handedness.
chlorophyll – major antenna for absorption of sunlight in plants.
citric acid cycle – set of coupled reactions in the mitochondrial matrix that oxidize acetyl groups and generate CO_2 and reduced intermediates used to make *ATP*; a.k.a. Krebs cycle and tricarboxylic acid (TCA) cycle.
closed system – one that is permeable to heat but not matter. See *system*.

cold-denaturation – disordering of an ordered system, e.g. a protein, by cooling rather than heating.
collision theory – one explanation of chemical reactivity. Compare *transition state theory*.
competitive inhibition – blocking of a biochemical interaction by an inhibitor through direct competition with the ligand for the ligand binding site.
configuration – arrangement of particles in a *system*.
conformational change – alteration of the three-dimensional structure of a molecule but not of its covalent bonds.
conformational entropy – entropy change associated with fixing the three-dimensional structure of a molecule.
conservation of energy – apparently fundamental principle of physics that *energy* is neither created nor destroyed in any physical, chemical, or biological *process*. See *First Law*.
cooperativity – degree of "concertedness" of a change in conformation or arrangement of particles in a system.
coupled reaction – an overall spontaneous reaction made so by the product of an unfavorable reaction being a reactant in a more favorable one.
critical concentration – concentration of actin monomers below which polymerization will not occur under specified conditions.
Dalton's Law – overall pressure of a gas is the sum of the partial pressures of the constituent gases.
degeneracy – number of distinguishable states of the same *energy* level.
differential scanning calorimetry (DSC) – device for measuring the heat exchanged at constant pressure as a function of temperature.
diffusion – random movement of particles at a given *temperature*. Also called *passive diffusion* in net movement of a chemical species across a membrane at a *rate* proportional to the concentration gradient. Compare *facilitated diffusion* and *active transport*.
dissipation – expenditure of *free energy* or increase of entropy in which no work is done. See also *substrate cycling*.
dissociation constant – concentration of ligand at which half of all sites are occupied.
distribution – real or conceptual dispersal of something in space and time.
disulfide bond – covalent linkage between two sulfur atoms, each of which is donated by the amino acid cysteine.
Donnan equilibrium – equilibrium involving a semi-permeable membrane, permeant ions, and impermeant ions (usually a biological macromolecule).
dynamic equilibrium – state of no net change (not no change), as in any chemical equilibrium.
effector – ion or molecule involved in allosteric regulation of enzymatic activity.
efficiency – ratio of work done by a system to heat added to the system.
electromotive force – synonym of *voltage*.
electroneutrality – condition of a net charge of zero.
electroweak interactions – interparticle interactions mediated by the electroweak *force*, one of the fundamental forces of nature; electromagnetic and weak nuclear interactions.
endergonic reaction – one which does not occur spontaneously at constant *temperature* and *pressure* unless *work* is done on the *system*. Antonym of *exergonic reaction*.

endothermic reaction – one which involves the absorption of heat. Antonym of *exothermic reaction*.
energy – the most fundamental concept of science; the capacity to have an effect, the capacity to do work.
energy well – local minimum in the *free energy* surface.
enthalpy – thermodynamic state function usually measured as heat transferred to or from a system at constant pressure.
enthalpy of binding – enthalpy difference between the bound and unbound states of a ligand–macromolecule system.
enthalpy of denaturation – enthalpy change of protein unfolding at a given temperature.
enthalpy–entropy compensation – phenomenon observed in weakly stable systems in which changes in enthalpy are attended by changes in entropy but little or no change in *free energy*.
enthalpy of hydration – enthalpy change on solvation of an ion or molecular compound.
entropy – thermodynamic state function that is a measure of disorder.
equilibrium – condition of no further net change in a closed system; not to be confused with *steady state*.
equilibrium constant – provides a means of calculating the standard *free energy* change for a reaction by measuring the amounts of products and reactants in a *system*.
ergodic hypothesis – assumption that the short term behavior of a large collection of identical objects is equivalent to the long term behavior of a small collection of such objects.
evolution – gradual change in genetic material with time.
EX1 mechanism – rate of hydrogen exchange limited by the intrinsic rate.
EX2 mechanism – rate of hydrogen exchange limited by the rate of exposure of the labile hydrogen to the exchange medium (solvent).
exergonic reaction – one which does occur spontaneously at constant temperature and pressure in the absence of work being done on the system. Antonym of *endergonic reaction*.
exothermic reaction – one which involves the release of heat. Antonym of *endothermic reaction*.
extremophile – bacterium that thrives in a harsh physical or chemical environment.
extrinsic property – quantity that does depend on amount of substance present, for example, *energy*. Compare *intrinsic property*.
facilitated diffusion – membrane protein-aided transport of an ion or molecule across a membrane and down its concentration gradient. Compare *diffusion* and *active transport*.
feedback inhibition – in metabolism, down-regulation of a metabolic pathway by interaction between a product of the pathway and one of its enzymes.
First Law of Thermodynamics – statement of the conservation of *energy*.
first-order reaction – one in which reaction rate is proportional to the first power of the concentration of reactant.
force – in mechanics, physical agency that changes the velocity of an object of constant mass.
frequency factor – reaction rate parameter at a given temperature.
function – variable quantity related to one or more other variables in terms of which it may be expressed or on the value of which its own value depends.

Gibbs free energy – thermodynamic potential for a system under the constraints of constant temperature and constant pressure.
glucose – predominant source of chemical *energy* in cells.
glycolysis – anaerobic conversion of sugar to lactate or pyruvate with the production of *ATP*.
group-transfer potential – driving force for the chemical transfer of a given type of chemical group, e.g. phosphoryl group.
half life – time required for half of a given amount of reactant to be converted into product.
half-reaction – conceptual reduction reaction showing the transfer of electrons explicitly.
heat – *energy* transfer by random motion. Compare *work*.
heat capacity – change in enthalpy per unit change in temperature.
heat capacity at constant pressure – change in enthalpy per unit change in temperature at constant pressure. The heat capacity specifies the temperature dependence of the enthalpy and entropy functions.
heat engine – system that uses heat transfer to do work.
heat sink – thing which absorbs thermal *energy*.
heat source – thing which radiates thermal *energy*.
helix propensity – one of several definitions is the relative probability that an amino acid type is found in helical structure in the folded states of proteins.
Henderson–Hasselbalch equation – mathematical relationship between pH and *acidity constant*.
Hess's Law – additivity of independently determined enthalpies; a statement of the First Law.
heteroallostery – allosteric regulation in which the *effector* is a different chemical species from the *substrate*.
Hill equation – mathematical relationship between free ligand concentration, binding constant, number of cooperative subunits, and fractional saturation of binding sites; named after Archibald Hill.
Hill plot – popular but non-ideal graphical representation of binding data which can be used to determine the cooperativity of binding, named after Archibald Hill.
homoallostery – allosteric regulation in which the *effector* is the same chemical species as the *substrate*.
hydrogen electrode – standard for measurement of redox potential.
hydrogen exchange – *acid*- and *base*-catalyzed chemical *process* in which one labile hydrogen atom is exchanged for another, generally donated by the solvent.
hydrophobic interaction – in biological macromolecules, particularly proteins, favorable intermolecular interaction between apolar moieties, e.g. aliphatic amino acid side chains.
ideal gas law – quantitative relationship between pressure, volume, temperature, the number of moles of ideal gas in a closed system.
information content – minimum number of binary decisions required to construct a message.
information theory – science of data communication.
inherent meaning – meaning of a message that is completely independent of an observer.
interaction – effect of one object on another.
internal energy – thermodynamic *state function* that measures the *energy* within the *system*.

intrinsic property – quantity that does not depend on amount of substance present, for example pressure. Compare *extrinsic property*.
intrinsic rate of exchange – rate of exchange of a specific labile hydrogen atom with solvent, usually in a completely unstructured polypeptide.
irreversibility – dissipation or destruction.
isoelectric point – pH at which the net charge on a macromolecule (usually a polypeptide) is zero.
isolated system – one permeable neither to heat nor matter.
isosbestic point – wavelength at which the value of a spectroscopic variable is independent of the structure of the system (usually a macromolecule in solution).
isothermal system – one at constant temperature.
isothermal titration calorimetry (ITC) – measures heat exchanged at constant temperature and pressure in binding experiment.
kinetic barrier – synonym of *activation energy*.
Kirchoff's enthalpy law – mathematical relationship between reference state enthalpy change, heat capacity change, temperature change, and overall enthalpy change.
KNF model – one of the two most popular models of allosteric regulation, named after Koshland, Némethy, and Filmer. See *MWC model*.
Langmuir adsorption isotherm – mathematical relationship between the free ligand concentration, the association constant, and degree of saturation of binding sites at constant temperature; named after Irving Langmuir.
latent heat – the enthalpy of a phase change, i.e. of the reorganization of the state of matter.
law – in science and philosophy, a theoretical principle stating that a particular phenomenon always occurs if certain conditions are met.
Le Châtelier's principle – system at equilibrium responds to a disturbance of a system by minimizing the effect of the disturbance.
life – qualitative property of highly organized matter, essential features of which are: growth, development, metabolism, and reproduction under the control of a genetic program, and reproduction by means of transmission of genetic material.
Lifson–Roig model – popular model of helix–coil transition theory.
ligand – ion or molecule (other than an enzyme substrate) that binds (usually specifically) to a macromolecule (usually a protein).
linking number – parameter describing the number of complete turns of the DNA backbone within defined boundaries.
local unfolding – fluctuation of structure, usually a protein, not complete unfolding. May result in some amide protons becoming available for exchange but not others.
machine – structure of any kind; an apparatus for applying power.
macroscopic system – comprises such a large number of particles that measured properties at equilibrium are approximately constant in time and fluctuations are relatively small.
Marcus theory – widely accepted mathematical description of the energetics of electron transfer.
mass action – in a system at equilibrium, a change in the amount of reactants (products) results in a compensating change in the amount of products (reactants), so that the relative proportion of reactants and products is minimized.

mass action ratio – ratio of the product of the activities (concentrations) of products to the product of the activities (concentrations) of reactants.
mean chemical potential – chemical potential of a chemical species whose activity is calculated as a *mean ionic activity*.
mean ionic activity – geometric mean of the activities of the ions of an electrolyte.
methanogens – bacteria that live only in oxygen-free milieus and generate methane by the reduction of carbon dioxide.
microscopic system – one member of a large ensemble of identical objects, for example, one protein molecule in a concentrated solution of identical protein molecules.
minus end – synonym of *pointed end*.
mean free path – average distance between collisions of particles.
melting temperature – temperature at which a solid undergoes a phase transition to the liquid state; in the case of proteins, temperature at which denaturation occurs.
metabolism – the biological enzymatic breakdown of molecules.
Michaelis–Menten equation – useful mathematical relationship of enzyme kinetics, named after the biochemists who described it.
molecular motor – *energy*-consuming protein molecule involved in force generation.
molecular switch – protein molecule whose conformation and biological function is controlled by binding (e.g. of protons, dissolved gas molecules, inorganic ions, small organic compounds, proteins, nucleic acids . . .).
molten globule – partly-ordered state of proteins, characterized by compactness, intact secondary structure, and fluctuating tertiary structure.
momentum – mass times velocity.
MWC model – one of the two most popular models of allosteric regulation, named after Monod, Wyman, and Changeux. See *KNF model*.
nanotechnology – development of systems and devices at a length scale below 100 nm.
Nernst equation – mathematical relationship between electrical potential across a membrane and the ratio of the concentrations and valences of ions on either side of the membrane, named after Walther Nernst.
non-competitive inhibition – inhibition of enzyme activity resulting from the binding of an inhibitor to a location on the surface of the enzyme other than the site where the substrate binds (active site).
non-equilibrium thermodynamics – thermodynamic concepts and relationships pertinent to systems that are not at *equilibrium*.
nucleation – in helix–coil transition theory, the formation of the first $i,i+4$ hydrogen bond of an α-helix; with regard to microfilaments, the formation of a sufficiently stable complex of actin monomers to enable polymerization (see *polymerization assays*).
number of ways – number of distinct arrangements of particles in a system.
observable quantity – measurable property of a system.
observer – key component of any scientific experiment, from deciding what the aim of the experiment will be, to how the experiment is designed, to how the data are analyzed, and what the data mean. The role of the observer is highlighted in information theory.
open system – permeable to heat and matter.

optimization – process whereby an observable quantity of a system is made as large or as small as possible within given constraints.
order – non-arbitrary arrangement of things. Compare *random*.
order of reaction – sum of the powers of the molar concentrations of the reactants in the *rate law* of a reaction.
osmosis – movement of water across a semi-permeable membrane from a region of low to a region of high impermeant solute concentration.
osmotic pressure – *pressure* arising from *osmosis*, e.g. by the displacement of a quantity of solvent against the force of gravity.
osmotic work – (mechanical) *work* done by *osmosis*.
oxidant – synonym of "oxidizing agent," electron acceptor. Compare *reductant*.
panspermia – plausible hypothesis that life on Earth originated at a remote location.
partition function – sum of all relevant statistical weights of a system.
passive transport – diffusive movement of an ion or molecule across a membrane, often through a protein pore.
path function – thermodynamic properties that relate to the preparation of the state.
phase – a state of matter that is uniform throughout, both in chemical composition and physical state.
phosphates – important constituent of many biological molecules, e.g. *ATP*, DNA, ...
phosphoanhydride bond – type of bond that is cleaved when *ATP* is hydrolyzed to ADP.
photosynthesis – biological *process* by which photosynthetic bacteria and plants convert the *free energy* of photons into chemical energy.
plus end – synonym of *barbed end*.
pointed end – minus end of actin filament, where ADP-bound actin monomers dissociate from polymer. So named from appearance of myosin S1 fragment-bound actin filaments by electron microscopy.
polymerization assays – means of testing the effect of a potential actin-binding protein on the polymerization of actin.
potential information – in information theory, maximum possible information content of a message. Compare *actual information*.
precursors – subunits of which a polymer is made, e.g., free amino acids in the case of a polypeptide.
pressure – force per unit area.
process – course of action or succession of actions, taking place or carried on in a definite manner.
propagation – in helix–coil transition theory, lengthening of the helix following nucleation.
protection factor – ratio of intrinsic rate of exchange to measured rate of exchange of polypeptide backbone amide proton.
proton motive force – proton concentration gradient across a membrane and the membrane electrical potential.
quenched-flow pulse labeling – technique for measuring the rate of stabilization of structure in proteins.
random – not sent, guided, or arranged in a discernibly special way. Compare *order*.
rate constant – proportionality constant between *rate of reaction* and molar concentrations of reactants.
rate-determining step – slowest step of a multi-step chemical reaction.

rate law – experimentally determined mathematical relationship between molar concentrations of reactants and *rate of reaction*. See *rate of reaction*.

rate of reaction – mathematical expression in terms of molar concentrations of reactants and *rate constant*.

rectangular hyperbola – shape of ligand-binding curve when saturation is plotted as a function of free ligand concentration and there is no binding *cooperativity*, as in the case of myoglobin.

redox – chemical *process* in which electrons are transferred from the reductant to the oxidant. Important in metabolic reactions. See *redox couple* and *standard redox potential*.

redox couple – electron donor and acceptor.

reductant – synonym of "reducing agent," electron donor. Compare *oxidant*.

reference state – the most stable state of an element under defined conditions; alternatively, any set of defined conditions.

respiration – biological *process* by which oxygen is used as an electron acceptor in the metabolism of food, principally glucose.

reversibility – *process* runs equally well backwards or forwards and is always in a near equilibrium state.

RNA world – hypothesis that living organisms were preceded on Earth by abiotic synthesis of RNA.

salt bridge – energetically favorable electrostatic interaction between an ionized acid and an ionized base.

salting in – increased solubility of protein in low ionic strength aqueous solution relative to pure water. Compare *salting out*.

salting out – decreased solubility of protein in high ionic strength aqueous solution relative to low ionic strength solution. Compare *salting in*.

saturation – complete filling of available binding sites by *ligand*.

Scatchard plot – popular but non-ideal graphical representation of binding data which can be used to determine the binding affinity and number of ligand binding sites.

Second Law of Thermodynamics – statement regarding the increase of the entropy of the universe for any real process.

second messenger – intracellular signaling molecule the concentration of which rises or falls in response to *binding* of an extracellular *ligand* to a receptor.

second-order reaction – one in which reactant rate is proportional to the second power of the concentration of reaction rate.

self-organization – spontaneous appearance of order on a local level.

semantic level – levels of meaning.

sliding filament model – model of interaction between actin and myosin underlying force generation in skeletal muscle.

solubility – the extent to which a chemical species, for instance a metabolite or protein, will dissolve in a solvent, usually water in the life sciences.

somatic mutation – major mechanism by which *antibody diversity* is generated; involves point mutations in B cells.

somatic recombination – major mechanism by which *antibody diversity* is generated; involves genetic recombination in B cells.

specific heat – the heat capacity per unit mass of material.

spontaneity – tendency of a chemical reaction to proceed in a certain direction without the addition of *energy*.

stability – difference in *free energy* between states, usually between the unfolded and folded states of a protein.
stability curve – variation of stability with some independent variable (usually temperature but often denaturant concentration).
standard redox potential – measured voltage difference between a half cell consisting of both members of a *redox couple* (an electron donor and acceptor) in their standard states and a standard reference half cell (usually a *hydrogen electrode*).
standard state – unambiguous reference state.
standard state enthalpy change – enthalpy change for a *process* under standard conditions.
state – thermodynamic state of a system, for example, the folded conformation or unfolded conformation of a protein, or the liquid or solid state of water.
state of the system – specified by values of state variables.
state function – thermodynamic quantity whose value depends only on the current *state of the system* and is independent of how the *system* was prepared, e.g. internal *energy*, *enthalpy*, *entropy*.
state variable – thermodynamic quantity under the control of the observer which, when fixed, determines the state of the system, e.g. pressure, volume, temperature.
statistical factors – binding constant coefficients related to the number of ways in which ligands can associate with and dissociate from a macromolecule.
statistical weight – synonym of *Boltzmann factor*.
steady state – condition of an open system in which the rate of flow of *energy* or a substance into the system is identical to the rate of flow out of the system. Compare *equilibrium*.
steady state assumption – in enzyme kinetics, assumption that the time rate of change of concentration of enzyme–*substrate* is zero.
substrate – chemical compound on which an enzyme acts.
substrate cycling – in metabolism, the formation and breakdown of a certain molecular compound that results in a net change in the concentration of *ATP* but not of the molecular compound itself.
sulfate-reducing bacteria – prokaryotic organisms that can grow and reproduce at temperatures as high as 100 °C and under very high pressure.
supercoiling – coiling of circular double-stranded DNA.
surroundings – the part of the universe that is not the system.
symbol – abstract representation of a thing. For instance, "G" can represent the base guanine, the amino acid glycine, or the Gibbs free energy. The meaning of the sign will of course depend on the context.
system – part of universe chosen for study.
temperature – measure of *thermal energy*, or how fast molecules are moving.
temperature of maximum stability – temperature at which the free energy difference between the folded and unfolded states of a protein at a given pressure is a maximum.
thermal energy – the average energy of a particle at a given temperature.
thermodynamic potential function – measures the *free energy* difference between states and is therefore an indicator of whether a reaction will be spontaneous.

thermodynamics – the study of the nature of heat and its relationship to other forms of *energy*.

thermostability – the *free energy* difference between states, usually of a macromolecule, under specified conditions, especially *temperature*.

Third Law of Thermodynamics – the *entropy* of a *system* approaches zero as the *temperature* goes to absolute zero (0 K).

titration – gradual filling up or removal of a *ligand* from a binding site. See *dissociation constant*.

transfer free energy – *free energy* change on transfer of a compound from one medium to another, for example from an organic solvent phase to aqueous solution.

transition – structural change of a system, e.g. the unfolding of a protein from conformation (e.g. the folded state) to another (e.g. a denatured state).

transition state – the crucial configuration of atoms at which the potential energy of reactants is a maximum.

transition state theory – a means of identifying the main features governing the size of a rate constant with a model description of a chemical reaction.

transition temperature – temperature at which a phase change occurs; in the context of protein denaturation, the temperature of the midpoint of a folding/unfolding transition.

transmission coefficient – the proportionality constant when the rate of passage of the activated complex through the transition state is assumed to be proportional to the vibrational frequency along the reaction coordinate.

treadmilling – simultaneous polymerization and depolymerization of a filamentous structure, e.g. an actin microfilament.

turnover number – the number of catalytic reactions per enzyme molecule per unit time.

twist – parameter describing the frequency of turns of the DNA double helix.

two-state approximation – in order–disorder transitions, characterization of a structural change in terms of two states (e.g. folded and unfolded, native and denatured), and in ligand binding, characterization of the equilibrium in terms of just the bound and unbound states of the ligand.

van der Waals interaction – type of intermolecular interaction named after van der Waals.

van't Hoff enthalpy – the *enthalpy* change calculated from the *temperature* dependence of the *equilibrium constant*.

van't Hoff graph – a plot of the equilibrium constant versus temperature, from which the van't Hoff enthalpy can be obtained.

velocity – speed of motion in a certain direction (mechanics) or rate of enzyme catalysis (enzyme kinetics).

voltage – synonym of *electrical potential*, named after Alessandro Volta.

work – *energy* transfer by organized motion. Compare *heat*.

writhe – *parameter* describing the pathway of the DNA backbone in space in the supercoiling of circular DNA.

Zeroth Law – simple argument by which the concept of *temperature* is justified.

Zimm–Bragg model – one of the two most popular models of helix–coil transition theory, named after the persons who were the first to describe it.

Index of names

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