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.

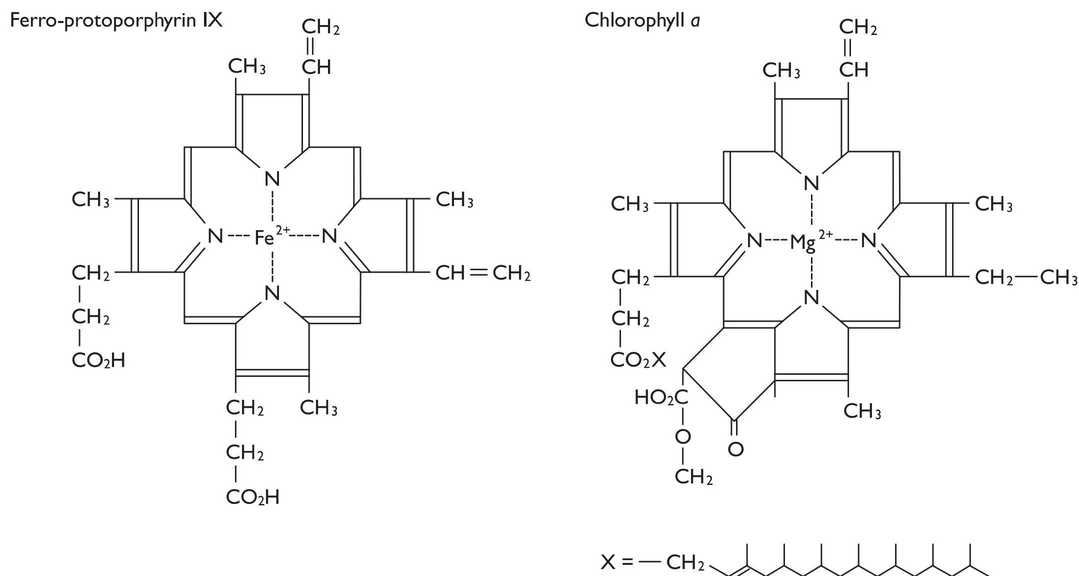


Fig. 5.1 Molecular formulas of the heterocyclic ring systems of ferro-protoporphyrin IX (heme) and chlorophyll *a*. The bound iron of heme is normally in the Fe(II) (ferrous, 2+) oxidation state regardless of whether oxygen is bound. The structure of chlorophyll *b* is nearly identical to that of chlorophyll *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.

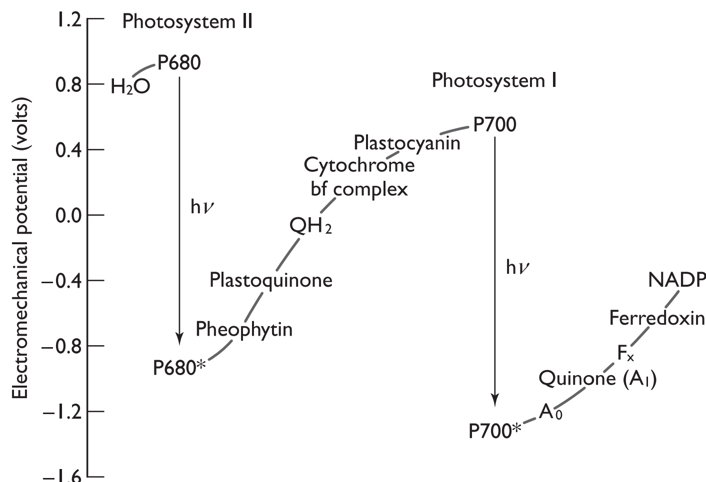


Fig. 5.2 Schematic diagram ("Z-scheme") of the energetics of electron transport in photosynthesis. The electrochemical potential (free energy) is measured in volts. Electrons tend to flow spontaneously from a state of higher to lower free energy. In terms of electrochemical potential, electrons migrate 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).

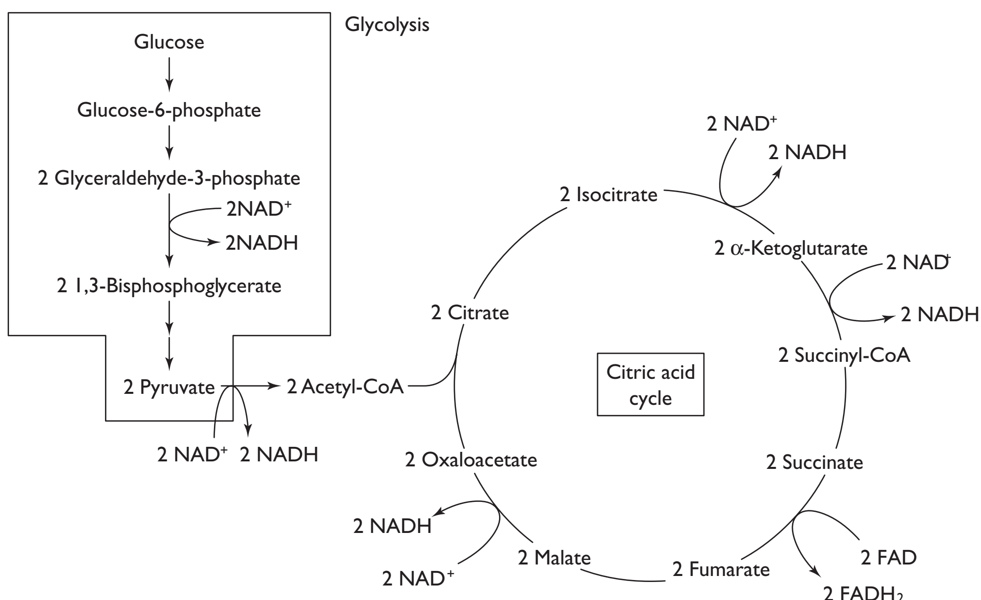


Fig. 5.3 Schematic diagram of glycolysis and the citric acid cycle. The figure shows the points at which the electron carriers NAD^+ and FAD are reduced by electron transfer to form NADH and $FADH_2$.

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

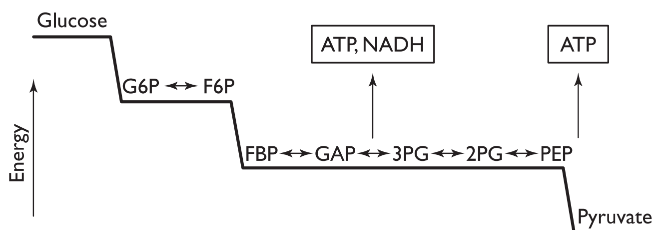
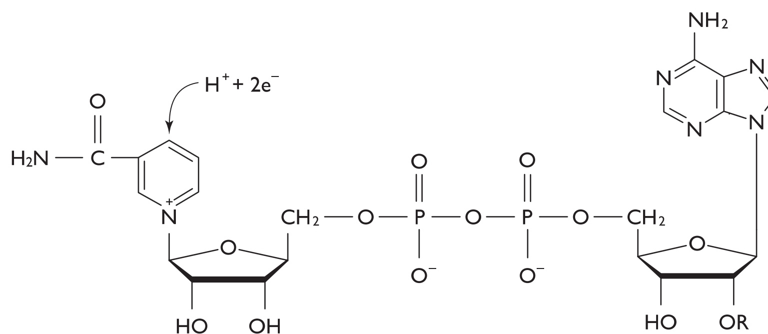


Fig. 5.4 Control by phosphofructokinase (PFK) of the flux of glycolysis breakdown products. Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose bisphosphate; GAP, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate. The 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).

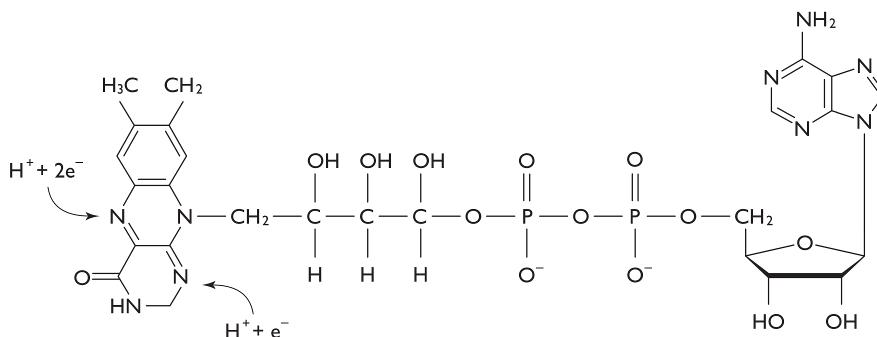
conversion of pyruvate to ethanol and CO_2. In the presence of oxygen, the three carbons of pyruvate are completely oxidized to CO_2.

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

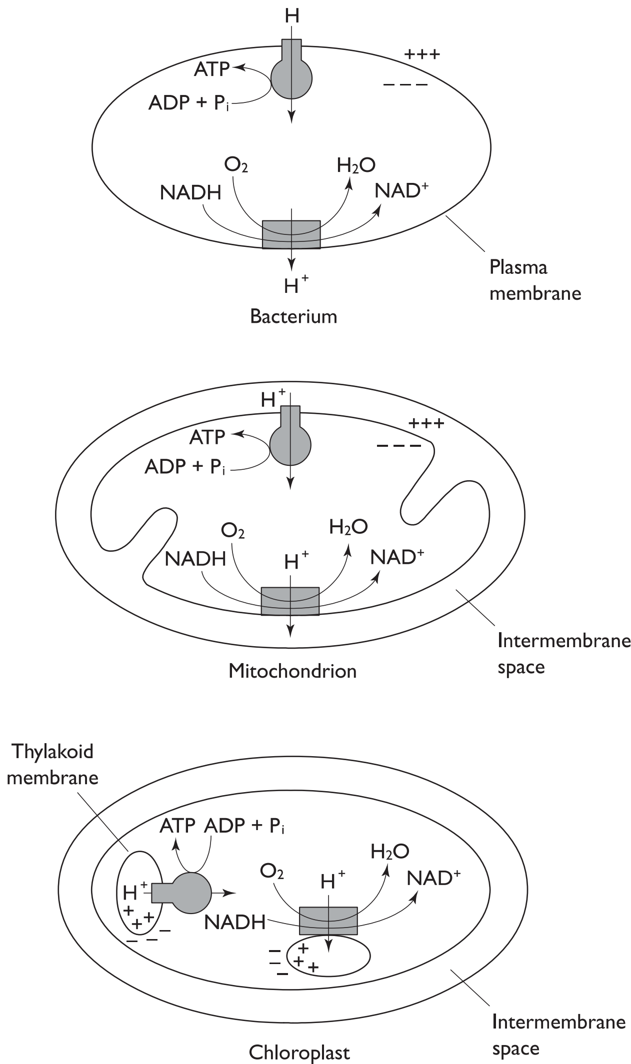


Fig. 5.6 Proton movement in bacteria, mitochondria, and chloroplasts. Note the similarities and differences in membrane orientation and direction of proton movement. In bacteria, mitochondria, and chloroplasts, the protein complex in which ATP is synthesized in situated on the cytosolic face of the membrane. 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).

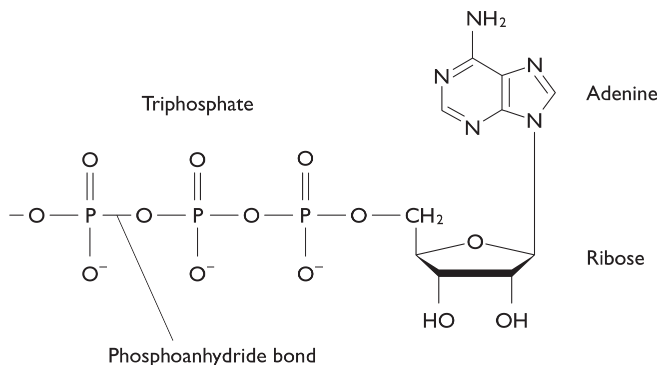


Fig. 5.7 The structure of adenosine triphosphate. There are three main components: adenine, a base found in RNA and DNA; ribose, a sugar; and triphosphate. In most biochemical reactions in which it is involved, ATP is hydrolyzed to ADP and inorganic phosphate. The bond broken in this reaction is a phosphoanhydride 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

$$ATP + H_2O \Leftrightarrow ADP + P_i + H^+. \quad (5.3)$$

Using Eqns. (4.32) and (4.38), the free energy change for this reaction can be expressed as

$$\Delta G = \Delta G^{\circ\prime} + RT\ln[ADP][P_i]/[ATP]. \quad (5.4)$$

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} × 298 K × ln(0.010)] = -7 kcal mol^{-1} − 2.7 kcal mol^{-1} ≈ -10 kcal mol^{-1} ≈ − 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 ~50 × [AMP] and ~10 × [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 $\Delta H^\circ = -4\ \text{kcal mol}^{-1}$. 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 $-3\ \text{kcal mol}^{-1}$. 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.

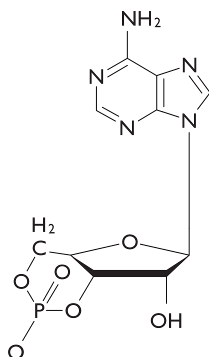


Fig. 5.8 Cyclic AMP. This molecule, which is synthesized from ATP, plays a key role in a variety of cellular processes. Principal among these is the control of glycogen metabolism in muscle. Glycogen is the highly branched high molecular mass glucose polysaccharide that higher animals synthesize to protect 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

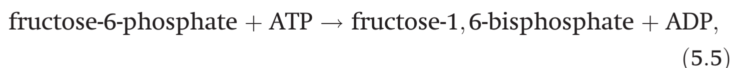
Box 5.1. Cont.

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.

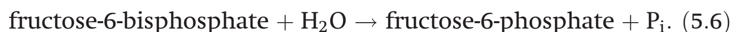
D. Substrate cycling

The reaction catalyzed by the glycolytic enzyme phosphofructokinase is highly exergonic. Under physiological conditions,



$$\text{fructose-6-phosphate} + \text{ATP} \rightarrow \text{fructose-1,6-bisphosphate} + \text{ADP}, \quad (5.5)$$

with $\Delta G = -25.9 \text{ kJ mol}^{-1}$. 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:



$$\text{fructose-6-bisphosphate} + H_2O \rightarrow \text{fructose-6-phosphate} + P_i. \quad (5.6)$$

This also occurs spontaneously because $\Delta G = -8.6 \text{ kJ mol}^{-1}$, 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.

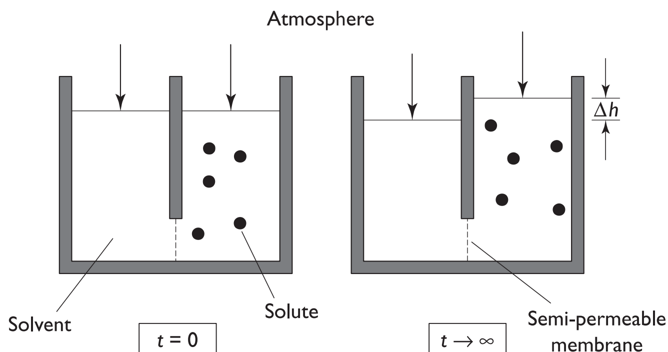


Fig. 5.9 A simple osmometer. A solute can move freely in a fraction of the total volume of solvent. The solution is separated from pure solvent by a membrane that is permeable to the solvent but not the solute. There is a net flow of solvent from the pure solvent to the solution, resulting in the development 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 $\mu_1 - \mu_1^{\text{o}} = \Delta\mu < 0$ (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

$$\Delta \mathrm{G}/\Delta n_1 = \mu_1 = V_{\mathrm{m},1}\Delta p, \tag{5.9}$$

where $V_{\mathrm{m},1}$ 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 $V_{\mathrm{m},1} \approx V_1^{\circ}$, 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, $\mu_1 - \mu_1^{\text{o}} = 0$. 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):

$$\pi = \frac{C_2RT}{M_2}(1 + B_1(T)C_2 + B_2(T)C_2^2 + \ldots). \tag{5.12}$$

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

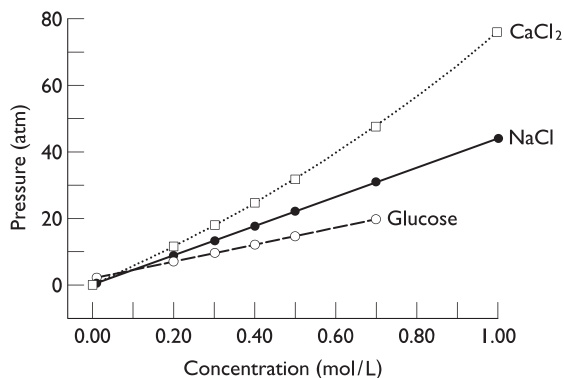


Fig. 5.10 Osmotic pressure measurements. Osmotic pressure increases with concentration of solute, as predicted by the van't Hoff law. The pressure at a given concentration of solute depends significantly on the solute. If the solute is a salt, dissociation in aqueous solution will result in a greater 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:

$$\pi_{\text{total}} = \pi_1 + \pi_2 + \cdots + \pi_n = \Sigma\pi_i. \quad (5.13)$$

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, $\mathbf{P}$ $(mT)^{\frac{1}{2}}$. In other words, the more massive the particle, the greater its momentum. Note that $\mathbf{P}$ 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).

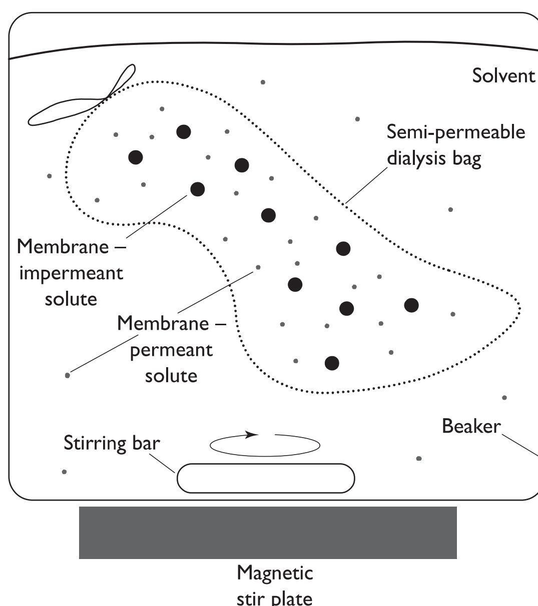


Fig. 5.11 Dialysis. A dialysis bag containing a membrane-impermeant solute is submerged in solvent in a beaker. Membrane-permeant solute appears on both sides of the membrane. The dialysis bag-solvent system is not at equilibrium. At equilibrium, the concentration of membrane-permeant solute will be 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

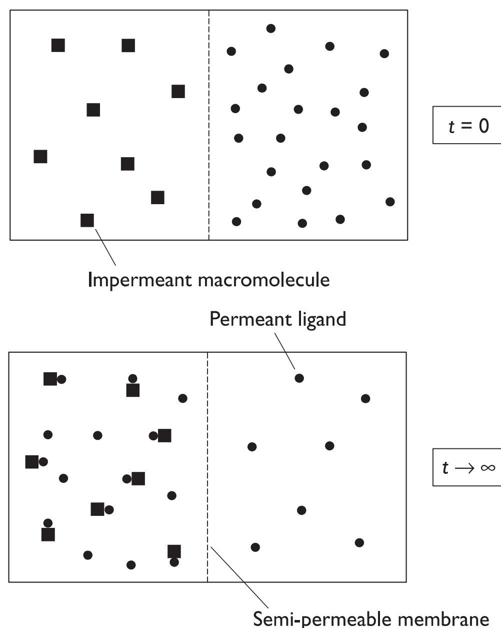


Fig. 5.12 Equilibrium dialysis. At the beginning of the experiment ($t = 0$), the membrane-impermeant macromolecule and membrane-permeant ligand are on opposite sides of a semi-permeable dialysis membrane. The two-chambered system is not at equilibrium. After a long time ($t \to \infty$), the concentration 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 $[\text{DNA}] \rightarrow 0$. 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, $[\text{Na}^{+\beta}] > [\text{Na}^{+\alpha}]$. 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

$$\mu^{\alpha}_{\text{NaCl}} = \mu^{\beta}_{\text{NaCl}}. \tag{5.14}$$

Let's keep things simple and assume that the solution is ideal. The chemical potential of the salt is

$$\mu_{\text{NaCl}} = \mu^{\circ}_{\text{NaCl}} + RT\ln[\text{Na}^+][\text{Cl}^-]. \tag{5.15}$$

At equilibrium, the standard state chemical potential must be the same in both phases, so

$$[\text{Na}^{+\alpha}][\text{Cl}^{-\alpha}] = [\text{Na}^{+\beta}][\text{Cl}^{-\beta}]. \tag{5.16}$$

And the net charge of each phase must be equal to zero, a condition known as *electroneutrality*, which is expressed mathematically as

$$[\text{Cl}^{-\alpha}] = [\text{Na}^{+\alpha}] \tag{5.17}$$

$$z[\text{DNA}^{\beta}] + [\text{Cl}^{-\beta}] = [\text{Na}^{+\beta}], \tag{5.18}$$

where z is the number of negative charges on the DNA. With a bit of algebra, these equations can be combined to give

$$[\text{Na}^{+\beta}] = [\text{Na}^{+\alpha}]\left(1 + \frac{z[\text{DNA}^{\beta}]}{[\text{Cl}^{-\beta}]}\right)^{1/2} \tag{5.19}$$

$$[\text{Cl}^{-\beta}] = [\text{Cl}^{-\alpha}]\left(1 - \frac{z[\text{DNA}^{\beta}]}{[\text{Na}^{+\beta}]}\right)^{1/2}. \tag{5.20}$$

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

$$\begin{aligned}[I]_i/[I]_o &= \exp(-nF\Delta \mathbf{V}/RT)\\ &= \exp[(-96.5\ \text{kJ}\,\text{V}^{-1}\text{mol}^{-1} \times 0.12\ \text{V})/(8.314\,\text{J}\,\text{mol}^{-1}\,\text{K}^{-1} \times 300\,\text{K})]\\ &= 0.01.\end{aligned} \tag{5.25}$$

$[I]_o$ 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,

$$\begin{aligned}\Delta\mu = {} & [2.3 \times 8.314\,\text{J mol}^{-1}\,\text{K}^{-1} \times 298\,\text{K} \times (-0.75)] \\ & + [1 \times 96\,500\,\text{J V}^{-1}\,\text{mol}^{-1} \times (-0.17\,\text{V})],\end{aligned} \tag{5.27}$$

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

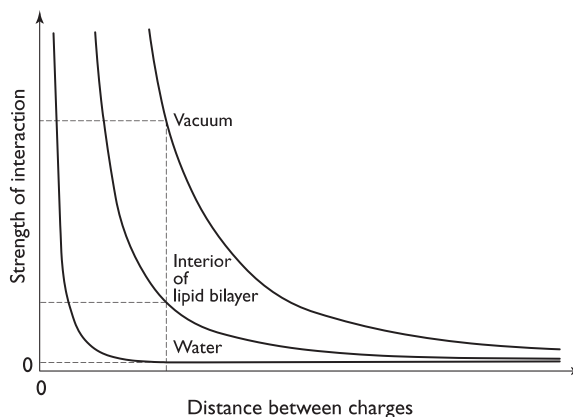


Fig. 5.13 Dependence of electrostatic energy on distance between charges and medium. The energy is inversely proportional to distance, so attraction or repulsion is greatest when the charges are close to each other. The energy also depends substantially on the stuff between the charges, varying inversely 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

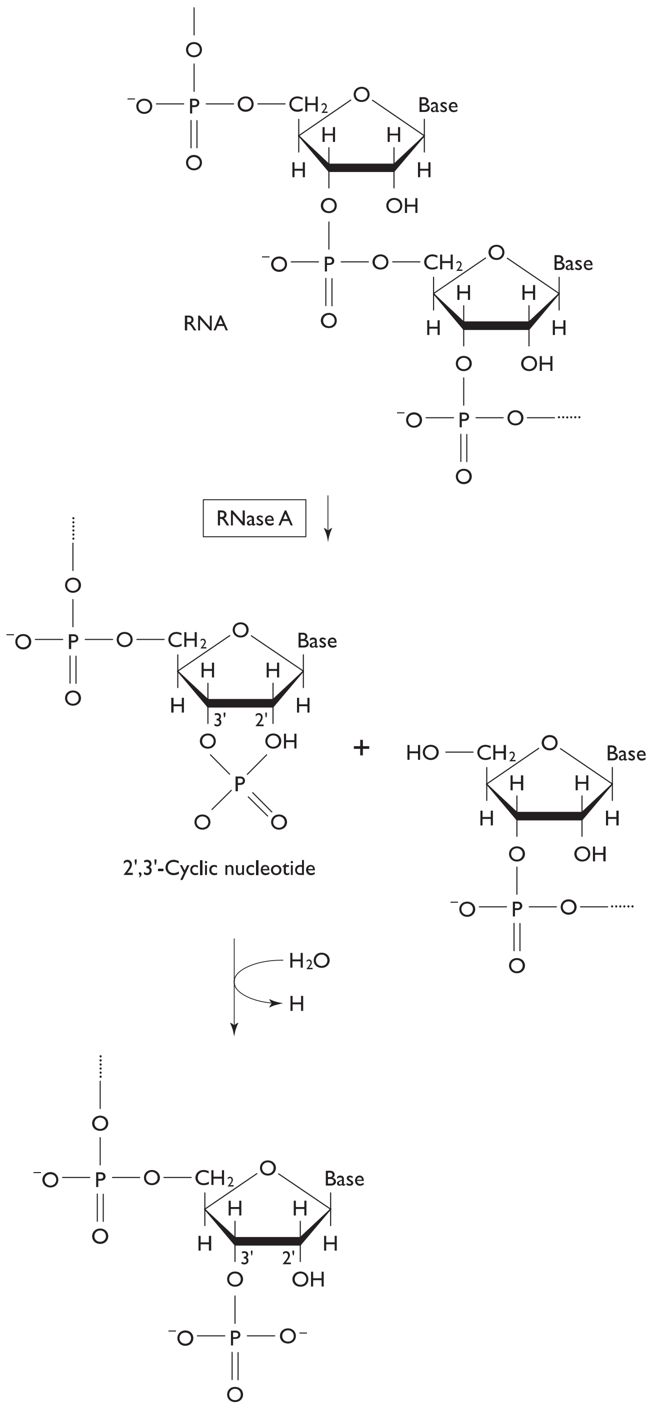
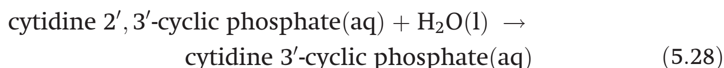


Fig. 5.14 Mechanism of RNase A activity. Bovine pancreatic ribonuclease A is an example of enzyme-mediated acid–base catalysis. The enzyme hydrolyzes RNA to its component nucleotides. The reaction scheme is based on the experimental finding that 2′,3′-cyclic nucleotides are present in RNase digests of 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



$$\text{cytidine } 2', 3'\text{-cyclic phosphate(aq)} + \text{H}_2\text{O(l)} \rightarrow \text{cytidine } 3'\text{-cyclic phosphate(aq)} \quad (5.28)$$

How might one determine ΔG° for this reaction?

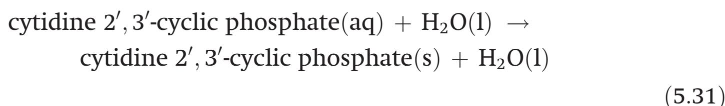
From Eqn. (4.32),

$$\Delta G = \Delta H - T\Delta S. \quad (5.29)$$

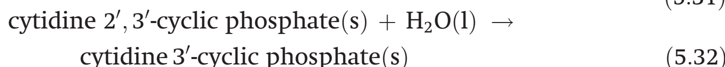
If the products and reactants are in the standard state, the thermodynamic relationship is

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ. \quad (5.30)$$

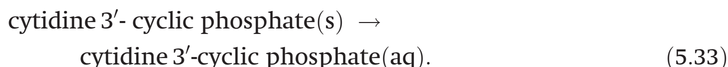
We'll need to know ΔH° and ΔS° to calculate the energy. The enthalpy change of the reaction, which can be estimated by calorimetry, is – 2.8 kcal mol^{-1}. But what is ΔS°? One approach would be to make use of the fact that S is a state function and combine measurements that, when summed, give ΔS° for Eqn. (5.28). The reaction scheme might look like this:



$$\text{cytidine } 2', 3'\text{-cyclic phosphate(aq)} + \text{H}_2\text{O(l)} \rightarrow \text{cytidine } 2', 3'\text{-cyclic phosphate(s)} + \text{H}_2\text{O(l)} \quad (5.31)$$



$$\text{cytidine } 2', 3'\text{-cyclic phosphate(s)} + \text{H}_2\text{O(l)} \rightarrow \text{cytidine } 3'\text{-cyclic phosphate(s)} \quad (5.32)$$



$$\text{cytidine } 3'\text{- cyclic phosphate(s)} \rightarrow \text{cytidine } 3'\text{-cyclic phosphate(aq)}. \quad (5.33)$$

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 K × 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

$$R + L \Leftrightarrow R \bullet L, \tag{5.34}$$

where R is the receptor, L signifies *free* ligand molecules, and $R \bullet L$ 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

$$K_a = [R \bullet L]/([R][L]) \tag{5.35}$$

and the *dissociation* constant is

$$K_d = K_a^{-1} = [R][L]/[R \bullet L] = ([R]_T - [R \bullet L])[L]/[R \bullet L], \tag{5.36}$$

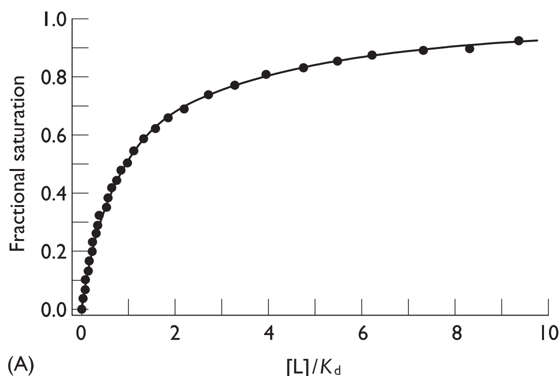
where $[R]_T = [R \bullet L] + [R]$ is the total receptor concentration. The fractional occupancy of ligand-binding sites, F_b, is

$$F_b = [R \bullet L]/[R]_T = [L]/(K_d + [L]). \tag{5.37}$$

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

$$[R \bullet L]/[L] = ([R]_T - [R \bullet L])/K_d. \tag{5.38}$$



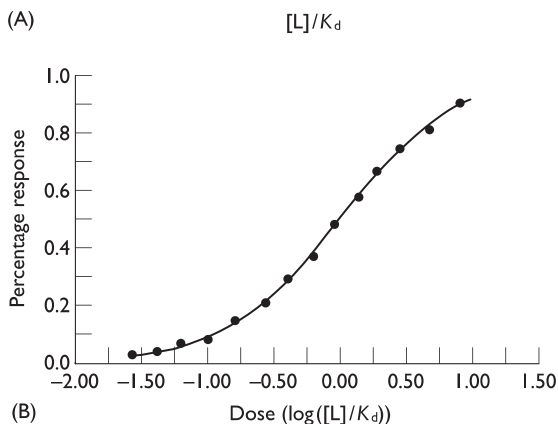


Fig. 5.15 Binding. In panel (A) the circles are experimental data points, the solid line is a theoretical description of binding. There is one ligand-binding site per macromolecule. In such cases, the mathematical relationship between the bound and free ligand concentrations is a rectangular hyperbola. 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.

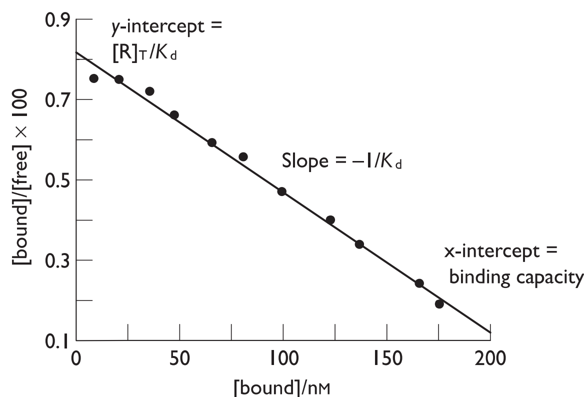


Fig. 5.16 Scatchard plot. The concentration of bound ligand divided by the concentration of free ligand is plotted against the concentration of bound ligand (nM). When binding data are presented in this way, the slope measures the negative inverse of the dissociation constant ($-1/K_d = -K_a$). The vertical 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.

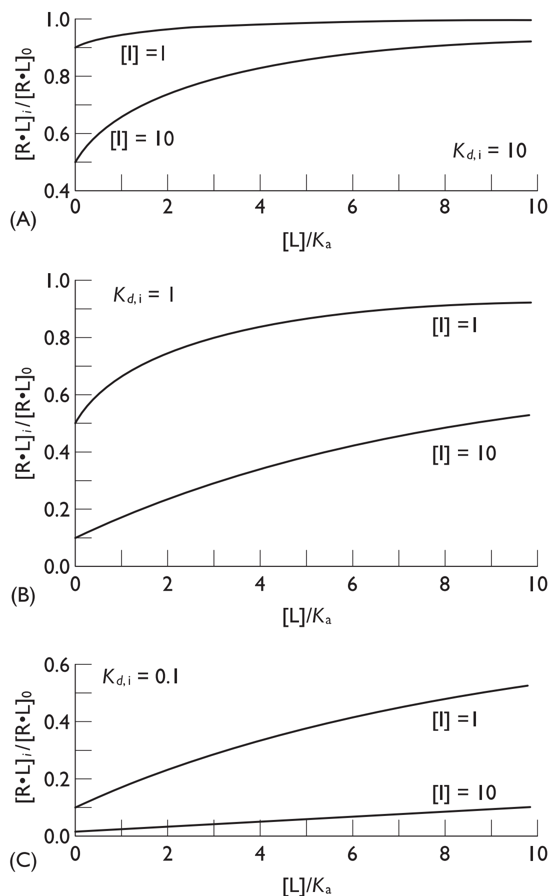


Fig. 5.17 Effect of inhibitor on ligand binding. When the concentration of inhibitor i is low and the inhibitor dissociation constant is high, as in panel (A), $[R\bullet L]_i/[R\bullet L]_0$ is nearly 1 even at low concentrations of ligand. Competition between ligand and inhibitor is more evident when 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.

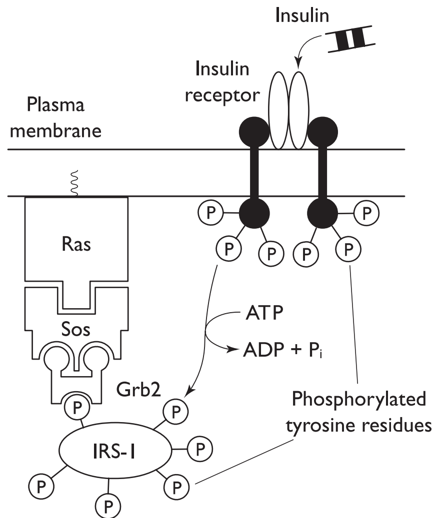


Fig. 5.18 Protein–protein interactions and phosphoryl transfer in signal transduction. The extracellular concentration of the hormone insulin, a peptide signal, is communicated across the plasma membrane by means of dimeric insulin-specific transmembrane receptor molecules. The binding of insulin to 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	$\Delta G°$ (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

$$Hb(aq) + O_2(g) \Leftrightarrow HbO_2(aq). \tag{5.43}$$

From experiments it is known that $K = 85.5\ \text{atm}^{-1}$ for the reaction as written. At 19 °C, $\Delta G = -2580\ \text{cal mol}^{-1}$. 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):

$$\begin{aligned}\Delta G &= G(O_2, 0.2\ \text{atm}) - G°(O_2, 0.2\ \text{atm}) - (G(O_2, 1\ \text{atm}) - G°(O_2, 1\ \text{atm}))\\ &= RT\ln(O_2, 0.2\ \text{atm}) - RT\ln(O_2, 1\ \text{atm})\\ &= RT\ln(0.2/1)\\ &= -930\ \text{cal mol}^{-1}.\end{aligned} \tag{5.44}$$

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:

$$Hb(aq) + O_2(g) \Leftrightarrow HbO_2(aq) \tag{5.45}$$

$$O_2(aq) \Leftrightarrow O_2(g), \tag{5.46}$$

which, when summed, give

$$Hb(aq) + O_2(aq) \Leftrightarrow HbO_2(aq). \tag{5.47}$$

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).

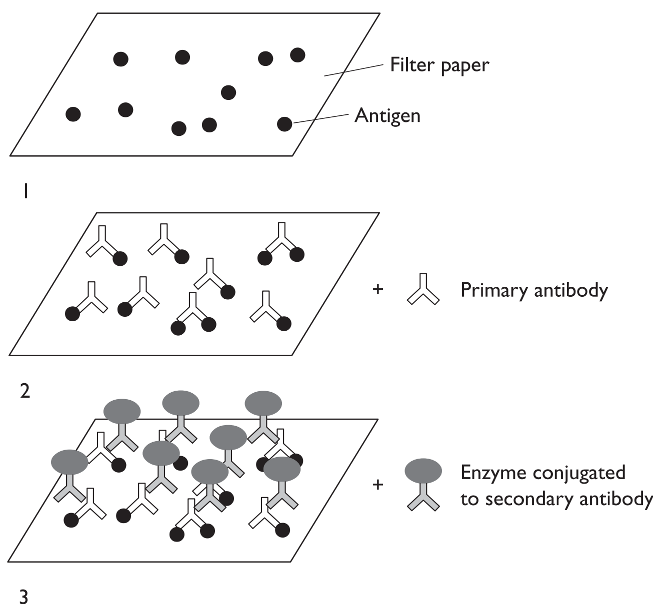


Fig. 5.19 ELISA. This very useful laboratory technique consists of three basic steps. First, the protein antigen is adhered to a solid support, often a nitrocellulose filter. This partially denatures the antigen. Next, (primary) antibodies are allowed to bind to the antigen. Finally, (secondary) antibodies 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

$$D \Leftrightarrow S. \tag{5.48}$$

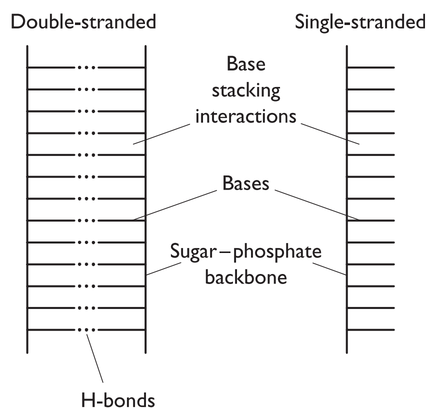


Fig. 5.20 Double-stranded and single-stranded DNA. DNA is composed of bases attached to a sugar–phosphate backbone. (See Fig. 5.14 for a higher resolution view of polynucleic acid.) There are two major types of interaction that stabilize double-stranded DNA: intermolecular hydrogen bonds (polar) and 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

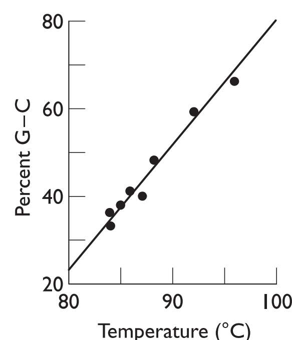


Fig. 5.21 Influence of G–C content on DNA melting temperature. As the percentage of G–C pairs increases, the number of intermolecular hydrogen bonds per base pair increases. The stabilizing effect on double-stranded DNA is reflected in the relationship between G–C content and melting temperature.

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.

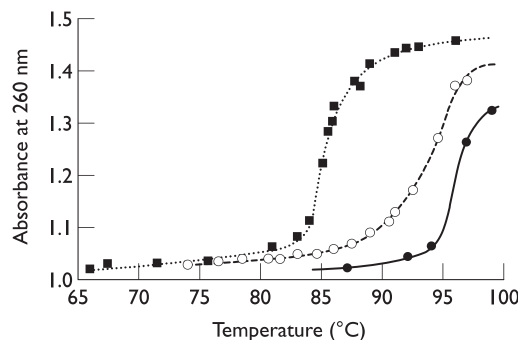


Fig. 5.22 DNA melting curves. The melting temperature varies not only with G–C content but also with size. In other words, a 100 base pair-long double-stranded DNA molecule will have a higher melting temperature than a 50 base pair-long double-stranded DNA for a given percentage and distribution of G–C pairs. Differences in G–C content and distribution and molecular size lead to differences in melting temperature.

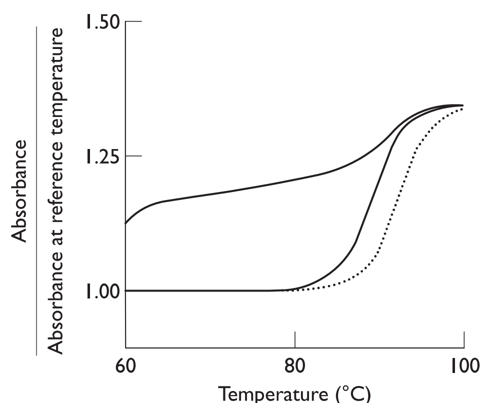


Fig. 5.23 Melting and cooling profile for double-stranded DNA. Solid line. The melting of double-stranded DNA is cooperative. Relatively few base pairs are broken below the melting temperature. Once melting has begun, however, relatively small increases in temperature result in the rupture of a relatively 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

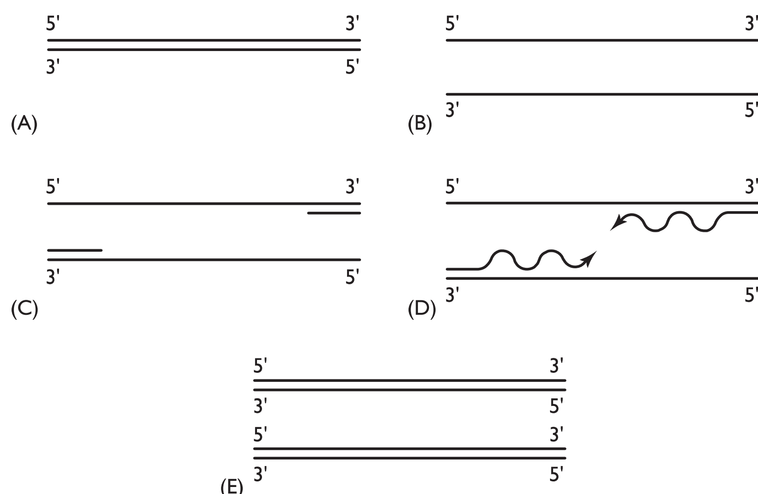


Fig. 5.24 Schematic diagram of PCR. When heated, a double-stranded template DNA (A) melts into two single-stranded molecules (B). If cooling is rapid, the template will not be able to reform. In the excess of complementary oligonucleotide primer, however, binding will occur at temperatures below the 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.

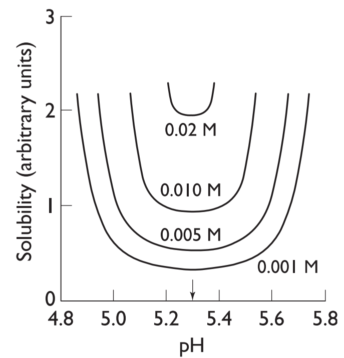


Fig. 5.25 Protein solubility. This depends not only on the net charge of the protein, which varies with pH, but also on ionic strength and temperature. The data shown are for a milk protein called β-lactoglobulin at different concentrations of NaCl. Solubility is very low at pH 5.3, the approximate isoelectric point of the protein. At this pH, the solubility of β-lactoglobulin increases exponentially with increasing ionic strength (solubility $\approx 0.255e^{101pH}$). 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

$$\mathrm{F} \Leftrightarrow \mathrm{U}. \qquad (5.56)$$

The equilibrium constant (Eqn. (4.11)) is then

$$K_{eq} = [\mathrm{U}]/[\mathrm{F}]. \qquad (5.57)$$

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

constant data. Now let's consider protein denaturation in terms of free energy. At constant temperature, Eqn. (4.2) becomes

$$\Delta G_d^\circ = \Delta H_d^\circ - T\Delta S_d^\circ, \tag{5.58}$$

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

$$\Delta G_d^\circ(T) = \Delta H_d^\circ(T_r) + \Delta C_p(T - T_r) - T[\Delta S_d^\circ(T_r) + \Delta C_p \ln(T/T_r)], \tag{5.59}$$

where the subscript "r" means "reference." $\Delta G_d^\circ(T)$ is not $(\Delta G_d^\circ \times T)$ 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 ΔC_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}. $\Delta G_d^\circ(T)$ 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 $\Delta G_d^\circ(T)$ 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

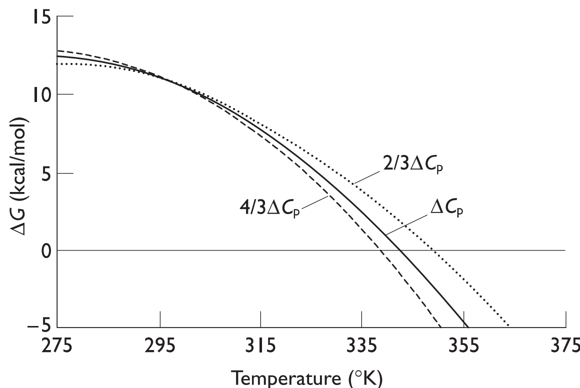


Fig. 5.27 Protein stability curves. Each one is a plot of ΔG versus T (or some other independent variable). This gives the free energy difference between the unfolded state and the folded state (the minimum amount of work that must be done to induce a transition from the folded state to the unfolded 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

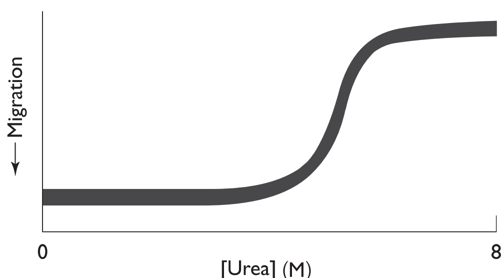


Fig. 5.28 Urea denaturation gel electrophoresis. Structural transitions in protein can be studied by polyacrylamide gel electrophoresis. The horizontal axis is a linear gradient in urea concentration. When the concentration is sufficiently high, the protein unfolds. This is detected as a change in the 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

$$\Delta(\Delta G_d)/\Delta(\text{pH}) = 2.3RT\Delta Q, \tag{5.61}$$

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

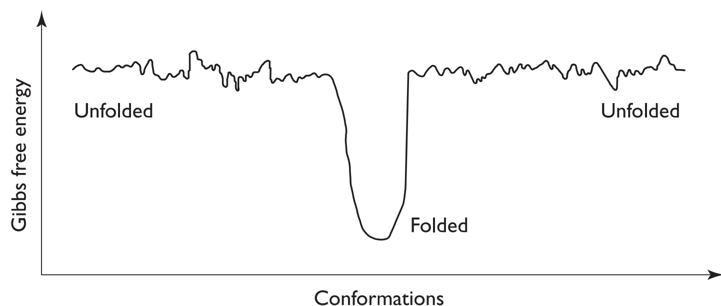


Fig. 5.26 Free energy profile of a "well-behaved" small protein. There are only two stable states: the folded state and the unfolded state. The number of unfolded conformations is vastly greater than the number of folded conformations. Although the unfolded conformations differ in energy, these differences 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.

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!

T. References and further reading

Adair, G. (1925). The osmotic pressure of hæmoglobin in the absence of salts. *Proceedings of the Royal Society of London A*, **109**, 292–300.

Anfinsen, C. B. (1973). Principles that govern the folding of protein chains. *Science*, **181**, 223–30.

Arakawa, T. & Timasheff, S. N. (1985). Theory of protein solubility. *Methods in Enzymology*, **114**, 49–77.

Atkins, P. W. (1994). *The Second Law: Energy, Chaos, and Form*, ch. 8. New York: Scientific American.

Baker, T. A. (1999). Trapped in the act. *Nature*, **401**, 29–30.

Barth, R. H. (1992). Dialysis. In *Encyclopedia of Applied Physics*, ed. G. L. Trigg, vol. 4, pp. 533–5. New York: VCH.

Ben-Shem, A., Frolow, F. & Nelson, N. (2003). Crystal structure of plant photosystem I. *Nature*, **426**, 630–5.

Bergethon, P. R. (1998). *The Physical Basis of Biochemistry: the Foundations of Molecular Biophysics*, ch. 13.1. New York: Springer-Verlag.

Brandts, J. F. (1964). The thermodynamics of protein denaturation. I. The denaturation of chymotrypsinogen. *Journal of the American Chemical Society*, **86**, 4291–301.

Brandts, J. F. (1964). The thermodynamics of protein denaturation. II. A model of reversible denaturation and interpretations regarding the stability of chymotrypsinogen. *Journal of the American Chemical Society*, **86**, 4302–14.

Bridger, W. A. & Henderson, J. F. (1983). *Cell ATP*. New York: John Wiley.

Brønsted, J. N. (1923). *Recueil des Travaux Chimiques des Pays-Bas*, **42**, 718–28.

Chothia, C. (1984). Principles that determine the structure of proteins, *Annual Review of Biochemistry*, **53**, 537–72.

Christensen, H. N. & Cellarius, R. A. (1972). *Introduction to Bioenergetics: Thermodynamics for the Biologist: A Learning Program for Students of the Biological and Medical Sciences.* Philadelphia: W. B. Saunders.

Conti, B., Sanchez-Alavez, M., Winsky-Sommerer, R., Morale, M.C., Lucero, J., Brownell, S., Fabre, V., Huitron-Resendiz, S., Henriksen, S., Zorrilla, E.P., de Lecea, L. & Bartfai, T. (2006). Transgenic mice with a reduced core body temperature have an increased life span. *Science*, **314**, 825–8.

Cooper, A. (2001). Thermodynamics of protein-protein and peptide interactions. In Aggeli, A., Boden, N. & Zhang, S. (eds.) *Self-assembling Peptide Systems in Biology, Medicine and Engineering*, pp. 47–64. Dordrecht: Kluwer Academic.

Cramer, W.A. & Knaff, D.B. (1991). *Energy Transduction in Biological Membranes. A Textbook of Bioenergetics*. New York: Springer-Verlag.

Creighton, T.E. (1991). Stability of folded proteins. *Current Opinion in Structural Biology*, **1**, 5–16.

Dawes, E.A. (1962). *Quantitative Problems in Biochemistry*, 2nd edn, ch. 1. Edinburgh: E. & S. Livingstone.

Donnan, F.G. (1911). Title. *Zeitschrift für Elektrochemie und Angewandte Physikalische Chemie*, **17**, 572.

Encyclopædia Britannica CD 98, "Colligative Property," "Dialysis," "Metabolism," "Photosynthesis," "Saturation," and "Vapour Pressure."

Epstein, I.R. (1989). The role of flow in systems far-from-equilibrium, *Journal of Chemical Education*, **66**, 191–5.

Fersht, A.R. (1999). *Structure and Mechanism in Protein Science: a Guide to Enzyme Catalysis and Protein Folding*. New York: W. H. Freeman.

Fox, S. & Foster, J.S. (1957). *Introduction to Protein Chemistry*, p. 242. New York: John Wiley.

Franks, F. (1995). Protein destabilization at low temperatures. *Advances in Protein Chemistry*, **46**, 105–39.

Frauenfelder, H., Parak, F. & Young, R.D. (1988). Conformational substates in proteins. *Annual Review of Biophysics and Biophysical Chemistry*, **17**, 451–79.

Freeman, B. (1995). Osmosis. In *Encyclopedia of Applied Physics*, ed. G.L. Trigg, vol. 13, pp. 59–71. New York: VCH.

Fröhlich, H. (1969). Quantum mechanical concepts in biology. In *Theoretical Physics and Biology*, ed. M. Marios. Amsterdam: North-Holland.

Fruton, J.S. (1999). *Proteins, Enzymes, Genes: the Interplay of Chemistry and Biology*. New Haven: Yale University Press.

Garrett, J. (1990). Thermodynamics in sheep. *Education in Chemistry*, **27**, 127.

George, P. & Rutman, R.J. (1960). The 'high energy phosphate bond' concept. *Progress in Biophysics and Biophysical Chemistry*, **10**, 1–53.

Gillispie, Charles C. (ed.) (1970). *Dictionary of Scientific Biography*. New York: Charles Scribner.

Girandier, L. & Stock, M.J. (eds.) (1983). *Mammalian Thermogenesis*. London: Chapman & Hall.

Gutfreund, H. (1949). In *Hæmoglobin: a Symposium Based on a Conference Held at Cambridge in June 1948 in Memory of Sir John Barcroft*, ed. F.J.W. Roughton and J.C. Kendrew, p. 197. London: Butterworths.

Haase, R. (1969). *Thermodynamics of Irreversible Processes*. New York: Dover.

Harold, F.M. (1986). *The Vital Force: a Study of Bioenergetics*. New York: W. H. Freeman.

Harris, D.A. (1995). *Bioenergetics at a Glance*, ch. 1. Oxford: Blackwell Science.

Hatefi, Y. (1985). The mitochondrial electron transport and oxidative phosphorylation system. *Annual Review of Biochemistry*, **54**, 1015–69.

Haynie, D.T. (1993). *The Structural Thermodynamics of Protein Folding*, ch. 4. Ph.D. thesis, The Johns Hopkins University.

Hinckle, P. C. & McCarty, R. E. (1978). How cells make ATP. *Scientific American*, **238**(3), 104–23.

Karplus, M. & McCammon, J. A. (1986). Protein dynamics. *Scientific American*, **254**(4), 30–9.

Katchalsky, A. & Curran, P. F. (1967). *Nonequilibrium Thermodynamics in Biophysics*. Cambridge, Massachusetts: Harvard University Press.

Kauzmann, W. (1958). Some factors in the interpretation of protein denaturation. *Advances in Protein Chemistry*, **14**, 1–63.

Klotz, I. M. (1986). *Introduction to Biomolecular Energetics*, cc. 3–7. Orlando: Academic Press.

Kondepudi, D. & Prigogine, I. (1998). *Modern Thermodynamics: from Heat Engines to Dissipative Structures*, ch. 8.2. Chichester: John Wiley.

Kyoguko, Y., Lord, R. C. & Rich, A. (1969). An infrared study of the hydrogen-bonding specificity of hypoxanthine and other nucleic acid derivatives. *Biochimica et Biophysica Acta*, **179**, 10–17.

Lamm, M. S., Rajagopal, K., Schneider, J. P. & Pochan, D. J. (2005). Laminated morphology of nontwisting b-sheet fibrils constructed via peptide self-assembly. *Journal of the American Chemical Society*, **127**, 16 692–700.

Lodish, H., Baltimore, D., Berk, A., Zipursky, S. L., Matsudaira, P. & Darnell, J. (1995). *Molecular Cell Biology*, 3rd edn, cc. 2, 4 & 21. New York: W. H. Freeman.

Lowell, B. B. & Spiegelman, B. M. (2000). Towards a molecular understanding of adaptive thermogenesis. *Nature*, **404**, 652–60.

Makhatadze, G. I. & Privalov, P. L. (1995). Energetics of protein structure. *Advances in Protein Chemistry*, **47**, 307–425.

Matthews, B. W. (1995). Studies on protein stability with T4 lysozyme. *Advances in Protein Chemistry*, **46**, 249–78.

McCammon, J. A. & Harvey, S. C. (1987). *Dynamics of Proteins and Nucleic Acids*. Cambridge: Cambridge University Press.

Millar, D., Millar, I., Millar, J. & Millar, M. (1989). *Chambers Concise Dictionary of Scientists*. Cambridge: Chambers.

Mitchell, P. (1976). Vectorial chemistry and the molecular mechanisms of chemiosmotic coupling: power transmission by proticity *Biochemical Society Transactions*, **4**, 398–430.

Morowitz, H. J. (1978). *Foundations of Bioenergetics*, ch. 3E. New York: Academic Press.

Nicholls, D. G. & Ferguson, S. J. (1992). *Bioenergetics 2*, ch 3. London: Academic Press.

Noyes, R. M. (1996). Application of the Gibbs function to chemical systems and subsystems. *Journal of Chemical Education*, **73**, 404–8.

O'Brien, C. (1994). New enzyme structure reveals cell's rotary engine. *Science*, **265**, 1176–7.

Osyczka, A., Moser, C. C., Daldal, F. & Dutton, P. L. (2004). Reversible redox energy coupling in electron transfer chains. *Nature*, **427**, 607–12.

Pardee, G. S. & Ingraham, L. L. (1960). Free energy and entropy in metabolism. In *Metabolic Pathways*, ed. D.M. Greenberg, vol. I. New York: Academic Press.

Pauling, L. C. (1970). Structure of high energy molecules. *Chemistry in Britain*, **6**, 468–72.

Pepys, M. B., Hawkins, P. N., Booth, D. R., Vigushin, D. M., Tennent, G. A., Soutar, A. K., Totty, N., Nguyent, O., Blake, C. C. F., Terry, C. J., Feest, T. G., Zalin, A. M. & Hsuan, J. J. (1993). Human lysozyme gene mutations cause hereditary systemic amyloidosis. *Nature*, **362**, 553–7.

Peusner, L. (1974). *Concepts in Bioenergetics*, cc. 3, 5, 6, 7 & 10–8. Englewood Cliffs: Prentice-Hall.

Plum, G. E. & Breslauer, K. J. (1995). Calorimetry of proteins and nucleic acids. *Current Opinion Structural Biology*, **5**, 682–90.

Prigogine, I. (1967). *Introduction to Thermodynamics of Irreversible Processes*. New York: John Wiley.

Prigogine, I. (1969). Structure, dissipation and life. In *Theoretical Physics and Biology*, ed. M. Marios. Amsterdam: North-Holland.

Prigogine, I., Nicolis, G. & Babloyants, A. (1972). Thermodynamics of evolution. *Physics Today*, **25** (11), 23–8.

Prigogine, I., Nicolis, G. & Babloyants, A. (1972). Thermodynamics of evolution. *Physics Today*, **25** (12), 38–44.

Record, M. T., Jr, Zhang, W. & Anderson, C. F. (1998). Analysis of effects of salts and uncharged solutes on protein and nucleic acid equilibria and processes: a practical guide to recognizing and interpreting polyelectrolyte effects, Hofmeister effects, and osmotic effects. *Advances in Protein Chemistry*, **51**, 281–353.

Rich, P. (2003). Chemiosmotic coupling: The cost of living. *Nature*, **421**, 583.

Roberts, T. J., Marsh, R. L., Weyland, P. G., & Taylor, C. R. (1997). Muscular force in running turkeys: the economy of minimizing work. *Science*, **275**, 1113–15.

Rondelez, Y., Tresset, G., Nakashima, T., Kato-Yamada, Y., Fujita, H., Takeuchi, S. & Noji, H. (2005). Highly conserved ATP synthesis by F1-ATPase single molecules. *Nature*, **433**, 773–7.

Rovner, S. L. (2004). The secrets of aging. *Chemical & Engineering News*, **82**(34), 30–5.

Ruelle, D. (2001). A departure from equilibrium. *Nature*, **414**, 263–5.

Schellman, J. A. (1987). The thermodynamic stability of proteins. *Annual Review of Biophysics and Biophysical Chemistry*, **16**, 115–37.

Schnitzer, M. J. (2001). Doing a rotary two-step. *Nature*, **410**, 878–81.

Secrest, D. (1996). Osmotic pressure and the effects of gravity on solutions. *Journal of Chemical Education*, **73**, 998–1000.

Segal, I. H. (1976). *Biochemical Calculations: How to Solve Mathematical Problems in General Biochemistry*, 2nd edn, ch. 3. New York: John Wiley.

Shavit, N. (1980). Energy transduction in chloroplasts. *Annual Review of Biochemistry*, **49**, 111–39.

Shaw, A. & Bott, R. (1996). Engineering enzymes for stability. *Current Opinion in Structural Biology*, **6**, 546–50.

Shortle, D. (1996). The denatured state (the other half of the folding equation) and its role in protein stability. *Federation of the American Societies for Experimental Biology Journal*, **10**, 27–34.

Smith, C. A. & Wood, E. J. (1991). *Energy in Biological Systems*, cc. 1.3 & 1.4. London: Chapman & Hall.

Snell, F. M., Shulman, S., Spencer, R. P. & Moos, C. (1965). *Biophysical Principles of Structure and Function*, ch. 8. Reading, Massachusetts: Addison-Wesley.

Spolar, R., Livingstone, J. & Record, M. T., Jr. (1992). Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water. *Biochemistry*, **31**, 3947–55.

Tanford, C. (1968). Protein denaturation (parts A and B). *Advances in Protein Chemistry*, **23**, 121–282.

Timasheff, S. N. (1993). The control of protein stability and association by weak interactions with water: how do solvents affect these processes? *Annual Review of Biophysics and Biomolecular Structure*, **22**, 67–97.

Timasheff, S. N. (1998). Control of protein stability and reactions by weakly interacting cosolvents: the simplicity and the complicated. *Advances in Protein Chemistry*, **51**, 355–432.
Timbrell, J. A. (1991). *Principles of Biochemical Toxicology*, 2nd edn, ch. 7. London: Taylor & Francis.
van Holde, K. E. (1985). *Physical Biochemistry*, 2nd edn, cc. 2.1, 2.3, 2.4, 3.4 & 3.5. Englewood Cliffs: Prentice-Hall.
Voet, D. & Voet, J. G. (1995). *Biochemistry*, 2nd edn, cc. 3, 4, 15–4–15–6, 16, 18–1, 19–1, 20, 22, 28–3, 28–5A & 34–4B. New York: John Wiley.
Weber-Ban, E. U., Reid, B. G., Miranker, A. D. & Horwich, A. L. (1999). Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. *Nature*, **401**, 90–3.
Williams, S. (1999). Life as a part-time plant. *Wellcome News*, **20**, 38.
Williams, T. I. (ed.) (1969). *A Biographical Dictionary of Scientists*. London: Adam & Charles Black.
Woodcock, A. & Davis, M. (1978). *Catastrophe Theory*. Harmondsworth: Penguin.
Wrigglesworth, J. (1997). *Energy and Life*, cc. 3, 5.7.2, 7.1, 7.3 & 7.5.1. London: Taylor & Francis.
Yasuda, R., Noji, H., Yoshida, M., Kinosita, K., Jr & Itoh, H. (2001). Resolution of distinct rotational substeps by submillisecond kinetic analysis of F_1-ATPase. *Nature*, **410**, 898–904.
Youvan, D. C. & Marrs, B. L. (1987). Molecular mechanisms of photosynthesis. *Scientific American*, **256** (6), 42–8.
Zouni, A., Witt, H.-T., Kern, J., Fromme, P., Krauß, N., Saenger, W. & Orth, P. Crystal structure of photosystem II from Synechococcus elongates at 3.8 Å resolution. *Nature*, **409**, 739–43.

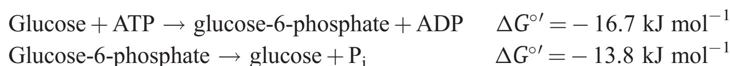
U. Exercises

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 $[M]/[M]_T \rightarrow 1$ as $[M]_T \rightarrow 0$, and that $[M]/[M]_T \rightarrow 0$ as $[M]_T \rightarrow \infty$. 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 $(kg\ H_2O)^{-1}$.

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

$$T_m = 41.1 X_{G+C} + 16.6 \log[Na^+] + 81.5, \quad (5.62)$$

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?