9 Protonmotive energy-transducing systems: some physical principles and experimental approaches

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9.1 Introduction and scope

If the metabolites are extracted from a cell, or observed *in situ* using a technique such as nuclear magnetic resonance, a morass of different compounds will, of course, be seen. Questions which one might wish to ask about groups of these compounds (Fig. 9.1) include:

(a) are they direct precursors or products of each other;
(b) if so what are the pathways by which they are interconverted; and
(c) does their intracellular activity differ substantially from their concentration (as estimated on the basis of (n)mol compound per unit membrane-enclosed volume)?

If they *do* bear a direct precursor–product relationship to each other, we might then enquire:

(d) as to the nature and mechanism of the enzymes catalysing their interconversion; and
(e) whether the metabolites are organized as a diffusible pool or whether there is a direct transfer of the protein-associated product of one reaction to the enzyme catalysing the 'next' reaction.

To give an answer to these types of question, the *criteria* which are involved in answering these questions must be considered, since a proper understanding of such criteria underpins much of what follows. The first part of this chapter, then, will address these points at a relatively fundamental level, seeking to indicate the difficulties which attach even to the simple, textbook types of question above. Since the present work constitutes an overview of bacterial bionenergetics, there will be special cause to ponder the fact that, whilst similar schemes to those of Figure 9.1 may be written for *bioenergetic* systems, there is an important additional difficulty. In contrast to metabolic substructures such as carbon skeletons, *free energy* is *not* perfectly conserved, and the degree of its conservation is not independent of time (Welch and Kell, 1986; Kamp and Westerhoff, 1987; Westerhoff and Kamp, 1987). Thus, even while clarifying the type of description sought for metabolic systems of the type given in Figure 9.1, it needs to be known to what extent such a description is suitable, even in principle, for bioenergetic systems. In other words, a distinction must be sought between *fundamental* and *phenomenological* descriptions of bioenergetic systems.

In the present context, this chapter enquires to what extent a minimal chemiosmotic scheme of the type shown in Figure 9.2 provides an adequate description of energy coupling in protonmotive systems. As the main example will be taken the ideas and data that pertain to the overall process of electron transport phosphorylation, not only in bacteria, but in other,

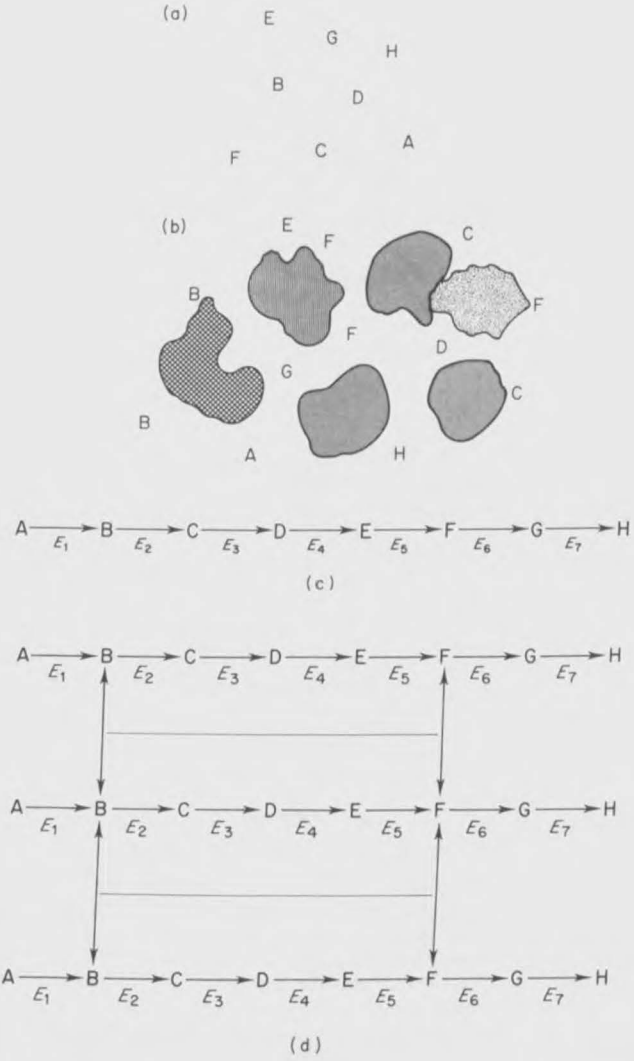


Fig. 9.1 (*a*) An ensemble of molecules as might be extracted from a cell. (*b*) *In vivo* some molecules are bound and some are free. Both their activity coefficients and standard chemical potentials may differ from those of the dilute aqueous extract in (*a*). (*c*) *In vivo* the molecules may form part of a metabolic pathway which operates in a particular order and a particular direction. The order is determined by the thermodynamics of the reaction A . . . → . . . H, the catalysts being enzymes of particular specificities. (*d*) Although one may observe a metabolic flux from A to H, as in (*c*), this does not show whether metabolites are free, diffusible, 'pool' intermediates (such as B and F here) or whether their transfer from enzyme to enzyme is direct, and not via a pool with a macroscopically definable activity or concentration.

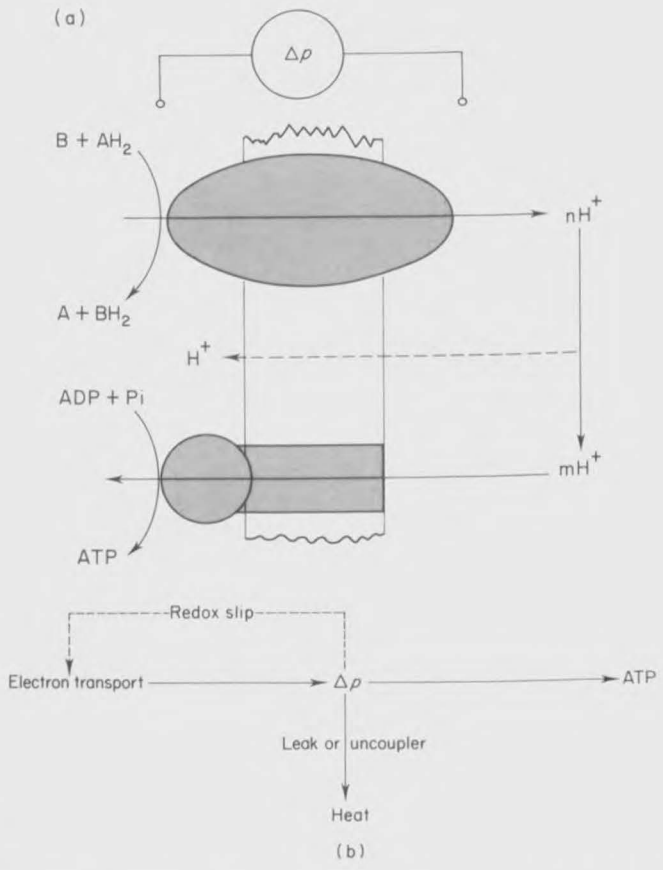


Fig. 9.2 A chemiosmotic coupling scheme. (*a*) A (redox-linked) primary proton pump creates a protonmotive force (pmf, Δp) across a (relatively) ion-impermeable coupling membrane. Protons may leak back across the membrane, a process stimulated by so-called uncoupler molecules, or may drive an otherwise endergonic reaction catalysed by a secondary proton pump, in this case an H^+-ATP synthase. No topological relationships between the primary and secondary proton pumps are specified since coupling is via a pmf. (*b*) The coupling between the chemical reactions and proton transfer catalysed by the H^+ pumps themselves may not be perfect, a phenomenon known as 'redox slip'.'Leak' refers to any H^+ pumped across the membrane by the primary pump but not coupled to the chemical reaction catalysed by the secondary H^+ pump, so that this definition includes slip within the secondary H^+ pump. Δp is by definition a delocalized intermediate, in equilibrium with the pmf between the two bulk aqueous phases that the coupling membrane serves to separate. It is thus freely available to all enzymes in the membrane vesicle. In the stationary state, the relation $z\mathrm{F}\Delta p \geqslant \Delta G_\mathrm{p}$ holds, where ΔG_p is the phosphorylation potential in the phase to which the ATP synthase is adjacent. *F*, Faraday's constant; *z*, the number of protons passing through the ATP synthase during the synthesis of one molecule of ATP (see also Table 1.2, p. 12).

evolutionarily related, free-energy-conserving membrane systems such as mitochondria and chloroplast thylakoids. Since the subject and its relevant literature are broad, if not limitless (Frank Harold's recent and beautifully structured overview required some 600 pages; Harold, 1986), and the shades of opinion, arguments and counterarguments ever-changing, it is necessary to be selective about the topics covered in any detail, notwithstanding a substantial list of references.

The major themes of this chapter are:

(a) that there are reasons strongly to doubt that the simplest purely chemiosmotic scheme for energy coupling is an adequate description of reality as reflected in the existing data;
(b) that something approximating the most extreme alternative viewpoint is as useful and defensible a hypothesis as is the scheme of Figure 9.2; i.e. That *electron transport-linked* phosphorylation *in vivo* is in *no* case *coupled* via a delocalized protonmotive force;
(c) that there are experiments and predictions which follow from some of the more plausible alternative schemes that are not simply consequential upon the chemiosmotic description embodied in the scheme of Figure 9.2.

At the more fundamental level it will be argued that it is unlikely *in principle* that the types of question that dominate the current bioenergetic literature are, in fact, altogether pertinent to the types of answer genuinely required. This is a much more difficult area, not for dwelling on here were it not that it is believed that the present book may foster the thinking and experimentation of the bioenergetics and bioenergeticists of the future. This said, it is time to look a little more closely at the metabolic system of Figure 9.1 and the energy coupling system of Figure 9.2, beginning with a discussion of some relevant chemical thermodynamics. A brief introduction to this topic is given in Chapter 1 (pages 9–11).

9.2 Elementary chemical thermodynamics; chemical and electrochemical potentials

9.2.1 Chemical potentials

The chemical potential of a substance i, usually denoted μ_i, is an intensive property of a system which describes the force which causes that system to relax to equilibrium. It may be thought of as the increase in the free energy of the system when one mole of compound i is added to an infinitely large quantity of the mixture so that the mixture does not significantly change its

composition. The chemical potential is, in fact, the partial derivative corresponding to the change in Gibbs free energy per unit change in the number of molecules of type i under conditions in which all other parameters such as temperature, pressure and the number of molecules of substances other than i remain constant (see e.g. Smith, 1982). The change in Gibbs free energy dG associated with a particular chemical reaction occurring in a single thermodynamic phase is given by:

$$dG = VdP - SdT + \Sigma\mu_i dn_i \tag{9.1}$$

where V, P, S and T are respectively the volume, pressure, entropy and temperature of the (closed) system and dn_i the change in the number of molecules of i. For almost all the biochemical reactions needed to be considered, temperature and pressure are controlled. Thus the first two terms on the right-hand side of equation (9.1) may be ignored (although remember that this is not so for special cases such as those involving changes in osmotic pressure in one or more phases), giving:

$$dG = \Sigma\mu_i dn_i \tag{9.2}$$

For a pure substance dissolved in water at a mole fraction x_i and forming an ideal solution, the chemical potential is:

$$\mu_i = \mu^\circ{}_i + RT \ln x_i \tag{9.3}$$

$\mu^\circ{}_i$ is the *standard chemical potential* of the substance in the phase to which the substance i is being added. This standard chemical potential may be defined in various ways, but the most usual is to define it for a solution of unit molality. For a *real* solution, equation (9.3) does not hold, due mainly to interactions between solute and solvent molecules (Bockris and Reddy, 1970). Thus the *activity* a_i of substance i is defined in terms of its chemical potential in the solution and in its standard state:

$$\mu_i = \mu^\circ{}_i + RT \ln a_i \tag{9.4}$$

The activity may be considered to represent the *effective concentration* per unit of component i relative to that in its standard state. They are related to each other by the activity coefficient γ_i:

$$a_i = \gamma_i x_i \tag{9.5}$$

For an ionic solution, such as a KCl solution in which the KCl molecules are fully ionized, there is a *different* standard state for each of the components, so that the chemical potential of a KCl solution is:

$$\mu_{KCl} = \mu^\circ{}_{K+} + \mu^\circ{}_{Cl-} + RT \ln a_{KCl} \tag{9.6}$$

It is particularly important to note that, in a mixture, the *standard chemical*

potential of a substance depends upon the chemical potentials of *all* other substances in the thermodynamic phase under consideration. Particular difficulties arise here in knowing what the actual activities of different compounds in the aqueous cytoplasm and in other intracellular or intra-organellar phases are, and what proportion of the molecules of a given type are bound or complexed. An introduction to these topics may be found in Clegg (1984), Ling (1984) and Welch and Clegg (1987). Parenthetically, it should also be mentioned that although one may speak about the activity coefficients of single ions, e.g. a_{K+}, such a thing cannot ever be measured since single ions cannot be added to a solution without also adding their counterions. Thus the well-known definition of pH, viz.:

$$\mathrm{pH} = -\log_{10} a_{H+} \tag{9.7}$$

is not a thermodynamic definition but merely a convention.

For a chemical reaction such as A + B ↔ C + D, the *extent of reaction* χ, which may have a value between 0 and 1, may be defined. If the position of the reaction is such that the only molecules present are those on the left-hand side of the reaction $\chi = 0$, whilst if the position of the reaction is such that the only molecules present are those on the right-hand side of the reaction then $\chi = 1$. For an actual chemical *change* such as A + B → C + D, we may refer to the change in Gibbs free energy per extent of reaction $dG/d\chi$, a variable that is often called ΔG (see, however, Welch, 1985). At equilibrium, $\Delta G = 0$ and the equilibrium constant of the reaction K_{eq} is given by the ratio of the activities of the products to those of the reactants when $\Delta G = 0$. The *standard* free energy change for the reaction, $\Delta G^{0\prime}$, is thus:

$$\Delta G^{0\prime} = -RT \ln K_{eq} \tag{9.8}$$

The prime indicates that the standard state is defined in a way that differs from that usual in chemistry, where the standard states are all of unit activity. Since this would mean considering for protons a pH $\simeq$ 0, a more convenient standard state is used for biochemical work, i.e. pH = 7.0. For reactions in which the mass action ratio (i.e. the product of the activities of the products divided by the product of the activities of the reactants) is not equal to the equilibrium constant, the *actual* free energy change ΔG associated with the transformation of a certain amount of reactants to the appropriate (stoichiometric) amount of products differs from the standard free energy change. In general:

$$\Delta G = \Delta G^{0\prime} + RT \ln \{(\Pi a_{\mathrm{PRODUCTS}})/(\Pi a_{\mathrm{REACTANTS}})\} \tag{9.9}$$

Therefore, as is well known, the direction in which a reversible reaction such as A + B ↔ C + D will go spontaneously depends upon the mass-action ratio of the different compounds involved and the equilibrium constant for

the reaction. The direction of a particular reaction may thus be changed simply by changing the standard chemical potential or the activity (coefficient) of one of the reactants. Thus the *primary criterion* by which the possible metabolic interconversions of the molecules in the system of Figure 9.1 is determined is whether or not such an interconversion is thermodynamically favourable.

9.2.2 Electrochemical potentials

If the reaction in which one is interested involves separate thermodynamic phases, the difference in pressure between those thermodynamic phases (if applicable) must be included and, for reactions involving charged compounds, an electrical term added. So far, the contribution of electrical factors to thermodynamic potentials has been ignored since thus far only isolated, macroscopically homogeneous thermodynamic phases have been considered in which significant differences in electrical potential may be assumed not to exist. Strictly, however, the *electro*chemical potential (denoted $\tilde{\mu}$) of an ion is what is of interest, and this differs from the chemical potential (equation 9.4) by the electrical potential (ψ) times the charge on the ion (z) times Faraday's constant (F):

$$\tilde{\mu}_i = \mu_i + z_i F\psi = \mu^\circ{}_i + RT \ln a_i + z_i F\psi \qquad (9.10)$$

The splitting up of the electrochemical potential into a standard chemical potential and an electrical part is not strictly necessary; the electrical term could just as easily be incorporated into the standard chemical potential. However, whilst this separation is considered controversial in some quarters, it has become common in bioenergetics to adopt this experimentally convenient convention.

Since *absolute* values of the electrical potential are inaccessible, it might be wondered whether the ψ term has any physical meaning; this point is discussed further by Walz (1979), who gives a helpful description of much of the relevant chemical thermodynamics. What is especially important for our purposes is that *differences* in electrical potential between two thermodynamic phases *may* be measured, using reversible reference electrodes (such as the Ag/AgCl electrode); differences in the *electrochemical* potential of an ion between such phases may be measured using an electrode reversible to that ion (such as a potentiometric glass pH electrode for protons). If there is no difference in the standard chemical potential of the ion in the two phases, differences in *chemical* potential may thus be obtained by difference, by means of equation 9.9. Similarly, since the standard chemical potential of an ion in a particular thermodynamic phase is only separated from its measured activity coefficient by convention (it cannot be separated experimentally), a

further simplification is acceptable: a system of interest may be allowed to come to equilibrium (so that there is no difference in electrochemical potential of the ion of interest between the two thermodynamic phases); the differences in *concentration* of the ion in the two phases can be measured; the standard chemical potential of the ion in the two phases be *assumed* to be the same; and all differences in concentration be *ascribed* to differences in the activity coefficients for the ion in the two phases. These points are illustrated, from both a theoretical and experimental standpoint, in Figure 9.3 and Table 1.2 (page 12).

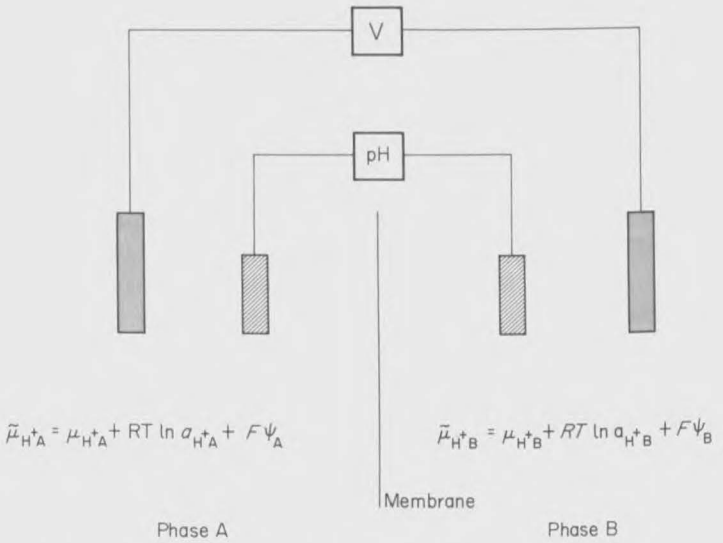


Fig. 9.3 The electrochemical potential of the proton in two adjacent thermodynamic phases separated by a membrane. The electrochemical potential of the proton in each phase is given (Eq. 9.9) by the expressions in the figure. However, since $pH = -\log_{10} a_{H+}$, $RT \ln a_{H+} = -2.303\ RT\ pH$ (since $2.303 = \ln 10$). Thus the difference in the electrochemical potential of the proton in the two phases is:

$$\Delta\tilde{\mu}_{H+\,(A-B)} = \Delta\mu^{\circ}_{H+\,(A-B)} + F\Delta\psi_{(A-B)} - 2.303\ RT\ \Delta pH_{(A-B)}.$$

If the standard chemical potentials are assumed to be the same on either side of the membrane:

$$\Delta\tilde{\mu}_{H+\,(A-B)} = F\Delta\psi - 2.303\ RT\ \Delta pH_{(A-B)}, \text{ with the units in kJ.}$$

If the membrane is permeable to ions other than protons, to allow the electrical potentials to be defined, $\Delta\psi$ may be measured with a voltmeter connected to reversible electrodes (such as Ag/AgCl), $\Delta\tilde{\mu}_{H+}$ with glass pH electrodes (which themselves contain reversible Ag/AgCl electrodes) and ΔpH by difference. For further details, see text, and also Table 1.2 (page 12).

Thus (Fig. 9.3) there is a definition of the difference in proton electrochemical potential between two phases which is not only thermodynamically correct but experimentally useful. Similar expressions may be written for the difference in (electro)chemical potential of any other ion between two such phases.

The 'membrane' in Figure 9.3 was used just as a permeability barrier, to stop the equilibration of the electrochemical potential difference of the protons in the two phases, and no other properties were ascribed to it. Similarly, the individual compartments, which the membrane served to separate, were considered as themselves to be homogeneous and at electrochemical equilibrium. Whereas the second description may be acceptable for present purposes, the first one is not sufficient, since there is always an *interfacial region* in which strict electroneutrality is not maintained. Especially in the case of a membrane which possessses fixed charges, and thus a surface potential (Fig. 9.4), the adjacent molecular layers will be excessively populated by ions of opposite charge, to form a so-called electrical double layer. The structure and thickness of this electrical double layer depend in a complex fashion upon the surface charge density, surface potential and number and valency of the ions of the bulk phase (e.g. Bockris and Reddy, 1970; McLaughlin, 1977; Barber, 1980, 1982; Pethig, 1986).

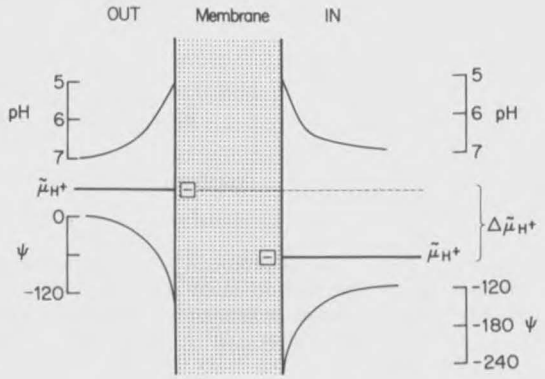


Fig. 9.4 The existence of fixed (surface) charges at the interface between a biomembrane and solution implies the existence of a surface potential. Under equilibrium conditions, this surface potential will change the extent to which Δp is distributed between $\Delta\psi$ and ΔpH but not the magnitude of Δp.

It is convenient initially to consider this interfacial region as one or more separate thermodynamic phases, and to think about it in the following way. As a negative charge is approached, the local electrical potential becomes more negative; however, the negative charge is also increasingly attractive to

protons (for electrostatic reasons) so that the *chemical* potential of the proton is increased. Thus, provided the system on one side of the membrane is *in equilibrium* ($\Delta G = 0$), the *electrochemical* potential of the proton will not be changed from that in the bulk phase as the surface is approached, whilst its local distribution between ψ and pH may be changed (see e.g. Junge, 1977; Nicholls, 1982). This is experimentally useful (provided the equilibrium assumption holds), since measurements in 'bulk' phases are generally more convenient and reliable than those in complex and heterogeneous interphases. However, and this is most important, spectroscopic (or other) probes *cannot* properly be used to measure the electrochemical potential difference between two bulk phases if the probes are at the same time bound to membranes or interfaces. *Only* if it is *known*, (a) that the system *is* in electrochemical equilibrium throughout the phases on a given side of the membrane, *and* (b) that the probes of the electrical and chemical parts of the proton electro-chemical potential difference bind in *identical* places, can success be hoped for. It is unlikely that either (let alone both) of these conditions is met in practical situations of interest to the bioenergeticist.

Acquaintance has now been made with the most important equations of macroscopic, equilibrium thermodynamics that are relevant to the questions to be discussed here, in particular the criterion to be used to decide whether it is *possible* that a given (named) reaction is taking place: is it associated, as written, with a negative change in (electro)chemical potential (or Gibbs free energy) of the system under the conditions prevailing? Of course, as is well known, the thermodynamics of initial and final states cannot tell us anything about reaction *rates*, but at least there is a criterion for deciding whether a particular rate may be expected to exceed zero! It now needs to be considered how (electro)chemical reactions may be coupled to each other.

9.3 Energy coupling of reactions and phenomenological non-equilibrium thermodynamics

The important reaction $ADP + P_i \rightarrow ATP + H_2O$ has a typical standard free energy change *in vivo* of some $+31.8$ kJ/mol. The actual value depends, for reasons given above, upon the pH, Mg^{++} concentration and other factors (Thauer *et al.*, 1977). In other words, ATP synthesis is a highly endergonic process, and *in vivo* is poised so that its free energy change is positive by some 43.9 kJ/mol; Thauer *et al.*, 1977). One might wonder, then, how this is possible. The answer is, of course, that ATP synthesis is coupled to the performance of reactions whose free energy change is yet more *ex*ergonic. Consider a reaction such as $S \rightarrow P$, with a ΔG value under a

particular set of conditions equal to -53 kJ/mol. Allowing these reactions to be coupled to each other means that the overall reaction:

$$S + ADP + P_i \rightarrow P + ATP + H_2O$$

has a free energy change that is -9.1 kJ/mol and thus favourable.

If the coupling is strictly stoichiometric, the equilibrium distribution of S, P, ADP, P_i and ATP may be calculated from a knowledge of the starting mixture and the free energy change of the constituent reactions. In real systems, however, energy coupling is rarely strict, since there are likely to be side reactions and uncoupled partial reactions (e.g. ATP hydrolase activity which leads only to heat production). To maintain a particular series of reactions at a particular poise (mass action ratio), then, a continuing input of free energy is necessary, and the system cannot attain a true (global) equilibrium. To begin to describe such reactions, the formalism of non-equilibrium thermodynamics (NET) must be used. A full introduction to this and related topics is given in the authoritative monograph of Westerhoff and van Dam (1987).

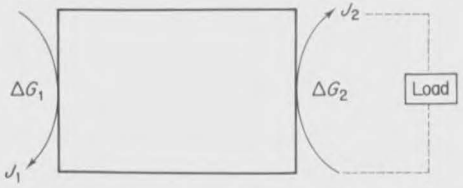


Fig. 9.5 A phenomenological, 'black box', non-equilibrium thermodynamic view of energy coupling. Here only input and output forces and fluxes are considered. For further details, see text.

A 'black box' may be considered (Fig. 9.5) catalysing an input reaction with a flux (i.e. at a rate) J_1 and held (by unspecified means) so that it has a free energy change ΔG_1. When the system reaches a stationary (i.e. steady) state, an output reaction may also be observed occurring at a rate J_2 and associated with a ΔG_2. (Obviously the ΔG of the output reaction is constantly changing, and times for which ΔG_2 has a 'constant' value are therefore considered short.) In the formalism of NET, an affinity X is defined, which is the negative of the free energy change (per extent of reaction). The efficiency of energy coupling in the system of Figure 9.5 is then defined as $-J_2X_2/J_1X_1$ (see e.g. Westerhoff *et al.*, 1982, 1983). Such a definition, based upon the observations of the effects upon the outside world of the 'black box' energy converter, is purely phenomenological, and cannot

therefore of itself provide mechanistic answers to questions such as *why* a particular energy converter is more or less efficient than any other one. It is, however, interesting to note that, in contrast to what may be proved rigorously or expected from first principles (Kubo, 1969, 1986; Nicolis and Prigogine, 1977), it is often found that the 'domain of linearity' between macroscopic forces (affinities) and their concomitant fluxes in bioenergetic systems is very broad (e.g. Rottenberg, 1973; Caplan and Essig, 1983; Stucki *et al.*, 1983). The fundamental (and mechanistic) reasons for this are not understood, but one might speculate that they are perhaps related to the ability of such proteinaceous coupling devices to exhibit many more degrees of conformational freedom than can a small molecule.

Phenomenological NET certainly allows a more accurate description of living processes than does classical thermodynamics, but there is neither space here to do the subject justice nor in fact does it seem to have greatly aided the resolution of the central problems of membrane bioenergetics addressed in this chapter. Apart from an NET approach to the description of proton pumps (see later), it must suffice to cite several of the more relevant articles and books which must serve as an introduction to the literature for the interested reader (Stucki, 1978, 1980, 1982; Rottenberg, 1979a,b; Westerhoff and van Dam, 1979, 1987; Caplan and Essig, 1983; Stucki *et al.*, 1983). However, the following ideas from these mainly phenomenological descriptions are particularly important:

(a) that the efficiency of coupling in non-equilibrium systems that behave according to these NET principles is always less than 100%;
(b) that this efficiency depends upon each of the terms J_1, X_1, J_2 and X_2 (and therefore upon the load on the energy converter); and
(c) that the optimal degree of coupling for any type of coupling system depends upon whether it is J_2, X_2 or their product that it is desired to maximize.

The available evidence indicates, interestingly enough, that microorganisms have in general evolved to permit a maximum *flux* at the expense of yield (Westerhoff *et al.*, 1983; Kell, 1987a,b).

While, as shall be seen, there are reasons to doubt the genuinely rigorous basis even of the simplest NET treatments when applied to proton pumps, a number of authors have derived detailed specific models to describe the energy coupling of protonmotive systems of the type displayed in Figure 9.2. These models form some of the more rigorous descriptions of how one should *actually* expect a purely chemiosmotic system to behave. For this reason, they shall be treated in a separate section. However, it is first worthwhile considering the relationship between macroscopic and microscopic systems generally.

9.4 Statistical thermodynamics and the relationship between microscopic and macroscopic descriptions of bioenergetic systems

Thus far only macroscopic systems have been considered. What this means is that it has been assumed that it is suitable to treat an ensemble of molecules of a given type as though they were behaving identically. Of course, experimentally, huge ensembles of particles are of necessity what are observed. In fact, the term 'ensemble' has a special meaning in this context; it is used specifically to describe 'a random collection of systems, each of which corresponds to the "same" macroscopic thermodynamic state but which has a different microstructure'. This, however, leads us to problems of great difficulty, since modern thermodynamics is still built upon the foundations laid by pioneers such as Boltzmann in the nineteenth century (Brush, 1976, 1983). What is known from the work of Maxwell, Boltzmann and others is that an ensemble of gas molecules of a given type (at temperatures above absolute zero) is constituted such that individual molecules do not possess the same instantaneous energy since, even at thermodynamic equilibrium, they are exchanging heat energy quanta between themselves and the walls of the isothermal heat bath in which they are contained (of a magnitude equal to $\frac{1}{2}k_BT$ per degree of freedom, where k_B = Boltzmann's constant = 1.38×10^{-23} J/K). The factor k_BT thus has the units of energy, and is taken as a yardstick by which free energy changes of interest are compared with the randomizing thermal forces pervading a system; at 25°C it is equal to some 2.48 kJ/mol or, in electrical units, 26 mV.

Imagine such a heat bath, containing a mixture of molecules of H_2, Cl_2 and HCl whose composition is such that it is at chemical equilibrium and further assume that the chemistry involves interconversions written as:

$$H_2 + Cl_2 \leftrightarrow 2\ HCl \tag{9.11}$$

If it was observed that, despite the direction in which the reaction was started, the system evolved spontaneously and monotonically to give an identical mixture of molecules one might be confident that a true chemical equilibrium had been attained (Denbigh, 1981). This would, of course, be a *dynamic* equilibrium, for reversible chemical changes are known to be constantly taking place as thermally activated molecules cross and re-cross the 'energy barrier' separating them (see later). Since the system is (macroscopically) unchanging it would be assumed correctly (at equilibrium) that for every molecule participating in the reaction from left to right a stoichiometrically equal number is doing the reverse.

If one asks what is happening as a function of time to any *individual* molecule, the realms of statistical mechanics and statistical thermodynamics

are entered (see e.g. Hill, 1960; Finkelstein, 1969; Gassier and Richards, 1974; Gopal, 1974; Knox, 1978; Brush, 1983). Their fundamental importance to the whole of thermodynamics and hence bioenergetics means that one must dwell here briefly, to develop one particular set of arguments. Given the present state of knowledge, however, it is not necessary to be acquainted with the mathematical foundation of the subject to acquire an understanding of the relevant physical behaviour and points at issue.

Statistical mechanics and thermodynamics consider the motion of particles through *phase space*, a mathematical construct in which the (generalized) three-dimensional position of a particle is plotted against the generalized momentum of the particle. According to Boltzmann's *ergodic hypothesis*, any particle in a closed ensemble of particles will sooner or later have the opportunity of passing through (or arbitrarily close to) all regions of phase space. It will have the opportunity of meeting and reacting with all other particles in the system and thus of experiencing (populating) any of the 'states' possible to the system and that determine *in toto* the system's physical and chemical constitution. Because the classical equations of motion are unchanged by time-reversal, Tolman (1938) was able to argue that direct interconversions between the various individual cells (points) making up phase space satisfy Boltzmann's conditions of the ergodic hypothesis and, from a statistical point of view, the equilibrium condition is the most probable one because sooner or later it was inevitably attained, and maintained (in the absence of outside influences) (Tolman, 1938).

Because the macroscopic state of the system is unchanging, under equilibrium conditions, *individual* quantal processes and their reverses must occur at equal rates and, since they are quantal, they must occur by the same pathway in each direction; this principle is known as *microscopic reversibility*. An extension of this principle states that equilibrium can therefore be maintained by balancing the number of particles moving into or out of a particular *region* of phase space; a principle known as the principle of *detailed balance* (Tolman, 1938). Whilst it is usually considered that microscopic reversibility holds for equilibrium and non-equilibrium systems, detailed balance holds only at or very close to equilibrium (Morrissey, 1975; Haken, 1977) (though fundamental problems remain even here, Zukav, 1980; Primas, 1981; Landsberg, 1982; Davies and Brown, 1986). Why is this important?

It is possible to calculate *from first principles* the dynamics of small gas molecules in a closed thermodynamic phase: to show that at equilibrium these dynamics display both microscopic reversibility and detailed balance; to derive the statistical distribution of these states; and to show both that this distribution is Boltzmannian and that it is the state of lowest energy available to the system throughout the entirety of phase space. In other

words, the equilibrium observed represents a *global* free energy minimum and the above ideas have a rigorous foundation for small molecules forming an ideal gas at equilibrium.

Unfortunately the same cannot be said even about an ensemble of 'identical' aqueous globular protein molecules isolated in a heat bath *and which is believed to be at equilibrium*. The reasoning runs as follows (e.g. Jaenicke, 1984; Kell, 1987a, 1988). A protein of molecular mass 20 kD can, in principle, possess some 10^{80} conformational states (i.e. positions in phase space), but since the Universe is 'only' some 10^{17} seconds old (Barrow and Silk, 1983), even if one allows the protein to explore these states at a rate of 10^{15} per second, it cannot conceivably explore all of them in passing from the unfolded to the folded state. Thus, given experimental realities, even a protein isolated in a heat bath of solvent molecules at 'equilibrium' is not an ergodic system. Furthermore, *it cannot be known* whether it is at a local or a global free energy minimum and it seems probable that the relevant *macroscopic* thermodynamic properties cannot even be *measured* properly (Lumry, 1986). What is known, assuming that it *is* at 'equilibrium', is that the protein is exploring many areas of phase space and fluctuating wildly between conformations whose free energy differences are roughly those to be expected from an ensemble of particles at equilibrium in a heat bath. In other words, any conformation or conformational 'state' of a protein is constituted by a large variety of microstates whose energy lies within $k_B T$ of the average energy of the system.

One consequence of the ergodic hypothesis is that one should be able to make statements about an *individual* molecule, which might itself be isolated (but at thermal equilibrium) in a *microscopic* thermodynamic phase. Since a particle at equilibrium explores all regions of phase space with equal probability, the time-average of the energy of such molecules will be equal to the instantaneous average of the energies of an ensemble of such molecules. This is the central dogma of equilibrium statistical thermodynamics, and allows macroscopic concepts (such as concentration) legitimately to be used to describe the different states of small equilibrium systems (Hill, 1963). For non-equilibrium systems, however (which nonetheless for systems *near* equilibrium exploit the assumption of local equilibrium by virtue of the fluctuation-dissipation theorem (Kubo, 1969, 1986; Kreuzer, 1983), the situation is entirely different: the ergodic hypothesis does not hold, and, if the system *works* microscopically (i.e. significant parts of the system do *not* interact during the relevant time of observation), then it is *not* appropriate to describe that system macroscopically (Welch and Kell, 1986). In fact, the Boltzmannian derivation of the ergodic theorem contains the assumption (usually referred to as the *Stosszahlansatz*; Chester, 1969) that the motions of the molecules constituting the system *are uncorrelated before each collision*

that takes place. Whilst this is acceptable for a dilute gas, it is obviously incorrect for a protein molecule in which the conformational flexibilities of individual atoms are weakly or highly correlated with those of other atoms. Thus, the fact that protein molecules themselves are individually so complex gainsays any rigorous application of macroscopic equilibrium thermodynamic principles to non-equilibrium systems. Whether this matters from an *experimental* standpoint or not depends simply upon the extent to which individual but isoenergetic ($\pm k_B T$) areas of phase space are explored by any particular molecule of interest over the relevant time-scale. Consider the 'ensemble' of protein molecules. Let them be present at 1 mM (i.e. 6×10^{17} molecules in 1 ml). Let each one explore 6×10^{12} *overall* conformations per second. Even in 1 millisecond (a typical turnover time for an enzyme) only 1 in 100 molecules even has a chance to encounter (for a miniscule fraction of the time) the same conformational state as any other. Since the real numbers are vastly greater than this, it should be obvious that the forward pathway taken by an enzyme during a reaction is *most unlikely* to be identical to that taken by it during any reverse reaction. This is just another way of saying that the principle of detailed balance cannot be applied *a priori* to working protein molecules (although experimentally such deviations *may* not be manifest). This point applies both to proteins and to organisms. A simple example, relevant to bacterial bioenergetics and based loosely on the famous thought experiment of Schrödinger's (1935) cat (Primas, 1981), may be used to illustrate this.

Consider an individual, obligately aerobic respiratory, bacterium whose behaviour depends upon (a) whether it spends half of its life exposed to 100 μM O_2 and half of its life anaerobic, or (b) whether it spends its entire life exposed to 50 μM O_2. Notwithstanding the difficulty of defining 'life' in the case of a microorganism (see e.g. Harris and Kell, 1985a; Mason *et al*, 1986), it would be agreed that after a greater or lesser period of anaerobiosis, prior to any re-exposure to oxygen, the microorganism would have 'snuffed it'. The probability of this microorganism 'dying' depends upon the *length of time* for which it experiences anaerobiosis. Given experimental realities connected with the accuracy and response time of oxygen electrodes, however, the two sets of oxygen tensions might be made to appear identical *from a macroscopic point of view*. In other words, because an oxygen electrode is rather slowly responding, its *mean* signal after a long time will be the same in the two cases because in each case the 'mean' does correspond to 50 μM O_2. Only *after* exposing this bacterium to one of the two regimes of oxygenation would one be able to tell if it were 'alive' or 'dead'. Similarly, the state of a system consisting of a culture of bacteria in which one half of the bacteria are exposed to 100 μM O_2 and one half anaerobic will differ from that of a system in which all bacteria are uniformly exposed to 50 μM

O_2. Since oxygen reduction is irreversible, individual cells work 'in isolation' and cannot share the free energy made available by respiratory electron flow. Again, one would be able to have two macroscopically stationary states which were apparently identical but which led the system, because it works *microscopically*, to behave in two entirely different ways! The time average is not equal to the ensemble average (Welch and Kell, 1986).

This general point, which underlies the so-called 'problem of scale-up' in biotechnological systems (Kell, 1986a), means that macroscopic descriptions of far-from-equilibrium systems, even in apparently macroscopically stationary states, do not of themselves give an adequate picture of the systems they are seeking to describe. Indeed, an overzealous application of macroscopic considerations to microscopic coupling systems may lead to apparent violations of the second law of thermodynamics. An example of relevance to membrane energy coupling is that given by Westerhoff and Chen (1985), and relevant considerations for purely electrical fields are given by Kell *et al.* (1988).

The general conclusion to be drawn is that non-equilibrium systems, containing ensembles of molecules with many degrees of freedom, individually may explore different parts of conformation space when working in the forward and reverse direction, i.e. when crossing and re-crossing an energy barrier. Since there is by definition a loss in free energy in one of the directions, and there cannot be a gain in free energy in the 'other' direction, irreversibility is to be seen as a property of individual molecules, and not of ensembles. This explains in another way why the principle of detailed balancing does not, in general, apply to microscopic systems of macromolecules transducing free energy *via* states which are far from thermal equilibrium (Steinberg, 1986).

Parenthetically, it is worth mentioning that while there is much lively and current debate about the philosophical status and interpretation of the behaviour of quantum mechanical systems (e.g. Bohm, 1980; Zukav, 1980; Gal-Or, 1981; Primas, 1981; MacKinnon, 1982; Popper, 1982; Wheeler and Zurek, 1983; Bohm, 1984; Garden, 1984; Davies and Brown, 1986), at the time of writing little of this debate (e.g. McClare, 1971; Blumenfeld, 1983; Welch and Kell, 1986; Kamp and Westerhoff, 1987; Westerhoff and Kamp, 1987) has filtered through to the fields of chemical thermodynamics and bioenergetics. However, it seems implausible that this unsatisfacatory state of affairs be allowed to continue indefinitely, and it can be concluded from this section that, while thermodynamics is often presented as beyond debate or discussion, a proper formalism for describing the energy states of even equilibrium proteinaceous systems, let alone those in the act of catalysing free energy transduction, is not yet to hand. This point will be considered when we discuss the degree of coupling within individual proton pumps.

9.5 Transition state theory

The *rates* of (electro)chemical reactions which possess favourable thermodynamics must now be considered. It is first necessary to distinguish transition states from systems exhibiting non-thermally activated collective behaviour.

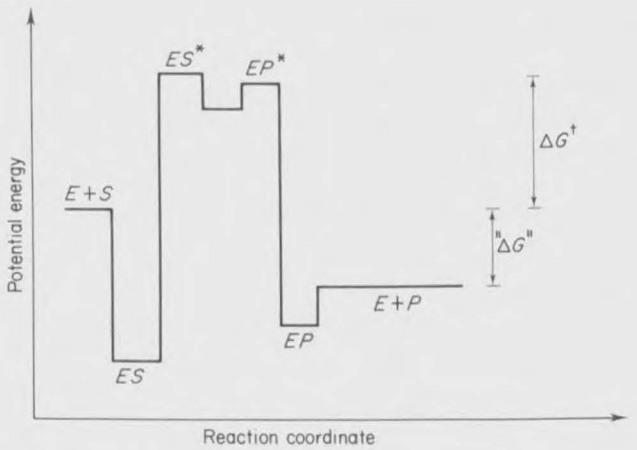


Fig. 9.6 The energetics of enzymes as assessed by transition-state theory. For further details, see text.

Whilst chemical kinetics may perhaps best be approached in terms of collision theory (Atkins, 1978; Knox, 1978), it is nowadays usual to treat the kinetics of enzyme-catalysed reactions in terms of absolute reaction rate theory (ART), commonly called transition-state theory (TST) (e.g. Glasstone *et al.*, 1941; Jencks, 1969; Laidler, 1969; Thornton and Thornton, 1978; Blumenfeld, 1981; Wharton and Eisenthal, 1981; Somogyi *et al.*, 1984; Fersht, 1985). The salient features of TST are summarized in Figure 9.6, which plots the potential energy of a system against the reaction coordinate (i.e. the physicochemical nature of the system at a given point in the reaction sequence). In Figure 9.6, the system chosen is an enzyme obeying Michaelis–Menten kinetics and catalysing the reaction $S \rightarrow P$ according to the reaction scheme:

$$E + S \leftrightarrow ES \leftrightarrow ES^* \rightarrow EP^* \rightarrow E + P \qquad (9.12)$$

Starred states represent 'activated complexes' or 'transition states' and are at the peaks of such a diagram, while unstarred states represent more-or-less stable intermediates of the reaction sequence such as enzyme–substrate

complexes. TST addresses the question of how *mechanistically* the overall reaction takes place and what factors underlie the *rate* of such a reaction. Theories such as TST therefore seek to bridge the gap between free energy terms (i.e. thermodynamics) and reaction rates (i.e. kinetics).

The central idea in transition-state theory is that reactions between molecules proceed via an activated complex whose rate of formation is a function of the absolute temperature and the so-called activation energy. Once formed, the activated complex, in this case ES^*, decays rapidly to give the products of the reaction. Neglecting second-order effects and assuming that every molecule of activated complex decays to give products (i.e. that the so-called transmission coefficient = 1), the rate of formation of the activated complex is given by the equation:

$$k = k_B T/h \exp(\Delta S^{\dagger}_0/R) \exp(-\Delta H^{\dagger}_0/RT) \tag{9.13}$$

where h is Planck's constant ($=6.63 \times 10^{-34}$ J s), R is the gas constant (8.31 J mol^{-1}K^{-1}) and $\Delta S^{\dagger}_0$ and $\Delta H^{\dagger}_0$ are respectively the entropy and enthalpy of activation, related to $\Delta G^{\dagger}_0$ (the free energy of activation) by the well-known general equation (applied at constant volume):

$$\Delta G = \Delta H - T\Delta S \tag{9.14}$$

The existence of T in the exponent of equation 9.13 (which is derived from statistical mechanics) indicates that the production of this activated complex is caused by the absorption of thermal energy from the surroundings, and such a reaction is said to be *thermally activated*. Equation 9.13 resembles the Arrhenius rate law:

$$k = A \exp(-\Delta E_a/RT) \tag{9.15}$$

in which ΔE_a is an *experimentally determined* 'activation energy' (and might better be referred to as ΔE_{expt}) whereas the entropy and enthalpy of activation in equation 9.14 are thermodynamic variables which may or may not be related to it, and which may or may not have some genuine meaning. It should be noted that the above assumes thermal and thermodynamic equilibrium between the states $E + S$ and ES^*, so that their concentrations may be calculated from their differences in free energy (via Eq. 9.8) and that the exchange of heat quanta is so rapid that we may treat these states macroscopically as being in equilibrium with an ensemble of microstates of the energy $\pm k_B T$. Taking natural logarithms and differentiating equations 9.13 and 9.15 with respect to temperature:

$$d \ln k/dT = \Delta E_{expt}/RT^2 = 1/T + \Delta H^{\dagger}_0/RT^2 \tag{9.16}$$

$$\text{i.e. } \Delta E_{expt} = \Delta H^{\dagger}_0 + RT \tag{9.17}$$

Thus, whilst the temperature-dependence of rate constants is often used experimentally to obtain the value of $\Delta H^{\dagger}_{0}$ and $\Delta S^{\dagger}_{0}$ from equations 9.13 and 9.14, the former equals the Arrhenius activation energy only at absolute zero and, as Blumenfeld (1981, p. 66) has put it, 'there is no physical meaning in substituting [Eq. 9.17] into [Eq. 9.13] with "T" as a variable.'

It has been seen that enzymes in a given 'state' (i.e. collection of conformational microstates with a free energy difference $\leqslant k_B T$) exhibit thermal fluctuations. In transition-state theory the idea is that thermal energy (derived mainly from the solvent heat bath) is used to drive the enzyme-substrate complex into a transition state whose energy may differ from that of the ground state by *many times* $k_B T$. Since the assumption of equilibrium between the ground state and the transition state means that the ordinate actually represents Gibbs Free Energy in a diagram such as Figure 9.6, it might be wondered how heat quanta, *under macroscopically isothermal conditions*, might be permitted to raise the free energy of a molecule, albeit transiently, without breaking the second law of thermodynamics. As Kemeny (1974) has put it, however, 'this does not mean that heat energy is turned into free energy but rather that the transduction of internal energy and heat exchange with the reservoir are part of the same mechanism.' This leads to the idea of *enthalpy–entropy* compensation (see Somogyi *et al.*, 1984; Lumry, 1986), i.e. the experimental finding that small changes in protein molecules (or their substrates) are often accompanied by equal and opposite changes in the values of ΔH_0 and ΔS_0 for a particular step such as ligand binding. The molecular meaning of this compensation behaviour remains unknown (Lumry, 1986), but it seems probable that it has the same fundamental basis as the linear free energy relationships (LFER) first observed for an enzymatic reaction by Fersht and his colleagues in engineered derivatives of the tyrosyl t-RNA synthetase of *Bacillus stearothermophilus* (Fersht *et al.*, 1986).

Linear free energy relationships (LFER) are well known in organic chemistry in the form of Hammett or Brønsted plots of the log of the rate constant for a particular reaction step versus the log of the equilibrium constant for that step (i.e. a ΔG term) (Jencks, 1985). The finding that LFER may be observed with (conservative) changes in the structure of an enzymatic active site (Fersht *et al.*, 1986) provides powerful (though not conclusive) evidence for the thermally based mode of energy transduction of at least some steps in an enzyme that simply catalyses the approach of a reaction to equilibrium. In particular, such a finding may be taken to bolster the case for a macroscopic treatment of enzyme kinetics (at least at room temperature), assuming microscopic reversibility and detailed balance for interconversions between macrostates (i.e. intermediates) and ignoring the microstates which indubitably contribute to any given 'state'. Against this,

however, is the finding by Bechtold *et al.* (1986) that the pathway of electron transfer in a cytochrome *c* derivative is different in the forward and reverse directions. Whiist this may be just a clear-cut example of a general truth, Williams and Concar (1986) remark that 'if . . . [the data do not go away,] . . . we must re-examine a considerable part of our thinking about protein energy states and not just about electron transfer.' Similarly, in the carefully studied example of myoglobin (Ansari *et al.*, 1985), it has been shown that the transduction of free energy from initially non-equilibrium states does not pass through microstates that are in thermal equilibrium (Frauenfelder and Wolynes, 1985; cf. Bialek and Goldstein, 1985). Indeed, it is worth remarking that, while much is known about the dynamics of relatively simple synthetic polymers (Hedvig, 1977; Doi and Edwards, 1986) in which local, but not *large-scale* (i.e. cooperative) motions obey the Arrhenius activation law (Hedvig, 1977), much remains to be learned about the more complex, and above all *coupled* internal motions of proteins. In particular, formalizing the relationships between enzymatic fluctuations between microstates, the relevant thermodynamics and their functional consequences, remains an important goal (Somogyi *et al.*, 1984; Welch, 1986).

In summary, then, transition-state theory describes the behaviour of chemical reactions activated by the absorption of thermal energy and in internal thermal equilibrium. It should be noted, however, that linear free energy relationships are linear plots of *log k* versus ΔG (times a constant), whereas the linear non-equilibrium thermodynamic relationships are between forces (equivalent to ΔG) and rates (i.e. k rather than log k). The significance of this important difference remains unknown, but *might* be related to whether a particular step is thermally activated or not. What are non-thermally activated processes?

9.6 Non-thermally activated processes

Although many processes exhibit behaviour of the type concentrated on above, and which is consistent with the view that their activated states are in internal thermal equilibrium with the ground states from which they arise, this is by no means true of all physical systems. A well-known example is the laser (see e.g. Haken, 1977). Why then is a laser not like a light-bulb (i.e. a tungsten filament lamp)?

Figure 9.7a gives the relevant schematic diagram of a simple electrical circuit containing a light-bulb; the latter is simply an electrical resistor of magnitude R Ohms. Before the switch is turned on, the system is in thermal equilibrium with its heat bath. Since the electrical resistor consists of atomic particles, they exhibit Brownian motion, and, being electrically charged their

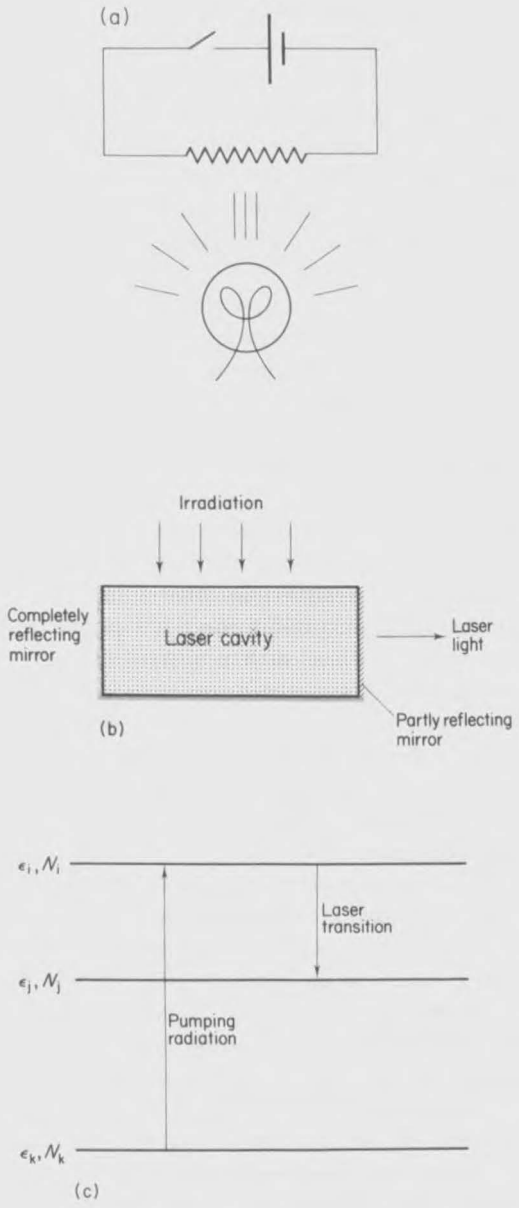


Fig. 9.7 A laser and a light bulb; examples of two very different energy-transducing systems in which the output is a flux of protons. (*a*) A light bulb is simply a resistor, transducing but dissipating the free energy input from the voltage source. (*b*) Block diagram of the action of a laser. (*c*) The three-level laser whose operation is outlined in the text.

motions give rise to a fluctuating displacement current. Since these currents are carried in an electrical resistor, they may be detected as a fluctuating voltage ('noise'), if the terminals of the resistor are connected to a voltmeter of high input impedance. Because of the equipartition of states, according to a Boltzmann distribution and consequent upon the stated thermal equilibrium, this noise is independent of frequency (i.e. 'white'). It was discovered by Johnson in 1927 and explained in these terms by Nyquist the following year (see Johnson, 1928; Nyquist, 1928; Beck, 1976; van der Ziel, 1976; De Felice, 1981).

If a steady current is allowed to flow through the resistor, an excess noise appears. This is not simply because the resistor becomes hotter (for such noise would still be white), since it has an approximately inverse dependence upon frequency. This so-called $1/f$ noise reflects the fact that, as energy flows through the system, some localized states with a higher-than-average energy appear with a probability that is increased the longer the period of observation. Nonetheless, the ratio of the noise voltages to the voltage across the resistor is miniscule, and little error (from a macroscopic standpoint) results in assuming that the current flowing is strictly proportional to the voltage drop produced across the resistor, i.e. that Ohm's Law holds. In other words, although the thermal equilibration (i.e. equipartition) of states is not *perfect* it is reasonable to treat such a system as if it were genuinely in internal thermal equilibrium.

In the laser, by contrast, the phenomenon of stimulated emission occurs (Fig. 9.7b and see e.g. Jones, 1969). In a solid-state laser, the active material can absorb photons, forming excited states. These can decay either back to the ground state or they may be stimulated to do so by absorption of radiation of frequency equivalent (via $E = h\nu$) to the difference in energy levels between the states. The simplest possible three-level laser consists of three energy levels (ε_i, ε_j and ε_k) populated with a number of atoms (N_i, N_j and N_k) (Fig. 9.7c). In this arrangement, the active part of which might be a crystal of ruby, the atoms in an energy state k may be induced to absorb radiation such that they are excited to a higher energy state i. The decay of atoms from this state to the states of lower energy may be by spontaneous or stimulated emission. In the case of spontaneous emission, the decay rate is simply proportional to the number of atoms in state k. In stimulated emission, by contrast, the decay is stimulated by absorption of radiation so that the decay is proportional both to the number of atoms in state i and to the intensity of the irradiation at this particular frequency (ν_{ij}). If spontaneous emission from state j to state k is much more rapid than that from state i to state k then an excess of population of atoms in state i over those in state j will occur in the steady state (known as a 'population inversion'). States of higher energy become more populated than those of lower energy

and the system is evidently *not* in thermal equilibrium. (Application of the Boltzmann expression actually implies the existence of a negative temperature, which is simply misleading.) The build-up of this population inversion is what is responsible for the laser transition of frequency ν_{ij}, since the absorption of radiation of this frequency stimulates suitably excited atoms to emit radiation at the same frequency. Ultimately, the build-up of photons of the relevant frequency inside the laser cavity leads to the amplification characteristic of laser action. Readers interested in exploring this topic further may find details in Smith and Sorokin (1966), Haken (1970, 1977) and Jenkins and White (1981).

What then distinguishes a laser from a light bulb is that in the laser the population of excited states exceeds that to be expected on the basis that they are in thermal equilibrium with the ground states of the system. This can lead to modes of free energy transduction that are *not* based upon the formation and decay of activated states that are in thermal equilibrium with the ground states from which they arise. Such effects are widespread in physics, and underlie many other important phenomena such as superconduction. The type of behaviour in which collections of molecules do not act independently from each other is usually referred to as collective behaviour (e.g. March and Parrinello, 1982). Evidently, since the distinction between 'thermal' and 'non-thermal' processes is so fundamental in physics, the question arises as to whether or not biological systems might possess non-thermal means of free energy transduction, especially in electron transport-linked phosphorylation, and introductory discussions of this distinction as applied to proteins and free energy-transducing molecular machines may be found in several recent articles (Blumenfeld, 1983; Somogyi *et al.*, 1984; Welch and Kell, 1986; Kell, 1987a, 1988; Westerhoff and Kamp, 1987).

Many of the collective states described above may arise even from linear processes, i.e. the rate of their production in response to a stimulus is a linear function of the extent to which that stimulus exceeds its starting value. There are other modes of free energy transductions which are highly non-linear, and two which are presently attracting widespread interest and discussion will be discussed below: solitary excitations (solitons) and Fröhlich processes.

As mentioned above, proteins, like other macromolecules, are highly dynamic entities, and contain a vast plethora of internal motions (e.g. Careri *et al.*, 1979; Gurd and Rothgeb, 1979; Welch *et al.*, 1982; Englander and Kallenbach, 1984; Somogyi *et al.*, 1984; Ringe and Petsko, 1985; Welch, 1986). Motions of individual groups or segments are in many cases highly correlated, and may be thought of as the motions of real or quasi-particles in a local potential well or a particular region of phase space (Welch and Smith, 1987). Such motions will generally contain both acoustic (mechanical) and electric modes. Acoustic modes are known as phonons. A soliton is a special

kind of phonon or wave packet which, because of the type of wave equation to which it conforms, can carry energy over a 'long' distance in an essentially *dispersionless* fashion, i.e. without losing its energy by thermal exchange or viscous damping. It thus maintains its energy at a level greater than that expected on the basis of a Boltzmannian distribution of vibrational energies at the ambient temperature. Solitons are thus non-thermally excited modes. None of the workers who have considered solitons in a reasonably biological context has considered what happens when the motion of a soliton is tightly coupled to that of an 'energized' proton (e.g. Bilz *et al.*, 1981; Blumenfeld, 1983; Davydov, 1983; Jardetzky and King, 1983; Scott, 1983; Yomosa, 1983; Careri and Wyman, 1984; Carter, 1984; Chou, 1984; Del Giudice *et al.*, 1984; Lomdahl, 1984; Lomdahl *et al.*, 1984; Somogyi *et al*, 1984). However, as discussed elsewhere (Kell and Westerhoff, 1985; Kell, 1987a), if a proton is part of a solitary excitation, its free energy will be both inseparable from it and inadequately described by a macroscopic electrochemical potential. Since these are exactly the properties that it is necessary to invoke in order to solve many of the current problems of bacterial bioenergetics, we should not ignore the probability of this type of behaviour being of crucial importance.

Another type of collective behaviour or 'coherent excitation' which may be important in bioenergetic systems is that developed over a number of years by Fröhlich (see e.g. Fröhlich, 1968, 1969, 1980, 1986; Bilz *et al.*, 1981; Fröhlich and Kremer, 1983; Kell and Hitchens, 1983; Del Giudice *et al.*, 1984; Kell and Westerhoff, 1985; Kell, 1987a, 1988). In the most general terms, Fröhlich showed that *non-linear* coupling between the electric and acoustic modes of a system (such as an energy coupling membrane) with the thermal energy of a heat bath could lead to the condensation of many of the various possible modes into a single mode of a particular energy, a phenomenon equivalent to the Einstein condensation of a gas of bosons. This excited mode could then serve as a source of free energy which might be transduced in a *dispersionless* fashion, as with a soliton above. Fröhlich discusses in particular the idea that relevant frequencies of the exciting modes might be of the order of 10^{11} Hz, based upon the velocity of sound (phonons) in condensed media (ca. 10^3 ms^{-1}) and the thickness of biomembranes (ca. 10^{-8} m). However, many types of mode softening are possible through appropriate interactions, and the model may thus be generalized to take particular cases into account. Various types of experimental evidence, such as the effects of very weak microwaves on cellular growth (see Fröhlich and Kremer, 1983) and the laser Raman spectroscopic studies of Webb (1980; and see Del Giudice *et al.*, 1984), have been invoked in support of the Fröhlich model, and do not lend themselves to any other simple explanation. Suffice it to say that, given its generality, the Fröhlich model provides another good example of a *well-developed* physical model in which non-

thermal behaviour has been seen as an important part of biological free energy transduction. Therefore, whereas most existing models of bioenergetic systems are thermally based, future workers might fruitfully bear in mind that such models represent only one subset (and perhaps the least interesting one) of the approaches that colleagues in the physical sciences have found necessary to describe the properties of condensed matter.

Despite the foregoing discussion, thermally based, macroscopic models are by far the most familiar to workers in the biological sciences, and the 'fundamental' or more theoretical part of this chapter is concluded by drawing attention to two particularly relevant and well-founded macroscopic descriptions of relevant biochemical systems: metabolic control analysis and Hill diagrams.

9.7 Metabolic control theory

In a metabolic system, such as that of Figure 9.1, the question often arises as to which step in the pathway A → H is rate-limiting when one is measuring a steady-state flux through it. This is, in fact, an inappropriate question, since each enzyme (and other parameters) contributes to the control of a particular flux. The last few years have seen a renaissance of interest in the metabolic control theory developed from the work of Kacser and Burns (1973) and Heinrich and Rapoport (1974). The formalism, which will be referred to as 'control analysis', considers only small departures from (asymptotically stable) stationary states, and is thus an *exact* theory. This formalism has been reviewed extensively several times recently so no more than its chief tenets need be mentioned here (Groen *et al.*, 1982; Westerhoff *et al.*, 1984a; Derr, 1985, 1986; Porteous, 1985; Kell and Westerhoff, 1986a,b; Kell, 1987; Westerhoff and van Dam, 1987).

Unfortunately, as with all theories, the relevant concepts come with names attached, i.e. there is a jargon to be learned. However, the minimal time necessary to acquire a working knowledge of the formalism is far outweighed by the improved understanding of metabolic systems that it brings. The treatment ascribes, by means of the flux-control coefficients, *quantitative measures* of the degree to which any step in a metabolic pathway is flux controlling. The elasticity coefficients provide a mechanistic basis for the contribution of a particular enzyme (or other external parameter) to flux control, and are related to the flux-control coefficients by means of the connectivity theorems. Other theorems relate to the relationship between metabolite concentrations and enzymic elasticities.

For present purposes, the main benefits of the control analysis are that it

is exact (this is refreshing enough in itself) and that it provides an appropriate formalism for the treatment of stationary metabolic systems. Additionally, it serves to relate the properties of *systems* to those of the subsystems from which they are constructed. It implicitly contains one major assumption: that intermediates exhibit pool (i.e. macroscopic or delocalized) behaviour. Thus, any apparent *failure* of the system of interest to conform to the tenets of control analysis provides an excellent and *rigorous* set of criteria for the failure of the pool assumption in that particular case. Since chemiosmotic coupling systems are simply metabolic systems (or may be treated thus), and the protonmotive force is by definition a macroscopic, delocalized, pool intermediate, it is obvious that the application of control analysis to putatively chemiosmotic coupling systems may be particularly rewarding. A later section covers some relevant applications. What it means, however, is that such traditional ill-phrased questions as, 'is metabolite X "the" intermediate of process Y?' or 'is the rate of ATP synthesis controlled by the protonmotive force?', are quite inappropriate, and that special procedures and arguments must be applied for the proper description of stationary processes. One special procedure that has been applied to the description of proton pumping systems is the Hill diagram method, and especially since it gives one a useful intuitive feel for certain of the points at issue in bioenergetics, it is introduced here.

9.8 Hill diagrams and the 6-state proton pump

One way to consider biological free energy transduction is by means of the King-Altman (1956) method popularized by Hill (1977) and illustrated, for a typical proton pump, in Figure 9.8. This shows in schematic form the different macroscopic conformational states that may be adapted by a protein catalysing the transmembrane, electrogenic pumping of a proton coupled to the performance of a redox reaction. Each of these states, of course, comprises a multitude of micro- or sub-states, which are *assumed* (by definition) to be in internal equilibrium, and which therefore exhibit both microscopic reversibility and detailed balance. The population of the states may be calculated on the basis of a knowledge of the forward and reverse rate constants for each of the transitions between the intermediate 'states' (Hill, 1977). There is as yet little certainty regarding the strict validity of these assumptions for real proton pumps, and whether, in view of the discussion above, simple models containing small numbers of conformational states are applicable even in principle to large protein molecules. In any event, models such as that in Figure 9.8 have often been applied to the proteins thought to be involved in electron transport-linked phosphorylation.

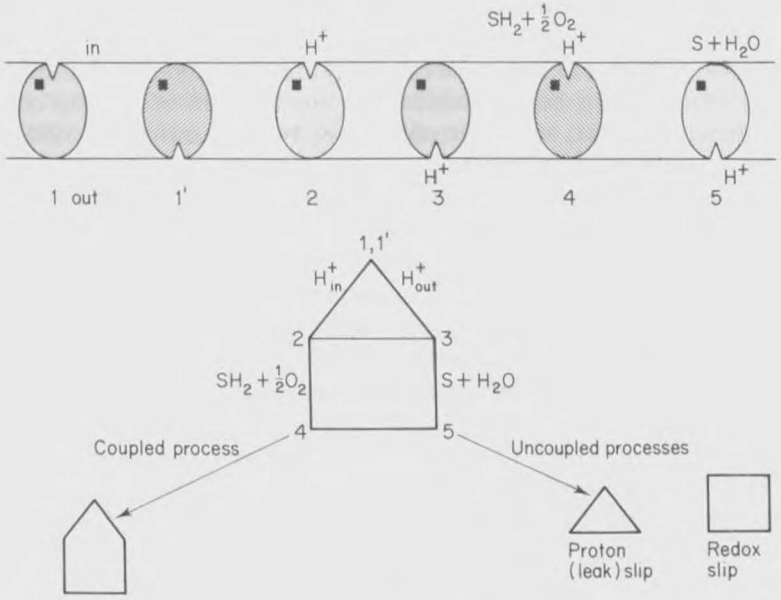


Fig. 9.8 A Hill diagram illustrating the operation of a redox-linked proton pump. The top part shows some of the plausible and identifiable conformational states of a membrane-located, redox-linked proton pump whilst the lower part shows a cycle diagram indicating the pathways of protein–ligand interactions. It is evident that there is one coupled cycle and two uncoupled ones.

Figure 9.8 also illustrates the fact that the protein may indulge in both coupled and uncoupled cycles between the macroscopic conformational states, in a fashion that may depend upon the prevailing protonmotive force. A similar diagram may be drawn for a proton pump coupled to ATP synthesis/hydrolysis. The idea is that in the uncoupled cycles, a proton pump may catalyse a redox (or ATP hydrolase) reaction without pumping a proton, or it may let a proton leak through without reversal of the scalar reaction. In the coupled cycle there is 'perfect' coupling with the so-called 'mechanistic' stoichiometry. The overall efficiency is varied by balancing the proportion of coupled and uncoupled cycles. An explicit version of this type of proton-pump model is given by Pietrobon and Caplan (1985b).

One important corollary of this 'balancing' between coupled and uncoupled cycles is that it permits a great deal of freedom in the modelling of chemiosmotic coupling systems. In other words, whatever the *mechanistic* stoichiometry of the coupled cycle of a redox-linked proton pump and an ATP synthase-linked proton pump embedded in the same coupling membrane, the observable stoichiometry will be modified in a fashion which

depends upon the prevailing value of Δp (and *vice versa*). Indeed, it was in particular the unexpected properties of the relationship between the rate of electron transport and the apparent Δp (judged by ion-distribution techniques) which caused the invocation and development of the slip concept (Pietrobon *et al.*, 1981, 1982; Pietrobon *et al.*, 1986; Zoratti *et al.*, 1986). The general reasoning runs as follows.

In the stationary state, the net rate of formation of the pmf is zero; i.e. there is an exact balance between the generation and utilization of the pmf. The rate of generation of the pmf in electron transport-linked phosphorylation (J_{GEN}) is given by the rate of electron transport times the $\rightarrow H^+/e^-$ ratio at the prevailing value of Δp; and the rate of utilization of the pmf (J_{DIS}) is given by the pmf times the conductance of the membrane. If the conductance is itself a function of the pmf, the membrane is said to exhibit non-ohmic conductance. If this is accompanied by a variable extent of uncoupled transport of charge across the membrane, the phenomenon is referred to as non-ohmic leakage. At the time of writing, most of the evidence based upon measurement of the relation between the rate of phosphorylation (J_0) and the apparent Δp suggests that slip within redox pumps is of special importance in modulating the apparent stochiometries of oxidative phosphorylation (Pietrobon *et al.*, 1981, 1982, 1986; Zoratti *et al.*, 1986). Other evidence, particularly that based on spectroscopic measurements of J_{DIS} from the group of Jackson (Jackson, 1982; Clark *et al.*, 1983; Cotton *et al.*, 1984), would ascribe a more significant role to non-ohmic leaks. The data from these latter studies suggest that phosphorylation-coupled charge transport across the plane of the membrane may account for more than 90% of the utilization of the energized state set up by electron transport (Clark *et al.*, 1983).

The Hill diagram approach has the great merit of having an essentially rigorous basis (incorporating the assumptions of non-equilibrium thermodynamics described above), for the description of systems that actually work according to macroscopic chemiosmotic coupling principles. As with metabolic control analysis, any failure of these descriptions to exhibit self-consistency may be taken as good evidence against the significance of Δp in energy coupling. Thus another view (e.g. Welch and Kell, 1986; and see later) would have it that, whilst both slips and leaks in the sense of Figure 9.8 are of fundamental importance to the understanding and operation of protonmotive devices, their extents are not determined by Δp but by an *alternative* 'high energy' intermediate, which itself remains to be established (see Welch and Kell, 1986; and later). Whereas a 'chemiosmotic' analysis of Hill diagrams can account for much of the available data, there are other data for which the analysis cannot account. This author's view, particularly considering the difficulties in obtaining a correct value for the magnitude of

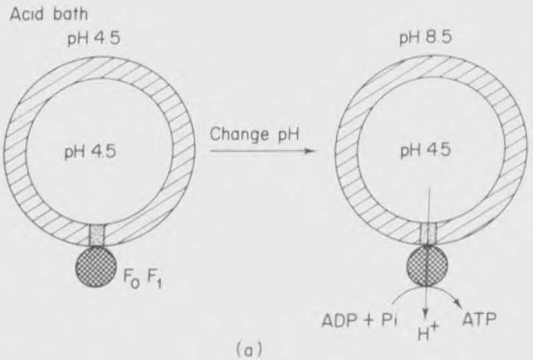
the pmf (see later), is that these overall observations are best interpreted as yet further evidence that one of the axioms which they incorporate, (the primacy of the pmf as an energy coupling intermediate) is the simplest one to jettison in order most readily to accommodate the available data. Since this view leaves open the question of exactly what it is that many probes are measuring when they purport to measure an energetically-significant Δp, one must now enquire more closely into the methods which are generally used for the estimation of Δp, and the extent to which they are self-consistent and are likely to be accurate.

9.9 Is the protonmotive force an energetically significant intermediate in electron transport-driven phosphorylation?

9.9.1 Introduction

Although the above question is frequently asked, the answer obtained depends upon what one thinks the question actually means. In common with Ort and Melandri (1982), for instance, I shall take as a 'straw man' (model for destruction) the idea that the only free energy-transducing interactions between primary (redox-linked) and secondary (ATP synthase) proton pumps in energy coupling membranes are mediated via a delocalized protonmotive force (pmf). In this context 'delocalized' means macroscopic and implies that the 'energized state' set up by a given electron transport-linked (primary) proton pump may be used by all secondary proton pumps in the same membrane vesicle. Actually, this definition of 'delocalized' as 'macroscopic' already raises difficulties since, as seen above, the definition of a macroscopic thermodynamic force such as the pmf is based upon the acceptance of detailed balance and the attainment of a macroscopic stationary state. This restriction might appear unnecessarily limiting, because there is no doubt that energy-transducing membranes can make ATP under transient conditions; nor does it seem likely that they would do it differently under transient and steady-state conditions. One way round the problem is to consider a transient as a superposition of short-lived stationary states, so that one might compare the *instantaneous* value of the pmf (for example) with the instantaneous value of an output flux such as the rate of phosphorylation. It is implicitly assumed, on this basis, that one is averaging the pmf over all the vesicles in the system and for the time equivalent to the turnover time of an ATP synthase. It should be noted that, although it may be helpful in some cases, this has no rigorous quantitative basis, because the equivalence of a time average with an ensemble average follows from the ergodic hypothesis which requires an arbitrarily long time, and not a stated, limited time.

It was noted above that thermodynamic criteria are the most rigorous by which to decide whether or not a particular variable is an intermediate in a particular process. In order to gain an idea of what magnitude of pmf must be created by primary proton pumps in order to make credible the idea that energy coupling takes place via the intermediacy of a delocalized pmf some studies of phosphorylation driven by *artificially applied* pmf will be considered. This will then enable an assessment to be made of some of the methods actually used for estimating the pmf under steady-state conditions.



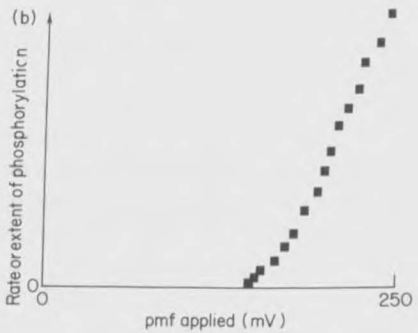


Fig. 9.9 Phosphorylation driven by an artificial pmf. (*a*) A typical experimental protocol, in which an 'inverted' vesicle (with the F_1 part of the ATP synthase which it contains facing outwards) is incubated in a medium of low pH and then immersed in a medium of high pH, possibly concomitant with the generation of a diffusion potential. It is thought that phosphorylation may be driven during the passage of H^+ through the H^+-ATP synthase, down their electrochemical potential. (*b*) The typical 'threshold' behaviour (of some 150 mV) found when the rate or extent of phosphorylation is varied by varying the magnitude of the pmf applied.

9.9.2 Phosphorylation driven by an artificially applied pmf

If a pmf couples electron transport to phosphorylation, it might be predicted that an artificially-applied pmf should also drive phosphorylation (Fig. 9.9). The key criterion is that it *should* do so, without a significant lag and at rates commensurate with those observed *in vivo*. Following the pioneering experiments of Jagendorf and Uribe (1966), many investigators have found that an artificially applied pmf, generated across the bulk aqueous phase that the coupling membrane serves to separate, can indeed drive phosphorylation catalysed by energy coupling membrane vesicles, and even by purified ATP synthases reconstituted in liposomes (e.g. Thayer and Hinkle, 1975a,b; Smith *et al.*, 1976; Wilson *et al.*, 1976; Sone *et al.*, 1977; Gräber, 1981; Hangarter and Good, 1982; Maloney, 1982; Mills and Mitchell, 1982; Gräber *et al.*, 1984; Horner and Moudrianakis, 1985; Schmidt and Gräber, 1985). The assumption in such experiments is that, whilst the incubation conditions may not be *identical* to those *in vivo*, there should be a combination of conditions (of pH, adenine nucleotide concentration, etc.) which should permit phosphorylation at approximately *in vivo* rates (or better). The following take-home messages may be distilled from the above references:

(a) rates of ATP synthesis driven by an artificially imposed pmf may indeed be made to approach or exceed those driven by electron transport, provided that the applied pmf is high enough;
(b) the initial rate of phosphorylation is very much more rapid than that measured after a few seconds;
(c) the relationship between the applied Δp and the rate of phosphorylation (J_P) or the phosphorylation yield (since ATP hydrolysis is slow under the conditions of a very low ATP:ADP ratio used) is highly non-linear;
(d) there is a 'threshold' value typically amounting to 150 mV or even higher, below which *no* phosphorylation takes place.

Finding (d) means that, since ΔG_P is less than $z\ \Delta p$ then z is not infinitely variable, and values greater than 3 may not be invoked (Kell, 1986b). (ΔG_P is the free energy change in the ATP synthase reaction and z the number of protons moving through the ATP synthase per ATP molecule synthesized.) Similarly, findings (b) and (c) mean that imperfect rapid-mixing and -quenching techniques used to initiate and terminate the reaction will tend to blur both the existence of the threshold, and the sharpness of the distinctions to be made between localized and delocalized coupling (Kell, 1986b). It is also worth pointing out that the 'threshold' behaviour observed may really reflect a more fundamental behaviour of a protonmotive system as a

molecular energy machine (Welch and Kell, 1986). (For a very unusual and probably unimportant exception to the 150 mV threshold, see van Walraven *et al.*, 1985, 1986).

The general conclusion from this section is that we should expect the protonmotive force generated by electron transport to exceed at least 150 mV in order to obtain any phosphorylation at all, and that the steady-state values should be substantially higher to be consonant with reasonably rapid rates of phosphorylation. To what extent is this borne out in practice?

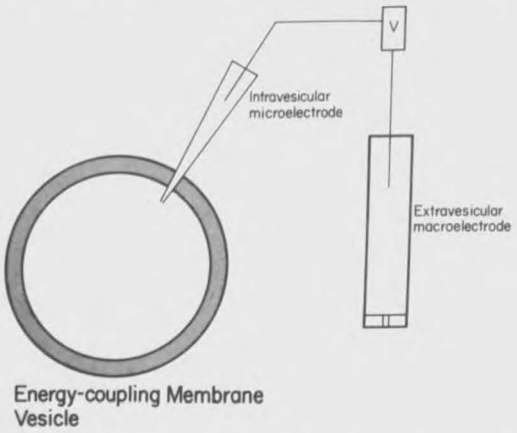


Fig. 9.10 Estimation of $\Delta\psi$ and/or ΔpH by means of microelectrodes. This type of approach is essentially that described in Fig. 9.3 by which the relative contributions of $\Delta\psi$ and ΔpH to Δp may be assessed. Care must be taken to ensure that a diffusion potential at the electrode tip does not contribute to the assessed potentials. Similarly, the necessity to puncture the energy coupling membrane means that care must also be taken to ensure that any low values measured are not due simply to a leak induced by the electrode puncture.

9.9.3 Direct estimation of the pmf using microelectrodes

As intimated in Figure 9.3, the most rigorous means by which one might aim to estimate the magnitude of the protonmotive force across an energy coupling membrane is to measure the electric membrane potential and the pH differential directly by means of (micro)electrode techniques (Fig. 9.10). This experiment is technically very difficult since the small size of mitochondria (say) places great demands upon electrode placement. Generally, therefore, only the membrane potential is measured, but this type of experiment has been performed over a number of years in a variety of

laboratories, particularly that of Tedeschi (Bulychev and Vredenberg, 1976; Vredenberg, 1976; Giulian and Diacumakos, 1977; Maloff *et al.*, 1978a,b; Tedeschi, 1980, 1981; Bulychev *et al.*, 1986; Tamponnet *et al.*, 1986). The results are quite clear and consistent in each case: the electron transport-linked, delocalized membrane potential across the mitochondrial or thylakoid membrane is energetically insignificant (< 50 mV), even under conditions in which a significant pH gradient either was not or could not have been formed. In the case of the single, procaryotic exception, the estimated membrane potential never extrapolated (after correction for the transmembrane conductance) to more than 140 mV (negative inside), which is still below the required threshold of 150 mV and even though the incubation conditions (of pH) were such that a pH gradient would have been absent (Felle *et al.*, 1980). Further, in this work it is by no means certain that the membrane potentials were generated metabolically, since the effect of uncoupler was not tested and the values measured were very sensitive to the external ionic incubation conditions (Felle *et al.*, 1978), strongly suggesting that an ionic diffusion potential was contributing to some extent to the values of $\Delta\psi$. This important experiment has not yet been reproduced.

A variety of explanations has been offered to account for the generally negligible values of $\Delta\psi$. Two of the more plausible were that the electrode is not inside the mitochondrion (or other vesicle), or that the mitochondrion is membrane-leaky (i.e. uncoupled); but these explanations have been discounted by experiments showing interior impalement of single mitochondria capable of making ATP. Many (but not all) of the experiments cited above required that the mitochondria be prepared from rats fed on cuprizone, which causes the formation of 'giant' mitochondria. Grinius (1986) has suggested that pinching off of the cristae in such mitochondria (Wakabayashi *et al.*, 1984) might allow net ATP synthesis by inverted, intramitochondrial vesicles when the parent mitochondria are incapable of forming a pmf. Obviously, this explanation can be invoked only for the case of mitochondria from cuprizone-fed rodents. Evidently, the simplest conclusion from all of these measurements is that it is most likely that the delocalized pmf is energetically insignificant. Astonishingly enough, however, this remains a minority viewpoint, largely since other methods purport to give values of the pmf at total variance with those observed with microelectrodes. As Ferguson (1985) has put it, 'some biochemists outside the immediate field of bioenergetics are puzzled why the apparent absence of a membrane potential according to microelectrodes does not create more concern amongst those working in the field of bioenergetics.' One can only agree, and trust that the future will bring further studies of this crucial topic from independent laboratories, perhaps using *really* giant mitochondria (1 mm diameter) produced by electrofusion (Zimmermann, 1982).

Patch clamping (Sakmann and Neher, 1983, 1984; Dwyer, 1985) is a related technique in which the electrical behaviour of (say) energy-transducing membranes may be measured by direct electrophysiological techniques, particularly since the conductivity of the membrane patch may be made exceedingly low. Hamamoto *et al.* (1985) did in fact make patch clamp measurements of the $\Delta\psi$ generated by cytochrome *o* from *Escherichia coli* incorporated into a black lipid membrane. The membrane potentials they measured were 2–4 mV, rather lower than the 200 mV expected and significantly lower than the threshold of 150 mV. Nonetheless, these authors (Hamamoto *et al.*, 1985) chose to argue that their low values were due to the low electrical resistance of the membranes as measured under *DC* conditions. As discussed in more detail elsewhere (Kell, 1986b) this is an entirely incorrect line of argument (since a resistance 1000 times greater than that measured would mean (by Ohm's law) a potential of 2–4 V, greater than the therymodynamic driving force for the reaction!). Future workers trying to perform patch clamp measurements on protonmotive systems should be aware that the potential generated by a putatively electrogenic source embedded in a membrane depends upon the impedance of the source *and* of the membrane, at the frequency of operation of the enzyme. Such impedances contain capacitive terms which it is not permitted to ignore (see below).

The existence and approximate biological constancy of the membrane capacitance per unit area (see Cole, 1972; Kell and Harris, 1985) provides one of the more clear-cut means for determining the veracity of the chemiosmotic coupling concept. An electrical potential between two electrodes causes the induction of an electrical potential *difference* ($\Delta\psi$) across the relatively ion-impermeable membrane of a spherical vesicle suspended between such extracellular electrodes (Fig. 9.11). For a DC field, this $\Delta\psi$ is given by $1.5\ E_0\ r \cos\theta$, where θ is the angle between a particular portion of the bilayer and the field direction, E_0 is the electric field strength (equal to the potential difference between the electrodes divided by the distance between them) and r is the radius of the vesicle (e.g. Zimmermann, 1982; Kell and Harris, 1985; Tsong and Astumian, 1986). Below an apparent 'threshold' potential no damage results (but see Glaser, 1986). However, above this threshold there is a reversible or irreversible electrical breakdown (depending upon the length of time for which the electrical field is applied), caused by electromechanical forces and/or by the formation of conducting aqueous pores or 'electropores', a phenomenon for which the theoretical basis is reasonably well developed (see e.g. Zimmermann and Vienken, 1982; Chernomordik *et al.*, 1983; Tsong, 1983; Dimitrov and Jain, 1984; Sugar and Neumann, 1984; Glaser, 1986; Powell *et al.*, 1986). The $\cos\theta$ relation between the field and the induced potential, coupled with the 'threshold'

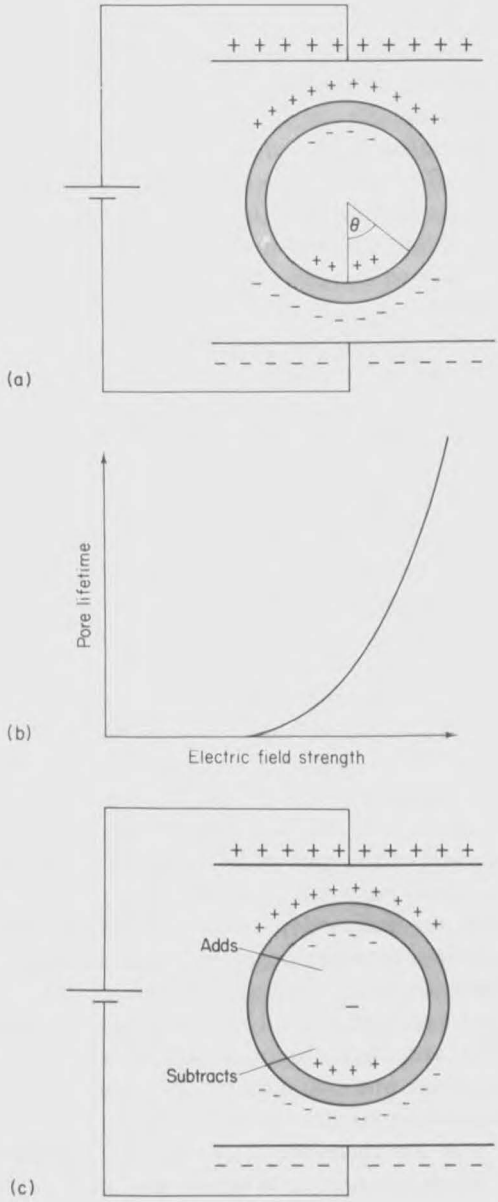


Fig. 9.11 (*a*) Induction of a transmembrane potential in a spherical vesicle by means of an electric field between two macroscopic electrodes. (*b*) The pore lifetime increases non-linearly with the potential induced. (*c*) If there is a pre-existing or metabolically induced electrical potential across the vesicle membrane of interest it will on one side *add to*, and on the other side *subtract from*, that induced by the extracellular electrodes, permitting an estimation of whether any such natural potential difference exists at a significant magnitude.

behaviour of the electrical breakdown voltage, means that (a) electrical breakdown just above the 'threshold' will be confined to regions of the vesicle facing the poles opposite the electrode faces and (b) *the existence of any electric membrane potential caused by other processes such as electron transport will have the effect of inducing an anisotropy in the electroporation process* (Fig. 9.11). Since the *true* field-induced potential may be observed optically (Gross *et al.*, 1986), and electroporation and its sidedness may be observed in the microscope by the release of appropriate fluorescently tagged molecules (Mehrle *et al.*, 1985; Sowers and Lieber, 1986), the presence or absence of a transbilayer electrical potential should severely modify, quantitatively and qualitatively, both the field/electroporation spectrum and in particular its anisotropy (Mehrle *et al.*, 1985). It should be noted that any negative intravesicular *surface* potential will also tend to induce a degree of anisotropy in the electroporation of individual vesicles, so that the *lack* of such an effect would constitute powerful evidence against the existence of an energetically significant, electron transport-linked bulk phase-to-bulk phase $\Delta\psi$. Obviously uncoupler-treated cells or vesicles provide appropriate controls, since the passive electrical properties of biological membranes observed with extracellular electrodes are only *very* weakly dependent upon the transmembrane conductance (see e.g. Harris and Kell, 1985b). This would seem to provide a potent and novel means of assessing the magnitude of any $\Delta\psi$ without having to attempt to measure it by the use of intracellular electrodes or, as shall now be seen, extrinsic probe molecules.

It has been seen that the threshold behaviour observed in 'artificial pmf' experiments means that it is of the first importance to have an accurate method for the estimation of the pmf under stationary conditions, so as to establish whether the signal purporting to reflect the pmf is consistent with the view that the pmf is a thermodynamically competent intermediate in processes such as electron transport phosphorylation. Readers will be aware that a great many methods have been used for this purpose, each with its problems; the different approaches tend to give different values under the 'same' conditions and are therefore possessed of a different degree of credibility depending upon one's standpoint, prejudices and the types of experiment with which one is familiar. Some of these methods and the type of results that they give are therefore discussed. Perhaps the primary or commonest method, often regarded as a benchmark method, is the ion- or acid/base-distribution method; it is therefore given special treatment.

9.9.4 Ion- and acid/base-distribution methods for estimating the pmf

First are considered ion-distribution methods that may be used to estimate the electrical potential difference between two thermodynamic phases.

Figure 9.11 shows a vesicle in which the inside lumen (bulk phase) has a *pre-existing* electrical potential that is maintained at a value positive with respect to the bulk phase outside the vesicle. Surface potentials of the type described in Figure 9.4 are not considered here. If the vesicle membrane is permeable to a particular charged 'probe' anion (X^- in Fig. 9.9), the ion will flow down its electrochemical potential until it is in equilibrium with the pre-existing or maintained electrical potential. (For this to occur, it must cross the membrane purely by uniport, and not be taken up or extruded by other means.) Under such equilibrium conditions, the transmembrane electrical potential $\Delta\psi$ is related to the internal and external concentrations (strictly activities, of course) of the ion by the equation:

$$\Delta\psi = -2.303\frac{RT}{F}\log\left([a_x\text{-}]_{in}/[a_x\text{-}]_{out}\right) \qquad (9.18)$$

This equation follows from equation 9.10 on the basis that the criterion for electrochemical equilibrium is that there is no difference in the electrochemical potential of the 'probe' ion in the internal and external phases when equilibrium is attained between its chemical potential difference across the membrane and the electrical potential difference across the membrane. Since the standard electrochemical potential of the probe ion is not known in either the inner or the outer phase it is assumed that it is the same for each phase and that all differences in thermodynamic factors *other* than the electrical potential are incorporated into the activity term. Evidently, then $\Delta\psi$ can be estimated by measuring the activity ratio of such probe ions inside and outside the vesicle of interest. (For reviews, see Rottenberg, 1975, 1979, 1985; Ferguson and Sorgato, 1982; Nicholls, 1982; Azzone *et al.*, 1984; Jackson and Nicholls, 1986).

It is generally assumed that the activity coefficients of probe ions are the same inside and outside the vesicle of interest. Whilst this assumption can never be checked, it probably does not introduce *enormous* errors; a twofold difference in the activity coefficients would introduce an inaccuracy into the estimation of $\Delta\psi$ (if calculated on the basis of free internal and external *concentrations*) of some 18 mV (equation 9.18). If one wishes to interpret certain data in terms of strictly delocalized chemiosmotic coupling, it is necessary to assume that a variation of Δp of this magnitude is sufficient to cover all rates of phosphorylation (from zero to maximal). In such cases it is obvious that an ignorance of the true values of $\Delta\psi$ of even this magnitude precludes any sensible interpretation of the relationship between, say, the rate of phosphorylation and the protonmotive force. Naturally, errors in the estimation of $\Delta\psi$ due to different causes are additive, and it is necessary to take all of them into account. On the (perhaps doubtful) assumption that the principle of the method described in Figure 9.11 actually holds for energy

coupling membrane vesicles, the sources of such errors should be considered, so that one may be aware of the limited significance of reported values, which are often given, without error bars, to the nearest millivolt! (for those who like this sort of thing, a tabulation is given by Kashket, 1985).

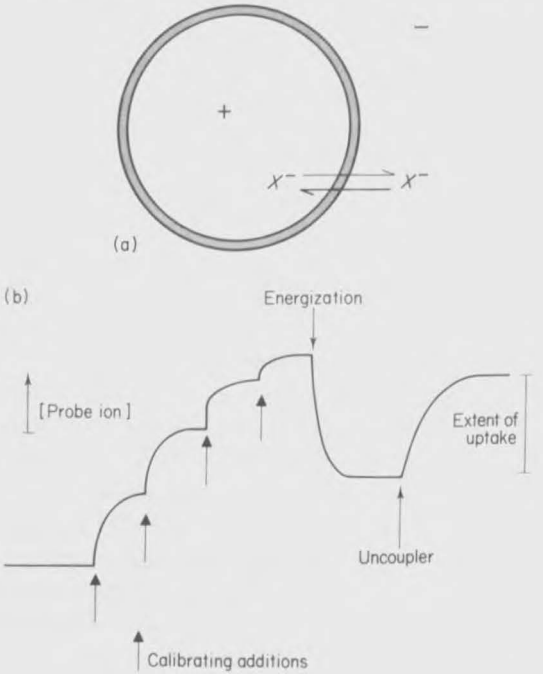


Fig. 9.12 (*a*) Principle of the estimation of $\Delta\psi$ by means of the ion-distribution technique, as described in the text. (*b*) A typical trace of the extracellular activity of the ion of interest, as measured with an electrode, showing how one may calculate the extent of energy-dependent ion uptake/binding.

With the exception of such techniques as nuclear magnetic or electron spin resonance, which have not been widely used in this context (Cafiso and Hubbell, 1981), most approaches are capable of measuring only the *amount* of probe ion taken up by the vesicle of interest. It is thus necessary to know the volume of the internal phase to convert amounts to concentration. Further, what matters is the activity of probe ions that are free in the lumen of the vesicle, so that any binding must be corrected for. The extent of *energy-dependent* binding cannot be corrected for, since one has no knowledge of the 'real' membrane potential which is what one is trying to measure. At least in bacterial cells, however, the energy-*in*dependent binding of probe ions can be very extensive; corrections for such binding cause the calculated

values of $\Delta\psi$ to fall very sharply, to values well below the 150 mV 'threshold' (e.g. Lolkema *et al.*, 1982, 1983; Elferink *et al.*, 1985, 1986). In other vesicular systems, binding or energy-dependent extrusion (Midgley, 1986) of the probe may be sufficiently great to cause the estimated $\Delta\psi$ to be of the opposite *sign* to the 'real' one assessed (and in *these* cases generally accepted!) on the basis of microelectrode measurements (Ritchie, 1982; Barbier-Brygoo *et al.*, 1985). Controls which can help to alleviate these difficulties include: showing that variation of the concentration of vesicles or probe ion gives no change in apparent $\Delta\psi$; observing equal extents of uptake using probe ions having the same charge but which differ in their rate of uptake; and comparing the membrane potential estimated by probe methods with one of known magnitude generated artificially by means of a diffusion potential.

To avoid becoming enmeshed in the details of particular cases, only the following general findings are stated: sometimes different methods agree and sometimes they do not, and in no case known to the author have different methods, including all the controls listed above, been performed on the same system with a resulting $\Delta\psi$ exceeding 150 mV. Sometimes authors will pick one method in preference to another (say Rb^+ uptake in preference to the uptake of methyl triphenylphosphonium$^+$). Obviously there is no rational basis for such choices. Kaback (1986) continues to claim, apparently on the basis of unpublished observations, that $\Delta\psi$ values estimated from the steady-state accumulation of a variety of lipophilic cations are independent of the concentration of these cations 'over submicromolar to millimolar ranges'. For a $\Delta\psi$ of 120 mV, this implies internal concentrations of hundreds of millimolar lipophilic cation. Such values exceed the aqueous solubility of these cations and it is necessary to regard such a claim as a 'Churchillian' terminological inexactitude.

Since the imposition of an artificial pmf has the merit of more certainly representing what it claims to, it is particularly important to compare the ability of an artificial pmf to stimulate phosphorylation with that of what is claimed to be a real, *electron transport-generated* pmf of the same magnitude. This is rarely done in the same system at the same time. However, in alkalophilic bacteria in which an apparent pmf of significantly less than 50 mV is observed in spite of an unimpaired ability to synthesize ATP, the apparent $\Delta\psi$ measured by standard ion-distribution techniques is *not* equivalent to a diffusion potential of the same magnitude so far as its ability to drive phosphorylation is concerned (Krulwich and Guffanti, 1983; Guffanti *et al.*, 1984, 1985). One interpretation of this is that the ion-distribution method, whilst reliable in cases in which there *is* a pre-existing $\Delta\psi$, is *falsely* suggesting the existence of a significant, energy-dependent $\Delta\psi$ across the bacterial cytoplasmic membrane (and other membranes) for reasons yet to

be explained (but see later). Since this is consistent with the general lack of a membrane potential estimated directly, as described above, and with other findings to be discussed later, this is an acceptable, simple interpretation of an otherwise inexplicable phenomenon.

The principle of the acid/base-distribution method for measuring ΔpH is similar to that of the ion-distribution method for estimating $\Delta\psi$, except that here the membrane of interest must be permeable only to the probe in its *uncharged* form (see e.g. Rottenberg, 1975a,b, 1979; Nicholls, 1982). At least in bacteria, the values of ΔpH are generally rather small (exceptions are acidophiles; Cobley and Cox, 1983), and are believed to be fairly reliable, not least since approaches such as ^{31}P-NMR give values similar to those estimated by acid/base-distribution (e.g. Nicolay *et al.*, 1981). What is generally wished to be known is the functional relationship between what is believed to represent the total pmf and the rate or extent of an output process such as ATP synthesis. To help sharpen the argument, and to decrease the number of variables that must be measured, it is often helpful to attempt to estimate $\Delta\psi$ under conditions (controlled by external pH or by the addition of an ionophore such as nigericin) in which ΔpH is zero, since it is well known that bacteria maintain a relatively constant internal pH (Padan *et al.*, 1981; Booth, 1985; Kaback, 1986). The fact that both output forces and fluxes are generally rather independent of the existence of a ΔpH (acidophiles are again an exception here; Cobley and Cox, 1983), might be taken as a further indication of the irrelevance of ΔpH as an energy coupling intermediate in electron transport phosphorylation in bacteria.

9.9.5 Spectroscopic methods for estimating $\Delta\psi$ in bacteria

Although most commentators regard probe-distribution as the method of choice for estimating Δp (despite the many technical difficulties raised above), this method suffers the grave disadvantage of being relatively slow to respond. Optical methods, such as the electrochromic response of carotenoids (see Jackson, Chapter 7), can be very rapidly responding and may give information unobtainable from stationary-state measurements. However, even here, for reasons which are not yet understood (Jackson and Nicholls, 1986), the steady-state value of $\Delta\psi$ estimated from ion-distribution methods is about half that estimated on the basis of the electrochromic carotenoid response (Ferguson *et al.*, 1979; Clark and Jackson, 1981; McCarthy and Ferguson, 1982). Evidently, any attempts to draw quantitative conclusions from the latter method concerning the relationship between $\Delta\psi$ and the rate of phosphorylation, while ignoring the fact that the $\Delta\psi$ given by ion distribution is below the 150 mV threshold, are doomed to failure. Until we can be more sure of what the various methods for estimating '$\Delta\psi$' are

actually measuring there seems no hope of significant progress in solving the central problem of whether an energetically significant $\Delta\psi$ actually *exists* between the bulk aqueous phases that energy coupling membranes serve to separate.

Notwithstanding this gloomy prognosis, and the carotenoid/ion-distribution discrepancy is as marked as any, many workers have carried out measurements of the pmf under various conditions, titrating it with inhibitors of electron transport, with ionophorous uncouplers or with other molecules, and comparing these values with either the rate or extent of phosphorylation. These studies are briefly commented on below.

9.9.6 Relationships between the apparent pmf and rates and extents of phosphorylation

9.9.6.1 Introduction

On the highly questionable assumption that the Δp values measured by any given method are at least semi-quantitatively reliable, studies of the effects of variation in Δp on the rate or extent of ATP synthesis can give mechanistic information concerning the number of protons translocated across the membrane per ATP synthesized, and on the efficiency of coupling of Δp to phosphorylation.

It has been widely observed that the rate of phosphorylation (J_P) depends more upon the rate of electron transport than upon Δp. In some cases the relationship is entirely arbitrary. These findings have themselves led some reviewers to invoke 'parallel coupling' or alternatives to Δp as an intermediate in energy coupling (see e.g. reviews in Kell, 1979; Ferguson and Sorgato, 1982; Westerhoff *et al.*, 1984a; Ferguson, 1985; Rottenberg, 1985). However, since the rates of reactions may depend upon many factors besides the thermodynamic poise of an energy coupling intermediate (see above), and even on the relative magnitudes of $\Delta\psi$ and ΔpH at constant Δp (Boork and Wennerström, 1984; Sanders, 1987), such findings alone are probably not very informative as to the veracity of any coupling scheme. What *is* worth stressing, however, is that if one can see a significant rate of phosphorylation during a titration at a value of Δp that is well below the 'threshold', it is difficult to argue that this Δp is actually reflecting the Δp which is applied during an 'artificial pmf' experiment. Since this is exactly what has been done in many cases, albeit unknowingly, the titration studies do have the merit of pointing to some of the inadequacies of the methods for estimating Δp.

In a similar vein, there has been a recent controversy (Woelders *et al.*, 1985; Petronilli *et al.*, 1986) as to the relationship between Δp and ΔG_P

under static head conditions (actually in this case equilibrium is claimed) and whether or not this is a function of Δp as the latter is diminished with uncouplers. Whatever the reality, these studies show that continuing ATP synthesis can occur during the approach to static head under conditions in which the Δp (estimated by ion-distribution techniques) is well below 100 mV. Not only does this lead to unrealistic values of the H^+/ATP ratio (Petronilli *et al.*, 1986) but it is *quite inconsistent* with the view that the apparent values of Δp reflect the real values given the thresholds observed in artificial pmf experiments. Similar data are obtained with *Paracoccus denitrificans* (Kell *et al.*, 1978; McCarthy and Ferguson, 1983a,b). Noting the difficulties encountered above with the ergodic hypothesis of equilibrium in complex, heterogeneous structures, it might be prudent to realize how insecure is the idea that stationary extents of probe ion uptake correspond to an equilibrium with any putative $\Delta\psi$ across an energy-coupling membrane.

In view of all these difficulties, and the circularities inherent in attempting to *test* the role of Δp (i.e. delocalized chemiosmotic coupling) by trying to estimate Δp alone, many authors have tried to approach this problem using methods that do not require such an estimation. Some of these are considered next.

9.9.6.2 Respiration-driven proton translocation

The addition of a pulse of oxygen (as a small volume of air-saturated KCl) to a weakly buffered, anaerobic suspension of aerobic respiratory bacteria such as *Paracoccus denitrificans*, held in a reaction vessel whose pH is constantly monitored, might lead to the production of one of the pH traces of the type shown in Figure 9.13. In other words, there might be a slowish decrease in pH which then stays constant (trace a); or a rapid and larger decrease in pH which then turns into a pH increase towards the baseline (trace b); or no effect whatsoever (trace c). In fact, all three such traces have been observed by different workers studying suspensions of *Paracoccus denitrificans*, albeit with different interpretations of their underlying bases (Scholes and Mitchell, 1970; Kell and Hitchens, 1982; Hitchens and Kell, 1984). What are the specific conditions that determine which of these traces is observed?

The first trace is typically observed when cells are used 'as isolated' and when held in a medium containing KCl as osmotic support. The second trace is observed when a compound such as valinomycin is also present or (more commonly with bacteria) when a proportion of the KCl in the suspending medium is replaced by KSCN. The last trace is observed when an appropriate concentration of an uncoupler such as FCCP is present. Taken together, these results are interpreted within a chemiosmotic coupling

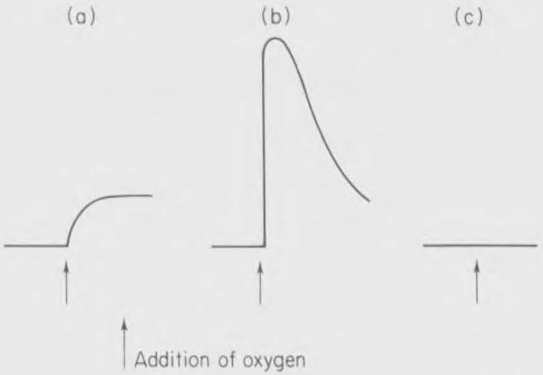


Fig. 9.13 Typical traces observed in an O_2-pulse experiment. For further details, see text.

framework as follows (Fig. 9.14). The energy coupling, bilayer membrane may be represented as an electrical capacitor, whose magnitude may be calculated, and is observed, to be some $1 \pm 0.5\ \mu F/cm^2$ (see e.g. Cole 1972; Harris and Kell, 1985b; Kell and Harris, 1985; Kell, 1986b; Pethig and Kell, 1987 and reference therein). If the primary, electron transport-linked proton pump is electrogenic in the sense that it pumps protons *electrogenically* from the inner bulk phase to the outer bulk phase of the suspension, the result of moving such electrically charged particles across the membrane capacitance is that the 'plates' of this capacitance (represented by the aqueous interfaces on either side of the membrane) become charged, the membrane potential V being given by $Q = CV$ where C is the total capacitance and Q the total number of charges moved. If the cells are spherical or of a known shape, and their number and hence their membrane area is known, the maximum number of charges necessary to obtain a given membrane potential may be estimated (Gould and Cramer, 1977; Kell and Hitchens, 1982; Kell, 1986b, 1987b).

In a typical arrangement such as that of Figure 9.13, the number of protons measured (if pumped *electrogenically*) is more than sufficient to charge fully the membrane capacitance, given that an excessive value of V leads simply to dielectric breakdown (see above). This, in turn, leads either to a redox slip or a non-ohmic leak such that the average, net number of protons observably pumped into the bulk aqueous phase is a function of the average membrane potential during the O_2 pulse. The presence of a substance such as the SCN^- ion, which can cross biological membranes in the charged form (e.g. Kell *et al.*, 1978), dissipates the membrane potential and permits the 'true', limiting stoichiometry of H^+ translocation to be observed. The presence of FCCP causes a rapid back-flow of protons before

the pH electrode can respond, and the fact that the observable $\rightarrow H^+/O$ stoichiometry drops to zero is taken to mean that all H^+ are vectorially pumped, and not scalar protons accompanying unwanted chemical side-reactions.

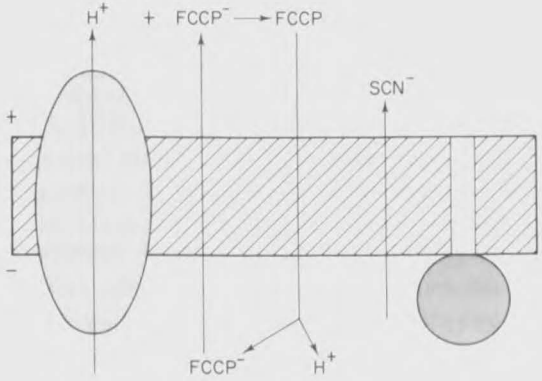


Fig. 9.14 Classical chemiosmotic explanation of the type of data observed in Fig. 9.13, as discussed in the text.

Such reasoning can explain the general features of respiration-linked proton translocation seen in a typical 'O_2-pulse' experiment of the type outlined. If asked to choose between 'slip' and 'non-ohmic leak' as the major explanation for the 'low' $\rightarrow H^+/O$ stoichiometry observed in the absence of SCN^- with a chemiosmotic framework one should plump for the former, since the observable rates of proton back-flux in the presence of SCN^- are very much greater than in its absence, a condition which should be accompanied by an *enhanced* pmf and which is already somewhat difficult to explain (Ferguson, 1985; Kell, 1986b). [It is expected that the electrical capacitance of the coupling membrane is very much smaller than the so-called differential buffering power (Mitchell, 1968).]

This explanation, however, whilst apparently plausible, is open to a more stringent test than that posed by the simple '$\pm$ SCN^-' experiment just described (Gould and Cramer, 1977; Kell and Hitchens, 1982; Hitchens and Kell, 1984). If the translocation of a small number of protons causes the build-up of a membrane potential sufficient to induce a substantial slip or non-ohmic leak across the coupling membrane, so that the net observable $\rightarrow H^+/O$ stoichiometry is submaximal, then doubling the size of the O_2 pulse should not allow the appearance of further protons, since the membrane potential (or pmf) is supposedly already saturated. In practice, this prediction is not borne out (Gould and Cramer, 1977; Gould, 1979; Kell and

Hitchens, 1982; Hitchens and Kell, 1984); the $\rightarrow H^+/O$ stoichiometry is independent of the size of the O_2 pulse over a wide range, before secondary phenomena such as an unfavourable internal pH and an inadequacy of internal anions, come into play. One may take this experiment further, and seek to vary the rate of electron transport (Kell and Hitchens, 1983) or the extent of any putative non-ohmic leak (Hitchens and Kell, 1984) with the intention of comparing the $\rightarrow H^+/O$ stoichiometry under the various conditions using O_2 pulses of different sizes. When conditions are arranged such that this $\rightarrow H^+/O$ stoichiometry is independent of the size of the O_2 pulse, one may treat the system as though it has attained a macroscopic stationary state. In such a system, the pathways of electron transport are independent of the size of the O_2 pulse. What is found (Hitchens and Kell, 1984) is that the presence of venturicidin, a compound which inhibits ATP synthase (Ferguson and John, 1977) and which should also decrease any non-ohmic leak (Cotton *et al.*, 1981), *raises* the observable $\rightarrow H^+/O$ stoichiometry above that seen in its absence (and in the absence of SCN^-). Since this should raise the membrane potential (Kell *et al.*, 1978), it appears that slip is irrelevant to these observations (since a higher $\Delta\psi$ should exacerbate slip and *decrease* the observable $\rightarrow H^+/O$ stoichiometry). Thus (in spite of the argument in the previous section concerning the rate of H^+ backflux), non-ohmic leak is the only free variable to permit these observations to be explained within the framework of a delocalized, chemiosmotic coupling scheme. Unfortunately, as seen above, the stationary state conditions mean that the extent of non-ohmic leak should be a strictly inverse function of the observable $\rightarrow H^+/O$ stoichiometry. This is not the case, and so it must be construed that the lack of observable protons in the absence of SCN^- occurred not because they were not pumped (i.e. slip occurred), nor because they returned across the coupling membrane before the electrode could respond (non-ohmic leak), but simply because they were not pumped in to the bulk aqueous phase.

The calculations concerning the number of protons necessary to make a membrane potential of, say, 200 mV suggest that the great majority appearing in the bulk phase could not be appearing there electrogenically. They must therefore have got there electroneutrally, in symport or antiport with another ion. The question thus arises as to whether *any* of the observable protons were pumped electrogenically to the bulk phase, and were thus capable of creating an energetically significant, delocalized electric membrane potential. This question, which obviously relates to the question of the magnitude of the electron transport-linked $\Delta\psi$ estimated by ion-distribution methods, may be addressed by turning the above reasoning on its head (Gould and Cramer, 1977; Conover and Azzone, 1981; Kell and Hitchens, 1982; Kell, 1986b, 1987).

If an energetically significant $\Delta\psi$ *is* feeding back on the electron transport

chain, then decreasing the number of electron transfer events per cell, and hence the number of H^+ pumped per cell, will decrease the maximum $\Delta\psi$ attainable (i.e. even if all observable protons are pumped electrogenically) to an insignificant value. If this is the case, then the net observable $\rightarrow H^+/O$ stoichiometry should be the same whether SCN^- is present or absent (since its presence can hardly dissipate an already negligible $\Delta\psi$). Again, when attempts are made to perform the experiment under conditions approximating a stationary state, the $\rightarrow H^+/O$ stoichiometry is independent of the size of the O_2 pulse over a wide range, and apparently strictly dependent upon the natural permeability of the membrane to ions other than protons (Gould and Cramer, 1977; Conover and Azzone, 1981; Kell and Hitchens, 1982; Kell, 1986b). There is only one conclusion it seems possible to draw from these types of observations (e.g. Kell and Westerhoff, 1985; Kell, 1986b; and see also Tedeschi, 1980, 1981): the apparent absence of an energetically-significant $\Delta\psi$ (as observed by microelectrodes) is due to the fact that in the stationary state all protons pumped by the respiratory chains of protonmotive systems do not appear in the bulk phase unless the 'primary macroerg' (Blumenfeld, 1983) or 'energized state' set up by electron transport is dissipated by the inclusion of a membrane-permeant ion which then allows each proton pumped across the membrane into an 'invisible' space to enter the bulk phase *electroneutrally*. It should be evident that such an explanation is not consistent with the generation, and hence intermediacy, of a chemiosmotic membrane potential as a part of electron transport-linked phosphorylation.

Experiments related to the above have recently been performed by Jackson and colleagues in photosynthetic bacteria, with similar results (in terms of the discrepancies between 'expected' and 'observed' $\rightarrow H^+/e^-$ ratios) but with, in part, a somewhat different interpretation (Taylor and Jackson, 1985a,b; Myatt and Jackson, 1986). In view of the discrepancies between the number of protons measured with a glass electrode and a pH indicator (approx. 43 and 144 in Fig. 2 of Taylor and Jackson, 1985b), and between the number of charges allegedly necessary to create the maximum membrane potential (approx. 5) judged by carotenoid spectroscopy, and these observed (approx. 2; Myatt and Jackson, 1986), one would retain the conclusion that the data obtained to date, using observations of respiration-linked translocation of protons, are consistent with the view that essentially *all* protons observed in the bulk phase are deposited there electroneutrally and thus cannot contribute to the generation of a delocalized membrane potential.

In this regard, it is worth drawing what I have found to be a useful analogy when discussing some of the points at issue in this chapter (Fig. 9.15). It is not disputed that the source of free energy both for charging a car

battery via a dynamo and for turning the wheels is the engine (Fig. 9.15). Similarly, it is not conventional to draw a bidirectional arrow between the wheels and the dynamo to suggest that the latter may be an intermediary energy source for the former. Nevertheless, there is a remarkable tendency, faced with what amounts to the same logical structure, to assume that any ion or proton movements accompanying electron transport and entering the bulk phase might subsequently be coupled to otherwise endergonic processes such as ATP synthesis. It should be clear (Fig. 9.15) that this is not a logical procedure! The fact that bulk-phase proton movements accompany electron transport, and thus contribute to the creation of a small pH gradient, tells us nothing whatever about whether they may subsequently be used to drive phosphorylation. This rests on other criteria, which, when tested, are found wanting.

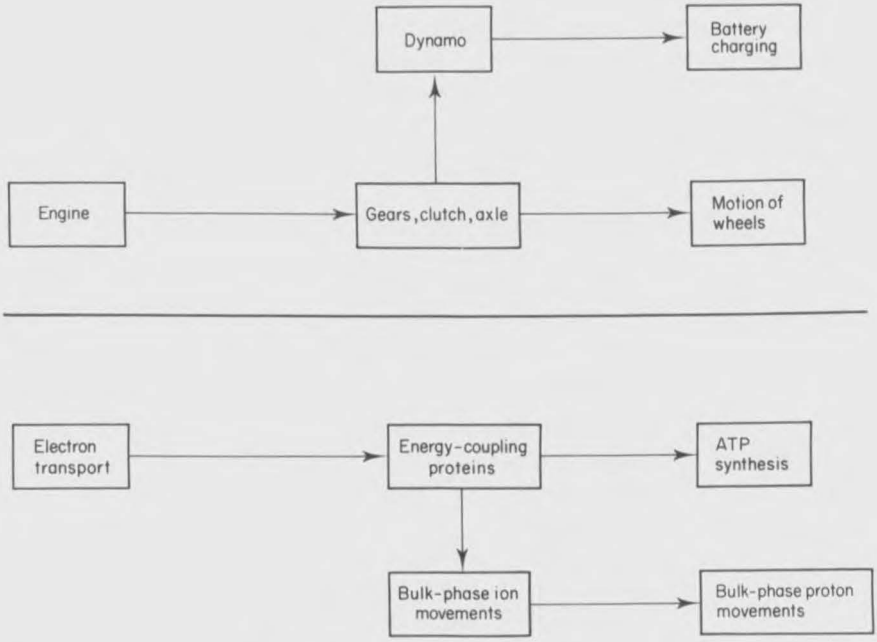


Fig. 9.15 Phenomena which may be observed concomitantly do not necessarily bear a given cause-and-effect relationship to each other. Turning on the engine of a car causes both dynamo and wheels to move, but it is not the dynamo which is driving the wheels. Similarly, electron transport causes both phosphorylation and observable movements of (bulk phase) protons and other ions to occur. This does not of course mean that the proton movements are driving phosphorylation!

Since everything seen so far might well have caused one to have the gravest suspicions about whether or not any signal purporting to represent $\Delta\psi$ actually leads one logically to the view that the latter is of sufficient magnitude to be an intermediate in electron transport-linked phosphorylation, other approaches which do not rely upon the measurement of Δp but exploit its delocalized *nature* have been sought. One such approach is that known as 'double-inhibitor titrations'.

9.9.7 Double-inhibitor titrations

The general principle of the double-inhibitor titration (DIT) method was first expounded in a bioenergetic context by Kahn (1970) and by Baum (Baum *et al.*, 1971; Baum, 1978). Motivated by related experiments carried out by Venturoli and Melandri (1982), the approach was taken up and extended by Hitchens and Kell (1982a,b; 1983a,b) and by a number of other workers (Parsonage and Ferguson, 1982; Cotton and Jackson, 1983; Berden *et al.*, 1984; Krasinskaya *et al.*, 1984; Mills, 1984; Westerhoff *et al.*, 1984b; Davenport, 1985; Ferguson, 1985; Herweijer *et al.*, 1985, 1986; Pietrobon and Caplan, 1985a; van der Bend and Herweijer, 1985; van der Bend *et al.*, 1985; Chen, 1986; Kell, 1986b,c; Kell and Walter, 1986; Westerhoff *et al.*, 1986; Pietrobon and Caplan, 1986a,b; Kell, 1987b; Petronilli *et al.*, 1988; Slater, 1987; Westerhoff and Kell, 1988).

The idea behind these types of approach runs essentially as follows (Fig. 9.16).

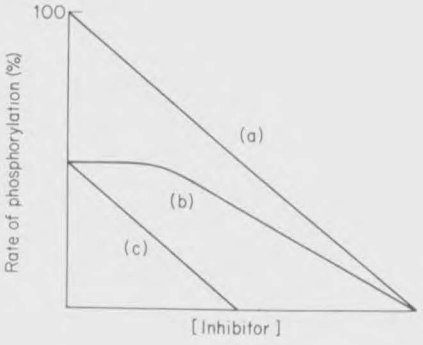


Fig. 9.16 Possible diagrammatic traces observed in a double-inhibitor titration using an inhibitor of electron transport in the absence (a) or in the presence (b,c) of a titre of ATP synthase inhibitor sufficient to inhibit the 'starting' rate of phosphorylation by 50%.

If a delocalized intermediate (such as the pmf) serves to link electron transport with phosphorylation, the addition of a titre of a tight-binding ATP synthase inhibitor will tend to raise the pmf and thus the titre of an inhibitor of electron transport or of an uncoupler necessary to inhibit phosphorylation, provided that these act to inhibit phosphorylation by decreasing the pmf, and that the rate of phosphorylation is controlled solely by the pmf. In practice, it is observed, under appropriate conditions, that such a treatment tends to leave unchanged the inhibitory titre of an electron transport inhibitor and actually *decreases* the titre of uncoupler necessary to inhibit phosphorylation by a given amount, results initially thought quite incompatible with a delocalized energy coupling scheme.

However, as seen above, when considering metabolic control analysis, metabolic fluxes are rarely, if ever, controlled by single parameters (and never by variables), and the later commentaries on this topic have invoked, or drawn attention to, conditions (of varying degrees of plausibility) in which the data observed *may* in fact be made to fit a delocalized energy coupling scheme. For instance, there may be redox-linked *activation* of the ATP synthases, or the elasticity of the primary proton pumps towards the pmf may be significantly different from that of the ATP synthase towards the pmf, so that uncouplers might (counterintuitively) be expected to work better when ATP synthases are partially inhibited by an appropriate titre of energy transfer (ATP synthase) inhibitor. Thus a largely quantitative analysis is required, although preferably one which does not of itself *rely* upon the accurate estimation of the values of the pmf.

The most recent and comprehensive analyses of this topic (Pietrobon and Caplan, 1987a,b; Petronilli *et al.*, 1988; Westerhoff and Kell, 1987), based on metabolic control analysis and permitting arbitrary (but *stated and self-consistent*) relations to exist between Δp and the partial reactions with which it is supposed to be coupled, lead to the following conclusions:

(a) no delocalized model can account for the finding that the titre of uncoupler necessary for *full* uncoupling is lower in the presence of a partially-inhibitory titre of ATP synthase inhibitor than in its absence;
(b) qualitatively, linear and non-linear chemiosmotic models can explain some but not all of the data; and
(c) when the P/2*e* ratio is taken into account, the degree of leakiness required to explain the data on a delocalized coupling basis is not consistent with that found.

It is worth remarking in this context that the above analysis is confined *strictly* to genuinely stationary states. It is of interest nonetheless that studies on the prestationary *initiations* of phosphorylation in thylakoids (Hangarter and Ort, 1985) show that this may, under some, but not all conditions

(Chiang and Dilley, 1987), be strongly controlled by cooperation between electron transfer chains.

Finally, the DIT approach opens up a further interesting means of testing whether a particular method such as ion uptake might plausibly be measuring a delocalized intermediate such as a membrane potential. The idea in such an experiment is that, given the relatively low electrical capacitance of coupling membranes discussed above, the pmf rapidly attains its 'steady-state' value. The uptake of 'probe' ions which supposedly monitor $\Delta\psi$ is then a slow response to this essentially 'clamped' $\Delta\psi$. Since in all cases, purely energy-transfer inhibitors tend to raise the $\Delta\psi$ estimated in the stationary state, it is to be expected that neither the rate nor extent of ion uptake should be decreased, during an uncoupler titration, by the inclusion of an energy transfer inhibitor in the reaction mixture.

A reductionist approach to biochemistry would have it that the best way to decide what is necessary for a given process, and thus what may be its mechanism, is to isolate, purify and reconstitute the enzymes believed to be involved in that process. Purportedly according to such reasoning, another means by which some authors have sought to establish the veracity of delocalized chemiosmotic coupling is by co-reconstitution experiments. However, since the theory and the data (as with several other aspects of the present topic) are utterly divergent (Kell and Westerhoff, 1985), it seems appropriate to draw attention to them here.

9.9.8 Co-reconstituted systems: the emperor is not yet clothed

The idea behind the co-reconstitution approach may be illustrated with reference to Figure 9.17; it is that the co-reconstitution, in a lipid vesicle, of a 'primary' proton pump, capable of creating a pmf, together with a 'secondary' proton pump, capable of using a pmf to drive a reaction, should permit the energy coupling of the chemical reactions catalysed by the two types of enzyme (complex). Following the initial experiments of Racker and Stoeckenius (1974) with bacteriorhodopsin and a very impure ATP synthase preparation, it is widely believed that such a successful co-reconstitution has been amply demonstrated, with the implication that energy coupling is, indeed, via a chemiosmotic type of mechanism. As stressed elsewhere (Kell and Morris, 1981; Kell and Westerhoff, 1985; Kell, 1986b) and as I shall briefly review once more, the presently available data actually justify rather the opposite conclusion.

In many of these co-reconstitution experiments, the assumption is made that because the reversible primary and secondary proton pumps do actually pump protons, then any coupled phosphorylative activity (for example) that is observed must also be taking place via a delocalized pmf. As seen above

(Fig. 9.15), this is an extraordinary piece of logic which may be expunged from our considerations. Any phosphorylative activity observed in a co-reconstituted system may be occurring via any type of mechanism, which may or may not be the same as that occurring *in vivo* and may or may not be via a pmf. (Enhanced and possibly free-energy-transducing collisional interactions between the proteins in the more fluid milieu of a liposome are one obvious possibility.) The question which should at the very least be asked in order to gain mechanistic information about the coupling in a co-reconstituted system is: what is the *turnover number* of the secondary proton pump (typically an ATP synthase preparation)? This, and not the absolute flux (in terms of nmol/minute/mg protein), is what can show that these systems are actually working according to the 'simple interpretation' (Kell and Westerhoff, 1985) given in Figure 9.17.

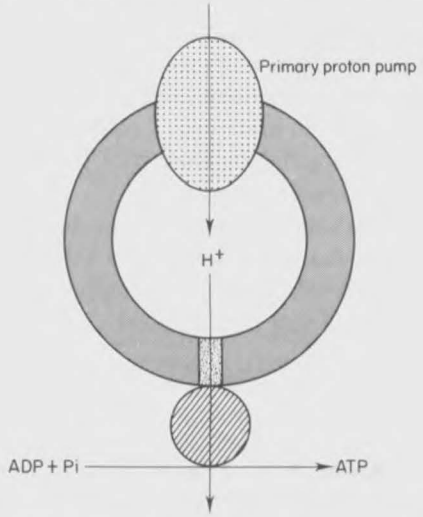


Fig. 9.17 The 'simple interpretation' of co-reconstitution experiments in which it is assumed that any phosphorylation observed is occurring via the generation of a pmf between *purified* and *spatially separate* proton pumps.

In one of the more thoughtful studies of that era (Hauska *et al.*, 1980), it was shown in a system co-reconstituted from a photosystem I preparation and a thylakoid CF_0F_1 that, whereas the *rate* of photophosphorylation was some 30% that of the rate *in vivo*, the *turnover number* of the ATP synthase molecules was *less than 1% of that in vivo*; i.e. a value that in other contexts is considered to be more or less zero. Since even in these relatively purified systems the components were certainly not 99% pure, one can as well argue that *purified* co-reconstituted systems can *not* in fact make ATP. As surveyed

and discussed elsewhere (e.g. Kell and Morris, 1981; Casey, 1984; Kell and Westerhoff, 1985; Kell, 1986b), the data of Hauska *et al.* (1980) are quite typical for co-reconstituted systems using more-or-less *purified* components. Recent small improvements in turnover numbers (van der Bend *et al.*, 1984), and studies of the effects of alternating the ratio of primary to secondary proton pumps, have not qualitatively altered these conclusions; i.e. that energy coupling here is (i) extremely inefficient and (ii) not via a pmf of any credible magnitude.

9.10 Concluding remarks

Readers will be aware that the present overview differs from many in bioenergetics in seeking to argue a self-consistent case to the effect that the protonmotive force across energy coupling membranes catalysing electron transport-linked phosphorylation is energetically insignificant. Reasons of space have meant that many pertinent arguments (Kell and Westerhoff, 1985) could not be developed but hopefully, it has been conveyed, at the very least, that the subject of bacterial bioenergetics is alive, lively and offers plenty of scope for further research and study.

Finally, the Editor wishes me to add here that his aim in including this final chapter is to indicate that, although this is the close of the book, the book of bioenergetics is not closed, and also to emphasize that we should not believe all that we read (including this chapter).

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