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Catalytic Facilitation and Membrane Bioenergetics

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> It seems to me that there is no single idea in biology which is hard to understand, in the way that ideas in physics can be hard. If biology is difficult, it is because of the bewildering number and variety of things that one must hold in one's head.
>
> (Maynard-Smith, 1977)

I. INTRODUCTION

What is a cell like? The simplest attempt at a conceptual subdivision contrasts those (soluble) enzymes and molecules which, upon cell disruption, are released into the supernatant of a high-speed centrifugation step (105,000 × *g*; 1 h) with those (membrane bound) which are not. Now, while the evidence for the organization, aggregation, and indeed membrane association *in vivo* of many enzymes, such as those of glycolysis (Gorringe and Moses, 1980; Masters, 1981), is now extensive (e.g., Welch 1977; Welch and Keleti, 1981; Clegg, 1983a,b), this topic has been widely reviewed and is also excellently covered by other contributors to this volume.

Equally, just as a prime purpose of glycolysis is to synthesize ATP by substrate-level phosphorylation, a prime purpose of many biological membranes (*energy coupling membranes*) is also to catalyze ATP synthesis. These coupling membranes are exemplified by the inner mitochondrial membrane, the chloroplast thylakoid membrane, and the plasma (cytoplasmic) membrane

of respiratory and photosynthetic bacteria. Our main purpose in this chapter will thus be to describe the nature, role, and organization of the free energy-transducing devices that these energy-coupling membranes contain, placing special emphasis upon the process of electron-transport phosphorylation. The literature is covered comprehensively through 1983.

Since many membranes as isolated catalyze electron-transport phosphorylation at rates, and with efficiencies, similar to those implicated *in vivo*, we regard it as permissible to consider such membranes in isolation from their normal "cytosociological" (Welch and Keleti, 1981) habitat. To this end, we next consider the gross structural features of typical membranes catalyzing electron-transport phosphorylation. We shall concentrate in particular upon the inner mitochondrial membrane, although when necessary or appropriate we shall address ourselves to other systems. We shall see that in electron-transport phosphorylation as well, enzymes that were previously thought to operate independently turn out to function in rather tightly linked units.

II. STRUCTURAL DYNAMICS OF ENERGY-COUPLING MEMBRANES

A. The Occurrence and Nature of Oligomolecular Complexes in Energy-Coupling Membranes

1. Introduction

As is now well known (e.g., Stryer, 1981; Lehninger, 1982), the best *general* biomembrane model (Singer and Nicolson, 1972; Finean *et al.*, 1978; Houslay and Stanley, 1982) visualizes biological membranes as consisting of a fluid phospholipid bilayer in, on, and through which are dispersed protein molecules, either as monomers or, more commonly in energy-coupling membranes, as oligomeric, polytopic (Blobel, 1980; Brock and Tanner, 1982) complexes. Thermodynamic considerations dictate (e.g., Tanford, 1978, 1980; Jähnig, 1983) that the major organizing force determining the degree of penetration of such complexes into or through the bilayer is the favorable free energy of transfer of the hydrophobic areas of proteins from an aqueous to a lipidic environment. There are also reasons to suppose that "the hydrophobic domain of a transmembrane protein is vertically delimited at both its upper and lower ends by two collars of charged amino acid residues, which interact with the polar head groups of lipids" (Montecucco *et al.*, 1982). Such interactions can be of great importance to the catalytic activity of such proteins (Johansson *et al.*, 1981a,b).

The likely arrangement of the polypeptide chain in, say, bacteriorhodospin (e.g., Engelman *et al.*, 1980) is consistent with the probable generality of this

view, and, since this is not, perhaps, to be regarded as unexpected, we do not pursue it here. We may add, at this stage, that the flip-flop motion of even phospholipids (Kornberg and McConnell, 1971), let alone proteins, is negligible on a time scale relevant to electron-transport phosphorylation. Finally, there is abundant calorimetric and other evidence that the gel–liquid phase transition of membrane phospholipids usually occurs well below physiological temperatures, as documented, for instance, for the membranes of thylakoids (Murato and Fork, 1975), mammalian mitochondria (Hackenbrock *et al.*, 1976), and respiratory (McElhaney, 1974; Mechler and Stein, 1976) and photosynthetic (Fraley *et al.*, 1978; Kenyon, 1978; Kaiser and Oelze, 1980) bacteria (for possible exceptions see Raison, 1973). The gel–liquid phase transition does, however, occur over a range of temperatures, which tends to indicate heterogeneity in the fluidity of biological membranes (Vaz *et al.*, 1982).

We may therefore proceed from our general fluid mosaic picture to ask some slightly more detailed questions concerning the structural dynamics of (generally unenergized) energy-coupling protein complexes in biological membranes. The insertion and assembly of these complexes in the membrane, though a fascinating area of study, is beyond the scope of this chapter (see e.g., Brock and Tanner, 1982). Because of its special properties, we shall in general treat the quasi-crystalline "membrane" bacteriorhodospin as a separate complex.

2. How Much Phospholipid Bilayer Is There in Energy-Coupling Membranes?

Energy-coupling membranes are among the richest in protein. Thus, the inner mitochondrial membrane contains approximately 75–80% protein and 20–25% phospholipid (e.g., Tzagoloff, 1982; Kröger and Klingenberg, 1970), and similar ratios are found in other energy-coupling membranes (e.g., John and Whatley, 1977). It is sometimes remarked (e.g., Ling, 1981) that this low percentage of phospholipid cannot be sufficient to form a continuous bilayer. Now, many proteinaceous complexes pass right through the plane of the bilayer, for example, bacteriorhodopsin (Henderson and Unwin, 1975; Ovchinnikov *et al.*, 1979; Engelman *et al.*, 1980) and cytochrome oxidase (e.g., Henderson *et al.*, 1977; Fuller *et al.*, 1979; Azzi, 1980; Brunori and Wilson, 1982; Capaldi, 1982a,b), and while the former is fairly flush with the plane of the membrane, the latter extends well beyond it, as do many other complexes that we shall discuss, such as the H^+-ATP synthase (Soper *et al.*, 1979) and ubiquinol–cytochrome *c* reductase (EC 1.10.2.2) (e.g., von Jagow and Engel, 1980). Thus, a figure of, say, 20% for the percentage of phospholipid in an energy-coupling membrane provides a minimum for the percentage of surface

area that may be in a bilayer configuration, since energy-coupling membranes are not thought to contain large areas of protein–phospholipid monolayer.

In any event, NMR work (e.g., Arvidson *et al.*, 1975; Cullis *et al.*, 1980; de Kruijff *et al.*, 1982) shows very clearly that the great majority of phospholipids of the inner mitochondrial membrane are in a bilayer configuration, and we do not find much experimental evidence (but cf. Sjöstrand, 1978) to dissuade us from this interpretation. Whereas it is of interest that rather extensive delipidation of mitochondria causes no gross structural changes in the inner membrane (Fleischer *et al.*, 1967), as judged by electron microscopy, our own present interest must be centered primarily upon systems that are capable of carrying out free energy transduction, and we shall not consider these observations further, save to mention that they simply indicate that lipid–protein interactions may be substituted for by hydrophobic protein–protein interactions under appropriate conditions.

Studies using differential scanning calorimetry and freeze-fracture electron microscopy by Hackenbrock, Höchli, and colleagues (Hackenbrock *et al.*, 1976; Höchli and Hackenbrock 1976, 1977; Hackenbrock, 1976, 1981) show rather clearly that the total protein mass of the native inner mitochondrial membrane occupies less than one-half of the total lateral area of the membrane, and freeze-fracture work by others indicates a similar picture for the thylakoid membrane (e.g., Anderson, 1975; Arntzen, 1975, 1978). Most importantly, however, quite small changes in physical parameters have been shown to exert a marked effect upon the state of distribution of the intrinsic protein complexes of the inner mitochondrial membrane (Hackenbrock, 1976), and this leads us nicely to two important ideas that form the basis of the next two sections: proteins exist, and can move, as oligometric complexes in the plane of energy-coupling membranes. Topics related to this question, such as phospholipid organization (e.g., Cullis and de Kruiff, 1979; Israelachvili *et al.*, 1980; Blaurock, 1982; Davis, 1983) and asymmetry (e.g., Etemadi, 1980), are largely beyond our present scope.

3. Complexes of the Inner Mitochondrial Membrane

The inherent complexity of living systems in general, and of the inner mitochondrial and other energy-coupling membranes in particular, has led many workers to seek to isolate enzymes and/or complexes that carry out defined, partial reactions of processes such as oxidative phosphorylation. As is well known, pioneering work in the laboratories of Green, Hatefi, and Racker led to the isolation and characterization of proteinaceous complexes of the mitochondrial electron-transport chain (complexes I to IV) and the H^+-ATPase (complex V) components that could catalyze defined electron transfer or ATPase–ATP-P_i exchangease reactions, respectively. Not surprisingly,

each complex has provided a rich area of study in its own right, and this approach has been extended by a great many workers to broadly comparable complexes in thylakoid and bacterial energy coupling membranes. The reviews by De Pierre and Ernster (1977), by Hatefi (1976), and by Capaldi (1982b) (see Table I) and the books by Tedeschi (1975), by Tzagoloff (1982), and by Nicholls (1982) provide useful introductions to the voluminous mitochondrial literature.

As pointed out by Saraste (1983), the criteria usually used in assessing the polypeptide composition of a particular complex often leave much to be desired. Fortunately, we shall largely be able, for our present purposes, to treat the complexes, especially complexes I to IV, more or less as black boxes, and will tend to assume that they contain those polypeptides ascribed to them as a consensus by the authorities in this field. The "complexes" of beef heart mitochondria are given in Table I.

The crucial, if well-known, message for our present purposes is this: oligometric protein complexes, which can be visualized by electron microscopy, exist as defined entities in energy-coupling membranes and catalyze reactions coupled to macroscopically observable chemical changes such as electron transport or ATP hydrolysis. Since, to preempt some of our later discussion, we shall wish to consider in detail the degree of localization of free energy transfer between different electron-transport and ATPase complexes, it

TABLE I
SOME GENERALLY RECOGNIZED COMPONENTS OF THE BEEF HEART MITOCHONDRIAL INNER MEMBRANE[a]

Components	Concentration range (nmol/mg membrane protein)	Approximate molecular weight of monomer (kD)
Complex I (NADH–ubiquinone oxidoreductase)	0.06–0.13	700
Complex II (succinate–ubiquinone oxidoreductase)	0.19	200
Complex III (bc_1 complex) (ubiquinol–cytochrome *c* oxidoreductase)	0.25–0.53	300
Complex IV (cytochrome *c* oxidase)	0.6–1.0	160
Complex V (ATP synthase)	0.52–0.54	500
Cytochrome *c*	0.8–1.0	12
ADP–ATP translocase	3.4–4.6	30
Transhydrogenase	0.05	120
Ubiquinone	6–8	—
Phospholipid	440–590	—

[a] Data after Capaldi (1982b).

is of interest to know the rate at which such complexes may collide with each other, a topic to which we now turn.

4. Lateral Diffusion of Protein Complexes in Energy-Coupling Membranes

That oligomeric protein complexes can, in many cases, diffuse laterally at rather rapid rates in the plane of energy-coupling and other biological membranes, although an important implicit corollary of the concept of the fluid mosaic membrane has now been demonstrated directly by a variety of experimental techniques (e.g., Edidin, 1974; Poo and Cone, 1973; Jaffe, 1977; Poo and Robinson, 1977; Webb, 1977; Evans and Hochmuth, 1978; Finean *et al.*, 1978; Cherry, 1979; Barber, 1980; Sowers and Hackenbrock, 1981; Anderson and Anderson, 1982; Houslay and Stanley, 1982; Vaz *et al.*, 1982; Webb *et al.*, 1982; Zimmerman, 1982; Zimmerman and Vienken, 1982; Axelrod, 1983; Barber, 1983; Kell, 1983; Robertson, 1983). The different apparent mobilities of complexes I–IV in the inner mitochondrial membrane have been tabulated by Hackenbrock (1981) (Table II). Thus, even the least mobile complexes can move nearly 0.05 μm during the passage of a single electron through them. The distance diffused by ubiquinone molecules per turnover is some 40-fold greater, and this phenomenon can evidently account for the "Q-pool" behavior established by Kröger and Klingenberg (1973) in mitochondria, and the well-established and broadly comparable function of plastoquinone in thylakoids as a mobile electron-transfer agent between the spatially separate (Anderson, 1981; Andersson and Haehnel, 1982), proteinaceous light-harvesting complexes containing photosystems I and II. It is worth stressing that one may make these conclusions based on experimental data such as the rates of electron transfer in mitochondria (Hackenbrock, 1981) and the amount of spillover between the two photosystems in thylakoids (Barber 1980, 1982b; Anderson and Andersson, 1982) that have negligible interpretational complexities relative to those of certain more physical, as opposed to functional, approaches. However, we should mention that the exact status of the "Q-pool" concept (Heron *et al.*, 1978; Gutman, 1980; Yu and Yu, 1981; Haehnel, 1982; Rich, 1982; Trumpower, 1982; Hauska *et al.*, 1983) represents an area of active debate and great uncertainty.

For our discussions below it is important that Hackenbrock (1981) claims that the different protein complexes (i.e., complex I, II, III, and IV (Tables I and II) diffuse independently of one another. This might be extrapolated to a suggestion that also complex V (i.e., the H^+-ATPase) diffuses independently. We wish to point at a number of uncertainties in the basis of such a claim. First, we are not aware of any experimental evidence indicating diffusion of H^+-ATPases independent from electron-transfer chain components. Secondly,

TABLE II

EXPECTED LATERAL DISTANCE DIFFUSED PER UNIT TIME BY REDOX COMPONENTS OF THE INNER MITOCHONDRIAL MEMBRANE[a]

Redox component	Approximate stoichiometry	Lateral diffusion coefficient in cm^2/s	Electrons transferred in one turnover	One turnover in state 3 in ms	Distance diffused during one turnover in nm[b]	Distance diffused during 20 ms in nm
Complex I	1	8.3×10^{-10}	2	5.5	43	81
Complex II	2	8.3×10^{-10}	2	11.0	60	81
Complex III	3	8.3×10^{-10}	1	8.6	53	81
Complex IV	7	8.3×10^{-10}	1	20.0	81	81
Cytochrome *c*	9	1.0×10^{-8}	1	28.0	335	280
Ubiquinone	63	1.0×10^{-8}	2	780.0	1760	280

[a] Data after Hackenbrock (1981).

[b] Distances are based on the lateral diffusion coefficients given and a state 3 respiratory rate for succinate oxidation of 50 electrons s^{-1} $heme^{-1}$ aa_3.

Hackenbrock bases his claim on the effect of fusion of liposomes to mitoplasts (i.e., mitochondria depleted of their outer membrane), which is reduction of the rates of redox reactions that involve two or more of the complexes. It should be noted that the explanation alternative to Hackenbrock's (who proposes that the rates are reduced because the proteins have to diffuse over longer distances), that is, that low-molecular-weight (lipid-soluble) ecofactors such as ubiquinone are diluted out, has not been fully ruled out. Moreover, mitoplasts fused to liposomes are immensely different from intact mitochondria with their much more folded inner membrane (cf. Sjöstrand, 1978); the former have been shown not (Hackenbrock, 1981; Westerhoff, unpublished observations) to catalyze oxidative phosphorylation. Thirdly, the diffusion coefficients measured by Sowers and Hackenbrock (1981) may be representative of a case where the existing protein–protein interactions have been disrupted (see below).

For our present purposes, however, we may state three facts: (1) electron-transport events may take place in isolated, reconstituted systems *in vitro*, containing only the recognized complexes of the appropriate region(s) of the electron transport chain(s) of interest; (2) such complexes do not need to be membrane incorporated to express such activity (e.g., Lam and Malkin, 1982); and (3) *electron-transport* events can be reconstituted using protein complexes from different types of energy-coupling membrane (e.g., Packham *et al.*, 1980). Such findings give weight to the widely held belief in the close functional relationships between electron-transfer components in different types of membrane (see e.g., Hauska *et al.*, 1983), as well as to the permissibility of treating these complexes as black boxes.

5. Rotational, Bending, and Stretching Behavior of the Components of Energy-Transducing Membranes

It is now well known that the rotation of individual complexes is generally rather fast (e.g., Cherry, 1979; Muller *et al.*, 1982) and occurs on a time scale similar to that of the exchange of so-called boundary lipid (e.g., Vanderkooi, 1978; Jardetzky and Roberts, 1981; Gennis and Jonas, 1977; Chapman *et al.*, 1982; Marsh, 1983) with that of the bulk lipid of the bilayer. These rates are far more rapid than the turnover of the apparatus of electron-transport phosphorylation, but we will not pursue this issue here.

In modelling a biomembrane, it is common to draw it as a flat sheet, as though there were no mobile deformations in a plane perpendicular to the plane of the bilayer. This is certainly an unjustified and erroneous oversimplification (e.g., Evans and Hochmuth, 1978; Haines, 1979; Miller, 1981; Crilly and Earnshaw, 1983; Brown *et al.*, 1983; Robertson, 1983); however, the extent to which such motions are of significance in energy coupling remains,

regrettably, as yet unknown, although Haines (1979) proposes a causal role of lipid-mediated potential compaction waves in oxidative phosphorylation (see also Haines, 1982).

6. Fluctuational Behavior of Energy-Transducing Proteins

It is now widely recognized that proteins exhibit thermally activated motions of varying degrees of cooperativity, on a time scale from picoseconds upwards (e.g., Gurd and Rothgeb, 1979; Clementi and Sarma, 1983; McCammon and Karplus, 1983; Welch, 1985). There is also evidence of very slow conformational transitions in energy transducing ATP synthases, on a time scale of minutes (e.g., Slooten and Branders, 1979). However, since our main interest lies in the organization of these membranes during the time necessary to synthesize a single ATP molecule, we shall confine our thoughts to the time scale below 100 ms. Some of the typical motions that have been characterized for globular proteins are noted in Fig. 1. Our main purpose here is (1) to indicate the extensive nature of these fluctuations, (2) to remind readers of the probability that these fluctuations play an *essential* role in enzyme catalytic processes (Welch *et al.*, 1982; Careri *et al.*, 1979; Welch, 1985) and (3) to state that a complete description of electron-transport phosphorylation should preferably take such fluctuational behavior into account. The philosophical problems inherent in the description of free energy-transducing devices of molecular size, which are subject to such thermal fluctuations yet work under macroscopically isothermal conditions, are explored elsewhere (Welch and Kell, 1985; Somogyi *et al.*, 1984).

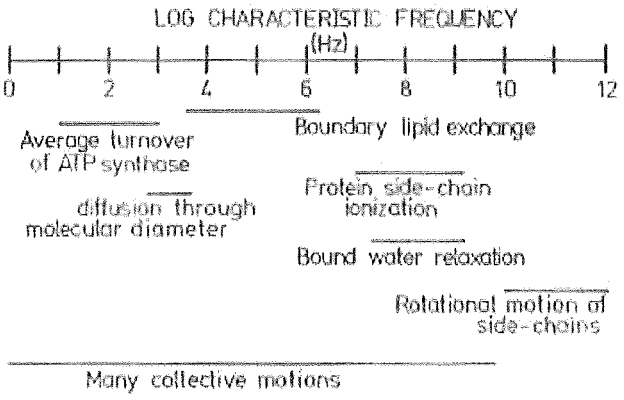

Fig. 1. Some time events of biomembrane proteins of relevance to electron transport phosphorylation. For many further details, and the evidence for such motions, see Gurd and Rothgeb (1979), Careri *et al.* (1979), and Welch *et al.* (1982). It is evident that the overall process of electron transport phosphorylation occurs on a very slow time scale relative to that of other molecular motions.

7. Disposition of Protein Complexes under Energized Conditions

In the absence of electron transport- or ATP-derived free energy, it is to be assumed that the disposition of protein complexes in the fluid mosaic membrane follows a more or less random distribution, that is, that their topological relationship to each other constitutes an equilibrium, or quasi-equilibrium process. The question arises, therefore, as to what infuence, if any, the input of free energy (whether from electron transport or from ATP) exerts upon the disposition of the energy-transducing protein complexes themselves.

Remarkably enough, there is relatively little direct evidence to indicate, whether such free energy inputs can promote the (transient) association of such protein complexes that may be of relevance to the free energy-transducing processes themselves. However, several lines of circumstantial evidence indicate that this indeed may be the case. Energization was shown to affect the distribution of complexes in the inner mitochondrial membrane (Hackenbrock, 1972), and Goodchild *et al.* (1983) have shown that electron transport does indeed result in a somewhat closer association of intramembranous particles, including the light-harvesting and the cytochrome *b/f* complexes, in chloroplast thylakoids.

A number of recent detailed and elegant studies have shown that appropriately oriented electrical fields can drive the migration of intramembrane protein complexes (see e.g., Jaffe, 1977; Poo and Robinson, 1977; Sowers and Hackenbrock, 1981; Poo, 1981; Zimmermann, 1982; see also Kell, 1983). This phenomenon has been termed *lateral electrophoresis*, and it has been shown that the clustering of certain membrane components can have significant effects upon their catalytic properties (Young and Poo, 1983). In particular, since, as we shall see shortly, electron-transport and ATP hydrolase reactions are accompanied by the generation of (initially) localized electrical fields, it does not seem unreasonable that some type of topological arrangement of electron transport and ATP synthase complexes, *inter alia*, is of significance to the energy-coupling process.

We may consider the recent elegant work of Zimmermann and colleagues (see Zimmermann *et al.*, 1981; Zimmermann, 1982; Zimmermann and Vienken, 1982, for reviews) on electrically mediated cell–cell fusion. In this process, cells are apposed by dielectrophoresis in a medium of low electrical conductivity, and then fused by the application of a short (microsecond) electrical pulse of high field strength. Molecularly, it is thought that the area of cell–cell contact (strictly, protoplast–protoplast contact) involves solely phospholipid bilayer areas of the membrane, and that, *ergo*, part of the role of dielectrophoretic induction involves the lateral electrophoresis of membrane proteins (and/or charged phospholipids (Zimmermann and Vienken 1982; Kell, 1983)). Most significantly, it was shown using *Avena sativa* protoplasts

that while the adenylate energy charge of the cells was irrelevant to fusion, the time taken for the fused double cell to round up was essentially inversely proportional to the ATP/ADP ratio (Verhoek-Köhler *et al.*, 1983). Although, as the authors mention, this may be due to the requirement for phosphorylation of one or more membrane proteins, it seems possible that ATP energization of the plasma membrane may be required for the normal effective disposition of proteins in these membranes (as well as the rearrangement of the cytoskeleton).

We may also consider the isolation by simple mechanical disruption of chromatophores from the plasma membrane of photosynthetic bacteria. As is well known, these chromatophores contain all the apparatus of electron-transport phosphorylation, yet possess a composition quite distinct from that of the other parts of the cytoplasmic membrane (Garcia *et al.*, 1981; Dierstein *et al.*, 1981; Kaufmann *et al.*, 1982; Oelze and Drews, 1981). Given the apparent absence of any anchorage between membrane proteins and cytoplasmic structures in these prokaryotes (Drews, 1982), one is virtually bound to conclude either that the complexes bind to each other or to other membrane proteins as an equilibrium process and/or that some factors, requiring the input of free energy, act to array the complexes of electron-transport phosphorylation in chromatophores in a reasonably close spatial relationship to each other *in situ* in the intact cell. This conclusion is very strongly reinforced by the elegant observations of a highly *nonrandom* segregation of membrane components between daughter cells in a variety of dividing microorganisms (Kepes and Autissier, 1972; Poole, 1981; Edwards, 1981; Lloyd *et al.*, 1982).

To put this point on a quantitative footing, let us take a fairly typical protein complex diffusion coefficient (D) of 10^{-9} cm^2/s (Hackenbrock, 1981). The relaxation time (τ) for the randomization of such a complex in a spherical shell membrane of radius R is given by $\tau = R^2/2D$ (Huang, 1973; Benz and Zimmermann, 1981; Sowers and Hackenbrock, 1981; Zimmermann, 1982). As remarked elsewhere (Kell, 1983), this equation assumes that the complexes take up a negligible volume fraction of the bilayer; for a given τ this will overestimate D (or underestimate R), but we will ignore this. Thus the relaxation time for randomization of the disposition of a protein complex in a spherical bacterial cell of radius 0.5 μm ($= 5 \times 10^{-5}$ cm) is $25 \times 10^{-10}/2 \times 10^{-9}$ s, that is, 1.25 s. Since the fastest doubling time for a microorganism, $\sim$20 min, is some 1000-fold greater, there would seem to be an extremely serious discrepancy between (1) the more biophysical studies indicating rapid and random diffusion of electron-transport complexes and (2) the more biochemical studies demonstrating highly *nonrandom* segregation of membrane components. Obviously, such calculations depend greatly upon the value chosen for D. However, the lowest value we have found for an energy-

coupling membrane ($D = 10^{-11}$ cm^2/s) is that given by Barber (1982b) in thylakoids, based on chlorophyll fluorescence induction. We can perceive one possible resolution of this problem which seems consistent with the available data. Using the same equation as Sowers and Hackenbrock (1981), Kell (1983) obtained, from dielectric measurements in chromatophores, a diffusion coefficient of 1.35×10^{-7} cm^2/s, on the assumption that individual complexes could diffuse freely within the entire chromatophore membrane; this value is evidently far too large and forces one to the conclusion that the radius one should construe is not the actual geometric radius of the chromatophore but a more restricted domain. [Actually, as stressed by Peter Rich (personal communication), the dielectric method cannot alone distinguish lateral and rotational movements of charged or dipolar groups; however, the relative effective dipole moments involved will mean that lateral, rather than rotational, motions should, if present, dominate the dielectric response.] In these dielectric measurements the applied field was approximately 50 mV/cm (Kell, 1983), while in the experiments of Sowers and Hackenbrock (1981) the applied field was approximately 650 V/cm, a value large enough actually to drive ATP synthesis in particles of this size (e.g., Vinkler and Korenstein, 1982; Hamamoto *et al.*, 1982; Schlodder *et al.*, 1982). Thus the energy in the field induced across the membranes in the experiments of Sowers and Hackenbrock (1981) could easily have been enough to rupture noncovalent, intercomplex interactions, while that induced in the dielectric measurements (which will be very substantially below kT) was not. Obviously the free energy available in energized membranes could promote *different* intercomplex associations from those existing under nonenergized conditions. Dielectric measurements at widely varying field strengths (see Delalic *et al.*, 1983) might shed light on this possibility.

8. Summary

The foregoing sections may be summarized as follows:

1. Electron transport and ATP synthase components exist as discrete complexes in energy-coupling membranes.
2. These complexes, in common with other globular proteins, exhibit a variety of intramolecular fluctuations that may be cooperative and may be of crucial significance to their catalytic activity.
3. The complexes can apparantly diffuse at rates sufficient to account for the role of bimolecular collisions in controlling the rate of electron transport.
4. Under energized conditions there is a significant amount of evidence that the disposition of these complexes in energy-coupling membranes is not random; this may be important in regulating the transfer of free energy between them.

5. There are some serious discrepancies between many *biophysical* measurements of membrane protein diffusion coefficients and those obtained from more biochemical approaches (Kell, 1984).

III. PROTONMOTIVE FUNCTIONS OF ELECTRON TRANSPORT AND ATP SYNTHASE COMPLEXES

A. Introduction

As is now well known, P. Mitchell (see e.g., Mitchell, 1966, 1968, 1979, 1981; Nicholls, 1982), in his chemiosmotic coupling hypothesis, proposed that, *inter alia*, the function of electron transport and ATP synthase complexes was to catalyze the vectorial translocation of protons across the coupling membrane in which the complexes are embeded. We take as axiomatic the fact that such activity is now proven. However, there are two areas of outstanding controversy in relation to the general problem of so-called protonmotive systems.

1. What *type* of general mechanism is exploited by these complexes in catalyzing protonmotive activity?;
2. What pathway is taken by energized protons translocated across the coupling membrane by the electron-transport-linked proton pumps *en route* to sinks such as the H^+-ATP synthase?

To a certain extent, these two questions may be dealt with separately. However, as mentioned by others (e.g., Wikström and Krab, 1980; Wikström *et al.*, 1981; Ferguson and Sorgato, 1982), it is not easy to see how a direct type of protonmotive mechanism might exhibit other than an orthodox, "delocalized" chemiosmotic coupling activity. What does this mean? The original version of the chemiosmotic coupling hypothesis posited direct mechanisms by which the reactions of electron transport might be coupled to proton motive activity. The simplest version, the redox loop concept, rests on the appropriate spatial relationship between electron-transport carriers and the alternation of hydrogen and electron carriers (Fig. 2a). Such mechanisms, including the so-called Q-cycle (Mitchell, 1976), have been referred to as *direct* mechanisms (Mitchell, 1977a), a semantic convention which we shall adopt. By contrast, the possibility exists (see e.g., Papa, 1976; Wikström and Krab, 1980; Fillingame, 1980; von Jagow and Engel, 1980; Wikström *et al.*, 1981; Kell *et al.*, 1981a; Vignais *et al.*, 1981; Nicholls, 1982) that the protonmotive and electron-transport–ATP synthase–hydrolase activities are coupled by a purely indirect, conformational type of mechanism (Fig. 2b).

Although there are other differences between the various types of model, such as the protonmotive stoichiometries with which they are consistent, in

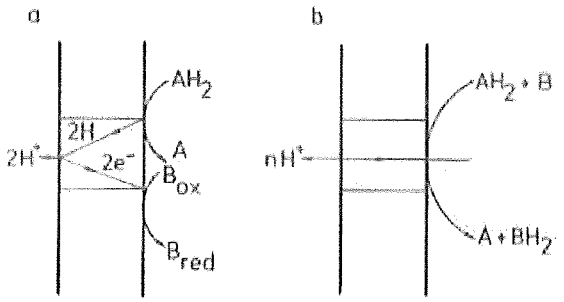

Fig. 2. Direct and indirect mechanisms of redox-linked protonmotive activity: (a) The classical direct method: the simplest possible redox loop depends upon the alternation of hydrogen (H) and electron (e^-) carriers at opposite faces of the coupling membrane. This type of model has, *for a given pathway of electron transfer*, an invariant $\rightarrow H^+/2e^-$ stoichiometry of, in this case, 2. (b) An indirect, conformationally coupled proton pump. In this case the redox reactions are functionally linked, through conformational changes of the protein complex, to transmembrane proton translocation. The $\rightarrow H^+/2e^-$ stoichiometry may in this case be variable due to incomplete coupling (slip) at the molecular level. This type of model is compatible both with delocalized chemiosmosis (as drawn) or with more localized energy-coupling theories. This type of device requires some kind of molecularly ratcheted proton channel within the protein complex.

our view a crucial difference between them is as follows: in the case of redox-linked protonmotive systems the direct mechanism, for a given pathway of electron transport between redox centers, requires an invariant ratio of protons translocated per electron passing through the region of interest, i.e., a constant $\rightarrow H^+/e^-$ ratio. By contrast, redox-linked proton pumps *sensu stricto* (i.e., those operating via an indirect mechanism) are more likely to exhibit what has been termed *slip* (Rottenberg, 1973; Baccarini-Melandri *et al.*, 1977; Hill, 1977; Pietrobon *et al.*, 1981, 1982; Kell and Morris, 1981; Stucki, 1982; Walz, 1983). That is, the electron- and proton-transport reactions themselves may be incompletely coupled at the molecular level. This concept is illustrated in a simplified fashion, using a so-called Hill diagram (Hill, 1977), in Fig. 3.

By and large, we shall be able to avoid the controversy concerning the direct/indirect type of mechanism of protonmotive activity. Nevertheless, a few comments are in order.

1. Endogenous slip in redox-linked (Pietrobon *et al.*, 1981) and ATP synthase-linked (Pietrobon *et al.*, 1983) proton pumps has been noted in rat liver mitchondria (see, however, Westerhoff *et al.*, 1983c, 1984b).
2. Slip in redox-linked proton pumps has been demonstrated directly in a number of systems (e.g., Casey *et al.*, 1980; Anderson *et al.*, 1981; Phelps and Hatefi, 1981; Tu *et al.*, 1981; Wikström and Penttilä, 1982; Price and Brand, 1983; Walz, 1983, and see later.)
3. There has been a historical tendency in some quarters to interpret experimental data solely in terms of direct mechanisms; readers should not

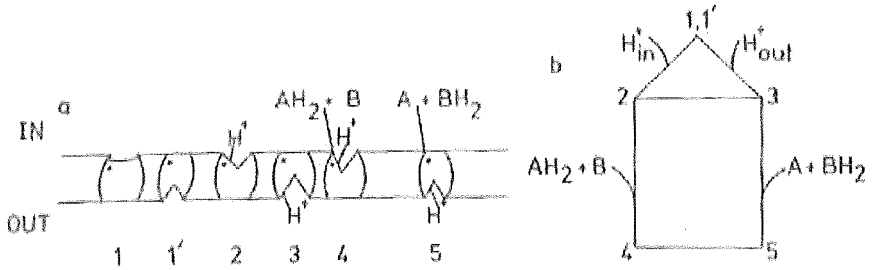

Fig. 3. Coupled and uncoupled (slip) protonmotive cycles in a redox-linked proton pump. (a) Diagrammatic representation of a redox-linked proton pump in an energy-coupling membrane. The protein contains one (shown) or more key proton-binding amino acid side chains on each face of the membrane and can adopt various types of conformational state (1 to 5) depending upon the redox level and degree of energization. The bonds between H^+ and the protein indicate localized coupling; for diagramming delocalized chemiosmosis the bonds are relaxed and the protons are free to come into electrochemical equilibrium with those in the bulk aqueous phases. (b) Hill (1977) diagram for this type of redox-linked proton pump. For a fully coupled cycle (an $\rightarrow H^+/2e^-$ cycle of only 1 is shown in the diagram), the cycle of the Hill diagram is 1–2–4–5–3–1'. There are two other, uncoupled slip cycles. 1–2–3–1' is a proton leak (proton slip: charges separated during the protonmotive activity relax without concomitant reversal of electron transfer). *Redox slip*, cycle 2–4–5–3, occurs when the redox reactions occur without concomitant protonmotive activity. Other reactions, such as the interaction of such a device with membrane-permeant ions, are not considered here.

think that this reflects the current status of this controversy, which possibly favors the universality of the indirect pump type of mechanism (Kell *et al.*, 1981a; Nicholls, 1982).

4. Slip is often referred to loosely as a variable stoichiometry in these proton pumps; we shall use the terms interchangeably.

Given our intention to treat these protonmotive complexes more or less as black boxes (i.e., we take a coarse-grained approach), we may turn to the problem of the localization of the free-energy transfer and proton current pathway during electron-transport phosphorylation.

Independently of Mitchell, R. J. P. Williams (see e.g., Williams, 1961, 1978a,b, 1982) proposed a more general view on protonic coupling in electron-transport phosphorylation. Our purpose here, however, is not to distinguish the views of Mitchell and Williams (see e.g., Mitchell, 1977b; Williams, 1978a) in explicit detail nor historical context. It is because Mitchell's ideas generated a much greater number of testable predictions that most workers in the field of membrane bioenergetics have come to regard them as the more useful framework for experimental activity. For our part, we will take it that chemiosmosis (or delocalized chemiosmosis) constitutes a special case of Williams' more general ideas (Williams, 1978a).

What then is the crucial feature of chemiosmotic coupling? It is usually taken as the central dogma of the chemiosmotic coupling model (Nicholls, 1982) that under stationary-state conditions the proton electrochemical

potential difference $\Delta\tilde{\mu}_{H^+}$ or protonmotive force ($\Delta p = \Delta\tilde{\mu}_{H^+}/F$) possesses the properties appropriate to its being the high-energy intermediate of electron-transport phosphorylation (and other processes). This concept is illustrated in Fig. 4a. Quantitative calculations concerning proton diffusion rates indicate that in the time range of redox or ATP synthetic reactions, no significant protonic potential differences can be maintained between points in space (in the same aqueous compartment) at a distance corresponding to the size of a

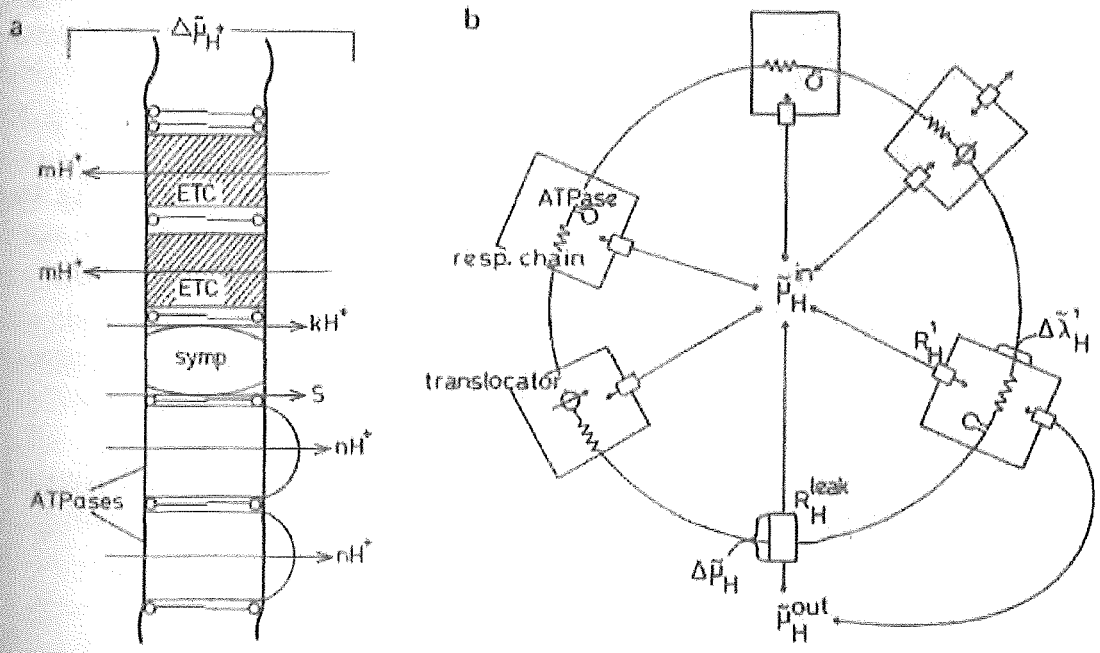

Fig. 4. (a) The principle of delocalized chemiosmotic coupling. The diagram shows a phospholipid bilayer energy-coupling membrane incorporating two redox-linked proton pumps (ETC), an electrogenic substrate (S) carrier (symp), and two ATP synthases (ATPases). The proton motive activities of the ETC set up a proton electrochemical potential difference ($\Delta\tilde{\mu}_{H^+}$) across the coupling membrane, and this proton electrochemical potential difference acts to drive H^+ back through the ATP synthases and to carry out active transport by various means, etc. Since these membranes are isolated as topologically closed vesicular structures, the proton electrochemical potential difference is homogeneous, in the sense that free energy released by a particular electron-transport chain passes via $\Delta\tilde{\mu}_{H^+}$ and may therefore be utilized by any of the ATP synthases embedded in the same coupling membrane. Membrane leakiness or the presence of ionophoric compounds leads to a decrease in $\Delta\tilde{\mu}_{H^+}$, and hence to a decrease in the rate of phosphorylation (or active substrate transport). The membrane itself is thus viewed, in the chemiosmotic model, simply as an inert, insulating phase that prevents uncoupled proton backflow and does not itself become energized during electron-transport phosphorylation. (b) The principle of mosaic protonic coupling. Free-energy coupling is visualized as occurring in n independently operating coupling units. Each of these units is organized in a fashion broadly comparable to that in Fig. 4a, except that the number of proton pumps per unit is confined to two. Because the combination of proton pumps may be different for each coupling unit, and because there are also leak units (the lower unit in Fig. 4b), energy coupling is essentially proposed to be mosaic in nature. The nature of the functional connections between the different proton pumps, and the relative proton current carried via the separate pathways, remain as yet undefined.

bacterium or an intracytoplasmic organelle (Mitchell, 1981; Melandri *et al.*, 1983; contrast Hong and Junge, 1983; but see Westerhoff, 1983), and experimental measurements (Gutman *et al.*, 1981, 1982, 1983) confirm this. Further, as pointed out previously (Kell and Hitchens, 1983; Hitchens and Kell, 1982a, 1983b), the fact that the $P/2e^-$ ratio is for practical purposes independent of electron transport over a wide range (e.g., van Dam and Tsou, 1970; Ernster and Nordenbrand, 1974; Ferguson *et al.*, 1976; Gräber and Witt, 1976; Graan *et al.*, 1981; Jackson *et al.*, 1981) indicates that any distinction between delocalized chemiosmosis and alternative, more localized energy-coupling schemes may be regarded as clear-cut. Thus the proton electrochemical potential difference constitutes a macroscopic, delocalized, ensemble property of the two aqueous phases that coupling membranes serve to separate (Walz, 1983).

In common with Ort and Melandri (1982), it is this minimal model (Fig. 4a) which we use as a "straw man" to assess the agreement between experimental results and the predictions of chemiosmosis in the following sections. As we shall see, the agreement is not good when the experimental tests which are applied are stringent, and it will be necessary to enquire as to what minimal changes we may make to the chemiosmotic model in order to make it accord more fully with experimental findings.

We begin by considering the protonmotive properties of systems catalyzing electron-transport phosphorylation as observable in (phases in equilibrium with) bulk aqueous phases.

B. Measurement of Proton Translocation by Systems Catalyzing Electron-Transport Phosphorylation

1. Respiratory Systems

Following pioneering experiments with mitochondria (see Mitchell & Moyle, 1967), the oxygen pulse method was applied to suspensions of the respiratory microorganism *Micrococcus* (now *Paracoccus*) *denitrificans* by Scholes and Mitchell (1970). In this method (see e.g., Mitchell *et al.*, 1979; Reynafarje *et al.*, 1979; Wilkström and Krab, 1980; Kell and Hitchens, 1982; Nicholls, 1982), a pulse of O_2, as air-saturated saline, is added to a well-stirred, weakly buffered, anaerobic suspension of the membrane vesicles of interest, and the resultant pH changes in the external aqueous phase monitored with a sensitive glass electrode. The ratio of the measured number of H^+ translocated across the membrane to the number of O atoms reduced, the $\rightarrow H^+/O$ ratio, is greatly increased in the presence of compounds such as SCN^- or K^+/valinomycin that are believed to cross biological membranes rapidly in

their charged forms, as are the rates of H^+ transfer to and from the bulk aqueous phase in equilibrium with the measuring electrode.

According to the conventional chemiosmotic explanation of this behavior (see e.g., Mitchell, 1967, 1968; Scholes and Mitchell, 1970; Kell, 1979; Archbold *et al.*, 1979; Conover and Azzone, 1981; Kell and Morris, 1981; Heinz *et al.*, 1981; Kell and Hitchens, 1982, 1983; Hitchens and Kell, 1984), the relatively low static electrical capacitance of the membrane means that the transmembrane transfer of a rather small number of electrically uncompensated H^+ ions leads to the formation of a large bulk–bulk phase membrane potential, which either inhibits further pumping or drives H^+ ions back across the membrane before they can be detected. SCN^- or K^+/valinomycin acts to dissipate this membrane potential and thus allows all the protons translocated into (or from) the bulk phase external to the microorganisms to be detected and to remain there sufficiently long to be measured as a true, limiting stoichiometric $\rightarrow H^+/O$ ratio.

A simple prediction that should be fulfilled if this explanation is correct is as follows: in the absence of added "permeant" ions the measured $\rightarrow H^+/O$ ratio should be a monotonically decreasing function of the size of the O_2 pulse, provided that the smallest size does not induce the maximum membrane potential (Sorgato and Ferguson, 1979; Heinz *et al.*, 1981). This is because, if the supposed membrane potential that has been set up by the translocation of a small fraction of the pumped H^+ is acting to inhibit further proton motive activity (and thus keep the $\rightarrow H^+/O$ ratio submaximal) as described above, this membrane potential should stop the further translocation of any more H^+ if the size of the O_2 pulse is increased, and thus lower the $\rightarrow H^+/O$ ratio. However, oxygen pulse experiments in mitochondria (Archbold *et al.*, 1974, 1979; Conover and Azzone, 1981), in *Escherichia coli* (Gould and Cramer, 1977a; Gould, 1979), and in *Paracoccus denitrificans* (Hitchens and Kell, 1982b, 1984; Kell and Hitchens, 1982) demonstrate that this prediction is not fulfilled; the $\rightarrow H^+/O$ ratio in the absence of added "permeant" ions and at low oxygen concentrations is independent of the size of the O_2 pulse. This type of observation has been interpreted by the above authors to indicate that there must be at least two types of proton circuits in these membranes, only one of which, not seemingly coupled to phosphorylation [but presumably concerned with pH regulation (Padan *et al.*, 1981; Booth and Kroll, 1983)], enters the bulk aqueous phase external to the membrane vesicles. Further, the half-time for H^+ ejection under many of these circumstances greatly exceeds the half-time of O_2 reduction, obviating arguments based on non-ohmic links or slips.

A converse experiment to the foregoing studies the $\rightarrow H^+/O$ ratio under conditions in which the number of O atoms added per cell (vesicle) present is so small that the maximum membrane potential that could be set up if chemiosmotic mechanisms are operative is so small as to be energetically

insignificant. Under these conditions, the $\rightarrow H^+/O$ ratio should be the same whether or not "permeant" ions are present. Again, however, experiment shows that in the absence of added "permeant" ions the $\rightarrow H^+/O$ ratio remains submaximal (Gould and Cramer, 1977a,b; Hitchens and Kell, 1982b; Kell and Hitchens, 1982) consistent with the findings described above and with the view that the H^+ that *are* seen are not responsible for inhibiting the transfer of further protons under the typical conditions used.

2. Photosynthetic Systems

Photosynthetic electron transport in both thylakoid and bacterial membranes is also more or less tightly coupled to the reversible transmembrane translocation of protons. We will confine our discussion in this section to studies of chromatophores (inverted cytoplasmic membrane vesicles) from photosynthetic bacteria, although our remarks are essentially equally valid for thylakoids, with the exception that thylakoids as isolated generally have a much greater passive ion permeability than chromatophores. H^+ uptake by thylakoids has been reviewed, for example, by Murakami *et al.* (1975).

The relationship between photosynthetic electron flow and concomitant reversible proton uptake was first reported, in chromatophores for *Rhodospirillum rubrum* (von Stedingk and Baltscheffsky, 1966; von Stedingk, 1967). The extent of uptake was highly pH dependent, being negligible at the alkaline pH values characteristic of the intact cell interior. This is not inconsistent with a teleological view that such proton movements are normally concerned with the regulation of internal pH. The cyclic nature of chromatophore electron transport has in general (but cf. Jackson *et al.*, 1981) precluded the measurement of steady-state rates of electron transport, which might be correlated with the (quasi-)steady-state H^+ uptake which may be observed under conditions of continuous illumination so as to establish more clearly the molecular events underlying the macroscopically observable H^+ uptake. However, it is now understood (Cogdell & Crofts, 1974) hat these H^+ movements are elicited by the action of one or more electron-transport-linked H^+ pumps that act to move H^+ across the chromatophore membrane against their electrochemical potential.

This cyclic nature of chromatophore electron transport has promoted the use of light flashes of a duration sufficiently short to induce single turnovers of the chromatophore electron-transport chain and/or its associated phosphorylation apparatus (e.g., Crofts and Wood, 1978; Dutton and Prince, 1978; Wraight *et al.*, 1978; Baccarini-Melandri *et al.*, 1981; Junge and Jackson, 1982; Ort and Melandri, 1982; Prince *et al.*, 1982). However, despite the extensive and elegant use of this technique, there is currently a great deal of uncertainty concerning the exact nature and extent of the relationship between electron-

transfer events and the observable H^+ movements in (phases in equilibrium with) the bulk aqueous phase external to illuminated chromatophore suspensions.

When chromatophores are illuminated with trains of single-turnover flashes of different frequencies, one can be sure that one is detecting all the H^+ pumped from the external aqueous phase, using the pH electrode technique, if this number is frequency independent. This was done by Kell and Hitchens (1983; see also Cogdell and Crofts, 1974), with results comparable to those described above for the measurement of respiration-driven H^+ translocation: for small numbers of flashes, the (submaximal) $\rightarrow H^+$/flash ratio observed in the absence of added ionophores was largely independent of the number of flashes (and their frequency) applied. Thus the conclusion again is that there must be at least two types of proton circuit in this type of system, only one of which enters the bulk phase external to the membrane vesicle of interest. Since chromatophores are inside out with respect to whole cells, the conclusion may be drawn that it is permissible to treat the two bulk phases that the coupling membrane separates as more or less equivalent. The protons which were *not* seen must still have been pumped *across* the membrane [as judged, e.g., by electrochromic measurements of membrane-located carotenoids (see e.g., Wraight *et al.*, 1978)]; some, however, must eventually have returned across the membrane without driving concomitant phosphorylation, presumbly via a slip pathway within the primary (electron-transport-linked) proton pump, since similiar behavior is observed with isolated electron-transport complexes reconstituted into liposomes.

Thus the experiments discussed in this and the preceding section show that one must be very careful to distinguish bulk-phase from non-bulk-phase proton movements in discussions of energy coupling, since *both* are coupled to electron transport. What we wish to know is which of them act in the entire energy-coupling process of electron-transport phosphorylation. It is, of course, a logical absurdity to ascribe a causal status as an intermediate between two processes to a process that is merely concomitant with them.

Lastly, it is worth reminding readers, in this section, that concentrations of ionophores sufficient to inhibit phosphorylation completely have relatively little effect upon the extent of light-induced H^+ uptake in bacterial chromatophores (Nishimura and Pressman, 1969; Pressman, 1972).

3. Summary

The discussion in the preceding two sections may be summarized as follows:

1. Electron-transport reactions are coupled to H^+ movements to and from bulk aqueous phases in contact with the coupling membranes in which the electron-transport reactions take place.

2. The measured kinetics and stoichiometeries of these H^+ movements, in the absence of specially added "permeant" ions, and under conditions in which, were ADP and phosphate present, they would have sufficient free energy to make ATP, are not easily consistent with the view that the *observable* H^+ movements might serve as an intermediate in electron-transport phosphorylation.

3. The addition of certain compounds ("permeant ions") indicates that "extra" H^+ were pumped but were not seen in the absence of such compounds; it is these protons that must be presumed to be a possible intermediate in electron-transport phosphorylation.

Similar statements to the foregoing may be made concerning ATP-linked transmembrane H^+ translocation, although all the pertinent experiments do not appear to have been done explicitly. In addition to the slip concept discussed earlier, then, one should thus be aware that variable stoichiometry may be construed to have different properties depending upon whether one is measuring redox-linked H^+ movements in bulk phases or more functional processes such as phosphorylation itself (for excellent examples of the latter, see, e.g., Heber and Kirk, 1975; Stucki, 1982).

Such studies on electron-transport-linked H^+ movements lead naturally to studies which have attempted to measure the thermodynamic magnitude of the proton motive force itself. This type of study forms the subject of the following section.

IV. THE PROTONMOTIVE FORCE AS AN INTERMEDIATE IN ELECTRON-TRANSPORT PHOSPHORYLATION?

> "Reality" is what we take to be true. What we take to be true is what we believe. What we believe is based upon our perceptions. What we perceive depends upon what we look for. What we look for depends upon what we think. What we think depends upon what we perceive. What we perceive determines what we believe. What we believe determines what we take to be true. What we take to be true is our reality.
>
> Zukav (1980)

A. Introduction

As indicated earlier, it is usually taken as the central dogma of the chemiosmotic coupling model that the proton motive force or proton electrochemical potential difference between two bulk aqueous phases is "the" (or at the very least "a") high-energy intermediate in processes such as electron-transport phosphorylation. Thus a great many studies have been devoted to an assessment of the role of this variable. However, the first problem arises in the *measurement* of the protonmotive force (pmf) ($\Delta\tilde{\mu}_{H^+}$).

As shown, the reactions of electron transport (and ATP hydrolysis) *are* linked to the vectorial, transmembrane translocation of protons between the two bulk aqueous phases that the coupling membrane serves to separate, it is evident that bulk phase pH gradients *are* produced by such reactions. (This does not, of course, tell us whether such gradients can serve as an intermediate between the two processes.) Methods for measuring such pH gradients are now well established, and the coincidence between different methods in the same system (e.g., Nicolay *et al.*, 1981) lends confidence to the view that certain methods such as weak acid/base distribution can measure the transmembrane pH difference quantitatively. Such methods have been reviewed by a number of workers (e.g., Kell, 1979; Rottenberg, 1979; Ferguson and Sorgato, 1982; Nicholls, 1982; Azzone *et al.*, 1984), and are not here considered further.

A bigger problem arises with the magnitude of the bulk-to-bulk phase membrane potential which is set up, in the chemiosmotic model, as an accompaniment to proton motive activity. The only absolute method, in principle, is measurement with microelectrodes. These very difficult measurements have indeed been reported for a number of systems. Tedeschi and colleagues have, for a number of years, in experiments of increasing refinement, reported that the metabolically induced membrane potential in mitochondria is energetically insignificant (see Tedeschi, 1980, 1981; Ling 1981) to account for ATP synthesis observed in the same mitochondrion (Maloff *et al.*, 1978). These important measurements represent the only direct methods used in mitochondria to date.

Vredenberg, Bulychev, and colleagues have used microelectrodes in giant chloroplasts (see, e.g., Vredenberg, 1976), and found again that the potential did not exceed a few tens of millivolts in the light, even under transient conditions. They also have given arguments indicating that the process of inserting microelectrodes did not damage the membrane significantly. Thus in this system, too, even in the first few turnovers following the initiation of illumination, the bulk-to-bulk phase membrane potential was far too small, assuming an $\rightarrow H^+/ATP$ ratio of 3, to permit ATP synthesis.

Only in giant *Escherichia coli* cells did Slayman and colleagues (Felle *et al.*, 1978, 1980) establish some degree of consonance between the membrane potential measured with microelectrodes and that calculated from the distribution of membrane-permeating lipophilic cations. However, it is by no means clear that the membrane potentials measured with the microelectrodes in these studies resulted from metabolically dependent processes, since (1) the effect of uncouplers or other inhibitors was not tested, (2) the dependence of the measured membrane potential on the external sodium concentration was semi-Nernstian (Felle *et al.*, 1978) (consistent with the view that it was at least partly a simple diffusion potential), and (3) the coincidence between the measurements with the microelectrodes and the permeant ion uptake methods

was obtained with organisms suspended in *wholly distinct*, and thus non-comparable, media. Thus the status of these otherwise crucial experiments, which regrettably, have not yet been reproduced, is somewhat uncertain.

Partly on the basis of calibration with induced diffusion potentials, a great many workers (e.g., Westerhoff *et al.*, 1981) have assumed that, under common experimental conditions, and with appropriate corrections, ion-distribution methods are successfully measuring the transmembrane electrical potential difference. Although it is easily possible to envisage mechanisms of ion uptake that are independent of a bulk-to-bulk phase electrical potential difference, we may proceed, initially, by assuming that the generally used methods are measuring the bulk-to-bulk phase pH gradient and membrane potential components of the $\Delta\tilde{\mu}_{H^+}$. Even with this assumption, we find two major sets of anomalies between the data obtained and those expected upon a commonsense reading of the chemiosmotic model, incorporating appropriate additions such as a slightly variable $\rightarrow H^+/ATP$ stoichiometry and/or a non-ohmic leak of energy-coupling membranes to protons. Since we have co-authored a number of articles on this topic (e.g., Kell and Hitchens, 1983; Melandri *et al.*, 1983; Westerhoff *et al.*, 1983a, 1984b, we confine ourselves to some summary statements.

B. Limited Correlation between the Apparent $\Delta\tilde{\mu}_{H^+}$ and Rates of Electron Transfer or of ATP Synthesis

It was observed by many workers, under a variety of conditions, that rates of electron transport or of phosphorylation are not uniquely dependent upon the value of the apparent protonmotive force, but in many cases depend more upon the number of active electron-transport chains or ATP synthases (e.g., Padan and Rottenberg, 1973; Baccarini-Melandri *et al.*, 1977, 1981; Casadio *et al.*, 1978; Sorgato and Ferguson 1979; Kell *et al.*, 1978a; Melandri *et al.*, 1980; de Kouchovsky and Haraux, 1981; de Kouchovsky *et al.*, 1982, 1983; Zoratti *et al.*, 1982; Wilson and Forman, 1982; Mandolino *et al.*, 1983). Some of these observations have been taken to indicate that there may exist kinetic interactions between redox- and ATP synthase-linked proton pumps that are not free energy transducing. However, there is another type of otherwise comparable anomaly which is not subject to this type of interpretation.

C. The Force Ratio under Static Head Conditions Is Not Constant at Different Values of the Apparent $\Delta\tilde{\mu}_{H^+}$

If an energy-transducing system is allowed to synthesize ATP until no *net* ATP formation is observed, the maximal free-energy change for ATP synthesis may be evaluated and compared with the magnitude of the simultaneously

measured $\Delta\tilde{\mu}_{H^+}$. Such stationary-state measurements are said to take place at *static head*. It is generally assumed that the ratio of the two free-energy terms may be used, in the chemiosmotic model, to give a minimal measure of the $\rightarrow H^+/ATP$ ratio. It is found, however, in a variety of experiments, that this ratio is heavily dependent upon the magnitude of the apparent pmf, and achieves wholly unrealistic values when the pmf is severely decreased (e.g., Wiechmann *et al.*, 1975; Kell *et al.*, 1978b,c; Azzone *et al.*, 1978; Guffanti *et al.*, 1978, 1981; Decker and Lang, 1977, 1978; Westerhoff *et al.*, 1981; Baccarini-Melandri *et al.*, 1977; Mandolino *et al.*, 1983). Since, at least in some of these experiments, the reaction catalyzed by the ATP synthase was apparently in *equilibrium* with the measured pmf, no kinetic arguments may be invoked (Westerhoff *et al.*, 1981).

These two main types of anomaly, which have been widely observed, have led many workers to assess the competence of the $\Delta\tilde{\mu}_{H^+}$ in energy coupling using methods that do not depend on the accuracy of methods that purport to be measuring the $\Delta\tilde{\mu}_{H^+}$ generated by electron transport.

D. Indirect Means Used to Assess the Competence of the Protonmotive Force in Energy Coupling

1. "Acid Bath" Experiments

Following the pioneering experiments of Jagendorf and Uribe (1966), a number of workers, in a variety of systems, have demonstrated that the imposition of an *artificial* $\Delta\tilde{\mu}_{H^+}$ (acid bath), sometimes partly constituted by a diffusion potential, can drive the synthesis of ATP (see e.g., Thayer and Hinkle, 1975; Schuldiner, 1977; Gräber, 1981; Schlodder *et al.*, 1982; Maloney, 1982; and contrast Malenkova *et al.*, 1982). Most workers find a sharp value of the applied pmf, typically 150 mV, below which the rate of ATP synthesis is negligible. When the value of the applied $\Delta\tilde{\mu}_{H^+}$ is very great, the rates of phosphorylation induced are as great as those driven by electron flow (e.g., Thayer and Hinkle, 1975; Smith *et al.*, 1976), indicating that were the electron-transport-induced $\Delta\tilde{\mu}_{H^+}$ as great as the artificially applied one, $\Delta\tilde{\mu}_{H^+}$ could serve as a kinetically competent intermediate in electron-transport phosphorylation. Unfortunately, the coincidence of $\Delta\tilde{\mu}_{H^+}$ values under the comparable conditions has not yet been tested adequately (see Schlodder *et al.*, 1982) to arrive at a conclusion on this point. It is obviously this comparison, of rates of phosphorylation with electron flow causing a properly measured $\Delta\tilde{\mu}_{H^+}$ equal to that applied in acid-bath experiments, which is the only meaningful one.

Parenthetically, it is worth mentioning that D_2O substitution for H_2O does not significantly affect acid bath-induced phosphorylation in *Streptococcus*

V4051 (Khan and Berg, 1983), while, at least in thylakoids, such a substitution markedly inhibits electron-transport phosphorylation (de Kouchovsky and Haraux, 1981). This would seem to argue against a pmf being the sole intermediate in the latter process.

It is usually assumed that the decay of the pmf applied in acid-bath experiments is concomitant with the phosphorylation, as expected in the chemiosmotic model. However, in an important series of experiments, Hangarter and Good (1982, 1984) have strongly called this assumption into question. They observed that the ability of a preilluminated thylakoid suspension to phosphorylate ADP decays exponentially, as does the light-induced H^+ uptake, and was not, in contrast to acid bath-induced phosphorylation, increased by the imposition of a potassium diffusion potential. The exponential dependence of the decay is actually wholly unexpected given the highly pH-dependent buffering power of the inner thylakoid space and the threshold pmf demonstrated in the acid-bath experiments. Thus the "energized state" set up by preillumination of thylakoids is not the same as that caused by the imposition of an acid bath. It is the presumed equality of these states that has provided for many a convincing demonstration of the accuracy of the chemiosmotic model in describing photophosphorylation. It can only be concluded that the *actual* pmf caused by electron transport is lower than the threshold observed in acid bath experiments when it is created in response to electron flow.

Blumenfeld (1983) also discusses many of the points raised in Section III in his outstanding monograph, which readers are urged to consult for fuller details.

2. "Antacid Bath" Experiments

If a pH gradient in thylakoids were to be driving ATP synthesis *in vivo*, then decreasing it, under conditions of a constant (or, better, negligible) membrane potential should affect the ability of such thylakoids to phosphorylate. The ways in which such experiments have been performed is to study the yield of ATP as a function of the number of single turnover flashes. Consistent with chemiosmotic expectations (Mitchell, 1968), given the reasonably significant ion permeability of thylakoids and the low electrical capacitance relative to the differential buffering power, it is found that the addition of valinomycin leads to a lag in the capacity for ATP formation (Ort *et al.*, 1976; Davenport and McCarty, 1980; Vinkler *et al.*, 1980; Graan *et al.*, 1981; Ort and Melandri, 1982). The lag is independent of the concentration of K^+ between 20 and 40 m*M*, indicating that under such conditions the pH gradient should be the sole contributor to $\Delta\tilde{\mu}_{H^+}$ (Graan *et al.*, 1981; but contrast Boork and Baltscheffsky, 1982, for chromatophore experiments of this type). However, it

is found that if the thylakoids are preloaded with hydrophilic, non-uncoupling buffers, the lag remains essentially unaffected (Graan *et al.*, 1981). The pH gradient that should have been produced in the presence of the buffer can be calculated from H^+ uptake measurements and may be arranged to be significantly less than the threshold required in acid bath experiments. The conclusion to be drawn from this type of "antacid" bath experiment is that the protons used for making ATP did not pass through a space in equilibrium with the internal thylakoid space accessible to the buffer molecules. Thus this type of observation complements the findings discussed under the acid bath experimental section and indicates that the localization of the high-energy intermediate is more microscopic than implied in the delocalized chemiosmotic model.

A number of other workers have also sought a functional approach that considers whether the free energy released by electron transport is freely available to every enzyme molecule of a given type in a given vesicle, as assumed in the chemiosmotic model (Fig. 4a), approaches which form the subject of the next section.

E. The Intermediate Is Not a Pool: Dual Inhibitor Titrations

The principle underlying the general methods of double inhibitor titrations may be expounded with reference to Fig. 5a. This figure models the process of electron transport phosphorylation as four redox-linked H^+ pumps (ETC) plus four H^+-ATP synthases (written ATP in the figure) that are coupled via a high-energy intermediate ($\sim$). The box indicates a model in which the free energy released by a particular electron-transport complex may be used by any ATP synthase in the membrane vesicle preparation, as in the delocalized

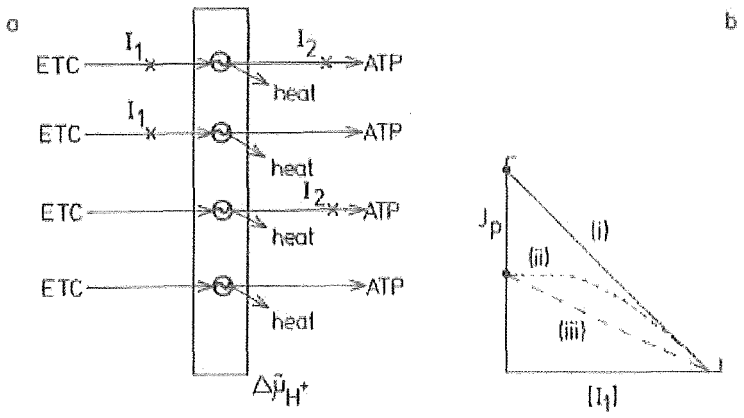

Fig. 5. Principle of dual inhibitor titrations using inhibitors of electron transport and ATP synthase enzymes. For discussion and explanation, see text.

chemiosmotic model. I_1 and I_2 represent tight-binding and specific inhibitors of electron transport and the ATP synthase, respectively. "Heat" represents all free-energy-dissipating processes.

As first expounded by Baum and colleagues (1971; Baum, 1978), a simple analysis (Fig. 5b) of this situation would suggest that partial inhibition of the overall flux of the system (i.e., the rate of ATP synthesis, J_p) using an I_2-type inhibitor [Fig. 5b(i), (ii)] should increase the titer of an I_1-type inhibitor initially necessary to inhibit phosphorylation relative to that in the absence of the I_2 inhibitor if energy coupling is delocalized (ii) (and see Section IX). Conversely, if energy coupling is fully localized, the titers will be unchanged (iii). As mentioned earlier, the very common finding of an independence of the $P/2e^-$ ratio on the rate of electron transport over a wide range in well-coupled systems indicates that energy leaks are not especially relevant to this type of analysis. Such a finding also militates against the thoughtful suggestions of Parsonage and Ferguson (1982) concerning putative $\Delta\tilde{\mu}_{H^+}$ values during such titrations. Experimentally, it was found by several groups, in a number of systems, that the behavior observed corresponded to that expected for a fully localized system (Baum *et al.*, 1971; Lee *et al.*, 1969; Baum, 1978; Venturoli and Melandri, 1982; Hitchens and Kell, 1982a,c; Kell and Hitchens, 1983; Westerhoff *et al.*, 1982, 1983a,c; Westerhoff, 1983; Welch and Kell, 1985). It is also worth remarking that the selection for *unc* phenotypes in aerobically grown *E. coli*, based on aminoglycoside resistance, follows from the assumption that their cytoplasmic membranes are *less* energized than those of *unc*$^+$ strains. In the case of the *unc*A phenotype, which should exert a lower drain on the energized state than the wild type, this observation is not easily reconciled with delocalized coupling, but is to be expected from considerations of the results of I_1/I_2 titrations discussed above. The finding (Schreiber and Del Valle-Tascon, 1982; Blumenfeld, 1983) that there is no nonzero threshold rate of electron transport necessary to drive ATP synthesis under level flow conditions has also been taken to indicate a localized energy-coupling interaction between redox chains and ATP synthase enzymes.

A variety of experiments following, in some aspects, this broad strategy has also been performed by Dilley and his colleagues (see Dilley *et al.*, 1982), with results incompatible with a delocalized energized state caused by electron transport (cf. Haraux *et al.*, 1983). It is also germane to draw attention to some beautifully done expositions of this type of approach in relation to the interaction between electron-transport chains (Heron *et al.*, 1978; Hächnel, 1982; Mar *et al.*, 1982; Packham and Barber, 1983).

Other dual inhibitor titrations using combinations of uncoupler and I_2-type inhibitors may be found in work by the present authors and others (e.g., Hitchens and Kell, 1982c, 1983a,b; Kell and Hitchens, 1983; Westerhoff *et al.*, 1982, 1983a,b; Westerhoff, 1983; Herweijer *et al.*, 1984). The crucial observation in these experiments was that inhibition of the output (secondary)

proton pump decreased the titer of uncoupler required to uncouple the energy-linked reactions completely. A similar observation, interpreted somewhat differently, was earlier made in the case of the uncoupling antibiotic leucinostatin plus the F_o inhibitor venturicidin by Lardy *et al.* (1975) and by Reed and Lardy (1975).

Cotton *et al.* (1981) made a similar observation concerning the release of respiratory control in intact cells of *Rps. capsulata*; less uncoupler was required to induce loss of respiratory control in cells which had been treated with venturicidin, an H^+-ATP synthase inhibitor, than in cells which had not been so treated. These authors argued that the uncoupler might have decreased the pmf in a fashion which would have led to an inhibition of substrate uptake sufficient to explain the result in terms of delocalized coupling. Unfortunately, they did not seek to test this hypothesis in cells respiring on endogenous substrate.

The same group (Cotton and Jackson, 1983) has criticized some of the experiments of Hitchens and Kell (1982c, 1983a,b) in that, especially in the presence of energy-transfer inhibitors, they were unable to obtain linear rates of phosphorylation. They argued (Cotton and Jackson, 1983) that such effects might serve to explain the observations of Hitchens and Kell (1982c, 1983a,b). With regard to these criticisms, the following remarks, *inter alia*, may be made:

1. In contrast to the results of Cotton and Jackson (1983), linear rates of photophosphorylation *could* easily be obtained by Hitchens and Kell; these were also observed, with the same resultant uncoupler titers, when uncoupler titrations were carried out on a succession of samples, with the uncoupler being added at the same time after illumination (Hitchens and Kell, unpublished).
2. Figure 1 of Cotton and Jackson (1983) indicates that even in the *absence* of oligomycin the apparent uncoupler potencies measured by these authors could vary by a factor of approximately two in the same chromatophores; according to their Fig. 1, the uncoupler is *less* potent the longer it is present.
3. The rapid initial proton uptake cannot be due to light-induced vectorial proton translocation since it is fully sensitive to energy transfer inhibitors (Hitchens and Kell, 1982c).
4. The decrease in H^+ uptake after 8 min illumination cannot be due to the buildup of a high phosphorylation potential since the decrease in rate under *these* (latter) circumstances (using ADP, but not UDP) can only be observed very shortly before the attainment of static head (Hitchens & Kell, unpublished).
5. Similar trends in uncoupler titrations are also observed in sub-mitochondrial particles using an entirely different assay (Westerhoff *et al* 1982, 1983a,b; Westerhoff, 1983; Herweijer *et al.*, 1984).

Thus these purely functional approaches seem to indicate that electron-transport phosphorylation cannot be proceeding via a delocalized intermediate, and that some kind of catalytic facilitation in some kind of functionally organized multienzyme complex is an integral part of these processes. We shall return to this concept in more general terms at the end of this review (Sections IX and X). Our next task is to look at studies that have aimed to take apart and reconstitute the enzymes catalyzing electron-transport phosphorylation.

V. THE EFFECTIVENESS OF RECONSTITUTED SYSTEMS IN CATALYZING ATP SYNTHESIS

> We find that the theories of physicists constantly undergo modification, so that no prudent man of science would expect any physical theory to be quite unchanged a hundred years hence. But when theories change, the alteration usually has only a small effect so far as observable phenomena are concerned.
>
> (Russell, 1966)

A. Mitchondrial Oxidative Phosphorylation

It is widely believed (see, e.g., Racker, 1976; Tzagoloff, 1982) that the successful co-reconstitution of purified electron-transport complexes (or bacteriorhodospin) plus a *purified* ATPase complex in a liposomal system capable of catalyzing electron-transport phosphorylation at rates (and with efficiencies) characteristic of those *in vivo* has been amply demonstrated. In particular, the experiments of Racker and Stoeckenius (1974) on co-reconstitution of bacteriorhodopsin plus an oligomycin-sensitive ATPase preparation are regularly cited as the classic demonstration that energy coupling occurs via a purely (i.e., delocalized) chemiosmotic mechanism. This notion, which we shall here refer to as the simple interpretation, is illustrated in Fig. 6. To what extent does this notion actually fit the available data?

Kagawa and Racker (1971) isolated amorphous membrane fragments ("hydrophobic protein fraction"), depleted in phospholipids and cytochrome oxidase, from beef heart mitochondria, which could be reconstituted with phospholipids to catalyze ATP–P_i exchange activity. In the same year, Racker and Kandrach (1971) added cytochrome *c* and cytochrome *c* oxidase during the reconstitution of this hydrophobic protein fraction preparation and found that the new preparation would catalyze oxidative phosphorylation with a P:O ratio of 0.13. In his 1972 review, Kagawa (1972, p. 330) cautioned, "it should be stressed that the intrinsic membrane proteins used in the reconstitution are still crude preparations. Further purification and character-

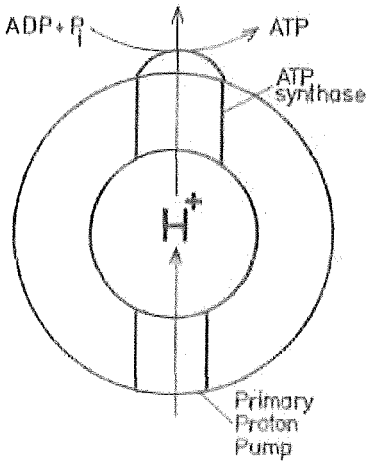

Fig. 6. The simple interpretation of reconstitution experiments involving proton-motivated ATP synthesis. In this interpretation, it is assumed that purified ATP synthases plus a purified primary proton pump (bacteriorhodopsin or a redox-linked proton pump) have been co-reconstituted into relatively ion-impermeable liposomes with the appropriate mutual polarity. According to a delocalized chemiosmotic viewpoint, the primary proton pump sets up a proton-motive force, which is then used to drive ATP synthesis at a rate characteristic of those *in situ*. Although no special spatial interaction between the complexes is required, whether one in fact occurs is not usually clarified. The extent to which the available data indicate that such a preparation catalyzes phosphorylation with turnover numbers akin to that *in vivo* is discussed in the text.

ization of the proteins essential to phosphorylation or respiration is [sic] necessary before we can discuss the molecular architecture of this biomembrane and the mechanism of oxidative phosphorylation." Regrettably, this advice has not always been followed.

Meanwhile, Racker and Kandrach were optimizing their technique, and by 1973 (Racker and Kandrach, 1973) had achieved a P:O ratio of 0.5 with ascorbate *plus* phenazine methosulfate (PMS) as electron donor to cytochrome oxidase, and Ragan and Racker (1973) had achieved a similar P:$2e^-$ ratio using electron flow through the NADH–ubiquinone–oxidoreductase segment of the respiratory chain. Such yields, rather close to those *in vivo*, might be (and indeed are widely) thought to have proven the simple interpretation. However, *every one* of these studies as well as that of Racker and Stoeckenius (1974) included the hydrophobic protein fraction; these pioneering reconstituted particles were *not purified* (i.e., pure) *preparations*.

What does the hydrophobic protein fraction contain? We know of only one attempt properly to characterize this material, that of Capaldi *et al.* (1973), who found that even a one-dimensional SDS–polyacrylamide gel separated a massive number of components; their Fig. 1 (Capaldi *et al.*, 1973) would seem to indicate, assuming a Gaussian peak shape with the width of the best

resolved peak, 20 at the very least. How many of these might be ascribed to the F_oF_1-ATPase (complex V)? There seems to be a general consensus (e.g., Baird and Hammes, 1979; Galante *et al.*, 1979; Kagawa, 1979; Senior, 1979; Berden and Hennecke, 1981; Pedersen *et al.*, 1981; Pedersen, 1982; Senior and Wise, 1983) that one-dimensional SDS gels can separate approximately 11–13 components in *purified* beef heart mitochondrial complex V. [Up to 18 may be seen in two-dimensional gels (Ludwig *et al.*, 1980; Capaldi, 1982b), although the role of many of these remains unknown.]

It may be concluded that the hydrophobic protein fraction may contain many polypeptide components that are not part of those of purified complex V.

B. Reconstitutions Using *Purified* Systems

How efficient are reconstitutions of phosphorylation performed without a hydrophobic protein fraction or something analogous from another energy-coupling membrane? We can give the answer at once: not at all. A few quite typical examples serve to illustrate this.

Winget *et al.* (1977) reconstituted bacteriorhodopsin and the chloroplast CF_oF_1-ATPase. Their best (highest) rates of photophosphorylation were approximately 1 nmol ATP min^{-1} mg^{-1} CF_oF_1 (their Fig. 6). This is already very low compared with that in thylakoids *in vivo*, but, as remarked by Hauska *et al.* (1980) and by Kell and Morris (1981), what we need to know is the turnover number of the ATP synthase, since these preparations are enriched in the sole proteins supposedly required (in the simple interpretation) for free-energy transduction. If we assume a fairly rough molecular weight for the CF_oF_1 of 500,000 daltons (Pick and Racker, 1979), 1 mg CF_oF_1 = 2 nmol CF_oF_1, so that the turnover number of photophosphorylation in the reconstituted system is approximately 0.5/min, i.e., about 0.01/s. Since the turnover number of the CF_oF_1 during photophosphorylation by thylakoids *in vivo* is approximately 100/s (Hauska *et al.*, 1980), and this figure is essentially accurate for energy-coupling membranes generally (Kell and Morris, 1981), the reconstituted system has an efficiency of 0.01%, hardly consistent with the simple interpretation discussed above!

Oren *et al.* (1980) co-reconstituted bacteriorhodopsin and a purified F_oF_1 preparation from *Rhodospirillum rubrum*; their highest rates of photophosphorylation (their Table I) were approximately 8 nmol min^{-1} mg^{-1} F_oF_1, similar to those found by Ryrie *et al.* (1978) using bacteriorhodopsin plus a purified yeast F_oF_1 preparation and those of Berden *et al.* (1981) using bacteriorhodopsin plus beef heart complex V. Using the assumed molecular weight for the ATPase complexes given in the previous paragraph, we obtain a turnover number of about 0.08/s, again less than *0.1%* of the *in vivo* rate. It

seems that van de Bend and colleagues may now have somewhat increased this value (van de Bend *et al.*, 1984).

Finally, we may consider the detailed experiments of Hauska *et al.* (1980), who carefully studied the ability of liposomes containing a purified photosystem I preparation and a purified CF_oF_1-ATPase preparation to catalyze photophosphorylation in the presence of phenazine methosulphate. Their best turnover number was 0.4% of that in the thylakoids from which the preparations were obtained, and, as far as we are aware, this is the highest published rate obtained in any system reconstituted with "purified" components to date. It may be mentioned that the proton tightness of these proteoliposomes was just as great as that of the thylakoids, as judged, insofar as is possible, by 9-aminoacridine fluorescence measurements (Hauska *et al.*, 1980).

We are, of course , aware that unidentified features such as a lack of proper orientation of some components may be responsible for the poor rates observed in these purified reconstituted systems. Nevertheless, it may be concluded that the simple interpretation of these admittedly difficult and elegant reconstitution experiments is, on the basis of presently available data, wholly incorrect and misleading. Further, the success of reconstitutions using the hydrophobic protein fraction may well indicate, in our view, that "for rapid and efficient protonmotivated energy coupling, something else is required in addition to the protonmotive sources and sinks [i.e., primary and secondary proton pumps] and an intact insulating coupling membrane" (Kell and Morris, 1981). We shall return to this shortly, but digress briefly to comment upon the role of so-called structural protein in mitochondria.

C. Structural Protein

Many early studies (e.g., Criddle *et al.*, 1962; Richardson *et al.*, 1964) indicated the presence of a so-called structural protein, which could be isolated from mitochondria, which might have a crucial role in oxidative phosphorylation, and which might yet be distinct from the (now) generally recognized protonmotive protein complexes. This concept was effectively nullified by the work of Schatz and Saltzgaber (1970), who showed that these preparations of structural protein contained, *inter alia*, large amounts of denatured F_1 protein. Since the publication of this article (Schatz and Saltzgaber, 1970), the literature has been essentially devoid of discussion of the role of some kind of (quasi-) structural protein (but cf. Sjöstrand, 1978). We suspect here that the baby may have been thrown out with the bathwater, since the studies summarized in the two previous sections, which are *wholly self-consistent*, indicate that some uncharacterized proteins important to energy coupling are now routinely being neglected. Let us look at some further studies

which bolster this conclusion still further and indeed may shed important light upon why energy coupling may *not* be easily reconstituted in liposomes, using purified proton pumps alone.

D. Phospholipid-Enriched Mitochondria and Chromatophores

The fusion of liposomes with the inner mitochondrial membrane (as mitoplasts) to produce lipomitochondria, with the same protein composition but a greatly enhanced lipid: protein ratio, was reported in two elegant articles by Schneider *et al.* (1980a,b). The activities of individual electron-transport and ATPase complexes remained unchanged (or were slightly increased), while the rate of electron-transport events between the substrate-linked dehydrogenases and cytochrome-containing complexes was decreased by a factor in rough proportion to the diffusional mean free path of the complexes. This was taken to indicate (see also Hackenbrock, 1981; Schneider *et al.*, 1982; but cf. our discussion in Section I,4) that there was (at least in lipomitochondria) a diffusion-limited step in intercomplex electron transfer. This limitation could be relieved by the incorporation of lipophilic ubiquinone homologs (Schneider *et al.*, 1982).

However, although the ATP hydrolase activity of these lipomitochondria was unimpaired or slightly increased, and remained oligomycin sensitive, the activity was insensitive to uncouplers, and the membranes were incapable of oxidative phosphorylation (Schneider *et al.*, 1980b; Westerhoff, unpublished). These workers indicated (Schneider *et al.*, 1980a,b) that the lipomitochondria were rather nonspecifically permeable to charged molecules. We find it a little difficult to concur with this conclusion for two reasons: (a) liposomes are routinely highly ion impermeable; indeed, they are significantly more so than typical energy coupling membranes; (b) lipochromatophores produced from *Rhodopseudomonas sphaeroides* by the incorporation of extra phospholipids (Casadio *et al.*, 1982) are just as ion impermeable as their parent chromatophores. The finding (Casadio *et al.*, 1982) that lipid enrichment did not affect the extent of the spectral carotenoid signal induced by K^+/valinomycin diffusion potentials in the dark, but greatly decreased that of the light-dependent signal, adds weight to the finding (Ferguson *et al.*, 1979) that ion distribution and the carotenoid signal monitor *different* aspects of the energized state.

Although there was evidently a certain amount of scrambling (inversion) of protein complexes in the lipomitochondria, in that their ATP hydrolase activity was only partially sensitive to atractylate (Schneider *et al.*, 1980b), we wish to mention an alternative explanation for the incapability of this system to catalyze oxidative phosphorylation: that the normal functioning of the apparatus of oxidative phosphorylation requires some kind of special,

supramolecular, topological relationship between the energy-transducing membrane components additional to an appropriate polarity.

In these sections we have been adducing circumstantial evidence that energy-coupling membranes require (a) some kind of special lateral arrangement of the protein complexes *known* to be involved in electron-transport phosphorylation and (b) one or more proteins distinct from those in the generally recognised quasi-reversible proton pumps. We will now turn to further evidence implicating such additional proteins, which have been termed *protoneural proteins* (Kell *et al.*, 1981b; Kell and Morris 1981).

VI. HOW THEN MIGHT ENERGY COUPLING PROCEED?

A. Introduction

In the preceding sections we have given a picture of the properties of energy-coupling membranes. In Section IV we reviewed the evidence showing that the electrochemical potential difference for protons between two supposedly homogeneous, aqueous phases bordering the energy-coupling membrane is not competent to act as the free-energy intermediate in free-energy transduction. Thus, the (delocalized) chemiosmotic coupling hypothesis put forward by Mitchell (1961), which had this implication, would not seem to be a sufficient account of how free-energy transduction takes placce. A number of authors have proposed modifications of the chemiosmotic coupling hypothesis. In recent reviews (Melandri *et al.*, 1983; Westerhoff, 1983; Westerhoff *et al.*, 1983a–c, 1984b) we have discussed these and have distilled a minimum hypothesis (called *mosaic protonic coupling*) for the mechanism of membrane-linked free-energy transduction. This hypothesis gives a functional definition of a device that should be present among the membrane properties reviewed above. In Section VII we shall discuss a possible structural identity of this device. Here we shall summarize the minimum hypothesis and discuss the consequences that are most relevant in the context of this volume.

B. Mosaic Protonic Coupling: A Feasible Minimum Hypothesis for Energy Coupling

To be compatible with the results of numerous experiments designed to prove the delocalized chemiosmotic coupling hypothesis (see above), it is necessary to retain most elements of the latter in any alternative scheme. Consequently, (see Fig. 4b) the mosaic protonic coupling hypothesis assumes that free-energy-coupling membranes contain proton pumps that are driven by chemical reactions. Important ones are electron-transfer-driven proton pumps and F_oF_1-type proton-translocating ATP synthases. Just as in the

delocalized chemiosmotic coupling hypothesis, free-energy transduction is proposed to materialize as a proton current: a primary proton pump (e.g., an electron-transfer chain) pumps protons across a membrane. As a consequence there is an increase in proton concentration (or rather occurrence) and in electric potential difference across that membrane itself. However, in mosaic protonic coupling, free energy remains stored in the membrane, as well as in the proton gradient. Thus the average tendency of the pumped protons will be to return across the membrane. Through this tendency the protons can reverse the ATP hydrolysis-driven proton pump (the secondary proton pump). This then leads to the synthesis of ATP.

The difference between the delocalized and the mosaic chemiosmotic coupling hypotheses is that the former proposes that there is one single compartment (domain) into which all individual proton pumps pump (the high-free-energy protons form a pool), whereas the latter proposes that, functionally, and on a given turnover, every primary proton pump has its own compartment, which it shares with only one (or a very limited number of) secondary proton pump(s). For different proton domains to be as functionally independent as proposed, there should be an isolation between these domains and the bulk aqueous phase bordering the membrane. This barrier should be such that it prevents proton equilibration between the local domain and the bulk aqueous phase for at least the turnover time of free-energy transduction (typically some 1–100 ms, but in certain cases for several seconds). Moreover, there should be a significant proton leakage, draining the proton electrochemical potential difference between the two aqueous bulk phases (i.e., $\tilde{\mu}_{H^+}$ "in" minus "out"). Figure 4b gives a scheme for this mosaic protonic coupling hypothesis: the coupling membrane essentially contains a multitude of independently operating and possibly heterogeneous (i.e., a mosaic of) coupling units.

C. Implications of Mosaic Protonic Coupling

1. Explanation of the Incompetence of the PMF ($\Delta\tilde{\mu}_{H^+}$)

In terms of the mosaic protonic coupling hypothesis it is readily understood why observed magnitudes of the proton electrochemical potential difference between the bulk aqueous phases bordering the membrane ($\Delta\tilde{\mu}_{H^+}$, or pmf) have often been too low to account for the free energy necessary for (the observed) synthesis of ATP: due to leakage of protons through R_H^{leak} in Fig. 4b, there is a continuous dissipation of $\Delta\tilde{\mu}_{H^+}$ such that the latter will always be lower than the proton gradient at the "site" of the proton pumps, which is denoted $\Delta\tilde{\lambda}_{H^+}$ (see Fig. 4b). It is this $\Delta\tilde{\lambda}_{H^+}$, and not $\Delta\tilde{\mu}_{H^+}$, that should be competent for the observed ATP synthesis.

2. Some Statistical Aspects of Energy Coupling

It can be estimated (Westerhoff, 1983) that if indeed the proton pumped by the electron transfer-linked proton pump remains localized to the coupling unit consisting of that proton pump plus an H^+-ATPase (i.e., the pumping of on the order of two protons) would create a local proton potential (or rather, see below, the energetic equivalent thereof) that would be energetically sufficient to drive ATP synthesis. The fact that this number is so small has important effects on the characteristics of energy coupling. First, a proton domain is either in the energized state (i.e., contains one or two protons), or in the unenergized state (i.e., contains no protons). If, say, 50 proton domains are energized and 50 others are not, then this cannot be approximately described by saying that 100 domains are half energized. The system is not ergodic (Welch and Kell, 1985).

To illustrate the relevance of this phenomenon, we shall give a brief description (for a more refined treatment see Westerhoff *et al.*, 1984b) of the time dependence of energization in a system where the electron-transfer chains are regularly, and simultaneously, pumping protons at a frequency of $1/\tau$. If the number of coupling units is denoted by n and the probability of escape of the energized proton is represented by the rate constant κ, then the number of energized proton domains n^* as a function of time is given by Eq. (1).

$$n^*(t) = n^*(0)\,e^{-\kappa t} \qquad (\text{for } t < \tau) \tag{1}$$

At $t = \tau$ this number would again jump back to n. The probability that the H^+-ATPase present in an energized coupling unit would make ATP may be described by (cf. Van Dam *et al.*, 1983; Westerhoff and Van Dam, 1985) the rate constant $K_p[1 - f(\Delta G_p)]$, which indicates that at increasing magnitudes of the phosphate potential (ΔG_p) this probability should be reduced. The amount of ATP synthesized per coupling unit can be calculated:

$$(\delta \mathrm{ATP}/n) = (n/\kappa)(1 - e^{-\kappa\tau})K_p[1 - f(\Delta G_p)] \tag{2}$$

Equation (2) shows that at low turnover rates [compared to the rate at which the energized proton leaks out of the proton domain, i.e., $(1/\tau) \ll \kappa$], the ATP yield per coupling unit per turnover of the electron-transfer chain tends to become independent of the turnover rate, or, in other words, the $P/2e^-$ ratio tends to become independent of the rate of electron transfer. In many free-energy transducing systems this is indeed observed (e.g., Van Dam and Tsou, 1970; Ernster and Nordenbrand, 1974; Ferguson *et al.*, 1976; Venturoli and Melandri, 1982; and see earlier).

We may also consider what would happen if a fraction α_p of the H^+-ATPase or a fraction α_e of the electron-transfer chains would be eliminated at random, e.g., through the addition of irreversible inhibitors. The ATP yield per

turnover of the electron-transfer chain would then become:

$$(\delta ATP/n) = (n/k)(1 - e^{\kappa\tau})K_p[1 - f(\Delta G_p)](1 - \alpha_e)(1 - \alpha_p). \quad (3)$$

Importantly, both types of inhibition would have the same effect, simply reducing the number of coupling units (n). Consequently, inhibition of electron-transfer chains is not expected to lead to a reduction in the titer of the inhibitor of the H^+-ATPase (nor vice versa). The delocalized chemiosmotic coupling hypothesis would generally predict such a reduction in titer. The experimental results seem to plead in favor of mosaic protonic coupling (see Section IV,E).

If the effect of an uncoupler would be to increase the probability of escape of a "local proton" (i.e., increase κ), then inhibition of the H^+-ATPases would be expected to have no effect on the titer of the uncoupler in its inhibitory action on ATP synthesis. If some (F_1-type) ATPase activity would be present in the system, then the titer would even be reduced. This is in contrast to the predictions of the hypothesis of delocalized chemiosmotic coupling, but in keeping with the experimental results (cf. Section IV,E).

Thus, it seems that precisely the statistical (or quantal; see Welch and Kell, 1985) properties entailed by the mosaic chemiosmotic coupling hypothesis (Westerhoff *et al.*, 1983a,c, 1984b) can account for most observations that were at odds with the delocalized chemiosmotic theory. Consequently, mosaic protonic coupling is a feasible minimum hypothesis. However, it lacks a definitive postulate concerning the structural basis for the extra device(s) that keep(s) the energized proton from equilibrating with the bulk aqueous phase adjacent to the membrane, and thus from forming a pool with its fellow energized protons. In the next section we will discuss the indications for the existence of proteinaceous devices of that kind that are distinct from the proteinaceous proton pumps themselves.

VII. PROTONEURAL PROTEINS?

A. Introduction

> How odd it is that anyone should not see that all observation must be for or against some view if it is to be of any service.
>
> (Darwin, 1903, cited by Howard, 1981)

A commonsense (and usual) reading of the delocalized chemiosmotic coupling hypothesis indicates that all that is required for electron-transport phosphorylation is a primary (redox-linked) H^+ pump and a secondary (ATP synthase-linked) H^+ pump embedded with the appropriate polarity in a suitably ion-impermeable membrane, as indicated in the simple interpretation

of reconstitution experiments (Fig. 6). In the previous sections, we concluded that more localized coupling theories permit (and, according to the calculations of H^+ transfer rates given in Section I, probably *mandate*) the existence of additional, presumably proteinaceous, devices that are required for energy coupling in electron-transport phosphorylation and related processes.

Hong and Junge (1983) have claimed that high local buffer capacities would produce localization of the high-free-energy proton. This proposal has been criticized (Westerhoff, 1983) as being insufficient to explain indications for mosaic behavior in steady-state experiments. Van Dam *et al.* (1983; cf. Sjöstrand, 1978) proposed that localization effects might be due to close apposition of the external surfaces of the inner mitochondrial (cristae) membrane. An uncertainty here is how this proposal could be extended to intact bacteria and submitochondrial particles. An alternative is the presence of special proteins in energy-coupling membranes constituting the so-called protoneural networks (Kell *et al.*, 1981b). Here we shall elaborate on this latter possibility, which was first explicitly stated, to our knowledge, by Ji (1976).

How complex really are these energy-coupling membranes, then? Have we already identified all the polypeptides which they contain? The answer again is: not at all. Lest any doubt remain concerning the very great, if often unrecognized, complexity of energy-coupling membranes, it may suffice to draw attention to the number of polypeptides resolvable in SDS gel electrophoretograms of some of these membranes that have been published to date. Thus, one-dimensional SDS gels of bacterial chromatophores contain at least 33 polypeptides (Gabellini *et al.*, 1982; Kaufmann *et al.*, 1982), few of which have as yet been identified, while two-dimensional gels of thylakoid membranes (Fig. 3 of Roscoe and Ellis, 1982) and the cytoplasmic membrane of *Escherichia coli* (Fig. 6 of Gibson, 1983) indicate numbers in excess of 50 [or 130 (Buetow and Gilbert, 1982)] and 100 polypeptides, respectively. Thus, in our search to identify the putative protoneural proteins, one can hardly claim that all the polypeptides of these energy-coupling membranes have already been identified and characterized!

To return to mitochondria, then, let us take a leap in the dark, and see whether even those few membrane proteins encoded by the mammalian mitochondrial genome have been identified.

B. Unidentified Reading Frames in the Mammalian Mitochondrial Genome

The entire genomes of a number of mammalian mitochondria, that is, those of human (Anderson *et al.*, 1981; see also Borst *et al.*, 1981), bovine (Anderson *et al.*, 1982), and murine (Bibb *et al.*, 1981) mitochondria, have been completely

sequenced. The importance of these beautiful studies to our present considerations is that, in each case, the presence of eight unidentified reading frames (URFs), coding for rather hydrophobic proteins of unknown function, was demonstrated. As Davies *et al.* (1982) have put it, in our view prophetically, "the discovery of these putative genes in organisms as different as man and *A[spergillus] nidulans* suggests that they code for functional proteins that play a hitherto unsuspected role in mitochondria." While one may imagine many possible indentities for the proteins encoded by the URFs, such as translocases, or ribosomal proteins, or processing enzymes (Attardi *et al.*, 1982), we wish to stress a possibility that our molecular biological colleagues, who may not be fully aware of the bioenergetic literature, might otherwise neglect: that these URFs code for proteins with the role of the protoneural proteins alluded to above (Kell and Morris, 1981). It should be stressed that these URFs do code for functional mRNA molecules. Finally, we would mention that two-dimensional gel autoradiograms (which, in contrast to Coomassie Blue staining, do not discriminate against very hydrophobic proteins) indicate that the mitochondrial translation products of HeLa cells include as many as 26 polypeptides (Ching and Attardi, 1982).

It may be remarked, in conclusion, that the coincidence of the hydrophobic nature of the proteins coded for by the URFs and the importance of using Racker's hydrophobic protein fraction rather than more purified H^+-ATP synthase preparations in reconstituting oxidative phosphorylation seems beyond fortuity. We now turn to some studies of plant mitochondrial molecular biology which give us a more forthright clue as to the role of at least one unidentified mitochondrial protein in mitochondrial free-energy transduction.

C. The Interaction of *Helminthosporium* T Toxin with Corn Mitochondria

> "Unexpected, shocking and true"
>
> [P. P. Slonimski, May 17, 1981, describing the kind of results he hoped to see in mitochondrial research; quoted by Bendich (1982)].

Cytoplasmic male sterility (CMS) is a maternally inherited trait that prevents the production of functional pollen but does not affect female fertility in a number of plants of commercial significance (Edwardson, 1970). The genetic determinants which control CMS in maize (corn) are located on mitochondrial DNA (see e.g., Leaver *et al.*, 1982; Leaver and Gray, 1982). Since certain plant lines carry nuclear fertility-restoring genes, the CMS phenotype is now widely used in the commercial production of F_1 hybrid seed varieties to prevent the self-pollination of the parent.

Three types of CMS, namely CMS-T, CMS-S and CMS-C, have been described in the maize. Our attention is concentrated on the T (Texas) cytoplasm, which was used in over 85% of hybrid corn grown in the United States by 1970 (Leaver and Gray, 1982). In that year, an epidemic of southern corn leaf blight disease virtually wiped out the maize crop, causing losses in excess of $1 billion. The disease is caused by a polyketide toxin (T toxin) (for probable structure see Kono *et al.*, 1980) produced by the fungus *Helminthosporium maydis* Race T, which strongly affects corn with the CMS-T trait but has little effect on normal fertile (N) or the CMS-S and CMS-C types (Ullstrup, 1972; Gregory *et al.*, 1977). A wealth of physiological studies, summarized by Gregory and colleagues (1977, 1980) and by Leaver and colleagues (Leaver and Gray, 1982; Leaver *et al.*, 1982), leaves little room for doubt that the primary target for the T toxin is a mitochondrially encoded protein resident in the inner mitochondrial membrane and that the interaction of T toxin with this protein leads, *inter alia*, even at a T toxin concentration of 10 pmol/mg mitochondrial protein (Gregory *et al.*, 1980), to the "uncoupling" of sensitive mitochondria. It should be stressed that normal (N) mitochondria are quite resistant to doses of this toxin at concentrations as much as 1000-fold higher.

What is the nature of the target protein? Leaver and colleagues have studied the synthesis of proteins by mitochondria isolated from a number of strains of corn possessing N and the CMS traits (Forde *et al.*, 1978; Forde and Leaver, 1980; Leaver and Gray, 1982; Leaver *et al.*, 1982; Dixon *et al.*, 1982); analysis of the radiolabeled products of mitochondrial translation by one-dimensional SDS–polyacrylamide gel electrophoresis and autoradiography shows that at least 20 polypeptides in the range 8000–54,000 Da can be resolved, and, with the exception of a 44-kDa polypeptide, all are normally membrane bound. Most strikingly, a 21-kDa polypeptide synthesized by N mitochondria is not seen in T mitochondria, which instead possess, uniquely, a 13-kDa polypeptide. It seems, therefore, very likely that this 13-kDa polypeptide is the (or at least a) target of the T toxin. Since the result of the interaction of the toxin with this protein is some kind of uncoupling of the sensitive T mitochondria, it is evident that the role of the 13-kDa polypeptide normally lies in energy coupling in such mitochondria. Since this polypeptide is not thought to be part of complexes I to V of these mitochondria, an intriguing possibility, in harmony with the considerations in the rest of this section, is that this target protein possesses the properties of a protoneural protein, as proposed previously (Kell *et al.*, 1981b).

Although the T toxin is not proteinaceous, the physiological effects of its interaction with sensitive mitochondria bear many striking resemblances to those elicited by the interaction of a number of proteinaceous membrane-active bacteriocins with their sensitive target bacterial cells, a topic which forms the subject of the next section.

D. Effects of Membrane-Active Bacteriocins on Sensitive Cells

> Since the BLM [black lipid membrane] is an exquisitely sensitive tool with which to study the interaction of proteins with bilayers, it is important to keep in mind the possible molecular mechanisms underlying the observed conductance effects. True channel formation in a BLM may reflect the actual physiologic role of a particular protein. If, on the other hand, a protein is observed to increase conductance in a BLM, but in a manner suggestive of lipid perturbation, it may be quite misleading to declare it a channel-former and to suppose its biological action or function is carried out via discrete channel formation.
>
> (Blumenthal and Klausner, 1982)

The colicins are a heterogeneous group of proteinaceous bactericidal agents produced by a variety of bacteria and active against many strains of *Escherichia coli*. Their physiological effects have been described in a number of reviews (Holland, 1975; Konisky, 1978, 1982; Konisky and Tokuda, 1979; Kell *et al.*, 1981b; Cramer *et al.*, 1983). Many other bacteriocins have been described, such as butyricin 7423 (see Clarke *et al.*, 1982), which is active against the anaerobe *Clostridium pasteurianum*, that, like a number of the colicins, have the ability to deenergize the cytoplasmic membrane of sensitive cells. Since most of these membrane-active bacteriocins elicit broadly comparable effects upon sensitive cells, we shall confine our comments to those studies which have sought to infer the mechanism by which the target cell membrane is deenergized by membrane-active colicins.

Upon the addition to a sensitive *E. coli* suspension of an appropriate concentration of membrane-active colicin, the following effects, *inter alia*, may be observed: inhibition of certain respiration-linked active transport processes (Fields and Luria, 1969a), a lowering of cellular ATP levels (Fields and Luria, 1969b; Hirata *et al.*, 1969), a rapid efflux of actively accumulated intracellular K^+ (Luria, 1964; Nomura and Maeda, 1965; Dandeu *et al.*, 1969; Feingold, 1970; Wendt, 1970), and inhibition of the energy-linked transhydrogenase (Sabet, 1976). Respiration is unaffected.

From a chemiosmotic standpoint, it seemed reasonable to suppose that these colicins might be protonophorous, since the addition of a protonophorous uncoupler would be expected to inhibit all proton-motivated bioenergetic processes in such organisms. However, it was found that colicin E1, a typical membrane-active colicin, exhibited no protonophorous activity, and its action could not be mimicked by known protonophores (Feingold, 1970; Luria, 1973; Konisky *et al.*, 1975). Further, although uncoupling of proton-motivated energy coupling systems occurred, colicins did *not* act to dissipate the pH gradient across the cell membrane (Brewer, 1976; Weiss and Luria, 1978; Tokuda and Konisky, 1978). This is consistent with other data, discussed earlier, that may indicate that the role of the pH gradient lies (in bacteria and mitochondria) in pH homeostasis and not in energy coupling.

Rosen and Kashket (1978), whose thoughtful article was unfortunately not noted in an earlier discussion of this problem (Kell *et al.*, 1981b), concluded that the available data best fit a model in which the binding of colicin to a membrane component caused the formation of an anion channel, although in some of the experimental conditions used, one might then expect a substantial increase in the pH gradient, which is not observed. Further, the low-molecular-weight anion content of these cells is too low to account, in this model, for a steady-state dissipation of a membrane potential (Gould and Cramer, 1977a), in the absence of such a high ΔpH.

One of the widely discussed features of the action of membrane-active colicins is that they appear to exhibit "single-hit killing," that is, the titer of active colicin necessary to kill an entire cell is one molecule. Actually, the available data (see Holland, 1975; Konisky, 1978) would seem to be just as consistent with oligo-hit killing, but this particular point is not quantitatively crucial to the following discussion. In an influential paper, Schein *et al.* (1979) demonstrated that the addition of colicin K to a planar phospholipid bilayer membrane (BLM) could lead to the formation of a rather unselective, gated, ion-permeable channel. These workers (Schein *et al.*, 1979) further drew together a number of previous observations, including the rates of K^+ efflux observed by Wendt (1970), and suggested that the pathophysiology of colicin K action could be adequately accounted for by the view that it acts merely to create a poorly selective ion channel across the bacterial cytoplasmic membrane. In particular, in a delocalized chemiosmotic model, such a model might simply explain the single-hit killing property discussed above.

This view, which we will refer to as the "orthodox view," has gained a degree of currency such that two recent reviews on bacteriocins have been entitled "Colicins and other bacteriocins with established modes of action" (Konisky, 1982) and "The membrane channel-forming bacteriocidal protein, colicin E2" (Cramer *et al.*, 1983). It is our opinion (see also Kell *et al.*, 1981b) that the orthodox view is a highly premature baby, and our task here is to draw attention to some of the reasons that we think it is unlikely to survive into puberty. First, it is highly dependent upon findings in artificial liposomes and planar BLM. However, the data available even with these systems are not self-consistent, as noted by Cramer *et al.* (1983). In the original work of Schein *et al.* (1979) the BLM conductance was found to increase only if the applied voltage had a polarity that was positive in the compartment to which the bacteriocin was added. This was also observed with colicin Ib (Weaver *et al.*, 1981) and colicin A (Pattus *et al.*, 1983). However, neither the single-channel conductances measured (Weaver *et al.*, 1981; Cleveland *et al.*, 1983) in planar BLM, nor the efflux of radiolabeled substances from liposomes induced by a variety of colicins (Tokuda and Konisky, 1979; Kayalar and Luria, 1979; Uratani and Cramer, 1981; Weaver *et al.*, 1981), was significantly dependent

on the polarity of any applied membrane potential. Uratani and Cramer (1981) had to *incorporate* colicin E1 into liposomes in order to see any effect upon their permeability to low-molecular-weight compounds, which is hardly consonant with the situation *in vivo*. The rates of Rb^+ efflux caused by the addition of an unspecified amount of colicin K to liposomes (Kayalar and Luria, 1979) were virtually identical to those of Na^+ efflux elicited by the addition of albumin to liposomes (Kimelberg and Papahadjopoulos, 1971); it is therefore important to relate the amount of bacteriocin used in these *in vitro* experiments to that serving as the minimum lethal dose in intact bacterial cells.

As indicated above, colicins are extremely potent; a typical titer giving >99% killing might be 100 ng colicin/mg dry weight cells (or per 5×10^8 cells, approximately). The surface area of such a cell mass is well in excess of 10 cm^2 if we (conservatively) model the cells as spheres of diameter 1 μm. In a typical experiment with BLM (Schein *et al.*, 1979) the amount of bacteriocin added was 700 ng for a BLM of area 0.01 mm^2. Thus, it is evident that these *in vitro* experiments have been using amounts of bacteriocin far in excess of those constituting a minimal lethal dose in bacteria. The excellent review by Blumenthal and Klausner (1982) lists the proper criteria for deciding whether a *bona fide* channel is formed in BLM or not. The available data using colicins do not suggest that these criteria are likely to be met with an orthodox result. Thus, given the self-inconsistency of the BLM and liposome data, and the inability of these supposedly poorly selective channel formers to collapse a pH gradient *in vivo*, one is forced to conclude that the primary act of membrane-active colicins *in vivo* cannot be to knock a rather nonselective hole in the lipid bilayer portion of the target cell membrane. This conclusion is greatly strengthened by the studies carried out by a number of groups on mutant strains which are nontrivially resistant to the membrane-active bacteriocins, and which for some reason seem not to be considered by the proponents of the orthodox view.

Parenthetically, some of the studies referred to above as using "colicin K" were actually performed with the comparable membrane-active bacteriocin colicin A (Luria, 1982).

If, as we have indicated here, a simple biophysical insertion of a hydrophobic channel-forming colicin into the target cell membrane does not explain the effects observed *in vivo*, what then might be the target membrane protein of these deenergizing colicins? Hong *et al.* (1977) isolated a mutant of *E. coli* which was resistant to colicin K (but still sensitive to colicin E1) and concluded from its properties and those of revertants that the basis for colicin sensitivity lay in the membrane-located product of a gene termed *ecf*, which they had previously studied (Lieberman and Hong, 1974, 1976; Hong, 1977;

Lieberman *et al.*, 1977; see also Tomochika and Hong, 1978). The properties of these mutants, when transferred to nonpermissive conditions, including a fall in intracellular ATP and efflux of intracellular metabolites, were closely similar to those induced by the addition of colicin K to the wild-type strain. Such mutants were pleiotropically defective in the coupling of respiration to a variety of proton-linked active transport systems and it was concluded that the defect lay in an inability of the mutant to couple respiration-derived energy to active transport and ATP synthesis. The mutation maps (Lieberman *et al.*, 1977) at minute 65 on the recalibrated *E. coli* linkage map (Bachmann, 1983) and is thus quite distinct from the operon coding for the *E. coli* ATP synthase (Gibson, 1983). It should be mentioned that apart from that of Tomochika and Hong (1978) these strains showed no increase in permeability to protons. Unfortunately, it seems that the original isolates have now been lost. Thus, these very elegant studies show quite clearly that for energy coupling to take place in electron-transport phosphorylation and respiration-linked active transport a factor distinct from the primary and secondary proton pumps and from proton leakiness is required.

Plate and his colleagues have described a number of other mutant strains of *E. coli* which, although selected for resistance to the aminoglycoside neomycin, are also rather resistant to colicin K, grow poorly on respiratory substrates, and are impaired in the operation of a number of respiration-linked active-transport systems (Plate, 1976, 1979; Plate and Suit, 1981). The properties of these strains are similar to those of Hong described in the previous paragraph (see also Hengge and Boos, 1983; Booth *et al.*, 1984), with the exception that their *eup* lesion maps at minute 87.5–88. It was concluded by these workers (Plate and Suit, 1981) that the *eup* locus codes for a protein normally required for the coupling of H^+ movements to solute symport. These mutants were also quite unimpaired in their ability to maintain a pH gradient (Plate and Suit, 1981). Physiological and genetic studies are consistent with the view that the *eup* gene is related to the independently studied genes *ssd* (Morris and Newman, 1980; Newman *et al.*, 1981, 1982) and especially *ecf*B (Thorbjarnardóttir *et al.*, 1978). Finally, it was shown (Hitchens *et al.*, 1982) that the ability of an *eup* mutant to exhibit respiration-driven H^+ translocation was unimpaired relative to that of its otherwise isogenic wild-type parent. This latter study confirmed (a) that the lesion lay in an inability to *use* energized protons and (b) that the proton (and other ion) permeability of the mutants, at least from the external bulk aqueous phase, was no different from that of their parents. Studies by C. A. Plate (personal communication) have added some complexity to the simple interpretation discussed herein, and suggest to us that, in the absence of any *eup* genetic locus, yet other proteins may fulfil the role normally played by the *eup* gene product (Kell *et al.*, 1981b). These studies

tend to indicate rather suggestively that there is now fairly abundant, if widely scattered, evidence that "energy-transducing membranes normally contain a number of proteinaceous components whose role is to act co-operatively as conformationally switchable proton conductors, permitting fast, controlled, *lateral* proton transfer along the surfaces of such energy-transducing membranes, and acting as the major energetic links between the various protonmotive sources and proton-accepting sinks embedded in such membranes" (Kell *et al.*, 1981b).

We have mentioned uncoupling several times in this section. The orthodox view would assume, since the membrane potential and pH gradients of delocalized chemiosmosis act all over the membrane surface, that the more classical, ionophorous types of uncoupler do not *necessarily* have to act at specific sites in such membranes. More localized views predict the existence of more or less specific sites of uncoupler action. It is therefore important to determine the extent to which such sites may have been demonstrated, a survey which forms the subject of the next section.

VIII. MECHANISMS OF UNCOUPLING

A. Protonophorous Uncouplers

1. Introduction

As is now well known, the addition of low concentrations of any of a number of certain nonphysiological low-molecular-weight chemicals to well-coupled mitochondria respiring under static head (state 4) conditions leads to a dramatic increase in respiratory rate, and to a more or less concomitant loss in the ability to couple the exergonic reactions of electron transport to the endergonic reactions of ATP synthesis. This *respiratory control* is only rarely observed in bacteria (e.g., Scholes and Mitchell, 1970; John and Whatley, 1977; McCarthy and Ferguson, 1982) or in submitochondrial particles (e.g., Hinkle *et al.*, 1975) and is thus probably not crucial to the interpretation of the compounds; what is most important is that they uncouple electron transport from ATP synthesis, and they are thus known as uncouplers. That concentrations of uncoupler necessary to inhibit oxidative phosphorylation in well-coupled mitochondria do not decrease respiration distinguishes them from inhibitors of electron flow or energy-transfer (ATP synthase) inhibitors. Typical phenomena correlative to uncoupling in mitochondria include the inhibition of various exchange reactions such as ATP–$^{32}P_i$ exchange and stimulation of the apparent ATP hydrolase activity (e.g., Heytler, 1979). The concentration of uncoupler required for a half-maximal effect in a given

suspension depends upon the reaction being considered, which is consistent with the view that one effect of uncouplers is to cause slip in primary and/or secondary proton pumps and also not easily reconciled with the classical chemiosmotic explanation of respiratory control.

The classical uncoupler is 2,4-dinitrophenol (DNP) (e.g., Loomis and Lipmann, 1948), the curious and interesting history of which is discussed by Racker (1976). One of the most important and (in our view) persuasive predictions of the chemiosmotic hypothesis was that uncouplers such as DNP could catalyze the electrogenic transport of protons, to and from bulk aqueous phases, across biological, and particularly across energy-coupling, membranes, by virtue of the fact that they are lipophilic weak acids or bases. DNP and comparable uncouplers of this type, which are often referred to as classical or *bona fide* uncouplers, are therefore known as protonophores or protonophorous uncouplers. Typical protonophores include DNP, carbonyl cyanide *p*-trifluoromethyoxyphenylhydrazone (FCCP) (Heytler, 1963), 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB), pentachlorophenol (PCP), 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S-13), bis(hexafluoroacetonyl)acetone (1799), and, the most potent so far discovered (in published literature), 3,5-di-*tert*-butyl-4-hydroxy-benzylidene malononitrile (SF 6847) (Muraoka and Terada, 1972; Terada, 1981). Since most investigations on energy coupling use only these compounds (amongst the protonophores) we shall in general concentrate our attention on these compounds. For an overview of protonophores, the reader is referred to five excellent and comprehensive reviews (Hatefi, 1975; Hanstein, 1976a,b; McLaughlin and Dilger, 1980; Terada, 1981).

Given the well-known and abundant evidence that protonophorous uncouplers can indeed catalyze the electrogenic transfer of protons across natural and artificial bilayer membranes from one bulk aqueous phase to another, it might be thought that the mechanism of their action was well understood, and indicative that a delocalized chemiosmotic *coupling* mechanism indeed operates in energy-coupling membranes. However, we shall see that a study of the extensive literature concerning the effects of protonophores on proton motive systems reveals a much more complex state of affairs than may be adduced from the simple view that uncouplers uncouple simply by dissipating the bulk-phase $\Delta\tilde{\mu}_{H^+}$ by catalyzing electrogenic H^+ transfer across the bilayer portions of energy-coupling membranes. Thus we wish to know whether uncouplers uncouple by acting in the bilayer, phospholipid-containing parts of such membranes or whether some uncoupler–protein interaction(s) might be of significance to the uncoupling process. We first draw attention to a strikingly comparable problem that is being earnestly debated in the field of general anesthetics. Since a fuller discussion of this will be given elsewhere, some summary remarks will suffice.

2. A Digression: Where Do General Anesthetics Act?

As is widely understood, the structure–activity relationship among different volatile narcotics (general anesthetics) is weak almost to the point of nonexistence (e.g., Bowman *et al.*, 1969). This and other facts have led to the view that general anesthetics do not act by virtue of specific molecular interactions, but act purely by their possession of one or more biophysical properties (e.g., Halsey, 1974; Miller and Miller, 1975; Kaufman, 1977), in particular their ability, by insertion into lipid bilayers, to expand nerve membranes above a critical volume in such a way as to inhibit neurotransmission (see e.g., Miller *et al.*, 1973; Janoff and Miller, 1982). We refer to the critical volume hypothesis and other cognate hypotheses as *phospholipid hypotheses*. Since, in addition, it has been known for many years that narcotics can interact with soluble proteins (e.g., White, 1974), some workers, notably Franks and Lieb (1978, 1979, 1981), have adduced evidence from a variety of experimental approaches, especially one involving solvent correlations, that the site of narcotic action has both polar and apolar characteristics and may thus be assumed to be partly proteinaceous in nature. Such protein hypotheses were lent especially cogent, even conclusive, support by a very elegant, yet conceptually simple study (Fernandez *et al.*, 1982). This study showed that while chloroform actually *stimulated* the rate of translocation of the lipophilic dipicrylamine ion across the bilayer portion of a squid axon (as expected; see Section VIII,A,3,b), it inhibited the gating currents associated with the opening and closing of the proteinaceous Na^+ channel. Lenaz (1978) also summarized extensive work from his laboratory consistent with the view that the site of general anesthetic action may lie at the interface between lipids and integral membrane proteins. Thus, there is an interesting parallel in the narcosis field with a debate of more immediate interest to us in the uncoupler field, concerning whether the site of action of such xenobiotics in biomembranes is lipidic or proteinaceous in nature. In our view, it may be concluded (see e.g., Hille, 1980; Sandorfy, 1980; Trudell, 1980; Franks and Lieb, 1981; Fernandez *et al.*, 1982; but contrast e.g., Janoff and Miller, 1982) that the bulk of evidence and opinion has swung in favor of the view that the true site of anesthetic action is proteinaceous in nature (Franks and Lieb, 1984; Smith *et al.*, 1984).

To return us to our discussion of uncouplers, we draw attention to a very important article by Rottenberg (1983), who found that the narcotics chloroform and halothane, at significantly supraclinical concentrations, acted to uncouple oxidative phosphorylation in rat liver mitochondria. It was demonstrated, however, that this uncoupling was *not* accompanied by any diminution in the apparent $\Delta\tilde{\mu}_{H^+}$ (judged by ion distribution methods) and was ascribed to "interference with delicate intramembrane processes that mediate direct energy transfer between the electron transport components and

the ATPase" (Rottenberg, 1983). One might therefore invoke an interaction between the narcotics and putative protoneural proteins in this system. However, it was not absolutely excluded that the narcotics might induce a slip in the H^+-ATP synthases in an otherwise chemiosmotically operating system. This general topic is considered further in part C of this section.

3. Where do Protonophores Act? Bilayer and Protein Hypotheses

The great complexity of energy-coupling membranes has led many investigators to study simpler model phospholipid bilayer systems, of which the liposome (e.g., Bangham, 1968, 1972; Szoka and Paphadjopoulos, 1980) and the planar black or bilayer lipid membrane (e.g., Jain, 1972; Tien, 1974) systems remain both the most popular and the best characterized. Although, as widely recognized, studies with such systems cannot easily be extrapolated to the situation *in vivo*, many useful findings have emerged.

We take as our starting point the data discussed in the comprehensive review article by McLaughlin and Dilger (1980). Classical uncoupling (for a recent discussion, see Benz and McLaughlin, 1983) by the A^- class of uncouplers is illustrated in Fig. 7a. In general, the correlation (at a given pH

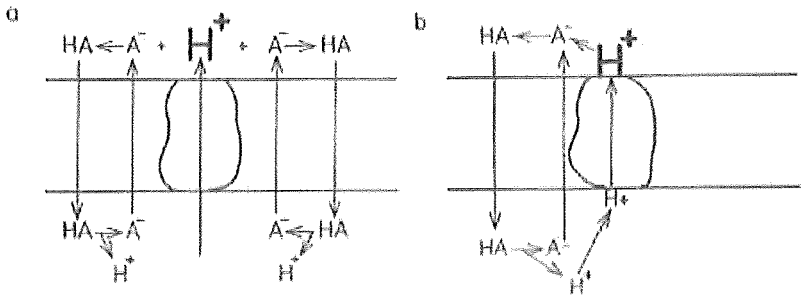

Fig. 7. Mechanisms of action of an A^- protonophore such as FCCP. (a) Classical, macroscopic picture, illustrated with reference to delocalized protonmotive activity by a single membrane-located primary proton pump. The chief feature of the uncoupler is that both charged and uncharged forms are membrane permeable. No special sites of action are required for the transmembrane passage of HA or of A^- during uncoupling, since the putative driving force is delocalized. (b) A more coarse-grained analysis, illustrated with reference to the concept (for a primary proton pump) of localized coupling. Localized charge separation occurs due to the activity of the primary proton pump. A *nearby* A^- molecule can then be driven across the membrane by the local field in a region of high permittivity at the protein–phospholipid interface. This causes the primary proton pump to lose its energization, releasing the proton which had been pumped. The protonophoric cycle is then completed as in (a). Note that the delocalized coupling model *permits* preferential movement of protonophore molecules near *any* membrane protein; localized coupling *requires* the rate-limiting steps of uncoupling to be driven only near the *energized* proteins. Within the framework of localized coupling, uncouplers may also possibly act to cause molecular slip (Westerhoff *et al.*, 1983c, 1984b); this is not illustrated in this figure. For further discussion, see text.

value) between (a) the effectiveness of uncoupling in rat liver mitochondria and (b) the ability of a compound to increase the conductance of BLM (which is generally ohmic up to supraphysiological values of transmembrane voltages), is extremely good (Skulachev, 1971; McLaughlin and Dilger, 1980). An important apparent exception in this context, however, is picric acid.

a. Uncoupling by Picric Acid. Hanstein and Hatefi (1974a) found that picrate could not uncouple mitochondria but was a good uncoupler of some submitochondrial particles (SMP, which are topologically inverted). They proposed that picrate was a (relatively) membrane-impermeant substance that exerted its effect by binding to the matrix side of the inner mitochondrial membrane. However, McLaughlin and colleagues (1978) showed that picrate is indeed protonophoric and permeates BLM in both the neutral and charged forms. They pointed out that since the presumed membrane potentials of mitochondria and SMPs are of opposite polarity, consideration of a typical kinetic model for an A^--type uncoupler can lead, in fact, to an analysis quite consistent with the chemiosmotic model. However, there are some severe quantitative problems with this view. In the model of McLaughlin *et al.* (1978) detectable uncoupling by picrate (10 μM) in SMP is observed (in the model) under conditions in which the bulk phase membrane potential was taken to be 175 mV and the pH gradient 1 unit. A similar model was used by Michels and Bakker (1981) in *E. coli*. However, it would seem that (a) the bulk-to-bulk phase proton permeability induced across the membrane of SMPs by this concentration of picrate is at least 1.5 orders of magnitude too low to account for the observed uncoupling in strictly chemiosmotic terms (Hanstein and Kiehl, 1981) and (b) uncoupling concentrations of picrate (10 μM) *do* decrease the membrane potential rather markedly, as judged by oxonol-VI fluorescence in SMP (Hanstein and Kiehl, 1981) or by SCN^- uptake in everted *E. coli* vesicles (Michels and Bakker, 1981). Similar remarks may be made concerning the nonprotonophorous tetraphenyl borate ion (Phelps and Hanstein, 1977), which is a significantly *more* potent uncoupler than is picrate in everted *E. coli* vesicles (Michels and Bakker, 1981). Thus examples seem to exist which clearly indicate that results in BLM are not easily extrapolated to native energy-coupling membranes, and that, by implication, some kind of uncoupler–protein interaction may be of importance in causing uncoupling.

b. Effect of Dielectric Permittivity on Uncoupler Fluxes. McLaughlin and Dilger (1980) drew attention to two interesting anomalies between the protonophorous properties of weak acid uncouplers in BLM and their ability to uncouple in mitochondria. The first is that although the correlation between these two types of property among different compounds is generally excellent, weak acid protonophores are two orders of magnitude more effective in mitochondria than in BLM. The second is that a weak acid for

which the bilayer conductance depends quadratically on the carrier concentration (i.e., an HA_2^--type protonophore) produces an uncoupling effect that depends linearly upon concentration (Dilger and McLaughlin, 1979). It was proposed (Dilger and McLaughlin, 1979; McLaughlin and Dilger, 1980) that the apparent anomalies are due to "a region of the bilayer component of the mitochondrial membrane having a higher dielectric constant than that of an artificial bilayer (p. 379)." We consider this extremely important and suggestive idea in some detail.

It is both reasonable and usual to treat a BLM as a homogeneous condenser of static permittivity ε_2; however, the extent to which this macroscopic simplification retains its utility in the heterogeneous milieu of a biomembrane is far from clear.

As indicated by a number of authors (e.g., Läuger and Neumcke, 1973; Andersen and Fuchs, 1975; Parsegian, 1975; McLaughlin and Dilger, 1980), the Born energy W required to transfer a monovalent, spherical, nonpolarizable ion of charge e and radius a from an aqueous phase of permittivity ε_1 into the center of a bilayer of permittivity ε_2 and thickness d is:

$$W = \frac{e^2}{8\pi\varepsilon_0 a}\left(\frac{1}{\varepsilon_2} - \frac{1}{\varepsilon_1}\right) - \frac{e^2}{4\pi\varepsilon_0\varepsilon_2 d}\ln\left(\frac{2\varepsilon_1}{\varepsilon_1 + \varepsilon_2}\right), \tag{4}$$

where ε_0 is the permittivity of free space. Thus the Born energy depends both on the thickness and the permittivity of the membrane. It was most elegantly shown (Dilger and McLaughlin, 1979; McLaughlin and Dilger, 1980; Benz and McLaughlin, 1983), by using the somewhat polar chlorodecane in the BLM-forming procedure, that the protonophore-induced conductivity of the BLM could be raised by approximately two orders of magnitude; changes in bilayer thickness played only a minor role. Further, under these conditions, the HA_2^- uncoupler DTFB (Cohen *et al.*, 1977) indeed behaved as an A^--type protonophore, since, from the Born charging equation, the relative decrease in Born energy induced by increasing the BLM permittivity will be greater for the smaller A^- ion. Similar observations on the membrane permeability were made using the membrane-permeable SCN^- and ClO_4^- ions (Dilger *et al.*, 1979; McLaughlin, 1981).

Now, it has of course been known for many years (see e.g., Pauly and Packer, 1960; Packham *et al.*, 1978; Pethig, 1979; Harris and Kell, 1983) that the *macroscopic* static permittivity of biomembranes is greater than that of BLM of comparable thickness by a factor of approximately two. Let us then take a coarse-grained approach (Fig. 7b). Given the great heterogeneity of energy-coupling membranes, it may then be stated quite clearly that, in view of the foregoing considerations alone, protonophores will tend to act preferentially in the regions of highest permittivity, that is, adjacent to membrane proteins. This does not of itself require that *specific* interactions with proteins

are part of the uncoupling process, nor does it, for instance, indicate of itself that the high-energy intermediate is confined to these preferential regions. To examine these questions, we must turn away from work with BLM.

c. *Uncoupler-Binding Proteins in Energy-Coupling Membranes.* We saw in the last section that there tended to be a preferential partitioning of charged uncoupling molecules into regions of highest local permittivity. Might some *specific* protein–uncoupler interactions be of significance to the uncoupling process? The structure–function relationships of homologous protonophores (e.g., Terada, 1981) do not lend much support to the idea that factors other than hydrophobicity and acid dissociation constant play a significant role in inducing uncoupling activity, so that steric effects, which might also play a role in putative protein–uncoupler interactions, are assigned a rather minor role (but see Büchel and Draber, 1972).

Now it is, of course, well known that uncouplers *can* interact with a great many proteins, and it is worth pointing out that delipidation of mitochondria with $CHCl_3$-methanol has a negligible effect upon the ability of mitochondrial proteins to bind a number of uncouplers (Weinbach and Garbus, 1965, 1969; Hanstein *et al.*, 1979). In general, energy-coupling membranes can bind much more uncoupler than is necessary to give maximal uncoupling, so that the question arises as to what fraction of the total bound uncoupler is actually uncoupling.

Hanstein and Hatefi (1974b) initiated an important approach to this question by synthesizing 2-azido-4-nitrophenol (NPA), a photoaffinity analogue of DNP with an uncoupling potency two to three times greater. In the dark, NPA bound to mitochondria both specifically and nonspecifically, the number of binding sites approximating one per ATPase. The specific binding was noncooperative and independent of the degree of energization of the mitochondria, and was competitively inhibited by other uncouplers such as DNP, PCP, CCCP, and S-13. When radiolabeled NPA was photolyzed in the presence of mitochondria, more than 90% of the label was associated with protein, and predominantly with (a) the F_1-ATPase and (b) one or more hydrophobic proteins of ~21 kDa, located in or near the F_o-ATPase, and referred to as the uncoupler-binding protein (Hanstein, 1978; Hanstein *et al.*, 1979; Drosdat *et al.*, 1982). This protein is located in carefully prepared complex V, an F_oF_1-ATPase preparation with *high* ATP-P_i exchangease activity (Hatefi, 1975; Kiehl and Hanstein, 1981; cf. Berden and Hennecke, 1981).

The photoaffinity labeling strategy was adopted by Kurup and Sanadi (1977) using NPA and by Katre and Wilson (1977, 1978, 1980), who synthesized 2-nitro-4-azidocarbonyl cyanide phenylhydrazone (N_3CCP). These latter workers obtained broadly comparable results to those of Hanstein and colleagues (op.cit.). However, the polypeptides labeled with

photolyzed N_3CCP apparently differed considerably from those labeled by NPA, raising the possibility that "... the ability of the weak acid protonophores to bind to proteins, and indeed to any hydrophobic surface, may be an epiphenomenon unrelated to their mechanism of action as uncouplers" (McLaughlin and Dilger, 1980). Competition between N_3CCP and NPA does not yet seem to have been tested, nor do protease inhibitors seem to have been included in the labeling experiments. However, it is probable that N_3CCP, being more hydrophobic, is more catholic in its binding to membrane proteins than is the relatively hydrophilic NPA (Hanstein, 1978). Since mitochondria covalently labeled with N_3CCP were completely uncoupled (Katre and Wilson, 1978), it seems improbable that the compound could have been acting as a mobile protonophore under these conditions. Nevertheless, it is probably fair to conclude that the very interesting observations made with these compounds do not alone yet allow us to assess the exact importance of the uncoupler-binding protein(s) in the mechanism of uncoupling by protonophores.

d. Resistance to Uncouplers. Resistance of mutant strains of biological cell systems to typical protonophores has so far been reported in *Bacillus subtilis* (Decker and Lang, 1977, 1978; Lang and Decker, 1978; Guffanti *et al.*, 1981), in *Escherichia coli* (Ito and Ohnishi, 1981; Ito *et al.*, 1983), and in the mitochondria of yeast (Griffiths *et al.*, 1972, 1974) and of Chinese hamster cells (Freeman *et al.*, 1980, 1983). In each of these studies, it was shown that the resistance was nontrivial, and the mutation seems to affect a protein lying in or near the F_o part of the ATPase as judged by cross-resistance to F_o inhibitors, increased ATP hydrolase activity, etc. In some cases (e.g., Griffiths *et al.*, 1972; Freeman *et al.*, 1980, 1983) mutant cells are as much as 10 times more resistant to selected protonophores than their wild-type parents, and Hitchens and Kell (unpublished observations, 1982) obtained similar data in *Rps. capsulata*. It seems, therefore, that *some* type of specific protein–uncoupler interaction, albeit transient (Hitchens and Kell, 1982c, 1983a,b), must be of functional significance in determining uncoupling by protonophorous uncouplers. This conclusion would be greatly strengthened by comparison of the titer of uncoupler-binding protein in various strains with the degree of resistance they possess to appropriate uncouplers. It may also be worth drawing attention to the ability of a variety of respiratory bacteria to grow on pentachlorophenol (Rao, 1978).

It has been proposed that uncouplers may cause redox-linked proton pumps to exhibit exacerbated slip (Pietrobon *et al.*, 1981, 1982; Walz, 1983), a proposal which is consistent with uncoupler titrations discussed above. These proposals (Pietrobon *et al.*, 1981, 1982) were based upon experiments measuring the apparent protonmotive force under various conditions. The

general conception of slip has been further strengthened (Pietrobon *et al.*, 1983) in experiments which contraindicated a role for a non-ohmic leak across the inner mitochondrial membrane. However, the latter experiments showed that since slip is not *only* a function of the apparent proton motive force then the energy coupling should not be presumed to be proceeding *via* the apparent proton motive force either.

It may be concluded that uncoupling does require some transient, specific, protein–uncoupler interactions. The substoichiometry of uncoupler–ATP synthase "binding" is simply explained by the fast *lateral* mobility of uncouplers (Hitchens and Kell, 1982c, 1983a,b; Kell and Hitchens, 1983).

B. Ionophorous Uncouplers

Most of what we have written in the previous section may be applied to the ionophorous type of uncoupler. For reasons of space, we will not give a detailed exposition, save to note that the synergism often found in uncoupling by electrogenic and electroneural ionophores reflects simply a requirement for a replenishment of the inner phase of the membrane vesicle suspension with the appropriate ion. The uncoupling step could be ascribed to a dissipation of the *localized* fields set up across the membrane in response to proton motive activity (Fig. 7b). Far more interesting from our point of view are a number of compounds, which we shall refer to as *decouplers*, which exhibit certain uncoupling properties yet which are not protonophorous.

C. Decouplers

A number of compounds have been described which, while not energy-transfer (ATPase) inhibitors, have the ability to inhibit ATP synthesis, to inhibit state 3 but not state 4 respiration, but not to release respiratory control in the sense that protonophores do. There does not seem to be an adequate role for such compounds in the chemiosmotic model, and they are thus generally ignored. We may mention biguanides (Schäfer and Rowohl-Quishoudt, 1975; Schäfer, 1981), fluorescein mercuric acetate (Southard *et al.*, 1974), tetraphenyl borate (Phelps and Hanstein, 1977; Michels and Bakker, 1981), and a variety of lipophilic cations often used in attempts to measure the membrane potential across energy-coupling membranes (Higuti *et al.*, 1978, 1980, 1981, and references therein; Zaritsky and MacNab, 1981). These types of compound, which should be distinguished from certain other types with comparable properties except that uncoupling *is* sensitive to ATPase inhibitors (Mai and Allison, 1983), also, in most cases, have the effect of increasing the number of H^+ translocated in the bulk phase external to the system. Authors who have worked with this type of compound have offered a variety

of interpretations of their findings. Just as with Rottenberg's (1983) study with anesthetics, however, we wish to stress the probability, discussed in more detail elsewhere (Kell and Hitchens, 1982), that this type of compound is interfering with the operation of the protoneural proteins, an interpretation greatly strengthened by work with an azido derivative of ethidium bromide (Higuti *et al.*, 1981) demonstrating specific labeling of certain hydrophobic proteins in the inner mitochondrial membrane. Unfortunately, however, the protein(s) identified by Higuti *et al.* (1981), which are distinct from the so-called DCCD-binding protein, have not yet been further characterized, although it may be mentioned that the apparent molecular weight given in the paper of Higuti *et al.* (1981) does not correspond to those putatively encoded by the URFs in the bovine mitochondrial genome (Anderson *et al.*, 1982).

D. Conclusions and Summary of Uncoupling Mechanisms

The more classical protonophorous types of uncouplers seem to require transient interactions with, *inter alia*, proteins in or near the F_o portion of the H^+-ATP synthase in energy-coupling membranes. Such interactions are required in some localized coupling theories, which are thus consistent with the available data. A variety of *nonprotonophorus* compounds also "uncouple" in some way; most workers believe that they interfere with the transfer of electron transport-derived free energy to the proton motive sinks, but that they cannot do so by affecting $\Delta\tilde{\mu}_{H^+}$, and they are not energy-transfer inhibitors *sensu stricto*. They could function by inhibiting the conformational transitions of the protoneural proteins which may be required in energy coupling.

IX. CONTROL THEORETICAL VIEW OF ENERGY COUPLING

Presently there is a revival of interest (see e.g., Groen *et al.*, 1982; Porteous, 1983; Tager *et al.*, 1983; Westerhoff, 1983; Westerhoff *et al.*, 1983b, 1984a; Westerhoff and Van Dam, 1985) in the metabolic control theories of Kacser and Burns (1973) and Heinrich and Rapoport (1974). One important application is the analysis of which enzymes control mitochondrial respiration. Groen and colleagues (1982b) demonstrated that this process is not controlled by one enzyme alone, but by at least three of the participating enzymes. Moreover, the control (coefficient) (or flux control) of respiration by, for instance, the adenine nucleotide translocase varied with the work load imposed on the mitochondria.

A crucial aspect of this approach is that the flux control coefficient is a mathematically defined, but experimentally readily determined, parameter indicating the percentage of the control on a metabolic flux exercised by a

certain enzyme (for review, see Westerhoff *et al.*, 1984a). One operational definition of this parameter is the percentage by which the flux is reduced upon a 1% inhibition of that enzyme. Importantly, the sum of the flux control (coefficient)(s) by all the enzymes exercised on a given flux, must, in this theory, equal one. As a consequence, flux control is shared among enzymes, and enzymes compete for flux control. This competition is the basis for the principle that when one enzyme in a system becomes rate-limiting [i.e., exercises a flux control (coefficient) close to one] the other enzymes must sacrifice their share of the flux control. Since partial inhibition of an enzyme usually increases the flux control (coefficient) by that enzyme, such inhibition is expected to reduce the flux control (coefficient) by the other enzymes in the system and thus the titers of inhibitors of those other enzymes. It is the latter principle that underlies the predictions of the results of dual inhibitor titrations of free energy transduction by the hypothesis of delocalized chemiosmosis [see Section IV,E and Fig. 5b(ii)].

Here we reach a point of special significance to the present volume. With respect to dual inhibitor titrations, the predictions of the mosaic protonic coupling hypothesis are at variance with those of delocalized chemiosmotic coupling and, as we have just seen, also with the predictions of control theory. Moreover, the experimental results are in line with the predictions of the hypothesis of mosaic protonic coupling (Section IV,E).

Control theory models metabolic systems exclusively as consisting of enzymes that are mutually independent, except through their interactions with common metabolites, which form pools. The pool property of the metabolites implies that a metabolite molecule is not confined to react with one individual enzyme molecule, but can react with all enzymes in the system with a given catalytic specification (e.g., an ATP molecule can react with all the H^+-ATPase molecules present in the system). In such a system, a 1% increase in the activities of all the enzymes will lead to unaltered concentrations of all metabolites and, consequently, to a steady state that is identical to the original one, except that all fluxes will have increased by the same 1%. Consequently, the sum of all flux control coefficients in such a system is equal to one (as already stated above).

A mosaic coupling system does not conform to this modeling of a metabolic system: each energized proton can only react with one H^+-ATPase molecule. Consequently, one may wonder whether the (summation) theorem that the sum of the flux control coefficients must equal one should hold in mosaic systems (Groen *et al.*, 1982; Kell and Hitchens, 1983). In fact we can easily show that this law is not valid in a mosaic coupling system: from Eq. (3) it follows that a 1% inhibition of the electron-transfer chains or 1% inhibition of the H^+-ATPase *both* lead to a 1% inhibition of ATP synthesis, that is, both

enzymes exercise a flux control (coefficient) of one, so that the sum of the flux control (coefficient)s is close to two, rather than to the one following from control theory. [It may be noted that this (in contrast to the case treated in Westerhoff and Arents, 1984) is not due to the presence of a leak with a control coefficient of minus one.]

In principle, an assessment of whether the magnitude of the sum of the flux control coefficients exceeds one can constitute an important criterion for localized, or mosaic, versus delocalized free-energy transduction or metabolism. Promising as it seems, this approach has not yet been taken by many authors. It may, however, be noted that in mitochondrial oxidative phosphorylation there was in fact a tendency for the total flux control to exceed one (Groen *et al.*, 1982b). A similar, though different, example is found in the work of Reddy and Pardee (1983), in which a variety of specific inhibitors of enzymes isolated from an *E. coli* replicase complex acted additionally to inhibit other enzymes in the replicase complex.

From the above it will be clear that if localization effects are indeed present (Welch, 1977; Clegg, 1983a,b) in metabolism, they require thoughtful application of the metabolic control theories (cf. Kell and Hitchens, 1983; Westerhoff *et al.*, 1983b): if two enzymes are operating as a coupling unit, then their flux control (coefficients) should be averaged rather than summed when summing all flux control coefficients in the system, if one wishes to establish a total flux control of one.

The implications of localization effects for the control of fluxes in biological systems are extremely important: rather than having to share the control, so that each enzyme can only control any flux to a small extent, the enzymes in a completely mosaic system all have a complete control (i.e., exercise a flux control coefficient of one) over their "own" flux (cf. Williams, 1978b; Westerhoff *et al.*, 1983b). If, for instance in a bacterium, the transport rate of a given substance is increased, because the concentration of that substance in the external medium increases, then the use of free energy for this transport process would, in the case of delocalized chemiosmotic coupling, lead to an inhibition of all the other free-energy requiring process, because $\Delta\tilde{\mu}_{H^+}$ would drop. In the case of mosaic protonic coupling, the other free-energy-requiring processes would not necessarily be affected.

From a biotechnological standpoint, however, the metabolic control theories make one important point: if one wishes to maximize the flux through a metabolic pathway, there may be *more than one* bottleneck (rate-limiting step), and strategies to enhance productivity, even if semiempirical, should be directed at a variety of control points. A good example is given in a recent discussion of strain improvements during the development of commercial lysine fermentations (Tosaka *et al.*, 1983).

X. ON THE "ORGANIZATION" OF ENERGY-COUPLING MEMBRANES AND OTHER ORGANIZED MULTIENZYME SYSTEMS

> There is an epoch in the growth of a science during which facts accumulate faster than theories can accommodate them.
>
> Medawar (1982)

A. Introduction

The foregoing, rather extensive survey of current knowledge of electron-transport phosphorylation has, we regret, been somewhat iconoclastic in nature. This is due, of course, to the still widespread belief in the simplest, delocalized chemiosmotic scheme that we set up as a straw man at the beginning of our survey. Those readers who are still with us may justifiably wonder, therefore, where we can go next: obviously, we must endeavor to provide some kind of more explicit model that accounts for the data we have thus far discussed.

As foreshadowed in Section VI, we find it necessary to modify the delocalized chemiosmotic view in the sense that, while we retain the proton pumps, their mutual activity is coupled via protons (in concert with some kind of membranous phonon wave) that do not equilibrate with the proton electrochemical potential in the bulk phases to which the given faces of the coupling membrane are adjacent. We have sought to adduce evidence that there exist, in these coupling membranes, proteinaceous devices whose role is to transfer the protonic free energy along the plane of the membrane, although we are not yet in a position to speculate explicitly upon their exact biophysical mechanism. Let us remind ourselves of the salient structural features of these energy-coupling membranes.

The key feature is that, although these membranes are organized as fluid mosaics, there is now a significant amount of evidence that the disposition of proteins involved in free-energy transduction, although they are isolatable as individual complexes, is such that the lateral movement of these complexes is not easily viewed as occurring by free diffusion under conditions approximating those *in vivo*.

We have seen that even a single turnover of the primary, redox-linked proton pumps causes an initial, localized charge separation across the plane of the membrane. The energetic magnitude of this field may be calculated from simple electrostatic considerations (Malpress, 1981a,b) (Section VI) and turns out to be more than adequate to permit ATP synthesis given reasonable assumptions about the magnitude of the "local" permittivity. Such a static field will affect the conformational state of any dipoles to which it is adjacent, the change in free energy being dependent, *inter alia*, upon the dipole moment of

the induced protein in a very simple fashion (Schwarz, 1978a,b). Since typical energy-transducing membrane proteins have very large dipole moments, typically in excess of 400 D (e.g., Petersen and Cone, 1975; Keszthelyi, 1980; Todorov *et al.*, 1982; Tsuji and Neumann, 1983), it is easy to see how localized fields could transfer free energy directly between spatially adjunct protein complexes. However, life is not so simple; let us consider bacteriorhodopsin, with which three of these studies were concerned, in more detail.

It is assumed, in the chemiosmotic coupling model, that the steady-state proton electrochemical potential difference across a working proton pump itself is as great as that between the two bulk phases to which the surfaces of the proton pump are adjacent. In the case of isolated bacteriorhodopsin sheets, these two aqueous phases are one. Two very interesting studies measured the average surface potential of such sheets (Carmeli *et al.*, 1980; Tokutomi *et al.*, 1980) using a spin-labeled probe. As expected, the surface potential measured with this method varied, in the dark, in exact accordance with the requirements of Gouy–Chapman theory, as the ionic strength was varied. However, the *light-dependent changes* in surface potential were *ionic strength independent*, under macroscopically stationary-state conditions. This indicates that free energy is stored in the protein under illuminated conditions (Kell and Griffiths, 1981) and is thus not simply dissipated when the protein is taken from the otherwise insulating membrane in which it is normally embedded. Thus, during the photocycle, as elaborated in more detail elsewhere, this "energized" protein will have the effect of altering the standard chemical potential of any ligand with which it happens to interact (see Welch and Kell, 1985; Somogyi *et al.*, 1984). Welch and Berry (1983 and this volume) have considered the consequences of such behavior for modeling the energy metabolism in cells.

Consider for a moment a protein isolated in a heat bath, in thermal equilibrium with its surroundings. Such a protein might be an isolated bacteriorhodopsin sheet. The addition of energy to this system, let us say, for bacteriorhodopsin, by absorption of a photon of wavelength 570 nm, evidently leads to some kind of nonequilibrium state of the protein system, which may then relax "forward" to the ground state via a catalytic cycle (see e.g., Westerhoff, 1983; Westerhoff and Dancshazy, 1984). In bacteriorhodopsin, the half-time for this relaxation is of the order of a few milliseconds, although certain treatments may prolong this for as much as 30 s. Nevertheless (Fig. 1), as discussed, for instance, by Careri *et al.* (1979), many relaxational modes, such as H^+ transfer and thermal exchange with bound and free solvent molecules, take place on a time scale of nanoseconds to microseconds. How, then, can an energized protein escape a much more rapid relaxation to its ground state via a back-reaction? Obviously, one possible mechanism is that of a cascade of chemical reactions with rate constants of 100/s and greater. An

indication for this is the occurrence of a succession of different spectral forms of photoexcited bacteriorhodopsin (Lozier *et al.*, 1975). It should be noted, of course, that visible spectroscopy probes only the environment of the chromophoric retinal group itself, while knowledge of the conformational state(s) of the entire system is required for true thermokinetic analyses. It cannot therefore be excluded that the actual mechanism is much less classical than this.

A number of workers (see e.g., Del Giudice *et al.*, 1982; Blumenfeld, 1983; Scott, 1983; Davydov, 1983, and references therein) have pointed out that there exist a variety of methods of transferring such free energy by the formation and utilization of solitary excitations, which exhibit greatly increased lifetimes and a correspondingly increased ability to transport free energy over biologically significant distances. The formation of such solitary waves is absolutely dependent upon the nonlinear character of interatomic forces between different parts of the system of interest. In this regard, we would draw attention to a long-standing general model of Fröhlich (see Fröhlich, 1980, for a review) concerning the possibility of long-distance energy transport (coherent excitations) in biology and dependent upon appropriate nonlinear interactions between biomembrane proteins and their surrounding heat bath.

In particular, the considerations of Fröhlich (1980, and references therein) offer us an escape route from the dilemma of why an energized charged membrane protein may not fulfil the requirements of Gouy–Chapman theory. Fröhlich considered the relationship between the probable velocity of sound (phonons) in a membrane protein ($\sim$1000 m/s) and a typical membrane thickness of 10^{-8} m, concluding that interactions between the electrical and acoustic modes of such a charged, transmembrane protein might have a frequency of the order of 10^{11} Hz. At these frequencies, electrostatic screening is of course impossible. Thus, in contrast to what might be expected from static considerations alone, these "unexpected" findings are perfectly compatible with well-known physical laws.

Thus, as Scott (1983) and others have noted, one should expect to see important changes upon energization in the spectra of proteins (such as bacteriorhodopsin) in the range 10^{11} to 2×10^{12} Hz. It is probably also worth remarking that the conceptual subdivision between (a) the activities of the *primary* proton pump(s) and (b) the subsequent long-range transfer of free energy along the coupling membrane bear striking analogies to (i) the initial and (ii) later processes discussed in the generation of ferroelectric states in this context by Bilz *et al.* (1981). As discussed elsewhere (Kell and Hitchens, 1983), this type of general model is fully consistent with the data presently available on the systems catalyzing electron-transport phosphorylation. However, we will not pursue these issues further here. Our purpose is thus only to point out

the possibility of the excitation of nonlinear transitions in vibrational modes of membrane proteins during proton motive activity. We may therefore, in contrast to our earlier, iconoclastic survey, allow ourselves a rather more optimistic conclusion: that the recognition of these possibilities may usher in a new and exciting phase of research in membrane bioenergetics.

B. Biotechnological Considerations

The foregoing discussion has concentrated on what we may refer to as "pure scientific" aspects of membrane bioenergetics. In the spirit of this volume, however, we wish finally to mention some consequences of the behavior of such membranous systems for work with immobilized cells and artificial enzyme membranes.

The most striking consequence of metabolic microcompartmentation may be stated as follows. Imagine a permeabilized, immobilized microbial cell catalyzing a multistep reaction such as glycolysis. The volume taken up by the enzymes in relation to the total volume is negligible. Thus, if each intermediate is allowed to become "free," so that its effective activity approximates the number of molecules divided by the total volume of the system, the transit time of the system will be enormous, and the number of substrate molecules existing as intermediates, rather than products, might form a substantial fraction of the whole. However, if the enzymes are arranged so that intermediate molecules remain bound to the multienzyme complexes, this number will be equal only to the number of enzymic active sites present. Evidently, the latter situation is far more satisfactory from a biotechnological standpoint, and an understanding of this type of behavior *in vivo* is of great importance to the design of synthetic and analytical systems based upon immobilized biocatalysts. Kasche (1983) has reviewed the consonance between the behavior of natural and artificial systems in the diffusion-limited regime and found it excellent, and the modulation of immobilized enzyme activities by microenvironmental effects is discussed by several other authors in this volume. The improved control structures possible in mosaic systems have been discussed in Section IX. From the standpoint of the role of cellular membranes in biotechnology, it is worth drawing attention to their role in slowing the egress of products, both of low and high molecular weight. A classic example of this occurs in the glutamic acid fermentation catalyzed by *Cornyebacterium glutamicum*; growth of this organism under biotin-limited conditions markedly enhances the membrane permeability of this organism and glutamate productivity of this fermentation (see e.g., Dulaney, 1967). The means by which this organism catalyzes oxidative phosphorylation under these membrane-leaky conditions does not, however, seem to be a point of discussion in the biotechnological literature.

Our final purpose is therefore to consider one neglected possible consequence of the coherent behavior of cellular metabolism and multienzyme aggregates.

C. Thermophilic Microorganisms

The idea explicit in the use of the term *coherent excitations* in the previous section is that the motions of particles in one part of a system are dependent upon the motions of particles in other, spatially separate parts of the system, so that there is thus a functional linkage between the two.

Now, as pointed out, for instance, by Klibanov (1983), by far the most important means of enzyme inactivation is thermal, and Lapange (1978), Mozhaev and Martinek (1982), and Sonnleitner and Fiechter (1983) have given useful reviews of the thermally based routes of enzyme denaturation/inactivation. Thus, a great many workers have sought to exploit the greater thermostability shown by most, *though not all*, enzymes isolated from thermophilic microorganisms in biotechnological applications. From a bioenergetic standpoint, an excellent example is provided by Kagawa's studies on the H^+-ATPase synthase of the thermophilic *Bacillus* PS3 (see, e.g., Kagawa, 1979).

Nevertheless, a consideration of the literature on thermophily (see, e.g., Amelunxen and Murdock, 1977, 1978; Kushner, 1978; Shilo, 1978; Friedman, 1978; Zeikus, 1979) reveals that although a variety of modifications in the structure of isolated macromolecules have been exploited by thermophiles, none offers an easy explanation for the remarkable (Baross and Deming, 1983) thermostability exhibited *in vivo*. Qualitatively, it is obvious that the organization of enzymes into multienzyme aggregates, involving the formation of extra noncovalent bonds, can in principle greatly increase the thermostability of the individual enzymes, provided that the enzymes in the complexes act as mutual thermal buffers. Thus, it would be predicted from such considerations that the thermostability of the enzymes and other macromolecules in thermophiles rests additionally upon the organization of the *milieu intérieur* of such organisms. How might one seek to test such a notion? It has been known for many years (e.g., Pauly and Schwan, 1966; Marquis and Carstensen, 1973) that the internal electrical conductivity of a variety of cells is significantly less, usually between one-third and one-half, of that expected (and found, upon cell disruption) on the basis of the ionic content of the cells. We may refer to this finding as the conductance deficit. It seems, then, to be a logical corollary of the foregoing considerations that the conductance deficit should be greater in thermophiles than in comparable mesophiles if the functional linkage between their enzymes is greater than that expected on the basis that all are freely diffusing in the membranes or

cytoplasm of such cells. Unfortunately, the relevant experiments to test this conception do not yet appear to have been performed.

D. Concluding Remarks

> One could, without too much exaggeration, say of review articles what Mark Twain is supposed to have said about the weather—everybody talks about it but nobody *does* anything about it.
>
> Garfield (1977)

The evidence for catalytic facilitation through organizing single enzymes into functional multienzyme aggregates is now very great, as discussed by other contributors to this volume. We hope that we have been able to show that the same holds true for enzymes catalyzing free energy transduction in biomembranes. While we have largely confined ourselves to the process of electron-transport phosphorylation, the interpretations we have given may be taken to apply to a variety of other processes occurring in energy-coupling membranes. The consequences of this for an integrated understanding of cellular metabolism and bioenergetics will provide a most interesting phase in the evolution of our understanding of the behavior of such systems.

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REFERENCES

Amelunxen, R. E., and Murdock, A. L. (1977). *Dev. Ind. Microbiol.* **18,** 233–248.
Amelunxen, R. E., and Murdock, A. L. (1978). *CRC Crit. Rev. Microbiol.* **6,** 343–393.
Anderson, J. M. (1975). *Biochim. Biophys. Acta* **416,** 191–235.
Anderson, J. M. (1981). *FEBS Lett.* **124,** 1–10.
Anderson, J. M., and Andersson, B. (1982). *Trends Biochem. Sci.* **7,** 288–292.
Andersen, O. S., and Fuchs, M. (1975). *Biophys. J.* **15,** 795–830.
Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981). *Nature (London)* **290,** 457–470.
Anderson, S., de Bruijn, M. H. L., Coulson, A. R., Eperon, I. C., Sanger, F., and Young, I. G. (1982). *J. Mol. Biol.* **156,** 683–717.

Anderson, W. M., Fowler, W. T., Pennington, R. M., and Fisher, R. R. (1981). *J. Biol. Chem.* **256,** 1888–1895.
Andersson, B., and Haehnel, W. (1982). *FEBS Lett.* **146,** 13–17.
Archbold, G. L., Farrington, C. L., Gill, B. J. O., and Malpress, F. H. (1974). *Biochem. Soc. Trans.* **2,** 751–754.
Archbold, G. P. R., Farrington, C. L., Lappin, S. A., McKay, A. M., and Malpress, F. H. (1979). *Biochem. J.* **180,** 161–174.
Arntzen, C. J. (1975). *In* "Bioenergetics of Photosynthesis" (Govindjee, ed.), pp. 51–113. Academic Press, New York.
Arntzen, C. J. (1978). *Curr. Top. Bioenerg.* **8,** 111–160.
Arvidson, G., Lindblom, G., and Drakenberg, T. (1975). *FEBS Lett.* **54,** 249–252.
Attardi, G., Cantatore, P., Chomyn, A., Crews, S., Gelfand, R., Merkel, C., Montoya, J., and Ojala, D. (1982). *In* "Mitochondrial Genes" (P. Slonimski, P. Borst, and G. Attardi, eds.), pp. 51–71. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Axelrod, D. (1983). *J. Membr. Biol.* **75,** 1–10.
Azzi, A. (1980). *Biochim. Biophys. Acta* **594,** 231–252.
Azzone, G. F., Massari, S., and Pozzan, T. (1977). *Mol. Cell. Biochem.* **17,** 101–112.
Azzone, G. F., Pozzan, T., and Massari, S. (1978). *Biochim. Biophys. Acta* **501,** 307–316.
Azzone, G. F., Pietrobon, D., and Zoratti, M. (1984). *Curr. Top. Bioenerg.* **14,** 1–77.
Baccarini-Melandri, A., Casadio, R., and Melandri, B. A. (1977). *Eur. J. Biochem.* **78,** 389–402.
Baccarini-Melandri, A., Casadio, R., and Melandri, B. A. (1981). *Curr. Top. Bioenerg.* **12,** 197–258.
Bachmann, B. J. (1983). *Microbiol. Rev.* **47,** 180–230.
Baird, B. A., and Hammes, G. G. (1979). *Biochim. Biophys. Acta* **549,** 31–53.
Bangham, A. D. (1968). *Prog. Biophys. Mol. Biol.* **18,** 29–95.
Bangham, A. D. (1972). *Annu. Rev. Biochem.* **41,** 753–776.
Barber, J. (1980). *FEBS Lett.* **118,** 1–10.
Barber, J. (1982a). *Biosci. Rep.* **2,** 1–13.
Barber, J. (1982b). *Annu. Rev. Plant Physiol.* **33,** 261–295.
Barber, J. (1983). *Plant Cell Environ.* **6,** 311–322.
Baross, J. A., and Deming, J. W. (1983). *Nature (London)* **303,** 423–426.
Baum, H. (1978). *In* "The Molecular Biology of Membranes" (S. Fleischer, Y. Hatefi, D. H. MacLennan, and A. Tzagoloff, eds.), pp. 243–262. Plenum, New York.
Baum, H., Hall, G. S., Nalder, J., and Beechey, R. B. (1971). *In* "Energy Transduction in Respiration and Photosynthesis" (E. Quagliariello, S. Papa, and C. S. Rossi, eds.), pp. 747–755. Adriatica Editrice, Bari, Italy.
Bendich, A. J. (1982). *In* "Mitochondrial Genes" (P. Slonimski, P. Borst, and G. Attardi, eds.), p. 477. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Benz, R., and McLaughlin, S. (1983). *Biophys. J.* **41,** 381–398.
Benz, R., and Zimmermann, U. (1981). *Biochim. Biophys. Acta* **640,** 169–178.
Berden, J. A., and Hennecke, M. A. C. (1981). *FEBS Lett.* **126,** 211–214.
Berden, J. A., Hennecke, M. A. C., van der Bend, R. L., and van Dam, K. (1981). *In* "Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria" (F. Palmieri, E. Quagliariello, N. Siliprandi, and E. C. Slater, eds.), pp. 209–212. Elsevier, Amsterdam.
Bibb, M. J., van Etten, R. A., Wright, C. T., Walberg, M. W., and Clayton, D. A. (1981). *Cell* **26,** 167–180.
Bilz, H., Büttner, H., and Fröhlich, H. (1981). *Z. Naturforsch.* **36B,** 208–212.
Blaurock, A. E. (1982). *Biochim. Biophys. Acta* **650,** 167–207.
Blobel, G. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1496–1500.
Blumenfeld, L. A. (1983). "Physics of Bioenergetic Processes." Springer-Verlag, Berlin and New York.

Blumenthal, R., and Klausner, R. D. (1982). *In* "Membrane Reconstitution" (G. Poste and G. L. Nicolson, eds.), pp. 43–82. Elsevier, Amsterdam.
Boork, J. and Baltscheffsky, M. (1982). *EBEC Rep.* **2**, 365.
Booth, I. R., and Kroll, R. G. (1983). *Biochem. Soc. Trans.* **11**, 70–72.
Booth, I. R., Kroll, R. G., Findlay, M. G., Stewart, L. M. D., and Rowland, G. C. (1984). *Biochem. Soc. Trans.* **12**, 409–411.
Borst, P., and Grivell, L. A. (1981). *Nature (London)* **290**, 443–444.
Bowman, W. C., Rand, M. J., and West, G. B. (1969). "Textbook of Pharmacology." Blackwell, Oxford.
Brewer, G. J. (1976). *Biochemistry* **15**, 1387–1392.
Brock, C. J., and Tanner, M. J. A. (1982). *In* "Biological Membranes" (D. Chapman, ed.), Vol. 4, pp. 75–130. Academic Press, New York.
Brown, M. F., Ribeiro, A. A., and Williams, G. D. (1983). *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4325–4329.
Brunori, M., and Wilson, M. T. (1982). *Trends Biochem. Sci.* **7**, 295–299.
Büchel, K. H., and Draber, W. (1972). *ACS Adv. Chem. Ser.* **114**, 141–154.
Buetow, D. E., and Gilbert, C. W. (1982). *In* "Cell Function and Differentiation, Part B" (G. Akoyunoglou, A. E. Evangelopoulous, J. Georgatsos, G. Palaiologos, A. Trakatellis, and C. P. Tsiganos, eds.), pp. 139–148. Liss, New York.
Capaldi, R. A. (1982a). *Trends Biochem. Sci.* **7**, 292–295.
Capaldi, R. A. (1982b). *Biochim. Biophys. Acta* **694**, 291–306.
Capaldi, R. A., Komai, H., and Hunter, D. R. (1973). *Biochem. Biophys. Res. Commun.* **55**, 655–659.
Careri, G., Fasella, P., and Gratton, E. (1979). *Annu. Rev. Biophys. Bioeng.* **8**, 69–97.
Carmeli, C., Quintanilha, A. T., and Packer, L. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4707–4711.
Casadio, R., Baccarini-Melandri, A., and Melandri, B. A. (1978). *FEBS Lett.* **87**, 323–328.
Casadio, R., Venturoli, G., and Melandri, B. A. (1982). *EBEC Rep.* **2**, 185–186.
Casey, R. P., Thelen, M., and Azzi, A. (1980). *J. Biol. Chem.* **255**, 3994–4000.
Chapman, D., Gómez-Fernández, J. C., and Goni, F. M. (1982). *Trends Biochem. Sci.* **7**, 67–70.
Cherry, R. J. (1979). *Biochim. Biophys. Acta* **559**, 289–327.
Ching, E., and Attardi, G. (1982). *Biochemistry* **21**, 3188–3195.
Clarke, D. J., Morley, C. D., Kell, D. B., and Morris, J. G. (1982). *Eur. J. Biochem.* **127**, 105–116.
Clegg, J. S. (1983a). *In* "Coherent Excitations in Biological Systems" (H. Fröhlich and F. Kremer, eds.), pp. 162–177. Springer-Verlag, Berlin and New York.
Clegg, J. S. (1983b). *Am. J. Physiol.* **246**, R133–R151.
Clementi, E., and Sarma, R. H., eds. (1983). "Structure and Dynamics: Nucleic Acids and Proteins." Adenine, Guilderland, New York.
Cleveland, M. v. B., Slatin, S. Finkelstein, A., and Levinthal, C. (1983). *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3706–3710.
Cogdell, R. J., and Crofts, A. R. (1974). *Biochim. Biophys. Acta* **347**, 264–272.
Cohen, F. S., Eisenberg, M., and McLaughlin, S. (1977). *J. Membr. Biol.* **37**, 361–396.
Conover, T. E., and Azzone, G. F. (1981). *In* "Mitochondria and Microsomes" (C.-P. Lee, G. Schatz, and G. Dallner, eds.), pp. 481–518. Addison-Wesley, Reading, Massachusetts.
Cotton, N. P. J., and Jackson, J. B. (1983). *FEBS Lett.* **161**, 93–99.
Cotton, N. P. J., Clark, A. J., and Jackson, J. B. (1981). *Arch. Microbiol.* **129**, 94–99.
Cramer, W. A., Dankert, J. R., and Uratani, Y. (1983). *Biochim. Biophys. Acta* **737**, 173–193.
Criddle, R. S., Bock, R. M., Green, D. E., and Tisdale, H. (1962). *Biochemistry* **1**, 827–842.
Crilly, J. F., and Earnshaw, J. C. (1983). *Biophys. J.* **41**, 197–210.
Crofts, A. R., and Wood, P. M. (1978). *Curr. Top. Bioenerg.* **7**, 175–244.
Cullis, P. R., and de Kruijff, B. (1979). *Biochim. Biophys. Acta* **559**, 399–420.

Cullis, P. R., de Kruijff, B., Hope, M. J., Nayar, R., Rietveld, A., and Verleij, A. J. (1980). *Biochim. Biophys. Acta* **600**, 625–635.
Dandeu, J. P., Billault, A., and Barbu, E. (1969). *C. R. Hebd. Séances Acad. Sci.* **269**, 2044–2047.
Darwin, C. (1903). *In* "More Letters" (F. Darwin and A. C. Seward, eds.). Murray, London.
Davenport, J. W., and McCarty, R. E. (1980). *Biochim. Biophys. Acta* **589**, 353–357.
Davies, R. W., Scazzocchio, C., Waring, R. B., Lee, S., Grisi, E., McPhail Berks, M., and Brown, T. A. (1982). *In* "Mitochondrial Genes" (P. Slonimski, P. Borst, and G. Attardi, eds.), pp. 405–410. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Davis, J. H. (1983). *Biochim. Biophys. Acta* **737**, 117–171.
Davydov, A. S. (1983). *In* "Structure and Dynamics: Proteins and Nucleic Acids" (E. Clementi and R. H. Sarma, eds.), pp. 377–387. Adenine, Guilderland, New York.
Decker, S. J., and Lang, D. R. (1977). *J. Biol. Chem.* **252**, 5936–5938.
Decker, S. J., and Lang, D. R. (1978). *J. Biol. Chem.* **253**, 6738–6743.
de Kouchovsky, Y., and Haraux, F. (1981). *Biochem. Biophys. Res. Commun.* **99**, 205–212.
de Kouchovsky, Y., Haraux, F., and Sigalat, C. (1982). *FEBS Lett.* **139**, 245–249.
de Kouchovsky, Y., Haraux, F., and Sigalat, C. (1983). *Bioelectrochem. Bioenerg.* **11** (in press).
de Kruijff, B., Nayar, R., and Cullis, P. F. (1982). *Biochim. Biophys. Acta* **684**, 47–52.
Delalic, Z., Takashima, S., Adachi, K., and Asakura, T. (1983). *J. Mol. Biol.* **168**, 659–671.
Del Giudice, E., Doglia, S., and Milani, M. (1982). *Phys. Scr.* **26**, 232–238.
De Pierre, J. W., and Ernster, L. (1977). *Annu. Rev. Biochem.* **46**, 201–262.
Dierstein, R., Schumacher, A., and Drews, G. (1981). *Arch. Microbiol.* **128**, 376–383.
Dilger, J., and McLaughlin, S. (1979). *J. Membr. Biol.* **46**, 359–384.
Dilger, J. P., McLaughlin, S. G. A., McIntosh, T. A., and Simon, S. A. (1979). *Science* **206**, 1196–1198.
Dilley, R. A., Prochaska, L. J., Baker, G. M., Tandy, N. E., and Millner, P. A. (1982). *Curr. Top. Membr. Trans.* **16**, 345–369.
Dixon, L. K., Leaver, C. J., Brettell, R. I. S., and Gengenbach, B. G. (1982). *Theor. Appl. Genet.* **63**, 75–80.
Drews, G. (1982). *In* "From Cyclotrons to Cytochromes" (N. O. Kaplan and A. Robinson, eds.), pp. 355–366. Academic Press, New York.
Drosdat, H., Kiehl, R., Hoffman-Posorske, E., Kordt, S., and Hanstein, W. G. (1982). *EBEC Rep.* **2**, 19–20.
Dulaney, E. L. (1967). *In* "Microbial Technology" (H. J. Peppler, ed.), pp. 308–343. Academic Press, New York.
Dutton, P. L., and Prince, R. C. (1978). *In* "The Photosynthetic Bacteria" (R. K. Clayton and W. R. Sistrom, eds.), pp. 525–570. Plenum, New York.
Edidin, M. (1974). *Symp. Soc. Exp. Biol.* **28**, 1–14.
Edwards, C. (1981). "The Microbial Cell Cycle." Nelson, London.
Edwardson, J. R. (1970). *Bot. Rev.* **36**, 341–420.
Engleman, D. M., Henderson, R., McLachlan, A. D., and Wallace, B. A. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2023–2027.
Ernster, L., and Nordenbrand, K. (1974). *BBA Libr.* **13**, 283–288.
Etemadi, A. H. (1980). *Biochim. Biophys. Acta* **604**, 347–422, 423–475.
Evans, E. A., and Hochmuth, R. M. (1978). *Curr. Top. Membr. Trans.* **10**, 1–64.
Feingold, D. S. (1970). *J. Membr. Biol.* **3**, 372–386.
Felle, H., Stetson, D. L., Long, W. S., and Slayman, C. L. (1978). *In* "Frontiers of Biological Energetics" (P. L. Dutton, J. S. Leigh, and A. Scarpa, eds.), Vol. 2, pp. 1399–1407. Academic Press, New York.
Felle, H., Porter, J. S., Slayman, C. L., and Kaback, H. R. (1980). *Biochemistry* **19**, 3585–3590.
Ferguson, S. J., and Sorgato, M. C. (1982). *Annu. Rev. Biochem.* **51**, 185–217.

Ferguson, S. J., John, P., Lloyd, W. J., Radda, G. K., and Whatley, F. R. (1976). *FEBS Lett.* **62,** 272–275.
Ferguson, S. J., Jones, O. T. G., Kell, D. B., and Sorgato, M. C. (1979). *Biochem. J.* **180,** 75–85.
Fernandez, J. M., Bezanilla, F., and Taylor, R. E. (1982). *Nature (London)* **297,** 150–152.
Fields, K. L., and Luria, S. E. (1969a). *J. Bacteriol.* **97,** 64–77.
Fields, K. L., and Luria, S. E. (1969b). *J. Bacteriol.* **97,** 57–63.
Fillingame, R. H. (1980). *Annu. Rev. Biochem.* **49,** 1079–1113.
Finean, J. B., Coleman, R., and Michell, R. H. (1978). "Membranes and Their Cellular Functions," 2nd Ed. Blackwell, Oxford.
Fleischer, S., Fleischer, B., and Stoeckenins, W. (1967). *J. Cell Biol.* **32,** 193–208.
Forde, B. G., and Leaver, C. J. (1980). *In* "The Plant Genome" (D. R. Davies and D. A. Hopwood, eds.), pp. 131–146. John Innes Charity, Norwich.
Forde, B. G., Oliver, R. J. C., and Leaver, C. J. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 3841–3845.
Fraley, R. T., Jameson, D. M., and Kaplan, S. (1978). *Biochim. Biophys. Acta* **511,** 52–69.
Franks, N. P., and Lieb, W. R. (1978). *Nature (London)* **274,** 339–342.
Franks, N. P., and Lieb, W. R. (1979). *J. Mol. Biol.* **133,** 469–500.
Franks, N. P., and Lieb, W. R. (1981). *Nature (London)* **292,** 248–251.
Franks, N. P., and Lieb, W. R. (1984). *Nature (London)* **310,** 599–601.
Freeman, K. B., Yatscoff, R. W., and Mason, J. R. (1980). *In* "The Organisation and Expression of the Mitochondrial Genome" (A. M. Kroon and C. Saccone, eds.), pp. 343–346. Elsevier, Amsterdam.
Freeman, K. B., Yatscoff, R. W., Mason, J. R., Patel, H. V., and Buckle, M. (1983). *Eur. J. Biochem.* **134,** 215–222.
Friedman, S. M., ed. (1978). "Biochemistry of Thermophily." Academic Press, New York.
Fröhlich, H. (1980). *Adv. Electron. Electron Phys.* **53,** 85–152.
Fuller, S. D., Capaldi, R. A., and Henderson, R. (1979). *J. Mol. Biol.* **134,** 305–327.
Gabellini, N., Bowyer, J. R., Hurt, E., Melandri, B. A., and Hauska, G. (1982). *Eur. J. Biochem.* **126,** 105–111.
Galante, Y. M., Wong, S. Y., and Hatefi, Y. (1979). *J. Biol. Chem.* **254,** 12372–12378.
Garcia, A. F., Drews, G., and Reidl, H. H. (1981). *J. Bacteriol.* **145,** 1121–1128.
Garfield, E. (1977). *In* "Essays of an Information Scientist" (Vol. 2, pp. 170–171). Inst. for Scientific Information, Philadelphia, Pennsylvania.
Gennis, R. B., and Jonas, A. (1977). *Annu. Rev. Biophys. Bioeng.* **6,** 195–238.
Gibson, F. (1983). *Biochem. Soc. Trans.* **11,** 229–240.
Goodchild, D. J., Duniec, J. T., and Anderson, J. M. (1983). *FEBS Lett.* **154,** 243–246.
Gorringe, D. M., and Moses, V. (1980). *Int. J. Biol. Macromol.* **2,** 161–173.
Gould, J. M. (1979). *J. Bacteriol.* **138,** 176–184.
Gould, J. M., and Cramer, W. A. (1977a). *J. Biol. Chem.* **252,** 5491–5497.
Gould, J. M., and Cramer, W. A. (1977b). *J. Biol. Chem.* **252,** 5875–5882.
Graan, T., Flores, S., and Ort, D. R. (1981). *In* "Energy Coupling in Photosynthesis" (B. Selman and S. Selman-Reimer, eds.), pp. 25–34. Elsevier, Amsterdam.
Gräber, P. (1981). *Curr. Top. Membr. Trans.* **16,** 215–245.
Gräber, P., and Witt, H. T. (1976). *Biochim. Biophys. Acta* **423,** 141–163.
Gregory, P., Earle, E. D., and Gracen, V. E. (1977). *ACS Symp. Ser.* **62,** 90–114.
Gregory, P., Earle, E. D., and Gracen, V. E. (1980). *Plant Physiol.* **66,** 477–481.
Griffiths, D. E., Avner, P. R., Lancashire, W. E., and Turner, J. R. (1972). *In* "Biochemistry and Biophysics of Mitochondrial Membranes" (G. F. Azzone, E. Carafoli, A. L. Lehninger, E. Quagliariello, and N. Siliprandi, eds.), pp. 505–521. Academic Press, New York.
Griffiths, D. E., Houghton, R. L., and Lancashire, W. E. (1974). *In* "The Biogenesis of Mitochondria" (A. M. Kroon and C. Saccone, eds.), pp. 215–223. Academic Press, New York.

Groen, A. K., van der Meer, R., Westerhoff, H. V., Wanders, R. J. A., Akerboom, T. P. M., and Tager, J. M. (1982a). *In* "Metabolic Compartmentation" (H. Sies, ed.), pp. 9–37. Academic Press, New York.

Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., van der Meer, R., and Tager, J. M. (1982b). *J. Biol. Chem.* **257,** 2754–2758.

Guffanti, A. A., Susman, P., Blanco, R., and Krulwich, T. A. (1978). *J. Biol. Chem.* **253,** 708–715.

Guffanti, A. A., Blumenfeld, H., and Krulwich, T. A. (1981). *J. Biol. Chem.* **256,** 8418–8421.

Gurd, F. R. N., and Rothgeb, T. M. (1979). *Adv. Prot. Chem.* **33,** 73–165.

Gutman, M. (1980). *Biochim. Biophys. Acta* **594,** 53–84.

Gutman, M., Huppert, D., Pines, E., and Nachliel, E. (1981). *Biochim. Biophys. Acta* **642,** 15–26.

Gutman, M., Huppert, D., and Nachliel, E. (1982). *Eur. J. Biochem.* **121,** 637–642.

Gutman, M., Nachliel, E., Gershon, E., and Giniger, R. (1983). *Eur. J. Biochem.* **134,** 63–69.

Hackenbrock, C. R. (1972). *Ann. N.Y. Acad. Sci.* **195,** 492–505.

Hackenbrock, C. R. (1976). *In* "The Structure of Biological Membranes" (S. Abrahamsson and Pascher, eds.), pp. 199–234. Plenum, New York.

Hackenbrock, C. R. (1981). *Trends. Biochem. Sci.* **6,** 151–154.

Hackenbrock, C., Höchli, M., and Chau, R. M. (1976). *Biochim. Biophys. Acta* **455,** 466–484.

Haehnel, W. (1982). *Biochim. Biophys. Acta* **682,** 245–257.

Haines, T. H. (1979). *J. Theor. Biol.* **80,** 307–323.

Haines, T. H. (1982). *Biophys. J.* **37,** 147–148.

Halsey, J. J. (1974). *In* "Anesthetic Uptake and Action" (E. I. Eger, ed.), pp. 45–76. Williams & Wilkins, Baltimore, Maryland.

Hamamoto, T., Ohno, K., and Kagawa, Y. (1982). *J. Biochem.* **91,** 1759–1766.

Hangarter, R. P., and Good, N. E. (1982). *Biochim. Biophys. Acta* **681,** 397–404.

Hangarter, R. P., and Good, N. E. (1984). *Biochemistry* **23,** 122–130.

Hanstein, W. G. (1976a). *Biochim. Biophys. Acta* **456,** 129–148.

Hanstein, W. G. (1976b). *Trends Biochem. Sci.* **1,** 65–67.

Hanstein, W. G. (1978). *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 56, pp. 653–683. Academic Press, New York.

Hanstein, W. G., and Hatefi, Y. (1974a). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 288–292.

Hanstein, W. G., and Hatefi, Y. (1974b). *J. Biol. Chem.* **249,** 1356–1372.

Hanstein, W. G., and Kiehl, R. (1981). *Biochem. Biophys. Res. Commun.* **100,** 1118–1125.

Hanstein, W. G., Hatefi, Y., and Kiefer, H. (1979). *Biochemistry* **18,** 1019–1025.

Haraux, F., Sigalat, C., Moreau, A., and de Kouchovsky, Y. (1983). *FEBS Lett.* **155,** 248–252.

Harris, C. M., and Kell, D. B. (1983). *Bioelectrochem. Bioenerg.* **11,** 15–28.

Hatefi, Y. (1975). *J. Supramol. Struct.* **3,** 201–213.

Hatefi, Y. (1976). *In* "The Enzymes of Biological Membranes" (A. Martonosi, ed.), pp. 3–41. Plenum, New York.

Hauska, G., Samoray, D., Orlich, G., and Nelson, N. (1980). *Eur. J. Biochem.* **111,** 535–543.

Hauska, G., Hurt, E., Gabellini, N., and Lockau, W. (1983). *Biochim. Biophys. Acta* **726,** 97–133.

Heber, U., and Kirk, M. R. (1975). *Biochim. Biophys. Acta* **376,** 136–150.

Heinrich, R., and Rapoport, T. A. (1974). *Eur. J. Biochem.* **42,** 89–95, 97–102.

Heinz, E., Westerhoff, H. V., and van Dam, K. (1981). *Eur. J. Biochem.* **115,** 107–113.

Henderson, R., and Unwin, P. N. T. (1975). *Nature (London)* **257,** 28–32.

Henderson, R., Capaldi, R. A., and Leigh, J. S. (1977). *J. Mol. Biol.* **112,** 631–648.

Hengge, R., and Boos, W. (1983). *Biochim. Biophys. Acta* **737,** 443–478.

Heron, C., Ragan, C. I., and Trumpower, B. (1978). *Biochem. J.* **174,** 791–800.

Herweijer, M. A., Berden, J. A., and Kemp, A. (1984). *EBEC Rep.* **3,** 241–242.

Heytler, P. G. (1963). *Biochemistry* **2,** 357–361.

Heytler, P. G. (1979). *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 55, pp. 462–472. Academic Press, New York.
Higuti, T., Yokota, M., Arakaki, N., Hattori, A., and Tani, L. (1978). *Biochim. Biophys. Acta* **503**, 211–222.
Higuti, T., Arakaki, N., Niimi, S., Nakasima, S., Saito, R., Tani, L., and Ota, F. (1980). *J. Biol. Chem.* **255**, 7631–7636.
Higuti, T., Ohe, T., Arakaki, N., and Kotera, Y. (1981). *J. Biol. Chem.* **256**, 9855–9860.
Hill, T. L. (1977). "Free Energy Transduction in Biology." Academic Press, New York.
Hille, B. (1980). *Prog. Anesthesiol.* **2**, 1–5.
Hinkle, P. C., Tu, Y. L., and Kim, J. J. (1975). *In* "Molecular Aspects of Membrane Phenomena" (H. R. Kaback, H. Neurath, G. K. Radda, R. Schwyzer, and W. R. Wiley, eds.), pp. 222–232. Springer-Verlag, Berlin and New York.
Hirata, H. J., Fukui, S., and Ishikawa, S. (1969). *J. Biochem.* **65**, 843–847.
Hitchens, G. D., and Kell, D. B. (1982a). *Biochem. J.* **206**, 351–357.
Hitchens, G. D., and Kell, D. B. (1982b). *Biochem. Soc. Trans.* **10**, 261.
Hitchens, G. D., and Kell, D. B. (1982c). *Biosci. Rep.* **2**, 743–749.
Hitchens, G. D., and Kell, D. B. (1983a). *Biochem. J.* **212**, 25–30.
Hitchens, G. D., and Kell, D. B. (1983b). *Biochim. Biophys. Acta* **723**, 308–316.
Hitchens, G. D., and Kell, D. B. (1984). *Biochim. Biophys. Acta* **766**, 222–223.
Hitchens, G. D., Kell, D. B., and Morris, J. G. (1982). *J. Gen. Microbiol.* **128**, 2207–2209.
Höchli, M., and Hackenbrock, C. R. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1636–1640.
Höchli, M., and Hackenbrock, C. R. (1977). *J. Cell. Biol.* **72**, 278–291.
Holland, I. B. (1975). *Adv. Microbial. Physiol.* **12**, 55–139.
Hong, J.-S. (1977). *J. Biol. Chem.* **252**, 8582–8588.
Hong, J.-S., Haggerty, D. L., and Lieberman, M. A. (1977). *Antimicrob. Agents Chemother.* **11**, 881–887.
Hong, Y. Q., and Junge, W. (1983). *Biochim. Biophys. Acta* **722**, 197–208.
Houslay, M. D., and Stanley, K. K. (1982). "Dynamics of Biological Membranes." Wiley, New York.
Howard, J. C. (1981). *Nature (London)* **290**, 441–442.
Huang, H. W. (1973). *J. Theor. Biol.* **40**, 11–17.
Israelachvili, J. N., Marcelja, S., and Horn, R. G. (1980). *Q. Rev. Biophys.* **13**, 121–200.
Ito, M., and Ohnishi, Y. (1981). *FEBS Lett.* **136**, 225–230.
Ito, M., Ohnishi, Y., Itoh, S., and Nishimura, M. (1983). *J. Bacteriol.* **153**, 310–315.
Jackson, J. B., Venturoli, G., Baccarini-Melandri, A., and Melandri, B. A. (1981). *Biochim. Biophys. Acta* **636**, 1–8.
Jain, M. K. (1972). "The Biomolecular Lipid Membrane." Van Nostrand-Reinhold, Princeton, New Jersey.
Jaffe, L. (1977). *Nature (London)* **265**, 600–601.
Jagendorf, A. T., and Uribe, E. G. (1966). *Proc. Natl. Acad. Sci. U.S.A.* **55**, 170–177.
Jähnig, F. (1983). *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3691–3695.
Janoff, A. S., and Miller, K. W. (1982). *In* "Biological Membranes" (D. Chapman, ed.), Vol. 4, pp. 417–476. Academic Press, New York.
Jardetzky, O., and Roberts, G. C. K. (1981). "NMR in Molecular Biology," pp. 537–579. Academic Press, New York.
Ji, S. (1976). *J. Theor. Biol.* **59**, 319–330.
Johannsson, A., Keightley, C. A., Smith, G. A., Richards, C. R., Hesketh, T. R., and Metcalfe, J. C. (1981a). *J. Biol. Chem.* **256**, 1643–1650.
Johannsson, A., Smith, G. A., and Metcalfe, J. C. (1981b). *Biochim. Biophys. Acta* **641**, 416–421.
John, P., and Whatley, F. R. (1977). *Biochim. Biophys. Acta* **463**, 129–153.

Junge, W., and Jackson, J. B. (1982). *In* "Photosynthesis: Energy Conversion by Plants and Bacteria" (Govindjee, ed.), pp. 589–646. Academic Press, New York.
Kacser, H., and Burns, J. A. (1973). *Symp. Soc. Exp. Biol.* **32,** 65–104.
Kagawa, Y. (1972). *Biochim. Biophys. Acta* **245,** 297–338.
Kagawa, Y. (1979). *J. Bioenerg.* **11,** 39–78.
Kagawa, Y., and Racker, E. (1971). *J. Biol. Chem.* **246,** 5477–5487.
Kaiser, I., and Oelze, J. (1980). *Arch. Microbiol.* **126,** 187–194.
Kasche, V. (1983). *Enzyme Microb. Technol.* **5,** 2–13.
Katre, N. V., and Wilson, D. F. (1977). *Arch. Biochem. Biophys.* **184,** 578–585.
Katre, N. V., and Wilson, D. F. (1978). *Arch. Biochem. Biophys.* **191,** 647–656.
Katre, N. V., and Wilson, D. F. (1980). *Biochim. Biophys. Acta* **593,** 224–229.
Kaufman, R. D. (1977). *Anesthesiol.* **46,** 49–62.
Kaufmann, N., Reidl, H.-H., Golecki, J. R., Garcia, A. F., and Drews, G. (1982). *Arch. Microbiol.* **131,** 313–322.
Kayalar, C., and Luria, S. E. (1979). *In* "Membrane Bioenergetics" (C.-P. Lee, G. Schatz, and L. Ernster, eds.), pp. 297–305. Addison-Wesley, Reading, Massachusetts.
Kell, D. B. (1979). *Biochim. Biophys. Acta* **549,** 55–99.
Kell, D. B. (1983). *Bioelectrochem. Bioenerg.* **11,** 405–415.
Kell, D. B. (1984). *Trends Biochem. Sci.* **9,** 86–88.
Kell, D. B., and Griffiths, A. M. (1981). *Photobiochem. Photobiophys.* **2,** 105–110.
Kell, D. B., and Hitchens, G. D. (1982). *Faraday Discuss. Chem. Soc.* **74,** 377–388.
Kell, D. B., and Hitchens, G. D. (1983). *In* "Coherent Excitations in Biological Systems" (H. Fröhlich and F. Kremer, eds.), pp. 178–198. Springer-Verlag, Berlin and New York.
Kell, D. B., and Morris, J. G. (1981). *In* "Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria" (F. Palmieri, E. Quagliariello, N. Siliprandi, and E. C. Slater, eds.), pp. 339–347. Elsevier, Amsterdam.
Kell, D. B., Ferguson, S. J., and John, P. (1978a). *Biochem. Soc. Trans.* **6,** 1292–1295.
Kell, D. B., John, P., and Ferguson, S. J. (1978b). *Biochem. J.* **174,** 257–266.
Kell, D. B., Ferguson, S. J., and John, P. (1978c). *Biochim. Biophys. Acta* **502,** 111–126.
Kell, D. B., Burns, A., Clarke, D. J., and Morris, J. G. (1981a). *Specul. Sci. Technol.* **4,** 109–120.
Kell, D. B., Clarke, D. J., and Morris, J. G. (1981b). *FEMS Microbiol. Lett.* **11,** 1–11.
Kenyon, C. N. (1978). *In* "The Photosynthetic Bacteria" (R. K. Clayton and W. R. Sistrom, eds.), p. 281. Plenum, New York.
Kepes, A., and Autissier, F. (1972). *Biochim. Biophys. Acta* **265,** 443–469.
Keszthelyi, L. (1980). *Biochim. Biophys. Acta* **598,** 429–436.
Khan, S., and Berg, H. C. (1983). *J. Biol. Chem.* **258,** 6709–6712.
Kiehl, R., and Hanstein, W. G. (1981). *In* "Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria" (F. Palmieri, E. Qualiariello, N. Siliprandi, and E. C. Slater, eds.), pp. 217–222. Elsevier, Amsterdam.
Kimelberg, H. K., and Papahadjopoulos, D. (1971). *Biochim. Biophys. Acta* **233,** 805–809.
Klibanov, A. M. (1983). *Biochem. Soc. Trans.* **11,** 19–20.
Konisky, J. (1978). *In* "The Bacteria" (L. N. Ornston and J. R. Sokatch, eds.), Vol. 6, pp. 71–136. Academic Press, New York.
Konisky, J. (1982) *Annu. Rev. Microbiol.* **36,** 125–144.
Konisky, J., and Tokuda, H. (1979). *Zbl. Bakteriol. Parasit. Infekt Hyg Abt. I Orig. ReiheA* **244,** 1105–120.
Konisky, J., Gilchrist, M. J. R., Nieva-Gomez, D., and Gennis, R. B. (1975). *In* "Molecular Aspects of Membrane Phenomena" (H. R. Kaback, H. Neurath, G. K. Radda, R. Schwyzer, and W. R. Wiley, eds.), pp. 193–215. Springer-Verlag, Berlin and New York.

Kono, Y., Takeuchi, S., Kawarda, A., Daly, J. M., and Knoche, H. W. (1980). *Tetrahedron Lett.* **21**, 1537–1540
Kornberg, R. D., and McConnell, H. M. (1971). *Biochemistry* **10**, 1111–1120.
Kröger, A., and Klingenberg, M. (1970). *Vitam. Horm.* **28**, 533–674.
Kröger., A., and Klingenberg, M. (1973). *Eur. J. Biochem.* **39**, 313–323.
Kurup, C. K. R., and Sanadi, D. R. (1977). *J. Bioenerg. Biomembr.* **9**, 1–15
Kusher, D. J., ed. (1978). "Microbial Life in Extreme Environments." Academic Press, New York.
Lam, E., and Malkin R. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5494–5498.
Lang, D. R., and Decker, S. J. (1978). *In* "Spores VII" (G. Chambliss and J. C. Vary, eds.), American Society for Microbiology, Washington, D. C. pp. 265–270.
Lapange, S. (1978). "Physicochemical Aspects of Protein Denaturation." Wiley, New York.
Lardy, H., Reed, P., and Lin, C. C.-H. (1975). *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 1707–1710.
Läuger, P., and Neumcke, B. (1973). *In* "Membranes: A series of advances", G. Eisenman, ed.). pp. 1–59. Dekker, New York.
Leaver, C. J., and Gray, M. W. (1982). *Annu. Rev. Plant Physiol.* **33**, 373–402
Leaver, C. J., Forde, B. G., Dixon, L. K., and Fox, T. D. (1982). *In* "Mitochondrial Genes" (P. P. Slonimski, P. Borst, and G. Attardi, eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
Lee, C. P., Ernster, L., and Chance, B. (1969). *Eur. J. Biochem.* **8**, 153–163.
Lehninger, A. L. (1982). "Principles of Biochemistry." Worth, New York.
Lenaz, G. (1978). *In* "The Molecular Biology of Membranes" (S. Fleischer, Y. Hatefi, D. H. MacLennan, and A. Tzagoloff, eds.), pp. 137–162. Plenum, London.
Lieberman, M. A., and Hong, J.-S. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4395–4399.
Lieberman, M. A., and Hong, J.-S. (1976). *J. Bacteriol.* **125**, 1024–1031.
Lieberman, M. A., Simon, M., and Hong, J.-S. (1977). *J. Biol. Chem.* **252**, 4056–4067.
Ling, G. N. (1981). *Physiol. Chem. Phys.* **13**, 29–96.
Lloyd, D., Poole, R. K., and Edwards, S. W. (1982). "The Cell Division Cycle: Temporal Organization and Control of Cellular Growth and Reproduction." Academic Press, New York.
Loomis, W. F., and Lipmann, F. (1948). *J. Biol. Chem.* **173**.
Lozier, R. H., Bogomolni, R. A., and Stoeckenius, W. (1975). *Biophys. J.* **15**, 955–962.
Ludwig, B., Prochaska, L., and Capaldi, R. A. (1980). *Biochemistry* **19**, 1516–1523.
Luria, S. E. (1964). *Ann. Inst. Pasteur.* **107**, 67–73.
Luria, S. E. (1973). *In* "Bacterial Membranes and Walls" (I. Leive, ed.), pp. 293–320. Dekker, New York.
Luria, S. E. (1982). *J. Bacteriol.* **149**, 386.
McCammon, J. A., and Karplus, M. (1983). *Acc. Chem. Res.* **16**, 187–193.
McCarthy, J. E. G., and Ferguson, S. J. (1982). *Biochem. Biophys. Res. Commun.* **107**, 1406–1411.
McElhaney, R. N. (1974). *J. Mol. Biol.* **84**, 145–157.
McLaughlin, S. (1981). *In* "Chemiosmotic Proton Circuits in Biological Membranes" (V. P. Skulachev and P. Hinkle, eds.), pp. 601–609. Addison-Wesley, Reading, Massachusetts.
McLaughlin, S. G. A., and Dilger, J. P. (1980). *Physiol. Rev.* **60**, 825–863.
McLaughlin, S., Elsenberg, M., Cohen, F., and Dilger, J. (1978). *In* "Frontiers of Biological Energetics" (P. L. Dutton, J. S. Leigh, and A. Scarpa, eds.), Vol. 2, pp. 1205–1214. Academic Press, New York.
Mai, M., and Allison, W. S. (1983). *Arch. Biochem. Biophys.* **221**, 467–476.
Malenkova, I. V., Kupriov, S. P., Davydov, R. M., and Blumenfeld, L. A. (1982). *Biochim. Biophys. Acta* **682**, 179–183.
Maloff, B. L., Scordilis, S. P., and Tedeschi, H. (1978). *J. Cell Biol.* **78**, 214–226.
Maloney, P. C. (1982). *J. Membr. Biol.* **67**, 1–12.

Malpress, F. H. (1981a). *Nature (London)* **289,** 355.
Malpress, F. H. (1981b). *J. Theor. Biol.* **92,** 255–265.
Mandolino, G., De Santis, A., and Melandri, B. A. (1983). *Biochim. Biophys. Acta* **723,** 428–439.
Mar, T., Picorel, R., and Gingras, G. (1982). *Biochim. Biophys. Acta* **682,** 354–363.
Marquis, R. E., and Carstensen, E. L. (1973). *J. Bacteriol.* **116,** 1273–1279.
Marsh, D. (1983). *Trends Biochem. Sci.* **8,** 330–333.
Masters, C. J. (1981). *CRC Crit. Rev. Biochem.* **11,** 105–144.
Maynard-Smith, J. (1977). *In* "The Encyclopedia of Ignorance, Vol. 2, Life Sciences and Earth Sciences" (R. Duncan and M. Weston-Smith, eds.), pp. 235–242. Pergamon, Oxford.
Mechler, B., and Steim, J. M. (1976). *Annu. Rev. Biophys. Bioeng.* **5,** 205–238.
Medawar, P. (1982). *In* "Pluto's Republic," p. 29. Oxford Univ. Press, London and New York.
Melandri, B. A., Venturoli, G., de Santis, A., and Baccarini-Melandri, A. (1980). *Biochim. Biophys. Acta* **592,** 38–52.
Melandri, B. A., Venturoli, G., Casadio, R., Azzone, G. F., Kell, D. B., and Westerhoff, H. V. (1983). *Proc. Int. Photosynthesis Congr. 6th, Brussells.*
Michels, M., and Bakker, E. P. (1981). *Eur. J. Biochem.* **116,** 513–519.
Miller, I. R. (1981). *Top. Bioelectrochem. Bioenerg.* **4,** 162–224.
Miller, J. C., and Miller, K. W. (1975). *MTP Int. Rev. Sci. Biochem.* **12,** 33–75.
Miller, K. W., Paton, W. D. M., Smith, R. A., and Smith, E. B. (1973). *Mol. Pharmacol.* **9,** 131–143.
Mitchell, P. (1966). *Biol. Rev.* **41,** 445–502.
Mitchell, P. (1967). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **26,** 1370–1379.
Mitchell, P. (1968). "Chemiosmotic Coupling and Energy Transduction." Glynn Research, Bodmin, Cornwall.
Mitchell, P. (1976). *J. Theor. Biol.* **62,** 327–367.
Mitchell, P. (1977a). *Symp. Soc. Gen. Microbiol.* **27,** 383–423.
Mitchell, P. (1977b). *FEBS Lett.* **78,** 1–20.
Mitchell, P. (1979). *Science* **206,** 1148–1159.
Mitchell, P. (1981). *In* "Of Oxygen, Fuels and Living Matter" (G. Semenza, ed.), pp. 1–160. Wiley, New York.
Mitchell, P., and Moyle, J. (1967). *Biochem. J.* **105,** 1147–1162.
Mitchell, P., Moyle, J., and Mitchell, R. (1979). *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 55, pp. 627–640.
Montecucco, C., Smith, G. A., Dabbeni-Sala, F., Johannsson, A., Galante, Y. M., and Bisson, R. (1982). *FEBS Lett.* **144,** 145–148.
Morris, J. F., and Newman, E. B. (1980). *J. Bacteriol.* **143,** 1504–1505.
Mozhaev, V. V., and Martinek, K. (1982). *Enzyme Microb. Technol.* **4,** 299–309.
Muller, M., Krebs, J. J. R., Cherry, R. J., and Kawato, S. (1982). *J. Biol. Chem.* **257,** 117–120.
Murakami, S., Torres-Pereira, J., and Packer, L. (1975). *In* "Bioenergetics of Photosynthesis" (Gorindjee, ed.), pp. 555–618. Academic Press, New York.
Muraoka, S., and Terada, H. (1972). *Biochim. Biophys. Acta* **275,** 271–275.
Murato, N., and Fork, D. C. (1975). *Plant Physiol.* **56,** 791–796.
Newman, E. B., Morris, J. F., Walker, C., and Kapook, V. (1981). *Mol. Gen. Genet.* **182,** 143–147.
Newman, E. B., Malik, N., and Walker, C. (1982). *J. Bacteriol.* **150,** 710–715.
Nicholls, D. G. (1982). "Bioenergetics." Academic Press, New York.
Nicolay, K., Lolkema, J., Hellingwerf, K. J., Kaptein, R., and Konings, W. N. (1981). *FEBS Lett.* **123,** 319–323.
Nishimura, M., and Pressman, B. C. (1969). *Biochemistry* **8,** 1360–1370.
Nomura, M., and Maeda, A. (1965). *Zbl. Bakteriol. Abt. 1 Orig.* **196,** 216–239.
Oelze, J., and Drews, G. (1981). *In* "Organisation of Prokaryotic Cell Membranes" (B. K. Ghosh, ed.), Vol. 2, pp. 131–195. CRC Press, Boca Raton, Florida.

Oren, R., Weiss, S., Garty, H., Caplan, S. R., and Gromet-Elhanan, Z. (1980). *Arch. Biochem. Biophys.* **205,** 503–509.
Ort, D. R., and Melandri, B. A. (1982). *In* "Photosynthesis; Energy Conversion by Plants and Bacteria" (Govindjee, ed.), pp. 537–587. Academic Press, New York.
Ort, D. R., Dilley, R. A., and Good, N. E. (1976). *Biochim. Biophys. Acta* **449,** 108–124.
Ovchinnikov, Y. A., Abdulaev, N. G., Feigina, M. Y., Kiselev, A. V., and Lobanov, N. A. (1979). *FEBS Lett.* **100,** 219–224.
Packham, N. K., and Barber, J. (1983). *Biochim. Biophys. Acta.* **723,** 247–255.
Packham, N. K., Berriman, J. A., and Jackson, J. B. (1978). *FEBS Lett.* **89,** 205–210.
Packham, N. K., Tiede, D. M., Mueller, P., and Dutton, P. L. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 6339–6343.
Padan, E. and Rottenberg, H. (1973). *Eur. J. Biochem.* **40,** 431–437.
Padan, E., Zilberstein, D., and Schuldiner, S. (1981). *Biochim. Biophys. Acta* **650,** 151–166.
Papa, S. (1976). *Biochim. Biophys. Acta* **456,** 39–84.
Parsegian, V. A. (1975). *Ann. N.Y. Acad. Sci.* **264,** 161–174.
Parsonage, D., and Ferguson, S. J. (1982). *Biochem. Soc. Trans.* **10,** 257–258.
Pattus, F., Cavard, D., Verger, R., Lazdunski, C., Rosenbusch, J., and Schindler, H. (1983). *In* "Physical Chemistry of Transmembrane Ion Motions" (G. Spach, ed.), pp. 407–413. Elsevier, Amsterdam.
Pauly, H., and Packer, L. (1960). *J. Biophys. Biochem. Cytol.* **7,** 603–612.
Pauly, H., and Schwan, H. P. (1966). *Biophys. J.* **6,** 621–639.
Pedersen, P. L. (1982). *Ann. N.Y. Acad. Sci.* **402,** 1–20.
Pedersen, P. L., Schwerzmann, K., and Cintron, N. (1981). *Curr. Top. Bioenerg.* **11,** 149–199.
Petersen, D. C., and Cone, R. A. (1975). *Biophys. J.* **15,** 1181–1200.
Pethig, R. (1979). "Dielectric and Electronic Properties of Biological Materials." Wiley, New York.
Phelps, D. C., and Hanstein, W. G. (1977). *Biochem. Biophys. Res. Commun.* **79,** 1245–1254.
Phelps, D. C., and Hatefi, Y. (1981). *J. Biol. Chem.* **256,** 8217–8221.
Pick, U., and Racker, E. (1979). *J. Biol. Chem.* **254,** 2793–2799.
Pietrobon, D., Azzone, G. F., and Walz, D. (1981). *Eur. J. Biochem.* **117,** 389–394.
Pietrobon, D., Zoratti, M., Azzone, G. F., Stucki, J. W., and Walz, D. (1982). *Eur. J. Biochem.* **127,** 483–494.
Pietrobon, D., Zoratti, M., and Azzone, G. F. (1983). *Biochim. Biophys. Acta* **723,** 317–321.
Plate, C. A. (1976). *J. Bacteriol.* **125,** 467–474.
Plate, C. A. (1979). *In* "Microbiology—1979" (D. Schlessinger, ed.), pp. 58–61. Am. Soc. Microbiol., Washington, D.C.
Plate, C. A., and Suit, J. L. (1981). *J. Biol. Chem.* **256,** 12974–12980.
Poo, M.-m. (1981). *Annu. Rev. Biophys. Bioeng.* **10,** 245–276.
Poo, M., and Cone, R. A. (1973). *Nature (London)* **247,** 438–441.
Poo, M.-m., and Robinson, K. R. (1977). *Nature (London)* **265,** 602–604.
Poole, R. K. (1981). *In* "Diversity of Bacterial Respiratory Chains" (C. W. Jones, ed.), pp. 87–114. CRC Press, Boca Raton, Florida.
Porteous, J. W. (1983). *Trends Biochem. Sci.* **8,** 200–202.
Pressman, B. C. (1972). *In* "Biochemistry and Biophysics of Mitochondrial Membranes" (G. F. Azzone, E. Carafoli, A. L. Lehninger, E. Quagliariello, and N. Siliprandi, eds.), pp. 591–602. Academic Press, New York.
Price, B. D., and Brand, M. D. (1983). *Eur. J. Biochem.* **132,** 595–601.
Prince, R. C., O'Keefe, D. P., and Dutton, P. L. (1982). *In* "Electron Transport and Photophosphorylation" (J. Barber, ed.), pp. 197–248. Elsevier, Amsterdam.
Racker, E. (1976). "A New Look at Mechanisms in Bioenergetics." Academic Press, New York.
Racker, E., and Kandrach, A. (1971). *J. Biol. Chem.* **246,** 7069–7071.

Racker, E., and Stoeckenius, W. (1974). *J. Biol. Chem.* **249,** 662–663.
Ragan, C. I., and Racker, E. (1973). *J. Biol. Chem.* **248,** 2563–2569.
Raison, J. K. (1973). *Symp. Soc. Exp. Biol.* **27,** 485–512.
Rao, K. R. (1978). "Pentachloroplenol. Chemistry, Pharmacology and Environmental Toxicology." Plenum, New York.
Reddy, G. P. V., and Pardee, A. B. (1983). *Nature (London)* **304,** 86–88.
Reed, P. W., and Lardy, H. A. (1975). *J. Biol. Chem.* **250.**
Reynafarje, B., Brand, M. D., Alexandre, A., and Lehninger, A. L. (1979). *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 55, pp. 640–656. Academic Press, New York.
Rich, P. R. (1982). *Faraday Discuss. Chem. Soc.* **74,** 349–364.
Richardson, S. H., Hultin, H. O., and Fleischer, S. (1964). *Arch. Biochem. Biophys.* **105,** 254–260.
Robertson, R. N. (1983). "The Lively Membranes." Cambridge Univ. Press, London and New York.
Roscoe, T. J., and Ellis, R. J. (1982). *In* "Methods in Chloroplast Molecular Biology" (M. Edelman, R. B. Hallick, and N.-H. Chua, eds.), pp. 1015–1028. Elsevier, Amsterdam.
Rosen, B. P., and Kashket, E. R. (1978). *In* "Bacterial Transport" (B. P. Rosen, ed.), pp. 559–620. Dekker, New York.
Rottenberg, H. (1973). *Biophys. J.* **13,** 503–511.
Rottenberg, H. (1979). *In* "Methods in Enzymology" (S. Fleischer and L. Packer eds.), Vol. 55, pp. 547–569. Academic Press, New York.
Rottenberg, H. (1983). *Proc. Natl. Acad. Sci. U.S.A.* **80,** 3313–3317.
Russell, B. (1966). "Human Knowledge, Its Scope and Limits," p. 213. Allen & Unwin, London.
Ryrie, I. J., Critchley, C., and Tillberg, J.-E. (1978). *Arch. Biochem. Biophys.* **198,** 182–194.
Sabet, S. F. (1976). *J. Bacteriol.* **126,** 601–608.
Sandorfy, C. (1980). *Prog. Anesthesiol.* **2,** 353–359.
Saraste, M. (1983). *Trends Biochem. Sci.* **8,** 139–142.
Schäfer, G. (1981). *In* "Inhibitors of Mitochondrial Function" (M. Erecinska and D. F. Wilson, eds.), pp. 165–185. Pergamon, Oxford.
Schäfer, G., and Rowohl-Quisthoudt. (1975). *FEBS Lett.* **59,** 48–51.
Schatz, G., and Saltzgaber, J. (1970). *In* "Electron Transport and Energy Conservation" (J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, eds.), pp. 273–280. Adriatica Editrice, Bari, Italy.
Schein, S. J., Kagan, B. L., and Finkelstein, B. A. (1979). *Nature (London)* **276,** 159–163.
Schlodder, E., Gröber, P., and Witt, H. T. (1982). *In* "Electron Transport and Photophosphorylation" (J. Barber, ed.), pp. 105–175. Elsevier/North-Holland, Amsterdam.
Schneider, H., Lemasters, J. J., Höchli, M., and Hackenbrock, C. R. (1980a). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 442–446.
Schneider, H., Lemasters, J. J., Höchli, M., and Hackenbrock, C. R. (1980b). *J. Biol. Chem.* **255,** 3748–3756.
Schneider, H., Lemasters, J. J., and Hackenbrock, C. R. (1982). *J. Biol. Chem.* **257,** 10789–10793.
Scholes, P., and Mitchell, P. (1970). *J. Bioenerg.* **1,** 309–323.
Schreiber, U., and Del Valle-Tascon, S. (1982). *FEBS Lett.* **150,** 32–37.
Schuldiner, S. (1977). *Encycl. Plant Physiol. New Ser.* **5,** 416–422.
Schwarz, G. (1978a). *J. Membr. Biol.* **43,** 127–148.
Schwarz, G. (1978b). *J. Membr. Biol.* **43,** 149–167.
Scott, A. C. (1983). *In* "Structure and Dynamics: Proteins and Nucleic Acids" (E. Clementi, and R. H. Sarma, eds.), pp. 389–404. Adenine Press, Guilderland, New York.
Senior, A. E. (1979). *In* "Membrane Proteins in Energy Transduction" (R. A. Capaldi, ed.), pp. 233–278. Dekker, New York.

Senior, A. E., and Wise, J. G. (1983). *J. Membr. Biol.* **73.**
Shilo, M., ed. (1978). "Strategies of Microbial Lie in Extreme Environments." Verlag Chemie Weinheim.
Singer, S. J., and Nicolson, G. L. (1972). *Science*, **175**, 720–731.
Sjöstrand, F. S. (1978). *J. Ultrastruct. Res.* **64**, 217–245.
Skulachev, V. P. (1971). *Curr. Top. Bioenerg.* **4**, 127–190.
Slooten, L. and Branders, C. (1979). *Biochim. Biophys. Acta* **547**, 79–90.
Smith, D. J., Stokes, B. O., and Boyer, P. D. (1976). *J. Biol. Chem.* **251**, 4165–4171.
Smith, E. B., Bowser-Riley, F., Daniels, S., Dunbar, I. T., Harrison, C. B., and Paton, W. D. M. (1984). *Nature (London)* **311**, 56–58.
Somogyi, B., Welch, G. R., and Damjarovich, S. (1984). *Biochim. Biophys. Acta* **768**, 81–112.
Sonnleitner, B., and Fiechter, A. (1983). *Trends Biotechnol.* **1**, 74–80.
Soper, J. W., Decker, G. L., and Pedersen, P. L. (1979). *J. Biol. Chem.* **254**, 11170–11176.
Sorgato, M. C., and Ferguson, S. J. (1979). *Biochemistry* **18**, 5737–5742.
Southard, J., Nitisewojo, P., and Green, D. E. (1974). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33**, 2147–2153.
Sowers, A., and Hackenbrock, C. R. (1980). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **39**, 1955.
Sowers, A. E., and Hackenbrock, C. R. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6246–6250.
Stryer, L. (1981). "Biochemistry." Freeman, San Francisco, California.
Stucki, J. W. (1980). *Eur. J. Biochem.* **109**, 269–283.
Stucki, J. W. (1982). *In* "Metabolic Compartmentation" (H. Sies, ed.), pp. 39–69. Academic Press, New York.
Szoka, F., and Papahadjopoulos, D. (1980). *Annu. Rev. Biophys. Bioeng.* **9**, 467–508.
Tager, J. M., Wanders, R. J. A., Groen, A. K., Kunz, W., Bohnensack, R., Küster, U., Letko, G., Böhme, G., Duszynski, J., and Wojtczak, L. (1983). *FEBS Lett.* **151**, 1–9.
Tanford, C. (1978). *Science* **200**, 1012–1018.
Tanford, C. (1980). "The Hydrophobic Effect: Formation of Micelles and Biological Membranes," 2nd Ed. Wiley, New York.
Tedeschi, H. (1975). "Mitochondria: Structure, Biogenesis and Transducing Functions." Springer-Verlag, Berlin and New York.
Tedeschi, H. (1980). *Biol. Rev.* **55**, 171–206.
Tedeschi, H. (1981). *Biochim. Biophys. Acta* **639**, 157–196.
Terada, H. (1981). *Biochim. Biophys. Acta* **639**, 225–242.
Thayer, W. S., and Hinkle, P. C. (1975). *J. Biol. Chem.* **250**, 5336–5342.
Thorbjarnardóttir, S. H., Magnúsdóttir, R. Á, Eggertson, G., Kagan, S. A., and Andrésson, O. S. (1978). *Mol. Gen. Genet.* **161**, 89–98.
Tien, H. T. (1974). "Bilayer Lipid Membranes (BLM). Theory and Practice." Dekker, New York.
Todorov, G., Sokerov, S., and Stoylov, S. P. S. (1982). *Biophys. J.* **40**, 1–5.
Tokuda, H., and Konisky, J. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2579–2583.
Tokuda, H., and Konisky, J. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6167–6171.
Tokutomi, S., Iwasa, T., Yoshizawa, T., and Ohnishi, S. (1980). *FEBS Lett.* **114**, 145–148.
Tomochika, K.-I., and Hong, J.-S. (1978). *J. Bacteriol.* **133**, 1008–1014.
Tosaka, O., Enei, H., and Hirose, Y. (1983). *Trends Biotechnol.* **1**, 70–74.
Trudell, J. R. (1980). *Prog. Anesthesiol.* **2**, 261–270.
Trumpower, B. L., ed. (1982). "Function of Quinones in Energy Conserving Systems." Academic, New York.
Tsuji, K., and Neumann, E. (1983). *Biophys. Chem.* **17**, 153–163.
Tu, S.-I., Lam, E., Ramirez, F., and Marecek, J. F. (1981). *Eur. J. Biochem.* **113**, 391–396.
Tzagoloff, A. (1982). "Mitochondria." Plenum, New York.
Ullstrup, A. J. (1972). *Annu. Rev. Phytopathol.* **10**, 37–50.

Uratani, Y., and Cramer, W. A. (1981). *J. Biol. Chem.* **256,** 4017–4023.
van Dam, K., and Tsou, C. S. (1970). *In* "Electron Transport and Energy Conservation" (J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater, eds.), pp. 421–425. Adriatica Editrice, Bari, Italy.
van Dam, K., Woelders, H., Colen, A. M., and Westerhoff, H. V. (1983). *Biochem. Soc. Trans.* **12,** 401–402.
van der Bend, R. L., Cornelissen, J.B.W.J., Berden, J.A., and van Dam, K. (1984). *Biochim. Biophys. Acta* **767,** 87–101.
Vanderkooi, G. (1978). *In* "The Molecular Biology of Membranes" (S. Fleischer, Y. Hatefi, D. H. MacLennan, and A. Tzagoloff, eds.), pp. 29–55. Plenum, New York.
Vaz, W. L. C., Derzko, Z. I., and Jacobson, K. A. (1982). *In* "Membrane Reconstitution" (G. Poste and G. L. Nicolson, eds.), pp. 83–135. Elsevier, Amsterdam.
Venturoli, G., and Melandri, B. A. (1982). *Biochim. Biophys. Acta* **680,** 8–16.
Verhoek-Köhler, B., Hampp, R., Ziegler, H., and Zimmerman, U. (1983). *Planta* **158,** 199–204.
Vignais, P. M., Henry, M.-F., Sim, E., and Kell, D. B. (1981). *Curr. Top. Bioenerg.* **12,** 115–196.
Vinkler, C., and Korenstein, R. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 3183–3187.
Vinkler, C., Avron, M., and Boyer, P. D. (1980). *J. Biol. Chem.* **255,** 2263–2266.
von Jagow, G., and Engel, W. D. (1980). *Angew. Chem. Int. Ed.* **19,** 659–675.
von Stedingk, L.-V. (1967). *Arch. Biochem. Biophys.* **120,** 537–541.
von Stedingk, L.-V., and Baltscheffsky, H. (1966). *Arch. Biochem. Biophys.* **117,** 400–404.
Vredenberg, W. J. (1976). *In* "The Intact Chloroplast" (J. Barber, ed.), pp. 53–88. Elsevier, Amsterdam.
Walz, D. (1983). *In* "Biological Structures and Coupled Flows" (A. Oplatka and M. Balaban, eds.), pp. 45–60. Academic Press, New York.
Weaver, C. A., Kagan, B. L., Finkelstein, A., and Konisky, J. (1981). *Biochim. Biophys. Acta* **645,** 137–142.
Webb, W. W. (1977). *In* "Electrical Phenomena at the Biological Membrane Level" (E. Roux, ed.), pp. 119–156. Elsevier, Amsterdam.
Webb, W. W., Barak, L. S., Tank, D. W., and Wu, E.-S. (1982). *Biochem. Soc. Symp.* **46,** 191–205.
Weinbach, E. C., and Garbus, J. (1965). *J. Biol. Chem.* **240,** 1811–1819.
Weinbach, E. C., and Garbus, J. (1969). *Nature (London)* **221,** 1016–1018.
Weiss, M. J., and Luria, S. E. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2483–2487.
Welch, G. R. (1977). *Prog. Biophys. Mol. Biol.* **32,** 103–191.
Welch, G. R., ed. (1985). "The Fluctuating Enzyme." Wiley, New York.
Welch, G. R., and Berry, M. N. (1983). *In* "Coherent Excitations in Biological Systems" (H. Fröhlich and F. Kremer, eds.), pp. 95–118. Springer-Verlag, Berlin and New York.
Welch, G. R., and Keleti, T. (1981). *J. Theor. Biol.* **93,** 701–735.
Welch, G. R., and Kell, D. B. (1985). *In* "The Fluctuating Enzyme" (G. R. Welch, ed.). Wiley, New York.
Welch, G. R., Somogyi, B., and Domjanovich, S. (1982). *Progr. Biophys. Mol. Biol.* **39,** 109–146.
Wendt, L. (1970). *J. Bacteriol.* **104,** 1236–1241.
Westerhoff, H. V. (1983). Ph.D. thesis, University of Amsterdam.
Westerhoff, H. V., and Arents, J. C. (1984). *Biosci. Rep.* **4.**
Westerhoff, H. V., and Dancshazy, Zs. (1984). *Trends Biochem. Sci.* **9,** 112–117.
Westerhoff, H. V., and van Dam, K. (1985). "Mosaic Non-equilibrium Thermodynamics and the Control of Biological Free-Energy Transduction." Elsevier, Amsterdam, in press.
Westerhoff, H. V., Simonetti, A. L. M., and van Dam, K. (1981). *Biochem. J.* **200,** 193–202.
Westerhoff, H. V., de Jonge, P. C., Colen, A., Groen, A. K., Wanders, R. J. A., van den Berg, G. B., and van Dam, K. (1982). *EBEC Rep.* **2,** 267–268.
Westerhoff, H. V., Helgerson, S. L., Theg, S. M., van Kooten, O., Wilkström, M., Skulachev, V. P., and Dancshazy, Z. S. (1983a). *Acta Biol. Acad. Sci. Hung.* **18,** 125–149.

Westerhoff, H. V., Colen, A., and van Dam, K. (1983b). *Biochem. Soc. Trans.* **11**, 81–85.
Westerhoff, H. V., Melandri, B. A., Venturoli, G., Azzone, G. F., and Kell, D. B. (1983c). *FEBS Lett.* **165**, 1–5.
Westerhoff, H. V., Groen, A. K., and Wanders, R. J. A. (1983d). *Biochem. Soc. Trans.* **11**, 90–91.
Westerhoff, H. V., Groen, A. K., Wanders, R. J. A., and Tager, J. M. (1984a). *Biosci. Rep.* **4**, 1–22.
Westerhoff, H. V., Melandri, B. A., Venturoli, G., Azzone, G. F., and Kell, D. B. (1984b). *Biochim. Biophys. Acta* **768**, 257–292.
White, D. C. (1974). *In* "Molecular Mechanisms in General Anaesthesia" (M. J. Halsey, R. A. Millar, and J. A. Sutton, eds.), pp. 209–221. Churchill Livingstone, Edinburgh.
Wiechmann, A. C. H. A., Beem, E. P., and van Dam, K. (1975). *In* "Electron Transfer Chains and Oxidative Phosphorylation" (E. Quagliariello, F. Palmieri, E. C. Slater, and N. Siliprandi, eds.), pp. 335–342. North-Holland Publ., Amsterdam.
Wikström, M., and Krab, K. (1980). *Curr. Top. Bioenerg.* **10**, 51–101.
Wilkström, M., and Penttilä, T. (1982). *FEBS Lett.* **144**, 183–189.
Wikström, M., Krab, K., and Saraste, M. (1981). *Annu. Rev. Biochem.* **50**, 623–655.
Williams, R. J. P. (1961). *J. Theor. Biol.* **1**, 1–17.
Williams, R. J. P. (1978a). *FEBS Lett.* **85**, 9–19.
Williams, R. J. P. (1978b). *Biochim. Biophys. Acta* **505**, 1–44.
Williams, R. J. P. (1982). *FEBS Lett.* **150**, 1–3.
Wilson, D. F., and Forman, N. G. (1982). *Biochemistry* **21**, 1438–1444.
Winget, G. D., Kanner, N., and Racker, E. (1977). *Biochim. Biophys. Acta* **460**, 490–499.
Wraight, C. A., Cogdell, R. J., and Chance, B. (1978). *In* "The Photosynthetic Bacteria" (R. K. Clayton and W. R. Sistrom, eds.), pp. 471–511. Plenum, New York.
Young, S. H., and Poo, M.-m. (1983). *Nature (London)* **304**, 161–163.
Yu, C.-A., and Yu, L. (1981). *Biochim. Biophys. Acta* **639**, 99–128.
Zaritsky, A., and MacNab, R. M. (1981). *J. Bacteriol.* **147**, 1054–1062.
Zeikus, J. G. (1979). *Enzyme Microb. Technol.* **1**, 243–252.
Zimmermann, U. (1982). *Biochim. Biophys. Acta* **694**, 227–277.
Zimmermann, U., and Vienken, J. (1982). *J. Membr. Biol.* **67**, 165–182.
Zimmermann, U., Scheurich, P., Pilwat, G., and Benz, R. (1981). *Angew. Chem. Int. Ed.* **20**, 325–344.
Zoratti, M., Pietrobon, D., and Azzone, G. F. (1982). *Eur. J. Biochem.* **126**, 443–451.
Zukav, G. (1980). "The Dancing Wu Li Masters," p. 328. Fontana/Collins, London.