listed in the table. A judicious design of experimental systems can employ many predetermined rate constants. Such an approach will reduce the uncertainty of analytical results and expand the fundamental list of rate constants, so much needed for future studies.

Acknowledgments

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[40] Localized Protonic Coupling: Overview and Critical Evaluation of Techniques

By DOUGLAS B. KELL

Introduction

It is widely recognized that free energy transduction in many biological processes is accompanied by and may be effected (at least partially) by means of a current of "energized" protons. In the case of the membranous systems catalyzing oxidative and photosynthetic phosphorylation, many experiments have indicated that the protein complexes catalyzing both oxidoreductive and ATP synthetic/hydrolytic reactions are, or may be, protonmotive, i.e., that their activities are more or less tightly coupled to the vectorial translocation of protons between the bulk phases that the membrane in which they are embedded serves to separate. This statement is true both for energy coupling membranes as isolated and for artificial proteoliposomes containing purified components. Many other experiments have also indicated that the imposition of an artificial proton electrochemical potential difference (protonmotive force, pmf, Δp) across such systems can drive ATP synthesis at rates that are at least as great as those driven by electron transport. The conclusion that many have drawn from these and other observations summarized in Nicholls's monograph[1] is that it is possible to describe the salient features of free energy transduction by the scheme shown in Fig. 1A, or by the shorthand notation shown in Fig. 1B. However, in all cases that are known to me[2] of phos-

[1] D. G. Nicholls, "Bioenergetics. An Introduction to the Chemiosmotic Theory." Academic Press, London, 1982.

[2] D. B. Kell and H. V. Westerhoff, *in* "Organized Multienzyme Systems: Catalytic Properties" (G. R. Welch, ed.), p. 63. Academic Press, New York, 1985.

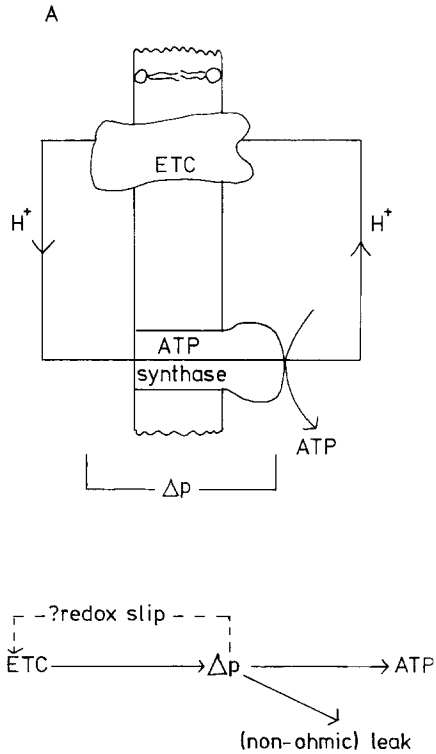

FIG. 1. The simplest, sophisticated, delocalized chemiosmotic coupling scheme for electron transport phosphorylation in which a primary protonmotive electron transfer chain or complex (ETC) generates, across the coupling membrane, a delocalized protonmotive force, which may be used by the ATP synthase to drive phosphorylation. (A) Diagrammatic picture: the topological proximity of the ETC and ATP synthase systems is not considered relevant. (B) Shorthand notation describing the free energy-transducing pathway and indicating that one may take cognizance of pmf-dependent leaks and slips in the system.

phorylation induced by an artificial pmf, there is in fact a very sharp threshold of applied pmf, equivalent to ~150 mV (in some cases 180 mV), below which no phosphorylation takes place (e.g., Refs. 3–10) and a

[3] E. Uribe, *Biochemistry* **11,** 4228 (1972).
[4] W. S. Thayer and P. C. Hinkle, *J. Biol. Chem.* **250,** 5336 (1975).
[5] N. Sone, M. Yoshida, H. Hirata, and Y. Kagawa, *J. Biol. Chem.* **252,** 2956 (1977).
[6] P. Gräber, *Curr. Top. Membr. Transp.* **16,** 215 (1981).
[7] P. C. Maloney, *J. Membr. Biol.* **67,** 1 (1982).
[8] E. Schlodder, P. Gräber, and H. T. Witt, *in* "Electron Transport and Photophosphorylation" (J. Barber, ed.), p. 105. Elsevier, Amsterdam, 1982.
[9] R. P. Hangarter and N. E. Good, *Biochim. Biophys. Acta* **681,** 397 (1982).
[10] R. P. Hangarter and N. E. Good, *Biochemistry* **23,** 122, (1984).

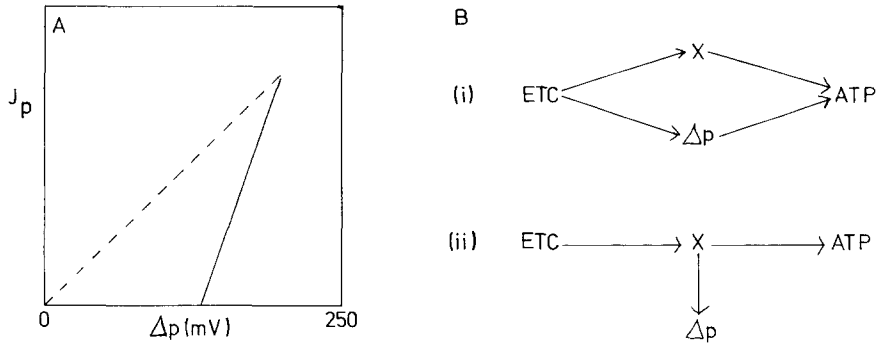

FIG. 2. The existence of a possible threshold for uptake ATP synthesis (J_p) driven by an artificial pmf [(A), solid line] raises the possibility that, in contrast to the nonthreshold case [(A), dotted line], free energy-transducing interactions may take place between ETC and ATP synthase complexes via a route additional to ("parallel coupling") (B, i) or independent of (B, ii) the pmf. For clarity, redox slips and nonohmic leaks driven by X and/or by the pmf are not diagrammed, but should also be considered. Non-free energy-transducing ("allosteric") interactions are not considered here. "X" represents any high-energy intermediate that does not come into equilibrium with Δp.

rather substantial applied pmf is required to obtain the crucial kinetic competence which would be required to persuade one of the veracity of the scheme shown in Fig. 1B.

The existence of the threshold pmf in artificial pmf experiments raises in particular two important points: (1) the very nonlinear relation between J_p (the rate of ATP synthesis), or the ATP yield, and the applied, and hopefully (quasi-) stationary, pmf means that any inhomogeneity or imperfection in the rapid mixing required in such an experiment, or any uncorrected adenylate kinase and other artifactual ATP synthetic or "background" activity,[11] will serve artifactually to blur the existence of the threshold, and (2) the threshold phenomenon (Fig. 2) serves strongly to sharpen the arguments concerning the veracity of the scheme of Fig. 1B; if the pmf actually generated by electron transport significantly exceeds the threshold value, then there is no reason on this basis to doubt that "delocalized" chemiosmotic coupling is an excellent approximation to reality, while if the pmf claimed to be or actually generated by electron transport is below the threshold seen in artificial pmf-driven phosphorylation experiments, then it seems to me that this should be taken to contraindicate the veracity *in vivo* of delocalized chemiosmotic coupling.[2,12,13]

[11] J. D. Mills and P. Mitchell, *FEBS Lett.* **144,** 63 (1982).

[12] D. B. Kell and G. D. Hitchens, *in* "Coherent Excitations in Biological Systems" (H. Fröhlich and F. Kremer, eds.), p. 178. Springer-Verlag, Berlin, 1983.

[13] H. V. Westerhoff, B. A. Melandri, G. Venturoli, G. F. Azzone, and D. B. Kell, *Biochim. Biophys. Acta* **768,** 257 (1984).

(Note that because of the threshold effect, this latter statement is not altered by the invocation of even enormous and variable $\rightarrow H^+/ATP$ stoichiometries.) This is why it is of the first importance to be able accurately to assess the value of the pmf under a variety of conditions.

Now it is widely recognized that the so-called delocalized chemiosmotic coupling hypothesis is in principle much more amenable to experimental falsification than are the localized coupling theories alluded to in the title of this article; the latter are usually invoked in the face of real or apparent failures of the predictions of delocalized chemiosmotic coupling.[2,12,14] Therefore, and since more specific mechanistic proposals concerning the latter have recently been given elsewhere,[2,12,13,15,16] I will assess in this chapter what are the likely or most credible values of the delocalized pmf (as defined in the chemiosmotic hypothesis) that are generated by electron transport during oxidative and photosynthetic phosphorylation, and whether or not they exceed the threshold alluded to above. Certain other points will be raised in relation to this process, but related matters such as the possible role of the pmf in active transport of molecules such as lactose will in general be omitted from consideration.

Since ion and weak acid/base distribution methods[1,17–19] are the most widely used, and in my view the only potentially credible, means for actually determining the pmf, I shall lay special emphasis on them and shall begin by addressing the intimately related matter of respiration-driven proton translocation.

Throughout this chapter I would wish readers to keep two crucial points in mind: (1) Qualitative arguments concerning whether a potential source of error is likely to cause a method to overestimate or to underestimate the pmf can be very helpful in forming a mental picture of whether the *actual* pmf is likely to be above or below the threshold; and (2) in all cases, we wish to know whether a particular method is actually responding to or reflecting one of the components of the protonmotive force as defined in the chemiosmotic theory or whether it is actually reflecting "membrane energization," since at all events the two phenomena may not be, and in my view are not, energetically the same thing.

I have attempted comprehensively to cover the literature through mid-1984.

[14] S. J. Ferguson, *Biochim. Biophys. Acta* **811,** 47 (1985).
[15] H. V. Westerhoff, B. A. Melandri, G. Venturoli, G. F. Azzone, and D. B. Kell, *FEBS Lett.* **165,** 1 (1984).
[16] D. B. Kell and G. D. Hitchens, *Biochem. Soc. Trans.* **12,** 413, (1984).
[17] S. J. Ferguson and M. C. Sorgato, *Annu. Rev. Biochem.* **51,** 185 (1982).
[18] H. Rottenberg, this series, Vol. 55, p. 547.
[19] G. F. Azzone, D. Pietrobon, and M. Zoratti, *Curr. Top. Bioenerg.* **14,** 1 (1984).

Respiration-Driven H^+ Translocation

In this method[20–24] a burst of respiration is initiated in a suspension of lightly buffered membrane vesicles or cells, usually by the addition of a small volume of air-saturated KCl to an anoxic system. The translocation of protons and sometimes of other ions is measured with ion-selective electrodes and calibrated with anaerobic standard solutions. Recent technical developments include the use of a fast-responding ($t_{1/2}$ = 1–10 msec) O_2 electrode to measure the duration of the respiratory burst[24–26]; the anodic iridium oxide film electrode[27] is insensitive to O_2, but responds to pH on a similar time scale.[28] According to chemiosmotic considerations, the principle of this method is that, given the low static electrical capacitance of the membrane, electrically uncompensated transfer of only a small number of H^+ across the membrane, between (phases in equilibrium with) the two bulk phases, will charge the membrane to its maximum potential, thereby causing redox slip and/or nonohmic leak (Fig. 1B). Since the capacitance of most energy coupling membranes is 1 ± 0.5 $\mu F/cm^2$,[29] the potential may be calculated[23,30] and should, if one wishes to know the absolute stoichiometry of proton translocation at level flow, be dissipated by the inclusion of an appropriate concentration of ionophore or of membrane-permeant ions. The pH changes usually observed are rather small; if the internal and external buffering powers, which are often arranged to be roughly equal, are known, the pH gradient formed may be fairly accurately calculated from the pH changes observed in the extravesicular phase.[31]

Qualitatively, it is well known that the $\rightarrow H^+/O$ ratio, as calculated (and perhaps underestimated[22]) from the extent of excursion of the pH trace at a time corresponding to the half-time of O_2 reduction, is indeed greatly increased by the presence of membrane-permeant ions. The delocalized chemiosmotic explanation[20] of this behavior is that the decrease in membrane potential caused by the transmembrane ion movement relieves

[20] P. Scholes and P. Mitchell, *J. Bioenerg.* **1,** 309, (1970).
[21] P. Mitchell, J. Moyle, and R. Mitchell, this series, Vol. 55, p. 627.
[22] M. Wikström and K. Krab, *Curr. Top. Bioenerg.* **10,** 51 (1980).
[23] D. B. Kell and G. D. Hitchens, *Faraday Discuss. Chem. Soc.* **74,** 377, (1982).
[24] G. D. Hitchens and D. B. Kell, *Biochim. Biophys. Acta* **766,** 222 (1984).
[25] B. Reynafarje, A. Alexandre, P. Davies, and A. L. Lehninger, *Proc. Natl. Acad. Sci. U.S.A.* **79,** 7218 (1982).
[26] L. E. Costa, B. Reynafarje, and A. L. Lehninger, *J. Biol. Chem.* **259,** 4802 (1984).
[27] F. L. H. Gielen and P. Bergveld, *Med. Biol. Eng. Comput.* **20,** 77 (1982).
[28] D. B. Kell, unpublished observations, 1983–1984.
[29] C. M. Harris and D. B. Kell, *Bioelectrochem. Bioenerg.* **11,** 15 (1983).
[30] J. M. Gould and W. A. Cramer, *J. Biol. Chem.* **252,** 5875 (1977).
[31] D. B. Kell and J. G. Morris, *J. Biochem. Biophys. Methods* **3,** 143 (1980).

the backpressure of the pmf on the respiratory chain and thereby permits the true, limiting stoichiometry to be observed. This is the theory and, as mentioned, it is open to experimental test, and perhaps falsification, as follows. If for a given cell concentration the $\rightarrow H^+/O$ ratio observed with an O_2 pulse of, say, 10 ng atom O is raised from 0.5 to 7.5 by the addition of a saturating concentration of permeant ions, then increasing the size of the O_2 pulse, in the absence of the added permeant ions, to, say, 20 ng atom O should not allow any more H^+ to be pumped, since the membrane potential has already supposedly reached its maximum value attainable under the prevailing conditions. In practice, when such an experiment is performed, the $\rightarrow H^+/O$ ratio remains the same,[23,24,30,32] so that the conclusion to be drawn[2,12,16,23,24,30] is that the observable protons are not those feeding back upon the respiratory chain to inhibit further protonmotive activity.

It is sometimes assumed that a very rapid, pmf-driven backflow of protons (nonohmic leak) may be the cause of the low $\rightarrow H^+/O$ ratios observed in the absence of permeant ions; however, observable pH decay rates in the absence of added "permeant" ions are almost immeasurably slow.[20,23,24,30,32] That the addition of the energy transfer inhibitor venturicidin, which should block any nonohmic leak through the ATP synthase,[33] preserves the independence of the $\rightarrow H^+/O$ ratio from the size of the O_2 pulse in bacterial protoplasts[24] indicates that pmf-driven redox slips and/or nonohmic leaks are not the cause of the low $\rightarrow H^+/O$ ratios seen in the absence of permeant ions. Further, as pointed out by Ferguson,[14] it is to be assumed on a chemiosmotic basis that since the pmf generated by a given size of O_2 pulse should actually be much greater in the absence of permeant ions than in their presence (see above), one might imagine that the observable pmf-driven back-decay rate of pumped H^+ should also be much greater in the absence of permeant ions than in their presence.[14] In practice, the opposite is true,[14,20,23,24,30] so that one is led to conclude that the rate of decay of observable H^+ back across the membrane is limited by the possible rate of decay of a co- or counterion to preserve electroneutrality. On this basis, it is to be assumed that the same holds true for the observable proton pumping to the bulk extracellular phase in the first place.[24,34] Thus, although the transmembrane movement of ions other than H^+ may be caused by the protonmotive activity of respiratory chains, the fact that *each* counterion taken up is apparently accompanied by the observable translocation of an "extra" proton does not give one confidence that ion-distribution methods (see later) are in fact reflecting a

[32] J. F. Myatt, M. A. Taylor, and J. B. Jackson, *EBEC Rep.* **3,** 249 (1984).
[33] A. J. Clark, N. P. J. Cotton, and J. B. Jackson, *Biochim. Biophys. Acta* **723,** 440 (1983).
[34] H. Tedeschi, *Biochim. Biophys. Acta* **639,** 157 (1981).

delocalized membrane potential of any energetic significance across energy coupling membranes.

The delocalized chemiosmotic riposte to the foregoing analysis runs essentially as follows: The membrane capacitance is *so* small that under the usual set of experimental conditions (cell concentration and O_2 pulse size), only, say, 1 ng ion of electrogenic H^+ translocation will charge the membrane to its maximum potential, while the total number of H^+ observably translocated may be, say, 100 ng ion. If valinomycin is present, we need to be able to distinguish 99 ng ion K^+ translocated from 100 ng ion K^+ translocated so as to be able to decide whether the membrane potential generated by the pulse of respiration is zero or attains its maximum value, a task that may be presumed to lie outside the attainable experimental precision. Thus, it would be argued, the ostensible numerical equivalence of H^+ and K^+ transport in this case[34] would be only *apparently* incompatible with the generation of an energetically significant delocalized membrane potential. Fortunately, this analysis is also open to experimental test and falsification.

Using the simple electrostatic equation ($Q = CV$) that relates the voltage V(volts) across a capacitor of C(farads) when it is charged by the movement of Q(coulombs) of charge, we have, for cells or vesicles, $\Delta\psi_{max} = en/C$, where e is the elementary electrical charge (1.6×10^{-19} C) and n the number of H^+ translocated across a single cell of capacitance C. If we treat the cells as spherical shell capacitors (no membrane invaginations) of capacitance 1 μF/cm^2, a typical bacterium of diameter 1 μm has a capacitance of 3×10^{-14} F.[23,30] If we measure, as usual, the total number of H^+ translocated and assume that all are electrogenic, then n may be calculated from a knowledge of the cell numbers. If these are obtained by viable counts,[23] they will tend to be underestimated,[35] so that this, as well as the assumptions concerning both the lack of membrane invaginations and the fullness of the electrogenicity of H^+ transfer, will all serve to underestimate C and hence to overestimate the maximum attainable membrane potential $\Delta\psi_{max}$. Therefore by using small O_2 pulses and large cell numbers, $\Delta\psi_{max}$ may be made arbitrarily small, so that according to chemiosmotic considerations, the $\rightarrow H^+/O$ ratio should now be as great in the absence of the added permeant ions as in their presence. In practice, again, this behavior is not observed,[23,30] so that the suggestion that the protonmotive activity of bacterial respiratory chains leads to a substantial electrogenic proton translocation into the external bulk aqueous phase seems to be falsified.

One further point needs to be raised concerning these[23,30] experiments. Although one may vary both the cell and O_2 concentrations to try to cover

[35] C. M. Harris and D. B. Kell, *Biosensors J.* **1**, 17 (1985).

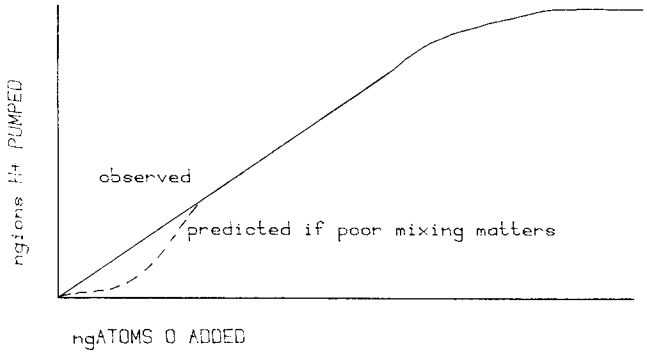

FIG. 3. A counterargument concerning the possibility that inhomogeneity of mixing in O_2 pulse experiments at low O_2 : cell ratios might obfuscate the conclusions to be drawn from the data observed. For further details, see text.

this point,[23] it is not easy to be certain, with very low O_2 : cell ratios, that imperfect mixing might not present a source of potential artifact, so that if only a small number of cells reduced all of the O_2 added, $\Delta\psi_{max}$ as calculated would now be grossly underestimated and an erroneous conclusion drawn. A further counterargument may be raised (Fig. 3) to help to exclude this possibility. Although the $\rightarrow H^+/O$ ratio is independent of the size of the O_2 pulse over a reasonably wide range (see above and op. cit.), a static head of nil net H^+ transfer is reached at yet higher O_2/cell ratios. Thus, if poor mixing is responsible for the results at low O_2/cell ratios, then a sigmoidal rather than the observed quasi-hyperbolic relation between H^+ translocation and added O_2 should be observed. Evidently, an important possible alternative is to study this problem using short bursts of saturating illumination in photosynthetic bacteria (or CO-inhibited respiratory organisms) where it may be noted that, perhaps surprisingly, a knowledge of the $\rightarrow H^+/e^-$ ratio is not required to assess the putative feedback role of a delocalized $\Delta\psi$. Knowledge only of the cell radii and numbers, together with the total H^+ movement, will suffice. The traces of Cogdell and Crofts,[36] who illuminated bacterial chromatophores with trains of single-turnover flashes, would seem to indicate that there is no change in the $\rightarrow H^+$/flash ratio under conditions in which the membrane potential should have varied over a wide range, while the full sensitivity of H^+ movement to nigericin is consistent with the view that all those H^+ moving from the bulk outer phase in either direction were doing so electroneutrally.

Despite the foregoing, ion-distribution methods have been widely used in attempts to measure the chemiosmotic membrane potential which, in all but thylakoids (see later), is supposed to dominate the pmf. Further,

[36] R. J. Cogdell and A. R. Crofts, *Biochim. Biophys. Acta* **347**, 624 (1974).

although there are apparently arbitrary relationships between the pmf values so measured and both rates and extents of phosphorylation,[2,12–15,17,19,37,38] the principles behind the ion- and weak acid/base-distribution methods have been widely discussed and mostly validated,[1,17–19,39–45] although it is worth remarking that almost all the potential sources of error such as energy-dependent probe binding[46] and low intravesicular activity coefficients will cause $\Delta\psi$ values to be *overestimated*. There is, however, one key point related to the putatively "passive" nature of ion uptake which has thus far mostly escaped discussion and which may serve to provide a stringent and decisive test for whether an energetically significant delocalized membrane potential is in fact generated by electron transport. A discussion of this point forms the subject of the following section.

Kinetics of the "Electrogenic" Secondary Uptake of Ions Used in $\Delta\psi$ Estimations

The rate of transmembrane field-driven secondary ion transport is a function both of any transmembrane potential and the "native" permeability coefficient. The principle of the ion-distribution method is that although $\Delta\psi$ is supposedly set up very rapidly (see above), the kinetics of the uptake of the permeant ions, which are commonly much slower than this,[40] should thus, in inhibitor titrations, be a function only of the $\Delta\psi$ that is calculated, as usual, when a steady state of nil net ion uptake is attained. Now, it has been widely observed[14,17,47–53] that partial restriction of

[37] D. B. Kell, *Biochim. Biophys. Acta* **549,** 55 (1979).
[38] D. B. Kell, P. John, and S. J. Ferguson, *Biochem. J.* **174,** 257 (1978).
[39] D. B. Kell, S. J. Ferguson, and P. John, *Biochim. Biophys. Acta* **502,** 111 (1978).
[40] D. B. Kell, P. John, M. C. Sorgato, and S. J. Ferguson, *FEBS Lett.* **86,** 294 (1978).
[41] E. Padan, D. Zilberstein, and S. Schuldiner, *Biochim. Biophys. Acta* **650,** 151 (1981).
[42] E. A. Berry and P. C. Hinkle, *J. Biol. Chem.* **258,** 1474 (1983).
[43] O. H. Setty, R. W. Hendler, and R. I. Shrader, *Biophys. J.* **43,** 371 (1984).
[44] R. J. Ritchie, *Prog. Biophys. Mol. Biol.* **43,** 1 (1984).
[45] E. R. Kashket, *Annu. Rev. Microbiol.* **39,** 219 (1985).
[46] J. S. Lolkema, K. J. Hellingwerf, and W. N. Konings, *Biochim. Biophys. Acta* **681,** 85 (1982).
[47] D. G. Nicholls, *Eur. J. Biochem.* **50,** 305 (1974).
[48] D. B. Kell, P. John, and S. J. Ferguson, *Biochem. Soc. Trans.* **6,** 1292 (1978).
[49] M. C. Sorgato and S. J. Ferguson, *Biochemistry* **18,** 5737 (1979).
[50] M. C. Sorgato, D. Branca, and S. J. Ferguson, *Biochem. J.* **188,** 945 (1980).
[51] M. Zoratti, D. Pietrobon, and G. F. Azzone, *Eur. J. Biochem.* **126,** 443 (1982).
[52] G. F. Azzone, V. Petronilli, and M. Zoratti, *Biochem. Soc. Trans.* **12,** 414 (1984).
[53] M. G. L. Elferink, K. J. Hellingwerf, M. J. van Belkum, B. Poolman, and W. N. Konings, *FEMS Microbiol. Lett.* **21,** 293 (1984).

the rate of electron transport does not significantly decrease the apparent $\Delta\psi$ measured either by ion-distribution methods (op. cit.) or by means of the electrochromic carotenoid response[54,55] (although the rate of phosphorylation is decreased essentially in parallel with the rate of electron transport). Under such conditions then, the rate of uptake of the ion used to estimate $\Delta\psi$ should similarly be unchanged by restricting the rate of electron transport in this way.[56] Provided then, that the apparent rate of uptake of the ion is limited by its permeability across the coupling membrane itself and not by the rate of generation of the putative $\Delta\psi$ (this may be checked by using a diffusion potential), by the rate of transfer across other barriers such as a bacterial cell wall, or by the rate of response of the measuring system (electrode, flow dialysis, etc.), a rather decisive experiment may be done.

If restricting the rates of electron transport under the above conditions decreases the rate of potential-measuring ion uptake without decreasing the apparent $\Delta\psi$ calculated in the steady state, then without a very extensive (and even mind-boggling) set of secondary hypotheses it would seem to me that it must be concluded to be membrane energization, perhaps viewed most simply as the "state" of the primary proton pumps, and not a delocalized membrane potential, which is driving the uptake of permeant ions usually used to estimate the latter. The effect upon our estimation of the *real* value of the delocalized membrane potential would then be to make it energetically insignificant; in all but thylakoids (see later) this would mean that the total pmf would be substantially below the threshold discussed above. Many systems exhibit the properties (listed above) which would permit the experiment to be performed, so that workers using such systems should report both the kinetics, and the step determining them, as well as the extent, of ion uptake. In the past, most workers have assumed that the kinetics are of no interest and have therefore just reported the $\Delta\psi$ values calculated from the uptake ratio in the steady state. I conclude that the discussion in the previous two sections would indicate that there are reasons strongly to doubt both the wisdom and the information content of this procedure.

Microelectrodes

Three separate laboratories using microelectrodes have routinely reported that the electron transport-linked, delocalized membrane potential across the mitochondrial or the thylakoid membrane is energetically insig-

[54] G. Venturoli and B. A. Melandri, *Biochim. Biophys. Acta* **680,** 8 (1982).
[55] A. J. Clark, N. P. J. Cotton, and J. B. Jackson, *Eur. J. Biochem.* **130,** 575 (1983).
[56] G. D. Hitchens and D. B. Kell, *EBEC Rep.* **3,** 243 (1984).

nificant (<50 mV), even under conditions in which a significant pH gradient either was not or would not have been formed, and even though phosphorylation can take place.[34,57–62] Against this, but a single laboratory,[63] using *Escherichia coli* and microelectrodes, did estimate a delocalized $\Delta\psi$ in the range 100–140 mV, under conditions (of pH) in which there was probably a negligible pH gradient. It is not clear in this work[63] to what extent, if any, the potential was metabolically generated, since the effect of uncouplers and ionophores was not tested. Even so, the potential estimated was still below that required to drive phosphorylation in artificial pmf experiments.[7] One might take it, then, that the tentative conclusion to be drawn on the basis of data using microelectrodes is that energy coupling via a pmf is not taking place during electron-transport phosphorylation.

Two recent technical advances in electrophysiology may help future workers to strengthen or disprove the foregoing conclusion: (1) Electrofusion of vesicles[64] to make giant mitochondria (d = 1 mm) would greatly decrease the technical demands on microelectrode placement and ATP synthesis assays; and (2) patch clamping[65] could be used to try to detect an energetically significant delocalized $\Delta\psi$ with macroelectrodes. These would seem to be exceptionally desirable experiments which, in order to obviate arguments concerning ΔpH,[1] should be done under conditions of no pH gradient.

Recently, Hamamoto *et al.*[66] did perform a patch clamp experiment on cytochrome *o* from *E. coli,* incorporated into a planar black lipid membrane (BLM). They found that a delocalized membrane potential of only 1–2 mV was observed instead of the 100–200 mV expected if the cytochrome was genuinely acting as a delocalized chemisomotic device. They sought to rationalize this by suggesting that the low value was due simply to the low resistance of their BLM (10 GΩ) such that if this resistance had been 1000 GΩ (it was argued), then the $\Delta\psi$ would indeed have attained 100–200 mV. By *reductio ad absurdam* it is easy to show that this argu-

[57] A. A. Bulychev and W. J. Vredenberg, *Biochim. Biophys. Acta* **423,** 548 (1976).
[58] W. J. Vredenberg, *in* "The Intact Chloroplast" (J. Barber, ed.), p. 53. Elsevier, Amsterdam, 1976.
[59] B. L. Maloff, S. P. Scordilis, and H. Tedeschi, *J. Cell. Biol.* **78,** 214 (1978).
[60] H. Tedeschi, *Biol. Rev.* **55,** 171 (1980).
[61] M. L. Campo, C. L. Bowman, and H. Tedeschi, *Eur. J. Biochem.* **141,** 1 (1984).
[62] D. Giulian and E. G. Diacumakos, *J. Cell Biol.* **72,** 86 (1977).
[63] H. Felle, J. S. Porter, C. L. Slayman, and H. R. Kaback, *Biochemistry* **19,** 3585 (1980).
[64] U. Zimmerman, *Biochim. Biophys. Acta* **694,** 227 (1982).
[65] B. Sakmann and E. Neher, *Annu. Rev. Physiol.* **46,** 455 (1984).
[66] T. Hamamoto, N. Carrasco, K. Matsushita, H. R. Kaback, and M. Montal, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 2570 (1985).

ment is, to say the least, inadequate: By extending the same reasoning, one has to say that if the resistance had been 10 TΩ, the membrane potential would have been 1–2 V, a value exceeding the thermodynamically available free energy in the driving redox reaction! The appropriate calculations for this type of situation must take at least the following into account: (1) whether the putative chemiosmotic device acts as a voltage source or a current source, (2) the relationship between the source impedance and the membrane impedance, (3) the fact that the possession of a capacitance by a BLM means that it can store charge, so that the membrane impedance is a time-dependent (or frequency-dependent) quantity. In other words, calculations of this type based simply on the DC resistance of the membrane are entirely misleading, and workers using the patch clamp technique in this kind of system should be aware that there are reasons to suppose that macroscopic membrane potentials exceeding, say, 100 mV are by no means the expected result, and that a systematic study incorporating the above features is required to come to a sensible decision concerning the real values of delocalized membrane potential actually generated by these putatively protonmotive proteins.

Thus far, I have concentrated largely on the values of the delocalized $\Delta\psi$ generated by electron transport. In many cases, the ΔpH values, although small, may be validated by independent means,[17] and are apparently reliable. In thylakoids, however, it is thought by many that a *large* ΔpH is generated by electron transport. We must now examine the evidence for such a view.

Light-Dependent pH Gradient across the Thylakoid Membrane

Many measurements based on the uptake of weak bases are apparently consistent with the view that the light-driven ΔpH in chloroplast thylakoids approaches and may exceed the threshold value of ~2.5 units necessary for initiating phosphorylation *in vitro*. (Measurements of Cl^- distribution give no evidence for a significant, delocalized steady-state membrane potential.[67] In these (former) experiments[68,69] the usual controls do not give any evidence for (although cannot exclude) gross probe binding and other such artifacts which would cause one substantially to overestimate ΔpH. In thylakoids, it would seem that one of the biggest problems lies in determining to what extent, if any, there actually *is* an intrathylakoidal bulk aqueous phase and to what extent there is electro-

[67] H. Rottenberg, T. Grunwald, and M. Avron, *Eur. J. Biochem.* **25,** 54 (1972).

[68] H. W. Heldt, K. Werdan, M. Milovancev, and G. Geller, *Biochim. Biophys. Acta* **314,** 224 (1973).

[69] A. R. Portis and R. E. McCarty, *J. Biol. Chem.* **249,** 6250 (1974).

chemical equilibrium (in the steady state) between membrane-bound, double-layer, and "bulk phase" ions, including probe ions. What little evidence there is[70] suggests that a great deal of methylamine may in fact be bound to the thylakoid membrane. Further, estimates of ΔpH based upon the kinetics of $P\text{-}700^+$ reduction, and not requiring the use of probes, suggest that ΔpH during photophosphorylation at pH 7.5 is only 0.5–1 unit.[71] It is unfortunate that pH-sensitive microelectrodes[72,73] have not yet been employed by workers with thylakoids. However, in view of the recent important demonstration[10] that, inter alia, an artificial pmf cannot contribute to postillumination phosphorylation, I am of the opinion that the weak base-distribution methods, though reliable in many other systems, are letting us down in thylakoids by causing us substantially to overestimate the light-dependent pH gradient by means of an as yet uncertain mechanism. Following problems raised by Dilley and colleagues,[74] Junge and colleagues[75] have also concluded that much of the protonmotive activity of thylakoids cannot be linked to the generation of a delocalized ΔpH.

One point worth mentioning in relation to the use of probe methods in thylakoids is that there is often, as judged by probe uptake, a substantial ΔpH in the dark in green plant photosynthetic systems. In the homogeneous phosphorylating chloroplast system of Heldt and colleagues,[68] this dark ΔpH amounted to 0.9 units, while the pH gradient in the light was judged to be 2.26 units. It is not clear, on the basis of present knowledge,[76,77] to what extent (if any) the light-dependent ΔpH should be corrected for that in the dark, although it should be noted that 2.26 units are already below the apparent threshold of 150 mV, let alone the pmf values required for rapid phosphorylation. Teleologically, it is reasonable that the "purpose" of light-dependent H^+, K^+, and Mg^{2+} movements is to activate the enzymes of the Calvin cycle.[68] It should be noted that in probe methods that rely upon the measurement of the disappearance of

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[71] A. N. Tikhonov, G. B. Khomutov, E. K. Ruuge, and L. A. Blumenfeld, *Biochim. Biophys. Acta* **637,** 321 (1981).

[72] H. J. Berman and N. C. Hebert (eds.), "Ion-Selective Microelectrodes." Plenum, New York, 1974.

[73] R. C. Thomas, "Ion-Sensitive Intracellular Microelectrodes: How to Make and Use Them." Academic Press, London, 1978.

[74] L. J. Prochaska and R. A. Dilley, *Arch. Biochem. Biophys.* **187,** 61 (1978).

[75] W. Junge, Y. Q. Hong, L. P. Qian, and A. Viale, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 3078 (1984).

[76] D. Walz, *in* "Biological Structures and Coupled Flows" (A. Oplatka and M. Balaban, eds.), p. 45. Academic Press, New York, 1983.

[77] D. Walz, *EBEC Rep.* **3,** 273 (1984).

probe from the outer phase, a dark ΔpH of 1 unit would likely be unmeasurable in terms of the extent of probe disappearance, while subtracting the calculated ΔpH would have a dramatic effect upon the estimated light-dependent ΔpH.

Finally, it is worth mentioning that the postulated large ΔpH in thylakoids can have effects, particularly on the kinetics of photophosphorylation, that are independent of any energetic considerations and would not occur if the ΔpH were substituted by $\Delta\psi$ of equivalent magnitude. In particular, the inhibitory effect of a low internal pH is pointed up by the experiments of Giersch[78] in relation to the stimulation of photophosphorylation by nigericin. Thus, the coexistence of both stimulatory and inhibitory effects on phosphorylation caused by the imposition of an acid internal pH in thylakoids may well account for the stimulation in ATP yield caused by the accumulation of permeant amines in the thylakoid lumen in postillumination phosphorylation experiments,[79,80] an observation which is otherwise to me most difficult to understand in other than delocalized chemiosmotic terms.

I do not intend to add to the abundant literature criticizing the use of the notorious 9-aminoacridine as a quantitative monitor of ΔpH in thylakoids, save to remind readers that it surely overestimates by at least 1–1.5 units.[37] Particularly in view of the threshold phenomenon (Fig. 2), therefore, I can only recommend that its use be discontinued for those experiments designed to assess whether the ΔpH generated by illuminating thylakoids is large enough to serve as an energy coupling intermediate in photophosphorylation.

The general conclusion is that none of the presently available methods for measuring the pmf gives one confidence that any value estimated therefrom which actually exceeds the threshold is in fact reliable. For these and other reasons, other approaches to assessing the veracity of delocalized chemiosmotic coupling in electron transport phosphorylation have been sought. In the next section I will briefly touch upon two of them: reconstitution experiments and double inhibitor titrations.

Reconstitution of Phosphorylation

Following the lead of Racker and Stoeckenius,[81] many workers have assumed that oxidative and photosynthetic phosphorylation has been re-

[78] C. Giersch, *Biochim. Biophys. Acta* **725**, 309 (1983).

[79] N. Nelson, H. Nelson, V. Naim, and J. Neumann, *Arch. Biochem. Biophys.* **145**, 263 (1971).

[80] W. A. Beard and R. A. Dilley, *EBEC Rep.* **3**, 221 (1984).

[81] E. Racker and W. Stoeckenius, *J. Biol. Chem.* **249**, 662 (1974).

constituted many times, with rates that are similar to those *in vivo*. As discussed at much greater length elsewhere,[2] when properly purified components are used, neither the turnover numbers of the F_0F_1 enzymes, nor the $P/2e^-$ ratios (where appropriate), nor the ability to build up a substantial phosphorylation potential remotely approach those observed in the "native" energy coupling membrane. Only when impure preparations are used can some of these things be observed. One conclusion that may be drawn is that this type of energy coupling requires additional proteinaceous components distinct from the primary and secondary proton pumps themselves.[2,82,83] It may be argued, if one accepts that purified reconstituted systems do not in fact work at rates remotely comparable to those *in vivo*, that they are not generating a large enough pmf, given the threshold requirement; no such demonstration to date exists.[84] Arguments based upon the rates of phosphorylation catalyzed by purified F_0F_1 in response to the artificial pmf do not serve greatly to clarify matters, since, as discussed above, the proper argument rests upon whether a comparably large pmf is actually generated by electron transport.

Given the foregoing, therefore, I am unable to conclude that the systems reconstituted to date, which contain a primary proton pump plus purified F_0F_1 as their "sole" protein components, may be used to argue in favor of the veracity of delocalized chemiosmotic coupling in electron transport phosphorylation. Rather, the failure of the purified systems compared with the relative success of those containing the so-called hydrophobic proteins[2] argues in favor of the view that components distinct from the appropriately oriented primary and secondary proton pumps plus a relatively ion-impermeable membrane are required for efficient protonmotive energy coupling.[2,83,84] That all the unassigned reading frames (URFs) of the mammalian mitochondrial genome code for hydrophobic proteins is a powerful hint that one or more of these URFs may serve the role of such "protoneural" coupling proteins.[2,83]

In reconstituted systems of phosphorylation, neither structural nor functional interactions between the particular proton pumps are usually properly specified or even considered. One approach to determining the localization of this type of functional linkage lies in the so-called double (dual)-inhibitor titrations, a topic which is now discussed.

[82] D. B. Kell, D. J. Clarke, and J. G. Morris, *FEMS Microbiol. Lett.* **11,** 1 (1981).

[83] D. B. Kell and J. G. Morris, *in* "Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria" (F. Palmieri, E. Quagliariello, N. Siliprandi, and E. C. Slater, eds.), p. 339. Elsevier, Amsterdam, 1981.

[84] G. Hauska, D. Samoray, G. Orlich, and N. Nelson, *Eur. J. Biochem.* **111,** 535 (1980).

Dual-Inhibitor Titrations

Leaving aside compounds such as "anisotropic inhibitors,"[85] other lipophilic ions,[86] general anaesthetics,[87] and other low-MW compounds of as yet uncertain action which do not appear to cause slip and which may be described as decouplers,[2,88] three types of inhibitors of electron transport phosphorylation may be recognized: electron transport inhibitors, energy transfer (ATP synthase) inhibitors, and uncouplers. Since both localized and delocalized coupling models can account for the synergism observed[55,89] between electron transport inhibitors and uncouplers, we will not discuss this here. Neither will we survey the literature on uncouplers per se in relation to observable protonophoric/ionophoric activity and/or whether they may induce slip in proton pumps, particularly since support for the latter possibility[90] is to date based solely upon experiments using the ion-distribution methods for determining the pmf, which have been criticized above. Thus we consider two types of dual-inhibitor titration which may in principle be used to distinguish localized and delocalized coupling models: those in which phosphorylation is titrated either with electron transport inhibitors or with uncouplers in the presence and absence of partially inhibitory titers of energy transfer inhibitors. The symmetrical experiments may also be considered; they give conceptually similar results.

The idea behind electron transport/energy transfer inhibitor titration experiments is broadly as follows: If energy coupling is delocalized, then decreasing the rate of the overall reaction with, say, the energy transfer inhibitor should make the other inhibitor less inhibitory.[2,12–14,17,54,91–98] In

[85] T. Higuti, *Mol. Cell. Biochem.* **61,** 37 (1984).
[86] A. Zaritzky and R. M. Macnab, *J. Bacteriol.* **147,** 1054 (1981).
[87] H. Rottenberg, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 3313 (1983).
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[89] G. D. Hitchens and D. B. Kell, *Biochem. J.* **212,** 25 (1983).
[90] D. Pietrobon, G. F. Azzone, and D. Walz, *Eur. J. Biochem.* **117,** 389 (1981).
[91] J. S. Kahn, *Biochem. J.* **116,** 55 (1970).
[92] H. Baum, G. S. Hall, J. Nalder, and R. B. Beechey, *in* "Energy Transduction in Respiration and Photosynthesis" (E. Quagliarello, S. Papa, and C. S. Rossi, eds.), p. 747. Adriatica Editrice, Bari.
[93] L. Ernster, *Annu. Rev. Biochem.* **46,** 981 (1977).
[94] G. D. Hitchens and D. B. Kell, *Biochem. J.* **206,** 351 (1982).
[95] G. D. Hitchens and D. B. Kell, *Biosci. Rep.* **3,** 743 (1982).
[96] H. V. Westerhoff, A. Coen, and K. van Dam, *Biochem. Soc. Trans.* **11,** 81 (1983).
[97] I. P. Krasinskaya, V. N. Marshansky, S. F. Dragunova, and L. S. Yaguzhinsky, *FEBS Lett.* **167,** 176 (1984).
[98] H. V. Westerhoff, S. L. Helgerson, S. M. Theg, O. van Kooten, M. Wikstrom, V. P. Skulachev, and Z. S. Dancshazy, *Acta Biol. Acad. Sci. Hung.* **18,** 125 (1984).

practice, no such decrease in potency is observed. In contrast, for electron transport phosphorylation, decreasing the rate of phosphorylation with a partially inhibitory titer of an energy transfer inhibitor actually increases the potency of uncoupler molecules.[89,95,99] If the decrease in the rate of phosphorylation caused by the two types of inhibitor (electron transfer inhibitor and uncoupler) is mediated via a decrease in the pmf, then the forms of the two types of titration curves should be changed in the same way when the rate of phosphorylation is initially decreased by the use of an energy transfer inhibitor. In other words, if the titer of electron transfer inhibitor stays the same, then so should the uncoupler titer, or if the former is increased, then so should the latter. That they are changed differently defeats any common-sense and self-consistent attempt to explain the two types of data in terms of the putative value of the pmf or any other delocalized coupling intermediate, so that these data are then explicable only in terms of localized coupling theories.

We now consider conceptual and methodological problems which have been or may be raised in this context and which might serve to cause the foregoing to be an erroneous conclusion, paying particular regard to our own experiments, and also providing the relevant counterarguments to the points raised.

1. The membranes have energy leaks[92] and/or are heterogeneous in their energy coupling properties; counterargument: the P/2e^- ratio (in the absence of added uncoupler) is independent of the rate of electron transport.[54,94,100–102]

2. The pmf-dependent binding of the inhibitors might obfuscate the analysis[98]; the counterargument: the titrations are symmetrical,[12,95] and further, in one case,[54] a covalent inhibitor together with trains of saturating light flashes were used.

3. Electron transfer or energy transfer inhibitors might be uncoupling or causing slip as well as acting in their primary role[98]; counterargument: if so, the former would be more potent when the energy transfer inhibitors are present, and vice versa; also, the latter behavior would be in marked contrast to that observed with energy transfer inhibitors generally.[99]

4. Adenylate kinase activity is present and may serve to obfuscate the truth; counterargument: diadenosine pentaphosphate (Ap_5A) is always present.

[99] G. D. Hitchens and D. B. Kell, *Biochim. Biophys. Acta* **723,** 308 (1983).

[100] S. J. Ferguson, P. John, W. J. Lloyd, G. K. Radda, and F. R. Whatley, *FEBS Lett.* **62,** 272 (1976).

[101] J. B. Jackson, G. Venturoli, A. Baccarini-Melandri, and B. A. Melandri, *Biochim. Biophys. Acta* **636,** 1 (1981).

[102] N. P. J. Cotton and J. B. Jackson, *FEBS Lett.* **161,** 93 (1983).

5. The uncoupler/energy transfer inhibitor titrations are an experimental artifact caused by the nonattainment of a stationary state[102]; counterargument: a continuous ATP synthase assay was used by us specifically to ensure that a strictly stationary state was attained,[94] including in these experiments; also, comparable findings concerning the potency of uncouplers in the presence and absence of inhibitors of the output proton pump were obtained by others using $^{32}P_i$ in thylakoids[103] and spectrophotometry in submitochondrial particles.[98,104] We remain unable to account for the inability of Cotton and Jackson[102] to obtain linear rates of phosphorylation in their system and stress again that their conclusions, based upon demonstrably nonstationary conditions, cannot be extended either to the work of Hitchens and Kell (op. cit.) or to the general case. It is worth noting (a) that their Fig. 1B[102] indicates that even under their "favorable" conditions, the percentage of inhibition of phosphorylation by uncoupler was not independent of the presence of the energy transfer inhibitor, and (b) the conditions of the experiments of these workers differed materially from those of ourselves in respect to the following: sucrose, K^+-acetate, ADP, K^+-phosphate, Na^+ succinate and H^+ concentrations, and buffering power. Other points relevant to this question are discussed elsewhere.[2]

6. The uncoupler/energy transfer inhibitor titrations do not hold for all uncouplers[104]; counterargument: even if localized coupling is occurring, an apparently delocalized result will be found if the phosphorylation-inhibiting, uncoupling step itself is not rate limiting in determining the uncoupler potency. In particular, van der Bend and colleagues[105] have argued that they expect, and find, that the uncoupler titer for inhibiting photophosphorylation in a coreconstituted system containing bacteriorhodopsin and a yeast H^+-ATP synthase is independent of the extent of inhibition of the latter. Not only is this system grossly inefficient to start off with (~2% of the *in vivo* turnover number, and see critique above), but the titer of uncoupler necessary for full uncoupling in this system (which was not in fact obtained) exceeded the concentration of ATP synthase by at least 30-fold! Only "substoichiometric" uncouplers are suitably used in this type of experiment, and therefore the findings in the above experiment (Ref. 105; Fig. 2) are both unsurprising and irrelevant to the debate.

Thus, the conclusion from the available data thus far using the double inhibitor–titration approach would seem to be that they provide strong

[103] J. W. Davenport, *Biochim. Biophys. Acta* **807,** 300 (1985).

[104] M. A. Herweijer, J. A. Berden, and A. Kemp, *EBEC Rep.* **3,** 241 (1984).

[105] R. L. van der Bend, J. Peterson, J. A. Berden, K. van Dam, and H. V. Westerhoff, *Biochem. J.* **230,** 543 (1985).

evidence for "localized coupling"[54] or "energy transfer domains."[93] Since the experiments involved are technically relatively straightforward, requiring only the measurement of rates of phosphorylation, other workers should attempt them in other systems. The uncoupler/energy transfer inhibitor experiments therefore add further weight to the arguments raised by Ort and Melandri[106] in their excellent and comprehensive demolition job on earlier (and influential) experiments in which the observation that one ionophore per thylakoid or per chromatophore would cause a certain amount of uncoupling was used in support of the delocalized chemiosmotic coupling concept. Finally, we should mention that the harmonization of double inhibitor–titration protocols within the framework of "metabolic control theory"[107] might constitute a particularly rigorous and rewarding approach to this problem.[2,12,108]

New Approaches

As discussed above, I have dwelt mainly on a critical analysis of experiments that pertain to or have as their theoretical framework the delocalized chemiosmotic coupling concept. It may be argued,[2,12,83] in view of the type of problem raised herein and elsewhere, that entirely new experimental approaches, such as laser Raman spectroscopy,[109] might shed more light on the energy coupling process. On the theoretical side, I do not believe that we have yet come adequately to grips with the problem that macroscopic measurements in stationary states cannot easily distinguish fast, infrequent events from slow but common ones, yet this distinction is of the first importance for far from equilibrium systems.[110] Experimentally, I have chosen to initiate a dielectric spectroscopic approach to the study of energy coupling membrane systems.[29,111,112] However, the main conclusion to date[111–113] is that with the present level of experimental

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[107] H. Kacser and J. A. Burns, *Symp. Soc. Exp. Biol.* **32,** 65 (1973).

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[112] C. M. Harris, G. D. Hitchens, and D. B. Kell, *in* "Charge and Field Effects in Biosystems" (M. J. Allen and P. N. R. Usherwood, eds.), p. 179. Abacus Press, Tunbridge Wells, 1984.

[113] D. B. Kell and C. M. Harris, *Eur. Biophys. J.* **12,** 181 (1985); C. M. Harris and D. B. Kell, *Eur. Biophys. J.* **13,** 11 (1985); D. B. Kell and C. M. Harris, *J. Bioelectricity* **4,** 317 (1985).

sensitivity, more is to be learned about the mobilities of proteins than of protons in this type of system. Perhaps the fact that this extent of protein mobility *in situ* appears[2,111-114] to be significantly lower than that envisaged in the original fluid mosaic model will turn out to be one of the more noteworthy features of the future evolution of the localized coupling concept.

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[41] Methods for the Determination of Membrane Potential in Bioenergetic Systems

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The measurement of membrane potential, strictly the electrical potential difference between two bulk phases separated by a membrane, is a central technique in bioenergetics. Since the small size of bioenergetic organelles prevents the direct application of microelectrode techniques, indirect methods must be resorted to. In this chapter we shall outline the practical details of membrane potential determination in a photosynthetic system, the bacterial chromatophore, and in two respiratory systems, the isolated mitochondrion and the *in situ* mitochondrion within an isolated nerve terminal. Although these systems are widely divergent, they serve to illustrate the approaches which are currently being taken to quantify this parameter, as well as the pitfalls which have to be avoided.

Measurement of Ionic Currents and Membrane Potentials by Electrochromism

The use of carotenoid and chlorophyll electrochromism for the measurement of the magnitude of the electric potential ($\Delta\psi$) across thylakoid membranes was developed by Junge and Witt.[1] The technique is espe-

[1] W. Junge and H. T. Witt, *Z. Naturforsch.* **23b,** 244 (1968).