REVIEW
Probes for Energy Transduction in Membranes

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Introduction *

Probes are small molecules, provided with environment-sensitive spectro-scopic properties. When inserted into membranes they report upon physical parameters of their microenvironment. For detailed and extensive analysis of the problem, a number of recent review articles is recommended [1–5]. The validity of the physical information obtained by the probe technique is subjected to the two following questions: Where are probes located in membranes? How much of what a probe is sensing is an environment created by itself? A detailed analysis of these problems would require a long list of examples for a number of probes and different membranes [3, 5]. It is sufficient here to state that in a number of cases the location of the probe in the membrane is rather safely identified. Evidence for such a location has been obtained by comparison with model membrane systems [6], from independent measurements [7, 8] (X-ray diffraction, nuclear magnetic resonance, electron spin resonance), from solvent perturbation data [2], and from the direct analysis of the probe emission data [9, 10]. When dealing with

* Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonic acid; As, 12-antranoylstearic acid; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; Nitroxide, nitroxyl group, sterically protected in the α positions, such as derivatives of 2,2,6,6-tetramethyl-piperidine-1-oxyl, 2,2,5,5-tetramethylpirrolidine-1-oxyl, or 4,4'-dimethyloxazoli-dine-N-oxyl derivative of a keto fatty acid; NCCD, N-(2,2,6,6-tetramethylpiperi-dine-1-oxyl)-N'-(cyclohexyl)carbodiimide.

the use of stoichiometric inhibitors provided with useful spectroscopic properties, the location of the probe-inhibitor molecule in the membrane is automatically identified with the site of inhibition [11–14].

As to the problem of probe-induced perturbation of the membrane system, despite the obvious alteration introduced by a foreign molecule, there are good reasons to believe that not all the information content of the measurement is lost.

X-ray diffraction analysis of lecithin dispersions [7, 15] indicate that ANS and AS do not perturb significantly, at low concentrations, the electron-density profile of the membrane. The study of phospholipid–protein complexes (cardiolipin–cytochrome-c, lysozyme–cardiolipin) by different techniques such as circular dichroism or X-ray diffraction has given information regarding the interactions and distances between protein and lipid, in quantitative agreement with those obtained by a fluorescent-probe analysis [16, 17]. Transition temperatures, obtained with fluorescent probes or spin-labels in natural and model membranes, are consistent with those obtained by light scattering [18].

The above examples suggest that despite the fact that local regions of disorder are created by the insertion of the probe in a membrane, useful information, is however, transmitted through the boundaries of the regions where the probes are located, and thus the probe may sense ordering, changes in fluidity, and other physical events. Therefore the structural differences between the probes and the components of phospholipid membranes do not reduce the information obtained from the probe.

The Information Content of the Probe Technique

The spectral analysis of fluorescent, absorbing, and spin probes permits an evaluation of the characteristics of the environment around them. The ANS-type probes have fluorescent quantum yields and emission maxima which are related to the polarity and relaxation time of solvent molecules [19]. From polarization and lifetime data it is possible to utilize fluorescent probes for measuring the fluidity of their environment, and in some cases microviscosity data can also be obtained [20].

Distances between a suitable pair of chromophores can be measured by using Förster energy-transfer theory [21, 22]. Fluorochromes having electric-potential-sensitive emission properties (electrochromism) [23] can be used for potential measurements. Potentials can be detected also by probes, which are transported or bound more or less firmly as a consequence of their electric charge (ANS⁻, ethidium⁺, Skulachev ions [24]. pH differences across membranes can be indicated by the distribution of fluorescent simple amines across the membrane, associated with emission changes [25].

The nitroxide derivatives permit evaluation of membrane fluidity from parameters derived from the lineshape of the spectrum [3, 4]. The distance between the spectral lines, a function of solvent polarity, may give information on this parameter [3, 4]. Paramagnetic interaction between spin-labels affords measurements of interprobe distances [26].

The potentialities of probe application to membrane studies have not been fully explored yet, and the possibility of measuring protein slow relaxation times by triplet probes or by special electron spin resonance analyses is only beginning to be investigated [27, 28].

The Microviscosity of the Coupling Membrane

In light of the theories [29, 30] describing the membrane as a fluid mosaic, in which the interactions between membrane components may strictly depend on the viscosity of the lipid moiety, data on the microviscosity of energy-transducing membranes and their changes are of great importance.

The probes devised for the study of membrane fluidity are of two types: fatty acids (or phospholipids) labeled at different distances from the polar head groups with a fluorescent or spin probe or alternatively hydrophobic molecules, such as perylene or pyrene or uncharged nitroxide derivatives. Data on the microviscosity of the mitochondrial membrane and for comparison of other natural and artificial membranes are reported in Table I.

Mitochondrial membrane at $37°$ C has a microviscosity of 95 cP [31]. Such a value is intermediate between that of egg lecithin, 73 cP, and that

TABLE I. Microviscosity of biological membranes

Membrane	Probe	Method	t($°$C)	Viscosity (cP)	Ref.
Dimyristoyl lecithin	Benzopyrene	excimer	32	56	32
	Pyrene	excimer	30	59	32
Dipalmitoyl-Lecithin	Perylene	polariz.	37	390	33
	Perylene	polariz.	45	94	33
Egg Lecithin	Perylene	polariz.	37	73	33
	Pyrene	excimer	20	57	32
	Perylene	polariz.	20	150	33
Erythrocyte	Perylene	polariz.	37	132	31
	Pyrene	excimer	25	62	32
Sarcoplasmic reticulum	Pyrene	excimer	20	29	32
Lymphocytes	Perylene	polariz.	37	109	31
Mitochondria	Perylene	polariz.	37	95	31
Synaptosomes	Perylene	polariz.	37	85	31

of erythrocytes, 132 cP, consistent with the lipid composition of the mitochondrial membrane, rich in unsaturated fatty acids and with no cholesterol content [34].

Some evidence that fluidity changes occur during energy transduction in mitochondria and chloroplasts has been also reported. Timberg et al. [35] observed that hydrophobic spin-labels partition differently during the cycle of an energy-dependent oscillation in rat liver mitochondria. Light induces in chloroplasts [36] a small change in the partitioning of a hydrophobic spin probe, equivalent to a 2°C temperature shift. The apparent rotational relaxation time of ANS in membrane fragments [37] is slightly, but significantly, increased by energy conservation.

Feeding rats on a diet rich in unsaturated fatty acids [38], results in an increased fluidity of mitochondrial membranes as revealed by the use of spin probes. The efficiency of energy conservation and of ion transport is not affected, however. Thus to a certain degree, membrane fluidity is not a critical parameter for oxidative phosphorylation.

On the other hand a number of reports indicate that parallel to changes in membrane fluidity (induced by varying the temperature), changes in enzymatic activities of the membrane also occur. Abrupt changes in the motion of spin-labeled fatty acids and in the activation energy of succinate oxidase and of ATPase occur at the same temperature [39, 40]. Since different reactions have identical breaks in their activation-energy profiles, changes in the physical properties of membrane phospholipid are considered of primary importance. An identity was also found between this critical temperature and that below which mitochondria from different animals and plants become injured upon exposure [41, 42].

Many of the abrupt changes in spin-label motion and enzymatic activity reported above are centered around 20°C for mammalian mitochondria, indicating that they are probable *not* a transition between crystalline and liquid-crystalline phases [43]. Such a transition for rat liver mitochondria is centered around 0°C.

The nature of the transitions observed by changing the temperature of mitochondrial membranes may be inferred from the studies of Lee et al. [44]. Using TEMPO (a hydrophobic spin-label), a transition temperature at 30°C was found in a dispersion of pure phospholipids (dioleoyllecithin), much above the crystalline to liquid-crystalline transition temperature of −22°C. The transition at 30°C can be interpreted in terms of the formation, at a critical temperature, of "clusters" of phospholipids, which, excluding the label, would simulate a transition to a crystalline state. Also in the presence of sarcoplasmic reticulum ATPase–dioleoyllecithin complex, a transition at 29°C was found, parallel to an abrupt change in the activation energy of the reaction. It is therefore probable that the transition occurring at 25°C in the native sarcoplasmic reticulum membrane reflects more a process of "cluster" formation than

a phase transition. If these results are extended to mitochondrial and chloroplast membranes, the conclusions that the observed transition temperatures reflect phase changes, or changes in the arrangement of phospholipids, with the formation of clusters are equally probable.

Further evidence for a lack of homogeneity in the physical state of phospholipids of mitochondrial membranes comes from the finding that the phospholipid molecules closely associated with the cytochrome c oxidase have a highly restricted motion as detected by spin-labeled fatty acids [45, 46] while the molecules further apart from the protein boundary behave as in a fluid bilayer. It appears clear, therefore, that probes, which are supposed to label the membrane randomly, may either report an average picture of different membrane local states, as previously discussed, or preferentially reflect the state of one region, with respect to others.

Specific Interactions between Membrane Components

The use of site-specific spin-labels or fluorescent probes has been introduced to study this problem. In mitochondria specific side chains of cytochrome-c can be spin-labeled by iodoacetamidenitroxide [47, 48]. The use of spin-labeled cytochrome derivatives has permitted the identification of the side of the protein where methionine 65 is located as the one interacting with the membrane, while the protein region where cysteine 103 (of yeast cytochrome-c) is located interacts preferentially with the solvent [47, 48]. Neither residue appears to interact with the isolated reductase or oxidase [49].

Specific labeling of mitochondrial ATPase and of its natural inhibitor has been also achieved [50]. Stoichiometric inhibitors of membrane enzymes, when provided with useful spectroscopic properties, can afford an excellent type of site-specific probe. Antimycin has been utilized to explore structural aspects of the $b-c_1$ segment of the respiratory chain of mitochondria [11], while aurovertin has been useful in elucidating structural facets of the mitochondrial soluble ATPase and its interaction with ligands, such as ADP, ATP, Mg ions, inorganic phosphate, and the ATPase natural inhibitor [12, 51]. In particular, structural changes have been inferred from the fluorescence intensity changes of the inhibitor associated with energy conservation [52].

Another ATPase inhibitor, a water-insoluble carbodiimide containing a nitroxide, NCCD, has been employed as a spin-label of the membrane-bound ATPase complex of mitochondria [13, 53]. The label was found to bind to a membrane proteolipid at a rigid site having low polarity. The conformation of the proteolipid was found to be strictly dependent on the presence of phospholipids. The binding site of the inhibitor has been

found to be at about 20 Å distance from the binding site of ATP on the complex.

The use of derivatives of acyl-CoA, with hydrocarbon chains labeled at different distances by a spin probe has been useful in exploring the environment of the ADP/ATP translocator in mitochondria. Acyl-CoA has proved to be an effective inhibitor of the translocation reaction. When the label is located between the first and eighth carbon atoms of the hydrocarbon chain, the immobilization of the spin-label was stronger than that of labels placed beyond the eighth position. The conclusion that the translocator extends only for a short depth into the membrane appears interesting [54].

The Generation of Electric Potentials

It was mentioned earlier that fluorescent probes may be utilized for the detection of membrane potentials. The dependence on "electric charge" of ANS binding to phospholipid model membranes and natural membranes was intuitively recognized since the first reports on the use of this probe [57–60]. Evidence in favor of the electrostatic interaction of ANS with membranes was based on the increase of ANS binding caused by cations, local anesthetics, and low pH [57–62].

Mitochondrial membranes in the presence of ANS or similar probes respond not only to changes of the ionic strength, the presence of divalent cations, and local anesthetics, but also in accordance with their metabolic activity [57]. When mitochondrial fragments are supplemented with oxidizable substrates which activate ATP synthesis (NADH, succinate or ascorbate *plus* phenazinemethosulfate) or with ATP itself, the fluorescence of ANS is increased several-fold [37, 57, 63, 65]. Thus ANS can monitor a situation in the membrane which can be produced either from the substrate or from the ATP side of the chain of energy-conserving reactions.

In mitochondrial fragments the fluorescence changes of ANS correlate with binding changes [63, 65, 66]. Evidence has also been produced that at relatively low ANS/ protein ratios, binding changes correspond quantitatively to fluorescence changes [63, 65].

Positively charged fluorescent probes such as ethidium bromide bind to mitochondrial fragments with a fluorescence increase, and energy conservation produces both a detachment of the bound probe molecules and a fluorescence decrease, as a consequence of a decrease of the affinity of the probe for the membrane [67–69].

The opposite behavior of anionic and cationic probes has suggested that mitochondrial fragments modify their membrane potential going from resting to energized states [67, 68].

The increased affinity of negative probes for energized mitochondrial fragments, the opposite behavior of some positive probes, the lack of changes for neutral probes, and the knowledge that ANS and ethidium binding are likely to be the consequence of potential changes [61, 62] have been considered as good evidence that the membrane potential becomes more positive in membrane fragments as a consequence of energy conservation.

Intact mitochondria also exhibit fluorescence changes of ANS and ethidium as a consequence of energy conservation but they are in the opposite direction with respect to membrane fragments [65, 67, 68, 71, 72], i.e., a decrease of fluorescence and binding of ANS follows ATP or substrate addition to intact liver, pigeon heart, and rat heart mitochondria.

It is conceivable that, as a consequence of membrane function, changes in the dissociation constant of surface groups may lead to a different charge density and an increase or decrease in the binding constants of charged probes. It obviously cannot be decided whether such a difference is the consequence of an increase in the negative groups or a decrease of the positive groups of the membrane, and thus hypotheses such as those proposed by Azzi [72], Chance [71], or Radda [64] are equally acceptable. It is of course appealing to consider that proton movements are associated with mitochondrial energy conservation and that a "membrane Bohr effect" [73], giving primary importance to proton association or dissociation from membrane groups, can account for changes in the surface-charge density of the membrane and in its relative affinity for charged probes. Such a possibility is supported by the pH dependence of ethidium binding to mitochondria in different states [74].

An alternative interpretation of ANS fluorescence changes during energy conservation in mitochondria is offered by Jasaitis et al. [75, 76] who consider the ANS fluorescence changes to be a consequence of electrophoretic movement of the probe in the electric potential generated across the mitochondrial membrane. The conclusion of Jasaitis et al. [75, 76] is based on the assumption that ANS can penetrate across the mitochondrial membrane and behaves analogously to the synthetic organic cations and anions utilized as probes for transmembrane potentials [24].

The possibility that ANS responds to either a surface potential or a transmembrane potential appears well documented. What is not clear is whether ANS can distribute across the membrane under the influence of the transmembrane potential (this appears difficult, at least in model systems, due to the impermeability of the pure phospholipid membranes to charged molecules), or rather is able to respond to a transmembrane potential by binding more tightly to one side of the membrane without

diffusing across it, or by responding to the modification of the surface potential induced by the transmembrane potential.

The question as to whether fluorescence changes of ANS, not accounted for by binding of the probe, are associated with energy conservation in mitochondrial membranes has been the object of long discussions. Brocklehurst et al. [37], by extrapolating the fluorescence of membrane fragments in the nonenergized state and succinate-energized state at infinite membrane concentration, found a different value in the two states. The energized state was characterized by higher quantum yields of ANS and longer lifetimes with respect to the nonenergized state. Azzi and Santato [67] have shown, however, that ATP does not lead to a change in extrapolated quantum yield. Thus energization of the membrane is not associated necessarily with a change in the environment of probe molecules. Moreover, Chance [73] has indicated that the lifetime of the fluorescence decreases, contrary to the observation of Brocklehurst et al. [37, 64] on energization. Also Brocklehurst et al. [77] arrived at the conclusion that for the ANS fluorescence changes to occur in mitochondrial membrane it is necessary that the probe be free to move. Covalently bound ANS or TNS derivatives do not in fact show fluorescence changes. Finally Azzi and Santato [67] have indicated that the biphasic kinetics of the transition produced by uncouplers do not reflect the existence of two pools of molecules bound to the membrane with different fluorescence quantum yields, but rather a technical artifact arising from a direct competition between the uncoupler and the probe for membrane binding. Thus the claim that energy conservation in mitochondria is associated with an increase in the quantum yield of bound ANS [2, 64] has not been substantiated, and the most likely conclusion is that energy conservation is associated with only changes in the binding of charged probe molecules, with increase in their fluorescence intensity, reflecting electric potential changes of the mitochondrial membrane.

Merocyanines [78] have been also employed in the detection of membrane potentials. After the demonstration that some merocyanines are able to follow (with fluorescence changes) very closely the changes in the action potential of giant squid axons [79, 80], several merocyanines were applied to mitochondrial membranes. Chance [78] found that merocyanine 1 and 2 responded with fluorescence changes to membrane energy conservation. On the assumption that the changes are electro-chromic, as demonstrated in model systems [81], it can be inferred that electric potential differences are present in the mitochondrial membrane during energy conservation. The part of the membrane across which the potential is established, however, could be its total thickness or only a small portion of it [78].

Excitation of chromatophores with different types of light or the induction of electrical potential differences across the chromatophores

membrane, by ionic gradients operating through ionophores or uncoupling agents, gives rise to a red shift of the carotenoid absorption bands [83–85, 87]. From the sensitivity to agents that catalyze electrophoretic ion movements, it has been concluded that the shift is a response to the membrane potential [82, 83]. Thus carotenoids in photosynthetic membranes can be considered an intrinsic probe of membrane potential.

The Generation of pH Gradients

The use of fluorescent amines to detect pH differences across the membrane of chromatophores and chloroplasts is based on the accepted method of measuring ΔpH by radioactive amines [86]. While the reasons for the fluorescence changes of the aromatic amines such as 9-amino-acridine following the distribution across a membrane in a pH gradient [87, 25] are not yet fully understood, the validity of the test was recently verified by Deamer et al. [88] in model systems in which controlled pH gradients were established. While the behavior of atebrin is not ideal (probably due to binding to phospholipids [89]), that of 9-aminoacridine has been considered to be always consistent with the theoretical expectations and can thus be considered the method of choice for transmembrane pH determination [90]. Recently, doubts on the quantitative relationship between 9-amino acridine quencing and transmembrane proton gradient have been raised by Fiolet et al. [91].

Conformational Changes

Acridines have been employed for the detection of conformational changes of membrane proteins. According to a series of studies from Azzone's group [92, 93] these dyes (with the exception of 9-amino-acridine which was not tested) are not able to detect pH differences across the mitochondrial membrane, but rather, as a consequence of energy conservation, they bind to nonpreexisting anionic sites of the membrane, in a low polarity environment. A consequence of binding would be the change in the absorbance properties of the dyes, whose spectra are similar to those obtained by binding to polyanionic molecules [92]. While the acridines interact with mitochondrial fragments only, safranines do so with intact membranes only [94], but their binding sites are considered to be the same. The reasons for this selectivity is not clear at the present moment.

Conclusions

It is possible to conclude, after the discussion on the application of different probes to energy-transducing membranes, that the efforts in the direction of the elucidation of the mechanism of energy conservation have not been wasted. Despite the lack of a final answer to the question whether the coupling device is chemiosmotic, chemical, or conformational in nature, a number of new important experiments have been performed which will finally contribute to the solution of the problem.

Apart from the important characterization of a number of physical parameters regarding the phospholipid environment and the interaction among proteins in the membrane, data concerning the onset of membrane potential(s) and pH gradients associated with energy conservation appear convincing. In fact, from different types of approach (lipophylic ions, charged fluorescent probes, carotenoids, and merocyanines) a common conclusion can be reached, namely that electrical potentials are set at the level of the mitochondrial and other energy-transducing membranes. There is still some controversy as to whether the potential is set across the entire thickness or only a small portion of the membrane.

It appears also well established that pH gradients are set across the coupling membrane during energy conservation and that pH gradients and membrane potentials have specific correlations. Whether the conclusions reached with the probe technique are perfectly in line with the chemiosmotic hypothesis, require some modification of it, or may also fit with a chemical hypothesis would go beyond the purpose of the present discussion. It seems, however, that some predictions of the chemiosmotic hypothesis have been, at least qualitatively, confirmed by the use of the probe approach described above.

References

1. D. Chapman and G.H. Dodd, in: *Structure and Function of Biological Membranes*, L.I. Rothfield (ed.), Academic Press, New York and London, 1971, pp. 13–81.
2. G.K. Radda and J. Vanderkooi, *Biochim. Biophys. Acta*, 265 (1972) 509–549.
3. A.D. Keith, M. Sharnoff, and G.E. Cohn, *Biochim. Biophys. Acta*, 300 (1973) 379–419.
4. S. Schreier-Muccillo and I.C.P. Smith, in: *Progress in Surface and Membrane Science*, J.F. Danielli, M.D. Rosenberg, and D.A. Cadenhead (eds.), Academic Press, New York and London, Vol. 9 (1973) pp. 318–390.
5. A. Azzi, *Q. Rev. Biophys.* 8 (1975) 237–316.
6. R.A. Badley, W.G. Martin, and H. Schneider, *Biochem.* 12 (1973) 268–275.
7. W. Lesslauer, J. Cain, and J.K. Blasie, *Biochim. Biophys. Acta*, 241 (1971) 547–566.

8. B. Chance, *Proc. Int. Congr. Biophys.* (1973) Moscow.
9. A.S. Waggoner and L. Stryer, *Proc. Natl. Acad. Sci., U.S.A.,* 67 (1970) 579–589.
10. J. Seelig, *J. Am. Chem. Soc.,* 92 (1970) 3881–3887.
11. J.A. Berden and E.C. Slater, *Biochim. Biophys. Acta,* 256 (1972) 199–215.
12. B. Chance, A. Azzi, I.Y. Lee, C.P. Lee, and L. Mela, in: *Mitochondrial Structure and Compartmentation,* FEBS Symposium, L. Ernster and Z. Drahota (eds), Academic Press, New York and London, vol. 17, (1969) pp. 233–273.
13. A. Azzi, M.A. Bragadin, A.M. Tamburro, and M. Santato, *J. Biol. Chem.,* 248 (1973) 5520–5526.
14. P.M. Vignais, P. Deveaux, and A. Colbeau, (1974) in: *Biomembranes: Lipids, Proteins and Receptors,* R.M. Burton and L. Packer (eds.), N.A.T.O. Advanced Study Institute, Espinho, Portugal, pp. 12.1–12.18.
15. W. Lesslauer, J.E. Cain, and J.K. Blasie, *Proc. Natl. Acad. Sci., U.S.A.,* 69 (1972) 1499–1503.
16. L. Letellier and E. Shechter, *Eur. J. Biochem.,* 40 (1973) 507–512.
17. T. Gulik-Krzywichi, E. Schechter, M. Iwatsubo, J.L. Ranck, and V. Luzzatti, *Biochim. Biophys. Acta,* 219 (1970) 1–10.
18. H. Traüble and E. Sackmann, *J. Am. Chem. Soc.,* 94 (1972) 4499–4510.
19. L. Brand and J.R. Gohlke, *Annu. Rev. Biochem.,* 41 (1972) 843–868.
20. M. Shinitzky, A.C. Dianoux, C. Gitler, and G. Weber, *Biochemistry,* 10 (1971) 2106–2113.
21. T. Förster, *Fluoreszenz organischer verbindung.* Vandenhoeck and Ruprecht, Göttingen, Germany (1951) p. 85.
22. L. Stryer and R.P. Haugland, *Proc. Natl. Acad. Sci., U.S.A.,* 58 (1967) 719–726.
23. J.R. Platt, *J. Chem. Phys.,* 34 (1962) 862.
24. L.L. Grinius, A.A. Jasaitis, Yu.P. Kadziauskas, E.A. Liberman, V.P. Skulachev, V.P. Topali, L.M. Tsofina, and M.A. Vladimirova, *Biochim. Biophys. Acta,* 216 (1970) 1–12.
25. S. Schuldiner and M. Avron, *FEBS Lett.* 14 (1971) 233–236.
26. J.S. Leigh, *J. Chem. Phys.,* 52 (1970) 2608–2612.
27. K. Razi-Naqvi, J. Gonzalez-Rodriguez, R.J. Cherry, and D. Chapman, *Nature New Biol. (London),* 245 (1973) 249–251.
28. J.S. Hyde and D.D. Thomas, *Ann. N.Y. Acad. Sci.,* 222 (1973) 681–692.
29. S.J. Singer and G.L. Nicholson, *Science,* 175 (1972) 720–725.
30. C. Gitler, *Annu. Rev. Biophys. Bioeng.,* 1 (1972) 51–92.
31. B. Rudy and C. Gitler, *Biochim. Biophys. Acta,* 288 (1972) 231–236.
32. J.M. Vanderkooi and J.B. Callis, *Biochemistry,* 13 (1974) 4000–4006.
33. U. Cogan, M. Shinitzky, G. Weber, and T. Nishida, *Biochemistry,* 12 (1973) 521–528.
34. G. Rouser, G.J. Nelson, S. Fleischer, and G. Simon, in: *"Biological Membranes,"* D. Chapman (ed.), Academic Press, New York and London, (1968) pp. 5–69.
35. H.M. Timberg, L. Packer, and A.D. Keith, *Biochim. Biophys. Acta,* 283 (1972) 193–205.
36. J. Torres-Pereira, R. Mehlhorn, A.D. Keith, and L. Packer, *Arch. Biochem. Biophys,* 160 (1974) 90–99.
37. J.R. Brocklehurst, R.B. Freedman, D.J. Hancock, and G.K. Radda, *Biochem. J.,* 132 (1970) 385–396.
38. M.A. Williams, R.C. Stancliff, L. Packer, and A.D. Keith, *Biochim. Biophys. Acta,* 267 (1972) 444–456.
39. J.K. Raison, J.M. Lyons, R.J. Mehlhorn, and A.D. Keith, *J. Biol. Chem.,* 246 (1971) 4036–4040.
40. G. Lenaz, G. Parenti-Castelli, A.M. Sechi, E. Bertoli, and D.E. Griffiths, in: *Membrane Proteins in Transport and Phosphorylation,* G.F. Azzone, M.E. Klingenberg, E. Quagliariello, and N. Siliprandi (eds.), North-Holland Publishing Co., Amsterdam and London (1974) pp. 23–28.

41. J.K. Raison and E.J. McMurchie, *Biochim. Biophys. Acta*, 363 (1974) 135–140.
42. W.B. McGlasson and J.K. Raison, *Plant Physiol.*, 52 (1973) 390–392.
43. E. Oldfield, K.M. Keough, and D. Chapman, *FEBS Lett.*, 20 (1972) 344–346.
44. A.G. Lee, M.J.M. Birdsall, J.C. Metcalfe, P.A. Toon, and G.B. Warren, *Biochemistry*, 13 (1974) 3699–3705.
45. P. Jost, O.H. Griffith, R.A. Capaldi, and G. Vanderkooi, *Proc. Natl. Acad. Sci.*, *U.S.A.*, 70 (1973) 480–484.
46. P. Jost, O.H. Griffith, R.A. Capaldi, and G. Vanderkooi, *Biochim. Biophys. Acta*, 311 (1973) 141–152.
47. A. Azzi, A.M. Tamburro, G. Farnia, and E. Gobbi, *Biochim. Biophys. Acta*, 256 (1972) 619–624.
48. H.R. Drott, C.P. Lee, and T. Yonetani, *J. Biol. Chem.*, 245 (1970) 5875–5879.
49. J. Vanderkooi and M. Erecinska, *Arch. Biochem. Biophys.*, 162 (1974) 385–391.
50. D.A. Harris, J. Rosing, and E.C. Slater, *FEBS Lett.*, 47 (1974) 236–240.
51. R.J. van de Stadt, K. van Dam, and E.C. Slater, *Biochim. Biophys. Acta* (1974) in press.
52. H.S. Penefsky and A. Datta, *Fed. Proc.*, 28 (1969) 2261.
53. C. Montecucco and A. Azzi, *J. Biol. Chem*, 250 (1975) 5020–5025.
54. P. Duveaux, A. Bienvenuë, A.D. Brisson, G. Lauquin, P.M. Vignais, and P.T. Vignais, *Biochemistry, N.Y.* (1974) (in press).
55. W.G. Hanstein and Y.Hatefi, *J. Biol. Chem.*, 249 (1974) 1356–1362.
56. Y. Hatefi and W.G. Hanstein in *Membrane Proteins in Transport and Phosphorylation*, G.F. Azzone, M.E. Klingenberg, E. Quagliariello, and N. Siliprandi (eds.) North-Holland Publishing Co., Amsterdam and London (1974) pp. 187–200.
57. A. Azzi, B. Chance, G.K. Radda, and C.P. Lee, *Proc. Natl. Acad. Sci. U.S.A.*, 62 (1969) 612–619.
58. B. Rubalcava, D. Martinaez de Munoz, and C. Gitler, *Biochemistry*, 8 (1969) 2742–2747.
59. B. Chance, A. Azzi, L. Mela, G.K. Radda, and H. Vainio, *FEBS Lett.*, 3 (1969) 10–13.
60. J.M. Wrigglesworth and L. Packer, *Bioenergetics*, 1 (1970) 33–43.
61. D.H. Haynes and H. Staerk, *J. Membr. Biol.*, 17 (1974) 313–340.
62. D.H. Haynes, *J. Membr. Bio.*, 17 (1974) 341–366.
63. K. Nordenbrand and L. Ernster, *Eur. J. Biochem.*, 18 (1971) 258–273.
64. G.K. Radda, *Biochem. J.*, 132 (1971) 385–396.
65. A. Azzi, P.L. Gherardini, and M. Santato, *J. Biol. Chem.*, 246 (1971) 2035–2042.
66. D.G. Layton, P. Symmons, and W.P. Williams, *FEBS Lett.*, 41 (1974) 1–7.
67. A. Azzi and M. Santato in *Biochemistry and Biophysics of Mitochondrial Membrane*, G.F. Azzone, E. Carafoli, A.L. Lehninger, E. Quagliariello, and N. Siliprandi (eds.), Academic Press, New York and London (1972) pp. 361–376.
68. A. Azzi and M. Santato, *Biochem. Biophys. Res. Commun*, 44 (1971) 211–217.
69. D. Layton, P. Symmons, and P. Williams, *Biochem. Soc. Trans. London*, 1 (1973) 418–421.
70 C. Gitler, B. Rubalcava, and A. Caswell, *Biochim. Biophys. Acta*, 193 (1969) 479–481.
71. B. Chance, *Proc. Natl. Acad. Sci.*, *U.S.A.*, 67 (1970) 560–571.
72. A. Azzi, *Biochem. Biophys. Res. Commun.*, 37 (1969) 254–260.
73. B. Chance, *FEBS Lett*, 23 (1972) 3–20.
74. C. Gitler in: *Biomembranes*, L.A. Manson (ed.), vol. 2, Plenum Press, New York and London (1971) pp. 41–73.

75. A.A. Jasaitis, V.V. Kuliene, and V.P. Skulachev, *Biochim. Biophys. Acta*, 234 (1971) 177–181.
76. A.A. Jasaitis, L. van Chu, and V.P. Skulachev, *FEBS Lett.*, 31 (1973) 241–245.
77. J.R. Brocklehurst, B.I.T. Cierkosz, and C.P. Lee, *Biochim. Biophys. Acta*, 314 (1973) 136–148.
78. B. Chance, *Fed. Proc.*, 32 (1973) 2569.
79. H.V. Davila, B.M. Salzberg, L.B. Cohen, and A.S. Waggoner, *Nature New Biol (London)*, 241 (1973) 159–160.
80. L.B. Cohen, B.M. Salzberg, H.V. Davila, W.N. Ross, D. Landowne, A.S. Waggoner, and C.H. Wang, *J. Membr. Biol.*, 19 (1974) 1–36.
81. H. Bücher, J. Wiegand, B.B. Snavely, H.K. Beck, and H. Kuhn, *Chem. Phys. Lett.*, 3 (1969) 508–511.
82. J.B. Jackson and A.R. Crofts, *FEBS Lett.*, 4 (1969) 185–189.
83. H.T. Witt and A. Zickler, *FEBS Lett.*, 37 (1973) 307–310.
84. H.T. Witt, *Q. Rev. Biophy.*, 4 (1971) 365–477.
85. M. Baltscheffsky, *Arch. Biochem. Biophys.*, 130 (1969) 646–652.
86. L. Packer and A.R. Crofts, in: *Current Topics in Bioenergetics*, D.R. Sanadi (eds.) vol. 2, Academic Press, New York and London, (1969) pp. 23–64.
87. R. Kraayenhof, *FEBS Lett.*, 6 (1970) 161–165.
88. D.W. Deamer, R.C. Prince, and A.R. Crofts, *Biochim. Biophys. Acta*, 274 (1972) 323–335.
89. S. Massari, *Biochim. Biophys. Acta*, 375 (1974) 22–34.
90. R. Casadio, A. Baccarini-Melandri, and A.B. Melandri, *Eur. J. Biochem.*, 47 (1974) 121–128.
91. J.W. Fiolet, E.P. Bakker, and K. van Dam., *Biochim. Biophys. Acta*, 368 (1974) 432–445.
92. P. Dell'Antone, R. Colonna, and G.F. Azzone, *Eur. J. Biochem.*, 24 (1972) 553–576.
93. S. Massari, P. Dell'Antone, R. Colonna, and G.F. Azzone, *Biochemistry*, 13 (1974) 1038–1043.
94. R. Colonna, S. Massari, and G.F. Azzone, *Eur. J. Biochem.*, 34 (1973) 577–585.