Proc. Natl. Acad. Sci. USA
Vol. 74, No. 2, pp. 570–574, February 1977
Biophysics

Surface localization of sites of reduction of nitroxide spin-labeled molecules in mitochondria

(spin-labels/electron transport)

ALEXANDRE T. QUINTANILHA AND LESTER PACKER

Membrane Bioenergetics Group, Lawrence Berkeley Laboratory and the Department of Physiology-Anatomy, University of California, Berkeley, Calif. 94720

Communicated by Manuel F. Morales, November 18, 1976

ABSTRACT **The relative rates of reduction of several spin-labeled molecules that partition differently across the hydrophobic-interface of inner membranes from rat liver mitochondria were investigated. Spin labels localized either deep in the hydrophobic region or in the aqueous phase are only slowly reduced; however, a spin-labeled analogue of the cationic detergent cetyltrimethylammonium bromide that partitions at the interface is rapidly reduced by coupled electron transport. Chemical studies on the reduction and oxidation of the spin label show that loss of signal is due to reduction and not destruction of the label. No evidence was found for flip-flop of the label in submitochondrial preparations.**

Spin reduction of respiring mitochondria, mitoplasts, or inverted submitochondrial preparations is inhibited by rotenone but is relatively insensitive to antimycin A and KCN. Because the midpoint potentials of the spin labels were found to be similar to that of ubiquinone, it is concluded that reducing equivalents of mitochondrial electron transport from this region of the chain are channeled to either membrane interface.

The electron transport chain of the inner mitochondrial membrane consists of a well-known series of redox components schematically reproduced in Fig. 1. Apart from cytochrome *c*, which is electrostatically attached, the various other components of the chain show strong hydrophobic interactions (1). Although specific lipids are required for the activity of electron transport complexes (2), ubiquinone is the only one that is known to participate in electron transfer.

During steady-state respiratory conditions (known as state IV respiration), the level of reduction of the various components of the electron transport chain is higher the further away the redox components are from the final acceptor O_2. Ubiquinone, for example, is about 50% reduced in the aerobic steady state (3). In an anaerobic state, the electron transport components become fully reduced. During steady-state respiration or aerobiosis, reducing equivalents are therefore accumulated at various "sites" along the electron transport chain. When uncoupled (state III respiration), this accumulation of reducing equivalents is decreased in most components of the chain except for cytochrome oxidase (a and a_3 in Fig. 1). Potentially, any such accumulation can reduce an added test substance (provided, of course, that the redox potentials of reductant and test substance are appropriately related). The test substance may be a spin label with the convenient property that it can report its own reduction (4–8). By manipulation of available O_2, with inhibitors specific for particular "sites" and uncouplers of respiration, it is sometimes possible to deduce the particular "site" at which the substance is being reduced. On the other hand, the test substance, e.g., a spin label, may have physicochemical properties ensuring that it will come to rest in a particular region of the mitochondrial structure. When both of these circumstances are realized, one can logically conclude that a transfer of reducing equivalents from the electron transport chain to the spin label goes on in the particular region of the mitochondrial structure where the spin-labeled molecule is localized. Our paper illustrates the foregoing strategy. To apply this strategy, we had to find a spin-labeled substance with the right properties (an analogue of cetyltrimethylammonium bromide, CDTAB) to show that after it was intercalated into the mitochondrial structure its signal decayed due to respiratory chain activity, and finally to show by manipulation of this activity that the reductant in the chain most likely to be affecting the spin label was ubiquinone.

Abbreviations: CDTAB, spin-labeled analogue of cetyltrimethylammonium bromide; SMP, submitochondrial preparation.

MATERIALS AND METHODS

Preparations. Rat liver mitochondria were prepared in a 0.25 M sucrose, 1 mM Tris·HCl and 1 mM EDTA medium at pH 7.4 as described (9). Mitoplasts were also prepared (10). Submitochondrial preparations (SMP) were made following a method described in ref. 11. Protein concentrations were determined by a standard method (12).

Spin Labels. The nitroxide spin-labeled molecules were provided by R. J. Mehlhorn (of this laboratory) and have the structures shown in Fig. 2. The labels Tempo, Tempol, Tempamine, Caprate, and CDTAB have a piperidine nitroxide ring whereas 2N3, 2N11, 7N14, A12NS, and A4NS have an oxazolidine nitroxide ring. Synthetic procedures for these labels have been described (13–15). The midpoint potential (pH 7.4) of the spin labels was determined from changes in line heights of electron paramagnetic resonance spectra (line width remains constant) by titration with ascorbic acid at 25° in the same test medium used in experiments. The midpoint potential of ascorbic acid at pH $\simeq$7.4 and 25° is approximately +44 mV.

Assays. Mitochondrial respiration was measured in a Rank oxygen electrode at 25° in an air-saturated medium containing 0.25 M sucrose, 5 mM Tris·HCl, 2 mM KCl, and 2 mM KH_2PO_4 at pH 7.4 (test medium) plus various spin labels as indicated in experiments at concentrations <20 nmol/mg of protein. Electron spin resonance spectra were obtained with a Varian E109 spectrometer at room temperature; power settings were generally 10 mW. Spectral changes of cytochromes (not shown) were recorded on a DW-2 Aminco spectrophotometer. Electron spin resonance measurements and spectral changes of cytochromes were carried out under exactly the same experimental conditions in which polarographic assays of mitochondrial O_2 consumption were performed in an aerobic test medium at protein concentrations of 3 mg/ml in the case of mitochondria and mitoplasts and 2 mg/ml for SMP.

RESULTS

Choosing an appropriate spin label

We first surveyed the behavior of various classes of spin labels (Fig. 2) toward the respiratory chain activity. CDTAB, a cation

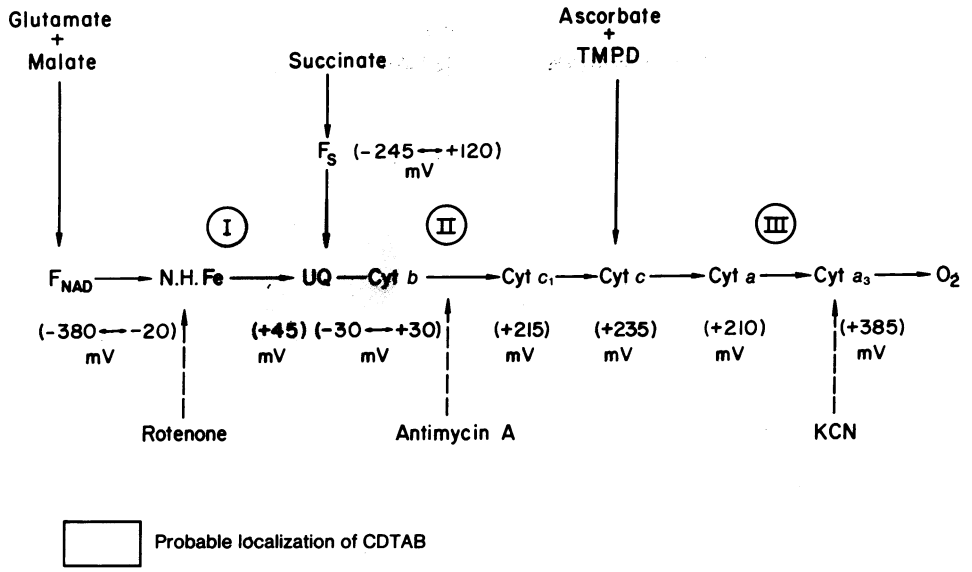

FIG. 1. Schematic representation of the electron transport chain in the inner membrane of mitochondria, showing midpoint potentials of components, the inhibitory sensitive regions of the chain (rotenone, antimycin A, and KCN), and the substrates used (glutamate + malate, succinate and ascorbate + TMPD). I, II, and III stand for the "coupling sites" along the chain. F_{NAD} denotes the NADH dehydrogenase; F_S, succinic dehydrogenase; N.H.Fe, nonheme iron; UQ, ubiquinone; Cyt, cytochrome. Midpoint redox potential ranges are indicated in mV. TMPD denotes *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

with a large hydrophobic moiety, was most readily reduced at a rate of 2 nmol/mg of protein per min; we therefore concentrated on its various interactions with mitochondrial and submitochondrial particles. The concentration at which these labels began to affect rates of O_2 uptake, respiration coupling, and swelling was approximately ≥20–30 nmol/mg of protein. Hence, for experiments spin-label concentrations were <20 nmol/mg of protein. Under the same conditions, the respiratory rate is approximately 8 nmol of O_2/mg of protein per min. The structure and charge of the CDTAB molecule and its electron paramagnetic resonance spectrum (13) indicate that the spin label is presumably located at the membrane interface. CDTAB is reduced simultaneously with O_2; hydrophilic labels are only appreciably reduced after the sample becomes anaerobic, and hydrophobic labels like 7N14 are only very slowly reduced.

Interaction of CDTAB with the chain

Next we tried to localize the "site" of action of CDTAB in the chemical kinetic scheme. Because CDTAB was reduced in the aerobic steady state, it was of interest to determine how it was reduced by electron transport. Artificial substrates like ascorbate and durohydroquinone reduce nitroxides chemically and therefore could not be used to study spin-label reduction, by electron transport. Mitochondria and mitoplasts metabolizing glutamate/malate and succinate show similar rates in CDTAB reduction. Comparison of CDTAB reduction in mitochondria and SMP (oxidizing NADH and succinate), are given in Table 1. Rotenone, antimycin A, and KCN are well known inhibitors of electron transport in the respiratory chain of mitochondria. The sites at which these inhibitors block electron transport are indicated in Fig. 1. Neither antimycin A nor KCN (acting downstream from ubiquinone in the electron flow) inhibit CDTAB reduction in mitochondria. On the other hand, rotenone (acting upstream from ubiquinone) appreciably inhibits the reduction of CDTAB by glutamate/malate. Rotenone does not inhibit succinate respiration (Fig. 1) and, as expected, spin reduction was not inhibited by rotenone in the presence of this substrate.

In SMP, rotenone completely inhibits spin reduction by NADH oxidation. Antimycin A or KCN partially inhibit spin reduction either by NADH, succinate, or choline chloride (not shown) oxidation. CDTAB reduction by NADH or succinate oxidation in SMP is 2.8% and 3.3–5%, respectively, of the corresponding rate of oxygen uptake. In mitochondria, CDTAB reduction by glutamate/malate proceeds at one third of the corresponding rate of oxygen uptake. The foregoing results suggest that CDTAB acts at the nonheme iron, ubiquinone region of the respiratory chain.

The slow spin reduction in the absence of added substrates in mitochondria and mitoplasts is probably due to the inability to completely deplete endogenous substrates. In the case of SMP, endogenous spin-label reduction is neglible. Similar rates of CDTAB reduction in mitochondria and mitoplasts indicate that the outer mitochondrial membrane is not involved in CDTAB reduction in these experiments.

Spectrophotometric studies

In the aerobic steady state, the level of reduction of cytochromes (Fig. 1) changes when the rate limiting steps of electron transport are modified. Because charged detergents can perturb membrane structure (16) and mitochondrial respiration (13), we studied the effect of CDTAB on the steady-state level of reduction of cytochrome b_{562}, b_{566}, *c*, and *a*. No effects were detected at CDTAB levels less than 20 nmol/mg of protein during glutamate/malate respiration.

Effect of uncouplers

In mitochondrial oxidative phosphorylation, both reactions of respiration and phosphorylation are firmly coupled to each other. One way agents such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 2,4-dinitrophenol are known to act as uncouplers is by dissipating proton gradients established across the inner membrane by the above reactions. In uncoupled mitochondria, the aerobic steady-state level of reduction of the components of the electron transport chain (Fig. 1) is significantly decreased particularly for the components near the substrate end of the sequence. In our experiments, both carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and

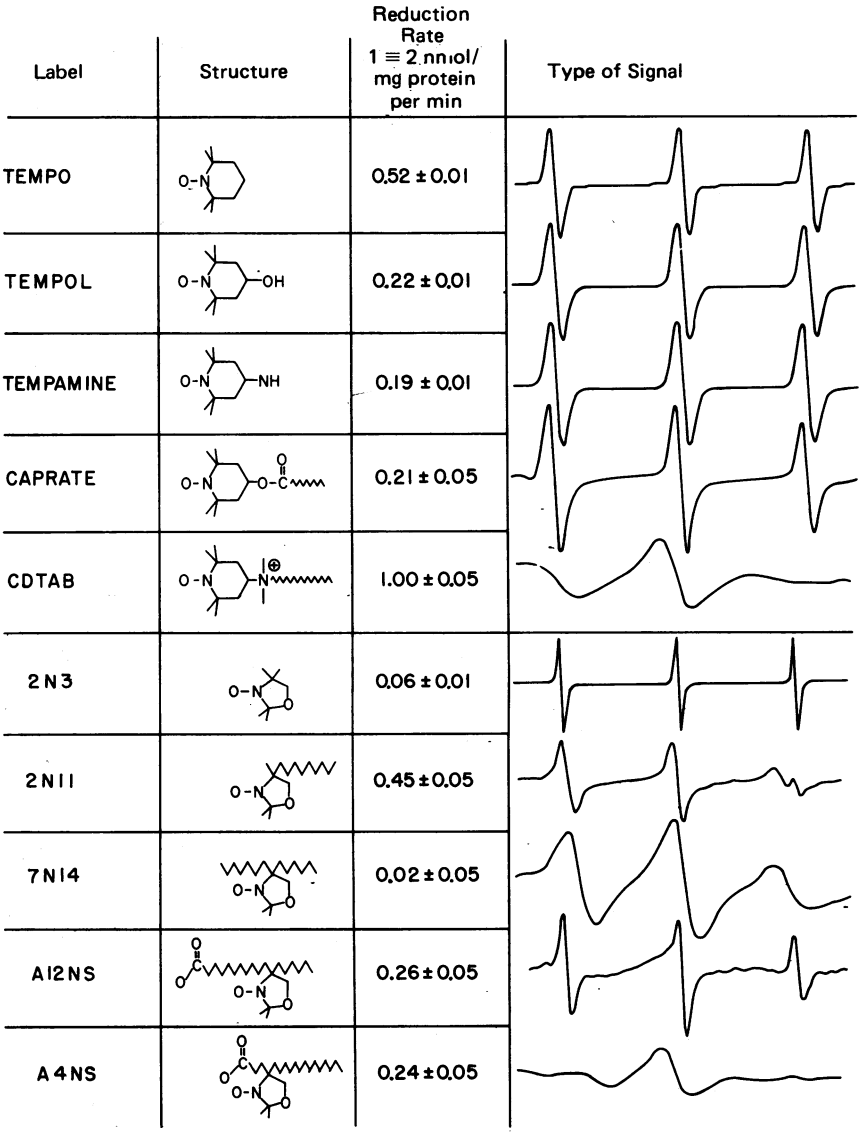

FIG. 2. Spin-labeled molecules used in this study, their structure, spectra in the presence of mitochondria at concentrations of 20 nmol/mg of protein, and rate of reduction by succinate when aerobic. TMPD denotes *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

2,4-dinitrophenol at concentrations of 0.3 μM and 10 μM, respectively, were effective in uncoupling glutamate/malate and succinate respiration. Under the same conditions CDTAB reduction was almost completely inhibited. However, after the sample became anaerobic, CDTAB reduction commenced again (at the same rate as seen aerobically in the absence of uncouplers). In uncoupled mitochondria the O_2 uptake versus spin-label reduction ratio was approximately 30 during glutamate/malate and 20–30 during succinate respiration. These experiments reinforce the notion that CDTAB is reduced (its signal is anihilated) by a steady-state accumulation of some reductant in the chain; this signal destruction disappears when the accumulations are abolished, e.g., by uncoupling.

Chemical reduction and oxidation

Mitoplasts and SMP have opposite membrane orientations. CDTAB is reduced by electron transport when added to either of these preparations. This could be due to the fact that reducing equivalents can be channeled to each membrane interface or because the labels can flip-flop in the perpendicular plane of the membrane. The following experiments make the flip-flop explanation unlikely. Kornberg and McConnell (17) have studied flip-flop rates of spin-labeled lipids by using the reducing capacity of Na ascorbate. Tonomura and Morales (18) have also used the reducing capacity of Na ascorbate to study the distribution of spin labels in sarcoplasmic reticulum vesicles. By using a concentration of SMP of 2 mg of protein per ml and concentrations of CDTAB of 20 nmol/mg of protein, we determined spin reduction by 5, 10, and 15 M equivalents of ascorbate for varying times after mixing CDTAB with SMP. The SMP were added to a medium containing CDTAB at levels below the critical micelle concentration, which is approximately 460 μM (13). Although the rate of CDTAB reduction increases with ascorbate concentration, there was no indication of biphasic kinetics of label reduction. Moreover, the rates of spin reduction by ascorbate do not change over a period of 24 hr after incubating the spin label with the SMP. It was found that the addition of the negatively-charged detergent, sodium dodecyl sulfate, inhibited the further reduction of the spin label in the presence of ascorbate (R. J. Mehlhorn, personal communication). This technique was then used to determine the amount of remaining unreduced label 15 min after treatment

Table 1. Reduction of CDTAB spin label by mitochondrial electron transport

	CDTAB reduction (nmol/mg of protein per min)	
	Mitochondria	SMP
Endogenous	0.4	0.05
NAD linked substrate*	1.0	3.0
+Rotenone	0.6	0.06
+Antimycin A	1.0	1.7
+KCN	1.0	1.7
Succinate	2.0	2.0
+Antimycin A	2.0	1.8
+KCN	2.0	2.0

* Glutamate/malate for mitochondria, and NADH for SMP.

with ascorbate; it was found that the percentage of unreduced label was independent of the time of incubation of SMP with label.

The following experiments were performed to show that ascorbate permeates across SMP membranes slowly enough to use this substance to study localization of nitroxides. External cytochrome *c*, in amounts comparable to the endogenous levels, was incubated with SMP for 30 min. KCN (1 mM) was introduced to block oxygen uptake. Ascorbate was then added and cytochrome *c* reduction was recorded at 550–540 nm. The kinetics of reduction clearly showed two distinct populations of cytochrome *c*, one reduced rapidly followed by a slower rate of reduction. Endogenous cytochrome *c* is known to be located on the internal side of the SMP membrane. The added cytochrome *c* was on the external side. Hence, the results indicate that two populations of cytochrome *c* on the two sides of the SMP membrane can easily be distinguished by their different rates of ascorbate reduction. Therefore, it may be concluded that reducing equivalents from ascorbate permeate SMP membranes slowly enough to use this substance to test for the localization of nitroxide spin labels.

It has been frequently observed that biological systems attenuate spin-label signals either by nitroxyl reduction or by other forms of chemical destruction. After complete disappearance of signal in the presence of CDTAB and respiring mitochondria, the signal could be completely restored by addition of stoichiometric amounts of oxidizing reagents like potassium ferricyanide. Thus, no detectable chemical destruction of spin label occurs in our experiments with mitochondria.

Mid-point potential measurements

Because the midpoint potential of most of the electron transport components is known (Fig. 1) it was of interest to determine the midpoint potential of the labels used in order to understand the spin reduction results. The midpoint potentials were determined as described in *Materials and Methods* starting with 100 μM of fully oxidized spin label; the midpoint potentials of the piperidine labels (measured both with tempo and CDTAB) in our test medium was around +60 mV which is slightly above that of ascorbate measured at room temperature and at pH 7.2. The midpoint potential of the oxazolidine labels (measured with 2N3) in our test medium was around +20 mV. Standard potentiometric techniques give the midpoint potential of methylene blue at pH 7 as +10 mV. By using our technique of titrating with ascorbic acid, we find the midpoint potential of methylene blue at pH 7 to be around 0 mV: our technique is therefore valid, yet accurate results will only be obtained where the midpoint potential of the test substance is close to that of ascorbic acid. Under our conditions, the midpoint potentials of oxazolidine and piperidine labels differ by 40 mV. By using different test conditions, previous workers have shown (19) that the midpoint potential of the tempo label varies over a considerable range depending on the pH, the concentration and nature of the buffers, and the ionic strength. The closest midpoint potential of a redox component of mitochondrial electron transport is that of ubiquinone which is +45 mV. We have found in spectrophotometric and electron paramagnetic resonance studies that ubiquinone in either its fully oxidized or reduced (quinol) state does not interact with CDTAB. However, if the semiquinone free radical species is formed in alkaline ethanolic solutions, it readily reduces CDTAB.

DISCUSSION

This investigation has shown that spin labels can be used to study the channeling of reducing equivalents from mitochondrial electron transport in the midpoint potential range +20 to +60mV. According to current concepts of electron transport, reducing equivalents are accumulated at various "sites" along the chain of electron carriers (Fig. 1). The action of uncouplers to inhibit spin-label reduction in the aerobic steady state is consistent with the decreased accumulation of reducing equivalents by the redox components of electron transport which are the electron donors for CDTAB reduction. Because CDTAB is the label that is reduced fastest in either mitochondria or SMP this indicates that reducing equivalents are preferentially channeled to the hydrophobic-hydrophilic interface on either side of the membrane.

CDTAB can flip-flop in certain systems, e.g., by using the assay method essentially of Kornberg and McConnell (17) it was found by Mehlhorn and Packer (20) that two populations of CDTAB were present in lecithin vesicles based upon their different rates of reduction after ascorbate treatment. The population of CDTAB reduced at a slower rate was virtually zero at the beginning of incubation of vesicles with CDTAB but it increased slowly with time until it corresponded to about 40% of the total amount of spin label present. The slowly reduced CDTAB should reflect CDTAB molecules that have moved on to the inner surface. Hence the rate of flip-flop of CDTAB has a half life of a few hours in this system. However, the studies of CDTAB reduction by ascorbate in SMP indicate that a final distribution of CDTAB molecules requires at most 15 min and no evidence exists for the presence of two populations of CDTAB present. However, the reduction of CDTAB with durohydroquinone which partitions in the membrane is very rapid but also shows only one population of label. Therefore, it seems unlikely that CDTAB flip-flops across the membrane as observed in lecithin vesicles.

Our results of CDTAB spin reduction in mitoplasts, SMP, and by the semiquinone free radical, similar inhibitor sensitivity, midpoint potential range and a certain degree of structural similarity, suggest that the piperidine label on CDTAB (+60 mV) can accept reducing equivalents from electron transport in the ubiquinone region at each membrane interface. The physical properties expected from the structure of CDTAB suggest that its hydrophobic moiety will locate inside the membrane with the cationic moiety directed toward either surface. In terms of the strategy outlined above, we are led to suggest that ubiquinone must reside wherever the nitroxyl of CDTAB resides, namely, toward either interface. Our results would seem to support some aspects of a recent proposal of Mitchell (21), termed the "proton motive Q cycle," whereby

the mobile uncharged semiquinone form of ubiquinone or "Q" mediates proton translocation across the inner mitochondrial membrane. This result is further reinforced by the finding that the semiquinone constitutes a relatively stable free radical in the mitochondrial inner membrane (22, 23).

We wish to thank Rolf J. Mehlhorn and Yoram Avi-Dor for many useful discussions. This research was supported by the Energy Research and Development Administration.

1. Green, D. E. & Goldberger, R. F. (1966) in *Molecular Insights Into the Living Process* (Academic Press, New York).
2. Fleischer, S., Brierley, G., Klouwen, H. & Slautterback, D. B. (1962) *J. Biol. Chem.* **237**, 3264–3272.
3. Palmer, J. M. & Hall, D. O. (1972) *Prog. Biophys. Mol. Biol.* **24**, 127–176.
4. Tinberg, H. M., Packer, L. & Keith, A. D. (1972) *Biochim. Biophys. Acta* **283**, 193–205.
5. Torres-Pereira, J., Mehlhorn, R., Keith, A. D. & Packer, L. (1974) *Arch. Biochem. Biophys.* **160**, 90–99.
6. Schreier, S., Stuhne-Sekalec, L. & Stanacev, N. Z. (1975) *Biophys. J.* **15**, 108a.
7. Baldassare, J. J., Robertson, D. E., McAfee, A. G. & Ho, C. (1976) *Biochemistry* **13**, 5210–5214.
8. Azzi, A., Bragadin, M. A., Tamburro, A. M. & Santato, M. (1973) *J. Biol. Chem.* **248**, 5520–5526.
9. Stancliff, R. C., Williams, M. A., Utsumi, K. & Packer, L. (1969) *Arch. Biochem. Biophys.* **131**, 629–642.
10. Schaitman, C. & Greenawalt, J. W. (1968) *J. Cell Biol.* **38**, 158–175.
11. Tinberg, H. M., Melnick, R. L., Maguire, J. & Packer, L. (1974) *Biochim. Biophys. Acta* **345**, 119–128.
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
13. Mehlhorn, R. J. & Packer, L. (1976) *Biochim. Biophys. Acta* **423**, 382–397.
14. Keana, J. F. W., Keana, S. R. & Beetham, D. (1967) *J. Am. Chem. Soc.* **89**, 3055–3056.
15. Williams, J. C., Mehlhorn, R. & Keith, A. D. (1971) *Chem. Phys. Lipids* **7**, 207–230.
16. Helenius, A. & Simons, K. (1975) *Biochim. Biophys. Acta* **415**, 29–79.
17. Kornberg, R. D. & McConnell, H. M. (1971) *Biochemistry* **10**, 1111–1120.
18. Tonomura, Y. & Morales, M. F. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 3687–3691.
19. Neimann, M. B., Mairanovskii, S. G., Kovarskaya, B. M., Rozantsev, E. G. & Gintsberg, E. G. (1964) *Izv. Akad. Nauk Uzb. SSR Ser. Khim. Nauk.* 8, 1518–1521.
20. Mehlhorn, R. J. & Packer, L. (1976) *Biophys. J.* **15**, 108a.
21. Mitchell, P. (1975) *FEBS Lett.* **56**, 1–6.
22. Ingledew, W. J. & Ohnishi, T. (1975) *FEBS Lett.* **54**, 167–171.
23. Ruzicka, F. J., Beinert, H,, Schepler, K. L., Dunham, W. R. & Sands, R. H. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2886–2890.