Membrane-potential-dependent changes of the lipid microviscosity of mitochondria and phospholipid vesicles

Paul S. O'SHEA, Sylvia FEUERSTEIN-THELEN and Angelo AZZI
Medizinisch-Chemisches Institut, Universität Bern, Postfach-3000, Bern, Switzerland

(Received 20 December 1983/Accepted 8 March 1984)

1. The effects of a transmembrane potential difference upon the lipid microviscosity of cytochrome oxidase vesicles (COVs) and rat liver mitochondria (RLM) were investigated. COVs and RLM were labelled with the fluorescent probe 1,6-diphenylhexa-1,3,5-triene (DPH). The fluorescence polarization of the probe was then measured when potentials of different magnitudes were induced across the membranes of these particles. 2. It was shown that the absolute value of the microviscosity changes to quite a significant extent, owing to the imposition of large membrane potentials. On relaxation of the membrane potential the lipid microviscosity was also shown to return to the value before the induction of the potential. 3. The largest change in lipid microviscosity was observed when coupled respiration was initiated. This occurred in both the COV system and the RLM system. The absolute value of the lipid microviscosity was shown to change by as much as 22% with the induction of membrane potentials, owing to respiration. 4. To confirm the viscosity measurements made with DPH, lipid microviscosity was also measured with the spin-labelled fatty acid 5-doxyl stearate. Measurements of the order parameters indicated that, in agreement with the results of fluorescence experiments, viscosity changes occurred that were due to the induction of a membrane potential. 5. The significance of these findings to the regulation of metabolism is briefly discussed, the main conclusion being that, although there is certainly a significant variation of lipid microviscosity with electric field, mechanistic interpretations will require further studies.

In recent years, changes of membrane structure have been implied to play a role(s) in the mediation or regulation of many biological processes. A great diversity of mechanisms have been suggested to be involved in an equally large number of different biological events. It has been suggested, for example, that changes of membrane structure may be, in part, responsible for nervous conduction (Keynes, 1972). Furthermore, it is well established that anaesthetics mediate their effects through the agency of fluidity changes of the membrane (Johnson & Bangham, 1969). Moreover, membrane-structural alterations have also been suggested to be involved in the mediation of the physiological effects of hormones (Houslay *et al.*, 1981; Halestrap, 1982*a,b*). Thus it is clear that the mem-

brane is not just an insulatory device, but appears to be an important facility in the repertoire of biological regulatory mechanisms.

There have been many studies carried out on the putative effects of electric fields on membrane structure [see, e.g., Georgescould & Duclohier (1979)].

Among the most comprehensive studies of lipid fluidity are those that have employed fluorescence techniques. The use of the fluorescent probe DPH for microviscosity measurements has been pioneered by Shinitzky (see, e.g., Shinitzky & Inbar, 1976). DPH is known to dissolve in the hydrophobic phase of the membrane; as such, the rotational motion of the probe may be influenced by the viscosity of the membrane. Application of the technique known as fluorescence depolarization to DPH-labelled membranes will, therefore, provide information about the fluidity state of the membrane. Fairly recently, Shinitzky and co-workers (Corda *et al.*, 1982) have also applied DPH to the

Abbreviations used: DPH, 1,6-diphenylhexa-1,3,5-triene; COVs, cytochrome oxidase vesicles; PLVs, phospholipid vesicles; RLM, rat liver mitochondria; FP, fluorescence polarization; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

study of the influence of a transmembrane potential on the lipid microviscosity of artificial phospholipid vesicles. Our investigation will attempt to extend these observations to natural membranes and possibly shed some light on the effects of electric fields on the integrity of the membranes of mitochondria and the phospholipid vesicles used for protein reconstitution studies (see, e.g., Casey *et al.*, 1984). We applied the fluorescent probe DPH and spin-labelled fatty acids (see, e.g., Schreier *et al.*, 1978) to measure lipid microviscosity.

Such investigations are thought to be worthwhile, since several recent and diverse investigations have implied that microviscosity changes may be an important consideration for the interpretation of studies of reconstituted electrogenic electron-transport proteins (Casey *et al.*, 1984). These latter studies were made together with similar studies of mitochondria, since there has been a suggestion that lipid fluidity may be an important parameter for the hormonal regulation of oxidative phosphorylation (Halestrap, 1982a,b). It is our intention to provide a basis for further more-comprehensive studies by clarifying whether or not lipid microviscosity may be influenced by changes of the physiological membrane potentials in the above-described systems.

Materials and methods

All biochemical reagents were of the highest purity commercially available. DPH was obtained from Serva, Heidelberg, Germany. Spin-labelled fatty acids, *n*-doxyl stearates, were obtained from Syva, Palo Alto, CA, U.S.A.

COVs, PLVs and RLM were prepared as previously described (O'Shea *et al.*, 1984). The RLM were labelled for fluorescence measurements by the addition of 40 μ l of ethanolic DPH (2 mmol/litre) to each of the 50 ml centrifuge tubes used for the last of two centrifugation washes of the RLM. The RLM protein concentration in each centrifugation tube was approx. 4 mg/ml.

To 1 ml each of the suspensions of COVs and PLVs (40 mg of lipid/ml) was added 40 μ l of DPH (20 mmol/litre); these mixtures were vortex-mixed and then dialysed for 1 h against the last dialysis medium used for the preparation of the vesicles (see O'Shea *et al.*, 1984).

For spin-label mobility measurements RLM were labelled with 5-doxyl stearate [I(12,3); abbreviations follow the form: I(*m*,*n*) (see the structure below)] by complete evaporation of an ethanolic solution of the probe under O₂-free N₂ in a test tube, followed by the addition of the RLM

suspension such that the final probe/mitochondrial-protein ratio was 5 μ g/mg of protein.

Nitroxide spin-label group

Molecular structure of doxyl stearates

All treatments were carried out at 4°C.

The principles and measurements of lipid microviscosity

1. Fluorescence polarization

Among the many techniques applied to study the dynamics of lipid systems, fluorescence polarization (FP) has in the last decade become increasingly popular. This is partly due to the simplicity by which such measurements may be carried out, but it is often also the case that, unlike e.s.r. and n.m.r. measurements, the fluidity state of a lipid membrane when measured by FP may be recorded as a relative parameter. Of course, the FP technique is also applied to obtain absolute values for order parameters of the fluidity state of the membrane [for an extensive discussion of these latter points the reader is referred to the fairly recent review of Heyn (1979)]. For our purposes, however, the relatively simple 'steady-state' technique of measuring FP was applied. This technique adopts the strategy of treating the system as homogeneous; it is therefore possible to apply classical hydrodynamic expressions to describe microscopic lipid domains. The experimental protocol involves the measurement of the fluorescence intensity of a suitable probe (such as DPH) when the probe is excited with plane-polarized light. The intensity of the emission is then recorded by means of a photomultiplier with attached polarization filters.

The extent of depolarization of the plane-polarized light as measured by the photomultiplier equipped with appropriate polarization filters reflects the degree to which a population of photo-selected (those which become excited by the polarized light) fluorophores lose their initial orientation. When this value is compared with the so-called limiting anisotropy, which may be considered to be the extent of fluorescence depolarization in a frozen solution and therefore the upper theoretical limit of the polarization, it is possible to relate the depolarization or observed anisotropy of the emitted light to the relative state of the membrane fluidity or the lipid microviscosity. For a more rigorous treatment of the theory, practice and application of these techniques, the reader is referred to the publications of Shinitzky and co-workers (Shinitzky & Inbar, 1976; Shinitzky & Barenholz, 1978).

Determinations of the lipid microviscosity ($\bar{\eta}$) were carried out essentially as reviewed by Shinitzky & Inbar (1976). The absolute value of the $\bar{\eta}$ was calculated by application of the Perrin equation for rotational depolarization:

$$\frac{r_0}{r} = 1 + C(r) \frac{T \cdot \tau}{\bar{\eta}} \quad (1)$$

where r is the measured value of the fluorescence anisotropy; r_0 , the value of the limiting fluorescence anisotropy at the excitation wavelength 355 nm, is taken to be 0.362 (Shinitzky & Barenholz, 1978). The lifetime of the excited state (τ) is taken to be 8.8 ns at 25°C. The structural parameter $C(r)$ and τ are not considered to vary with r and electric field respectively. Eqn. (1) thus simplifies at constant temperature to eqn. (2):

$$\left(\frac{r_0}{r} - 1 \right)^{-1} = \bar{\eta} \cdot K \quad (2)$$

where K is a proportionality constant.

We have chosen to represent our results as plots of r as a percentage of r_0 against either time or membrane potential (see Figs. 2–4).

FP measurements were carried out with a Perkin-Elmer fluorescence spectrofluorimeter (Model MPF-2A) equipped with a magnetic stirring device, thermostat and polarization filters.

Any changes of r induced by perturbation of the reaction mixture were resolved within about 5 s. The excitation wavelength was 355 nm and the emission wavelength was 430 nm; polarization filters were changed manually.

2. E.p.r. spin-label mobility measurements

The mobility of the fatty acid spin label was monitored with a Varian E-line Century series 9.5 GHz spectrometer fitted with a refluxing device. This facility allowed additions to be made to the quartz measuring cell without directly disturbing the cell. The quartz-cell volume was 0.1 ml and the time resolution of the flow device allowed measurements of any changes of spin-label mobility (due to perturbations to the biological material) to be made within 10 s. Measurements were made with the following instrument settings: modulation amplitude, 32 mT (3.2 G); modulation frequency, 100 kHz; microwave power, 5 mW; time constant, 0.128 s. The variable-temperature controller was accurate to within $\pm 0.1^\circ\text{C}$. Calculations were performed essentially as described by Sauerhaber *et al.* (1977). The outer ($2T_{\parallel}$) and inner ($2T_{\perp}$) hyperfine splittings were determined from expanded spectra with amplified wings. Eqn. (3) indicates the standard relationship applied to evaluate the mobility of the spin label in the membrane:

$$S = \frac{(T_{\parallel} - T_{\perp})(an)}{(T_{zz} - T_{xx})(an')} \quad (3)$$

where T_{zz} and T_{xx} are the hyperfine splitting elements of the static interaction tensor (T) parallel to the static Hamiltonian ($\mathcal{H}$) principal nuclear hyperfine axes (x and z). $T_{\parallel}$ and $T_{\perp}$ are the outer and inner hyperfine splittings. (an') and (an) are the isotropic coupling constants of the crystal and the membrane respectively where: $an' = 1/3(T_{\parallel} + 2T_{\perp})$ and $an = (T_{zz} + 2T_{xx})/3$. The value of T_{xx} is taken as 61 mT and that of T_{zz} as 324 mT (Sauerhaber *et al.*, 1977).

The value of the S parameter is the mobility index of the fatty acid spin label in the membrane (see Table 1 and Fig. 5).

After being labelled with the various probes the vesicles and RLM were assayed for semi-permeability to ions as described by O'Shea *et al.* (1984). Membrane potentials were induced across the mitochondrial and vesicular membranes by imposing K^+ diffusion potentials. This for the case of the RLM was achieved by making use of the high (100 mmol/litre) intramitochondrial K^+ concentration. Vesicles were prepared with low (0.1 mmol/litre) intravesicular K^+ concentrations. Thus if RLM or vesicles were suspended in media of low and high K^+ concentrations respectively, then, with the addition of valinomycin, K^+ attempts to move into chemical equilibrium, with the resultant generation of a membrane potential ($\Delta\Psi$). By varying the K^+ concentrations of the various suspension media, at constant osmolarity, it was possible to vary the imposed membrane potentials, which were deduced by application of the Nernst equation.

Results and discussion

The relationship between lipid microviscosity and temperature is illustrated in Fig. 1. There is a clear dependence of the measured rotational depolarization of DPH on the ambient temperature, indicating that DPH responds in both RLM and COVs to the fluidity state of the membrane. These observations are in good agreement with those of Shinitzky and co-workers (Shinitzky & Barenholz, 1978), who further extended their studies by using DPH to investigate all manner of treatments which may affect lipid microviscosity.

It was thus possible to apply DPH to the measurement of microviscosity with our experimental materials. The relationship between the imposed membrane potential and the measured lipid microviscosity of both COVs and RLM is shown in Fig. 2. The values of the membrane potential (abscissa) were calculated from the

Fig. 1. Effect of temperature on the fluorescence polarization of DPH expressed as $\log \bar{\eta}$

Uncoupled RLM protein (0.1 mg/ml) was suspended in 0.1 M-KCl/20 mM-Hepes/0.1 mM-EGTA, pH 7.3. The ambient temperature was varied as indicated by 0.25°C increments of the thermostatically controlled heating device attached to the spectrofluorimeter. Measurements were taken as often as possible. The excitation (polarizer) wavelength was 355 nm, the emission (analyser) wavelength was 430 nm. Similar experiments (with DPH) have been performed on phospholipid vesicles by Shinitzky & Barenholz (1978). A similar single-phase temperature-dependency was found.

Fig. 2. Effect of a membrane potential (mV) on the relative lipid micro-viscosity measured by fluorescence polarization expressed as a percentage change of r with respect to r_0 ($\bar{\eta}$)

RLM (■) (0.1 mg of protein/ml) were suspended in media containing various K^+ concentrations. Choline chloride was present to maintain a constant osmolarity. Also present was 20 mM-Hepes and 0.1 mM-EGTA. A known membrane potential was generated by the addition of valinomycin (1 μ M final concn.). The measurement of r was then made as soon as possible. Similar experiments were performed with COVs (●). The ambient pH was 7.3 and the temperature was 15°C.

Nernst equation. After the addition of valinomycin, measurements of the fluorescence anisotropy were taken as soon as possible (~ 5 –10 s). Membrane potentials induced in this way, however, are of a transient nature. It has been previously shown (O'Shea *et al.*, 1984) that, owing to the electrogenic countermovement of protons, the induced potential is rapidly abolished and is almost completely dissipated after approx. 2 min.

It is clear from Fig. 2 that there is an increase of lipid microviscosity with the imposition of a membrane potential. The time-dependence of the relaxation of $\bar{\eta}$ is illustrated in Fig. 3. We make the assumption that the induced membrane potential has substantially decayed after about 2 min, which in our experience is usually the case. Furthermore, we should point out that a transient, large $\Delta\Psi$ decays relatively much more rapidly than a transient small $\Delta\Psi$ due to an exponential dependence of conductance (increasing) with applied voltage. Thus the $\bar{\eta}$ measurements made at the highest imposed $\Delta\Psi$ may not be as accurate as we would like.

In an attempt to circumvent this latter problem, coupled steady-state respiration was initiated by supplying substrates to the respiratory proteins. The aerobic respiratory-control ratios of the COVs and RLM were 5 and 6 with ferrocycytochrome *c* and succinate as substrates respectively. A physiological membrane potential was thus generated

Fig. 3. Time-dependence of the change of lipid micro-viscosity of RLM due to the transient imposition of a membrane potential

The experimental protocol was as in Fig. 2. Fluorescence measurements were, however, continued for up to 10 min. $\bar{\eta}$ was constant at zero time (0); this value was considered to be 0%. Immediately after this measurement, valinomycin was added to induce a membrane potential. Measurements were then taken as often as possible, i.e. all points after zero time. The following membrane potentials were induced: ■, 180 mV; ○, 160 mV; ●, 0 mV.

(see, e.g., O'Shea & Chappell, 1984). (No spin-label reduction was found to occur in the presence of these substrates.) The experiments described in Fig. 4 indicate the effect of a steady state $\Delta\Psi$ on the microviscosity of both RLM and COVs. It was presumed that these parameters were stable as long as a source of reducing equivalents and an electron acceptor (dioxxygen) was made available. With the removal or inhibition of any of these substrates, the membrane potential will rapidly fall; apparently, as a consequence of this, $\bar{\eta}$ also falls. It was not possible to initiate the collapse of the steady-state $\Delta\Psi$ with any uncouplers of oxidative phosphorylation (e.g. carbonyl cyanide *m*-chlorophenylhydrazone), since it was found that they directly interfered with DPH.

If we compare the value of $\bar{\eta}$ obtained due to the transient induction of a membrane potential, i.e. the $\bar{\eta}$ value (%) (see Fig. 2) with those obtained at zero time (0 s) in Fig. 4, it appears that the value obtained during the steady-state membrane potential is greater than that of the largest deduced membrane potential for both RLM and COVs. It has been estimated by O'Shea & Chappell (1984) that the $\Delta\Psi$ of coupled RLM during steady-state controlled respiration is about 160–170mV. Clearly, therefore, the relationship between $\Delta\Psi$ and $\bar{\eta}$ for large values of $\Delta\Psi$ is less precise (underestimation) than that with low induced values of $\Delta\Psi$.

One further point worth stressing is that Figs. 3 and 4 indicate that the half-time of the microviscosity relaxation process, after the transient imposition of a membrane potential, is not the same as that of the half-time of the relaxation of the induced membrane potential. The microviscosity relaxation process therefore does not kinetically follow the membrane-potential relaxation process (see below).

Analogous experiments to those described above were carried out with the e.p.r. probe I(12,3) and are shown in Fig. 5 and Table 1.

The time resolution of the measurement of viscosity changes reported by the spin label was of the order of 10s. It was not possible, therefore, to correlate the absolute viscosity with specific induced $\Delta\Psi$, although there was a significant change of the *S* parameter during the transition from steady-state respiration to anoxic or cyanide-inhibited conditions.

The e.p.r. probe reported changes of lipid microviscosity in the same qualitative manner as those found with the fluorescent probe. Fig. 5 indicates

Fig. 4. Steady-state coupled respiration initiated in RLM (■) and COVs (●) by the addition of 3mM-succinate ($\pm 1.0\mu\text{M}$ -rotenone) and 3mM-ascorbate + 200 μM -NNN'-tetramethyl-*p*-phenylenediamine respectively. Measurements of $\bar{\eta}$ were then taken until a consistent value was obtained, which is the indicated value at zero time (0s). Cyanide (2mM) was introduced into the reaction vessel at zero time or the reaction vessel was allowed to become anaerobic. Measurements were then taken at the indicated times.

Table 1. Effect of membrane potential on the microviscosity of RLM as measured by an e.p.r. probe. Well-coupled RLM were labelled with the spin label I(12,3) as described in the Materials and methods section. Reaction conditions were as follows (all concentrations are in mmol/litre): (1) KCl, 100; potassium Hepes, 20; EGTA, 0.1; succinate, 2; the mitochondrial suspension was aerated by continuously refluxing through the quartz measurement cell; (2) as (1) with cyanide (2.0) introduced into the cell; (3) sucrose, 200; lithium Hepes, 20; EGTA, 0.1; after the addition of valinomycin (0.001) resonance measurements were taken as soon as possible. The time resolution of any effect of membrane potential on fluidity was not less than 10s. The possibility that the membrane potential (3) had substantially dissipated after this time is acknowledged. The membrane potential in (1) is presumed to be about 160–170mV (O'Shea & Chappell, 1984).

Treatment	T_{zz}	T_{yy}	Order parameter (<i>S</i>)	Comment
1. Substrate (respiration)	28.063	9.415	0.674	Maximum rigidity
2. Inhibition (of respiration)	27.75	9.425	0.667	Maximum fluidity
3. 180mV (induced membrane) potential	28.025	9.450	0.671	—

Fig. 5. Temperature-dependence of the lipid microviscosity as measured by the e.p.r. probe I(12,3)

Reaction conditions were as follows: 100 mM-KCl, 20 mM-choline chloride and 0.1 mM-EGTA. The points show for RLM (0.1 mg of protein/ml) the effect of the absence (○) and presence (●) of 2 mM-cyanide. Instrument settings were as described in the Materials and methods and the principles and measurements of lipid microviscosity sections.

the response of I(12,3) during the course of a temperature titration of the microviscosity parameters. Under the conditions of this titration there was no membrane potential present.

Table 1 illustrates the effect of the imposition of a transient membrane potential (as discussed above) on the microviscosity parameters (S) (results with RLM only are shown), which is our index of fluidity. Also included are the steady-state membrane-potential measurements, which express a higher degree of rigidity during coupled respiration. The microviscosity data obtained with the transient generation of a membrane potential, therefore, are also underestimates of the 'true values', owing to the rapid decay of the potential.

It was considered feasible that the addition of cyanide to the RLM or COVs to inhibit electron transport and thus allow the membrane potential to decay, may interfere with the probe's response (Smith & Butler, 1976). To discriminate such an interaction, the temperature-dependency of the spin-label order parameters in the presence of cyanide ions were measured. Fig. 5 indicates that cyanide does not influence the response of the spin label. Furthermore, the effect of cyanide on the measured steady-state S -value was very similar to the effect of the transition from aerobic conditions to anaerobic conditions (results not shown).

A second criticism of our measurements of field-induced changes of lipid microviscosity could be the possibility that we are not, in fact, measuring the secondary effect of the lipid microviscosity on the probe but a direct effect of the electric field on the probe orientation itself. Two considerations are relevant: we have used two very different probes to analyse the microviscosity and obtained qualitatively the same results. Thus it is unlikely that the field vector would affect both probes in the same manner, since they have very different structures. Secondly, with respect to the fluorescence measurements, Corda *et al.* (1982) have pointed out that the average kinetic energy, U , of a field, F , imposed on a molecule of dipole moment μ , is given by (4):

$$U = \mu F \int_0^{2\pi} \cos \theta \, d\theta \approx \frac{1}{2} \mu F \quad (4)$$

where θ is the angle between the dipole vector and the electrical field vector. If we consider, therefore that DPH is highly polarizable in the excited state, $\mu^* = 1 \times 10^{-18}$ e.s.u. (electrostatic units)/cm for a $\Delta\Psi$ of about 100 mV. With a membrane thickness of about 4–5 nm (40–50 Å), U may be calculated to be approx. 3×10^{-23} J (3×10^{-16} ergs). For an alignment motion this energy would have to be much greater than the ambient thermal energy, KT . The value of KT at 25°C is, however, 5×10^{-21} J (5×10^{-14} ergs). The energy of the field vector, therefore, is not sufficient to cause any alignment of DPH with the imposed electric field. In fact the net effect of U would be to increase the motion of DPH rather than to order it.

The foregoing account of our preliminary studies indicate that our approach may provide useful avenues for the understanding of the physiological aspects of microviscosity changes as a result of membrane-potential changes. As far as we are aware there has not previously been any demonstration of membrane-potential-dependent changes of the lipid microviscosity of mitochondria or COVs.

The observations described above would tend to support the hypothesis of Halestrap (1982a,b) that membrane-fluidity changes may be a significant event for both the short- (respiratory control) and long-term (hormonal) regulation of oxidative metabolism. Clearly, however, the demonstration of membrane-potential-dependent fluidity changes do not unequivocally allow such conclusions.

We should briefly consider how the imposed electric field may alter the membrane microviscosity. In an attempt to quantify the effects of electric fields on membranes, Crowley (1973) proposed that an electromechanical compression

(Maxwell pressure) of the membrane takes place with the application of an electric field. This model was appealing, because many other pressure effects could then be rationalized and equated. For example, hydrostatic pressure and osmotic pressure have been found to affect lipid microviscosity (Johnson & Miller, 1975) such that an increase of the pressure vector resulted in a decrease of membrane fluidity. Furthermore, the well-established increase of lipid fluidity attributed to anaesthetics (Johnson *et al.*, 1973) may be reversed by an increase of either of the respective pressures. Thus, if we apply the Crowley model such that the applied membrane potential compresses the membrane, one of two possible effects would be expected: either the density of the membrane would increase with an equivalent decrease in volume, or the membrane could expand laterally at constant membrane density. As we are dealing with essentially spherical particles, an increase in the lateral packing density of the phospholipid is unlikely. The data shown in Fig. 2 indicate that the membrane viscosity, and hence density, has increased; thus we conclude that a compression of the membrane has taken place with no increase of volume.

We referred above to our observation that the relaxation rate of the lipid microviscosity did not occur in parallel with the relaxation rate of the transiently induced $\Delta\Psi$. This observation deserves a further comment. It has been extensively discussed that membranes exhibit the properties of both fluids and solids (Peters, 1984). The possible transition from the liquidus state to the solidus crystalline state is thought to require an activation energy. Once into the latter state, however, the relaxation back to the former state has been found to occur relatively slowly. We may anticipate, therefore, that the imposed $\Delta\Psi$ orders the membrane (due to Maxwell pressure) such that a transition from the liquidus state to the solidus state occurs that is presumably responsible for the change of microviscosity. When the $\Delta\Psi$ is removed, the solidus state relaxes back to the liquidus relatively slowly. It thus would seem that the techniques pioneered by Shinitzky and co-workers (Corda *et al.*, 1982) may provide a useful

tool for our further understanding of the relationship between membrane structure and metabolic function.

We thank Mr. M. Thelen for useful discussion. The financial support of The Royal Society, the Emil-Barrel stiftung and the European Science Foundation is gratefully acknowledged. P. O'S. is grateful to the European Molecular Biology Organization for the award of a Postdoctoral Fellowship.

References

- Casey, R. P., O'Shea, P. S., Chappell, J. B. & Azzi, A. (1984) *Biochim. Biophys. Acta* **765**, 30–37
- Corda, D., Pasternak, C. & Shinitzky, M. (1982) *J. Membr. Biol.* **65**, 235–242
- Crowley, J. M. (1973) *Biophys. J.* **13**, 711–724
- Georgescu, D. & Duclouier, H. (1978) *Biochem. Biophys. Res. Commun.* **85**, 1186–1191
- Halestrap, A. P. (1982a,b) *Biochem. J.* **204**, 37–47 and 49–59
- Heyn, M. P. (1979) *FEBS Lett.* **108**, 359–364
- Houslay, M. D., Dipple, I. & Gordon, L. M. (1981) *Biochem. J.* **197**, 675–681
- Johnson, S. M. & Bangham, A. D. (1969) *Biochim. Biophys. Acta* **193**, 92–104
- Johnson, S. M. & Miller, K. W. (1975) *Biochim. Biophys. Acta* **375**, 286–291
- Johnson, S. M., Miller, K. W. & Bangham, A. D. (1973) *Biochim. Biophys. Acta* **307**, 42–57
- Keynes, R. D. (1972) *Nature (London)* **239**, 29–32
- O'Shea, P. S. & Chappell, J. (1984) *Biochem. J.* **219**, 401–404
- O'Shea, P. S., Petrone, G., Casey, R. P. & Azzi, A. (1984) *Biochem. J.* **219**, 719–726
- Peters, R. (1984) in *Structure and Function of Cell Membranes* (Benga, G., ed.), pp. 1–41, CRC Press, Boca Raton, FL
- Sauerhaber, R. D., Gordon, L. M., Crosland, R. D. & Kuwahara, M. D. (1977) *J. Membr. Biol.* **31**, 131–169
- Schreier, S., Polnasek, C. F. & Smith, I. C. (1978) *Biochim. Biophys. Acta* **515**, 375–436
- Shinitzky, M. & Barenholz, Y. (1978) *Biochim. Biophys. Acta* **515**, 367–394
- Shinitzky, M. & Inbar, M. (1976) *Biochim. Biophys. Acta* **433**, 133–149
- Smith, I. C. & Butler, K. W. (1976) in *Spin Labelling: Theory and Applications* (Berliner, L. J., ed.), pp. 411–541, Academic Press, New York