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ROLE OF LIPIDS IN MITOCHONDRIAL ENERGY COUPLING: EVIDENCE FROM SPIN LABELING AND FREEZE-FRACTURE ELECTRON MICROSCOPY

HAROLD M. TINBERG, LESTER PACKER AND ALEC D. KEITH

Physiology Research Laboratory, Veterans Administration Hospital, Martinez, Calif. 94553, and the Department of Physiology-Anatomy, and Genetics, University of California, Berkeley, Calif. 94720 (U.S.A.)

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SUMMARY

The structure of lipid in mitochondria under oscillatory state conditions was investigated using spin labeling and freeze–fracture electron microscopy. An examination of the temperature dependence of the time course of mitochondrial oscillations disclosed a break in the Arrhenius plot at approximately 24 °C. A similar break was observed for the partitioning of the hydrocarbon spin label, 6NII, between hydrophobic and polar domains of the membrane. Spin labeling was also used to study mitochondria fixed by glutaraldehyde in contracted and expanded states. These studies revealed that: (a) glutaraldehyde fixation appeared to restrict the motion of membrane lipids; (b) 6NII displayed an enhanced polar partitioning in expanded samples relative to contracted preparations; and (c) the partitioning phenomenon in contracted mitochondria showed a break in the Arrhenius plot at about 24 °C while no discontinuity was observed for expanded samples.

Mitochondrial expansion in the oscillatory state resulted in a change in particle density and aggregation in the outer membrane, but not in the inner membrane as shown by freeze–fracture electron microscopy.

Our results, therefore, suggest an active involvement of lipid in the mitochondrial oscillatory state.

INTRODUCTION

Previous studies have shown that various membrane functions depend on lipids for optimal activity. These functions include the activity of various membrane-bound enzymes[1,2], mitochondrial electron transport[3], and sugar transport in bacterial systems[4].

The present investigation was undertaken to examine the role of lipids in the energy-linked accumulation of ions under oscillatory state conditions[5]. This system was chosen for this study because, under oscillatory state conditions, the mitochon-

drial population is homogeneous with respect to gross conformation and energy state[5].

Lipid structure was investigated by electron spin resonance (ESR) spectroscopy of lipid-soluble spin labels and freeze–fracture electron microscopy. ESR analysis of nitroxide spin labels can reveal certain aspects of the probe's microenvironment, such as viscosity and polarity[6]. Freeze–fracture replicas reveal the distribution of membrane particles in the lipid phase of mitochondrial membranes. The results with these techniques are related to functional states established in oscillatory suspensions of rat liver mitochondria. Our results suggest that mitochondrial lipids undergo changes in physical state at the molecular level coincident with changes in energy state during the oscillation process.

MATERIALS AND METHODS

Isolation of mitochondria

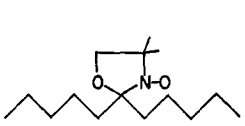
Mitochondria were isolated from the livers of Long Evans rats (Simonsen Labs, Gilroy, Calif.) using 0.33 M sucrose, 1.0 mM EDTA (pH 7.5) as the isolation medium as previously described[7]. The mitochondria were suspended at 25–75 mg protein per ml as determined by the method of Lowry *et al.*[8] using bovine serum albumin as a standard.

Mitochondrial oscillations

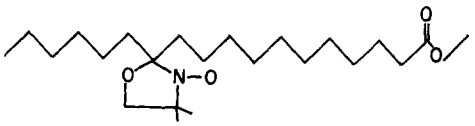
To an aerobic solution containing 0.10 M sucrose, 1.0 mM EDTA (pH 7.8) sequential additions of mitochondria (1.0–2.0 mg protein per ml) and sodium phosphate (0.5 M, 0.5 ml, pH 7.8) were made to a total volume of 8.0 ml. A damped sinusoidal oscillation of electron and energy transfer parameters was initiated by the addition of oxidizable substrate (sodium succinate 0.02 ml, 1.0 M, pH 7.85). Light scattering was measured at 90° as previously described[9] to measure changes in mitochondrial swelling and contraction accompanying the accumulation or loss of osmotically active ions. Mitochondria were fixed in various configurational states directly in the cuvette by the addition of glutaraldehyde (0.5 %) using the technique of Deamer *et al.*[10]. The preparations were put on ice for 15–30 min prior to centrifugation at 90000 $\times$ g·min in a Spinco No. 40 rotor. The fixed membranes were washed with a medium containing 0.10 M sucrose, 1.0 mM EDTA (pH 7.8) and centrifuged as before. The samples were resuspended in the same solution at 10–20 mg protein per ml.

Spin labeling

The two spin labels used in this study were added to mitochondrial suspensions (10–20 mg protein) to a bulk concentration of $4 \cdot 10^{-4}$ M. 6N11 is a hydrocarbon spin label which partitions between polar and apolar regions; consequently, it reveals information relevant to the relative state of the spin label in the two phases as a function of some variable. M12NS appears to be solubilized enti ely in hydrophobic domains of the mitochondrial preparations, and as a result yields information about hydrophobic zones which are probably further removed from the polar interface than those reported on by 6N11. Spectra were recorded on a JEOLCO X-band electron spin resonance spectrometer to an approximate accuracy of $\pm 0.5°$.



6NII



MI2NS

We define two parameters useful in analyzing ESR data in this study. The line height ratio of high-field line components, h_{-1H}/h_{-1P} is an empirical index of partitioning of the spin label between hydrophobic and polar domains and is defined in the insert to Fig. 3. The line heights of these two components do not give a quantitative value of partitioning but do give a relative indication. The motion parameter, τ_0

$$\tau_0 = 6.5 \cdot 10^{-10}\ \mathrm{s} \cdot W_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{\frac{1}{2}} - 1 \right]$$

has been defined previously[11].

No chemical reduction of the spin label was observed for either unfixed or fixed mitochondria, although other biological preparations, including crude mitochondrial preparations or preparations which have not been carefully washed, rapidly destroy the ESR signal.

Freeze-fracture electron microscopy

A drop of the sample was rapidly frozen in Freon 22 and replicas obtained according to Wrigglesworth *et al.*[12] on a Balzers freeze-etching apparatus. A stage which accepts four different samples was used. Specimens were fractured at -170 °C and immediately replicated. Replicas were examined using either a Siemens Elmiscope Ia (at the University of California, Berkeley) or an RCA EMU-3H (at the Veterans Administration Hospital, Martinez, Calif.) electron microscope. All photographs presented are positives, *i.e.* were processed so as to show shadows in white. Shadow direction is indicated by arrows in the figures.

RESULTS

Mitochondrial oscillations

In the presence of an energy source (succinate) and permeant ions (phosphate), rat liver mitochondria undergo damped volume oscillations (Fig. 1). Upon the addition of succinate, there is a decrease in 90° light scattering indicative of an expansion of the inner membrane compartment[5].

To ascertain the temperature dependence of oscillations, the process was examined between 15 and 40 °C. As the temperature increases, the time interval between the addition of succinate and maximum swelling (Phase A) decreases as does the time between maximum swelling and subsequent contraction (Phase B). This temperature dependence is displayed on an Arrhenius plot in Fig. 2. The plots are non-linear, displaying a change in activation energy at about 23–26 °C. For Phase A, an activation energy of 14.0 kcal/mole was determined for the range 25–35 °C and 21.9 kcal/mole between 15 and 22 °C. Although the magnitude of the activation

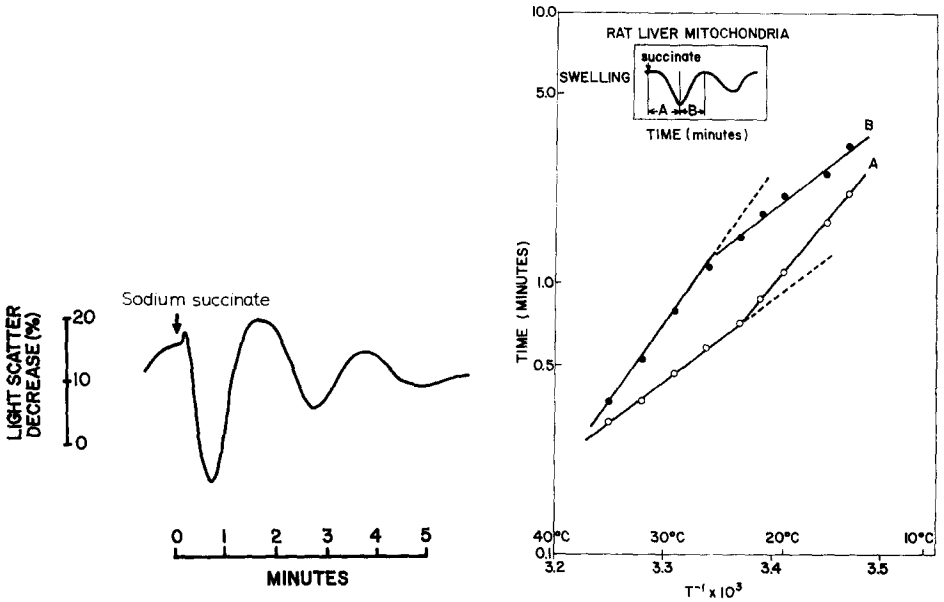


Fig. 1. The oscillatory state of mitochondria. To a solution containing 100 mM sucrose, 1.0 mM EDTA (pH 7.8) were added mitochondria (1.0–2.0 mg protein per ml) and sodium phosphate (0.5 M, 0.5 ml, pH 7.8). The oscillatory state was initiated by the addition of sodium succinate (0.02 ml, 1.0 M, pH 7.85). The total volume was 8.0 ml. Light scattering was measured at 90°. The system was thermoregulated at 25 °C.

Fig. 2. Temperature dependence of mitochondrial oscillations. Experimental conditions were identical to those described in Fig. 1. Insert: an oscillation showing the time intervals determined.

energies varied slightly between experiments, the discontinuity observed was reproducible.

Spin labeling

(*a*) *Model systems.* Idealized spin label partitioning with an infinitely small interfacial boundary between polar and nonpolar phases is shown in Fig. 3. The spectrum displayed in Fig. 3A results from 2NP in water, while that in Fig. 3B from 6NC in 1-octadecene. A composite spectrum (Fig. 3C) is obtained when one tube is placed within the other and analyzed. (By sample manipulation, composite spectra differing in high-field line ratios are obtained.) Fig. 4 shows the effect of temperature on the high-field line ratio (h_{-1H}/h_{-1P}) in a system such as that described in Fig. 3. An increase in temperature resulted in an increase in the ratio in all the systems examined. Linear Arrhenius plots are obtained over the temperature range of 20–50 °C. Since 1-octadecene has a bulk melting point of 17.5 °C (ref. 13) both solvents exist in a single phase over this temperature range. As the conditions of the experiment prevent partitioning, temperature-related changes in h_{-1H}/h_{-1P} in this system are due primarily to changes in bulk viscosity in the 1-octadecene.

In addition to viscosity, two other factors can influence high-field line ratios in heterogeneous systems. A change in the relative amount of spin label in the two phases (partitioning change) will also change the line height ratios. Furthermore, in

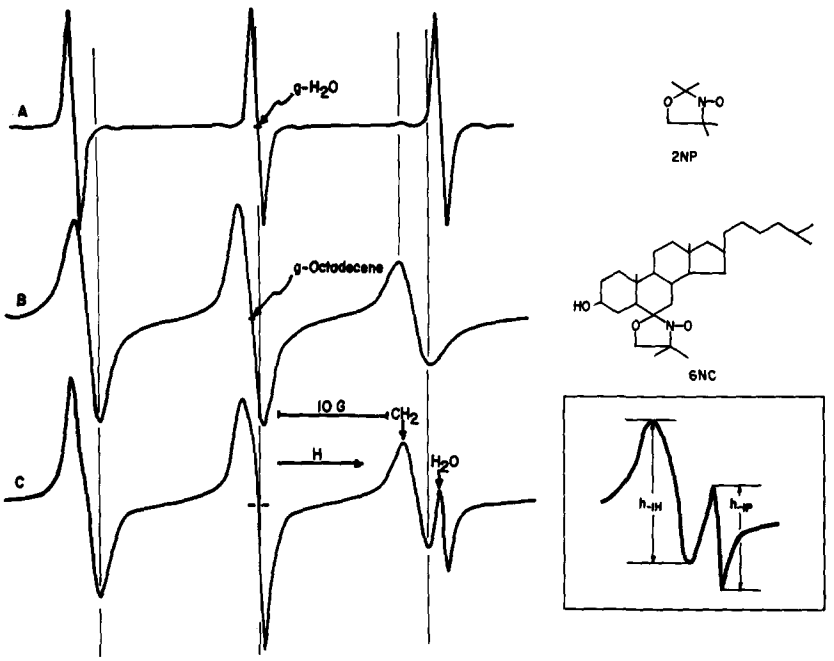


Fig. 3. Composite spectra from a polar–apolar mixed matrix. (A) The aqueous spectrum of 2,2,5,5-tetramethyl-*N*-oxyl oxazolidine (2NP) at room temperature in a 0.9 mm inside diameter capillary tube; (B) The hydrocarbon spectrum of 2,2-dimethyl-5-cholestane-*N*-oxyl (6NC) in degassed octadecene at room temperature in a 0.2 mm inside diameter capillary tube; (C) Capillary B is placed inside capillary A and the composite spectrum is shown. Insert shows the polar and hydrophobic components of the high-field line.

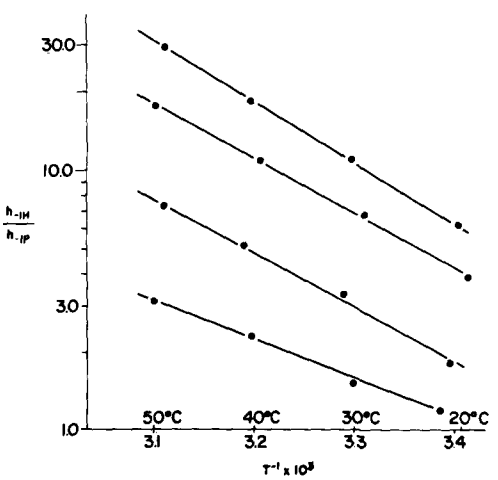


Fig. 4. Arrhenius plots of 2NP in water and 6NC in octadecene. Four mixtures were taken over a temperature range to verify that the line height ratios are linear in single phase media and that no partitioning changes take place.

most membrane dispersions, the hydrocarbon domain is very small compared to the water domain. Therefore, in these systems, local spin label concentrations may be very different, a situation which may result in line broadening due to electron exchange and/or magnetic dipole interactions. Any condition which results in dispersal or further aggregation of the spin label molecules may also change the h_{-1H}/h_{-1P} ratio as a result of concentration effects.

(*b*) *Mitochondrial membranes.* The spin label, 6NII, added to untreated mitochondria yielded a stable signal of the type shown in Fig. 3C for at least 1 h with no detectable loss in signal amplitude. A two-component signal is obtained, since (a) this spin label partitions in the membrane into hydrophobic and polar domains, and (b) the energy of absorption ($h\nu$) is solvent dependent.

In unfixed mitochondria, an increase in temperature causes h_{-1H}/h_{-1P} to increase indicating that (a) temperature increases result in an enhanced partitioning of 6NII preferentially into hydrophobic zones, or that (b) the apolar component narrows substantially while the width of the polar line remains unchanged. At all temperatures examined the two high-field lines are resolved indicating that the hydrophobic and polar domains retain their integrity. When displayed on an Arrhenius plot, the high-field line ratio exhibits a slope discontinuity at 23–26 °C (Fig. 5).

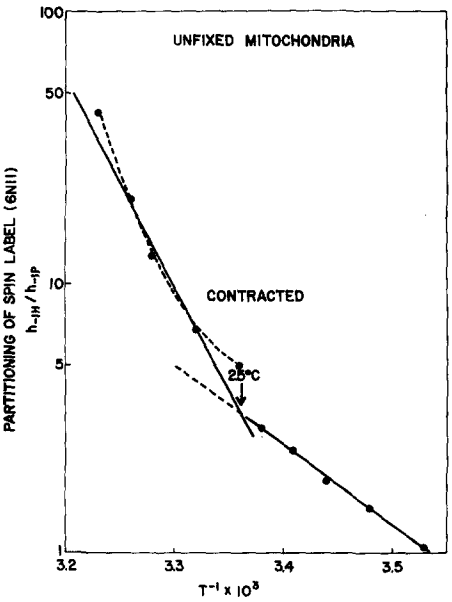


Fig. 5. Temperature dependence of spin label partitioning. Unfixed mitochondria (20 mg protein per ml) and 6NII (bulk concentration = $4 \cdot 10^{-4}$ M) were mixed and ESR spectra recorded at the temperatures indicated. The ratio of high-field line components, h_{-1H}/h_{-1P}, is an empirical index of spin-label partitioning.

We also examined mitochondria stabilized at various points during the oscillatory cycle. Stabilization was accomplished by the addition of 0.5 % glutaraldehyde to oscillating mitochondria, which resulted in the abolition of volume changes as monitored by 90° light scattering.

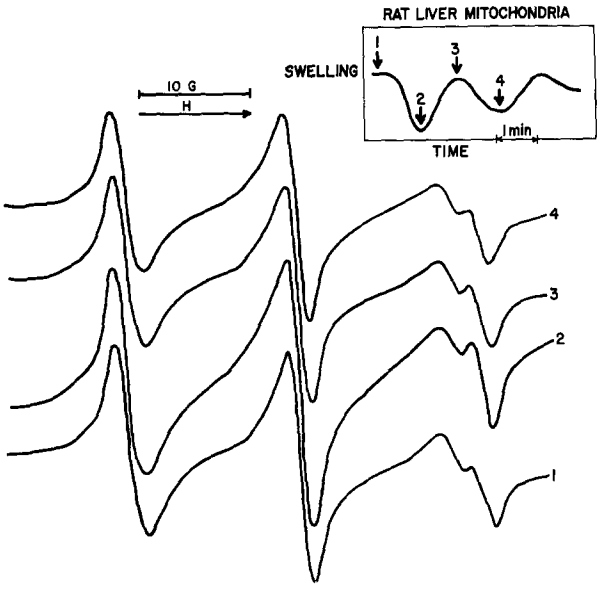


Fig. 6. ESR spectra of 6N11 in glutaraldehyde-fixed mitochondria. Mitochondria were placed in the oscillatory state as described in Fig. 1. Glutaraldehyde (0.5%) was added at the various points indicated in the insert. The preparations were placed on ice for 15–30 min prior to centrifugation at 90000 × g·min. (Spinco No. 40 rotor). The fixed mitochondria were washed with a medium containing 100 mM sucrose, 1.0 mM EDTA (pH 7.8) and centrifuged as before. The samples were resuspended in the same solution at 10–20 mg protein per ml. 6N11 was added at a bulk concentration of $4 \cdot 10^{-4}$ M. ESR spectra were recorded at 25 ± 0.5 °C on a JEOLCO X-band electron spin resonance spectrometer.

The addition of 6N11 to mitochondria fixed at various positions during the oscillation sequence resulted in the spectra shown in Fig. 6. The spectra were qualitatively similar to those obtained for unfixed preparations, indicating that fixation did not radically alter the partitioning of the spin label. An examination of high-field line ratios (Table I) disclosed that the greatest differences in partitioning existed between mitochondria fixed in the contracted state and in the expanded state. The temperature dependence of partitioning in the samples fixed in contracted and expanded systems is shown in Fig. 7. Both preparations exhibited increased high-field

TABLE I

PARTITIONING OF 6N11 IN MITOCHONDRIA FIXED IN VARIOUS OSCILLATORY STATES

Mitochondria were fixed in the various states of the oscillatory cycle (*cf.* Fig. 6, Insert) by the addition of 0.5% glutaraldehyde. Mitochondria and 6N11 were mixed and spectra taken immediately. The ratio (h_{-1H}/h_{-1P}) refers to the two components of the high-field line.

State	*Spin label partitioning* (h_{-1H}/h_{-1P})
1	0.57
2	0.37
3	0.54
4	0.54

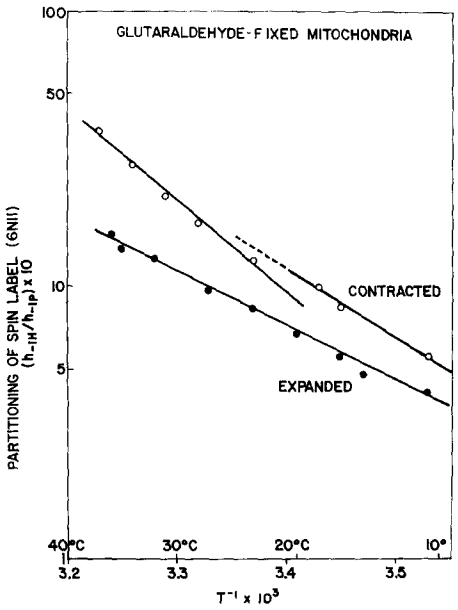
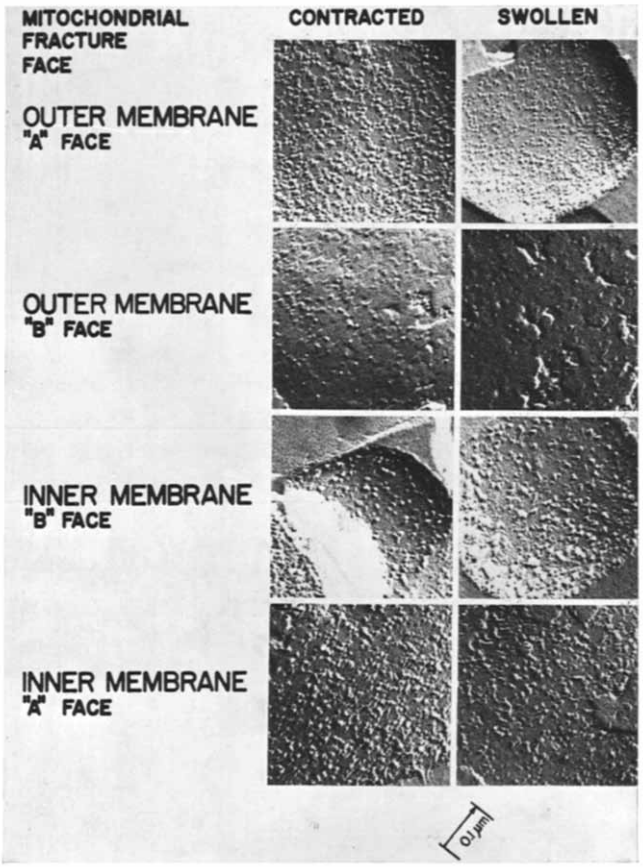


Fig. 7. Temperature dependence of spin label partitioning in glutaraldehyde-fixed mitochondria. Samples were fixed in contracted and expanded states during the oscillatory state with glutaraldehyde as described in Fig. 6. The contracted state was obtained by the addition of fixative prior to succinate. The expanded state was obtained by adding glutaraldehyde at the point of maximum light scattering decrease. For ESR measurements, the sample contained 10–20 mg protein per ml and $4 \cdot 10^{-4}$ M 6N11.

line ratios (h_{-1H}/h_{-1P}) with increasing temperature, although the activation energy associated with this process was less than that observed for unfixed mitochondria. The activation energy differences suggest that fixation of the membrane, while not grossly altering spin label partitioning, confines the alkyl domains and restricts the thermally induced disordering or expansion of these zones. At all temperatures examined, the contracted preparation exhibited higher high-field line ratios than did expanded mitochondria. Samples fixed in the contracted state displayed a discontinuous Arrhenius plot with a change in activation energy occurring at around 24 °C. Although some restriction of motion results from fixation, it appears that a phase change in the lipid alkyl chains does occur. No such break in the temperature plot was observed for samples fixed in the expanded configuration, suggesting the absence of a discrete thermal phase transition. It is conceivable that the alkyl chains in expanded mitochondria are fixed in a disordered state and therefore incapable of going through an order–disorder transition.

We also investigated the structure of lipid in fixed mitochondria using a spin label possessing no apparent polar high-field line component. This label, the methyl ester of 12NS, is localized in the hydrophobic domain of the membrane as reflected by a hyperfine coupling constant (A_n) of 14.2 G (A_n for M12NS is 14.2 G in octadecane and 16.1 G in water). Throughout the temperature range the contracted samples displayed higher τ_0 values than did expanded samples. It has been shown that τ_0 values vary smoothly with bulk viscosity, with higher values obtained at higher viscosities[14].

Spin labels are impurities in biological systems and therefore are capable of inducing local structural changes when dissolved in membrane matrices. However, addition of the label 5NIO (which differs from 6NII by one methylene group) at a level three times that used in the ESR experiments to mitochondria did not affect the oscillation phenomenon, suggesting the absence of significant probe-induced structural changes.



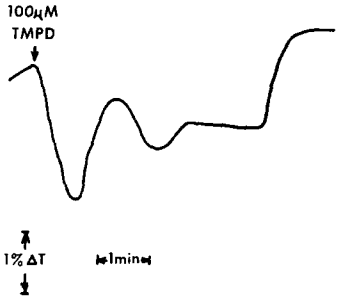


Fig. 8. Oscillations of rat liver mitochondria in 20% glycerol. Oscillations of mitochondria were induced under the following conditions: rat liver mitochondria (9.15 mg/ml), 100 mM sucrose, 1.05 mM EDTA, 20% glycerol, 8 μg/ml rotenone, 8 mM ascorbate, 28.6 mM sodium phosphate (pH 7.8), 25 °C. Samples were removed prior to and at the peak of the first oscillation, immediately frozen in liquid nitrogen, and replicas prepared for examination by freeze-fracture electron microscopy. TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

Fig. 9. Freeze–fracture replicas of outer and inner mitochondrial membranes. The characteristic fracture faces of the half-membranes that arise after fracturing contracted and expanded mitochondria (obtained from samples taken prior to and at the peak of the first oscillation (*cf.* Fig. 8) are illustrated. By convention, the heavy particle fraction face is designated as the "A" face; and the fracture face with few particles is the "B" face. In mitochondria, the A faces of the inner and outer membrane are oriented toward the cytoplasm and the mitochondrial matrix, respectively, and the B faces are adjacent to the intermembrane space. Particles in A faces are usually aggregated into particle networks which are frequently circular or hexagonal in the outer membrane and linear in the inner membrane. Particles of the B faces are usually single and few are observed as particle clusters.

Freeze–fracture electron microscopy

To examine directly changes in membrane organization accompanying gross morphological changes and changes in energy state, we performed the experiment shown in Fig. 8. In this instance, oscillations were performed in the presence of 20 % glycerol, a cryo-protectant which prevents ice crystal damage to mitochondria during the process of preparation of freeze–fracture replicas. The presence of 20 % glycerol does not significantly alter the oscillation process and serves to match the refractive index of the suspension. This permits higher concentrations of mitochondria to be employed, thus facilitating location of mitochondria in electron micrographs prepared from aliquots of the suspension removed at various time periods in the oscillation. Samples of mitochondria were taken prior to swelling and at the peak of the first oscillation when the membranes would be expected to be maximally unfolded and/or expanded. Samples were quickly frozen in liquid nitrogen.

Analysis of the characteristic half-membrane fracture faces (Fig. 9) showed that only the outer A face manifested significantly changed particle density as a consequence of swelling (Table II).

TABLE II

INFLUENCE OF OSCILLATORY STATE ON MEMBRANE PARTICLES OF RAT LIVER MITOCHONDRIA REVEALED BY FREEZE–FRACTURE ELECTRON MICROSCOPY

Oscillatory state	*Average area analyzed* (μm^2)	*Total number particles* *	*Particles per* μm^2
Outer membrane			
A face			
Contracted	0.05	225.3	4136 ± 107
Swollen	0.05	167.6	3404 ± 110
B face			
Contracted	0.117	152	1295 ± 90
Swollen	0.053	66	1256 ± 83
Inner membrane			
B face			
Contracted	0.06	126	1935 ± 41
Swollen	0.15	273	1891 ± 62
A face			
Contracted	0.092	400	4325 ± 392
Swollen	0.04	175	4367 ± 161

* Average of $\geqslant 3$ fracture faces.

Although conventional electron microscopy (*cf.* Deamer *et al.*[10]) has shown that the inner membrane undergoes marked configurational changes during swelling, these membranes displayed no apparent change in particle associations and particle distribution.

A change in the diameter of the particle network of the outer A face of the rat liver mitochondria was also observed. A particle network diameter of 0.0293 μm was found for contracted mitochondria, while expanded preparations exhibit a

diameter of 0.0327 μm. Hence, swelling of the inner membrane system may expand the outer membrane, causing a change in the clustering and distribution of membrane particles.

DISCUSSION

It is generally agreed that lipids play a central role in membranes: (1) they serve as a matrix for localization of protein components in membranes; (2) they affect the activity of membrane-associated enzymes; (3) they are important in permeability functions. In the present study, we have demonstrated that lipids are involved in all of these functions in mitochondria, and further, that they are involved in the process of energy coupling which occurs in the mitochondrion. Support for these functions of mitochondrial lipids has come from studies on uniform populations of mitochondria in the oscillatory state carried out with sensitive and specific techniques for examining the organization of the lipid environment.

Temperature dependence of mitochondrial functional parameters

Discontinuous Arrhenius plots have been obtained for a number of membrane-associated functions[4,15-18]. However, the factors responsible for these changes in activation energy are not fully understood. In some systems[4,15], the break in the Arrhenius plot has been attributed to a phase change in the lipid component of the membrane. This conclusion is supported by the work of Raison *et al.*[11], who used spin labeling to demonstrate the occurrence of a lipid phase change at a temperature similar to that at which an Arrhenius discontinuity in function was observed. We suggest that the 23 °C break in the Arrhenius plot of the oscillation time course also reflects a change in the lipid component of the membrane. An involvement of lipid in the oscillation phenomenon has also been shown by Stancliff *et al.*[7], who demonstrated that the oscillation period was sensitive to the fatty acid composition of the membrane. The loss of the 23 °C transition in swollen mitochondria is evidence for dynamic changes in the structure of membrane lipids coincident with changes in energy coupling during the oscillatory state. These results lead to the question: What is the molecular nature of these changes?

Lipid structural changes

Mitochondrial swelling during the oscillatory state resulted in structural changes in membrane lipid. These changes were manifested as a loss of a discrete thermal transition and an enhanced partitioning of the spin label, 6NII, into polar environments. It is conceivable that membrane expansion and subsequent spatial reorientation of lipids may reduce cooperative interactions between lipid molecules. This reduction in cooperativity may result in the abolition of the phase change. Raison *et al.*[15] showed that modification of lipid structure by detergent treatment abolished the phase transition normally displayed by succinoxidase activity.

A partitioning change as noted in the present study may be a consequence of the following factors: (1) an increased polar partitioning may be due to a decreased solubility of the spin label in the hydrophobic domain of the membrane; (2) intrusion of water into the membrane may increase the relative size of the polar domain giving rise to increased spin label localization in this zone. We suggest that both situations

may be responsible for the observed results. Support for this conclusion comes from studies on 8-anilino-1-naphthalene sulfonic acid fluorescence[19]. During the oscillation sequence, fluorescence changes have the same period but slightly precede those of light scattering. Swelling is associated with a decrease in 8-anilino-1-naphthalene sulfonic acid fluorescence indicating a decrease in the hydrophobicity of the probe's environment. An alteration of lipid structure as a result of mitochondrial swelling is also suggested by the studies of Utsumi and Packer[20], who demonstrated that swollen mitochondria lose the capacity to maintain proton gradients.

Protein influence on lipid structure

Glutaraldehyde fixation of the mitochondrial preparations is highly effective in stabilizing the various configurations attained during the oscillatory state[10]. In the present study, it was also demonstrated that fixation of the membrane restricted the mobility of membrane lipids. Due to the low concentration of fixative employed it is unlikely that the observed effect is due to a reaction of the fixative with phosphatidyl ethanolamine. Our results, therefore, suggest a strong relationship between the lipid and proteins of the mitochondrial membrane.

Wrigglesworth and Packer[21] demonstrated from optical rotatory dispersion and circular dichroism studies in the ultraviolet region that contracted and expanded glutaraldehyde-fixed mitochondria show aggregation–disaggregation changes in membrane proteins. This observation is consistent with the results of freeze–fracture electron microscopy in the present study which demonstrated changes in the organization of particle networks associated with aggregation of macromolecular protein components.

This investigation shows that changes in the physical state of lipids are coincident with the changes in the oscillatory state. Williams *et al.*[22] have demonstrated that changes in unsaturated fatty acid compositon of rat liver mitochondrial lipids modify the period of structural and functional parameters of oscillations. Hence, lipids have an intrinsic role in energy coupling in the oscillatory state of mitochondria. Furthermore, there appears to be considerable interdependence between lipid and protein in the mitochondrial membrane as shown by the observation that the mobility of membrane lipid can be modified by glutaraldehyde fixation of membrane proteins.

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REFERENCES

1 W. Fiehn and W. Hasselbach, *Eur. J. Biochem.*, 13 (1970) 510.
2 S. M. Duttera, W. L. Byrne and M. C. Ganoza, *J. Biol. Chem.*, 243 (1968) 2216.
3 S. Fleischer, G. Brierley, H. Klouwen and D. B. Slauterback, *J. Biol. Chem.*, 237 (1962) 3264.
4 G. Wilson and C. F. Fox, *J. Mol. Biol.*, 55 (1971) 49.

5 L. Packer, *Fed. Proc.*, 29 (1970) 1533.
6 M. E. Tourtellotte, D. Branton and A. D. Keith, *Proc. Natl. Acad. Sci. U.S.*, 66 (1970) 909.
7 R. C. Stancliff, M. A. Williams, K. Utsumi and L. Packer, *Arch. Biochem. Biophys.*, 131 (1969) 629.
8 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
9 L. Packer, J. M. Wrigglesworth, P. A. G. Fortes and B. C. Pressman, *J. Cell Biol.*, 39 (1968) 382.
10 D. W. Deamer, K. Utsumi and L. Packer, *Arch. Biochem. Biophys.*, 121 (1967) 641.
11 J. K. Raison, J. M. Lyons, R. J. Mehlhorn and A. D. Keith, *J. Biol. Chem.*, 246 (1971) 4036.
12 J. M. Wrigglesworth, L. Packer and D. Branton, *Biochim. Biophys. Acta*, 205 (1970) 125.
13 *Handbook of Chemistry and Physics*, Chemical Rubber Company, 47th edn, 1966–1967, p. c-432.
14 J. C. William, R. J. Mehlhorn, A. D. Keith, *Chem. Phys. Lipids*, 7 (1971) 260.
15 J. K. Raison, J. M. Lyons and W. W. Thomson, *Arch. Biochem. Biophys.*, 142 (1971) 83.
16 A. Kemp, Jr., G. S. P. Groot and H. J. Reitsma, *Biochim. Biophys. Acta*, 180 (1969) 28.
17 F. M. A. H. Schuurmans Stekhoven, B. P. Sani and D. Rao Sanadi, *Biochim. Biophys. Acta*, 226 (1971) 20.
18 W. P. Zeylemaker, H. Jansen, C. Veeger and E. C. Slater, *Biochim. Biophys. Acta*, 242 (1971) 14.
19 L. Packer, M. P. Donovan and J. M. Wrigglesworth, *Biochem. Biophys. Res. Commun.*, 35 (1965) 832.
20 K. Utsumi and L. Packer, *Arch. Biochem. Biophys.*, 121 (1967) 633.
21 J. M. Wrigglesworth and L. Packer, *Arch. Biochem. Biophys.*, 128 (1968) 790.
22 M. A. Williams, R. C. Stancliff, L. Packer and A. D. Keith, *Biochim. Biophys. Acta*, 267 (1972) 444.