MEMBRANE FLUIDITY AND FUNCTIONAL ORGANIZATION OF THE MITOCHONDRIAL MEMBRANES

by

ENRICO BERTOLI

May 1975

A dissertation submitted to the University of Warwick for the degree of Doctor of Philosophy.

Dedicated to my parents
and my friends

..... What are you working for? I maintain that the only purpose of science is to ease the hardship of human existence. If scientists, intimidated by self-seeking people in power, are content to amass knowledge for the sake of knowledge, then science can become crippled, and your new machines will represent nothing but new means of oppression. With time you may discover all that is to be discovered, and your progress will only be a progression away from mankind. .....

Berthold Brecht

(from The Life of Galileo)

Freedom of thought and expression is a condition necessary for a positive human growth and for a creative life. (May 1975)

ACKNOWLEDGMENTS

I would like to acknowledge the support and encouragement from my supervisor, Dr D. E. Griffiths.

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ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CAP	chloramphenicol
CE	cycloheximide
DCCD	dicyclohexylcarbodiimide
DNA	deoxyribonucleic acid
DPL	dipalmitoyl lecithin
E_a	energy of activation
EDTA	ethylenediamide tetra-acetic acid
ESR	electron spin resonance
LDM	lipid depleted mitochondria
MSL	4-maleimido-2, 2, 6, 6-tetramethylpiperidinooxyl
NCCD	N-(2, 2, 6, 6-tetramethyl-piperidyl-1-oxyl)-N-(cyclohexyl)-carbodiimide
NMR	nuclear magnetic resonance
OS-ATPase	oligomycin-sensitive adenosine triphosphatase
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PI	phosphatidyl inositol
RNA	ribonucleic acid
T_t	transition temperature
5NS	2-(3-carboxypropyl)-4, 4-dimethyl-2-tridecyl-3-oxazolidinyloxyl
12NS	2-(10-carboxydecyl)-2-hexyl-4, 4-dimethyl-3-oxazolidinyloxyl
M12NS	2-(10-carboxydecyl)-2-hexyl-4, 4-dimethyl-3-oxazolidinyl methyl ester
16NS	2-(14-carboxytetradecyl)-2-ethyl-4, 4-dimethyl-3-oxazolidinyloxyl

SUMMARY

Fluidity, existence of phase transitions and structural organization of lipoproteins in Saccharomyces cerevisiae promitochondria has been investigated by means of X-ray diffraction, differential scanning calorimetry, spin label techniques and freeze-etching.

Distinct phase transitions were found with these organelles which were shown to be partly dependent upon the lipid composition of the membranes.

X-ray diffraction patterns from these membranes show that two physical states of lipids were distinguished, a crystalline-hexagonal packing characterized by a sharp $4.2\text{\AA}$ diffraction band and a fluid (melted) state identified with a broad, diffuse band at $4.6\text{\AA}$.

At the growth temperature ($30^{\circ}C$) the promitochondria of the anaerobic cells contain lipids which are predominantly in the gel state with ordered lipid chains, whereas the mitochondria of the aerobic cells are in the liquid crystalline or fluid state with "melted" chains.

A comparison of the phase transitions of intact membranes and extracted lipids suggests that lipid-protein interaction contributes to the high lipid phase transition temperature of promitochondria. Analysis of the spin probe data indicates a different organization of lipid and protein in such membranes, whilst the greater fluidity in the polar head group and in the lipid core of the differentiated mitochondria reflects the dynamic properties of the membrane lipids on the newly assembled proteins.

Discontinuities in the ATPase Arrhenius plots are observed: with the promitochondria the discontinuity occurs near the beginning of the lipid phase transition and the ATPase enzyme may be associated with the most mobile lipid regions within the membrane. With the fully organized mitochondria it occurs well above the phase transition.

A significant difference has been found in the magnetic resonance spectra from mitochondrial membranes and purified OS-ATPase. The spin label incorporated in the crude membrane is located in a more fluid region than label in an OS-ATPase system, supporting suggestion of the immobilization of lipid in the immediate vicinity of the protein. These results lend suggestion that fluid lipid regions not interacting with proteins could exist in the differentiated mitochondrial membranes and/or specific phospholipids could be involved as physiological regulators of ATPase activity by inducing a proper conformation in the enzymic activity.

INTRODUCTION

1.1 Scope and Purpose of the Thesis

Mitochondrial membranes are highly organized structures, directly involved in essential life processes, such as protein biosynthesis, transport, respiratory activity and energy transduction (1 - 4). All understanding of such catalytic functions on a molecular level is of considerable importance and requires a knowledge of the extent to which lipids interact with other lipids, or with proteins within the membrane.

There is now a great interest in the new concept of membrane fluidity which is believed to control and to co-ordinate many metabolic processes in living systems, such as the regulation of enzyme activity, active transport, permeability and motion of lipid and protein components (5 - 8).

Recent evidence, indicates that membrane lipid bilayers play an important role in membrane structure and membrane fluidity, depending on the nature of protein, ionic strength, pH and temperature (9 - 11). In turn, changes in the physical state of lipids affect the biological activity of several membranes (12 - 14), as a consequence of lipid-protein interactions.

The mitochondrial membranes are heterogeneous structures with respect to both their lipid and protein components. The physiological importance of membrane structure has been

studied extensively in these organelles by dissociation and reconstitution of membrane into its components (15 - 18).

The aim of the present work was to study *in vivo* the association between membrane proteins and membrane lipids by combination of biochemical and biophysical approaches.

A facultative anaerobic yeast, such as *Saccharomyces cerevisiae*, provides an ideal system for these investigations. With this organism one can control *in vivo* the lipid and the protein components of the membrane, without extractive or substitutive techniques that may perturb the original structure of the membrane. This organism, can grow under both anaerobic and aerobic conditions (19 - 20), and this must imply the ability of the cell to accomodate different metabolic needs which involve the structural organization of the membrane.

In this study anaerobic yeast cells were grown in media with and without an unsaturated lipid supplement. A supplement of such lipids directly influences the membrane function as a consequence of the membrane lipid alteration (21 - 22).

A second approach described in this thesis was one in which mutants of *Saccharomyces cerevisiae* which lack the enzyme fatty acid desaturase were used (23). Such mutants which grow solely in the presence of unsaturated fatty acids, provide a means of studying under aerobic conditions, the relationship of manipulated membrane structure to mitochondrial enzyme activity.

1.2 Membrane Lipid and Membrane Protein in Promitochondrial Organelles

It seems reasonable at this stage that before asking questions about the role of membrane fluidity in the completely developed mitochondria, one needs to discuss some aspects of the role of proteins and lipids in promitochondria. The following initial questions can be posed:

(a) Which lipid molecules and which fatty acids occur in these organelles and what are the possible roles that they play in membrane fluidity?

(b) What is the functional role of the membrane proteins in the primitive stage of mitochondrial development?

(c) How can the mitochondrial structure and function be modified by changes in the environmental conditions, such as anaerobic growth?

During anaerobic growth yeast cells, which are glucose repressed, produce promitochondria (24), which lack the components of the respiratory chain, such as cytochromes, ubiquinone and iron sulphur proteins (25 - 26). On the other hand such organelles contain typical mitochondrial DNA, typical markers of the inner mitochondrial membrane such as OS-ATPase and succinate-dehydrogenase (27), and they possess a mitochondrial energy transfer system (28). The oligomycin-sensitive ATPase is an important component of the normal phosphorylative mechanism in intact mitochondria (29). Thus the possession by the anaerobic promitochondria of such a system indicates the ability of

the yeast cell to utilize glycolysis as the major source of energy (30) and to retain the major portion of their phosphorylative capacity even in the absence of the respiratory chain.

The membrane lipid is drastically affected by anaerobic growth of the yeast cell (31). Promitochondria derived from these cells grown without lipid supplement are characterized by an extremely low degree of unsaturation and by high content of short chain saturated fatty acids. Regardless of the lipid composition, promitochondria contain all the major phospholipid classes, typical of aerobic organelles, with a relative proportion of PI and PE significantly different. These observations of a concomitance of the high degree of lipid saturation coupled to lack of the respiratory chain, raises the question of the importance of all appropriate lipid membrane bilayers to accomodate newly synthesized proteins during the aerobic growth.

1.3 Mitochondrial Biogenesis and Membrane Structure

Yeast mitochondria can be considered as semiautonomous organelles, with a functional DNA-RNA synthesizing system (33). The biosynthesis of mitochondrial proteins occurs via co-operative interaction with the major cyto-nuclear protein apparatus, encoded in nuclear DNA (34 - 36).

Mitochondrial membranes are composed mainly of catalytic proteins and of lipids with functional and structural roles. A close co-ordination between membrane lipid and membrane protein biosynthesis has been observed

during the mitochondrial development (37 - 38). The molecular mechanism responsible for such co-ordination is still unknown. Otherwise, the most work on mitochondrial biogenesis is derived from biochemical and morphological studies (39 - 44). Only little attention has been given to how such studies relate to the physical behaviour of these membranes.

Knowledge of the biophysical properties of the promitochondria and mitochondria assumes now a particular importance for an understanding of the relationship between membrane function and membrane organization. In this prospect the most important question concerns how lipids and proteins are organized in the membrane and in what way they interact in the functional mitochondrial membrane.

1.4 Membrane Protein Organization

Membrane proteins are localized in many alternative ways with respect to the lipid bilayer. The association of protein and lipid, whether primarily polar or non polar is still uncertain. However, it seems probable that different membranes will have different proportions of ionic and hydrophobic interactions.

A variety of models have been proposed to describe the nature of such interactions (45 - 48). Experimental data some time do not agree with certain predictions of these models, regarding the arrangement of protein molecules in the plane of the membrane. Biochemical and biophysical studies (49 - 52) indicate that different membranes vary

significantly both in their composition and in their molecular arrangement. It may be that one type of interaction predominates in certain membranes, depending upon their function. For example the Danielli-type membrane, which is applicable to the myelin membranes, has lipid-lipid interactions with proteins restricted to the surface of a lipid bilayer (45). On the other hand, the globular type of proteins penetrating into a lipid bilayer may predominate in membranes having high catalytic activity, such as mitochondrial membranes, as proposed in the "fluid-mosaic" model (47).

The dominant interaction within mitochondrial lipoproteins is believed to occur primarily via hydrophobic forces (49), suggesting that such interactions are the most important in determining the function of the membrane. Evidence is available that anionic groups of phospholipids in native and reconstituted membranes are not shielded by membrane proteins via electrostatic forces. The reconstitution of LDM with phospholipids leads to a membrane having a lipid bilayer whose negative charges are available for ionic interaction with basic proteins, such as cytochrome c or lysosyme. Similar effects have been observed in the submitochondrial particles (50 - 52). These observations have been confirmed using different physical techniques. Differential scanning calorimetry studies of rat liver mitochondrial membranes indicate that the membrane lipids are influenced by the membrane proteins (53). This suggests that part of the lipid hydrocarbon chains are in contact with the proteins. NMR spectroscopy

studies also clearly show that in biomembranes, as compared to pure lipids, the lipid alkyl chains are immobilized by the presence of protein (54). Perturbation of lipid bilayers with detergents or lysolecithins leads to a sharp increase of the alkyl chain signal, indicating the weakening of the protein-lipid interaction under these conditions. This can only be understood on the basis of the hydrophobic penetration of the protein into the apolar region of the lipids.

1.5 Membrane Lipid Organization

There is a universal agreement that the lipids in biological membranes are in lamellar phases, consisting of bilayers separated by bilayers of water (55). A unique property of these lipid bilayers is their ability to exhibit a reversible number of phase transitions (polymorphism) in relation to changes in temperature (thermotropic) and water content (lyotropic). Order-disorder (gel to liquid crystalline) phase transition can be detected utilizing a variety of physical techniques. This indicates that below the T_t the hydrocarbon chains are in quasi crystalline arrangement, while above the T_t they are in a more fluid state (56). The basic work has been done by Chapman *et al.*, (57) using calorimetric studies. It has been proved that the temperature at which the phase transition occurs, depends upon the lipid polar head groups, the length of the hydrocarbon chain, the degree and type of unsaturation, the water content and the presence or absence of cholesterol.

The lipids with more unsaturated sites have a lower transition temperature than that of more saturated lipids; cis-unsaturated chains have a lower transition temperature than trans-unsaturated ones and longer chains have a higher transition temperature than shorter ones (58). Of particular interest is the role of cholesterol in preventing formation of crystalline gel areas in some membranes while also inhibiting the motion of hydrocarbon chains in more fluid regions (59).

Biological systems, however are constant in temperature; hence, conformational changes in vivo must be caused by alterations of ion concentrations, pH, water content at a constant temperature. Such structural changes at the phase transition have been found to affect some membrane functions such as the active transport (60), the permeability (61), the enzyme activity (62) being different below (solid phase) and above (liquid phase) the melting point of the membrane lipids.

In active membranes and bilayers formed from naturally occuring phospholipid mixtures, the hydrocarbon chains are in a mobile, fluid state (63). Recent studies of ESR with spin label probes and NMR, have shown that the fluidity of the bilayer varies with the distance from the interface, indicating that the molecular motion increases along the length of the hydrocarbon chain (64). The biological importance of these studies can be evaluated by studying the mobility of spin-label probes embedded in the membranes, compared to their motion in isolated lipid bilayers. These findings indicate a considerable similarity in the

solubility of the probes in bilayers and membranes of different biological systems (65 - 67), whilst at least in some membranes, like inner mitochondrial membrane the proteins tend to immobilize the motional freedom of the lipids (68). This observation, together with data obtained from X-ray diffraction and freeze-etching studies (69 - 71), supports the hypothesis of the hydrophobic association of proteins with the fluid apolar region of the lipids.

1.6 Membrane Lipid-dependant Enzymes

Several membrane-bound enzymes have been shown to require lipid for their physiological activity. The activity of most of the mitochondrial enzymes was found to be inhibited by detergents, solvents or phospholipases (72 - 74). The reactivation with added lipids has been successful in demonstrating the lipid requirement in membrane functions (75 - 77). The membrane lipids may have a structural role in the control of some membrane enzymes, by providing an alternative hydrophobic medium in which some catalytic reactions might occur. Such a situation may be a feature of oxidative phosphorylation, of energy linked transport of ions and of the respiratory chain (77 - 78). The degree of specificity of the phospholipid requirement for reactivation has been found to vary with the different enzymes. The mitochondrial -hydroxybutyric dehydrogenase is a unique enzyme showing an absolute specificity for one phospholipid type. Cytochrome

oxidase showed a relative specificity for certain phospholipids and the tightly bound cardiolipin seems involved in cytochrome oxidase activity.

The restoration of respiratory activity in lipid-depleted membranes, appears to be related to the nature and to the degree of unsaturated sites in the lipids, whilst saturated phospholipids, like DPL, are effective only above their transition temperatures (79). These observations suggest that not only the polar nature of the lipid-protein interactions are important in enzyme regulation, but also the property of the lipid moieties. That property is probably the fluid state of lipids in membrane systems. The membrane-lipids in biological membranes are in the liquid-crystalline state at the physiological temperature. The changes just a few degrees below the transition temperature could have a dramatic effect on many physiological processes, which are membrane-bound, as a consequence of temperature-induced changes in the structural properties of membrane.

Since the functional aspects of membrane are related to their structure which is dependent on the molecular organization of the lipid phase, the studies of temperature-dependence membrane activities are an important approach to establish such correlations. Membrane bound activities show a characteristic discontinuity in the Arrhenius plot, with a change in the E_a below a thermal phase change (80). The interpretation of the biphasic plot is not uniquivocal (81) and suggests some relation between the fluid state of the membrane and enzyme activity. A variety of mitochondrial

enzyme activities exhibit a biphasic Arrhenius plot with discontinuity occuring at around 20°C (82 - 83). No such transition is observed in mitochondria from poikilothermic animals. These observations were related to the peculiar lipid composition of poikilothermic animals, where the degree of unsaturation and hence the fluidity of the membrane can be easily varied with the environmental temperature. The temperature-induced change in the E_a of membrane-associated enzymes is considered a direct consequence of a temperature-induced change in the physical state of the membrane lipids. The physiological significance of these temperature-induced changes in the molecular organization of membrane lipids has been demonstrated by the corresponding change in E_a of the respiratory enzyme system associated with some biological membranes (84).

1.7 Membrane Fluidity

By this term is meant the motion of both lipid and protein in the membrane. There is evidence that at least certain proteins can move in the plane of the membrane and also to rotate in the plane. In this motion through a lipid bilayer the protein experiences the viscosity of the membrane, which can thus be measured (170-171). For example, the rotational rate of rhodopsin in the plane of the retinal-rod membrane indicates a medium of low viscosity equivalent to a light oil (172). Since the viscosity of the immediate environment of the rhodopsin molecules presumably determines their rotational diffusion, this highly fluid environment is certainly consistent with rapid lateral diffusion of the lipids. The biological significance of the surface dynamics of membrane components is also indicated by the rapid aggregation of immunoglobulins dispersed on the surface of the lymphocyte, these collecting at one pole of the cell to form a cap when treated with multivalent anti-immunoglobulin antibody or multivalent antigens (173).

The lipids also show fluidity. This fluidity is a function of the hydrocarbon chain motion, which can, in principle, be estimated in terms of rotational correlation time. The rotational correlation time (or relaxation time) has been defined by Debye (cited by Sinesky) (174) as "the time required for a system of orientated molecules to revert to a random distribution". The value of the rotational correlation time for a spherical molecule of radius r, related to the viscosity (η) of the medium, can be estimated by the Stokes relation in Eqn. 1 (175).

$$\tau_c = \frac{8\pi\eta r^3}{6KT}$$

where K is Boltzmann's constant and T is the absolute temperature. For example, at $20^{\circ}C$ the water has a correlation time of 10^{-12} sec.

The dominant mode of motional freedom of lipid chain appears to be rotations about hydrocarbon C-C bonds. Seeling et al (176) suggested that the barrier to rotation about these bonds is composed of two parts: an intrachain activation energy for bond rotation (ca. 3 Kcal/mol), and a structure factor due to interchain steric interactions providing additional activation energy (ca. 1 Kcal/mol). The relative intrachain energy of the various rotational configurations for normal lipid chain is seen with the aid of Newman projections, as represented in Fig. I (177).

Physical Methods Used in Detecting Lipid Fluidity

Amphapathic lipids undergo a phase transition upon raising the temperature. When the hydrocarbon chains of the component fatty acid "melt", the system passes from a crystalline to a liquid-crystalline state, where the fatty acyl chains are liquid-like, whereas the polar ends are still experiencing strong neutral attraction (178). At low temperatures, the hydrocarbon chains are most likely to be in the low energy all-trans configurations. As the temperature is raised, there will be some probability of gauche rotations about C-C bonds. However, the steric interactions between chains will prevent most single bond trans-gauche changes until relatively high temperatures when a cooperative melting of the hydrocarbon chains can take place (Fig. II). Such a cooperative "transition" is observed calorimetrically (109).

(a) Differential Scanning Calorimetry

Differential scanning calorimetry detects the endothermic transition which accompanies "melting" of the lipid hydrocarbon chains (179). The transition is abrupt in the case of a pure lipid component containing only one

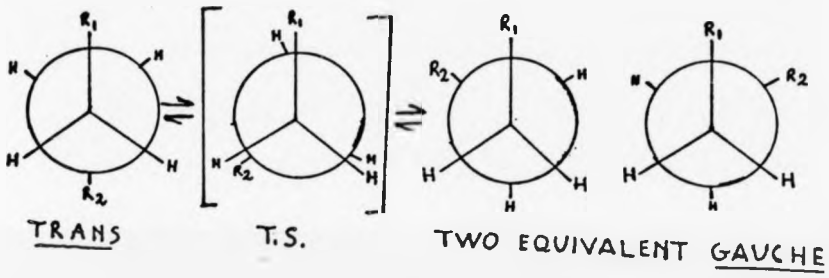


FIG. I Newman projections for C-C bond rotation in hydrocarbon chains. R_1 and R_2 are chain fragments. T.S. denotes a transition state. The conformation having R_1 and R_2 in trans relationship is of lowest energy since the R groups only experience protons on the opposite carbon. The two equivalent higher energy conformations with R_1 and R_2 in gauche relationship are found in chain-lipid bilayer above the phase transition. (From W. Plauchy et al, Biochemistry, (1974), 13, 4906).

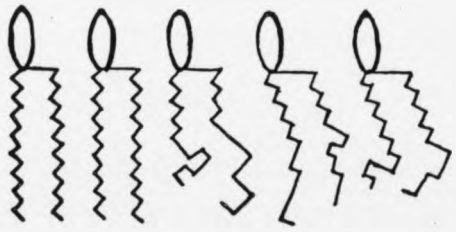


FIG. II One-half of the basic lipid bilayer. Ellipsoids represent the polar head groups and the wiggly lines the fatty acid chains. The chains on the left are in the all-trans conformation. The chains on the right have several gauche rotations in the lower half. (From J. F. Nagle, J. Chem. Phys., (1973), 58, 252).

fatty acid species; for example, hydrated dipalmitoyl lecithin has a transition temperature of about 41.5°C. Hydrated lipid mixtures in the form of liposomes or of vesicles obtained by sonication, which are often used as the most practical bilayer systems, show a very wide endothermic peak which is centred near 0°C in the case of liver mitochondrial phospholipids (180). Such a large range of melting temperature is considered to result from heterogeneity of the lipid molecules and their melting at different temperatures with phase separation (105). For pure phosphatidylcholine, a pretransition at lower temperature has been observed by scanning calorimetry. Such endothermic peak is probably associated with an increase in mobility of the polar head prior to the main transition.

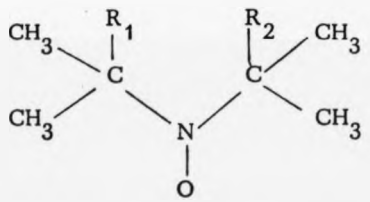
(b) X-ray Diffraction

The nature of the transition is most clearly revealed by X-ray diffraction experiments (13-114). Below the transition the hydrocarbon chains produce a sharp diffraction band which corresponds to a nearest neighbour chain distance of 4.2 Å Above the transition the band is quite diffuse as in liquid hydrocarbons and corresponds to a distance of 4.5-4.6 Å. In the case of heterogeneous lipids or membranes the two bands are separated by broad gradual transitions. Unfortunately, X-ray diffraction and thermal calorimetry give only a time-independant information of the membrane structure. Time-dependant motions of the molecular structures can be estimated by spectroscopic methods sensitive to motion in the range from 10^{-11} to a few seconds. This range is spanned by magnetic resonance techniques.

(c) E.S.R. Spectroscopy

Information relating to viscosity of local domains, polarity of local domains, states of molecular ordering and rotational or transitional motion can be studied by spin label method (181-183).

In order to be useful for biological studies, spin labels have to fulfill certain requirements, such as being stable at the condition of the experiment, and not constituting too much of a perturbation. The nitroxide molecules have been found to be convenient moiety and schematically they share the structural feature:



Most of the information arises from the interaction between the unpaired electron, localized primarily on the nitrogen atom, and the magnetic moment of the ^{14}N nucleus, giving rise to the hyperfine splitting (184).

In the case of isitropic motion and in the absence of intermolecular interaction, the line widths can be related to a correlation time, τ, which reports on the rotational rate of motion of the label. In order to faithfully inform about the macromolecule or aggregate to which it is attached or intercalated it is important that the label keeps little freedom of motion with respect to the macrostructure. Single bonds between the label and the site of attachment, and intramolecular isomerizations of the label moiety will contribute to an increased mobility of the latter with respect to the system, and calculated rotational correlation times may not correspond to that of the macrostructure.

When 2, 2, 6, 6-tetramethylpiperdinol-N-oxyl (TEMPOL) is dissolved in a solution at low viscosity as in water, the rate tumbling is fast on the time-scale of e.s.r. measurement, and three sharp lines are observed, as represented in Fig. III(a). This spectrum is commonly referred to as "freely mobile". When the viscosity of the solution is increased the tumbling rate is

decreased. As a consequence, the e.s.r. lines are shown in Fig. III broaden (b), the outer lines separate (c), and separation of $2A_{zz}$ (d). The limit of this situation is when the label is said to be immobilized (on the e.s.r. time scale) and the spectrum is called a rigid glass or "powder" spectrum. Spectral approaching the "powder" limit are frequently observed from spin labels in dried biological specimens or when rigidly bound to large proteins (185).

The correlation time, τ_c, inversely related to the tumbling rate of the nitroxide molecule can vary between 10^{-11} and 10^{-7} sec, corresponding to very fast and very slow tumbling, respectively. For nitroxides undergoing rapid tumbling, $\tau_c < 10^{-8}$ sec, correlation times may be calculated from the theory of Kivelson (186). This theory leads to an equation relating width differences to correlation times. If the line shapes are assumed to be single Larentzian curves, the correlation time can be calculated following Keith et al (187) by Eqn. 1.

$$\tau_c = 6.5 \times 10^{-10} W_o(\sqrt{h_o/h_{-1}} - 1) \qquad (1)$$

where W_o and h_o are the width and height of the first derivative centre line and h_{-1} is the height of the high field line.

Spectra obtained from almost completely immobilized spin labels (i.e. $\tau_c > 10^{-8}$ sec) are generally quite complicated to analyze. For example, the "powder" spectrum (Fig. III (d)), the line broadening Eqn. 1 cannot be applied to quantitatively calculate molecular mobility. However, an empirical relation based on suitable spectral parameter can be used to extend the range of fluidities in which useful data can be obtained from Eqn. 1. In this case the Equation of Stoke, which yields the rotational correlation time of an equivalent spherical molecule can be used.

$$\tau_c = \frac{4\pi\eta r^3}{3KT} \qquad (2)$$

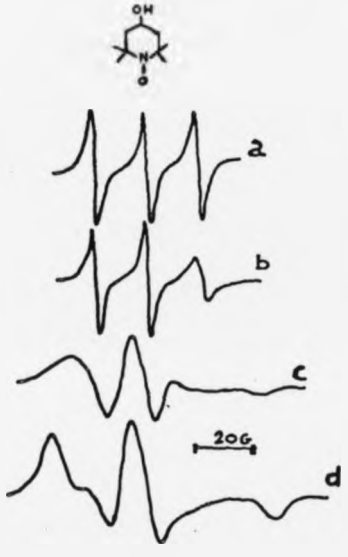


FIG. III Spectral effects of increasing glycerol-solution viscosities, (a) to (d), causing a decrease in the rotational motion of dissolved spin label TEMPOL. Spectra results from spin label motion (a) "free", (b) "partially" immobilized, (c) "strongly" immobilized, and (d) "powder" or completely immobilized.

The Stokes' Equation states that T_τ is proportional to viscosity η and hence, insofar as Eqn. 1 generates valid rotational correlation times, the time so calculated multiplied by the absolute temperature of measurement should be linear with the viscosity of the solvent. Experimental results indicate that Stokes' relationship is valid (at least for methyl-12-nitroxystearate) to rotational correlation time as long as 5 n sec (189).

The fluidity of a pure lipid bilayer or of a relatively fluid organised system, such as the lipid bilayer portion of certain biological membranes and phospholipids model membranes were first studied quantitatively by e.s.r. (189) Spin labelled fatty acids or phospholipids, leaving paramagnetic nitroxide groups at different positions in the hydrocarbon chain are used as probes of the lipid motion in the surrounding environment (130). The unpaired electron in the stable nitroxide group is sensitive to the degree of motional freedom allowed to the nitroxide group and the correlation time, τ_c, can quantify the rotational mobility of the lipids at least for times not higher than 10^{-9} sec. These experiments show that the nitroxide group has less freedom of motion when it is in the region of the bilayer close to the glycerol backbone than when it is at the centre of the bilayer.

The lipid mobility decreases with decreasing temperatures, and abrupt discontinuities or breaks are found at well-defined temperatures in the Arrhenius plots of the rotational correlation times or other motion parameters (65). The sharp breaks, however, do not correspond to the wide endothermic transitions detected by differential scanning calorimetry, although they usually fall in the range of such transitions. The reasons for such discrepancies are debated, but it is clear that the two methods detect different aspects of the same phenomena. Raison et al (190) have found two breaks for the motion of spin labelled fatty

acids in mitochondrial membranes, the first in the upper and the second in the lower range of the calorimetric phase transition. One reason is the preferential disposition of stearic acid spin labels in the more fluid areas of the bilayer having a heterogeneous physical state (137). Such preferential affinity will tend to squeeze out the labels into fluid bilayer areas when crystalline and liquid-crystalline domains co-exist in the same bilayer. A discontinuity in the upper range can be related to phase separation of crystalline from liquid-like domains when certain lipids begin to crystallize at decreasing temperature, while a discontinuity in the lower range will indicate completion of solidification.

(d) N.M.R. Spectroscopy

The trouble with studies of spin labels is that it is not clear how much such foreign molecules can locally perturb the system and thus, the results. Nuclear magnetic resonance (n.m.r.) methods have also been used to probe fluidity (191). They have a major advantage of not introducing foreign molecules in the membrane. A particular feature of the nuclear resonance technique is in the possibility of using selected nuclei for examination. Thus, it is possible to study ^{1}H nucleus, and all other nuclei in the system remain transparent. Alternatively, it is possible to look at ^{13}C, ^{2}H or ^{15}N when the motion of a particular grouping containing these nuclei is of interest. The application of the conventional n.m.r. to complicated structures such as the biological membranes has been limited by the -technical problems of low sensitivity and of the overlap of many resonances in broadened envelopes of signal from the nuclei present at natural abundance in membrane. With Fourier transformer n.m.r., which increases the effective instrument sensitivity, it is possible to study the motion in biological membrane quantitatively. The ^{13}C nucleus is particularly suitable, since its natural abundance is only 1.1% and selective enrichment by up to

90-fold can effectively simplify the spectra to single resonances from the enriched nuclei. The ^{13}C nucleus also has the technical advantage that the line widths of the resonances are much narrower than the corresponding proton resonances in structures with severely restricted motion, and hence, the effective resolution and sensitivity are increased (192-193). Quantitative information about the molecular motion of the lipids can be obtained from ^{13}C spin-lattice relaxation time (T_1), which can often be related to rotational correlation times for the individual ^{13}C nuclei in the lipid molecules. This provides a pattern of internal motions in the molecules that is sensitive to steric interactions with other membrane components. The ^{13}C relaxation times in the lecithin molecules increase from glycerol carbons towards the NMe_3 group on the terminal methyl carbons as represented in Fig. IV. This is consistent with e.s.r. observation where a gradient of increasing motion from the glycerol backbone towards the therminal methyl of the fatty acid chains has been found. These findings for lipid bilayers provide a preliminary information for comparison with lipids within the membrane. Since intermolecular interactions between lipid and protein are required to maintain the membrane structure, it is probable that constraints will be imposed on the molecular motion and organisation of at least some of the lipids in the membrane compared with the unperturbed bilayer. These intermolecular interactions may affect only those lipids in direct contact with the proteins, by affecting the packing of the lipids, or by causing separation into bilayer regions of differing lipid composition. Relaxation times of the lipid in sarcoplasmic-reticulum membranes have been related with the same lipids in sonicated vesicles after extraction from the membrane (194). The ^{13}C chain resonances have very similar T_1 values in both the membrane and vesicles preparations. The only significant difference is for the $N^+(CH_3)_3$ resonance,

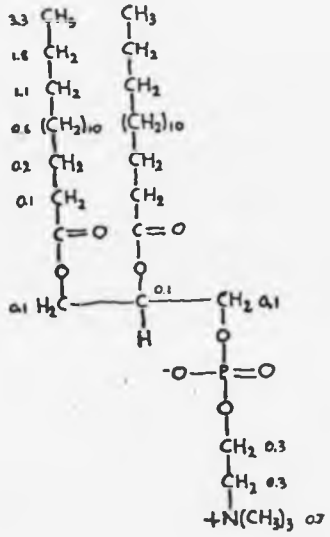


FIG. IV $^{13}CT_1$ relaxation times for dipalmitoyl lecithin in D_2O at 52°C.

where the value was shorter in the membrane by 35%, indicating some interaction with membrane protein.

Interesting information on lipid-protein organisation in erythrocyte membrane derive from studies using ^{1}H N.M.R. (195). It is of interest that $^{+}N(CH_3)_3$ protons are in similar environment to that observed with sonicated phosphatidylcholine. This is consistent with the idea that regions of the membrane containing lecithin or sfingomyelin do not strongly interact electrostatically with membrane protein. Significant difference is for the lipid hydrocarbon chains which are restricted in their molecular freedom. This may arise from lipid-lipid and lipid-protein interactions. Some restriction of the chain movements on the membrane may be caused by lipid-protein interactions as well as interaction with cholesterol. The absence of any cholesterol signals suggests that the cholesterol is in some anisotropic environment.

(e) Fluorescent Probes

Also fluorescent probes may be used as indicators of membrane properties and fluidity (196-197). There are two main kinds of fluorescent probes which can be used in membrane studies. One kind is represented by hydrophobic chromophores which are located in the non-polar core of a lipid bilayer, and their spectral properties are sensitive to microviscosity of the environment represented by the hydrocarbon/lipid chains. For example, polarisation of the fluorescence of perylene or 9-methylanthracene (198-199) increases as a function of viscosity and has been used as a sensitive probe for phase changes in biomembranes. On the other hand, amphipathic molecules like 1-anilinonaphthalene-8-sulphanate (ANS) are located in the polar region of the lipid bilayer near the water interface. ANS is strongly fluorescent in hydrophobic media, but its fluorescence is quenched in aqueous environments.

Also ANS fluorescence is sensitive to the viscosity of its microenvironment, and it has been used to detect phase changes in membranes (112). The main criticism that can be advanced against fluorescent probes and spin labels as well is that they are foreign molecules which are likely to perturb the lipid bilayer. However, the extreme sensitivity of fluorescence spectroscopy allows use of the probes at extremely low concentrations which may not affect the original packing of the membrane.

(f) Factors Affecting Molecular Fluidity

Van der Waals attractions between fatty acids decrease with decreasing chain length and increasing the number of cis double bonds. While lipids with more unsaturated chains have lower transition temperatures than more saturated ones (58), different lipid classes even having the same fatty acid compositions have different transition temperatures. In this case, restrictions must be exerted by the polar heads of the phospholipids onto the hydrocarbon chains. The existence of restrictions induced by the polar heads is quite evident by studying the rotational mobility of stearic acid spin labels having the nitroxide at varying distance from the carboxyl group and hence at varying depths in the lipid bilayer (200). The rotational mobility is highest in the centre of the bilayer and strongly decreases approaching the polar surfaces. Clearly, there is an immobilising effect of the polar groups on the nearest methylene groups.

The cholesterol content is an important factor in modulating membrane lipid fluidity (58). In model systems cholesterol abolishes the endothermic transition from a crystalline to a liquid-crystalline condition. In general, cholesterol appears to induce an intermediate degree of fluidity to bilayers (58). This effect of cholesterol can be of importance in the physiological properties and pathological deviations of plasma membranes which are rich in this

compound (201).

Of great importance appears the effect of proteins on lipid fluidity, since proteins together with lipids are the main membrane constituents. Comparison of rotational mobility of spin labels in membranes has shown that membrane protein exerts a strong immobilising effect on the lipids at all depths in the bilayer (202). Immobilisation by proteins has been directly shown by Jost et al (203) on isolated cytochrome oxidase. A stearic acid spin label becomes progressively more mobile as the lipid content of the enzyme complex is increased. Computer calculation shows that the mobility at any lipid content is the average of mobility of the label in the lipid-poor oxidase and in free bilayer, suggesting the existence of a strongly immobilized layer of PC interacting with the enzyme, while the surrounding lipids experience the fluidity of a protein-free bilayer. However, since spin labels prefer the most fluid areas in a heterogeneous environment (59), the increased mobility resulting from increasing the lipid content in cytochrome oxidase could be the result as well of progressive partition of the label into the lipids which are farther away from the protein and which are progressively less immobilized. This idea underlines the possibility of a cooperative effect of the immobilized PL molecules on their neighbours. Two possible interpretations can be made:

(i) the label undergoes an average mobility between a strongly immobilised lipid layer and fluid surrounding bilayer,

(ii) the label moves to the more mobile areas but they are not as fluid as free bilayer due to cooperative effects among the lipid chains.

Steroid spin label 3-doyl-5α-androstane, which is better suited to probe all types of lipid environment confirm the stearic probe studies, suggesting that

interpretation (i) may be correct. If this is the case, lipids "immobilized" by proteins do not exclude stearic acid spin labels as do crystalline lipids. Immobilization by proteins therefore may not be a real phase change, but this is rather an increase in viscosity of otherwise "fluid" (disordered) lipids. This interpretation is strengthened by differential scanning calorimetry and X-ray data.

Differential scanning calorimetry has been used to detect the effect of different kinds of proteins on lipid transitions. Cytochrome c, which interacts with phospholipids ionically, lowers the lipid transition of $10^{o}C$ (106, 169). The strongly shifted thermal transitions of lipids by proteins are probably related to the reorganisation of the polar groups by ionic interaction with the charged groups in fixed positions of the proteins, leading to less efficient packing of the lipid chains (169). It is possible that basic proteins immobilize the neighbouring segment of lipid chains, but at the same time decrease the overall packing of the fatty chains in the bilayer, leading to decreased transition temperature. Thus the possibility exists that the strongly immobilized layer of lipids surrounding the intrinsic proteins and detected by spin labelling techniques may not undergo the endothermic transition accompanying melting of the lipid chains; it may then behave as a disordered, yet highly viscous lipid region.

From these observations, the overall fluidity of a membrane system is the result of its microenvironments having varying ranges of fluidity, depending upon the following factors:

(a) intrinsic lipid microheterogeneity in the plane of the membrane,

(b) presence and localization of cholesterol,

(c) localization and nature of intrinsic and extrinsic factors,

(d) possibly, the presence of other compounds like quinones, chlorophyll, and the presence of bound water and ions,

(e) finally, the lipid mobility increases with the distance from the bilayer surface. Such situation itself may be locally perturbed by presence of proteins or other molecules.

Several compounds are known that alter membrane fluidity at different levels of the bilayer: adamantine acts on the hydrophobic region of the hydrocarbon chains(204), desiprimine on the polar headgroups (58), and polyene antibiotics by virtue of its ability to complex cholesterol (205). The treatment of disorders (206), caused by abnormal fluidity of membranes, with drugs capable of increasing or decreasing fluidity at different levels of the bilayer might be one of the hopes for the future.

EXPERIMENTAL PROCEDURES

2.1 Organism and Growth Conditions

The experiments were carried out with a wild-type of yeast, Saccharomyces cerevisiae strain D22. The strain was maintained on 2% agar slants, containing 1% Difco yeast extract, 2% Oxoid bacteriological peptone, and 4% glycerol. The yeast cells were grown in a Wickerman liquid medium (85), containing 1% Difco yeast extract, 0.5% bacteriological peptone, 10% glucose or 1% ethanol, with the addition of 5 g/l Tween 80 (polyoxyethylene sorbitan monolaurate) for the unsaturated fatty acid supplemented cultures. The composition of the fatty acids of the Tween 80 used in the experiments was determined by g.l.c. analysis of the methyl esters, and was 74% oleic acid, 12% palmitoleic acid and the rest a mixture of saturated fatty acids of chain lengths C_{10} - C_{14}. Aerobic and anaerobic cultures were inoculated from the aerobic early stationary phase ethanol or glucose grown precultures and grown at 30°C in a 10-liter New Brunswick rotary incubator stirred at 200 - 400 rev/min. High purity nitrogen gas, freed of traces of oxygen by Nilox gas purification apparatus (86) was bubbled through the culture to maintain anaerobic growth conditions. The yeast was allowed to grow to the early stationary phase.

The unsaturated fatty acid mutant of Saccharomyces cerevisiae (23), strain KD115, has also been used in the aerobic experiments. The cells were grown in the same

media as described above, on an ethanol (0.5%) or glucose (0.4 - 1%) as substrates in the presence of low concentration (50 - 100 µg/ml medium) and high concentration (2 - 4 mg/ml medium) of Tween 80. The cells were harvested at the beginning of stationary growth phase.

2.2 Preparation of Mitochondria

The anaerobic culture was chilled to 0°C and 4 mg/ml of chloramphenicol and 200 µg/ml of cycloheximide were added to the culture 15 min before harvesting. The anaerobic cells were washed twice in cold water containing 0.5 mM EDTA plus the same concentration of antibiotics as above, and once in cold medium containing 50 mM Tris-HCl (pH 7.2), 0.5 M Sorbitol and 0.5 mM EDTA. The same procedure was adopted for aerobic cells, but the antibiotics were omitted.

Washed yeast cells were suspended in EDTA-Sorbitol-Tris buffer with 0.1% of serum albumin and broken immediately. 20 ml of glass beads (0.45 to 0.5 mm diameter, B. Brown Helsunger Apparatus) were added to 10 ml of cell suspension in a Bronwill flask of 75 ml capacity, and homogenized at 4000 rev/min for 15 sec in a Bronwill mechanical cell homogenizer (Brown model MSK). The homogenate was decanted and the beads were washed twice with the above buffer. The combined homogenates were centrifuged at 2000 x g for 15 min to remove unbroken cells and nuclei. The resulting supernatant was centrifuged at 24000 x g for 20 min or 100000 x g for 60 min to separate mitochondrial or promitochondrial membranes, respectively.

These crude preparations were purified by flotation in a sucrose gradient (20 - 70% w/v) containing 50 mM Tris-HCl (pH 7.2) and 0.5 mM EDTA, in accordance with the procedure of Schatz (87). Alternatively mitochondria were purified on a discontinuous sucrose gradient consisting of a cushion of 4 ml of 70% (w/v) sucrose and 6 ml each of 50, 30 and 20% (w/v) sucrose. The sucrose was buffered with 50 mM Tris-HCl (pH 7.2) and 0.5 mM EDTA.

2.3 Preparation of OS-ATPase

Oligomycin sensitive ATPase was solubilized from yeast submitochondrial particles with 0.25% Triton X-100 and purified by centrifugation on a sucrose gradient (5 to 20%, w/v) as described by Tzagaloff *et al.*, (88). Triton X-100 was removed from a dispersed preparation of the OS-ATPase by absorption onto Bio-Beads SM-2, a neutral porous styrene-divinylbenzene copolymer (89).

2.4 Lipid Extraction and Fatty Acid Analysis

Lipids were extracted from purified mitochondria and promitochondria by the procedure of Bligh and Dyer (90) as modified by Ames (91). This extract will be referred to as total lipid.

Fatty acid methyl esters were prepared from the extracted lipids of the promitochondria and mitochondria using 0.5 M HCl-Methanol (92). The esters were analysed on a Perkin-Elmer F-11 gas-liquid chromatograph, with a polyethylene glycol succinate column operating at 180°C.

The column was calibrated with standard methyl esters of the different fatty acids.

2.5 Differential Scanning Calorimetry

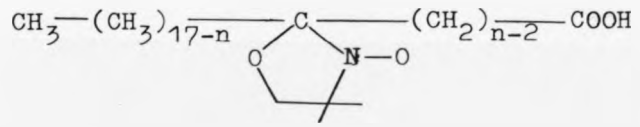
A Perkin-Elmer DSC-2 instrument was used to obtain the calorimetric heating curves and to reveal the lipid and membrane phase endothermic phase behaviour. Membranes were placed in specially constructed large pans to increase the signal strength. Ethylene glycol (50% by volume in water) was used to lower the freezing point of the free water in the membranes and lipids. The effect of ethylene glycol on a transition above $0^{o}C$ was found to be negligible. A temperature increase of 5K per min and heating rate of 0.5 mcal per sec were used.

2.6 Light Scattering

Light scattering at 90^{o} was measured by an EMI 9661B photomultiplier, the signal being amplified by a Keithley 610 C Electrometer. The output of the latter was fed to a Servoscribe (RE 541.20) potentiometric recorder. The light source was a 75 Watt iodine quartz lamp powered by lead storage batteries. Homogenized suspensions of membranes and sonicated lipid dispersions were contained in quartz cells. A slow rate of flow of dry nitrogen was passed over the cell to prevent condensation. The cell was placed in a metal block through which ran a supply of pre-heated water and a thermometer was placed very close to the cell inside the block.

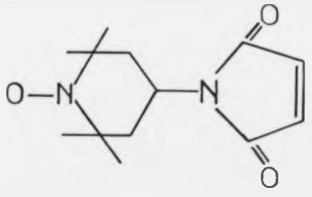
2.7 Electron Spin Resonance

(a) Membrane lipid spin-labelling. Promitochondrial and mitochondrial membranes were labeled with nitroxide derivative of stearic acid having the general structure



where the nitroxide ring is located on 4, 5, 12 or 16 carbons away from the carboxyl group. They are denoted by 4, 5, 12 or 16 NS, where the symbols NS stand for nitroxide stearate. The spin labels 4 NS and M12 NS (Methyl-12-nitroxide stearate) were a gift from Dr. A.D. Keith, whereas the 5 NS, 12 NS and 16 NS were obtained from Synvar (Palo Alto, California). Spin labels were introduced into the membrane suspensions (10 - 20 mg protein/ml) by mixing 5 µl of label (0.01 M in ethanol) with 0.5 ml of membrane. Destruction of the nitroxide spin probe was prevented by adding potassium ferricyanide (0.5 mM) to the membrane samples.

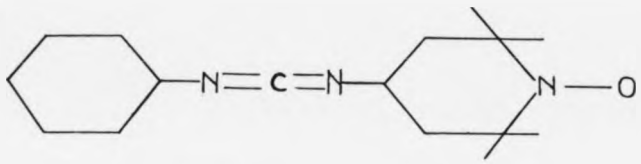
(b) Membrane protein spin-labeling. Promitochondria and mitochondria were labeled with nitroxide maleimide, N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl) maleimide (MSL).



Aliquots of membrane suspensions (10 - 20 mg protein/ml) were

incubated with 2×10^{-4} M of spin label at $4^{o}C$ overnight. After the incubation period, excess label was removed by washing four times in a medium of 0.25 M sucrose and 5 mM Tris-acetate (pH 7.4).

(c) Membrane-bound OS-ATPase spin-labeling. Promitochondrial and mitochondrial particles were spin labelled by the nitroxide analog of DCCD (dicyclohexyl carbodiimide) an inhibitor of mitochondrial ATPase. NCCD N,-(2,2,6,6-tetramethyl-piperidyl-1-oxyl)-N'-(cyclohexyl) carbodiimide is a spin label synthesized in our laboratory by Panos Ioannou, in accordance with the procedure of Azzi et al. (93).



NCCD

Membrane suspensions (25 - 30 mg protein/ml) were incubated with 5×10^{-4} M of NCCD at $4^{o}C$ for 24 to 48 hours. After the incubation, excess label was removed by several washings or by overnight dialysis against 5 mM Tris-acetate (pH 7.4).

The ESR spectra were recorded on a Varian E3 spectrometer or DECCA radar X1 with a Newport Instruments 7 in magnet. Samples were contained in thin glass capillaries and supported in a quartz tube. The temperature was controlled by a stream of pre-heated nitrogen and measured by a copper-constantan thermocouple immediately below the sample.

2.8 X-ray Diffraction

For X-ray diffraction studies, membranes were collected in a pellet at 100,000 x g for 3 h and a sample sealed in a thin walled glass capillary. The capillaries were placed in a thermally controlled chamber in which temperatures between -20° and 50°C could be maintained within ± 1°. In studying phase transition, diffraction patterns were recorded at 5° intervals. Diffraction patterns were recorded on Ilford Industrial G film in one hour exposures using an Elliot toroid camera filled with helium to reduce X-ray scatter. The X-ray generator was an Elliot GX6 rotating anode instrument at 47 kv and 20 mA.

2.9 Freeze Etching

Intact cells of *Saccharomyces cerevisiae* grown aerobically and anaerobically with and without a supplement of unsaturated fatty acids and mitochondria isolated from these cells were freeze etched. Samples were frozen directly in Freon 22, with no prior fixation or glycerination, and freeze-etched in a Balzer freeze-etch apparatus BAF 301. Etching was carried out for 1 minute at a temperature of -100°C and replicas were examined in an AEI-EM6B electron microscope.

2.10 Enzyme Assays

ATPase. The ATPase activity was carried out in the presence of an ATP-regenerating system, by coupling

the production of ADP to NADH reduction with pyruvate kinase and lactate dehydrogenase, as described by Pullman (94). The reaction mixture (final volume 1.1 ml) consisted of 50 mM Tris-sulphate (pH 9.4), 5 mM phosphoenolpyruvate, 5 mM $MgCl_2$, 2 mM ATP, pyruvate kinase (10 μg; Sigma type 11), lactate dehydrogenase (20 μg; Sigma type 11) and 25 μl membrane suspension (50 - 100 μg protein). The mixture was incubated for 5 minutes and the reaction was started by the addition of 0.15 mM NADH. Absorbance decrease was measured at 340 nm and an extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ for NADH was used. Freezing storage of the membranes caused an increase in the specific activity of the ATPase. Furthermore the break in the Arrhenius plot, temperature dependent of such enzyme, was not affected by freezing and storage of the membrane at $-20^{o}C$.

Succinate dehydrogenase. The succinate dehydrogenase was measured by a modification of the method of Arrigoni and Singer (95). The reaction mixture (final volume 1.1 ml) consisted of 50 mM potassium phosphate buffer (pH 7.2), 1 mM KCN and 50 - 200 μg of mitochondrial protein. The reaction was started by the addition of 0.05 mM dichlorophenol-indophenol and 0.5 mM phenozine metasulphate. Absorbance changes were measured at 600 nm and an extinction coefficient of 21 $mM^{-1}cm^{-1}$ for dichlorophenol-indophenol was used (96).

ATPase and succinate dehydrogenase are enzymes measured with a Cary 14 spectrophotometer connected to a Churchill thermocirculator. The temperature in the reaction

cuvette was recorded by a calibrate immersible thermocouple and dry nitrogen gas was flushed through the cell compartment to prevent condensation at the lower temperatures.

Respiratory Activity. The respiratory activity, measured as succinate oxidase, was determined polarographically (97) by using a Rank oxygen electrode (Rank, Cambridge, U.K.), connected to a Churchill thermocirculator. The assay medium (final volume 3.1 ml) contained 0.25 M sorbitol, 5 mM potassium phosphate, 1 mM EDTA, 10 mM Tris-maleate (pH 6.5), 0.1% bovine serum albumin and 1 - 3 mg of mitochondrial protein. The reaction was started by the addition of 0.5 mM ADP and 20 mM succinate as a substrate.

2.11 Analytical Methods

Cytochrome spectra. Yeast cells were washed twice in 50 mM potassium phosphate buffer (pH 7.4), and then suspended in 50% (w/v) sorbitol, 50 mM potassium phosphate buffer at 25 - 30 mg dry wt./ml. Cytochrome spectra were detected in a Unicam SP. 1800 spectrophotometer in 1 cm light path cells (final volume 2.5 - 3.0 ml) by using the turbid solution cell position and a slit width of 0.6 mm. The contents of the sample cell were reduced with dithionite and the reference was oxidised with 10 μl of H_2O_2 (98 - 99).

Protein determination. Protein was measured by the method of Lowry *et al.* (100), with bovine serum albumin as standard.

RESULTS AND DISCUSSION

3.1 Membrane Composition

It is well established that yeast cells change fatty acid composition with anaerobic growth (101). However, no correlation has been made between these changes and the importance of the hydrophobic portion of the lipids in maintaining the functional and structural properties of the membrane.

The fatty acid composition of the various membranes is shown in Table 1.

It has already been pointed out that in some organisms, such as E. coli and fatty acid desaturase mutants of S. cerevisiae, optimal growth requires a particular fluidity of the membrane. This in turn depends on the presence of lipids with fatty acids of various chain lengths and degree of unsaturation (102 - 103). As a consequence, the cells compensate by synthesizing primarily short chain fatty acids.

In promitochondria, as the long chain unsaturated fatty acids are decreased, the proportion of short chain saturated fatty acids in membrane lipids is increased. This increase will reflect a requirement for maintaining the hydrocarbon side chain portion of the membrane lipid in a fluid state. Similar behaviour was observed in anaerobic promitochondria from cells grown in the presence of unsaturated fatty acids. The increase of unsaturation was accompanied by a dramatic decrease in short chain fatty acids.

TABLE 1

FATTY ACID COMPOSITION OF THE PROMITOCHONDRIA AND MITOCHONDRIA

MEMBRANE	PERCENTAGE OF TYPES OF HYDROCARBON CHAINS		
	Short chain saturated ($C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$)	Long chain saturated ($C_{16:0}$, $C_{18:0}$)	Long chain unsaturated ($C_{16:1}$, $C_{18:1}$)
Promitochondria (depleted)[a]	55	36	9
Promitochondria (supplemented)[b]	3	34	63
Mitochondria	1	17	82

[a] Depleted: From cells grown anaerobically without added unsaturated fatty acids.

[b] Supplemented: From cells grown anaerobically in the presence of unsaturated fatty acids.

Table 2 shows the effect of short chain length of fatty acids in determining the ratio of saturated to unsaturated long chain fatty acids. The ratio of saturated to unsaturated fatty acids decreases in the order depleted-promitochondrial, supplemented-promitochondrial and mitochondrial membranes, and parallels the increasing fluidifying effects of incorporated or newly synthesized unsaturated fatty acids. In contrast, the phospholipid composition does not change significantly (101) indicating that the fatty alkyl chains in phospholipids would play the key role in controlling the fluidity of yeast membranes.

3.2. Physical State and Phase Transition of the Promitochondria and Mitochondria

(a) Differential Scanning Calorimetry Studies.

The dependence of mitochondrial enzyme activity upon membrane phospholipids (104) and the relative low lipid/protein ratio in promitochondria, raise the question: what is the influence of membrane proteins upon the physical state of membrane lipids?

This question was approached by measuring the thermal capacity of promitochondrial structures and that of the protein-free lipid extract, using differential scanning calorimetry.

Calorimetry was the first method used by Chapman *et al.* (57) to demonstrate lipid phase transitions in model membranes (105) and biological membranes (106). As a

TABLE 2

EFFECT OF SHORT CHAIN LENGTH FATTY ACIDS ON THE RATIO OF SATURATED TO UNSATURATED MEMBRANE FATTY ACIDS COMPOSITION

MEMBRANE	SHORT CHAIN SATURATED (%)	LONG CHAIN SATURATED / UNSATURATED
Promitochondria (depleted)[a]	55	4.0
Promitochondria (supplemented)[b]	3	0.54
Mitochondria	1	0.20

[a] Depleted: From cells grown anaerobically without unsaturated fatty acids.

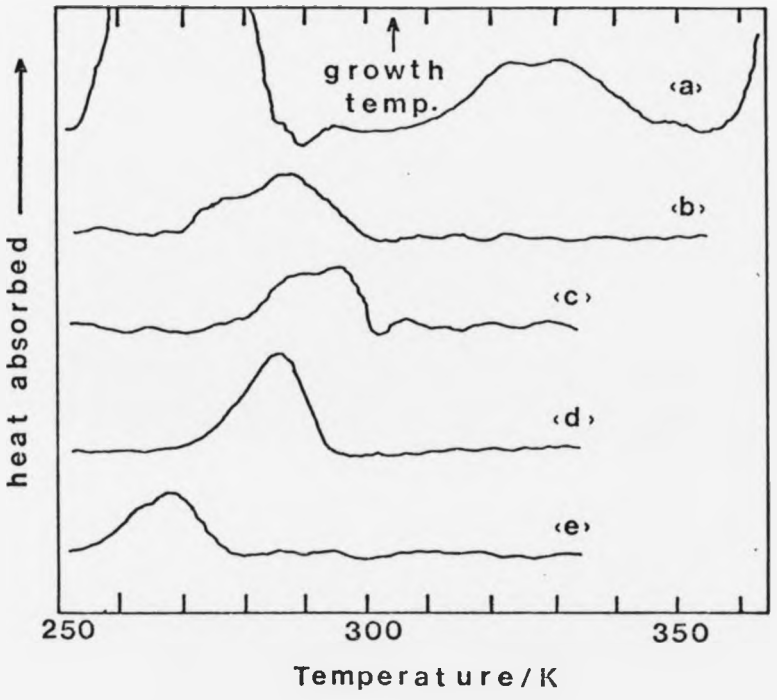
[b] Supplemented: From cells grown anaerobically in the presence of unsaturated fatty acids.

result of these studies it became apparent that aqueous phospholipid bilayers undergo a first-order thermal phase change from the gel phase to the liquid crystal phase. The temperature of the transition is chain length dependent, and increases with increasing hydrocarbon chain length. The positive entropy of transition ΔS increases linearly with chain length and this change can be attributed to new degrees of freedom available to the hydrocarbon chains in the liquid crystal phase when compared to gel phase (107). Since yeast aerobic mitochondria contain lipids with a higher content of cis-unsaturated hydrocarbon chains (oleic and palmitoleic fatty acids), phase transitions are expected to occur at relatively low temperature. On the other hand, the anaerobic promitochondria, which are characterized by a high degree of saturated alkyl chains, are expected to cause an increase in the transition temperature of membrane lipids.

Temperature-based thermograms of intact membranes and isolated lipids, derived from yeast cells grown aerobically or anaerobically in the presence or absence of unsaturated fatty acids are presented in Fig. 1 and the data summarized in Table 3.

The promitochondrial membranes, after cooling to -60°C, showed on heating well defined transitions and endotherms. The depleted promitochondria showed a phase change between 38°C and 70°C centered at about 54°C. The high temperature end of the scan was obscured by heat changes associated with protein denaturation effects. Such broad thermal transitions are characteristic of

FIGURE 1



Differential Scanning Calorimetry Curves for:
(a) depleted promitochondrial membranes, (b) supplemented promitochondrial membranes, (c) depleted promitochondrial-lipid extract, (d) supplemented promitochondrial-lipid extract, (e) mitochondrial lipid extract.

TABLE 3

PHASE TRANSITIONS OF MEMBRANES AND LIPIDS MEASURED BY D.S.C. CALORIMETRY

Temperatures (^{o}C) of the beginning and end of the transition are given, together with the temperature of the maximum heat flow in brackets.

MEMBRANE	INTACT MEMBRANE	LIPID EXTRACT
Promitochondria (depleted)[a]	35 to 60 (54)	8 to 32 (24)
Promitochondria (supplemented)[b]	-3 to 27 (14)	-5 to 20 (12)
Mitochondria	Not detectable	-21 to 5 (-3)

[a] Depleted: From cells grown anaerobically without unsaturated fatty acids.

[b] Supplemented: From cells grown anaerobically in the presence of unsaturated fatty acids.

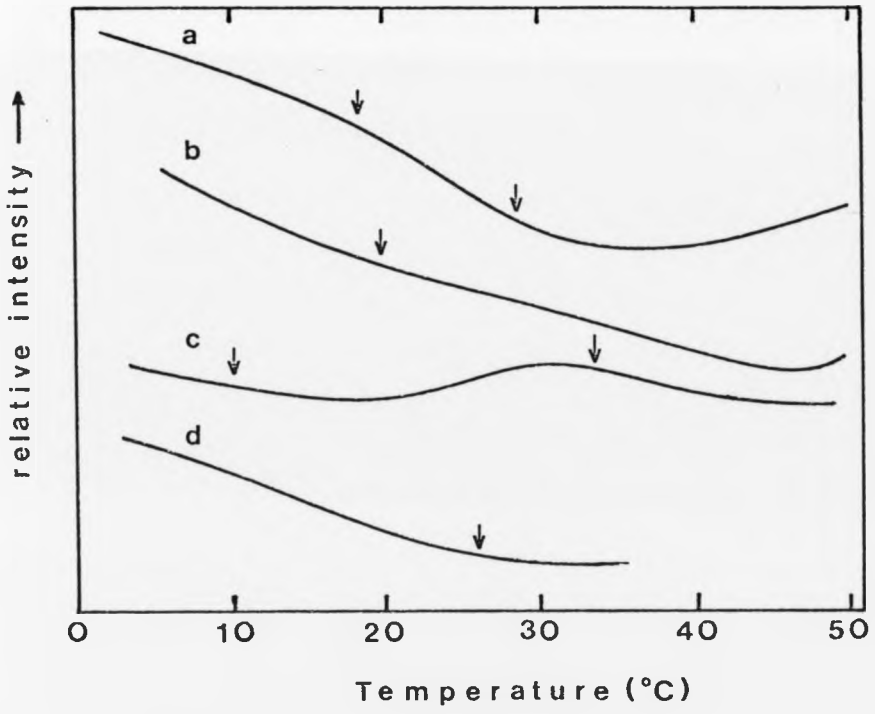
membranes and mixtures of lipids and are thought to be due to a gradual melting of the different types of lipids present in such systems (108 - 110).

As expected, the transition temperatures are profoundly influenced by the nature of the fatty acids esterified to the membrane lipids. Supplemented promitochondria, showed a thermal transition at temperatures between -3°C to 27°C, below those observed for unsupplemented promitochondria, and centered at about 14°C. The extracted lipids from these membranes also showed marked endotherms. The temperature ranges for these endotherms were substantially lower than the transition ranges for the depleted promitochondria. The membrane lipids, protein free, from unsupplemented promitochondria show a reversible endothermic transition between 8 and 32°C, centered at about 24°C, whereas in lipids from supplemented ones the transition is between -21 to 5°C centered at about -3°C. With the fully organized mitochondria the thermal transitions were not detected or were much smaller, although the lipid extract from this membrane did show a transition which occurred some 15°C below that observed with the lipid of the supplemented promitochondria. This is consistent with the fact that there is a greater proportion of unsaturated fatty acids in the lipids of the aerobic mitochondrial membranes. These studies demonstrate the existence in promitochondrial membranes of a gel-liquid crystalline phase transition which involves a transition from an ordered to a fluid lipid bilayer structure.

(b) Light Scattering Studies.

In general, the measurements of 90° light scattering agree with those observed from the calorimetric data. Light scattering measurements have been previously used to study phase transition in membranes and lipid dispersions (111 -112) and it is assumed that the sharper changes result from the beginnings or ends of phase transitions. A deviation from the linear decrease in intensity occurs at about 30°C (± 3) with the depleted promitochondria (Fig. 2). Deviations from linearity are detected readily because of the good signal to noise ratio obtained with the light scattering system, so this temperature may be a better estimate of the beginning of the phase transition for this membrane. Above 50°C, protein denaturation causes a large increase in light scattering and so the end of the membrane transition could not be clearly detected. (The large effect attributed to protein denaturation was confirmed by examining a solution of bovine serum albumin, heat changes occur at approximately the same temperature). There was also an increase of slope at about 20°C which could be due to a phase transition occurring with a small group of lipids of lower melting point. With the extracted lipids from promitochondrial membranes the transition occurred at a much lower temperature, the range being from 10°C to 33°C, similar to that observed using calorimetry. Only slight effects were observed with the supplemented promitochondria and the extracted lipids, with changes of slope occurring at about 17°C and 25°C, respectively (Fig.2).

FIGURE 2



Intensity of light scattering as a function of temperature in: (a) depleted promitochondrial membranes, (b) supplemented promitochondrial membranes, (c) depleted promitochondrial-lipid extract, (d) supplemented promitochondrial-lipid extract.

These temperatures are in reasonable agreement with the calorimetric measurements of the ends of the endothermic transitions. A determination of the beginning of the transition was not possible because of the lower limit of 2°C obtained with the light scattering apparatus. No transitions were detected with the mitochondrial membranes or their extracted lipids.

(c) X-Ray Diffraction Studies

X-ray diffraction has been used to study the structure of biological membranes by analysis of the differences in the electron density of the membrane components, such as lipid and protein. The average electron densities of proteins generally are considerably above the average level for water. The average densities in lipid head groups also are higher than water, but the density of liquid paraffin like fatty acid chains is considerably lower than for water (113).

Aqueous dispersions of phospholipids show a bimodal arrangement with a hydrated polar group orientated in the aqueous environment. X-ray diffraction analysis has revealed that the predominant structural arrangement of these lipid-water systems are lamellar consisting of lipid bilayers. The lipid bilayer has a hydrocarbon core with the fatty acid chains assuming either a crystalline or a liquid-like state. The change in the organization of the hydrocarbon chains from the crystalline to the liquid-like state can be evidenced by two important observations:

(i) the disappearance of the sharp 4.2Å X-ray

diffraction band (observed in solid paraffins), and

(ii) the appearance of a diffuse diffraction band at 4.6 Å (observed in liquid paraffins) at the calorimetrically determined transition temperature (114). The reflection of 4.2 Å is characteristic of a hexagonal packing of alkyl chains in the all-*trans* configuration in the plane of the bilayer. This has been observed in artificial phospholipids having two saturated alkyl chains and with natural phospholipids at low temperature (115). The reflection of 4.6 Å is characteristic of most biological membranes studied by means of X-ray diffraction (116). It has been shown that organization of the lipid molecules in these systems is liquid-like at the physiological temperatures but at lower than physiological conditions part of the lipid molecules may form ordered domain within the membrane (117 - 118).

That the phase transition involves a disordering of the phospholipid hydrocarbon chains in promitochondria and mitochondria is evidenced more directly by low-angle scattering from membrane dispersions. In each of these membrane transitions were observed in the X-ray patterns taken at different temperatures. Two physical states of lipid were distinguished, a crystalline hexagonal packing characterized by a sharp 4.2 Å diffraction band and a fluid (melted) state identified with a broad, diffuse band at 4.6 Å. In all cases the transitions were gradual and extended over several exposures. The temperature ranges for these transitions are shown in Table 4 and Figs. 2*a*, *b*.

TABLE 4

X-RAY DIFFRACTION PATTERNS FROM PROMITOCHONDRIAL AND MITOCHONDRIAL MEMBRANES

MEMBRANE	RANGE OF TRANSITION TEMPERATURE (°C) (Disappearance of 4.2 Å sharp ring)
Promitochondria (depleted)[a]	30 to 50
Promitochondria (supplemented)[b]	15 to 25
Mitochondria	-15 to -5

[a] Depleted: From cells grown anaerobically without unsaturated fatty acids.

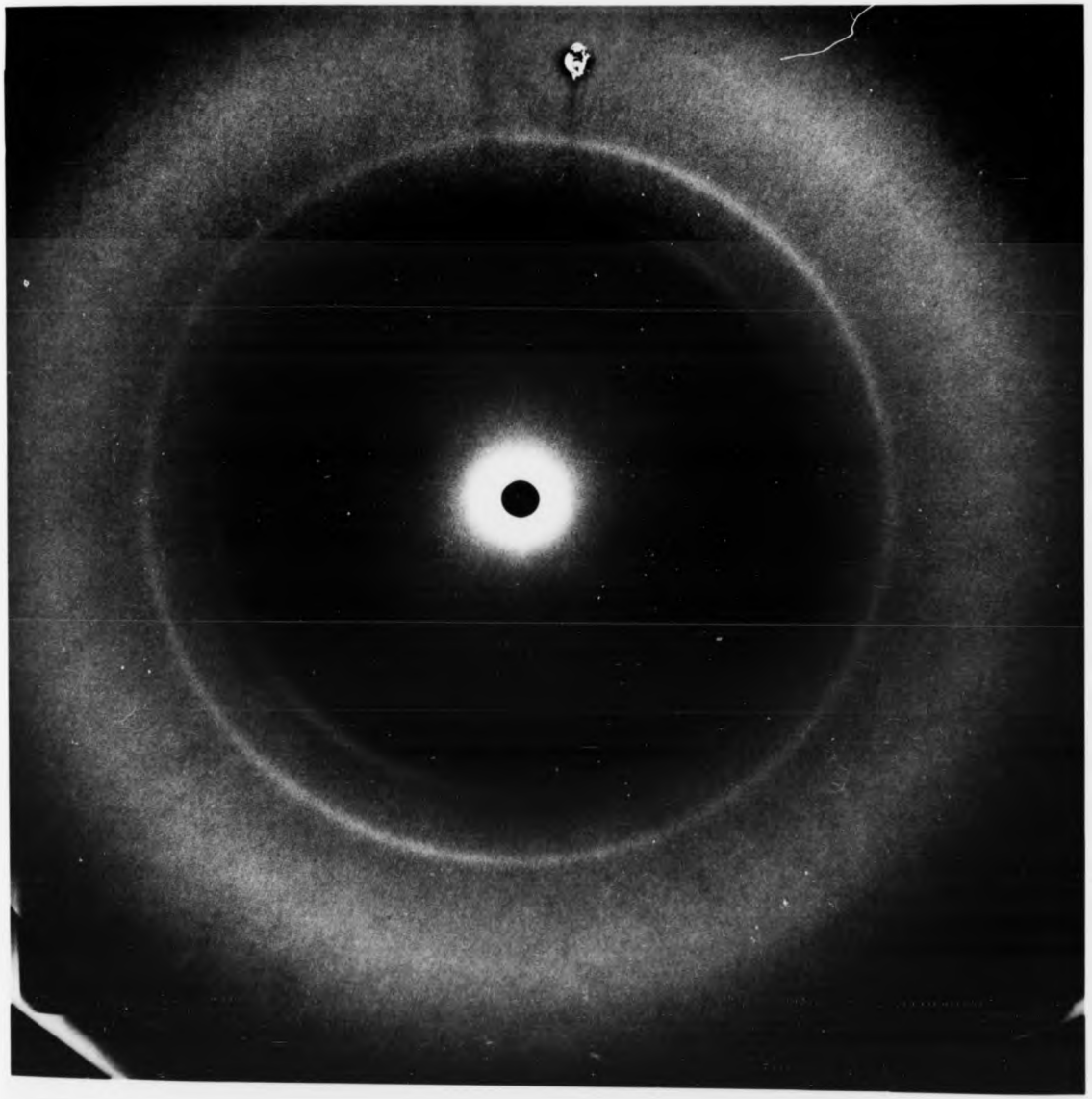
[b] Supplemented: From cells grown anaerobically in the presence of unsaturated fatty acids.

FIGURE 2a

X-ray diffraction pattern from anaerobic promitochondria, showing a sharp ring at 4.2Å, at the growth temperature of the yeast cell (gel state).

itochondria,

temperature of



mitochondria,

temperature of

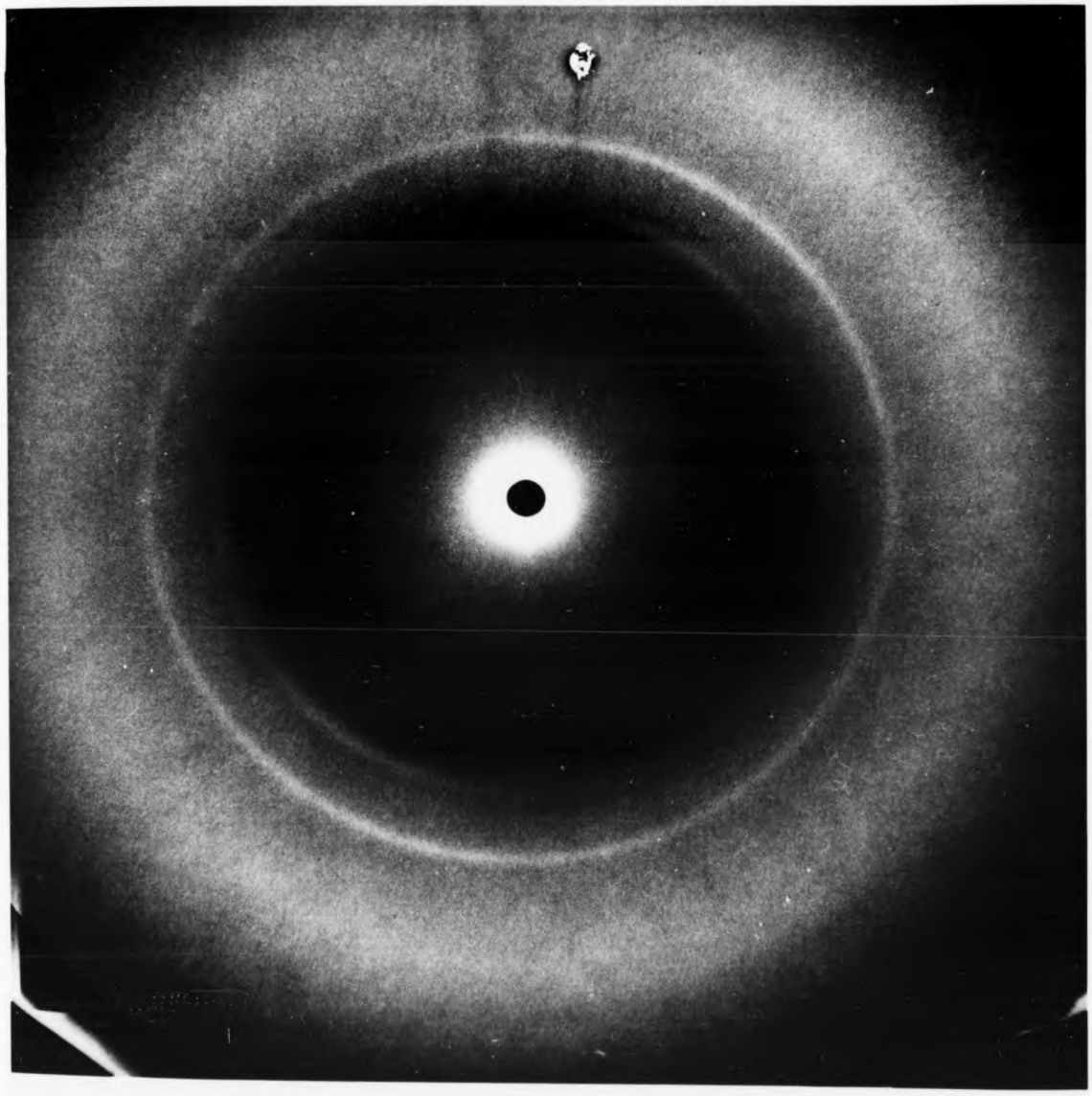
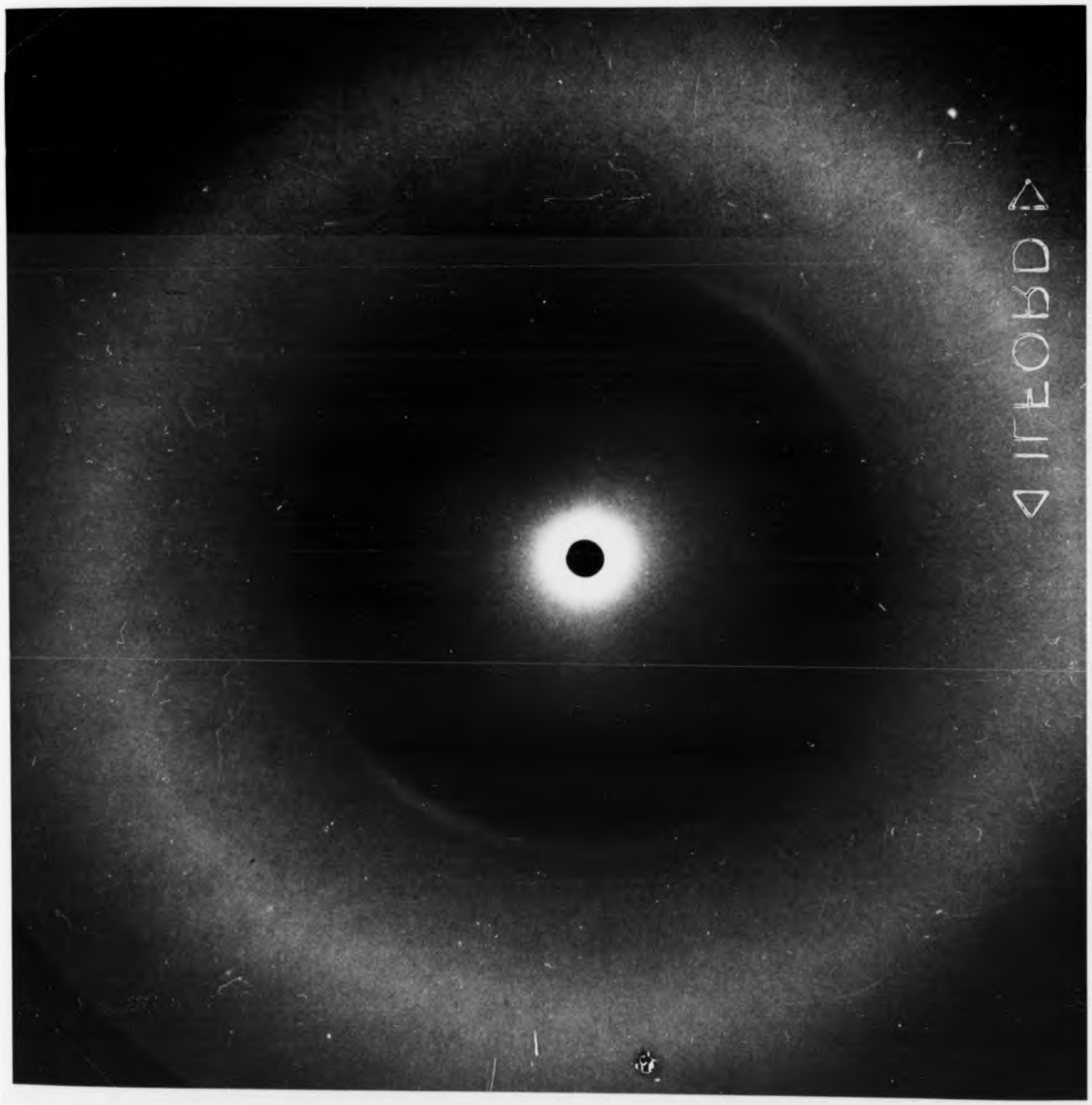


FIGURE 2b

X-ray diffraction pattern from aerobic mitochondria, showing a diffuse band at 4.6Å, at the growth temperature of the yeast cell, (liquid-like state).

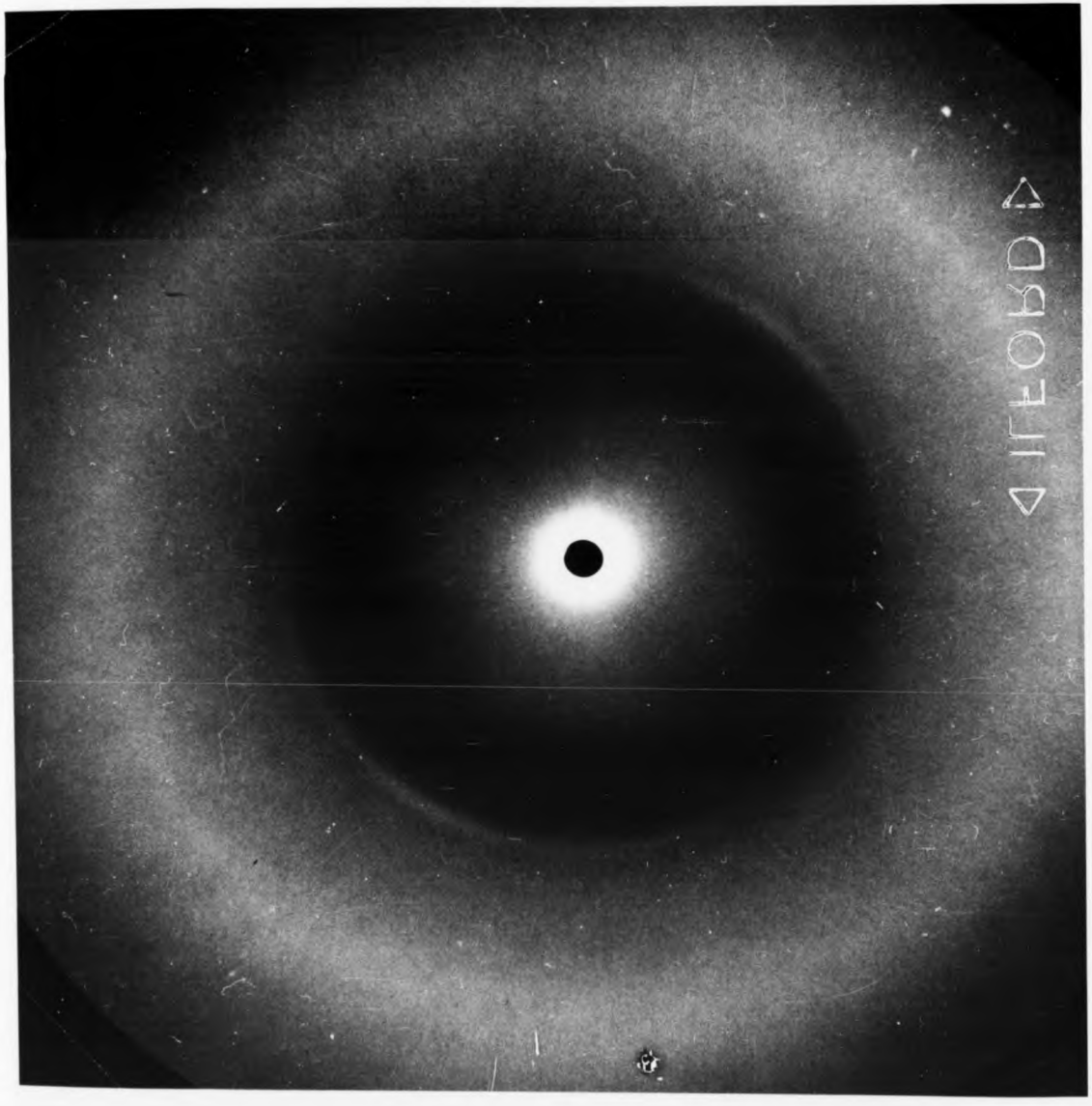
ondria,

temperature



ıondria,

ı temperature



From this table, two important observations can be evidenced: (i) the existence in promitochondria of sharp 4.2 Å band at the temperature of growth of the cell (30°C), and (ii) the effects of the unsaturated fatty acids used to support the anaerobic growth. In considering these effects, the thermal transition was shown to vary with the chemical structure of the fatty acids. The low degree of unsaturated hydrocarbon chain in the unsupplemented anaerobic promitochondria shifts the transition higher than aerobic mitochondria, with a gradual change from the sharp ring at 4.2 Å to a diffuse diffraction band at 4.6 Å. However the introduction of a fatty acid having a more fluid character (e.g. oleic acid), results in a decrease in the transition temperature and the anaerobic promitochondrial membranes become fluid-like at the temperature of growth of the cell.

3.3 Membrane Bound OS-ATPase

In order to correlate the physical changes of the promitochondria and mitochondria in response to environmental modifications, as described above, the activity of oligomycin-sensitive ATPase was studied as a function of increasing temperature. This study is an approach to understanding the dependence of membrane bound enzymes on the physical state of the membrane phospholipids (119 - 123). The OS-ATPase was chosen as a marker of inner mitochondrial membrane, because it is present in both systems anaerobic and aerobic mitochondria (27). Several

mitochondrial activities have been shown to exhibit discontinuities in the Arrhenius plots, indicating changes in activation energy of the reactions (124 - 125). These changes have often been related to distinct physical states of the lipids above and below the observed transition temperature. Above the break the lipids, at least those surrounding the enzyme, are in liquid-crystalline state and the kinetics of the reactions are characterized by a lower activation energy; below the break the lipids are primarily in gel-like state with higher activation energy. Different experimental approaches support the idea that a liquid crystalline state of the membrane is essential for a proper functioning of membrane bound enzymes (126 - 127). From these observations, the OS-ATPase of anaerobic promitochondria is expected to have an activation energy higher than that of supplemented ones or aerobic mitochondria. This has to be related to a high degree of saturated fatty acids which characterized the membrane lipids of anaerobic promitochondria (31).

The ATPase enzyme is present in the promitochondrial structures of anaerobic cells at much lower activity (128 - 129) and the increase of the activity in the derepressed aerobic cells may be related to lipid composition changes associated with mitochondrial development.
The lipid requirement of oligomycin-sensitive ATPase has been demonstrated (77). In order to have further evidence of the influence of lipid moieties on the ATPase system we have studied the temperature dependence of this enzyme activity in response to lipid alterations in the

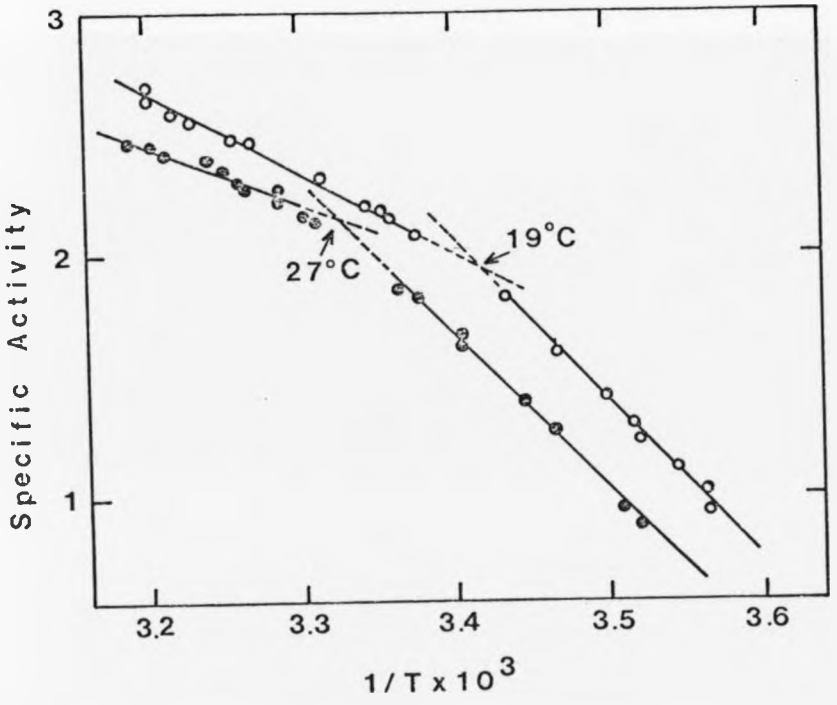
membrane. Discontinuities in the Arrhenius plots of the ATPase activities for the depleted and supplemented promitochondria indicate a change in activation energies of the reactions at temperatures near 28°C (26°C to 30°C) and 20°C (18°C to 21°C), respectively (Fig. 3). This demonstrates that the unsaturated fatty acids supplied to the growth medium has the effect of lowering the temperature of this discontinuity by about 10°C. The Arrhenius plot of ATPase activity in the fully organized mitochondria shows that this occurs near to that of the supplemented promitochondria at 19°C (17°C to 21°C). Thus the temperature at which these changes occur are influenced by the fatty acid composition of the membrane lipids.

3.4 Lipid-Protein Interaction in Promitochondria and Mitochondria

(a) Spin Label Studies.

The use of spin labelled probes has greatly contributed to the study of biological membranes (130 - 132). It consists basically of the introduction of a paramagnetic group into the system under examination, either by covalent binding (as in the case of most studies on proteins) or physical intercalation of a probe into the environment whose properties are being studied (as in the case of lipid bilayers). The degree of motion of a spin label will be evaluated by its ESR spectra. The most commonly used spectral parameters are the g-factor, the hyperfine splitting, and the linewidths and lineshapes (130, 133).

FIGURE 3



Arrhenius plots of the activity of OS-ATPase (oligomycin-sensitive).

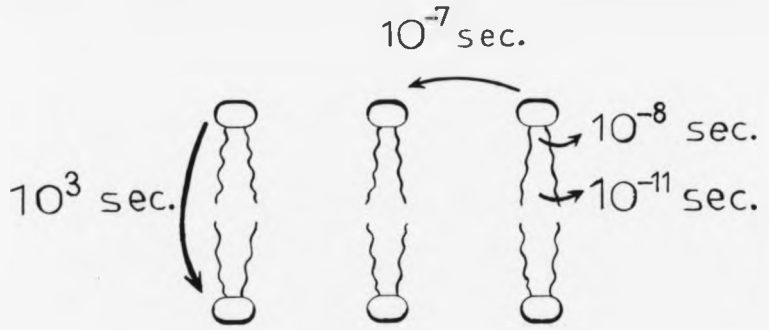
(a) supplemented promitochondrial membranes

(b) depleted promitochondrial membranes.

Nitroxide radicals are useful probes because their spectrum depends on the orientation of the $2p\pi$ orbital containing the unpaired electron with respect to the magnetic field. Since in these nitroxides the unpaired electron is to a large extent localized on nitrogen (50%), a simple three lines pattern will be observed by ESR analysis. However, due to the distribution of the electron density of the P_z orbital of the nitrongen atom, the ESR spectra can reflect a considerable amount of anisotropy in the hyperfine tensor and in the g-factor. When these spin labels are attached to macromolecules, the ESR spectra may be altered by changes in the rotational motion of the labels. The restriction of rotation results in a broadening of ESR spectral lines because of their dependence of anisotropy in both, the nitrogen hyperfine coupling and g-factor. These changes in probe mobility are important and may indicate changes in the environment associated with the spin label.

The possibility that the motion of spin-labelled lipids in fluid organized systems such as the lipid bilayer of certain biological membranes and phospholipid model membranes might be anisotropic was first exploited by Hubbel and McConnel (130). As a consequence of the amphiphilic character of phospholipid or fatty acid derivative spin labels, they orientate themselves in the membrane with their long molecular axes on the average perpendicular to the membrane surface. The polar head group is thought to be held rather firmly to the polar portion of the lipid-protein bilayer, while the hydrophobic tail of the molecule is less restricted

and can undergo rotational motion in the interior bilayer. These studies have demonstrated the existence of a mobility gradient in lipid bilayers, with an increase of motion in the fatty acyl chain as the terminal methyl group is approached. Quantitative estimation of the fluidity gradient has been done in terms of correlation time, depending on rotational motion (134), transverse motion and translational or lateral diffusion (135) of the lipid molecules. The values of rotational motions, as well as lateral and transverse diffusion of phospholipids in a bilayer are represented below. As a basis for comparison, water molecules rotate in 10^{-12} to 10^{-13} sec and a large protein in solution rotates on itself in about 10^{-7} sec (136).



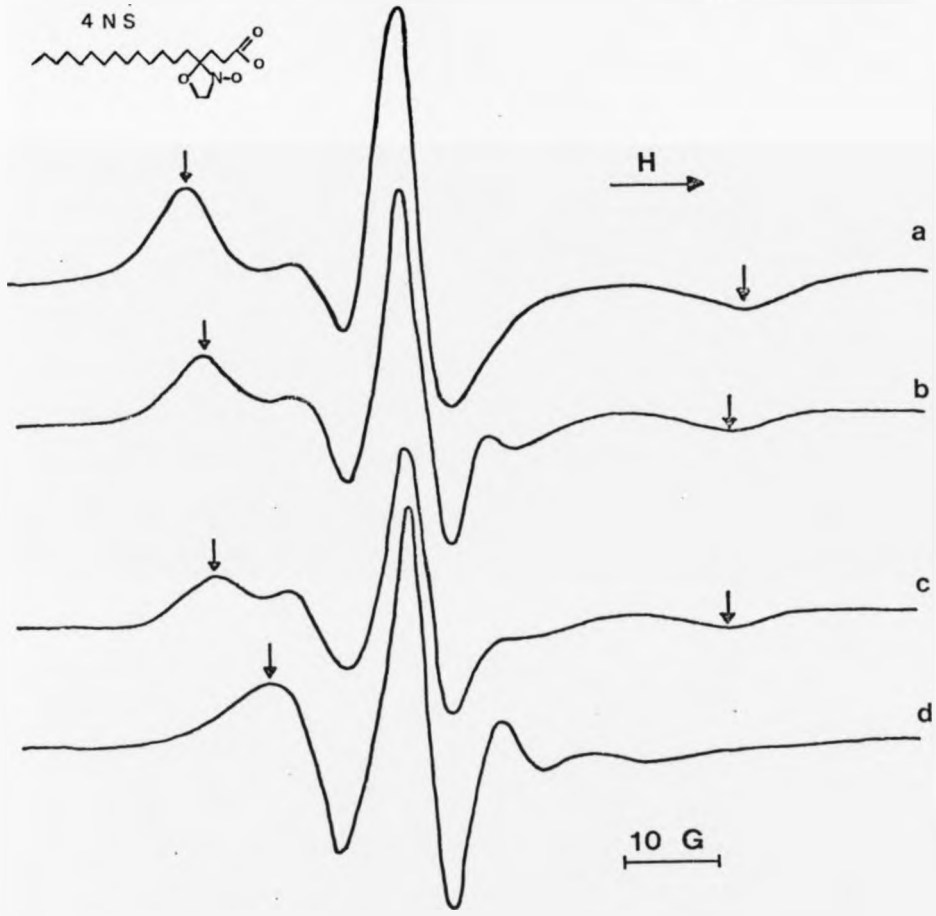
Explanation of diagram: Motion of phospholipid molecules in a bilayer: correlation time of $< 10^{-8}$ sec for the anisotropic rotation around carbon-carbon bonds; correlation time of 10^{3} sec for transverse motion (flip-flop); and lateral diffusion with a diffusion constant of 10^{-8} to 10^{-7} cm^2 sec^{-1}.

The ESR spectra of stearic acid spin labels were studied so as to make comparisons between the mobility of the membranes and their lipids and also to examine their phase transition behaviour. When the nitroxide group is near the polar end of the molecule, such as 4NS a 'partly immobilized' spectrum (132) is observed. This is indicative of restricted anisotropic motion (z-axis rotational correlation time $\tau_c \approx 10^{-8}$ sec), and the largest splitting $2T_{\parallel}$ which decreases as the motion increases, can be used as a measure of the molecular motion. This parameter has been related to the degree of order of the z-axis of the nitroxide and we find that measurement of $2T_{\parallel}$ can be made for degrees of ordering between 1 and 0.6. As the molecular motion increases, the spectrum passes through an "intermediate" stage and then becomes 'fully mobile' and the spectrum is similar to that which occurs in fast isotropic motion of a nitroxide. In this region it is convenient to measure the ratio of the line heights of the low fields (h_1) and middle lines (h_o), a parameter which can be related approximately to τ_c .

The ESR spectra of spin-labelled membranes and extracted lipid dispersions are shown in Fig 4. These spectra are indicative of spin label performing a rapid anisotropic motion and the parallel hyperfine splitting (parameter $2T_{\parallel}$) are measured as indicated in Fig. 4.

Promitochondrial organelles are characterized by a greater value of the hyperfine splitting $2T_{\parallel}$ which reflects restricted rotational motion and therefore indicates a

FIGURE 4



Electron spin resonance spectra of 4NS in (a) depleted promitochondrial membranes, (b) mitochondrial membranes, (c) depleted promitochondrial-lipid extract, (d) mitochondria-lipid extract. The spectra were measured at 15°C.

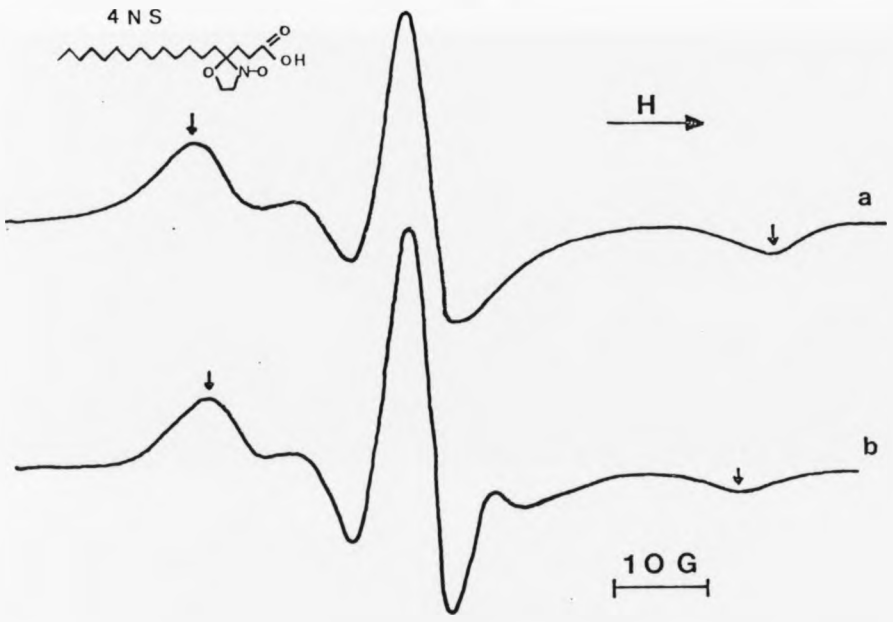
more viscous environment around the spin probe. The ESR spectra arising from spin label incorporated into membrane are significantly different from those of lipids. In fact, the motion of 4NS label was more free in the lipid dispersion than in the intact membrane, indicating some immobilizing effects of proteins which restrict the freedom of the lipids.

Yeast cells can grow well under anaerobic conditions only in the presence of unsaturated fatty acids. This might be related to the importance of a correct fluid state of the membrane at the physiological temperature of growth. However, an increase in membrane fluidity of anaerobic promitochondria derived from supplemented cells is found, as observed in the spectra represented in Fig. 5.

The temperature dependence of promitochondrial and mitochondrial membrane fluidity were studied.

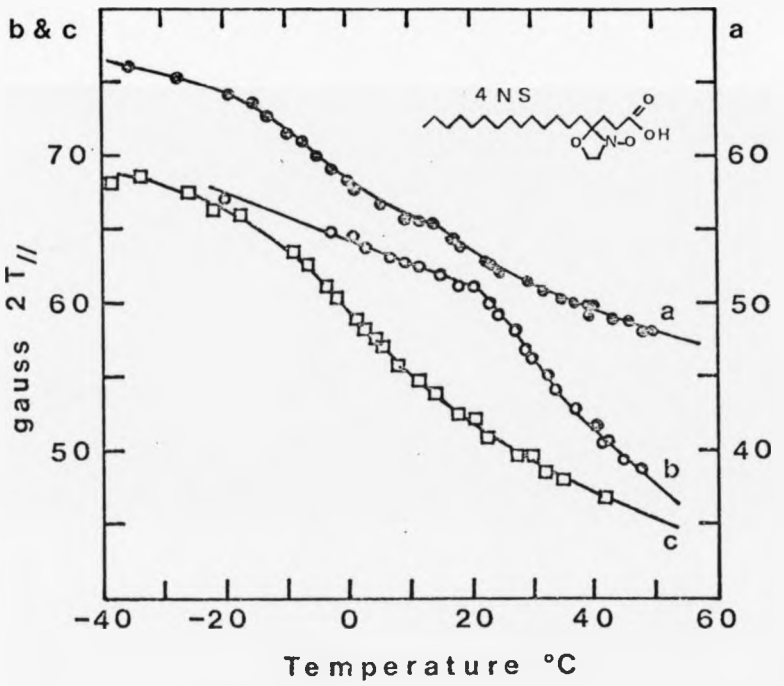
The spin label 4NS (parameter $2T_{\parallel}$) indicated that phase transitions occur in some of the membranes and lipid extracts. Fig. 6 shows three plots of the ESR parameter $2T_{\parallel}$ against temperature, and Table 5 summarises the results. The temperature range in which a rapid increase of the mobility of the spin label occurred was taken to indicate the occurrence of phase transitions. In the depleted promitochondrial membranes a transition occurred some 15°C lower than that observed using light scattering or calorimetric measurements. The extracted lipids show a considerable shift of lipid transition ($\sim$30°C) compared with that observed with the membrane. The supplemented promitochondrial membranes showed only

FIGURE 5



Electron spin resonance spectra of 4NS in (a) depleted promitochondrial membranes, (b) supplememnted promitochondrial membranes.

FIGURE 6



Plots of electron spin resonance parameter $2T_{//}$ against temperature of spin label 4NS in (a) mitochondrial membranes, (b) depleted promitochondrial membranes, (c) depleted promitochondrial-lipid extract.

TABLE 5

TEMPERATURE (^{o}C) OF BEGINNING AND END OF PHASE TRANSITIONS AS MEASURED BY ESR

MEMBRANE	INTACT MEMBRANE	LIPIDS
Promitochondria (depleted)[a]	19 to 40	-15 to 14
Promitochondria (supplememnted)[b]	2.5 to 24	-14 to -2
Mitochondria	-15 to 9	-16 to -2

[a] Depleted: From cells grown anaerobically without unsaturated fatty acids.

[b] Supplemented: From cells grown anaerobically in the presence of unsaturated fatty acids.

slight evidence of a phase transition but the extracted lipids showed a transition centred at about -7°C, some 15 to 20°C lower than that observed using calorimetric or light scattering data. However with the fully organized mitochondria, some evidence for phase change was detected between -15° and 9°C, with a plateau occurring just before 9°C whilst with the extracted lipids, a transition was observed between -16° and +2°C very close to the values observed by the use of the scanning calorimeter.

The possibility that spin labelling involves a major perturbation to the membrane lipids was investigated using the light scattering technique. With 4NS present at its maximum concentration for ESR measurements, no change in the transition temperature of the depleted promitochondria was detected. We have already noted that the change in light scattering at 20°C in the depleted promitochondria could be due to the melting of hydrocarbon chains of the most mobile group of lipid present. The observation of a change in the ESR parameter at this temperature suggests that the spin label may be situated in the most mobile part of the membrane, thus explaining the large discrepancy of the ESR results compared with other results.

The mobilities of 4NS and 16NS were compared in the membranes at the temperature of their ATPase activity break points (Table 6). The discontinuityfor enzyme activity occurs at about the same spin label mobility for the promitochondrial membranes (depleted and supplemented) but at a higher mobility with the fully organized

TABLE 6

MOBILITY OF SPIN LABELS AT THE TEMPERATURE OF THE BREAK IN ARRHENIUS ATPase ACTIVITY PLOT

MEMBRANE	4NS ($2T_{||}$)	16NS ($\frac{h_{-1}}{h_0}$)	ATPase Transition temperature (^{o}C)[c]
Promitochondria (depleted)[a]	55.8	0.677	26 to 30 (28)
Promitochondria (supplemented)[b]	56.2	0.680	18 to 22 (20)
Mitochondria	53.3	0.775	17 to 21 (19)

[a] Depleted: From cells grown anaerobically without unsaturated fatty acids.

[b] Supplemented: From cells grown anaerobically in the presence of unsaturated fatty acids.

[c] The value in brackets represents the mid point transition temperature.

mitochondrial structures.

Comparison of the mobility of spin labels in the membranes and their extracted lipids was also made. Measurements were made using using an equivalent viscosity parameter $\log_{10} \eta^{-}$. This is the logarithm of the viscosity of glycerol containing 4NS which results in the same ESR spectrum as the system under consideration (compared either by measurements of $2T_{\parallel}$ or line heights), These comparisons are shown in Fig. 7. In the supplemented promitochondria and the aerobic mitochondria, there is an increase in mobility in the extracted lipid compared with the membranes at 15°C when measured by 4NS (near the edge of the bilayer). In the depleted promitochondria, the lipid extract has a mobility similar to the other two but in the membrane 4NS was immobilised to a greater extent. When the interiors of the bilayers were examined by using the M12NS probe, it was found that compared with the lipid extracts all three membranes were immobilised to about the same extent (the effect being about the same as in the depleted promitochondria with 4NS). These comparisons were all made at temperatures below the transition temperature of the membrane or lipid extract.

The fluid state observed in the phospholipid bilayer of most of the biological membranes gives rise to a lateral diffusion of the individual phospholipid molecules (137 - 138). However, the lateral diffusion of lipid molecules within the plane of the membrane is important for an understanding whether protein or lipids are synthesized at distinct sites within the cell (139 - 140).

FIGURE 7

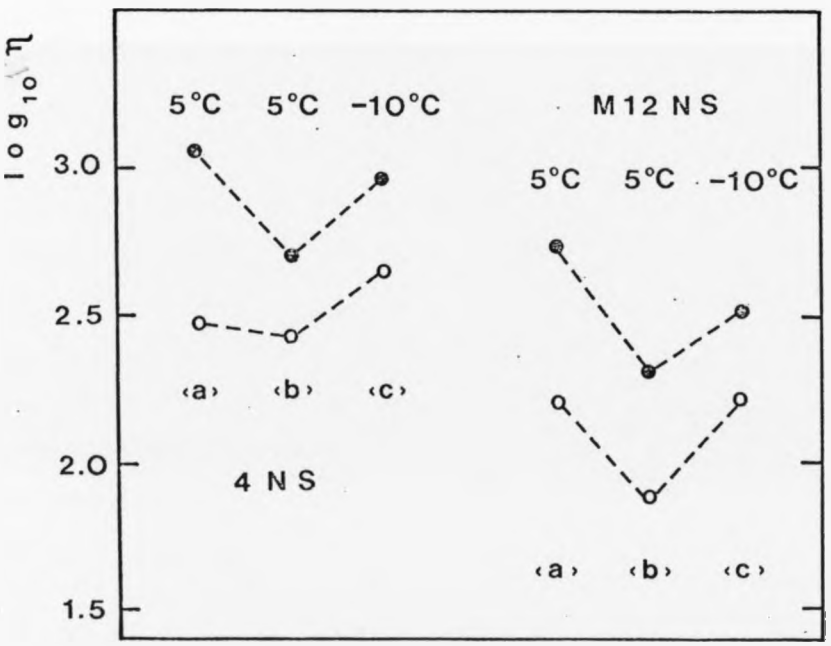


Diagram showing the mobility of spin labels 4NS and M12NS in membranes compared with lipid extracts. The mobility was measured by comparing the spectra with those of 4NS in glycerol at various temperatures and hence obtaining the equivalent viscosity (η) of glycerol. (a) from depleted promitochondrial membranes 5°C, (b) from supplemented promitchondrial membranes 5°C, (c) from mitochondrial membranes -10°C.

In turn, the fluid property of the lipid bilayer should be reflected in the behaviour of the associated protein molecules. The existence of a rotational degree of freedom has been recently observed in rhodopsin, an integral component of the disc membranes of the vertebrate rod outer segment (141). These studies have demonstrated that the distribution and mobility of this protein were dependent on the physical state of the membrane lipids.

Although most of the biological membranes appear fluid in character in regard to the lipid component, in some membrane the lipid and protein may not be particularly fluid or mobile (142). This appears to be the case with the anaerobic promitochondria, where the lipid components are in gel state. If this condition is a result of protein-lipid interactions, the protein components are expected to be in less mobile state in respect to the fully organized mitochondria.

In order to determine whether or not changes in the composition and state of the membrane lipids are reflected in the mobility of the proteins, promitochondria and mitochondria were labelled with maleimide spin label (MSL), which attaches primarily to the sulphydryl groups of the proteins (143). The ESR spectra of these membranes are shown in Fig. 8 and the data summarized in Table 7. It is a composite of at least two spectra, a "broad-line" spectrum and a "liquid-line" spectrum, indicating the existence of two types of label environment: one whose motion was severely hindered, and another which was only weakly immobilized. The weakly immobilized components

TABLE 7

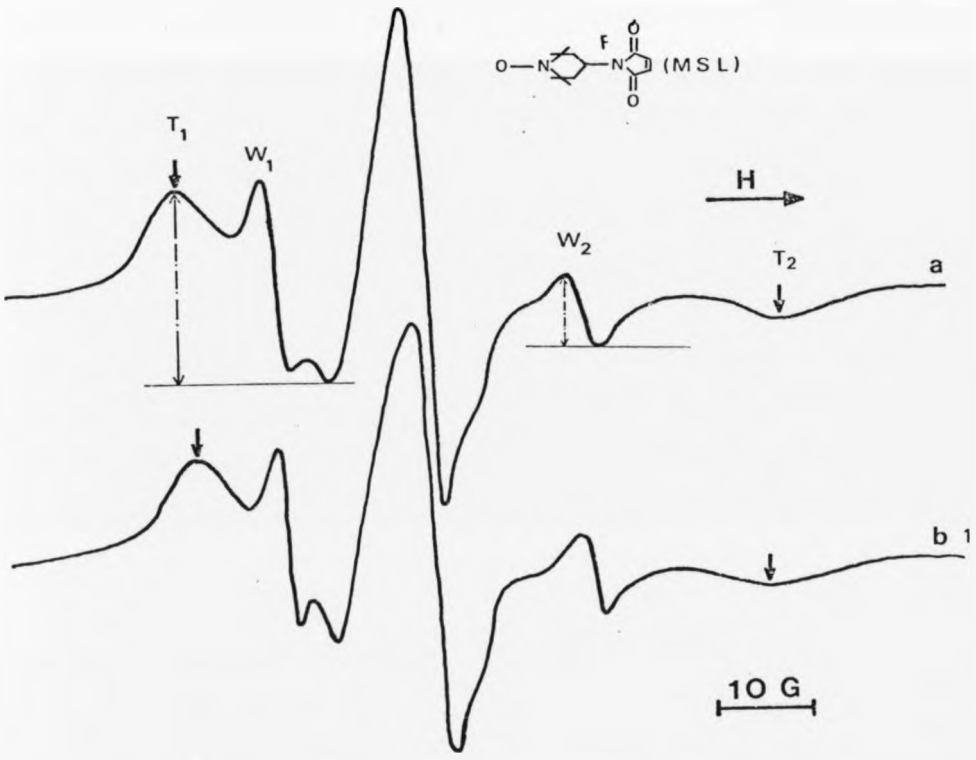
COMPARISON OF PROMITOCHONDRIA AND MITOCHONDRIA LABELLED WITH MALEIMIDE SPIN LABEL (MSL) AT THE TEMPERATURE OF GROWTH OF THE CELL

MEMBRANE	$2T_{\parallel}$	$\frac{W_2}{T_1}$ [b]
Promitochondria (depleted)[a]	63.5	0.396
Mitochondria	61.0	0.590

[a] Depleted: From cells grown anaerobically without unsaturated fatty acids.

[b] $\frac{W_2}{T_1}$ Taken as a measure of the ratio of two spin populations.

FIGURE 8



Electron spin resonance spectra of MSL in (a) anaerobic promitochondrial membranes, (b) aerobic mitochondrial membranes.

(W_1 and W_2) may result from spin labels bound on the membrane surface or inside the membrane in any region of high fluidity. The strongly immobilized components (T_1 and T_2) may arise from a label attached to a sulphydryl group on the surface of the membrane in such a way that its motion is severely restricted. An alternative explanation is that the label binds inside the membrane where its motion is restricted or is attached to groups other than sulphydryl ones. Experiments with spin labelled poly-L-lysine complexed with phospholipids, suggest that intermediate immobilization of the label may occur in non polar solvents and that weak immobilization is more characteristic of polar solvents. These results suggest that the strongly immobilized components of the spectra of the labelled membranes might be associated with spin labels located within the lipoprotein matrix. This might explain the higher immobilizing environment in promitochondria, indicating that the mobility of the protein components is a function of the composition and physical state of the lipid bilayer.

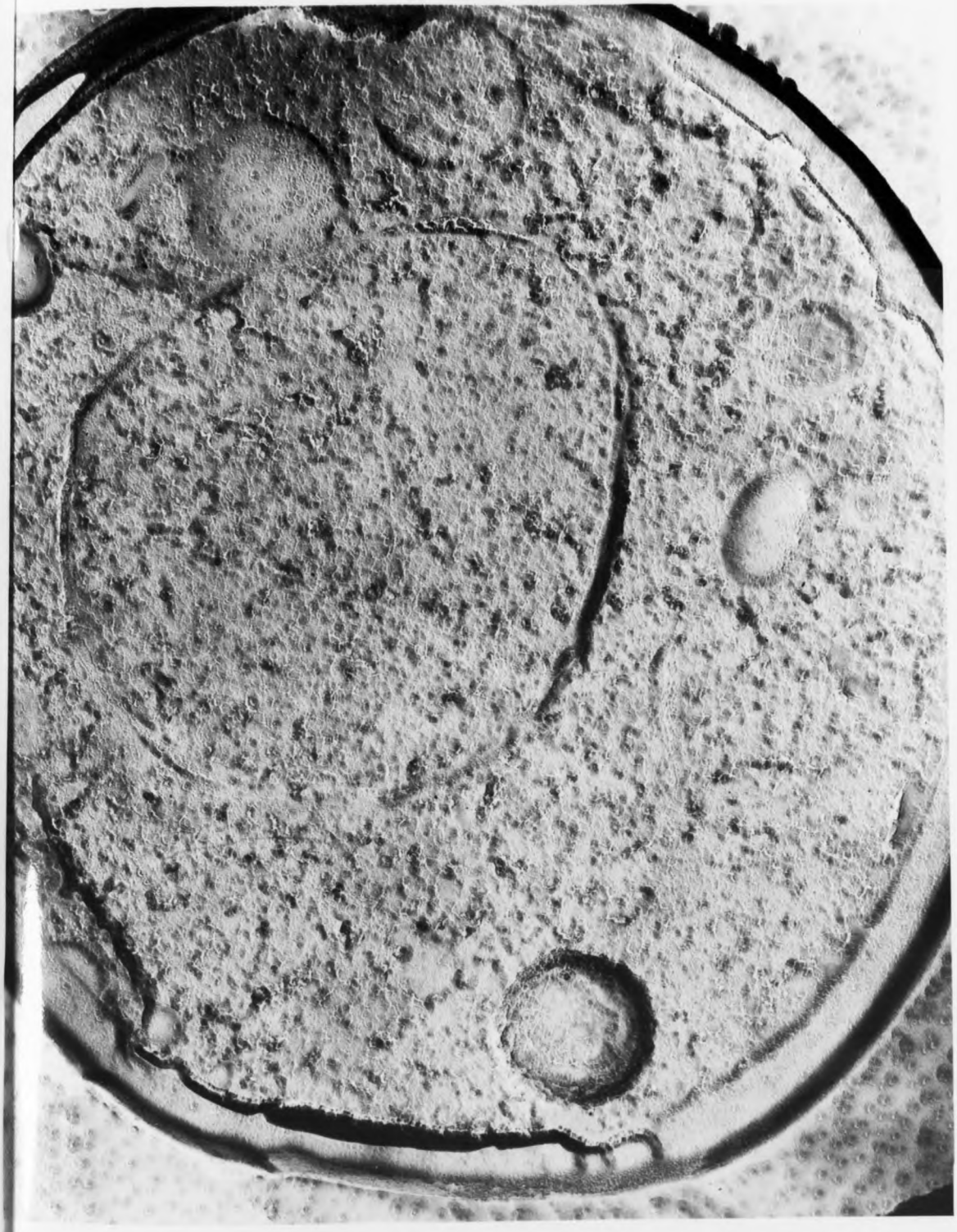
(b) Freeze-etching Studies

The introduction of freeze-etch techniques (144) has provided a new approach in the controversial debate on the membrane structure. Membranes are preserved by rapid freezing at liquid nitrogen temperature, instead of the chemical fixation and dehydration (145). One example is presented in Fig. 9, showing freeze-fracture replicas of

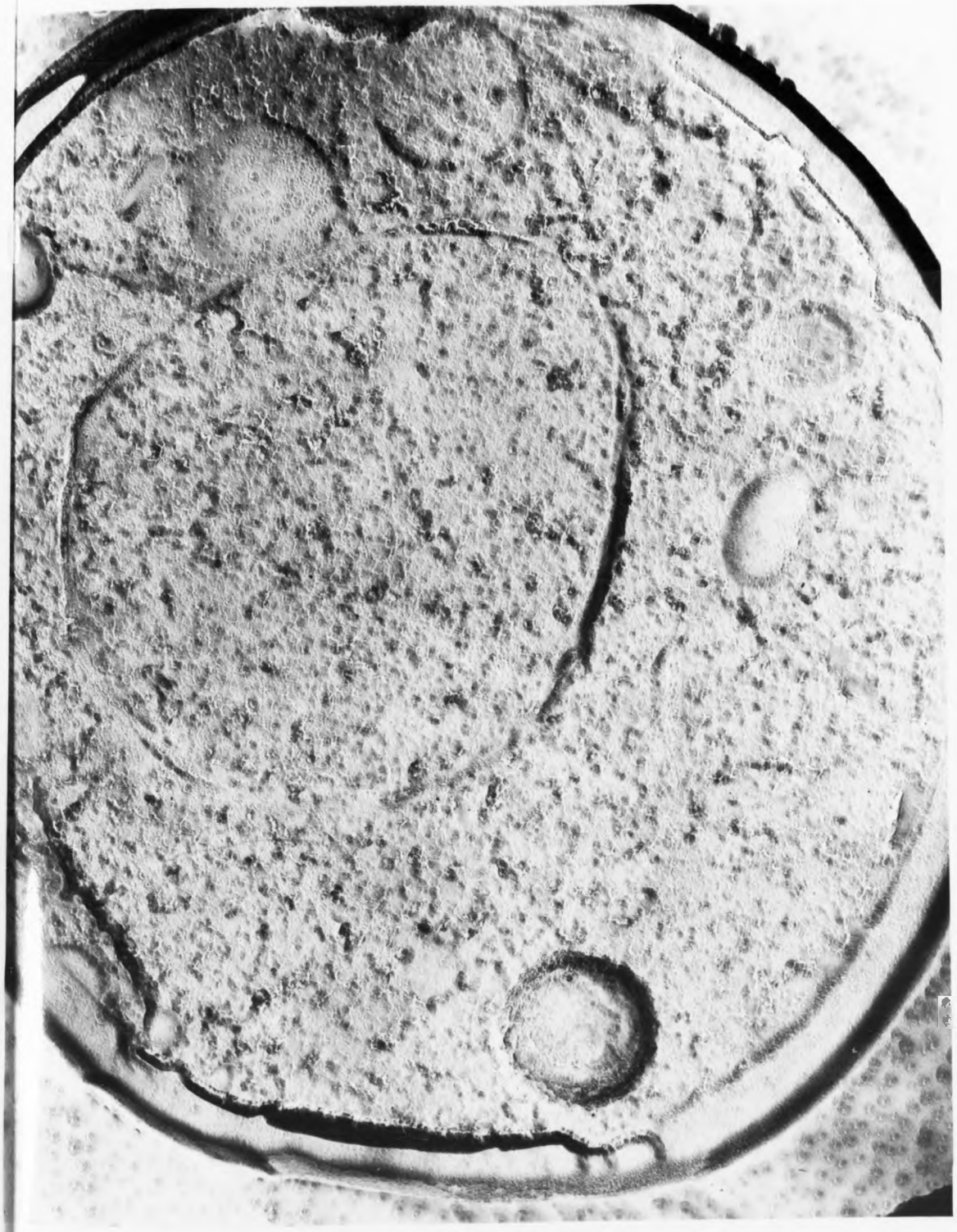
FIGURE 9

Freeze-etched preparation of Saccharomyces cerevisiae grown aerobically.

revisiae



erevisiae



an aerobic yeast cell.

The presence in the promitochondria of a lipid bilayer in gel state, with ordered hydrocarbon chains, raises the following questions:

(i) What is the arrangement of membrane proteins in promitochondrial organelles?

(ii) Can the proteins easily penetrate, by hydrophobic forces, the lipid bilayer that is in a gel state at the temperature of growth of the cell?

Freeze-etch is particularly appropriate for this purpose since at cleavage, the membrane is split along its lipid bilayer and reveals areas of the hydrophobic interior.

The freeze-etch studies show that membranes could be fractured and that these fractures depend upon the presence of the membrane lipids. This provides strong support for the idea that the planar continuity of biological membrane is dependent on the presence of a lipid bilayer, whose fatty acids provide a natural fracture plane between their hydrophobically interacting methyl groups (146).

In many membranes the bilayer is interrupted by characteristic particles as observed in freeze-fractured preparations. Evidence shows that these intramembrane particles represent proteins intercalated into hydrophobic regions of the membrane lipid bilayer (147 - 149). Recently, these observations have related these particles to specific proteins which may traverse the membrane. This is the case in the human red blood cell membrane, where these proteins are the basis for the antigenic action on

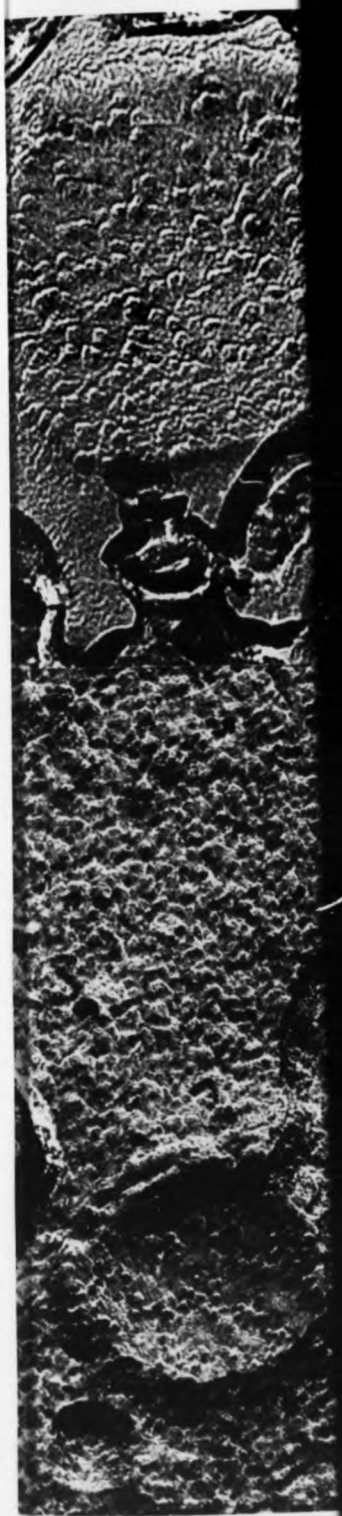
the erythrocyte surface (150 - 151).

The evidence of these particles by freeze-etch is important in order to establish their distribution in respect to lipid bilayers. In this context the pro-mitochondria and mitochondria are freeze-etched in order to gain some information on the arrangement of the proteins in such membranes. No apparent difference was observed in mitochondria from aerobically or anaerobically grown yeast cells (Figs. 10 and 11). Fracture faces of mito-chondrial membranes within the yeast cells were either smooth or particulate as was the case with mitochondria isolated from the aerobic and anaerobically grown cells. These results are in good agreement with ESR data and if the interpretation of the particle is correct, are in favour of some hydrophobic penetration of proteins into the lipid bilayer. In the controversial report on the ultrastructure of the anaerobic promitochondria the above observations also agree with evidence of organized cristae in such membranes (152).

3.5 Membrane Fluidity and Functional Assembly of The Respiratory Chain

The concomitance in the yeast cell of a higher saturation of the lipids with scarce or no respiratory activity, depending on the conditions of growth, raises the question of a casual or logical inter-relationship between the two processes. A possible dependence of the functional respiratory chain in the developing mitochondria on lipid unsaturation receives support by structural studies

FIGURE 10



Electron micrograph of a freeze-etched yeast cell grown aerobically. Mitochondria show particulate membrane faces, x55000. Inset shows a mitochondrion isolated from aerobically grown yeast cells, x 77,000.

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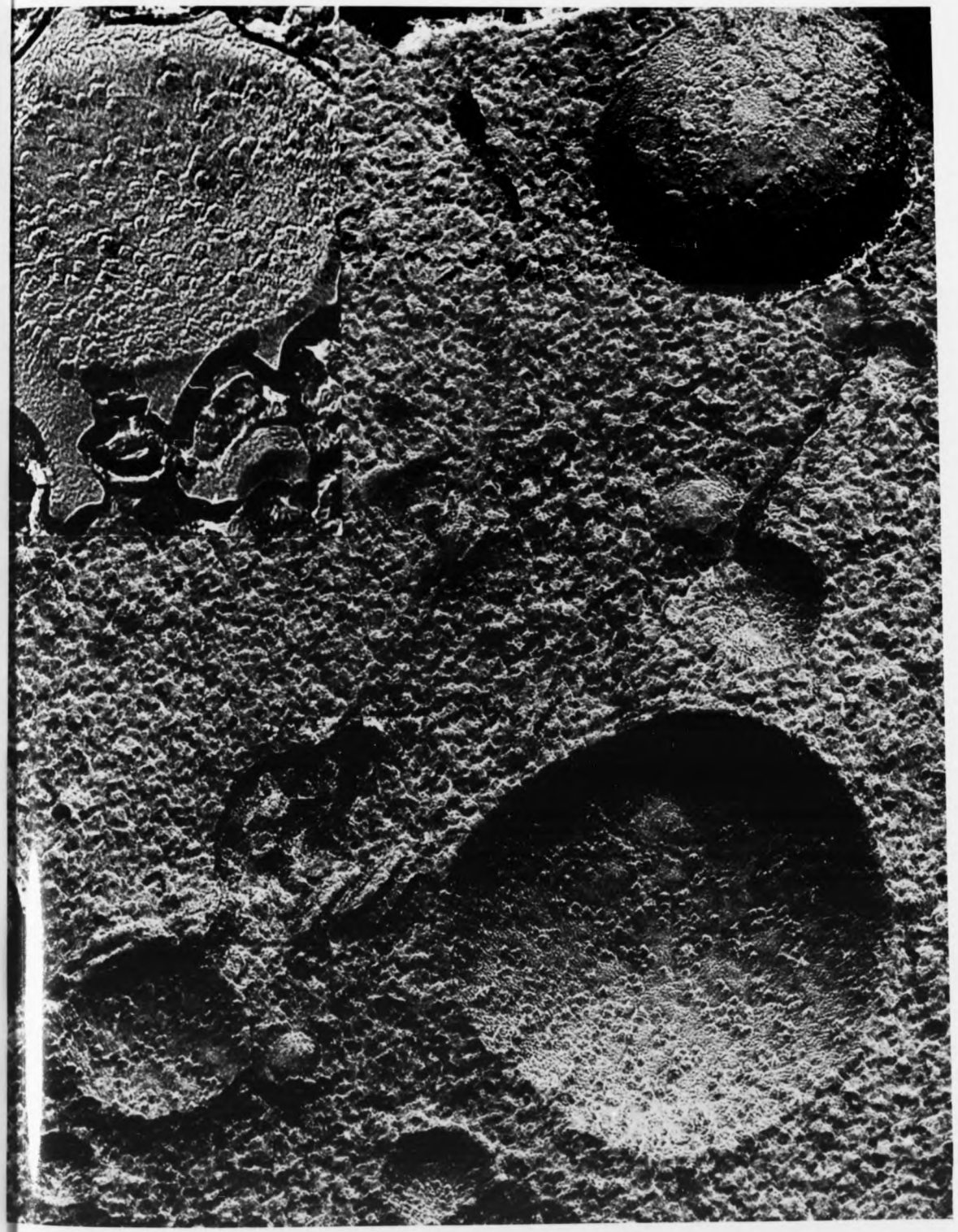

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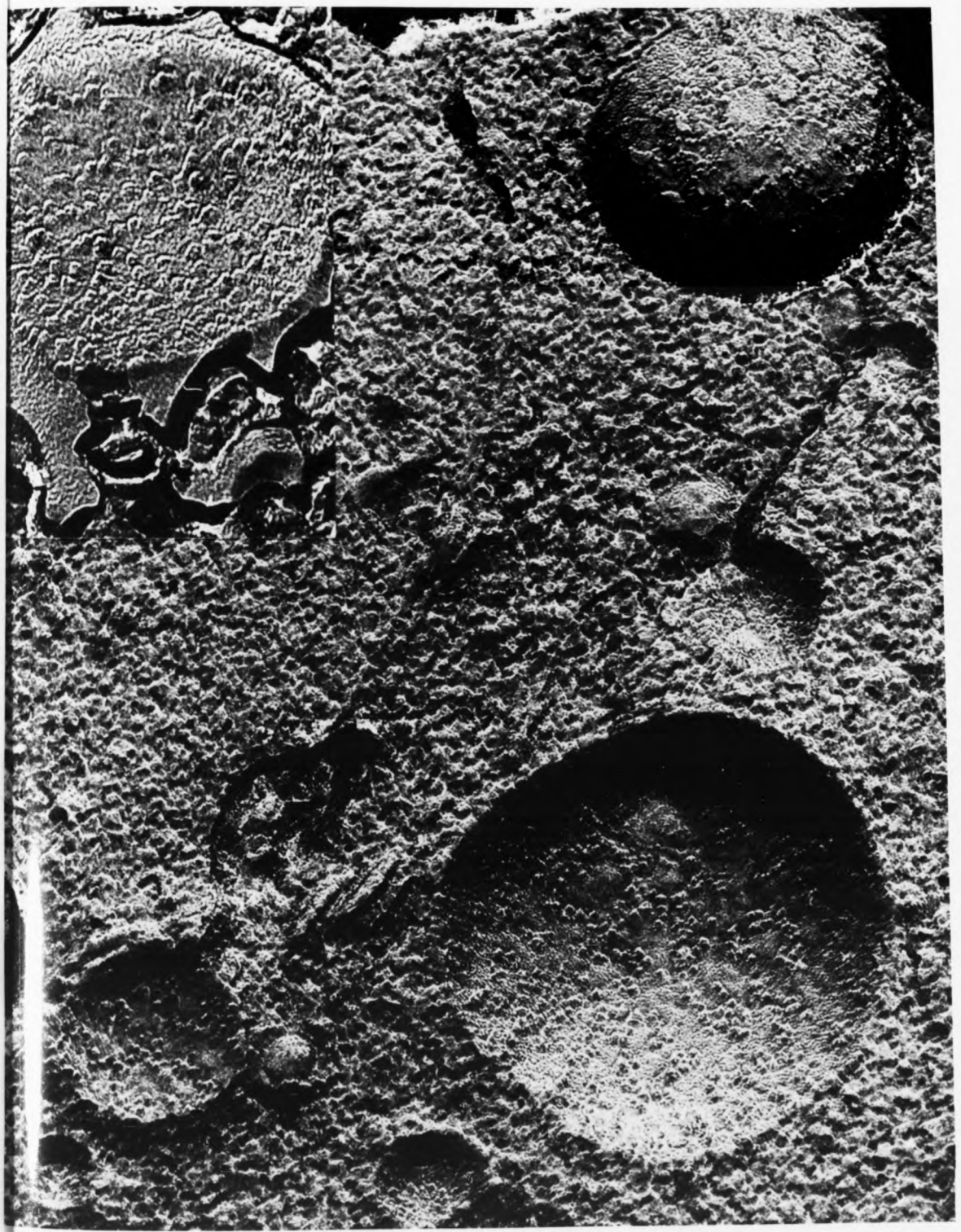

FIGURE 11

Electron micrograph of a freeze-etched yeast cell grown anaerobically in the absence of any unsaturated fatty acid supplement. Mitochondria show particulate membrane faces similar to those seen in yeast cells grown aerobically, x 55000. Inset shows a mitochondrion isolated from anaerobically grown yeast cells grown in the absence of unsaturated fatty acid supplement, x 77000.

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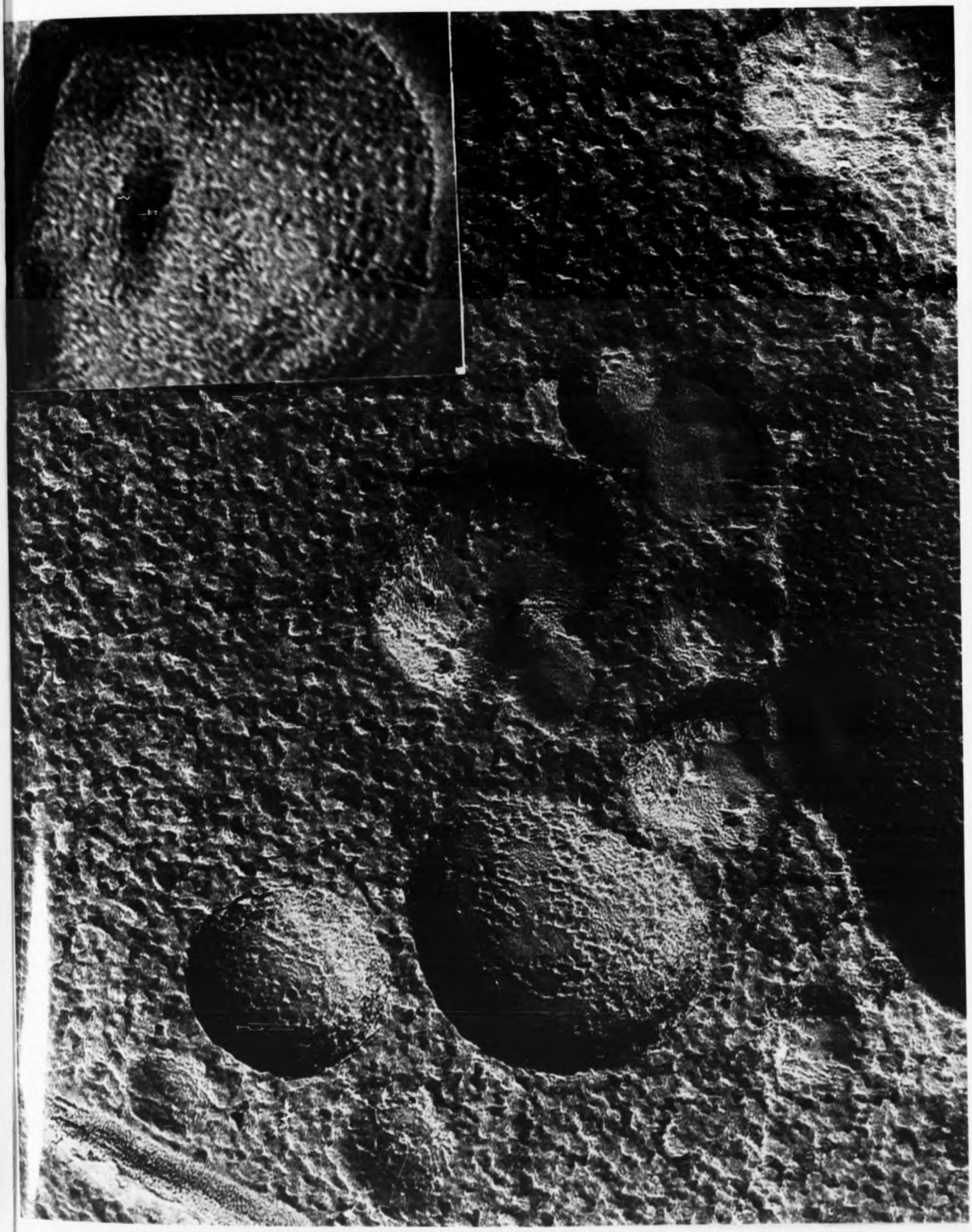

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indicating the importance of the hydrophobic interaction between lipids and proteins in the inner mitochondrial membrane (153 - 156). Correlations between unsaturated fatty acids in the phospholipids and the respiratory activity are in favour of the hydrophobic interactions which involve both protein and lipid molecules. In fact myelin phospholipids, which have lower unsaturation and longer hydrocarbon chains than physiological mitochondrial phospholipids, are much less effective in restoring succinoxidase and DPNH oxidase activities in beef heart mitochondria (157).

A mutant of Saccharomyces cerevisiae which cannot synthesize unsaturated fatty acids offers a good approach in order to have a greater insight into this problem. The advantage is that the membrane lipid can be manipulated in aerobic conditions by growing the organism in the presence of added unsaturated fatty acids (23). Relationships between growth conditions and unsaturated fatty acid content of the cell are represented in Table 8.

Growth of cells in ethanol in media low in unsaturated fatty acids produces cells which contain between 25 and 40% of unsaturated fatty acids. It was not possible to lipid deplete the desaturase mutant to values below about 25% of unsaturated fatty acids by using ethanol as a carbon source, without a concomitant loss in the viability of the cells. However, lipid depletion to values below 25% of unsaturated fatty acids was achieved by growing cells on glucose. Cells containing high proportions of unsaturated fatty acids (60 - 70%) were obtained by growth

TABLE 8

GROWTH CONDITION	UNSATURATED FATTY ACID SUPPLEMENTED (mg/ml)	SHORT CHAIN SATURATE ($C_{10:0}$, $C_{12:0}$, $C_{14:0}$)	LONG CHAIN SATURATED ($C_{16:0}$, $C_{18:0}$)	LONG CHAIN UNSATURATED ($C_{16:1}$, $C_{18:1}$)
0.5% Ethanol	0.1	17.3	54.1	28.6
	3.0	4.9	41.5	53.6
0.5% Glucose	0.1	21.9	59.6	18.5
	3.0	6.4	24.6	69.0

in media containing 2 to 4 mg of Tween 80/ml in ethanol (0.5%) or glucose (0.4 to 1.0%) as substrates. Correlation between synthesis of cytochromes and degree of membrane lipid unsaturation has been observed. Fig. 12 shows the cytochrome spectra of cells grown on ethanol in media containing low (50 µg Tween 80/ml) and high (3 mg of Tween 80/ml) content of unsaturated fatty acids. Absorption maxima corresponding to cytochromes a + a_3 (605 nm), cytochrome b (564 nm) and cytochrome c (550 nm) were observed in both cell types. However, the absorption peak of cytochromes a + a_3 was distinctly larger for lipid supplemented cells than that for lipid depleted cells. The difference in the content of cytochrome a + a_3 in the two types of cell was also clearly evident when the absorption bands in the Soret region were examined. The Soret band of cytochromes a + a_3 (445 nm) in the lipid depleted cells was only observed as a shoulder in the larger cytochrome b peak (430 nm). Cells, which were more extensively depleted of unsaturated fatty acids by growth on glucose, showed a much lower content of cytochromes a + a_3. The lower cytochrome content of the lipid depleted cells was also confirmed by measurement of O_2 consumption by intact cells, as presented in Table 9. A relationship between efficiency of oxidative phosphorylation and content of unsaturated fatty acids in the phospholipids has been observed in mitochondrial membranes, and oxidative phosphorylation was completely lost in membranes containing less than 20% of unsaturated fatty acids (158).

As was discussed before, membranes with a different

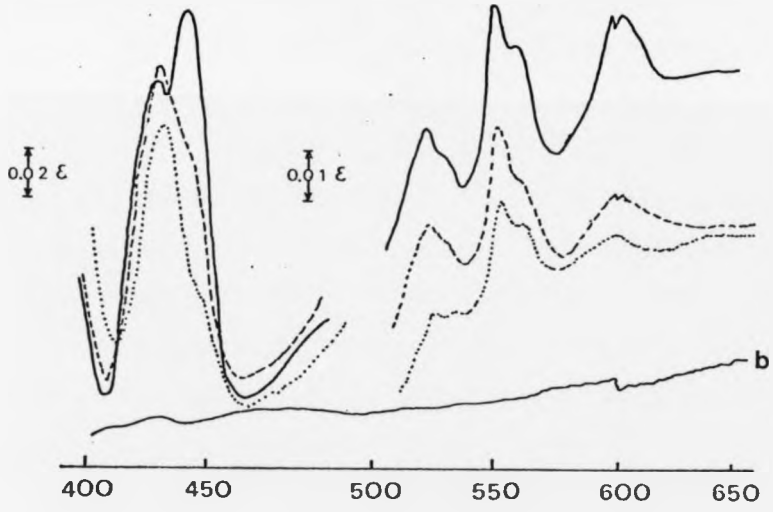
TABLE 9

EFFECT OF LONG CHAIN UNSATURATED FATTY ACID AND O_2 UPTAKE OF WHOLE CELLS

GROWTH CONDITIONS	UNSATURATED FATTY ACID (%)	O_2 UPTAKE (mg atom of oxygen/min/mg dry wt).
Ethanol (0.5%)		
Lipid depleted	31 ± 6 (6)	116 ± 40 (6)
Lipid supplemented	64 ± 7 (6)	159 ± 21 (10)
Glucose (0.4%)		
Lipid depleted	17 ± 5 (4)	82 ± 25 (4)
Lipid supplemented	68 ± 4 (4)	189 ± 30 (4)

( ) indicates the number of individual experiments from which the results were obtained,

FIGURE 12



Cytochrome ——— spectra of cells containing different proportions of unsaturated fatty acid.

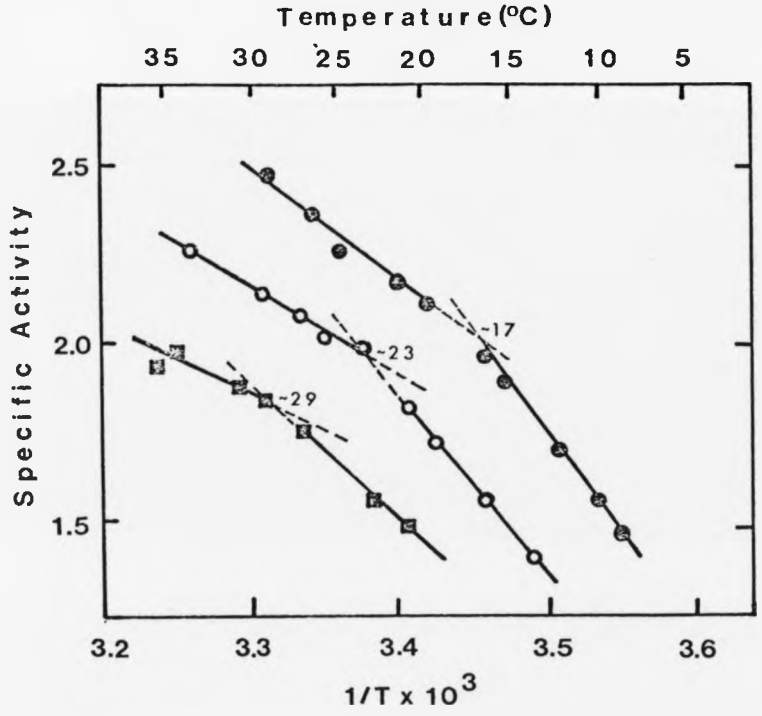
(——) Cells containing 62% unsaturated fatty acid; concentration 24.4 mg dry wt./ml; grown on 0.5% ethanol.

(- - -) Cells containing 32% unsaturated fatty acid; concentration 26.3 mg dry wt./ml; grown on 0.5% ethanol.

(·····) Cells containing 18% unsaturated fatty acid; concentration 28.3 mg dry wt./ml; grown on 0.5% glucose.

content of unsaturation in the phospholipids are physically different. Membranes with a high degree of unsaturation are in liquid like state, whilst those with lower (< 20%) are prevalently in gel state. In order to correlate these different physical states of the membrane with the physiological function, the OS-ATPase and succinate oxidase were studied, as a function of the temperature. The data are presented in the Arrhenius plots, as shown in Figs. 13 and 14, together with the temperature of discontinuity. In both cases, a non linear Arrhenius plot, with an increase in activation energy at lower temperatures was observed. The temperatures at which a discontinuity occurred were dependent on the degree of lipid unsaturation. The greater the degree of lipid unsaturation, the lower was the transition temperature. The specific activity was also dependent on the degree of unsaturation of the membrane lipids. In fact the succinate oxidase activity in mitochondria containing 67% of unsaturated fatty acids was approximately two fold higher than that of mitochondria containing 15%. For ATPase the highest specific activity was obtained for mitochondria with the highest proportion of unsaturated fatty acids. From these observations it seems clear that the liquid-crystalline state of the membrane is required for a functional assembly of both respiratory proteins and the ATPase enzyme system.

FIGURE 13



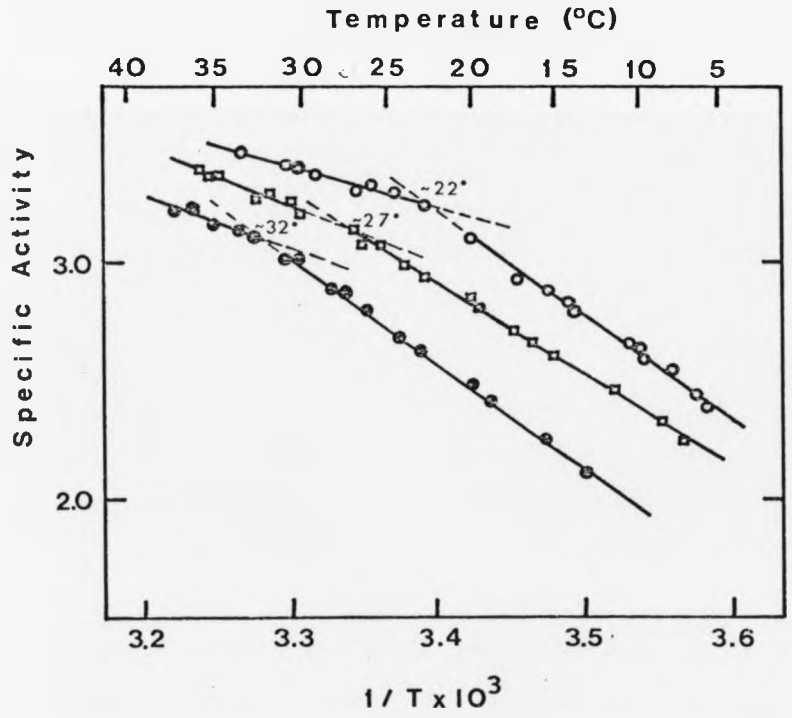
Arrhenius plots of succinate oxidase in mitochondria isolated from cells with different unsaturated fatty acid contents.

Mitochondria containing 67% unsaturated fatty acid

Mitochondria containing 31% unsaturated fatty acid

Mitochondria containing 15% unsaturated fatty acid.

FIGURE 14



Arrhenius plots of mitochondrial ATPase of cells with different unsaturated fatty acid contents.

○—○ Mitochondria containing 68% unsaturated fatty acid

□—□ Mitochondria containing 51% unsaturated fatty acid

●—● Mitochondria containing 21% unsaturated fatty acid.

3.6 Membrane Fluidity and OS-ATPase Microenvironment

(a) Spin Label Studies

The discrepancy between the phase transition observed in intact mitochondrial membrane and that in membrane bound ATPase activity raises these important questions:

(i) What is the distribution of lipids in the membrane?

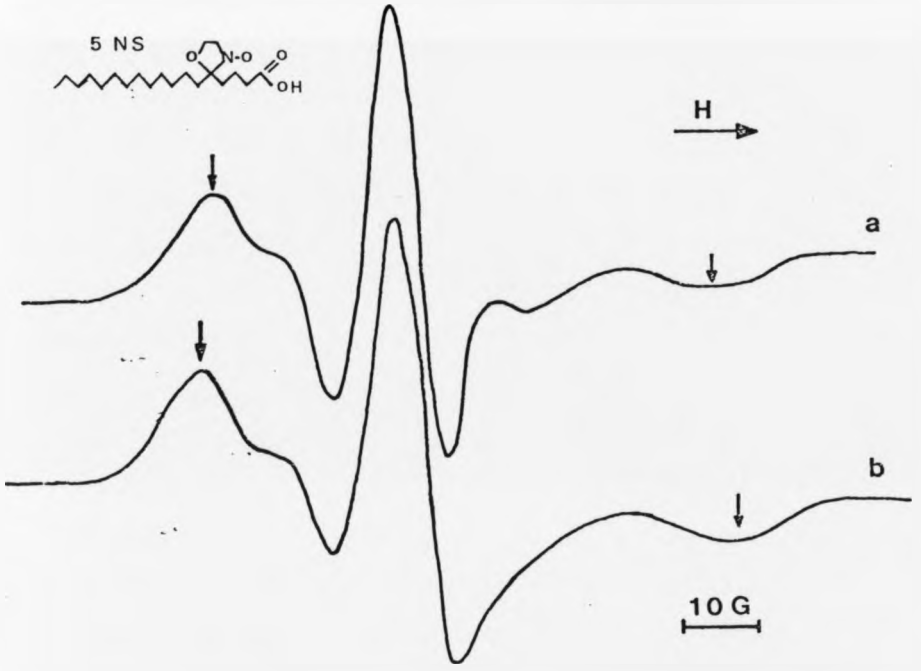
(ii) Are different membrane-bound activities associated with different microenvironments?

(iii) What is the role of the possible heterogeneous distribution of lipids on the membrane function?

The elucidation of these questions will require understanding, at the molecular level, of the interactions between lipid and protein in the isolated enzyme system. If the higher transition temperature found for OS-ATPase activity reflects the melting point of less mobile phospholipids associated with the enzyme, a large contribution of these lipid molecules to OS-ATPase phase transition will be expected. The OS-ATPase, isolated from aerobic yeast cells, still contains some of the phospholipids (88). The fluidity of this lipo-protein complex was investigated by ESR spectroscopy.

The ESR spectra of spin label stearic acid (5NS) incorporated into OS-ATPase and intact membrane is shown in Fig. 15. In the spectra, the arrows indicate the position of the outer hyperfine splitting measured as $2T_{\parallel}$. These data point out the presence of a strongly immobilized spin label in the OS-ATPase system compared to intact membrane, suggesting a more viscous lipid environment surrounding the enzyme. Some increase in fluidity was

Figure 15



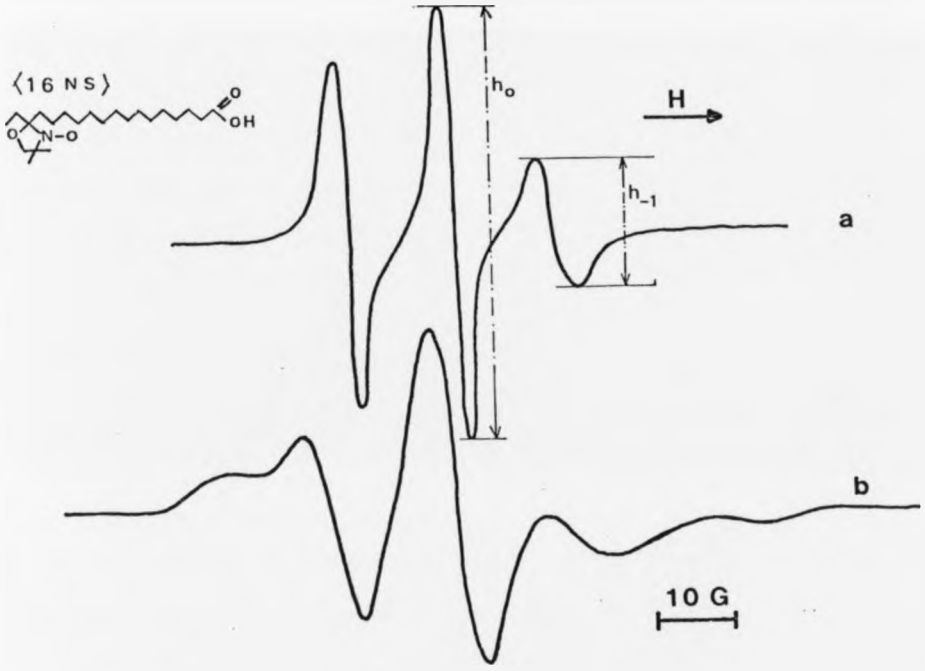
E.S.R. spectra of 5NS in;

(a) Mitochondrial membranes (b) isolated OS-ATPase.

observed as the label is moved further from the polar head group of the molecule towards the centre of the lipid core. The ESR spectra of OS-ATPase and native membrane labelled with 16NS are compared in Fig. 16. The spectra obtained demonstrate that the environment of the 16NS probe is less immobilized than that of 5NS near to the polar head group of phospholipids, but the degree of immobilization is still greater in OS-ATPase than that of intact mitochondria. These observations indicate the effect of enzyme protein extended at least to the middle of the membrane bilayer with a strong hydrophobic lipid-protein interaction. Such a strong immobilizing effect of lipids surrounding the enzyme has been observed in biological systems characterized by low content of lipid. This is the case in isolated cytochrome oxidase (157), where at low phospholipid/protein ratio (less than 0.2 mg of phospholipid per mg of protein) the lipid spin label probe is highly immobilized, whilst at higher phospholipid content the lipid bilayer assumes a characteristic fluid state. These studies were interpreted as evidence for a boundary of tightly bound lipids surrounding the hydrophobic region of the protein.

Another important point that has to be considered concerns the effects of detergent in maintaining the correct activity and conformation of the native protein. In fact, the soluble ATPase which exists as a free particle (88), retained a higher degree of catalytic activity. The observation of an increase in activity of the soluble enzyme, after detergent treatment, may indicate that the

FIGURE 16



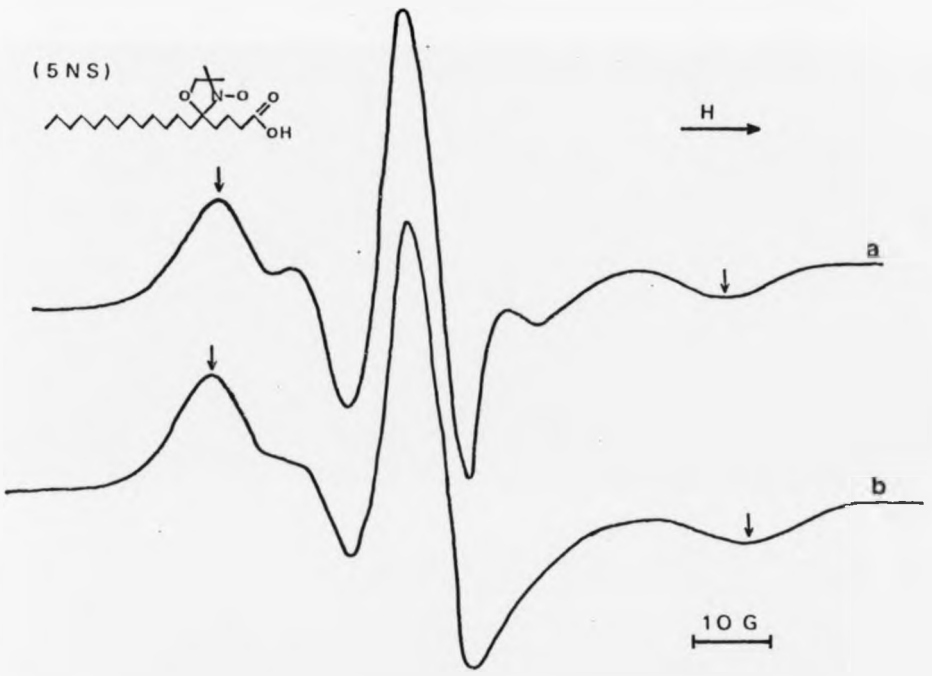
Electron Spin Resonance Spectra of 16NS in;
(a) Mitochondrial membranes (b) isolated OS-ATPase.

constraining effect of lipid could be removed by the action of detergent, perhaps involving some alterations in the enzyme microenvironment as observed in Fig. 17. From the analysis of these spectra it seems that Triton X-100 can maintain the OS-ATPase in its native conformation or something close to it, by increasing the fluid state of the lipoprotein system, near to that of the native membrane. This fluid state could be achieved at the expense of the decrease in E_a in the enzyme activity, perhaps through a conformation change of the protein, accompanied by a disappearance of the break in the Arrhenius plot of the activity as observed in Fig. 18.

(b) X-ray Diffraction Studies

Additional information on the structural organization of tightly bound lipids to OS-ATPase can be gained from X-ray diffraction analysis. The correlation between ESR and X-ray data are presented in Table 10. Thermal phase transition was observed in both ATPase and intact membrane. The transition was characterized in the X-ray diffraction patterns by a broad band centered at about 4.7Å (disordered lipid hydrocarbon chain) to a sharp reflection at 4.2Å (ordered lipid hydrocarbon chain) as the temperature was decreased. The data are represented in Table 11 and agree with observations in ESR experiments. A disorder-order transition of the hydrocarbon chains implies the existence of a segregation of the lipids, with distinct domains; those containing lipids with ordered hydrocarbon chains and those containing lipids with disordered paraffin chains. Microdensitometer traces

FIGURE 17

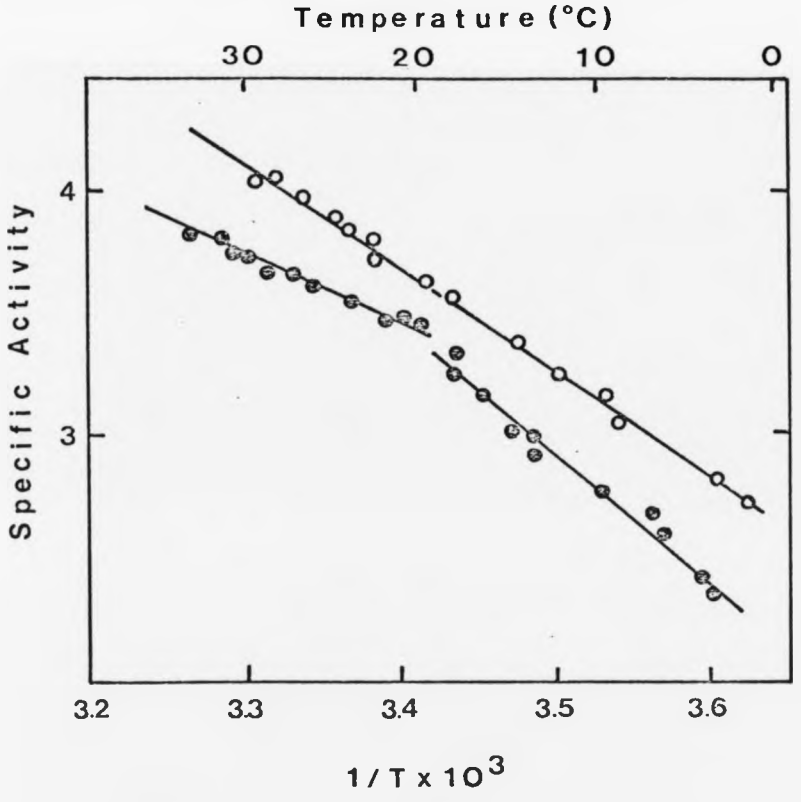


Electron Spin Resonance Spectra of 5NS in;

(a) OS-ATPase in the presence of Triton X-100

(b) OS-ATPase after treatment with Bio-Beads 5MZ.

FIGURE 18



The effect of Triton X-100 on the Arrhenius plots of OS-ATPase from mitochondria isolated from cells grown aerobically on 0.5% ethanol.

○—○ ATPase activity of aerobic mitochondria isolated from yeast cells grown aerobically on ethanol as substrate.

●—● Submitochondrial particles (20mg protein/ml) were treated with triton X-100 at a final concentration of 25%. The suspension was centrifuged at 105,000g for 15 min and the supernatant was used as the soluble ATPase.

TABLE 10

FLUIDITY AND X-RAY DIFFRACTION PATTERNS OF ISOLATED OS-ATPase AND NATIVE MEMBRANE

PREPARATION	5NS ($2T_{\parallel}$)	RANGE OF TRANSITION TEMPERATURE (Disappearance of 4.2Å sharp ring)
Mitochcndria	56.2	<-15 to -5
OS-ATPase	60.7	10 to 15

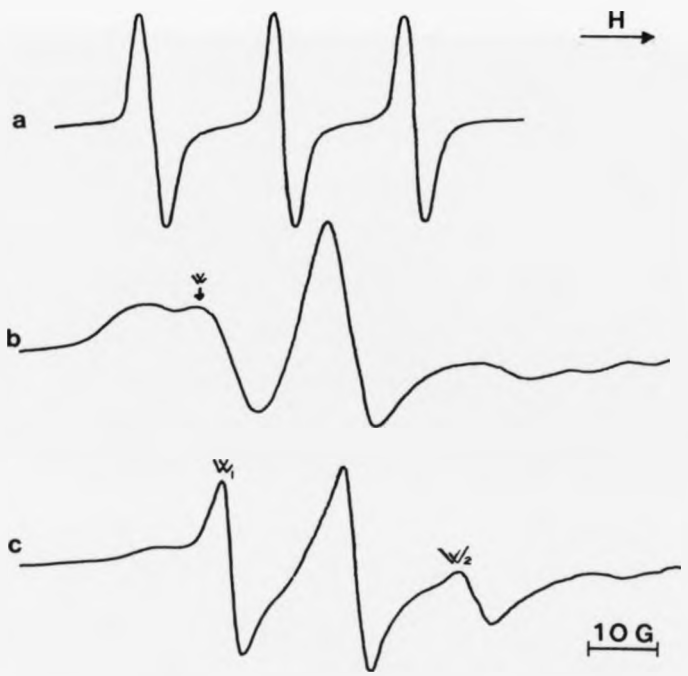
The ESR spectra were detected at environmental temperature.

and visual inspection of the diffraction patterns showed that the change took place over a temperature range of 5°C. The broadness of the transitions in the two systems is significantly different. For native membrane, the disorder-order transition extends over 10°C, beginning at -15°C and ending at -5°C. For OS-ATPase, the transition is considerably higher and relatively sharp, beginning at 10°C and ending at 15°C, approaching that of the membrane-bound enzyme activity. These differences can be understood in terms of both hydrocarbon chains and type of lipid-protein interactions. In heterogeneous systems during the disorder-order transitions, the totally unsaturated phospholipid will remain preferentially in the domains containing disordered hydrocarbon chains, while the saturated ones will preferentially crystallize within the domain containing ordered hydrocarbon chains. Under these conditions, the broadness of the transition for mitochondrial particles will depend mainly on its high degree of unsaturated phospholipid. With decreasing temperature, the transition will be expected to be broader with lipid that crystallizes out of the liquid domain, containing disordered hydrocarbon chains. The state of order of hydrocarbon chains is greater in OS-ATPase than in the corresponding native membrane. This difference could be interpreted as the result of interactions between penetrating enzyme molecules and hydrocarbon chains of the lipids and/or the association of one type of phospholipid which might be involved in controlling the physiological activity of the enzyme.

3.7 Membrane Fluidity and Conformational Change of OS-ATPase

Structural information concerning the hydrolysis and synthesis in mitochondrial membranes has been detected by using sensitive extrinsic probes which act at the catalytic sites of the ATPase system (159). A site-directed spin label NCCD has been used successfully in beef heart submitochondrial particles (160). NCCD is an analogue of dicyclohexylcarbodiimide, an inhibitor of mitochondrial ATPase (161), and conserves almost intact its inhibitory capacity (160). Conflicting studies have been reported on the nature of the binding site of this inhibitor (161 - 162). It is also well reported that DCCD binds to subunit 9 (163), a lipoprotein component of OS-ATPase, via a covalent bond. In order to have more insight into this problem we studied in different experimental conditions the microenvironment associated with subunit 9 of OS-ATPase in yeast and beef heart mitochondria using NCCD spin label and DCCD radiolabel. Two different types of yeast mitochondria, anaerobic promitochondria and aerobic mitochondria, were taken into consideration. NCCD spin label was incorporated into these membranes as described by Azzi *et al.*(160). The ESR signal characteristic of membrane-bound label was detected in all membranes, but the proportion of total signal in the anaerobic promitochondria and aerobic mitochondria was different, and only a weak signal was found in the latter. In Fig. 19a is reproduced the

FIGURE 19



Electron Spin Resonance Spectra of NCCD in

(a) distilled water

(b) incorporated into the mitochondrial membrane

(c) after treatment with n-butanol (100mM).

paramagnetic resonance spectrum of the NCCD in H_2O, showing a characteristic three sharp lines. In contrast, the spectrum of NCCD-labelled anaerobic promitochondria (Fig. 19b) shows three broad and relatively widely split peaks, indicating an immobilizing environment surrounding the binding site of the probe. Since the subunit 9 is a lipoprotein, the properties of this binding must be dependent on the nature of the lipid-protein interaction. The strong immobilization of the lipid molecules by the protein might affect the binding or the permeability of the inhibitor at the level of the action site. If this is true the perturbation of the lipid-protein interaction could have some effect on the environment where NCCD is located. It has been proved that the hydrophobic alcohols such as n-butanol, have the ability to disorganize the lipid-protein interactions in the membrane (164). Fig. 19c shows a dramatic change in the spectrum after treatment of the labelled membrane with butanol at moderate low concentrations. At low concentration of butanol the F_1 is not solubilized and the membrane ATPase is oligomycin-sensitive. The ESR spectrum shows a clear increase of mobile components (W_1 and W_2), indicating an increase in the local fluidity. On the other hand, beef heart mitochondria exhibit a different pattern and n-butanol, even at higher concentrations, does not change the characteristic strong immobilized spectra. Preliminary data show that the solvent does not displace DCCD from its hydrophobic site or prevent binding to it. These observations indicate that the organization of subunit 9

in yeast OS-ATPase is different from that of beef heart. In beef heart mitochondria the protein component must be directly involved in DCCD (and NCCD) binding. For yeast (pro)mitochondria it seems that some lipid component could also be involved in controlling the binding site. This might explain the fluidizing effect of n-butanol, perhaps acting via conformational change of the enzyme protein.

CONCLUSIONS AND PROSPECTIVES

4.1 General Remarks

Based on the information on yeast mitochondria and promitochondria available from X-ray diffraction, electron spin resonance, differential scanning calorimetry, freeze-etching and membrane-bound enzyme activity the following points emerge:

Membrane lipid exists in a bilayer array in promitochondria and mitochondria.

The continuity of lipid bilayer is interrupted in both promitochondria and mitochondria with hydrophobic protein particles.

Based on the considerations that the lipid molecules are immobilized at the level of the head groups and in the interior of the hydrophobic lipid core, the protein particles are not completely located in the interior of the lipid bilayer.

Promitochondrial membranes have at least some (or most) of the protein particles exposed to the exterior. These proteins are probably associated with other proteins or lipid head-groups via polar interactions.

Membrane fluidity is intimately correlated to functional activity of the membrane. The heterogeneous distribution of this fluidity is determined by lipid microenvironment of the protein molecules. This is the case of the OS-ATPase system.

4.2 Physiological Importance of Membrane Fluidity

Since the membrane lipids are closely related to membrane function, it is reasonable to suppose that the lipids in the membrane have both functional and structural roles. Heterogeneous distribution of lipids within the membrane could facilitate the insertion of newly synthesized protein components, such as respiratory proteins, without changing the original membrane area. In fact, the physiological importance of the liquid-expanded state of the lipid bilayer is dynamically evidenced by the behaviour of the promitochondria of yeast cells grown anaerobically. Under these conditions, yeast cells cannot synthesize unsaturated fatty acids, but they switch their biosynthetic mechanism in favour of saturated fatty acids of short chain length. These fatty acids would tend to increase the molecular area and hence to reduce the gel state of the membrane. The presence of unsaturated fatty acids in the medium provides optimum conditions for anaerobic growth. Under these conditions the membrane lipids are in the fluid state at the growth temperature. These observations demonstrate the importance of the appropriate lipid fluidity of the membrane to provide the best physiological conditions for growth. Nevertheless, it is of interest that anaerobic unsupplemented cells can grow with most of their lipids in a rigid or quasi crystalline character. The survival of the cell is critically dependent on the degree of unsaturation of the lipid molecules ($< 20\%$).

One possible explanation is that the temperature of growth falls below the midpoint of the membrane lipid phase transition. Physiologically significant proportions of lipids become solidified, thus not permitting the normal rate of growth. The optimum rate of growth can be achieved only when the cells grow at temperatures above their phase transition midpoint, as in the case of anaerobic supplemented cells and aerobic cells.

4.3 Membrane Phase Transition

In the present study when the results obtained by the various techniques (except those obtained with the spin-label methods) are compared, consistent values are obtained in indicating the range of lipid transitions of the membranes and lipids examined. The differences sometimes observed with the spin label method may arise because in certain instances the label situates itself in the most mobile region of a heterogeneous system of lipid molecules (137). It has been shown that this method can give reasonable results for transition temperatures with single pure lipid systems (165) but that with mixed lipid systems the label may distribute itself in the more fluid region making the transition temperature determination less certain. The use of probe molecules (130) to indicate molecular movement along the lipid chains and which are particularly marked at the methyl end of the chain (166) appears to be useful from the qualitative point of view but quantitative aspects are unsatisfactory as indicated by recent studies using deuterium magnetic resonance (167). These recent studies show a different

variation of the order parameter along the chains than that given by the spin probe data indicating effects of perturbation on the system (168). Thus we regard our deductions concerning mobility using these probes as providing qualitative information only on the organization of the lipids in the mitochondrial membranes.

A number of general comments relating to the factors underlying the phase transitions of cell membranes (106) require reconsideration to interpret the present data. Thus a comparison of the start of the lipid transition of a cell membrane with extracted lipids is of interest and can provide some information about possible protein-lipid interaction within the membrane system. Where large shifts occur between the transition temperature of the lipid and that of the membrane this may be indicative of lipid-protein interactions of an electrostatic nature occurring, or at least involving the polar groups of the lipids. (Various interactions of both an electrostatic and hydrophobic nature have been examined in model biomembranes to test effects on transition temperatures 29).

Where identical transitions are observed for membrane and lipid extract, strong electrostatic lipid interaction is probably not occurring. Such comparisons also assume similar lipid-lipid organization within the membrane and lipid extracts.

Furthermore, when a cell membrane is cooled slowly to lower temperatures (a process carried out prior to the heating runs) there is always the possibility that lipid

phase separation and crystallization will occur (137). When the higher melting lipids crystallize in the membrane the crystallization of the chains can exclude some proteins from that region into the remaining fluid region. Progressive cooling may then lead to protein aggregation and perhaps even total exclusion of protein from the bilayer structure (43). If this process does occur then the subsequent heating curves of the membrane will reveal phase transition behaviour of the lipids of the membrane from which the protein has been excluded. This needs to be remembered in consideration of the phase behaviour of those membranes such as promitochondria and mitochondria, where prior cooling of the membranes is essential for lipid phase behaviour to be observed during the subsequent heating run.

Finally, the wide nature of the observed transitions can be attributed to the "melting" of the various classes of phospholipids and associated fatty acids within the membrane system. Important biological functions such as membrane transport and growth as well as membrane-bound enzyme activity have been related to the lipid phase transition in the other biological membrane system (169, 127).

4.4 Membrane Fluidity and Lipid-Protein Interactions

The X-ray diffraction, light scattering and calorimetric data have shown that the beginning of the order-disorder transition in the depleted promitochondria takes place near the growth temperature of the cell. Some protein inter-

action with the lipid may be responsible for the large increase in transition temperature of the membrane compared with the extracted lipids. With mitochondria the X-ray diffraction data show that the phase transition occurs below $0^{o}C$ so that the hydrocarbon chains are in a fluid or liquid-like state at the growth temperature. A close correlation is observed with ESR spectroscopy which shows that the conformational state of the lipid chain is ordered or disordered in promitochondria or mitochondria, respectively. Magnetic resonance spectra also indicate differences in the motion of the polar and apolar regions of the lipids in the presence and in the absence of proteins. For the depleted promitochondria, the spin label data suggest that whilst there is a strong lipid-protein interaction inside the hydrophobic portion of the membrane lipids, there is also a strong immobilizing effect near the polar region. For the mitochondria the protein-lipid interactions may be similar to those which occur with the supplemented promitochondria, where the immobilization effect is considerably reduced near the polar region of the lipids and almost the same near the center of the bilayer. These observations suggest that the transition of the depleted promitochondria may be partly related to polar protein-lipid interactions.

4.5 Direct Evidence of Heterogeneous Fluidity in the Membrane

These studies show direct evidence that the mitochondria

are heterogeneous, containing domains of rigid and fluid lipids. Such convincing evidence arises from the behaviour of isolated OS-ATPase compared to its behaviour in the native membrane. Mitochondrial membranes are intrinsically more fluid with a phase transition below $0^{o}C$, whilst OS-ATPase enzyme is probably in an environment of a rigid domain of the membrane, with a phase transition well above $0^{o}C$ (10 to $15^{o}C$). The discrepancy between membrane phase transition and membrane bound activity, really reflects the presence of a particular microenvironment, whose characteristics are determined by the surrounding lipids and consequently the type of lipid-protein interaction. The fluidity within the membrane appears to be a basic property of the entire membrane, expressed in different ways in different regions for structural and functional reasons. The heterogeneous distribution of the fluidity within the mitochondrial membranes is of importance in relation to biogenesis of these membrane systems reflecting an asynchronous assembly of the different components of the membrane.

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