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Comparison of Membrane Organization in Mitochondria from Yeast and Rat Liver by Nuclear Magnetic Resonance Spectroscopy

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Summary. Proton magnetic resonance (PMR) and carbon-13 magnetic resonance (CMR) spectra of intact, unsonicated yeast and rat liver motochondria show differences which may be correlated with the composition of the membranes. High resolution PMR and CMR signals in intact yeast mitochondria have been assigned to regions of fluid lipid-lipid interaction on the basis of spectra of extracted lipid and protein, and the temperature dependence of NMR signals from the intact membrane. PMR spectra suggest that about 20% of total yeast phospholipid is in regions where both intramolecular fatty acid chain mobility and lateral diffusion of entire phospholipid molecules are possible. No such regions appear to exist in rat liver mitochondria. For both yeast and rat liver mitochondria, comparison of PMR and CMR spectra suggests that about 50% of phospholipid appears to be in regions where intramolecular fatty acid chain motion is considerable, but lateral diffusion is restricted. The remaining phospholipid appears to have little inter- or intramolecular mobility. Since NMR observation of lipid extracts from membranes indicates that phospholipid-sterol interactions do not account for the spectra of intact mitochondria, these effects are interpreted in terms of extensive lipid-protein interactions.

Current models of the structure of biological membranes differ, for the most part, in terms of the general organization and inter-relationship of lipid and protein components of the membrane (for review, *see* MacLennan, 1970). The extent to which lipids interact with other lipids, or with protein, is of considerable importance to an understanding of membrane function. One approach to this question has been to demonstrate the extent to which temperature-induced phase transitions in a membrane can be influenced by the lipid composition of the membrane (Chapman & Leslie, 1971), and in turn how the characteristics of reactions catalyzed by enzymes which are part of these membranes may be correlated with

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these alterations in lipid and thus with phase properties of the membrane (Haslam, Proudlock & Linnane, 1971). This evidence is very suggestive of lipid-protein interactions. On the other hand, there are important characteristics of fluidity which are explicable in terms of extensive domains of lipid-lipid interaction in biological membranes (Singer & Nicolson, 1972). Studies with model systems, involving phospholipid bilayers and similar structures, point to mechanisms which may regulate local changes in membrane fluidity; sterols may be important in this respect (Chapman & Leslie, 1971).

Detailed information concerning structural interactions in biological membranes has so far been difficult to obtain, primarily because the membrane often is not preserved in a native state during experimental analysis. Nuclear magnetic resonance techniques are particularly advantageous for native membrane studies because it is possible to observe a wide range of interactions while retaining the membrane system in an unmodified state. In this communication we report the results of a comparative study of mitochondria from yeast and rat liver using PMR [1] and CMR spectroscopy. The spectra obtained have been correlated with composition differences in these membranes to obtain information on the relative importance of lipid-lipid and lipid-protein interactions in the organization of the intact membranes.

Materials and Methods

Yeast Cultures

A wild-type, diploid strain of *Saccharomyces cerevisiae* was used in the present study. Cells were grown at 29 °C on a semi-synthetic medium with 2% (w/v) glucose as carbon source (Gordon & Stewart, 1971). Cells were collected after 18–20 hr of growth by centrifugation at 5,000 × *g* for 3 min, washed once with water and then with 0.1 M potassium phosphate buffer (pH 6.0).

Preparation of Mitochondria

Packed yeast cells were resuspended in an equal volume of 0.1 M potassium phosphate buffer (pH 6.0). The cell suspension (25 ml) was mixed with 25 ml of 0.5 mm glass beads and shaken at high speed for 30 sec in a Braun MSK homogenizer, using carbon dioxide as coolant to maintain the temperature near 0 °C. The cell homogenate was decanted from the beads and the beads were washed twice with 25 ml of buffer. The washings were added to the cell homogenate. The total homogenate was then centrifuged twice at 1,000 × *g* for

1 *Abbreviations used are:* PMR, proton magnetic resonance; CMR, carbon-13 magnetic resonance; TMS, tetramethylsilane; TFA, trifluoroacetic acid.

5 min to remove residual beads, unbroken cells and cell debris. Mitochondria were sedimented by centrifuging the cell-free homogenate at $20,000 \times g$ for 15 min. The pellet was washed twice with phosphate buffer, freeze-dried and stored at 0 °C. When required for NMR spectroscopic analysis, the mitochondria were resuspended at 60–70 mg protein/ml in D_2O. Samples of the mitochondrial suspension were taken for fatty acid, phospholipid, sterol and protein analyses.

In some cases, mitochondria were prepared as above except that the cells were broken in 0.2 M KCl solution adjusted to pH 6.0. The mitochondria were washed twice ($25,000 \times g$, 15 min) with 0.2 M KCl in D_2O at a pH meter reading of 6.0 and left overnight at 0 °C suspended in KCl/D_2O. After two more washes in KCl/D_2O the mitochondria were resuspended in KCl/D_2O solution (35–40 mg protein/ml) for NMR spectroscopy.

Rat liver mitochondria were prepared essentially as reported by Hogeboom (1955). The mitochondria were suspended in 0.2 M KCl/D_2O at a pH meter reading of 7.5, sedimented by centrifugation at $8,500 \times g$ and resuspended in KCl/D_2O. This washing procedure was repeated three times, and the mitochondria were finally resuspended at approximately 90 mg protein/ml for analysis by NMR spectroscopy.

NMR Spectroscopy

PMR spectra were obtained with a 100 MHz JEOL MH-100 spectrometer on line with a PDP 8/S computer to permit signal averaging when necessary. A capillary of tetramethylsilane (TMS) in carbon tetrachloride was used as an external reference unless otherwise noted. Proton decoupled CMR spectra were obtained on a Bruker HX-90 instrument with Fourier transform capability. External benzene was used as a reference and spectra converted to a TMS scale by use of the factor +128.7 ppm (Stothers, 1972). The position of all peaks is reported relative to TMS. Unless otherwise stated all spectra were recorded at 30 °C.

Lipid Extraction

Mitochondria which had been stored at −16 °C no longer than three days were used for phospholipid extraction and analysis. The extraction procedure combined features of the methods described by Letters (1968) and by Getz, Jakovcic, Heywood, Frank and Rabinowitz (1970). Mitochondrial pellets were thawed and an equal volume of water was added, followed by four volumes of absolute ethanol. The mixture was heated at 80 °C for 15 min, cooled, then shaken vigorously at room temperature overnight. The tubes were centrifuged for 5 min at $2,000 \times g$, and the supernatant decanted and held at 0 °C. Four volumes of chloroform/methanol (2:1, v/v) were added to the pellets and the tubes were shaken for 2 hr at room temperature. After brief centrifugation, the supernatants were decanted and added to the ethanol extract. The sediment was extracted once more with chloroform/methanol and the extracts pooled. Chloroform/methanol/conc. HCl (124:61:1, v/v/v) was used to extract the pellets once more by shaking overnight. After centrifugation the supernatant was neutralized with NaOH before being added to the earlier pooled supernatants.

Residual pellets were frozen for later analysis of the protein. The lipid extract was evaporated to dryness under vacuum at 40 °C and taken up in about 10 ml of chloroform/methanol (2:1, v/v). This was washed with one-fifth of its volume of 0.6% NaCl, centrifuged to break the emulsion and the upper aqueous layer removed. The interface was then washed with about 1 ml of upper-phase solvent according to Folch, Lees and Sloane Stanley (1957). Approximately 0.5 ml of methanol was added to resolve the system into a single phase.

This final extract was made up to a known volume with chloroform and samples were taken for quantitative analysis of ergosterol and phospholipid.

Dispersion of Lipid by Sonication

The total lipid extracted from approximately 100 mg of mitochondrial protein was evaporated to dryness under vacuum at 40° C and the residue was extracted three times with approximately 2 ml of chloroform. These extracts were pooled and the chloroform was removed at 40 °C under a stream of nitrogen. One ml of either D_2O (PMR studies) or H_2O (CMR studies) was then added. Nitrogen was used to flush out oxygen and the vials were shaken for 5 min at room temperature in a mechanical shaker. The resultant suspension was dispersed using a Branson S-75 sonicator (setting 3) for 10 min at 30 °C, under a stream of nitrogen. Samples of the sonicate were analyzed for phospholipid and sterol, and showed essentially the same ratios of lipids and sterols as in the initial extracts.

Quantitative Analyses

Analysis of fatty acids and sterol was by the methods described previously (Gordon & Stewart, 1971), except that the sterol fractions from the mitochondria were not subjected to a TLC purification step. Sterol was also estimated in some cases by the method of Searcy, Bergquist and Jung (1960). Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951), with bovine serum albumin as standard. Phospholipid was estimated by digesting samples in perchloric acid-sulfuric acid and assaying for phosphate (Ames & Dubin, 1960). Phosphatidyl choline was used as standard. Individual phospholipid species were separated and assayed as described by Getz *et al.* (1970) and Austin, Brown and Stewart (1975).

Results

NMR Spectra Obtained From Yeast and Rat Liver Mitochondria

In the PMR spectra in Fig. 1, unsonicated yeast mitochondria show a well-resolved, high intensity spectrum whereas the spectrum of rat liver mitochondria is of low intensity, very broad and poorly resolved (the peaks at 3.8–4.1 ppm are probably due to contaminating sugar residues). In the CMR spectrum, resolution is considerably improved for the rat liver mitochondria, but remains inferior to the CMR spectrum of the yeast mitochondria.

When compared with NMR studies of other natural membranes by both PMR and CMR (Chapman, Kamat, de Gier & Pankett, 1968; Jenkinson, Kamat & Chapman, 1969; Davis & Inesi, 1971; Robinson, Birdsall, Lee & Metcalfe, 1972, Sheetz & Chan, 1972*a*; Keough, Oldfield, Chapman & Beynon, 1973) the resolution of the yeast mitochondrial spectrum is remarkably good and in fact is comparable to that of model phospholipid bilayer systems. The spectra obtained from yeast mitochondria pre-

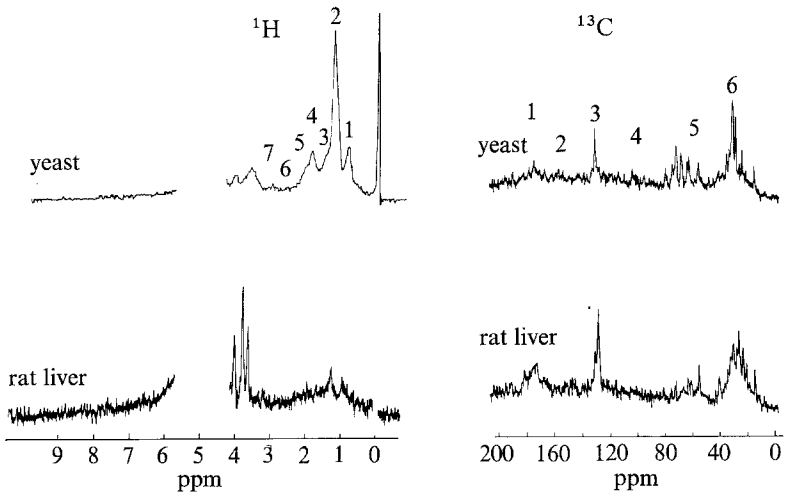


Fig. 1. PMR and CMR spectra of intact, unsonicated yeast and rat liver mitochondria. PMR spectra at 100 MHz in D_2O (the large residual HDO peak at 4–6 ppm has been omitted). CMR spectra in H_2O at 22.63 MHz with proton decoupling. Assignments for PMR spectra are given in Fig. 3 and for CMR spectra in Fig. 2.

sumably originate from components of the inner membrane since under the conditions of cell disruption used, the outer membrane becomes fragmented and is stripped from the organelle. This is evident from electron-microscopic examination of the mitochondrial preparation. It is unlikely that soluble proteins of the mitochondrial matrix would generate spectra with signal magnitudes of the type seen with these preparations. In the case of rat liver mitochondria, the preparation method used is such that outer membranes would remain intact and attached; it would be possible to distinguish signals generated by the two membrane systems of this organelle only by separating and examining them separately.

The broadening of the rat liver PMR spectrum precludes useful comparison with the yeast spectrum. The high field region (0-45 ppm) of the CMR spectrum of yeast mitochondria shows a few well-resolved resonances of large amplitude, whereas the equivalent region of the rat liver CMR spectrum appears much broader and may show a greater number of peaks. The region from 50–80 ppm shows several resonances in both the rat liver and yeast spectra, but there are differences in chemical shift. Both spectra exhibit a sharp peak at 131.5 ppm, while the rat liver spectrum shows a more intense peak at 129 ppm. The carbonyl peak (165–185 ppm) is considerably enhanced in the rat liver spectrum relative to the yeast spectrum. As will be shown below, these differences may

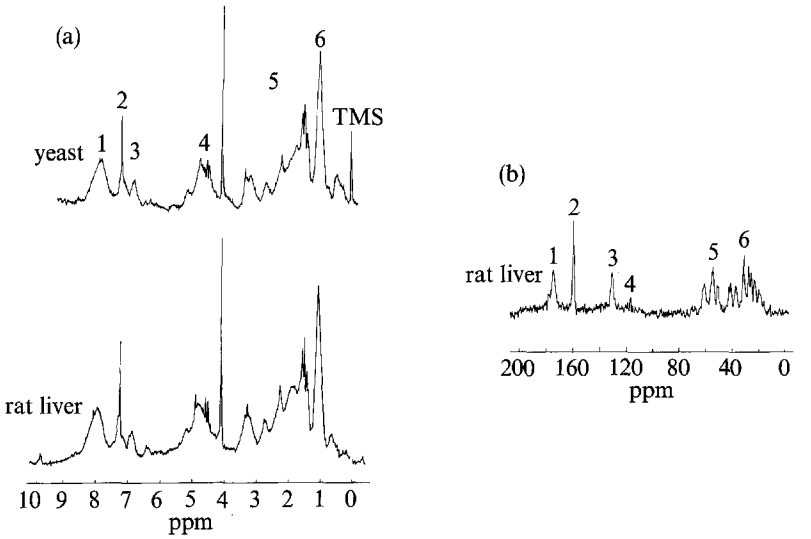


Fig. 2. (*a*) 100 MHz PMR spectra of yeast and rat liver mitochondrial protein in TFA. The sharp peaks at 4.0 and 7.3 ppm are due to extraction solvents. Assignments are: (1) amide; (2) aromatic; (3) guanidino, histidine N-H; (4) α-protons; (5) methine, methylene, methyl of Ala, Thr; (6) methyl. (*b*) 22.63 MHz CMR spectrum of rat liver mitochondria protein in 1 M HCl/6 M guanidinium chloride. Assignments are: (1) carbonyl; (2) guanidinium chloride; (3) Phe C-4, 5, 6; Tyr C-4; His C-2; Trp C-9; (4) His C-4, Tyr C-5; (5) α-C region; (6) others. Based on Stothers (1972); Cristl & Roberts (1972); Bradbury and Norton (1973)

be correlated with compositional and structural differences in the two types of mitochondria.

PMR and CMR Spectra of Extracted Protein and Lipids

The PMR spectra of the residual proteins after extraction of lipids from the two types of motochondria are shown in Fig. 2. The general similarity of these spectra indicates that if organizational differences are disregarded, the contribution of protein to the spectra of intact mitochondria should be similar for both membranes.

The rat liver mitochondrial protein dissolved only slowly in 1.0 HCl/6.0 M guanidinium chloride and even after stirring for one week remained viscous, thus causing broadening of the CMR spectrum. Based on the similarities of amino acid composition and PMR spectra, the yeast proteins should show a similar spectrum. The use of HCl/guanidinium chloride as solvent should denature the protein and remove any chemical shift

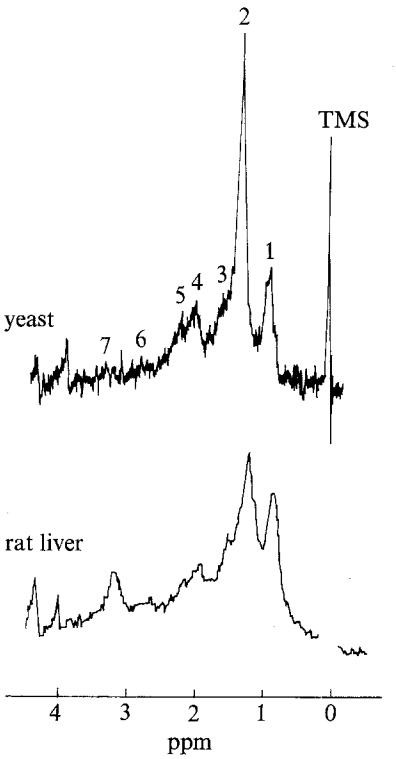


Fig. 3. 100 MHz PMR spectra of total lipid extracts from yeast and rat liver mitochondria, after sonication in D_2O. Resonance positions shown are for egg lecithin from Finer, Flook and Hauser (1972): (1) terminal CH_3; (2) $(CH_2)_n$; (3) CH_2-C-COO; (4) CH_2-C=C; (5) CH_2-COO; (6) C=C-CH_2-C=C; (7) $N(CH_3)_3$. No resonances were observable at low field

nonequivalence resulting from protein folding, which in any case should be small (Allerhand, Childers & Oldfield, 1973). Slight solvent-induced shifts relative to the intact rat liver mitochondria spectrum in D_2O may be introduced, and the relative intensities of the various peaks may differ from the folded protein intensities.

The total lipid extracts, containing phospholipid and sterol in ratios similar to those found in intact mitochondria, were sonicated to form dispersions of phospholipid bilayer vesicles (Hauser & Irons, 1972). The PMR spectra of the lipid preparations from yeast and rat liver mitochondria are similar (Fig. 3), except for a peak at 3.2 ppm (probably due to choline methyl protons) which is present only in the rat liver spectrum; the spectrum of the rat liver preparation is also broadened. This broadness may be due to decreased levels of unsaturated fatty acids in rat liver mitochondria relative to yeast mitochondria, and the replacement of cho-

Table 1. Phospholipid, fatty acid and sterol content of mitochondria from yeast and rat liver mitochondria

Mitochondria Type	Phospholipid					Sterol[a]
	Total (μg/mg protein)	PC	PE	PS+PI	Other	(μg/mg protein)
			(% by weight)			
Yeast	272	38	47	13	2	32
Rat[c]	128	41	35	8	16	6

Mitochondria Type	Fatty acids[b]			
	Total (μg/mg protein)	Short chain saturated	Long chain saturated	Long chain unsaturated
		(% by weight)		
Yeast	250	7	27	66
Rat[c]	110	<1	51	49

[a] Yeast: ergosterol; rat liver: cholesterol
[b] Short chain saturated: Yeast – 10:0, 12:0, 14:0; Rat Liver – 14:0.
Long chain saturated: Yeast – 16:0, 18:0; Rat Liver – 16:0, 18:0.
Long chain unsaturated: Yeast – 16:1, 18:1; Rat Liver – 16:1, 18:1, 18:2, 20:3, 20:4, 22:6.
[c] Values calculated from data of Colbeau, Nachbaur and Vignais (1971).

lesterol by ergosterol in yeast membranes (Table 1). The CMR spectra of the total lipid extracts from the two membrane types (Fig. 4) show similar but not identical spectra. Differences in the intensities of the various peaks are particularly noticeable and reflect the different fatty acids present. Thus, the increased proportion of fatty acids with multiple double bonds in rat liver mitochondria relative to yeast mitochondria is indicated by the large increase in the peak at the same resonance position as carbon 11 of linoleic acid (Fig. 4). This same difference is probably responsible for the slightly altered chemical shifts of the ethylenic carbons in the two membrane types. Neither membrane type shows significant peaks due to glycerol, carbonyl, or choline carbons, suggesting that the mobility of these residues has been decreased considerably. Previous studies (Metcalfe, Birdsall & Lee, 1972) have shown that incorporation of phospholipid into membranes does not greatly alter observed CMR chemical shifts, and hence model compound studies have been used to make the assignments shown (Birdsall, Feeney, Lee, Levine & Metcalfe, 1972; Stoffel, Zierenberg & Tunggal, 1972).

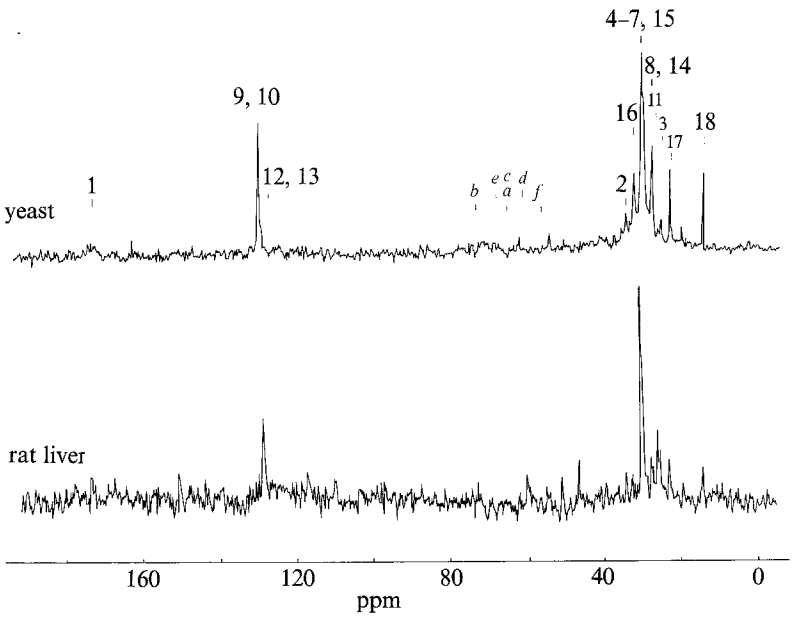
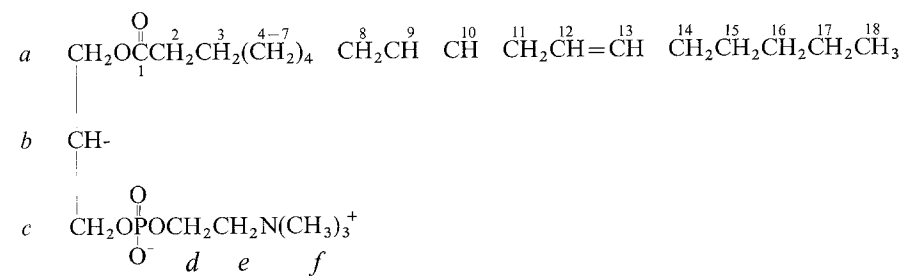


Fig. 4. 22.63 MHz CMR spectra of total lipid extracts from yeast and rat liver mitochondria, after sonication in H_2O. ^{13}C resonance positions shown for linoleic acid are from Birdsall, Feeney, Lee, Levine and Metcalfe (1972).



Protein and Lipid Contributions to PMR and CMR Spectra of Intact Mitochondria

Comparison of the spectra of the protein and lipid extracts with those of the intact mitochondria suggests that lipid is responsible for the major resonances present in the PMR and CMR spectra of the yeast mitochondrial membranes. Thus in the PMR spectra, no peaks from aromatic residues appear in the low field region. However, this is not conclusive since such aromatic peaks would be expected to be small; exchangeable amide, amino and guanidino proton resonances apparent in the spectrum in Fig. 2 of protein in TFA, would not be observable in D_2O. It is possible that protein makes some contribution to spectral intensity in the high field region, although this is obscured by the large resonances due to

lipid protons. The yeast CMR spectrum in Fig. 1 shows more clearly the predominance of lipid signals. As observed in the PMR spectrum there is very little intensity in the protein aromatic region (110–140 ppm), the major peak in this region being due to lipid ethylenic carbons. There may be some protein resonances in the α-carbon region although the major peaks are more likely due to lipid glycerol and choline peaks or possibly to sugar residues contaminating the mitochondria. In the high field region (0–45 ppm) the major peaks can be identified with lipid hydrocarbon chain resonances, with possibly some underlying broad protein resonances.

Interpretation of the CMR spectrum of rat liver mitochondria (Fig. 1) indicates that protein makes a greater contribution to the rat liver mitochondrial spectrum, and lipid considerably less, relative to the yeast mitochondrial spectrum. The intense peak at 129 ppm may contain resonances due to both lipid ethylenic carbons and protein aromatic carbons. The multiplet nature of this peak in Fig. 1 was not observed in the extracted lipid spectrum (Fig. 4), indicating that both lipid and protein contribute to the observed resonance. It is also notable that the intensity of the resonance at 129 ppm is considerably greater relative to the methylene peak at 29 ppm in the intact mitochondria (Fig. 1) than in the lipid extract (Fig. 4). This could be due to protein contributions at 129 ppm. Little signal can be observed for other protein aromatic carbons, but these also show low intensity in the extracted protein spectrum (Fig. 2). The peaks from 50–75 ppm appear to be predominantly lipid resonances with possibly some underlying protein contributions. In the upfield region there are broad peaks at 18, 36 and 40 ppm which are probably due to protein, and peaks at 14 and 34 ppm may be attributed to lipid, suggesting that both protein and lipid contribute to the observed spectrum. Keough *et al.* (1973) considered that most of the CMR signal from rat liver mitochondria is attributable to lipid.

Interaction of Protein and Lipid in Mitochondrial Membrane

The spectral differences observed for intact mitochondria from the two organisms suggest differences in composition and/or in organization of constituent material. Table 1 indicates that there are considerable differences in the amount and types of phospholipids and sterol present in yeast and rat liver mitochondria which may contribute to organizational differences within the membranes.

A wide variety of possible organizational states for lipid in membranes has been proposed (MacLennan, 1970), ranging between two extreme possibilities: (*i*) a phospholipid-sterol bilayer similar to that of model bilayer systems in which interactions are predominantly lipid-lipid; and (*ii*) a conformationally undefined state in which interactions are predominantly lipid-protein.

The observation of both PMR and CMR signals for constituents of membranes seems not to be clearly linked with sufficient local mobility of the relevant nuclei either through intramolecular conformational changes or intermolecular effects (Levine, Partington, Roberts, Birdsall, Lee & Metcalfe, 1972; Horwitz, Michaelson & Klein, 1973; Lee, Birdsall & Metcalfe, 1973). Attenuation of the resonances of lipid in rat liver mitochondria can thus be linked with a decrease in mobility of lipids and proteins constituting the membranes of this organelle. Either of the above two organizational states for lipid in membranes may restrict mobility of the lipid components. Thus, numerous studies using model bilayer systems have shown that phospholipid-sterol interactions reduce mobility and broaden NMR resonances (Darke, Finer, Flook & Phillips, 1972), and ESR studies with spin-labelled phospholipids have indicated that interaction with protein can also greatly reduce lipid hydrocarbon chain mobility (Jost, Griffith, Capaldi & Vanderkooi, 1973). The spectra described so far provide no means to differentiate between these states.

Effect of Temperature on PMR Spectra of Mitochondria

Fig. 5 shows PMR spectra as a function of temperature for intact rat liver mitochondria. The intense methylene peak at 1.3 ppm in the yeast mitochondria spectra (Fig. 1) which has been assigned to lipid, is completely absent in the rat liver mitochondria spectra at all temperatures. This is true even at temperatures far in excess of those for which phase transitions have been detected by other techniques (Raison, Lyons, Mehlhorn & Keith, 1971). Qualitative comparison of rat liver mitochondria PMR spectra with the spectra of extracted protein and lipid (Figs. 2 and 3) indicates a greater resemblance between the intact mitochondria and the extracted protein spectrum and suggests that the rat liver mitochondria PMR spectrum may be primarily due to protein. At higher temperatures the intensity of the spectrum increases, but the qualitative features remain unaltered, suggesting only greater protein contribution. This is supported by the observation that small resonances from protein aromatic

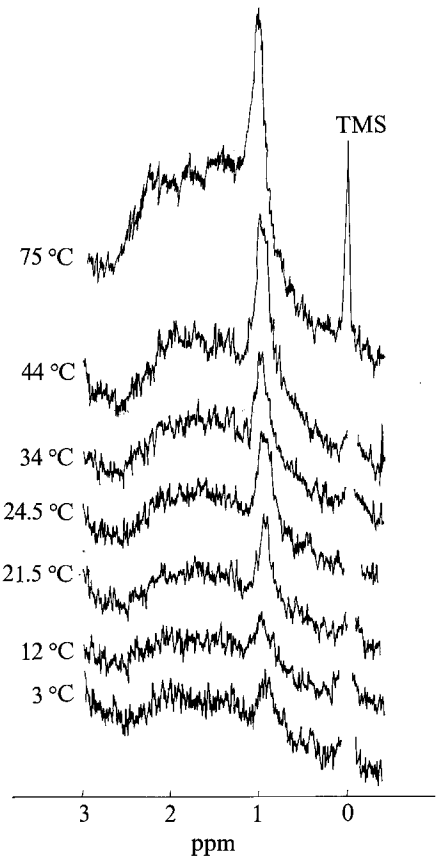


Fig. 5. 100 MHz PMR spectra of whole rat liver mitochondria in D_2O as a function of temperature

protons are apparent in the downfield region of the PMR spectra (not shown) at temperatures greater than 38 °C. It is possible that only the terminal methyl regions of the fatty acids hydrocarbon chains have sufficient mobility to give high resolution signals (Rothman & Engelman, 1972), suggesting that the prominent methyl peak in these spectra could still be due to lipid. A plot of peak height versus temperature for this peak shows only a gradually increasing peak height rather than the phase transition behavior described below for yeast mitochondrial spectra. This is not conclusive evidence against this peak being due to lipid, since high sterol to phospholipid ratios may prevent phase transition for the terminal methyl region (Rothman & Engelman, 1972). Similar results have been attributed to primarily protein resonances in PMR studies of whole human erythrocytes (Sheetz & Chan, 1972*a*). The CMR results on rat liver mitochondria described earlier also support a protein contribution to PMR spectra.

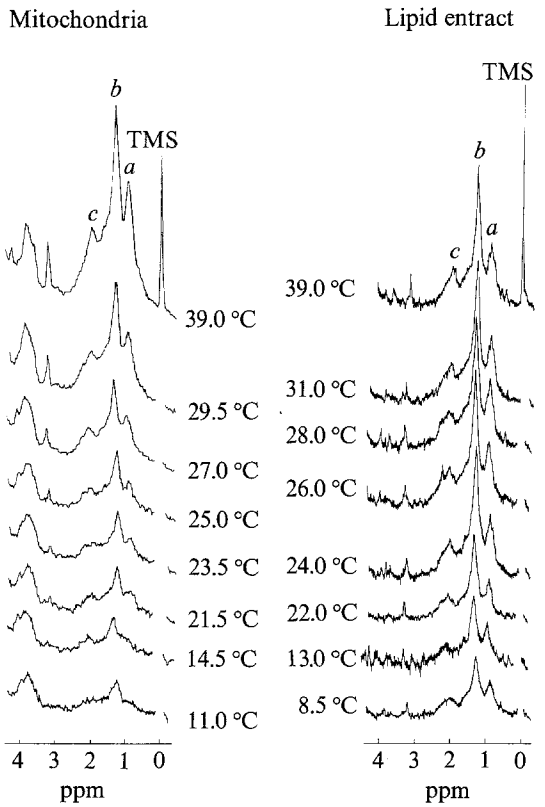


Fig. 6. 100 MHz PMR spectra of the upfield region as a function of temperature for yeast mitochondria in D_2O, and for total lipid extract from yeast mitochondria, sonicated in D_2O. *a, b, c* relate to those resonances (methyl, methylene) whose peak heights have been plotted as a function of temperature in Figs. 7 and 8

Fig. 6 shows PMR spectra as a function of temperature for intact yeast mitochondria, and for total lipid extract after sonication in D_2O. The two sets of spectra show similar temperature dependencies with broad spectra of small amplitude at low temperatures, and high intensity, well-defined spectra at higher temperatures. This is clearly shown in Figs. 7 and 8 where the heights of certain peaks are plotted as a function of temperature. In the case of the extracted lipids (Fig. 7), there is a gradual increase in peak heights between 9 and 22 °C, a rapid increase between 22 and 24 °C, then largely constant peak heights up to 40 °C. The enhancement of signal between 22 and 24 °C is characteristic of the phase transitions seen with phospholipid bilayer model systems. The behavior of intact yeast mitochondria is similar, though not identical (Fig. 8). Again there is a gradual increase in peak heights at lower temperatures, but the rapid increase in peak height now occurs over a higher and broader temperature

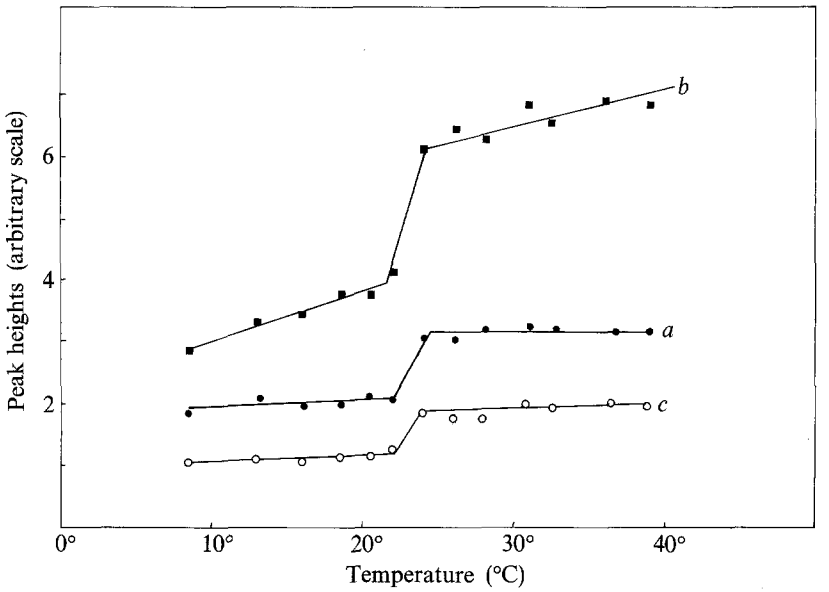


Fig. 7. PMR peak heights versus temperature for yeast mitochondria total lipid extracts. Peak assignments (*a*, *b*, *c*) from Fig. 6

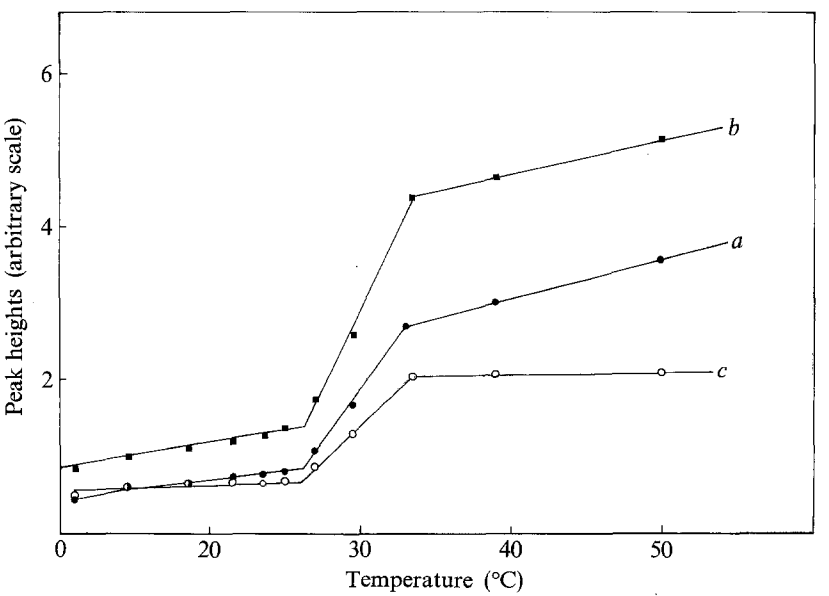


Fig. 8. PMR peak heights versus temperature for intact yeast mitochondria. Peak assignments (*a*, *b*, *c*) from Fig. 6

range, that is between 26 and 34 °C. Further small increases in peak height are observed at higher temperatures. The similarities of the intact mitochondria and the lipid dispersions, both in terms of the general appearance of the spectra and of the temperature dependence, support the view that lipid components, probably ordered in bilayers, account for most of the signal seen in yeast mitochondria spectra at 30 °C.

Discussion

The results presented above strongly suggest that the PMR spectrum of yeast mitochondria originates from a lipid bilayer structure within this membrane. Although there is now evidence that the small radius of curvature of sonicated liposomes may lead to properties and PMR spectra which are not found in lipid bilayers with less curvature (Scheetz & Chan, 1972*b*; Gent & Prestegard, 1974), in the present instance the PMR spectra of intact yeast mitochondria and of sonicated whole lipid extracts are very similar. Furthermore, a typical bilayer phase transition is observed for the yeast mitochondria as a function of temperature.

Possible explanations of the lack of signals consistent with lipid bilayer regions in the rat liver mitochondria PMR spectra include the absence of lipid bilayer regions, or decreased mobility or altered packing of the hydrocarbon chains in such regions. There is some evidence to support either contention. For example, recent studies have shown that protein is capable of interacting with lipid so as to greatly reduce mobility of the lipid. Thus, cytochrome oxidase complexed in vesicular bilayer structures with spin-labelled phospholipid immobilized a portion of the phospholipid amounting to 0.2 mg phospholipid/mg protein (Jost *et al.*, 1973). Phospholipid bilayer behavior was observed only when phospholipid/protein ratios were higher than this value. This was interpreted as being due to the decrease in mobility of lipid as a result of interaction with adjacent protein, and formation of fluid lipid bilayer regions at greater distance from the protein when sufficient excess phospholipid was present. Whether the phospholipid interacting with protein is also a bilayer is unknown, although the authors calculated that this ratio was approximately that necessary to form a bilayer of one phospholipid molecule thickness around the protein. On the other hand, there is some evidence suggesting that proteins may induce completely different phospholipid conformations (Hauser, Finer & Chapman, 1970).

If the above ratio of interacted phospholipid to protein is applied to the present results (Table 1), rat liver mitochondria do not possess sufficient lipid to show bilayer behavior, whereas yeast mitochondria do. This is also consistent with the observation based on the relative intensities of the PMR spectra of intact and TFA-dispersed yeast mitochondria, that only about 20% of the yeast lipids contribute to the high resolution PMR spectrum. This suggests that the bulk of the lipids in both yeast and rat liver mitochondria are in a state of decreased mobility as a consequence of interaction with protein, but that the yeast mitochondria possess a sufficient excess of lipid to also have limited regions of lipid bilayer which are not interacting with protein.

An alternative explanation is that the bulk of the lipids in these membranes have decreased mobility as a consequence of lipid-lipid interactions. Model system studies have shown that the type and ratio of phospholipids and sterol incorporated in a bilayer can have significant effects on the properties of the bilayer. In particular, it has been shown that increased ratios of sterol to phospholipid inhibit mobility (Darke *et al.*, 1972), that ergosterol is less effective than cholesterol in condensing phospholipid bilayers (Butler, Smith & Schneider, 1970; Demel Bruckdorfer & van Deenen, 1972; Ghosh & Tinoco, 1972; Hsia, Long, Hruska & Gesser, 1972), and that increased proportions of unsaturated fatty acids increase bilayer fluidity (Chapman & Leslie, 1971). For the last two reasons, lipid bilayers in yeast mitochondria would be expected to be more fluid and hence exhibit a more resolved NMR spectrum than similar regions in rat liver mitochondria. These effects have been demonstrated to a certain extent by PMR spectra of extracted lipids (Fig. 3).

The increased concentration of sterol in yeast mitochondria relative to rat liver mitochondria might be expected to increase broadening of the PMR spectrum. For model systems, approximately 1 : 1 ratios of sterol to phospholipid have been used to effect extensive broadening of PMR spectra (Darke *et al.*, 1972). The overall ratios observed for yeast and rat liver mitochondria are far less than this, but if sterol is not uniformly distributed, bilayer regions with high sterol/phospholipid ratios may still exist. This implies that the mobility of the bulk of the lipids must be decreased by other mechanisms, presumably by interaction with protein.

Lee *et al.* (1973) have recently shown that intermolecular effects are important in relaxation of phospholipid fatty acid chain protons. Such intermolecular interactions may be influenced by the packing of the hydrocarbon chains and/or lateral diffusion of the phospholipid molecules. Thus, it has been shown that the PMR signal of single bilayer vesicles

is dependent on the size (radius of curvature) of such vesicles (Scheetz & Chan, 1972*b*; Gent & Prestegard, 1974). If it is proposed that PMR signals are generated only by regions where the bilayer has high curvature, then about 20% of yeast mitochondrial lipids and virtually none of the rat liver mitochondrial lipids appear to be in such regions.

Alternatively, the differences in PMR signals from the two membrane types may be due to differences in lateral diffusion (Lee *et al.*, 1973). Such diffusion has been shown by ESR experiments for spin-labelled lecithin in lecithin multilayers (Devaux & McConnell, 1972), for spin labelled androstane in a phosphatidylcholine monolayer (Trauble & Sackman, 1972), and for spin labelled lecithin fused with sarcoplasmic reticulum membrane (Scandella, Devaux & McConnell, 1972). The lower limits on diffusion constants, calculated on the basis of PMR linewidths for native membranes which give high resolution signals, offer some support to the hypothesis that lateral diffusion dominates PMR relaxation of the phospholipid fatty acid chains (Lee *et al.*, 1973). If so, this implies that only the yeast mitochondria have a significant proportion of lipid in regions where such lateral diffusion is possible.

Because it has been shown that the relaxation of the ^{13}C nuclei of phospholipid fatty acid chains is dominated by directly bonded protons (Levine, Birdsall, Lee & Metcalfe, 1972), ^{13}C relaxation will depend on intramolecular motion of the hydrocarbon chains rather than on intermolecular effects. The proportion of lipids observable in CMR spectra will therefore reflect the proportion of lipid regions where the fatty acid chains have considerable intramolecular motion. For both yeast and rat liver mitochondria, such regions appear to be extensive (50–70% of the maximum ^{13}C intensity is observed). This indicates that regions must exist where the intramolecular motion is appreciable, but low intermolecular motion, or packing, broadens the PMR resonances. The present data does not allow differentiation between these mechanisms, but consideration of the relative compositions of the two membrane types, as discussed above, suggests that the ratio of lipid to protein in these membranes may be the determining factor in the difference in the PMR spectra between the two membrane types.

In conclusion, the remarkably good resolution of both PMR and CMR signals from the fluid regions of yeast mitochondria should make this system useful for investigating molecular motion of the hydrocarbon chains and possible interdependent protein-lipid conformational changes. A further advantage of yeast mitochondria is the possibility of manipulating membrane content either through use of mutant strains, or by varying

culture conditions. The latter approach has been used in the further investigation of the PMR spectra of yeast mitochondria (Austin *et al.*, 1975).

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