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Proton Magnetic Resonance Spectroscopy of Promitochondrial Membranes from Yeast Grown Under Different Regimes of Lipid Supplementation

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Summary. Promitochondrial membranes, prepared from *Saccharomyces cerevisiae* grown anaerobically under different conditions of lipid supplementation, have been examined by PMR spectroscopy. Promitochondria from cells cultured anaerobically in media containing both unsaturated fatty acid and sterol supplements, or containing unsaturated fatty acid alone, yield high resolution spectra similar to those which are characteristic of aerobic mitochondria. By contrast, promitochondrial membranes from cells grown only with sterol supplementation in order to deplete unsaturated fatty acid and total phospholipid content of the organelles, yielded PMR spectra which were very substantially broadened. These spectra are similar to those obtained with rat liver mitochondria.

PMR spectra of promitochondria from each cell type dispersed in trifluoroacetic acid, or of extracted lipids or residual proteins similarly dispersed, were different only in detail. It appears, therefore, that in the native state membranes of unsaturated fatty acid-depleted promitochondria are structurally different from promitochondria of the other two cell types. The difference may be a consequence of altered lipid-to-protein ratios, and thus of changes in the extent of lipid domain formation in the membranes of these organelles.

A variety of models have been proposed to describe the nature of the interaction of protein and lipids in biological membranes (Singer & Nicholson, 1972; Bretscher, 1973). Certain predictions of these models have been tested in simple systems containing a small number of components. Nuclear magnetic resonance (NMR) spectroscopy has been used with considerable effectiveness in some of these studies, providing information on the nature and degree of interaction between particular types of chemical groups within the synthetic membrane matrix, and at the membrane-solvent boundary. The more recent application of NMR spectroscopy to natural membranes has begun to yield valuable information on the more complex interactions occurring within these biological structures (Chapman, 1973). However, in order to test adequately predictions

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derived from simple model systems, it would be useful to have the means to alter the relative proportions of components of the native membrane *in situ,* preferably without resorting to extractive or substitution techniques that may perturb the normal interaction of the components.

In facultatively anaerobic yeasts, such as *Saccharomyces cerevisiae,* the relative amounts of sterol, phospholipid and protein in cell membranes can be manipulated by using appropriate regimes of growth of wild-type or auxotrophic strains (Jollow, Kellerman & Linnane, 1968; Keith, Resnick & Haley, 1969; Gordon & Stewart, 1971). Under anaerobic conditions of growth these yeasts do not synthesize sterol or unsaturated fatty acid. For growth to occur, the medium must be supplemented with these components, and these are incorporated into the membranes of the growing cells. The lipid composition of cells and promitochondria then reflects the nature of the growth supplements used (Bloomfield & Bloch, 1960; Proudlock, Wheeldon, Jollow & Linnane, 1968; Gordon & Stewart, 1971). Within certain limits, variation of the degree of supplementation, as well as the type of lipid provided, results in a corresponding change in the chemical composition of the membranes. Membranes from *Saccharomyces cerevisiae* may thus provide a useful system in which to examine specific intermolecular interactions involving fatty acids, sterol and proteins.

In the preceding paper (Brown, Bradbury, Austin & Stewart, 1975), we reported that mitochondria from aerobically grown yeast yield high resolution nuclear magnetic resonance spectra. The results indicate that the NMR spectra obtained reflect the mobility of a certain fraction of the lipid components of membranes.

This communication describes an examination, using proton magnetic resonance (PMR) spectroscopy, of promitochondrial membranes with altered proportions of phospholipid, sterol and protein from cultures of *S. cerevisiae* grown anaerobically under different regimes of lipid supplementation.

Materials and Methods

Growth Conditions and Preparation of Promitochondria

The strain of *Saccharomyces cerevisiae* used, and the general growth conditions have been described previously (Gordon & Stewart, 1971; Brown *et al.,* 1975). Where indicated, the growth medium was supplemented with ergosterol or cholesterol (30 mg/liter) and/or unsaturated fatty acid in the form of Tween 80 (polyoxyethylene sorbitan monoleate, 5 ml/liter); Tween 20 (polyoxyethylene sorbitan monolaurate) was substituted for Tween 80 when unsaturated fatty acid supplementation was not required. Cells grown with both Tween 80 and ergosterol added to the medium are referred to as *normal;* cells grown with Tween

20 and ergosterol are denoted *-UFA,* or unsaturated fatty acid-depleted cells; cells grown without ergosterol, but with Tween 80 in the medium, are denoted *-E,* or sterol-depleted cells. Cells were grown for 18–22 hr to optimize yield and to limit loss of viability that occurs with longer periods of growth. The cultures were inoculated with aerobic cells at an initial density of 0.03–0.04 mg dry wt/ml; cell densities at the time of harvesting were approximately 2 mg/ml for normal cells, and 1 mg/ml for *-UFA* and *-E* cells.

Cells were collected by pouring the cultures onto crushed ice. In some cases cycloheximide (10 μg/ml) was added to the medium immediately prior to harvesting, as well as to the washing solutions, to prevent aerobic adaptation by the cells. Cells were harvested by centrifugation at $5{,}000 \times g$ for 3 min, washed once with water, then with 0.1 M potassium phosphate buffer, pH 6.0.

Promitochondria were prepared from cell homogenates using the same methods described previously for preparation of mitochondria (Brown *et al.,* 1975).

Analysis of Phospholipids

Lipids were extracted from promitochondria and assayed as described previously (Brown *et al.,* 1975). Two-dimensional thin-layer chromatography was carried out as described by Getz, Jakovcic, Heywood, Frank and Rabinowitz (1970), except that the plates were not washed with diethyl ether between development in the first and second dimensions, since this step tended to remove material from the surface of the plates. Keiselgel G plates were prepared and activated by heating for 60 min at 100 °C one day before use. Phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol, and phosphatidyl ethanolamine were identified by comparison with appropriate standards (Koch-Light, U.K.). Components were identified from their staining behavior with molybdic acid spray, or with Dragendorf reagent (for phosphatidyl choline). The amount of phospholipid was estimated by scraping off spots, ashing without elution, and assaying for phosphate. Blank areas from the plates did not contain significant amounts of phosphate.

Membrane Aggregation and Solubilization

The extent of membrane aggregation was measured by centrifuging promitochondria, prepared as for NMR analysis, at $1{,}000 \times g$ for 15 min. The supernatant was then spun at $20{,}000 \times g$ for 15 min and the amount of protein in the two pellets and final supernatant estimated. Promitochondria were also centrifuged to equilibrium (30–60 min at $60{,}000 \times g$) in gradients of 30–70% (w/v) sucrose containing 10 mM Tris-Cl (pH 7.0).

PMR Spectroscopy

PMR spectra were obtained at 100 MHz and 30 °C using promitochondrial suspensions, or extracts and dispersion of these, as detailed previously (Brown *et al.,* 1975).

Results

Lipid Analysis of Promitochondria

Promitochondrial membranes prepared from cells grown under different regimes of lipid supplementation were analyzed for protein, phospholi-

Table 1. Phospholipid, fatty acid and sterol content of promitochondria from cells grown anaerobically under different conditions of lipid supplementation

Cell type	Phospholipid[a]					Ergosterol or cholesterol (μg/mg protein)
	Total (μg/ml protein)	PC	PE	PS+PI	Other	
		(% by weight)				
Normal	320	34	8	39	19	25
-E	340	48	8	30	14	12
-UFA	92	52	12	15	21	10
Normal (cholesterol)	320	–	–	–	–	51

Cell type	Fatty acids[b]			
	Total (μg/mg protein)	Short-chain saturated (% by weight)	Long-chain saturated	Long-chain unsaturated
Normal	330	3	15	82
-E	400	7	21	72
-UFA	130	50	17	33
Normal (cholesterol)	340	4	15	81

[a] PC: phosphatidyl choline; PE: phosphatidyl ethanolamine; PS: phosphatidyl serine; PI: phosphatidyl inositol.
[b] Short-chain saturated: $C_{10:0}$, $C_{12:0}$, $C_{14:0}$.
Long-chain saturated: $C_{16:0}$, $C_{18:0}$.
Long-chain unsaturated: $C_{16:1}$, $C_{18:1}$.

pid, sterol and total fatty acid. The total phospholipid content (protein basis) of the promitochondria was significantly affected only when unsaturated fatty acid was not included in the growth medium (Table 1). This particular growth regime also brought about a substantial decrease in the sterol and total fatty acid content of the membrane preparations. The proportion of the total fatty acid which was unsaturated in promitochondria from these cells was less than one-half that in normal promitochondria; this change was complemented by an increase in the proportion of short-chain saturated fatty acids. The effect of omitting ergosterol from normal growth medium was to cause an approximate halving of the sterol content of the membranes and a small increase in

the total fatty acid content; there was also a small change in the relative amount of saturated fatty acids at the expense of unsaturated acids. Gas-liquid and thin-layer chromatography (Gordon & Stewart, 1971) showed that more than 90% of the sterol in each of these membrane types is ergosterol.

The relative proportions of the major phospholipid classes are also shown in Table 1. The results for promitochondria from normal cells are comparable to those reported by Paltauf and Schatz (1969). Phosphatidyl choline is proportionally higher in the -E and -UFA types than in normal promitochondria. This was counterbalanced for the most part by a decrease in the relative amounts of phosphatidyl serine and phosphatidyl inositol.

PMR Spectra of Promitochondria

Electron-microscopic examination of promitochondria prepared after mechanical disintegration of cells, as described in this study, shows that the organelles are essentially devoid of outer membrane. Outer membrane in any case accounts for only approximately 10% of the protein of mitochondria prepared from yeast (Bandlow, 1972). The PMR spectra described below may be considered therefore to be primarily due to contributions by inner membrane and/or matrix components.

Spectra of the three types of promitochondria were obtained either immediately after preparation and washing in 0.2 M KCl in D_2O, or after lyophilizing and resuspending in D_2O, as detailed previously (Brown *et al.*, 1975). The differences in spectra were minor for the same type of promitochondria subjected to these two different preparative procedures. Since freeze-drying appeared equivalent to repeated washing in KCl-D_2O as a means for removing H_2O from the promitochondria, lyophilized preparations were, as a matter of convenience, used routinely.

Spectra obtained from normal promitochondria (Fig. 1) are similar to those described for mitochondria from aerobic cells (Brown *et al.*, 1975). Resonances in the spectrum of normal promitochondria at 0.9, 1.3 and 3.2 ppm have been assigned to protons in methyl, methylene, and choline N-methyl groups of lipids, respectively (Brown *et al.*, 1975). Less prominent resonances at 1.6 and 2.0 ppm have been assigned respectively to protons on methylene groups α to ester linkages, and to the methylene group β to unsaturated carbons in the fatty acid chains. There is no evidence of protein aromatic resonances, which would be expected in the region 6.5–8.5 ppm. The broad resonance at 3.7–4.0 ppm has been

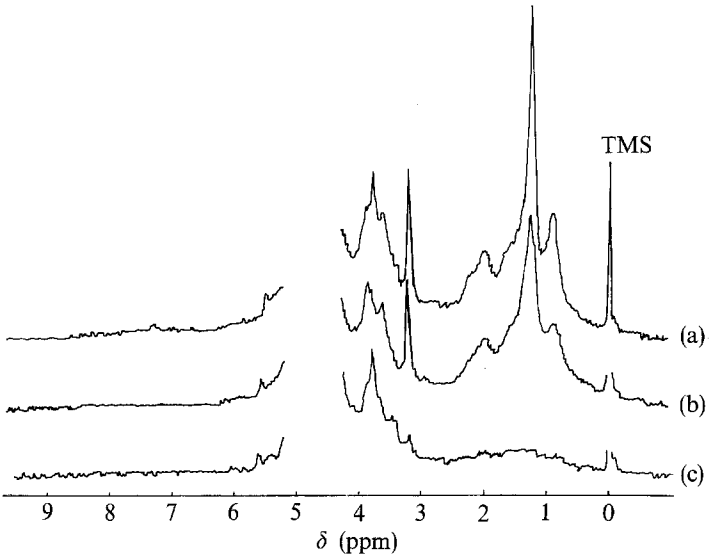

Fig. 1. PMR spectra of native promitochondria from (*a*) -E cells; (*b*) normal cells; (*c*) -UFA cells. After preparation, promitochondria were freeze-dried; for spectral determinations, the fractions were carefully redispersed in D_2O at protein concentrations of 30–40 mg/ml. Spectra were recorded at 30 °C, with external standard of tetramethylsilane. Assignments of chemical shifts are given in Brown *et al.* (1975)

tentatively assigned to protons in sugar residues (Brown *et al.*, 1975), and may be due to glycogen granules or ribosomes contaminating the promitochondria.

There are substantial quantitative differences in the high field region when the three different promitochondrial preparations are compared. The alkyl methyl and methylene protons of fatty acid chains, and choline N-methyl protons show the major intensity changes, from being very prominent in the spectra of normal and -E promitochondria, to very small or absent in -UFA spectra.

The possibility was considered that differences in spectra between the preparations examined might be due to solubilization of membrane protein, or to variation in particle size between the different suspensions. This was tested by differential centrifugation of promitochondrial suspensions and analysis of supernatant and pellet fractions for protein, and by sedimentation of the suspensions through sucrose density gradients. After dispersing the promitochondria in D_2O at concentrations similar to those used for PMR analysis (30–40 mg protein/ml) the suspensions were centrifuged at $1{,}000 \times g$ for 5 min, and the supernatant from this at $20{,}000 \times g$ for 15 min. The two sediments and final supernatant were

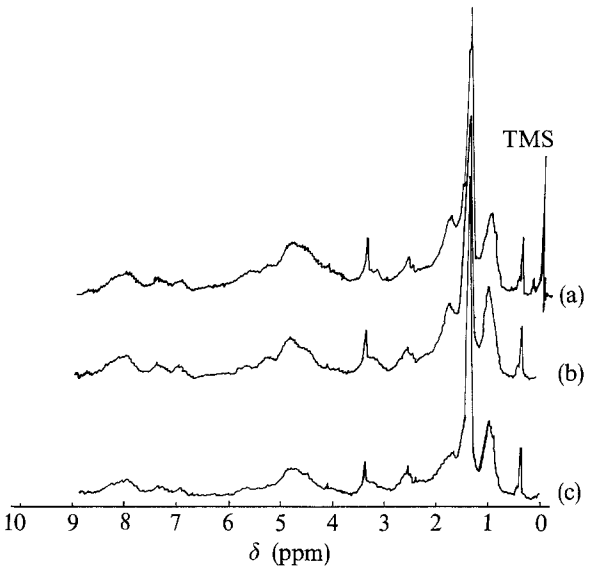

Fig. 2. PMR spectra of promitochondria dispersed in trifluoroacetic acid from (*a*) -E, (*b*) normal, (*c*) -UFA cells. Promitochondrial suspensions used to record the spectra shown in Fig. 1 were lyophilized, then dispersed in trifluoracetic acid. After 24 hr at room temperature in this solvent, spectra were re-recorded with tetramethylsilane as external standard. Peak at 0.4 ppm is due to a contaminant in the trifluoracetic acid used

assayed for protein. No significant differences between preparations were found: 75–79% of the protein sedimented at low speed (indicating considerable aggregation at the high concentrations of membrane used to generate the spectra), a further 15–20% sedimented at $20{,}000 \times g$, and 5–6% was recovered in the final supernatant. On sucrose density gradients the three preparations banded with little spreading at a density of 1.21–1.24 g/cm^3.

Spectra of Denatured Promitochondria

To test to what extent membrane organization leads to the observed differences in the spectra of the native promitochondria, the preparations used to generate the spectra shown in Fig. 1 were recovered by lyophilization and denatured by dispersing in trifluoroacetic acid. The spectra of the dispersions are shown in Fig. 2. These spectra appear to be weighted superimpositions of the denatured constituents. Both protein and lipid contribute to the spectra and peaks can be assigned to protons in each of these. Thus, in the high field region the dominant features are resonances

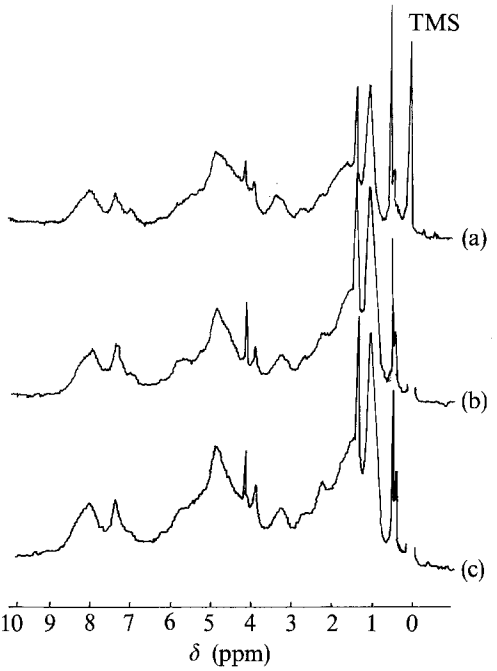

Fig. 3. Spectra of residual protein, dispersed in trifluoroacetic acid, after extraction of lipid from promitochondria prepared from (*a*) -E, (*b*) normal, (*c*) -UFA cells. Protein was dried under vacuum to remove solvent, then dispersed in trifluoroacetic acid for 24 hr at room temperature; protein concentration was 30–40 mg/ml. Peak at 0.4 ppm represents contaminant in trifluoroacetic acid

due to methyl and methylene protons; in the low field region broad resonances at approximately 4.6 ppm and from 6.8 to 8.6 ppm can be attributed to α-carbon, aromatic, amide, guanidino and amino protons of protein components of the membranes. It is notable that in contrast to the spectra of the native preparations there are no major differences in the spectra of these denatured dispersions of different promitochondrial types.

Spectra of Promitochondrial Protein

Fig. 3 shows NMR spectra in trifluoroacetic acid of the protein which remains as residue after extraction of lipid from each of the three types of promitochondria. Some phospholipid may remain in this residue as evidenced by the presence of the sharp methylene peak at 1.3 ppm. The presence of this residual lipid after exhaustive extraction indicates the possibility of very strong lipid-protein interactions in the native membrane.

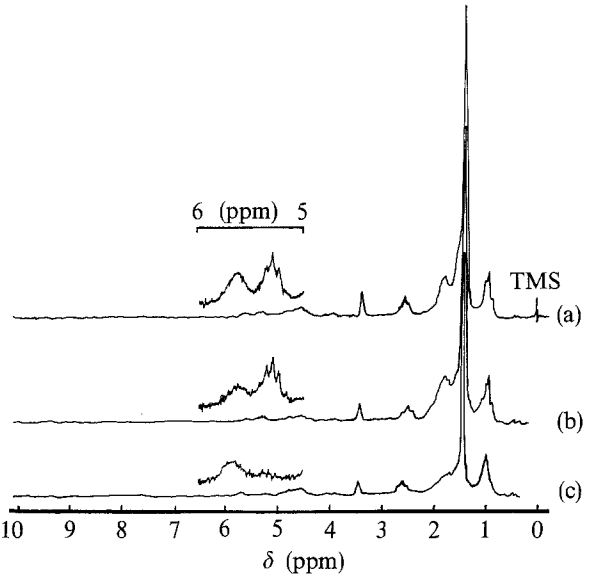

Fig. 4. Spectra of lipids extracted from promitochondria, and dispersed by sonication in D_2O. (*a*), (*b*) and (*c*) indicate lipids extracted from promitochondria prepared from -E, normal and -UFA cells, respectively. Lipid extracts were dried under vacuum, then sonicated in D_2O (Branson S-75, setting 3, 10 min, 30 °C, under nitrogen); phospholipid concentration, 20 mg/ml. Partial spectra on an expanded scate are shown for the region 5.0–6.0 ppm

Residual lipid does not appear to remain to the same extent after extraction of aerobic yeast mitochondria or rat liver mitochondria (Brown *et al.*, 1975).

The peaks at 3.9 and 4.2 ppm are probably due to sugar residues. The three protein spectra are virtually identical and similar to protein spectra from aerobic yeast mitochondria (Brown *et al.*, 1974), indicating that the overall composition of the proteins from the three promitochondrial preparations and mitochondria are similar. Amino acid analysis, and electrophoresis on polyacrylamide-SDS gels, of residual protein, also indicates that the protein constituents of the different types of promitochondria are essentially the same.

NMR Spectra of Promitochondrial Lipids

When total lipids were extracted from promitochondria, dried and sonicated in D_2O, well-defined spectra were obtained for each type of promitochondria (Fig. 4). This is in distinct contrast to the comparative spectra obtained from intact membranes, since -UFA promitochondria

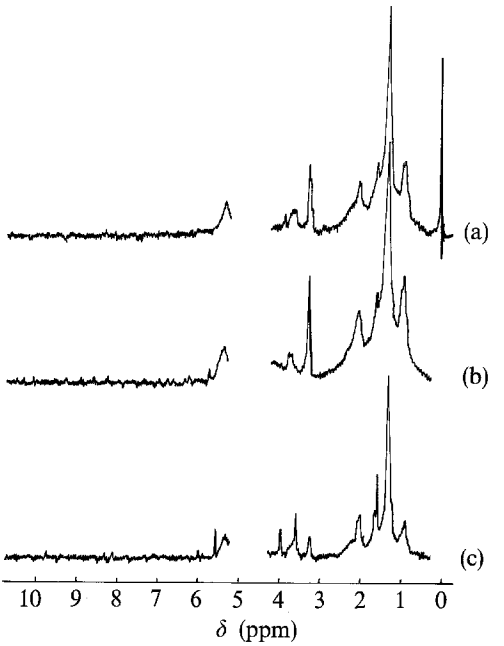

Fig. 5. Spectra of lipids extracted from promitochondria, and dispersed in trifluoroacetic acid. Lipid extracts were dried under vacuum then dispersed in trifluoroacetic acid and spectra obtained as indicated in Fig. 3. (*a*), (*b*), (*c*) indicate lipids extracted from promitochondria from -E, normal and -UFA cells, respectively

yielded practically no resonance signal. Considering the reduced proportion of unsaturated fatty acid and increased proportion of ergosterol in these promitochondria, the spectra of the lipid extracts might have been expected to be broader than for the other two membranes. In fact, lipid extracts from -UFA promitochondria yielded spectra with narrower line width for the main methylene peak. This may be due either to greater homogeneity of the fatty acids present (Table 1), or to the formation of smaller (higher curvature) vesicles in sonicates of these lipids (Sheetz & Chan, 1972; Gent & Prestegard, 1974). A small triplet at 5.3 ppm which is observable in the normal and -E preparations is practically absent from the -UFA spectrum. Since this resonance corresponds to ethylenic protons of unsaturated fatty acids its presence correlates with the amount of UFA in these membranes (Table 1). Spectra of trifluoracetic acid dispersions of lipid extracts were also obtained (Fig. 5). These spectra are also similar for the three membrane types.

The comparative behavior of these various promitochondrial preparations parallels the comparative behavior previously observed for aerobic yeast mitochondria and rat liver mitochondria (Brown *et al.*, 1975). Namely, in some cases there is no observable PMR signal from the intact membrane even though the spectra of extracted lipids or proteins give high resolution spectra.

Effects of Sterol on Promitochondrial Membrane

A major difference between mitochondria from rat liver and yeast is the substitution of ergosterol for cholesterol as the major sterol in yeast membranes. A number of studies have indicated that ergosterol and cholesterol differ in their ability to interact with phospholipid in model systems (Butler, Smith & Schneider, 1970; Demel, Bruckdorfer & van Deenen, 1972; Ghosh & Tinoco, 1972; Hsia, Long, Hruska & Gesser, 1972). In general, these studies indicate that cholesterol is more effective in condensing phospholipid bilayers and reducing their permeability. Hence, cholesterol may also be more effective in broadening PMR resonances in phospholipid bilayer systems. This was found to be the case when dispersions of egg lecithin were sonicated with cholesterol or ergosterol. In contrast to the pronounced broadening caused by cholesterol, the ergosterol had little effect on the PMR spectrum. To test whether similar differences may exist in intact promitochondrial membranes, ergosterol was replaced by cholesterol in the growth medium. Under these conditions, cholesterol becomes the predominant sterol in the membrane (Gordon & Stewart, 1971). Despite incorporation of twice as much sterol as in normal promitochondria without significant alteration of the distribution of fatty acid types (Table 1), spectra obtained from cholesterol-supplemented promitochondria were indistinguishable from the spectra of normal (ergosterol-supplemented) promitochondria shown in Fig. 1.

However, even with the doubling of sterol content in the cholesterol-supplemented promitochondria the mole ratio of sterol to phospholipid is only approximately 1:3. The other promitochondrial preparations have values of approximately 1:13 (-E), 1:6 (normal) and 1:4 (-UFA). Aerobic yeast show a molar ratio of about 1:4 and rat liver mitochondria about 1:10 (Brown *et al.*, 1975). As noted previously, approximately equimolar ratios of sterol to phospholipid have been used to effect extensive broadening of PMR spectra in model systems (Chapman & Penkett, 1966), so that the high resolution spectra observed in certain of the promitochondrial preparations may be a result of the low sterol-phospholipid ratio.

Discussion

A total of six intact membrane types of varying relative proportions and/or types of phospholipid, sterol and protein have been examined in this and the previous study (Brown *et al.*, 1975). The considerable differences in the PMR spectra obtained from these membrane preparations vary from virtually no signal for rat liver mitochondria and -UFA promitochondria, to extremely sharp resonances in -E promitochondria. These differences must be interpreted as being due to compositional and/or organizational differences in the membranes and hence consideration of the constituents of each membrane type in relationship to its PMR spectrum may be expected to give information on membrane architecture. It can be seen from the data in Table 1 of Brown *et al.* (1975) and Table 1 of this paper that there are very considerable differences in these constituents. For example, relative to the protein in the membrane, the ratio of phospholipid varies by three- to fourfold, the ratio of sterol by about eightfold, and the ratio of total fatty acids (free, triglyceride and phospholipid) by about fourfold. The changes in the relative amounts of the fatty acid classes is even greater. Thus, the ratio of saturated (short and long chain) to unsaturated fatty acids varies by up to ninefold.

Compositional differences in the lipid components of the membrane could thus be important in determining spectral differences between membranes, and these are considered in further detail below. Nevertheless, structural organization of the constituent proteins and lipids of the organelles is presumed also to be an important determinant of the nature of the PMR spectra of the preparations examined. This is most evident when the greatly enhanced spectra of membranes dispersed in trifluoracetic acid are contrasted with the spectra obtained from the corresponding suspensions of native preparations in D_2O. Significant contribution by protein protons to the spectra only become evident in preparations dispersed in trifluoroacetic acid (Brown *et al.*, 1975), and it is thus unlikely that protein-protein interactions account for differences between spectra of the native promitochondrial preparations.

Of greater interest are the interactions which lead to the substantial differences in lipid resonances in the NMR spectra of the membranes. The close similarity of the PMR spectra of the -E, normal and cholesterol-substituted normal promitochondria to that of aerobic yeast mitochondria indicates that the high resolution spectra of these promitochondrial membranes, like the spectrum of aerobic mitochondria, arise from lipid constituents (Brown *et al.*, 1975). It has been shown for all of the membrane types

examined (except cholesterol-substituted normal promitochondria, which were not tested) that the total lipid extracts exhibit high resolution NMR spectra after sonication in D_2O; this includes lipid extracted from -UFA mitochondria. While there is some question as to whether lipid vesicles with their small radius of curvature are adequate models for native membranes, where curvature may be much less pronounced (Sheetz & Chan, 1972; Gent & Prestegard, 1974), these extracts at least indicate that altered lipid-lipid interaction resulting from varied lipid content cannot explain the differences observed in native membranes. Moreover, in the membranes themselves, although there are marked differences in the lipid types between membranes, these do not appear to correlate with the observed NMR differences. Thus, for example, the ratio of sterol/unsaturated fatty acid is greater in -UFA promitochondria (broad spectrum) than in rat liver mitochondria (broad), but similar to that of cholesterol-substituted normal promitochondria (sharp). Similarly, the ratio of sterol/total fatty acids is similar in -UFA (broad) and normal promitochondria (sharp), but greater than in rat liver (broad). Thus, lipid-lipid interactions alone are insufficient to explain the differences in the PMR spectra of the native membranes.

The consistent difference which can be observed is that both membrane types which fail to show high resolution signals have much lower ratios of lipid/protein than the membranes showing high resolution signals. In this respect, it is of interest to note the experiments by Jost, Griffith, Capaldi and Vanderkooi (1973), which show that purified cytochrome oxidase is capable of complexing and decreasing the mobility of spin-labelled phospholipid in amounts up to 0.2 mg phospholipid/mg protein. Phospholipid bilayer behavior was observed only if the proportion of lipid exceeded this ratio. This was interpreted as being due to interaction of lipid in the immediate vicinity of the protein such that a substantial decrease in lipid mobility results; bilayer regions containing uninteracted lipid form only if sufficient excess phospholipid is present.

If this ratio of phospholipid to protein is applied to the present results, together with those given in Brown *et al.* (1975), rat liver mitochondria and -UFA promitochondria do not possess sufficient lipid (i.e. in excess of 0.2 mg/mg protein) to exhibit uninteracted bilayer behavior. Thus, for rat liver mitochondria and -UFA promitochondria, regions of lipid bilayer uninteracted with protein may not exist. In the other mitochondrial and promitochondrial preparations, the critical ratio of lipid to protein is exceeded and there may thus be fluid lipid regions not interacted with protein. This lipid may then have sufficient mobility to generate a high

resolution PMR spectrum. This agrees also with the observations that only about 20% of the total lipids contribute to the high resolution PMR spectra of aerobic yeast mitochondria (Brown *et al.*, 1975), and that there are essentially no differences for the various membranes in spectra of total extracted lipids sonicated in D_2O. Thus, even a relatively small decrease in the ratio of lipid to protein may result in a proportionally greater decrease in the amount of lipid in fluid lipid regions, with concomitantly large changes in the observable spectrum.

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