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CARBON-13 AND PROTON NUCLEAR MAGNETIC RESONANCE OF UNSONICATED MODEL AND MITOCHONDRIAL MEMBRANES

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Proton nuclear magnetic resonance (PMR) spectra at 270 MHz of aqueous dispersions of non-sonicated egg lecithin, dipalmitoyl lecithin, egg lecithin-cholesterol (1 : 1) and dipalmitoyl lecithin-cholesterol (1 : 1), together with PMR spectra of mitochondrial membranes and their extracted lipids, have been obtained.

Carbon-13 nuclear magnetic resonance (CMR) spectra at 25.2 MHz of egg lecithin, egg lecithin-cholesterol (1 : 1) and sphingomyelin, together with CMR spectra of chloroplast and mitochondrial membranes, and erythrocyte ghosts, have also been obtained. The results obtained using CMR appear very promising for further study of *intact* membrane structure.

It is suggested, on the basis of CMR evidence, that the *proteins* in mitochondrial membranes may be considerably less mobile than the *lipids*.

1. Introduction

Early studies of model membranes and biomembranes using proton nuclear magnetic resonance spectroscopy (PMR) were limited because of the low resolution and signal-to-noise ratios obtained with magnets of low field strength (1.4 T). The resolution obtained, particularly in the case of model systems, could be improved substantially by sonication of the specimens [1–3]. The mechanism(s) responsible for the appearance of high resolution signals in sonicated as opposed to unsonicated lipid-water dispersions is, however, still a matter of some controversy [4–7], some authors having suggested sonication causes a change in lipid packing # [4, 5, 7]. Also,

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the structural integrity of sonicated *bio*membranes may not always be unequivocal [8]. Recently these resolution difficulties have been partially overcome by the use of superconducting solenoid magnets and delayed Fourier transform NMR in PMR [9–12], and by the application of carbon-13 nuclear magnetic resonance spectroscopy (CMR), generally in pulse Fourier transform mode [13], to the study of these systems [14, 15].

In this report we present PMR spectra of some model and biological membranes obtained at a field strength of 6.7 T (270 MHz), and, for comparison, CMR spectra obtained at 2.3 T (25.2 MHz). For the reasons given, we have avoided sonic disruption in these studies. We show that excellent CMR spectra of unsonicated model and biomembranes can be obtained which are far superior in resolution to proton spectra obtained at much greater field strengths.

2. Experimental

Egg yolk lecithin (EYL) was prepared according to Singleton et al. [16]. Beef brain sphingomyelin and dipalmitoyl lecithin (DPL) were a product of Koch-Light Ltd. (Colnbrook, Bucks). Cholesterol was obtained from British Drug Houses Ltd. (Poole Dorset) and was recrystallised twice from ethanol. All lipid samples were hand dispersed with 50% by weight of water (or 99.7% D_2O) (Prochem Ltd., Croydon) except where noted. Lecithin-cholesterol samples were prepared as described previously [17].

Rat liver mitochondria were generously prepared by Dr. B.H. Robinson by a standard differential centrifugation technique [18]. They were twice subjected to osmotic shock in water and were recovered as a pellet after centrifugation at 150,000 × *g* for 1 hr. Where necessary the H_2O content was exchanged with D_2O five times, by centrifugation. Chloroplasts were prepared from lettuce using a minor modification of the method of Ridley and Leech [19], and were lysed in dilute buffer. Beef erythrocyte membranes were prepared essentially according to the method of Dodge et al. [20], using tris-HCl buffers (pH 7.6) which were 1 mM with respect to Ca^{2+} in order to minimise the loss of membrane lipid and protein components [21]. All membranes were analysed as thick suspensions obtained by high-speed centrifugation.

Continuous wave (CW) PMR spectra were obtained on the 270 MHz instrument of the Oxford Enzyme Group. Samples were observed at 18°C and the spectra were recorded over 8 kHz sweep widths, at a sweep rate of 500 Hz min^{-1}. Pulse Fourier transform CMR spectra were obtained using a JEOL PFT-100 spectrometer operating at 25.152 MHz, utilising a JEOL EC-6 spectrum computer. The pulse width was 6 μs and the computer delay-time was 100 μs. Data was collected in 4096 points and was Fourier transformed to give a 2048 point frequency domain spectrum. Proton noise decoupling was carried out over a bandwidth of 5 kHz and at a power of ca. 50 w. An external CW ^2H lock (D_2O capillary) was used for field-frequency stabilisation.

3. Results and discussion

3.1. Proton magnetic resonance

3.1.1. Model systems

Fig. 1 shows single scan PMR spectra obtained at 18°C, at a field strength of 6.7 T, using a field sweep of 8 kHz. In EYL-water (fig. 1a) the trimethylammonium group produces a sharp resonance situated on a broad envelope arising from the methylene chain signal. The terminal methyl signal is seen to be partially resolved from that of the methylene groups. The inclusion of cholesterol at a molar ratio of 1 : 1 with respect to EYL (fig. 1b) causes only slight broadening of the methylene chain signal and loss of resolution of the terminal CH_3 signal. This is presumably a consequence of the relatively small sweepwidths employed, resulting in a monitoring of predominantly those parts of the hydrocarbon chain situated farthest from the polar head-group, which are those expected to be less affected by the immobilising influence of the rigid steroid nucleus [22, 23].

With dipalmitoyl lecithin-water at 18°C, a broad featureless spectrum (fig. 1c) is obtained since the lipid is in the α-crystalline gel state. (The transition temperature of DPL is 41°C [24].) The inclusion of cholesterol in this system causes a pronounced increase in lipid mobility, with trimethylammonium and broad methylene chain signals now being apparent (fig. 1d). These results are consistent with those obtained using deuteron magnetic resonance (DMR) [17], unsonicated PMR at lower field strengths [25], and with results obtained by electron spin resonance [22, 23] and differential scanning calorimetry [26], indicating the production of a state of "intermediate fluidity" in the hydrocarbon chains. That is to say, cholesterol prevents flexing of the hydrocarbon chains in the liquid crystalline phase, though

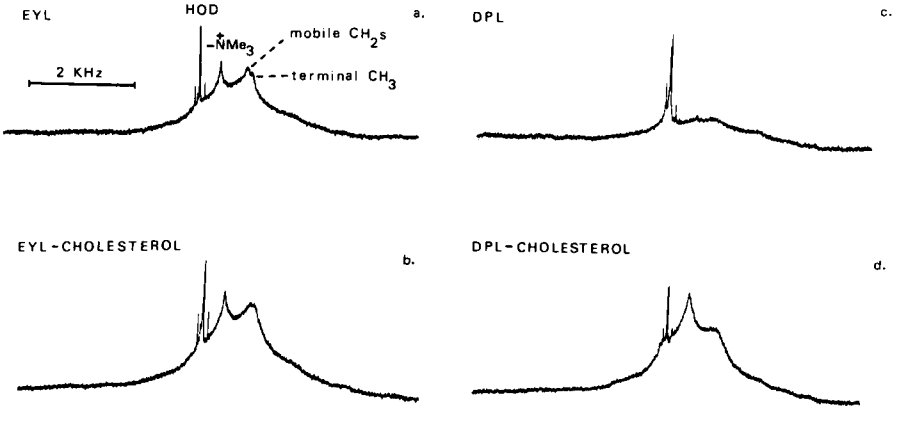


Fig. 1. 270 MHz proton nuclear magnetic resonance spectra at 18°C in D_2O of (a) egg lecithin, (b) egg lecithin-cholesterol (1 : 1), (c) dipalmitoyl lecithin, and (d) dipalmitoyl lecithin-cholesterol (1 : 1).

it *increases* their motion in the gel phase, by preventing chain crystallisation occurring (at 1 : 1 mole ratios).

There appears to be little effect of cholesterol on the linewidth or mobility of the trimethylammonium group in the liquid crystalline phase (figs. 1a, 1b). By contrast, however, there is a marked increase in the motional freedom of the trimethylammonium group on addition of cholesterol to the DPL gel (figs. 1c, 1d), consistent with weakened chain-chain and headgroup-headgroup interactions, due to the disruption of the crystal lattice by the steroid nucleus.

3.1.2. Biomembranes

In natural membranes containing a high degree of cholesterol and where lipid-protein interactions may further reduce lipid mobility, it might be expected that spectra without prominent lipid resonances would be obtained, even at 270 MHz. We have found this to be the case with beef erythrocyte and myelin membranes, studied at 18°C at 6.7 T, although some high resolution signals have been obtained from human erythrocyte membranes at higher temperatures [27, 28], and the $-NMe_3$ group in myelin has been reported to be resolvable [29] and hence fairly mobile.

Membranes which contain little or no cholesterol and which are observed above the thermal phase transition of their constituent lipids might be expected to provide better high resolution spectra. In fig. 2a we present the single scan spectrum at 18°C of rat liver mitochondrial membranes which satisfy both of these criteria since they contain relatively little cholesterol (about 6% of total lipid [30]) and also have highly unsaturated fatty acids [31]. A well resolved $-N(CH_3)_3$ signal superimposed on a broad methylene chain signal can be seen. In the membrane the chain methyl-

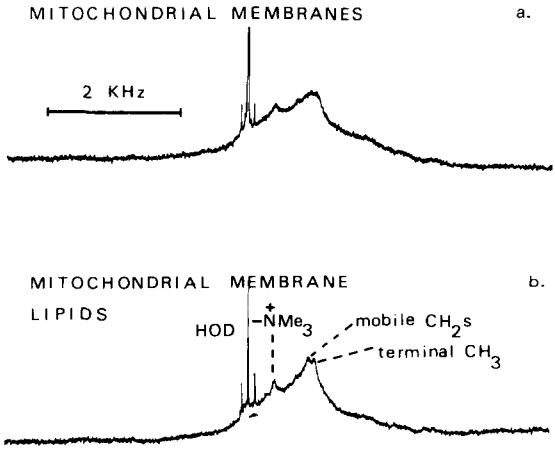


Fig. 2. 270 MHz proton nuclear magnetic resonance spectra at 18°C in D_2O of (a) mitochondrial membranes, and (b) mitochondrial membrane lipids.

ene and terminal methyl resonances are not well resolved. The small resonance on the low field shoulder of the methylene peak is probably due to CH_2–C = protons. The corresponding mitochondrial membrane lipid spectrum is shown in fig. 2b and the trimethylammonium resonance has become somewhat better resolved while the terminal methyl groups produce a signal separated from that of the chain. The broadening of the resonances in the membrane relative to those of the lipid may be the result of a decrease in motion by lipid-protein interactions.

3.2. Carbon-13 magnetic resonance

3.2.1. Model systems

In fig. 3 the CMR spectra of a number of *unsonicated* (and unhomogenised) lipid-water dispersions obtained at 25.2 MHz are presented and the striking increase in resolution over that obtained with PMR at 270 MHz is clear. The spectrum of EYL-water (fig. 3a) shows a large number of resonances. We assign the resonance at 173.5 p.p.m. downfield from external tetramethyl silane (TMS) to the carbonyl

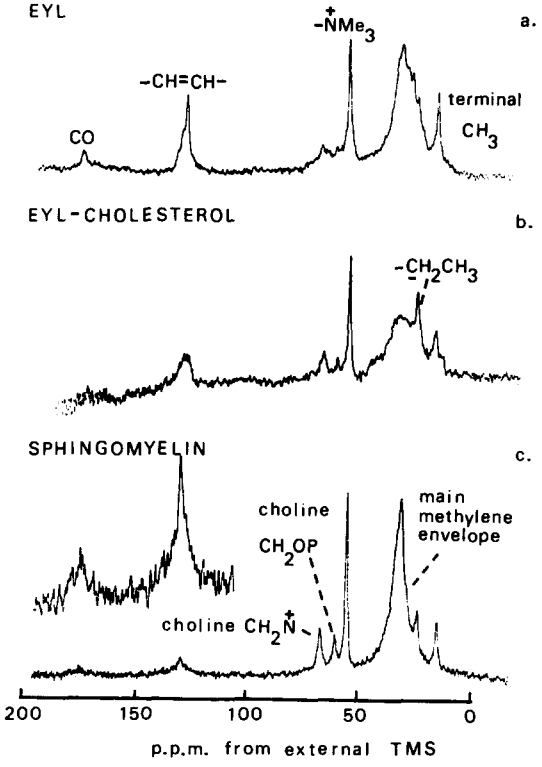


Fig. 3. 25.2 MHz carbon-13 nuclear magnetic resonance spectra of (a) egg lecithin, (b) egg lecithin-cholesterol (1 : 1), and (c) ox-brain sphingomyelin.

groups, and that at 128.0 p.p.m. to unsaturated carbons. The two carbons of the choline headgroup appear to be at least partially resolved from the resonances of the glycerol moiety, due to their greater mobility and hence narrower linewidths. By analogy to our previous ^{13}C assignments of choline chloride [22], and the results of Levine et al. [32], the resonance at 66 p.p.m. is assigned to the $-CH_2N-$ group, and that at 59.3 p.p.m. to the choline CH_2-OP group. These resonances are even better resolved in the spectrum of sphingomyelin (vide infra). Other assignments are, trimethylammonium group (54.4 p.p.m.), the hydrocarbon chain penultimate methylene group (23.1 p.p.m.) and the terminal methyl group (14.3 p.p.m.). The broad resonance at 30 p.p.m. is due to the bulk of the hydrocarbon chain methylene carbons, that at 25.8 p.p.m. appears to be due to the presence of unsaturation in the lipid, since it is also apparent in the spectrum given by Levine et al. [32] of dioleyl lecithin in solvent.

The EYL spectrum is considerably better resolved than our earlier spectra [22]. The increased resolution may be partially attributable to the wider decoupling bandwidth and higher proton decoupling power which we have now been able to use The observed linewidths are consistent with the glycerol moiety being the least mobile portion of the molecule with freedom of motion increasing in both directions away from the rigid glycerol backbone (the trimethylammonium and terminal methyl resonances have the smallest linewidths), consistent with our previous proton NMR measurements [10, 33], and the early PMR work of Veksli et al. [34].

The origin of the predominant linebroadening mechanism is of course an important factor in assessing the importance of linewidth measurements in the interpretation of molecular mobility. In proton NMR, when dipole-dipole interactions govern the observed linewidths, then linewidths and lineshapes are generally agreed to be a good reflection of the rates and types of mobility concerned [6, 9, 11, 33–35]. Charvolin and Rigny [36] have recently suggested that the relaxation time T_D [37], which can be regarded as the diffusional contribution to $T_{1\rho}$ (in zero H_1 field), rather than the relaxation time T_2 as normally measured from the linewidth or FID, may better describe the dynamics of lipid mobility. We feel that T_1, $T_{1\rho}$, T_2 and T_D (or T_x) measurements may be complementary. In this paper we shall confine ourselves to a discussion of molecular mobility using the observed linewidths. Since the observed *lineshapes* are not the characteristic powder patterns we might expect for a ^{13}C nucleus in an environment of lower than tetrahedral symmetry (see e.g. the DEFT [38] obtained $CaCO_3$ ^{13}C-spectrum of Pines et al. [39]) it would appear likely that any anisotropy of this tensor (which may of course be reduced) is obscured by dipolar coupling of the ^{13}C nuclei with the abundant protons. Levine et al. [32] have suggested that dipolar broadening accounts for the observed broad lines obtained in the gel phase of lipids, but have surprisingly ruled out this mechanism for the liquid crystalline phase.

Levine et al. [32] have also put forward the suggestion that only the outer lamellae of the unsonicated vesicles may give rise to the narrower (observable) signals of these vesicles (which are presumably superimposed on wider signals from the

remaining lipid). In the absence of any undecoupled integrated-intensity results [40] we see no compelling reason to take this view. If the results of Levine et al. [32] were obtained on decoupled spectra, then a reduced nuclear Overhauser enhancement (NOE) in the unsonicated lipid, due to gross deviations from the extreme narrowing condition could be important [41, 42]. The possible importance of this effect was emphasised in our previous communication [22].

In fig. 3b the CMR spectrum of a 1 : 1 mixture of EYL and cholesterol in water is shown. As can be seen by comparison with the spectrum of EYL (fig. 3a) that all the resonances except those associated with the choline headgroup have been broadened by the presence of cholesterol. The effect on the methylene chain signal is not dramatic, indicating that there must still be substantial motion in the parts of the chains distal to the steroid nucleus. Indeed the signals from the penultimate $-CH_2-$ and terminal $-CH_3$ groups are still well resolved although they show increased linewidth in comparison to those in EYL (table 1). This increase reflects the reduction in the cumulative motion along the chain brought about by cholesterol.

The introduction of the steroid could be expected to cause a substantial broadening of those ^{13}C resonances immediately adjacent, due to less efficient time-averaging of the $^{13}C-^{1}H$ dipole-dipole interactions. Carbons $1 \rightarrow (n-2)$ where n is the total carbon length of the chain in question and is generally 18, are appreciably broadened, though carbons n and $(n-1)$ are still quite free in their motion – and thus give rise to well resolved lines. As expected, carbons 9 and 10 (of the predominant olefinic *oleyl* residues) are appreciably affected, their half height ful-linewidths increasing from 71 ± 15 Hz to 161 ± 25 Hz on addition of equimolar quantities of cholesterol. Interestingly, the carbonyl carbon also appears immobilised on addition of cholesterol. This does not mean that the steroid skeleton is *necessarily* immediately next to the carbonyl group, since the immobilisation of carbons distal to C_1, and the relative immobility of the headgroup glycerol moiety (no defined signals are seen from the glycerol residue, with or without cholesterol) will lead to an immobilisation of C_1 by a cooperative effect, transmitted along the chain. At present it is not possible to assign resonances to the cholesterol carbons, so that a more detailed analysis is not yet possible. Many of the cholesterol carbons may be "lost" in the baseline due to their immobility.

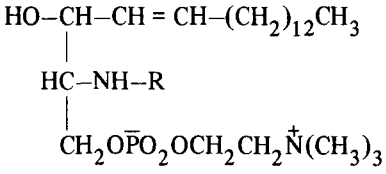
The effect of the steroid on the trimethylammonium group motion appears to be negligible and this agrees with the interpretation of PMR. It is interesting that the other choline headgroup carbons appear rather better resolved in the EYL-cholesterol spectrum than in the spectrum of EYL alone. This may be due to some immobilization of the glycerol moiety so that contributions from the glycerol carbons are less prominent in the spectrum in this region, or to a slight increase in motion in the headgroup now permitted by the increased spacing.

The spectrum shown in fig. 3c is of beef brain sphingomyelin

Table 1
^{13}C–Chemical shifts from external TMS in p.p.m. and half height linewidths in Hz for resonances in model and mitochondrial membranes.

	Sphingomyelin		Egg yolk lecithin		Egg yolk lecithin-cholesterol		Mitochondrial membrane	
	Shift	$W_{\frac{1}{2}}$	Shift	$W_{\frac{1}{2}}$	Shift	$W_{\frac{1}{2}}$	Shift	$W_{\frac{1}{2}}$
–C=0	177.2	210 ± 50	173.5	84 ± 20	175.5		177.0	
–CH = CH–	131.2	100 ± 25	128.0	71 ± 15	131.1	161 ± 25	128.5	76 ± 10
$CH_2\overset{+}{N}$	67.4	59 ± 10	66.0	100 ± 30	66.5	76 ± 10	(66.2)[a]	
CH_2OP	60.8	63 ± 10	59.3		60.4	42 ± 15	(61.4)[a]	
$\overset{+}{N}(CH_3)_3$	55.0	29 ± 6	54.4	34 ± 6	54.3	32 ± 6	54.7	40 ± 6
$-(CH_2)_n-$	30.7	162 ± 25	30.3	190 ± 40	30.6	305 ± 60	29.9	105 ± 30
Penultimate $-CH_2-$	23.1	74 ± 25	23.1		22.5	136 ± 35	23.0	75 ± 20
CH_3	14.4	49 ± 6	14.3	48 ± 6	14.3	68 ± 10	14.4	32 ± 6

[a] These resonances may also be due to protein.



where R = 18:0 40%
24:0 11%
24:1 25%

obtained at 70°C. The resonances assigned in the EYL spectrum (with the exception of the high field resonance due to the presence of unsaturated carbons) are all also evident here. This is a particularly well defined spectrum of a highly saturated lipid [43] above its transition temperature (this sample showed a broad thermal transition extending from approximately 35–55°C). Resolution is better than that observed previously with the saturated lipid dipalmitoyl lecithin, above its transition temperature [15]. Two points of difference between this and the EYL spectrum are apparent. First the carbonyl and unsaturated carbon resonances are shifted downfield in the sphingomyelin spectrum relative to those in the EYL spectrum (3.7 p.p.m. and 3.2 p.p.m. respectively). The shift in the carbonyl resonance is expected from the adjacent nitrogen in the amide bond of the fatty acid linkage, while that of the unsaturated carbon resonance is possibly due to deshielding by the vicinal hydroxyl of the double bond in the sphingosine which would be expected to be the major source of unsaturation in this compound. The other point of note is the large linewidth of the olefinic carbon resonance relative to that found with EYL (table 1). This is also expected since the double bonds in sphingomyelin are predominantly located in the sphingosine residue, near the polar/apolar interface, whereas in EYL they are mainly ca. 9 carbons along the acyl chain in the bilayer, and are hence more mobile.

3.2.2. Biomembranes

In fig. 4 the spectra of mitochondrial inner and outer membranes and beef erythrocyte membranes are presented.

Mitochondrial membranes give better resolved PMR spectra than erythrocyte membranes. The greatly increased resolution obtained with CMR should thus make this an excellent technique for studying these membranes in the "intact" state. The CMR spectrum of rat liver mitochondrial membranes at 37°C (fig. 4a) shows excellent resolution. The detail apparent in this spectrum is striking in comparison to that of the erythrocyte membrane spectrum (fig. 4b), with sharp signals from $-CH=$, $-N(CH_3)_3$ and $-CH_3$ groups being apparent. Resolution in the methylene chain region is good, with the penultimate methylene signal (23.0 p.p.m.) being partially resolved. The carbonyl resonance is very broad in the mitochondrial membrane spectrum indicating relative immobility (see fig. 5a).

The erythrocyte spectrum (fig. 4b) is similar to that obtained by Metcalfe et al. [15], and is included here primarily for comparison with the mitochondrial membrane spectrum to show the effect of cholesterol (and possibly lipid-protein interactions) on resolution. With the erythrocyte ghosts the resonance at 54.5 p.p.m. is

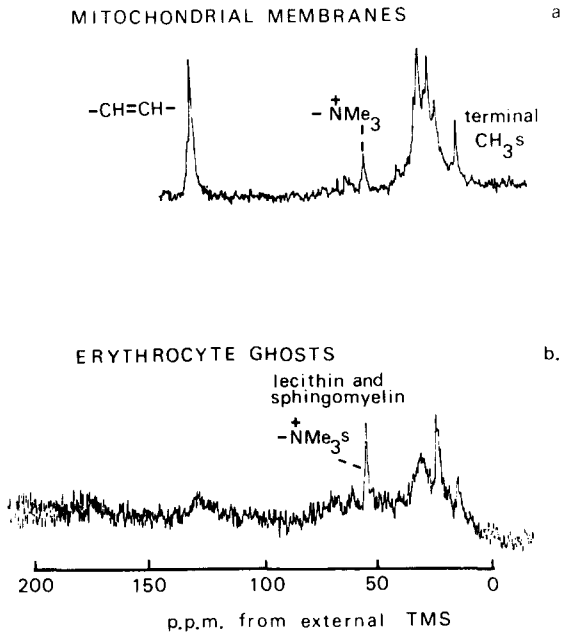


Fig. 4. 25.2 MHz carbon-13 nuclear magnetic resonance spectra at 37°C (a) mitochondrial membranes, and (b) erythrocyte ghosts.

ascribed to the $-N(CH_3)_3$ groups of lecithin and sphingomyelin (cf. Metcalfe et al. [15]), and those at 22.8 p.p.m. and 14.4 p.p.m. may be attributable to the penultimate $-CH_2-$ and terminal methyl groups of the lipids. The region of the spectrum associated with the fatty acids is qualitatively similar to that of EYL-cholesterol. It is likely, however, that contributions from amino acid side chains are also significant in this area. A very broad resonance centred at 128.8 p.p.m. is attributable to olefinic carbons and to carbons of protein aromatic residues.

The amino-acid composition of the mitochondrial membranes was determined, using the method of Atkin and Ferdinand [44, 45], and together with published lipid analyses [30, 31] and CMR chemical shifts [32, 46], the theoretical membrane spectrum was computed (fig. 5b).

As can be seen from a comparison of figs. 5a and 5b, the major contribution to the signals in the high field region of the spectrum is attributable to the lipid superimposed on a large number of overlapping protein side chain resonances. Contributions from aromatic side chains to the well defined resonance at 128.5 p.p.m. are expected to be small. We have assigned the resonance centred at 54.7 p.p.m. to the choline trimethylammonium groups of the lipid. The calculated spectrum shows that a substantial number of protein resonances, mainly from the α-carbons of the amino acids, are, however, also expected in this region. Three observations indicate that these do not likely contribute significantly to the sharp signal. First, spectra

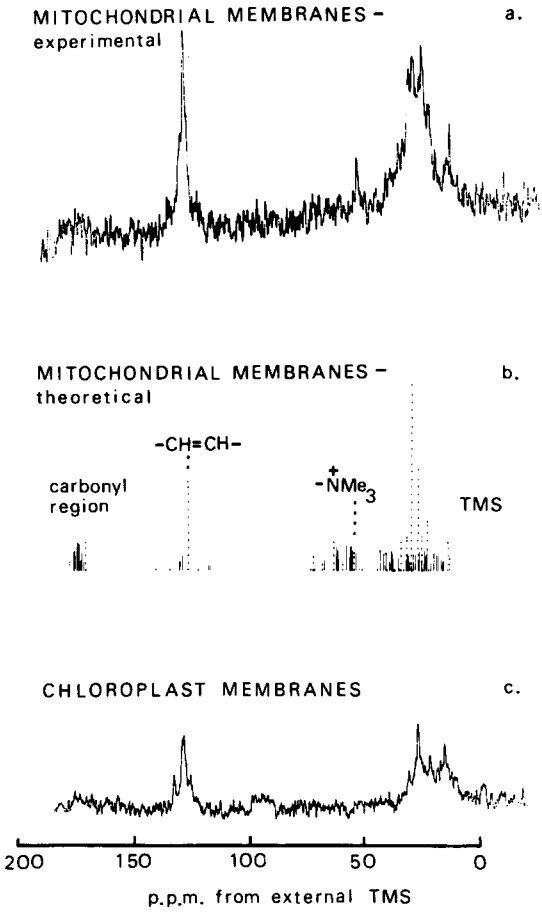


Fig. 5. (a) 25.2 MHz carbon-13 NMR spectrum of mitochondrial membranes at 37°C together with (b) computed spectrum of mitochondrial membranes using equal NOE's, X = lipid, – = protein, and (c) 25.2 MHz carbon-13 NMR spectrum of chloroplast membranes at 20° C.

obtained even after 60,000 pulses did not show well defined carbonyl resonances even though from the calculated spectrum a considerable signal might be expected in this region. This is undoubtedly because the carbonyl groups of the protein and probably the lipid are relatively immobile and produce very broad signals which are probably lost in the baseline of the spectra. A low NOE might also be expected [47]. The α-carbons, *also* located in the protein "backbone", would be expected to be similarly immobile and thus not contribute significantly to the sharp resonance [47]. Second, if this signal is due to the choline $-NMe_3$ groups, then it might be expected to be absent in a membrane where very little choline-containing lipid is present but where contributions from amino acid α-carbons would still be expected,

if these were significantly mobile. The chloroplast membrane spectrum (fig. 5c) is obviously extremely complicated in the hydrocarbon chain region, possibly because of resonance contributions from the chlorophyll molecules in this region [48, 49], but no signal appears at 55 p.p.m. Thirdly, the chemical shifts of the sphingomyelin $-NMe_3$ and egg lecithin $-NMe_3$ groups (55 p.p.m. and 54.4 p.p.m.) are *very* close to the 54.7 p.p.m. shift of the ($-NMe_3$) resonance in the mitochondria.

It thus appears that the most prominent features of the mitochondrial membrane spectra are ascribable to lipids. This may prove to be useful in experiments on conformational changes in oxidative phosphorylation.

3.3. Biophysical significance

By comparison of the experimental mitochondrial membrane spectrum with the computed "stick-diagram" (figs. 5a, b), it appears that the resonances from the protein carbonyl groups and α-carbon atoms are very broad, the observed spectra being attributable to resonances principally from the unsaturated lipid hydrocarbon chains and choline headgroups.

We suggest that this means that the peptide-backbones have considerably less motional freedom than the lipid molecules. The proteins are more "rigid" than the lipids. This may not be so with all biomembranes, e.g. *Acholeplasma laidlawii* B and *Escherichia coli* [50, 51], where proteins may be embedded in a more crystalline lipid-matrix (under some circumstances).

4. Conclusions

(a) Well-resolved natural abundance carbon-13 NMR spectra can be obtained from a variety of lipid and lipid-cholesterol systems. The resolution is considerably better than that obtained using proton NMR with comparable non-sonicated systems.

(b) Carbon-13 NMR spectra of egg-lecithin cholesterol systems indicate that cholesterol causes a significant decrease in mobility of carbons 1 to *ca.* ($n - 2$) of the hydrocarbon chain, but has little effect on the terminal CH_2 or CH_3 groups, or on the choline headgroup mobility.

(c) Well-resolved spectra have been obtained from "intact" mitochondrial membranes. The most prominent features are attributable to the *lipids*. The *proteins* appear therefore to be more rigid than the lipids in these membranes.

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