^{13}C NMR SPECTRA OF ACHOLEPLASMA MEMBRANES CONTAINING ^{13}C LABELLED PHOSPHOLIPIDS

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1. Introduction

Recently we have shown that aqueous suspensions of sonicated lecithin vesicles yield high resolution ^{13}C NMR spectra, which allow the spin lattice relaxation times (T_1) to be measured for all the resolved resonances in the structure [1, 2]. For example, dipalmitoyl lecithin T_1 values were obtained for 6 of the 16 fatty acid chain resonances resolved from the main methylene envelope (C4–13) and for all 6 of the non-equivalent choline and glycerol carbons. The T_1 values of ^{13}C nuclei in lecithin molecules in a bilayer increase from the glycerol group towards both the $-\overset{+}{N}Me_3$ polar headgroup and the terminal methyls of the fatty acid chains. The T_1 values were also found to depend on the chain structure of the lecithin molecule and its steric interactions with other lipid molecules in organic solvents in which different structural organisations of lecithin molecules are formed. These experiments indicate that the wide range of chemical shifts and relatively sharp resonances from ^{13}C nuclei are well suited for membrane studies. The ^{13}C nucleus also causes no perturbation of the lipid structure, and all the nuclei in the molecule can be examined by selective enrichment of the ^{13}C isotope from the natural abundance level of 1.1%.

This enrichment technique is important in extending the ^{13}C relaxation measurements to biological membranes, since the problems of overlapping resonances and poor signal to noise in the spectra of membranes at natural abundance can also be solved in principle by specific ^{13}C enrichment. For example, the spectra of erythrocyte membranes [1] and the microsomal membranes from the sarcoplasmic reticulum [16] consist mainly of broad envelopes of many resonances, and lack the resolution necessary to measure T_1 values for many of the lipid resonances. In the microsomal membrane spectrum, T_1 values could only be measured for the methylene $(CH_2)_n$ envelope, the terminal methyls of the chains, and the lecithin-$\overset{+}{N}Me_3$ resonances, and the low sensitivity required long spectral accumulation times. Specific enrichment of nuclei in the membrane lipids by biosynthetic incorporation should allow direct comparison of the relaxation times of the enriched nuclei in the intact membrane and in vesicles of the extracted lipids in bilayers. The results of the T_1 measurements on synthetic lecithins at natural abundance suggest that the relaxation times of lipid molecules in cyto-membranes should be sensitive to their steric interactions with the membrane proteins. It is possible that the proteins will impose a characteristic pattern of molecular motion on the lipids with which they are directly interacting, which will result in T_1 values which differ from those of the same lipids in a bilayer structure.

Here we report the ^{13}NMR spectra of membranes from *Acholeplasma laidlawii* grown on a medium supplemented with ^{13}C-enriched palmitic acid. The membrane spectra are simplified to a single well-defined resonance under conditions where no natural abundance ^{13}C resonances can be detected. The resonances from the enriched nuclei in the lipids are only observed above the thermal transition of the lipids in the membranes, and similar broadening and loss of intensity of the fatty acid chain resonances occurs in the spectra of the extracted membrane lipids in vesicles below the transition temperature. The same phenomenon has been observed in both the ^{13}C and the ^{1}H NMR spectra

of dipalmitoyl lecithin vesicles through the transition [2, 3].

The thermal transition in the membrane lipids was examined by measuring the partition of the spin label TEMPO [2, 2, 6, 6-tetramethyl piperidine-l-oxyl; ] into the membranes [4, 5]. The sharp increase in binding of TEMPO through the transition in dipalmitoyl lecithin coincides closely with the thermal transition temperature [6]. It was found that the binding of TEMPO to the Acholeplasma membranes and the extracted lipid vesicles also increased steeply through the temperature range in which the ^{13}C NMR spectra became visible. A similar proportion of TEMPO was bound to the membranes and to an equivalent concentration of the extracted lipid vesicles, whereas the separated membrane protein had a negligible binding capacity for TEMPO. As judged by this parameter, the organisation of most of the lipids in the Acholeplasma membrane is indistinguishable from the simple bilayer structure.

2. Materials and methods

Palmitic acid (1-^{13}C) was prepared by treating excess 1-bromopentadecane with $K^{13}CN$ (60% enrichment) in ethanol and subsequently hydrolysing the 1-cyanopentadecane with aqueous C_2H_5OH/H_2SO_4 [Yield 80%, mp. 61–62°].

A. laidlawii B (PG 9) were grown at 37° on a medium [7] containing 20 g Difco tryptose; 5 g NaCl; 5 g Tris; 10 g glucose; 10 ml Bacto PPLO serum (Difco); 2.5 g bovine serum albumin (Armour, fraction V), and 50,000 units of penicillin G made up to 1 litre at pH 8.4. The medium was supplemented with 50 mg/l of ^{13}C labelled palmitic acid in ethanol (10 mg/ml); the BSA was added to hold the palmitic acid in solution. The Acholeplasma were harvested by centrifugation at 10,000 *g* for 30 min and washed twice with β buffer (0.156 M NaCl, 0.05 M Tris, 0.01 M 2-mercaptoethanol, pH 7.4). The membranes were prepared by osmotic lysis in $\beta/20$ buffer as described previously [8] and the yield was approx. 50 mg of membranes per 1 of medium. The membrane lipids were extracted with $CHCl_3$/MeOH (2:1, v/v) after the NMR experiments and accounted for 35% of the membrane by weight, in agreement with the data of Razin et al. [9]. There was less than 5% free fatty acid present in the lipids examined by thin-layer chromatography on silica gel G ($CHCl_3$: CH_3OH: 7 N NH_4OH solvent, 69:27:4.5, by volume). The fatty acid composition of the phospholipids was estimated by transmethylation in methanolic sodium hydroxide [10]. The methyl esters were chromatographed on a Perkin Elmer F11 gas chromatograph using a diethylene glycol succinate column at 185°, and were identified from their retention times by comparison with known standards. The composition was 14:0, 0.5%; 16:0, 81.7%; 16:1, 1.0%; 18:0, 4.8%; 18:1, 6.8%; 18:2, 2.0%; unidentified, 3.2% (average of 3 experiments). The membranes contained very little carotenoid when grown at 37° and were only faintly yellow. The carotenoid content was increased on lowering the growth temperature to 30°. The Acholeplasma membrane proteins were solubilised (> 95%) by the butanol fractionation procedure of Maddy [11] as described previously [8].

The ^{13}C NMR spectra were obtained using a Varian XL 100 spectrometer locked on solvent deuterium (D_2O or $CDCl_3$) employing the Fourier transform technique and were accumulated in a Varian 620i computer as described previously [3]. The chemical shifts are corrected to dioxan as internal reference. The membrane samples (~7%, w/w) were suspended in 3 ml of $\beta/20$ buffer in D_2O in 12 mm NMR tubes. The aqueous lipid samples in the same D_2O buffer were sonicated in glass vials under nitrogen as described previously [1].

TEMPO binding measurements were made using a Varian E3 ESR spectrometer with a variable temperature controller at a TEMPO concentration of 5×10^{-4} M. Mercaptoethanol was omitted from the buffer for these experiments to prevent reduction of the nitroxide group. The bound TEMPO fraction was estimated from the difference in intensity of the high field resonance from TEMPO in the aqueous medium (a_n = 17.4 gauss) and of the corresponding resonance of a standard TEMPO solution of the same total concentration in the absence of the membranes. A more precise estimate of free TEMPO concentration can be obtained by correcting for the partial overlap of the bound component with the free signal, but the change in binding through the thermal transition in the membrane lipids is so pronounced that the small inaccuracy (< 5%) in the estimate of the bound fraction is unimportant in the present experiments. Any systematic error in the estimate of bound TEMPO concentrations will apply equally to

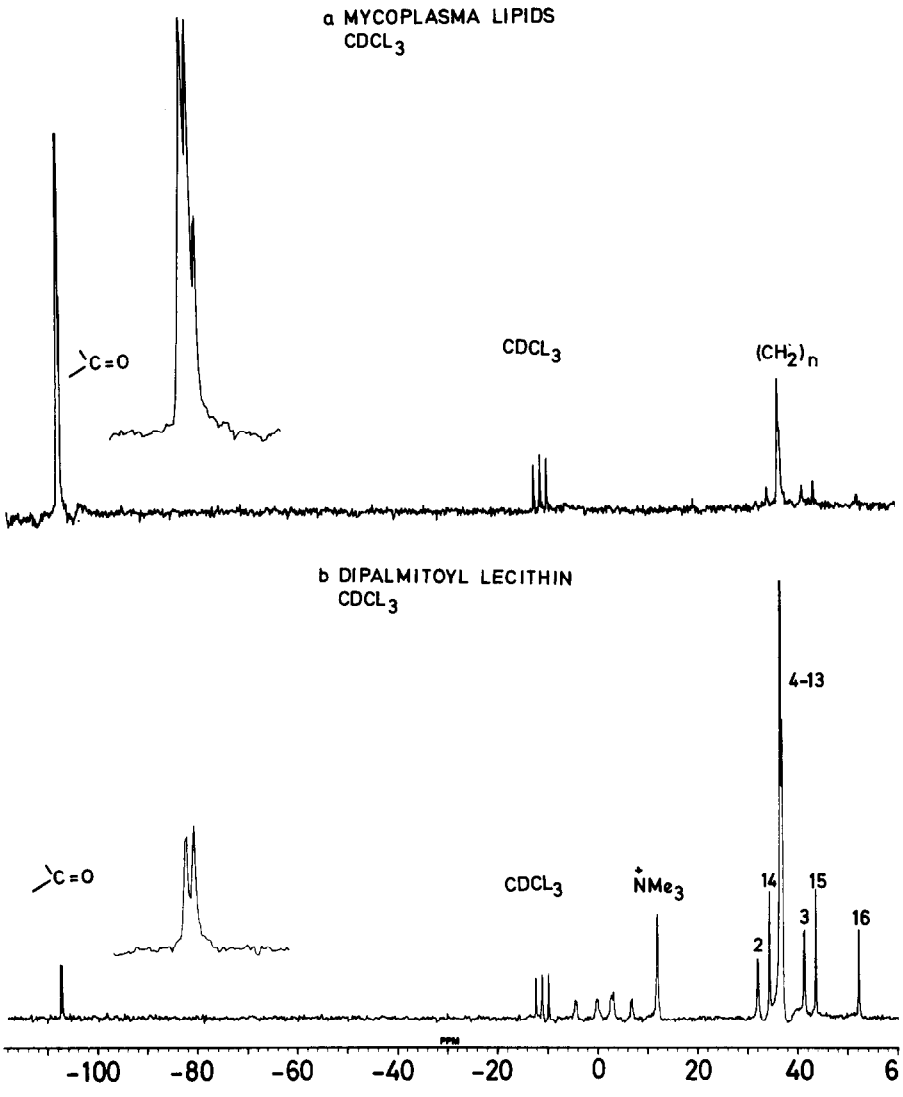

Fig. 1. ^{13}C NMR spectra of lipids in $CDCl_3$ at 30°: a) Acholeplasma lipids containing ^{13}C-enriched carboxyl nuclei in the fatty acid chains. b) Dipalmitoyl lecithin (290 mM) at natural abundance. The insert spectra are the carboxyl resonances on an expanded scale (× 5).

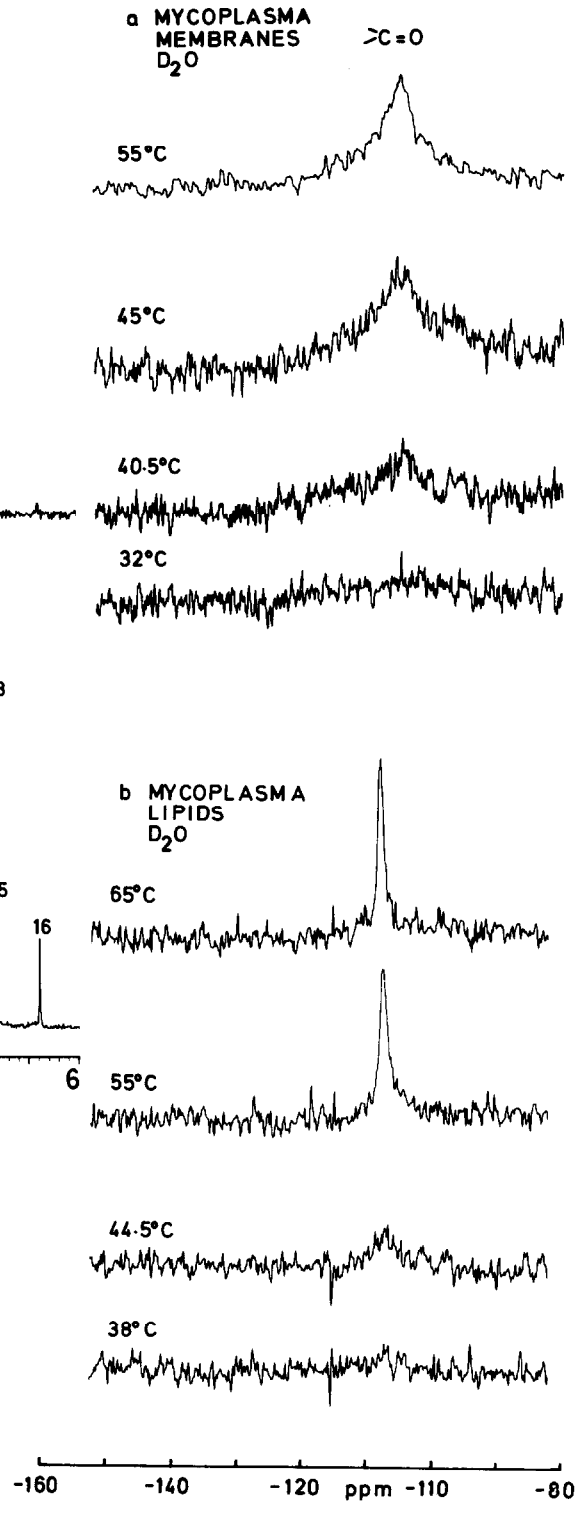

Fig. 2. ^{13}C NMR spectra of: a) Acholeplasma membranes containing lipids enriched with ^{13}C in the fatty acid chains (1-^{13}C) and b) sonicated vesicles of the lipids extracted from the same membrane samples (β/20 buffer in D_2O).

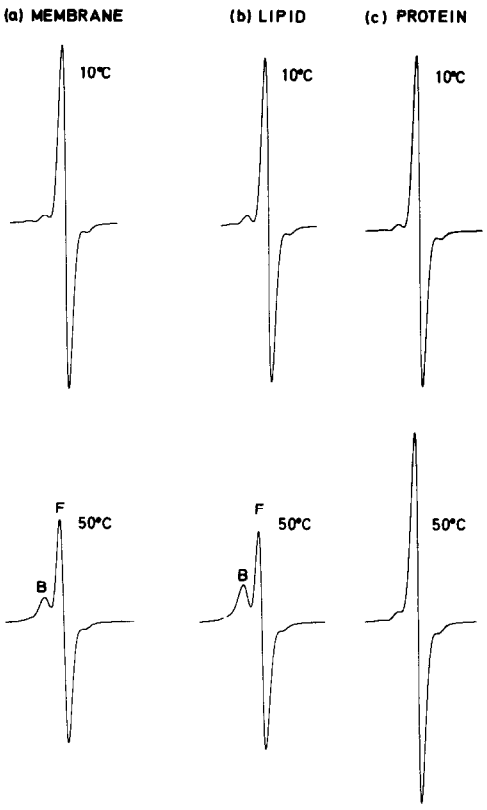

Fig. 3. ESR spectra of the high field resonance of 5×10^{-4} M TEMPO in the presence of: a) Acholeplasma membranes (6.0%, w/w). b) Extracted lipid vesicles (3.1%, w/w). c) Membrane protein separated by butanol fractionation (4.0%, w/w).

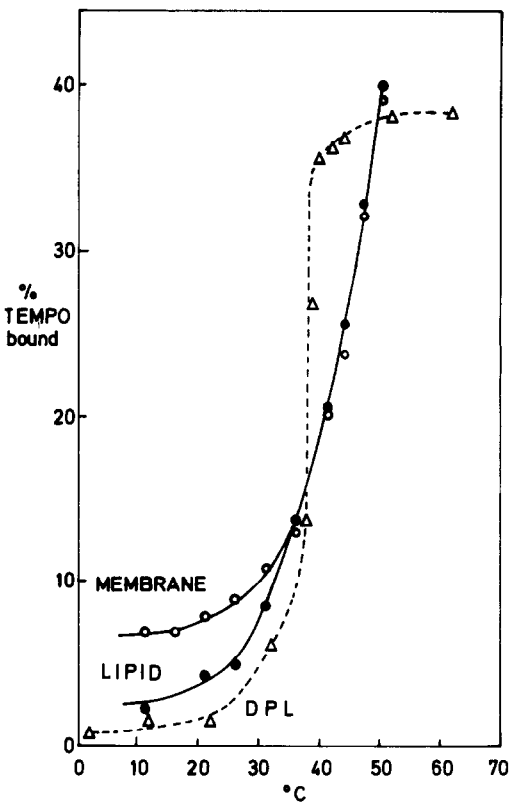

Fig. 4. Calculated % TEMPO bound by (○–○–○) Acholeplasma membranes (8.9%, w/w) and (●–●–●) extracted lipid vesicles (3.1%, w/w). These preparations contain equivalent lipid concentrations (3.1%, w/w). Total TEMPO concentrations 5×10^{-4} M. (Δ--Δ--Δ) is the % TEMPO bound by 3.1%, w/w, dipalmitoyl lecithin (DPL).

both membrane and lipid vesicle samples since the bound concentrations in the two preparations were closely matched.

3. Results and discussion

The spectrum from the extracted membrane lipids in $CDCl_3$ in fig. 1 (a) shows the enrichment of the $\rangle C{=}O$ resonance relative to the $(CH_2)_n$ resonance envelope at natural abundance. The relative intensity of these two resonances in the enriched Acholeplasma lipids is 1.5:1 ($\rangle C{=}O : (CH_2)_n$) and in the lipid spectrum at natural abundance the ratio is 0.11:1, implying a 14-fold enrichment of the $\rangle C{=}O$ group with ^{13}C and an overall incorporation of 24% of the added palmitic acid enriched to 60% with ^{13}C. The carboxyl resonance consists of a doublet of equal intensity from the two non-equivalent lipid chains, and an additional partially resolved resonance on the high field side of the doublet. The doublet is assigned by comparison with the chemical shift of the corresponding resonances in synthetic lecithins (see dipalmitoyl lecithin in fig. 1b), but the additional peak has not been assigned. It does not appear to be due to free fatty acid which is not present at high enough concentration to account for the intensity of the resonance.

The spectra from the membranes enriched with palmitic acid (1-^{13}C) are shown in fig. 2a. No spectrum is observed at 32°, but the carboxyl resonance becomes increasingly well-defined above 40° and the chemical

shift coincides with that of the carboxyl resonance of synthetic lecithin vesicles in D_2O [1, 2]. The linewidth at half height ($\Delta\nu_{\frac{1}{2}}$) of the carboxyl resonance in the membrane spectrum at 55° is approx. 130 Hz, which is substantially broader than the linewidth of 40 Hz of the corresponding resonance in sonicated vesicles of the membrane lipid. This resonance also disappears below the thermal transition (fig. 2b). Although the sonicated lipid suspensions do not become optically clear, they are stable and the spectral changes through the thermal transition are fully reversible. It is unlikely that the very high viscosity of the membrane suspension is responsible for the relatively broad linewidth of the carboxyl resonance since it does not appear to depend significantly on the membrane concentration. The carboxyl resonance in the membrane spectrum is virtually unaltered at 65°, although the membranes undergo a second irreversible transition at this temperature which has been attributed to the thermal denaturation of the membrane proteins [13]. This transition is not detected in the spectrum of the enriched lipid in the membranes, although the texture of the membrane suspension becomes friable after prolonged incubation at 65°. This is consistent with previous spin label experiments [14, 15] which show that the membrane proteins have only minor effects on the interaction between the lipids in intact Acholeplasma membrane, and that spin labelled lipids are insensitive to the thermal denaturation of the membrane protein. It is also clear from these experiments that the Acholeplasma are able to grow rapidly at a temperature towards the lower end of the thermal transition at which most of the lipid chains are in the crystalline form.

The spectra of 5×10^{-4} M TEMPO in the presence of the membranes, extracted lipid vesicles, and separated membrane protein are shown in fig. 3. Very little TEMPO is bound to the membranes or lipid vesicles below 30°, but at higher temperatures there is a progressive increase in the bound (B) component of the high field resonance which is partially resolved from the free (F) TEMPO in the aqueous medium. Previous work by Hubbell and McConnell has established that the bound component corresponds to TEMPO in a highly fluid hydrophobic environment within the membrane [4]. There is virtually no bound component in the spectrum of TEMPO in the presence of separated membrane protein over the temperature range of 10–50° (fig. 3c). However, this is not conclusive evidence that the protein does not bind TEMPO, since the binding sites on the separated protein may be exposed to the aqueous medium so that the bound TEMPO resonance is superimposed on the free TEMPO resonance. Equilibrium dialysis experiments confirmed that the separated membrane protein had less than 10% of the binding capacity of an equivalent concentration of membranes, so that the contribution of the protein to TEMPO binding in the intact membrane is likely to be negligible. The TEMPO bound to the membrane is therefore assumed to be localised almost entirely in the lipid regions of the structure.

The fraction of TEMPO bound by equivalent concentrations of lipid in the membranes and in separated lipid vesicles are compared as a function of temperature in fig. 4. At temperatures below 30° the membranes bind slightly more TEMPO than the lipid vesicles, although the partition coefficient is very low (< 2). As the partition coefficient increases through the thermal transition, the membranes and lipids bind very similar proportions of TEMPO. The transition is apparently much broader than for dipalmitoyl lecithin [6] for which our data are included in fig. 4 [12]. There is no indication that the increase in partition falls off at temperatures up to 50°, or that a region of constant partition is reached as for dipalmitoyl lecithin. The measurements were unreliable above 50° due to slow degradation of the nitroxide group and loss of resonance intensity. The sharp increase in TEMPO partition observed in these experiments is consistent with previous observations that the extent of TEMPO binding is critically dependent on the fluidity of the lipid bilayer [4, 5]. It is very probable, for example, that if the membrane protein significantly increased the packing of the lipids this would lead to a decrease in the partition of TEMPO into the lipid region of the membrane. This effect has been observed in the membranes of the sarcoplasmic reticulum which bind only 75% of the TEMPO bound by an equivalent concentration of the separated lipid vesicles [16]. The proportion of TEMPO bound by a membrane compared with its separated lipid is therefore a fairly sensitive technique for probing the state of the membrane lipids. In the present experiments, the Acholeplasma membrane has a similar TEMPO binding capacity over the accessible temperature range as its lipid component organised in bilayers in the vesicle form. This suggests

that at least 80% of the lipids in the membrane are in a bilayer structure, which is consistent with the evidence from differential calorimetry experiments [13]. However it has not been established that the lipids in the two structures have the same maximum binding capacity for TEMPO since the partition is still increasing beyond the accessible temperature range; membranes in which the thermal transition occurs over a lower temperature range can be used to characterise the maximal binding capacity of the lipids [12] which should be the same in the membranes and the vesicles if their structural organisation is the same.

We conclude that these experiments demonstrate that it will be feasible to make relaxation measurements on defined lipid resonances in the membrane and that the partition of TEMPO can be used to define the extent of the fluid hydrophobic bilayer region of the lipids in the membranes.

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