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PHYSICOCHEMICAL CHARACTERIZATION OF 1,2-DIPHYTANOYL-*sn*-GLYCERO-3-PHOSPHOCHOLINE IN MODEL MEMBRANE SYSTEMS

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Summary

We report here on a series of studies aimed at characterization of the structural and dynamical properties of the synthetic lipid diphytanoyl phosphatidylcholine, in multilamellar dispersions and vesicle suspensions.

This lipid exhibits no detectable gel to liquid crystalline phase transition over a large temperature range (-120°C to $+120^{\circ}\text{C}$).

Examination of proton nuclear magnetic resonance (NMR) free induction decays obtained from multilayer dispersions of diphytanoyl phosphatidylcholine provided an estimate of the methylene proton order parameter. The estimated magnitude of 0.21 is comparable to those determined for other phospholipids.

Sonication of aqueous dispersions of diphytanoyl phosphatidylcholine led to formation of bilayer vesicles as determined by the measurement of the outer/inner choline methyl proton resonances, vesicle sizes in electron micrographs, and comparison of proton NMR linewidths between multilayer and sonicated dispersions. Ultracentrifugation studies of diphytanoyl phosphatidylcholine vesicles in H_2O and $^2\text{H}_2\text{O}$ media yielded a value of 1.013 ± 0.026 ml/g for the partial specific volume of this lipid.

We have measured spin lattice relaxation rates for the methyl and methylene-methyne protons of the hydrocarbon chains of diphytanoyl phosphatidylcholine in bilayer vesicles over a range of temperatures and at two NMR frequencies (100 and 220 MHz). The observed relaxation rates for the methylene protons in this system were approximately twice those previously reported for

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dipalmitoyl phosphatidylcholine at comparable temperatures and resonance frequencies, whereas the relaxation rates measured for the methyl protons were greater than those of the straight chain lipid by an order of magnitude.

Measurement of the spin lattice relaxation rates of the hydrocarbon protons of the diphytanoyl phosphatidylcholine in a 10 mol% mixture of the branched-chain lipid in a deuterated host lipid, diperdeuteropalmitoyl phosphatidylcholine, showed a discontinuity in the temperature dependence of the proton NMR longitudinal relaxation rates of the branched-chain lipid in the region of the gel to liquid crystalline phase transition temperature of the deuterated dipalmitoyl phosphatidylcholine host lipid. This result may be taken as evidence of lateral phase separation of a liquid crystalline phase enriched in diphytanoyl phosphatidylcholine from a gel phase enriched in diperdeuteropalmitoyl phosphatidylcholine at temperatures below the phase transition temperature of deuterated host lipid. This conclusion is supported by the observation of an abrupt change in the hydrocarbon methylene linewidth (at 100 MHz) of 10 mol% diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine over the temperature range where lateral phase separation is taking place according to differential thermograms.

Introduction

Dihydrophytol, the reduced form of phytanic acid, has been shown to be the only detectable hydrocarbon chain in the diether analogues of glyceride-derived lipids found in the membranes of halobacteria [1]. It is known that the bacteriorhodopsin isolated from *Halobacterium halobium* is coupled in these lipids [2]. The physiological significance of the absence of other hydrocarbon moieties in the lipids of these bacterial membranes is unclear. It is possible that the bacterial lipids engender special permeation properties in these membranes; or perhaps the function of bacteriorhodopsin in the purple membrane is dependent on the presence of branched-chain lipids.

Phytanic acid has been implicated in a number of genetically related neural disorders in mammals. The presence of large concentrations of phytanic acid in the nervous tissues is thought to be the probable cause of the hindered propagation of nerve impulses symptomatic of these diseases [3,4]. Its mechanism of action is, however, unknown.

With the intent of clarifying the structure-function relationship of this particular acyclic diterpenoid molecule in membranes we have undertaken a study to elucidate the structural and dynamic properties of model membrane systems composed of or containing diphytanoyl phosphatidylcholine.

Experimental

Materials. 1,2-Diphytanoyl-*sn*-glycero-3-phosphocholine (diphytanoyl phosphatidylcholine) was synthesized following a procedure set forth in a published synthesis [5]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (dipalmitoyl phosphatidylcholine) was purchased from Calbiochem. 1,2-Diperdeuteropalmitoyl-*sn*-glycero-3-phosphocholine (diperdeuteropalmitoyl phosphatidylcholine) was

prepared as described in a previous publication [6]. All lipids were purified by extensive silicic acid chromatography.

Sample preparation. Samples for differential thermal analysis were prepared by dissolving weighed amounts of lipid (total sample size approx. 5 mg) in a minimum of chloroform and transferring with a Hamilton syringe into capillary tubes. These tubes were evacuated at high vacuum overnight to remove solvent. 5 μ l of deionized water was then added to each sample and the capillary tube sealed. The sealed tubes were incubated for several hours at approximately 45°C.

Unsonicated lecithin multilayers were prepared by addition of 0.4 ml Ringer solution [7] prepared with $^2\text{H}_2\text{O}$ ($^2\text{H}_2\text{O}$ /Ringer) to approximately 100 mg of lipid in an NMR tube. The NMR tube was then sealed and the sample agitated using a vortex mixer and allowed to stand overnight at approx. 42°C. The samples were again agitated before spectra were taken.

Vesicle suspensions of mixed diphytanoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine were prepared by dissolving weighed amounts of lipids in a minimum of chloroform in glass centrifuge tubes. The samples were evaporated nearly to dryness by placing the tubes in a sand bath at approximately 60°C and directing a stream of dry nitrogen into the tube. The tubes were then evacuated at high vacuum overnight. $^2\text{H}_2\text{O}$ /Ringer (1 ml) was added to each tube and the sample was either sonicated continuously at high power in an ice bath for 15 min or for the same period of time using an alternating 30 s on-30 s off cycle with an MSE 150 W ultrasonic disintegrator. Vesicle suspensions of pure lipid were prepared in the same fashion except that the chloroform step was omitted.

For electron microscopy the vesicle suspensions described above were diluted to 1–5 mg/ml in lipid. A drop of this solution was then applied to a 200 mesh copper grid coated with parlodion onto which a thin film of carbon has been evaporated. After approximately 30 s the excess liquid was blotted off and a drop of 2% (w/v) phosphotungstic acid (pH 7.4) was applied to stain the sample. After approximately 40 s the excess staining solution was blotted off and grid allowed to dry before observation.

Instrumentation. The differential thermal analyses were performed on a Dupont Model 900 Differential Thermal Analyzer. Operation at sub-ambient temperatures was achieved by cooling the heating cell with a stream of dry nitrogen which had been precooled by passing the gas through a coil submerged in a liquid nitrogen bath. A rate of heating of 7°C/min was used in the recording of the thermograms.

Hydrated multilamellar samples of diphytanoyl lecithin were examined under cross-polarized light using a Zeiss Standard WL polarizing microscope.

The electron microscopy grids prepared as described above were observed on a Phillips 201 C electron microscope operating at 60 kV at magnification level 10 (actual magnification on camera: 13 900).

The proton NMR free induction decay of a multilamellar sample was recorded at 14.1 kG (MHz) courtesy of Dr. R.W. Vaughan using pulse NMR equipment described elsewhere [8].

100 MHz proton NMR measurements were made using a Varian XL-100 system and measurements at 220 MHz were performed on a Varian HR-220

spectrometer. Both spectrometers are equipped with a Fourier transform accessory and interfaced with a Varian 620/i 16K computer. Probe temperature in both instruments was regulated to $\pm 1^\circ\text{C}$ by a Varian 4540 variable temperature unit. The probe temperature was determined using either a methanol or an ethylene glycol standard sample previously calibrated against a copper-constantan thermocouple.

Spin lattice relaxation time (T_1) measurements were made using a $(\pi - \tau - \pi/2)$ inversion recovery sequence. (Normally 36 transients were collected for vesicle suspensions of pure diphytanoyl phosphatidylcholine. One hundred transients were collected for diphytanoyl phosphatidylcholine diluted in the diperdeuteropalmitoyl phosphatidylcholine host matrix.) The spin lattice relaxation times were calculated from the slope of a plot of $\ln(h(\infty) - h(\tau))$ versus τ , where $h(\tau)$ is the peak height of the partially relaxed resonance line in the Fourier-transformed spectra.

Vesicle suspensions prepared as described above were also examined by ultracentrifugation in a Beckman-Spinco Model E analytical ultracentrifuge equipped with schlieren, interference, and scanning ultraviolet optical systems. (Only the schlieren optics were used.) The experiments were performed at 25°C in An-H rotor using double-sector cells with a 12 mm light path. The temperature was regulated by a RITC unit and the velocity of the run was controlled via the Electronic Speed Control unit.

Results

Differential thermal analysis

We have sought evidence for a thermal phase transition in multilayers of diphytanoyl phosphatidylcholine by examining differential thermograms of the lipid in a number of media: (i) deionized water; (ii) $^2\text{H}_2\text{O}$; (iii) 1/1 (w/w) sucrose/ H_2O mixture; (iv) 1/1 (w/w) ethylene glycol/water mixture. In the deionized water and glycol/water mixture the sample was scanned over a temperature range of -120°C to $+120^\circ\text{C}$. The temperature range covered for the other samples was -120°C to $+80^\circ\text{C}$. No lipid phase transitions were detected. Hydrated suspensions of diphytanoyl phosphatidylcholine viewed under cross-polarized light were birefringent and had an extinction angle of 0° . This result is consistent with the existence of a lamellar phase in the hydrated lipid.

Addition of diphytanoyl phosphatidylcholine to dipalmitoyl phosphatidylcholine was found to have a pronounced effect on the differential thermogram of dipalmitoyl phosphatidylcholine multilayers. A series of differential thermograms of mixtures of diphytanoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine in the multilamellar state are depicted in Fig. 1. It is seen that the addition of diphytanoyl phosphatidylcholine to dipalmitoyl phosphatidylcholine causes a broadening of the phase transition and the disappearance of the pretransition in the latter lipid. The onset temperature for the phase transition of dipalmitoyl phosphatidylcholine is shifted to lower temperatures, while the temperature at which the transition terminates is only slightly affected. This thermal behavior resembles that which would be expected if diphytanoyl phosphatidylcholine were only slightly soluble in dipalmitoyl phosphatidylcholine in the gel phase.

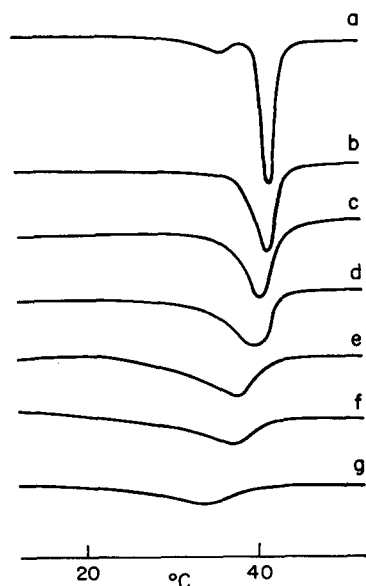


Fig. 1. Differential thermograms of mixtures of diphytanoyl phosphatidylcholine in dipalmitoyl phosphatidylcholine. Diphytanoyl phosphatidylcholine: (a) 1 mol%; (b) 4 mol%; (c) 7 mol%; (d) 11 mol%; (e) 18 mol%; (f) 22 mol%; (g) 46 mol%. The sample consisted of 5 mg lipid in 5 μ l of water.

Characterization studies of sonicated suspensions of diphytanoyl phosphatidylcholine and diphytanoyl phosphatidylcholine in dipalmitoyl phosphatidylcholine

Sonicated suspensions of diphytanoyl phosphatidylcholine were examined via electron microscopy, analytical ultracentrifugation and NMR spectroscopy to ascertain that bilayer vesicles were formed on sonication of this lipid. A typical electron micrograph of a sonicated suspension of diphytanoyl phosphatidylcholine is shown in Fig. 2. It is noteworthy that there is considerable heterogeneity in the vesicle sizes according to this electron micrograph. It was found that the average size and size distribution of diphytanoyl phosphatidylcholine vesicles depend on the ionic composition of the aqueous solution in which the vesicles are formed and, to a lesser extent, on the method of sonication (see Table I). Continuous sonication produces vesicles of an average size range of 280–360 Å in diameter in 5 mM $\text{La}(\text{NO}_3)_3$ compared with 220–330 Å in diameter in $^2\text{H}_2\text{O}$ /Ringer solution. The size distribution of vesicles is less homogeneous when cycling sonication is used. In contrast, continuous sonication of dipalmitoyl phosphatidylcholine or 10 mol% diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine produces vesicles with an average diameter of approx. 220 Å in 5 mM $\text{La}(\text{NO}_3)_3$ and approx. 250 Å in $^2\text{H}_2\text{O}$ /Ringer solution.

The heterogeneity of vesicle samples produced from sonication of diphytanoyl phosphatidylcholine was further examined via analytical ultracentrifugation. These results corroborate the electron microscopy findings. While the sedimentation boundary observed for dipalmitoyl phosphatidylcholine vesicles produced by continuous sonication is sharp, that observed for

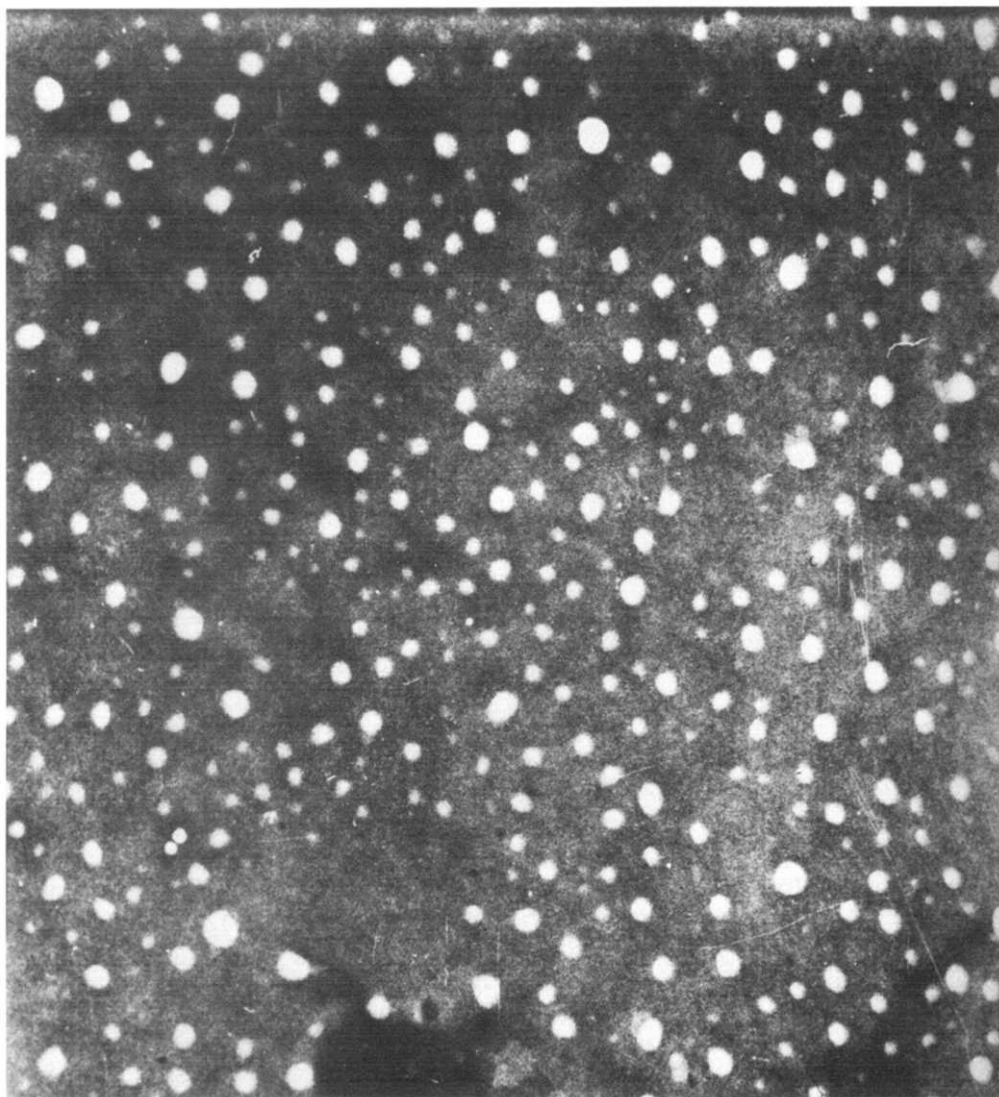


Fig. 2. A representative electron micrograph of diphytanoyl phosphatidylcholine vesicles prepared by continuous sonication in $^2\text{H}_2\text{O}$ /Ringer solution.

diphytanoyl phosphatidylcholine vesicles produced similarly is much broader (see Table I). However, the analytical ultracentrifuge results show that sonicated suspensions of diphytanoyl phosphatidylcholine sediment at the same rate and exhibit only one sedimentation boundary even when produced by cycling sonication, whereas, sonicated suspensions of dipalmitoyl phosphatidylcholine manifest multiple sedimentation boundaries and pronounced shoulders to the main peak when cycling sonication is employed to produce these vesicles.

In the ultracentrifuge diphytanol phosphatidylcholine vesicles undergo flotation in both H_2O and $^2\text{H}_2\text{O}$ /Ringer solution. Dipalmitoyl phosphatidylcholine vesicles, on the other hand, undergo flotation in only $^2\text{H}_2\text{O}$ /Ringer. The partial

TABLE I

AVERAGE SIZE OF VESICLES PRODUCED BY CONTINUOUS SONICATION IN VARIOUS MEDIA

La³⁺ 'mock Ringer' consists of Ringer solution in which the divalent salts have been replaced with sufficient La(NO₃)₃ to give the appropriate ionic strength.

Lipid	Aqueous media	Average diameter of vesicles (Å)
Diphytanoyl phosphatidyl choline	5 mM La(NO ₃) ₃	280–360
	20 mM La(NO ₃) ₃	300–400
	100 mM La(NO ₃) ₃	200–250
	La ³⁺ 'mock Ringer'	490–635
	² H ₂ O/Ringer	220–330
Dipalmitoyl phosphatidylcholine	² H ₂ O/Ringer	250

specific volume of the two lipids can be estimated from their sedimentation rates in the two media using the Svedrup equation. The value of the partial specific volume calculated for dipalmitoyl phosphatidylcholine vesicles is 0.950 ml/g at 25°C (literature value 0.948 ml/g at 25°C [9]), while that for diphytanoyl phosphatidylcholine vesicles is 1.013 ± 0.026 ml/g at 25°C.

Sonicated suspensions of diphytanoyl phosphatidylcholine were verified to be single-walled bilayer vesicles by proton NMR. When Eu(NO₃)₃ (5 mM) is added externally to a sonicated suspension of diphytanoyl phosphatidylcholine prepared in the presence of 5 mM La(NO₃)₃ solution, two choline methyl proton NMR signals are observed. On the basis of earlier work [9] these signals can be assigned to the choline methyl protons on the outer and inner sides of bilayer. The ratio of the intensities of the outer to the inner choline methyl proton resonances ranges from 1.4 to 1.7. Another determination of the number ratio is obtained by measuring the area of the choline methyl proton resonance in the absence and presence of Mn²⁺ in the extravesicular solution. In this latter experiment the ratio of the intensities of the outer/inner choline methyl signal is found to be approx. 1.7. These results suggest that the vesicles are approximately 300 Å in diameter. This same average size is found by electron microscopy.

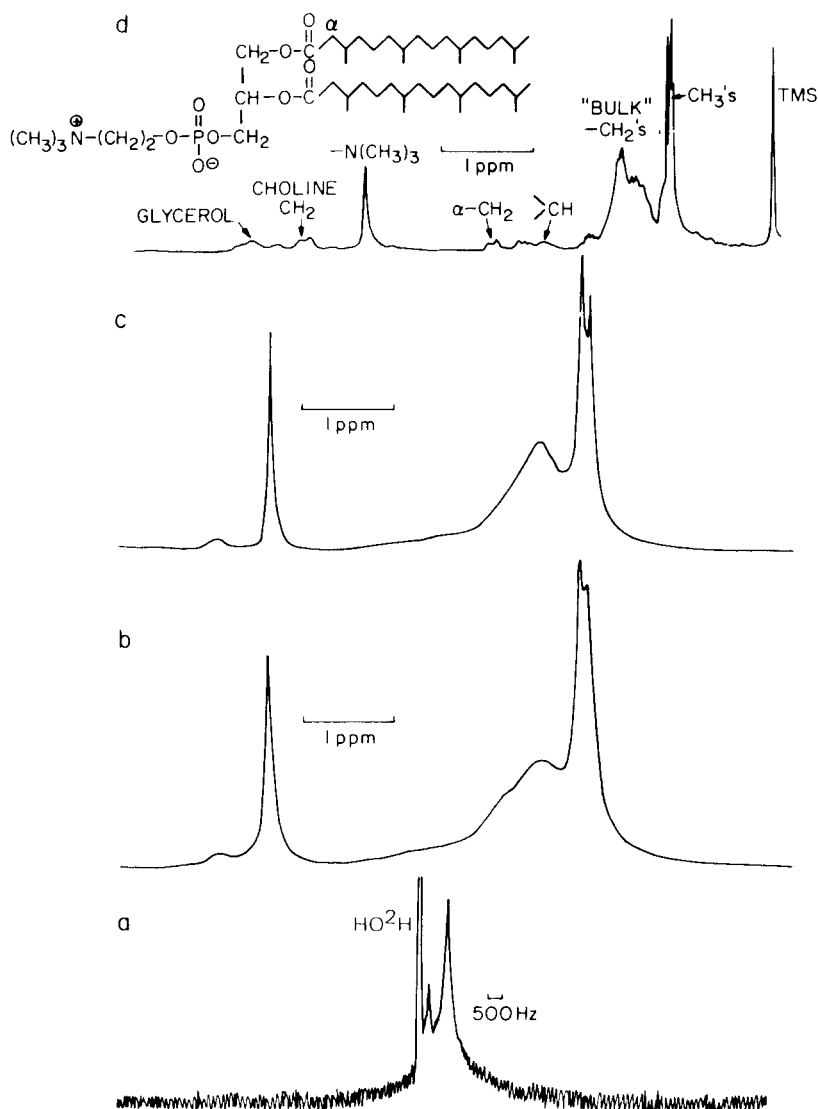
The permeability of the diphytanoyl phosphatidylcholine vesicles has also been determined from the time dependence of the separation between the outer and inner choline signals for a vesicle sample which has been prepared in 5 mM La(NO₃)₃ with Eu(NO₃)₃ added externally to a final concentration of 2.5 mM. When such a suspension is incubated for 75 h at 55°C, there is no significant change in the intensities or the separation of the inner and outer choline resonances showing that these membranes are impermeable to ions.

Nuclear magnetic resonance studies

Multilamellar dispersions. The frequency spectrum of a multilamellar suspension of diphytanoyl phosphatidylcholine at 65°C taken at 220 MHz in the continuous wave mode is shown in Fig. 3a. It consists of relatively sharp choline and hydrocarbon methyl resonances ($\Delta\nu \approx 350$ Hz) superimposed on a broad methylene-methyne proton signal ($\Delta\nu > 2000$ Hz). For comparison, we show in Fig. 3e the corresponding spectrum for a suspension of dipalmitoyl phos-

phatidylcholine multilayers. Note that the intensities of the chain and the choline methyl signals are quite comparable in the case of dipalmitoyl phosphatidylcholine, whereas the hydrocarbon chain methyls clearly dominate the continuous wave spectrum of the diphytanoyl phosphatidylcholine.

In an effort to discern the broader features of the diphytanoyl phosphatidylcholine spectrum we have also examined the proton NMR free induction decay of multilayer samples prepared from this lipid. A representative free induction decay taken at 57.4 MHz and at 20°C is shown in Fig. 4. The free induction decay appears to be comprised of two components, a rapidly decaying one and a much more slowly decaying one. As would be expected [9,10] the early portion of the decay is Gaussian (Fig. 5a). The effective decay constant, $T_{2(\text{eff})}$



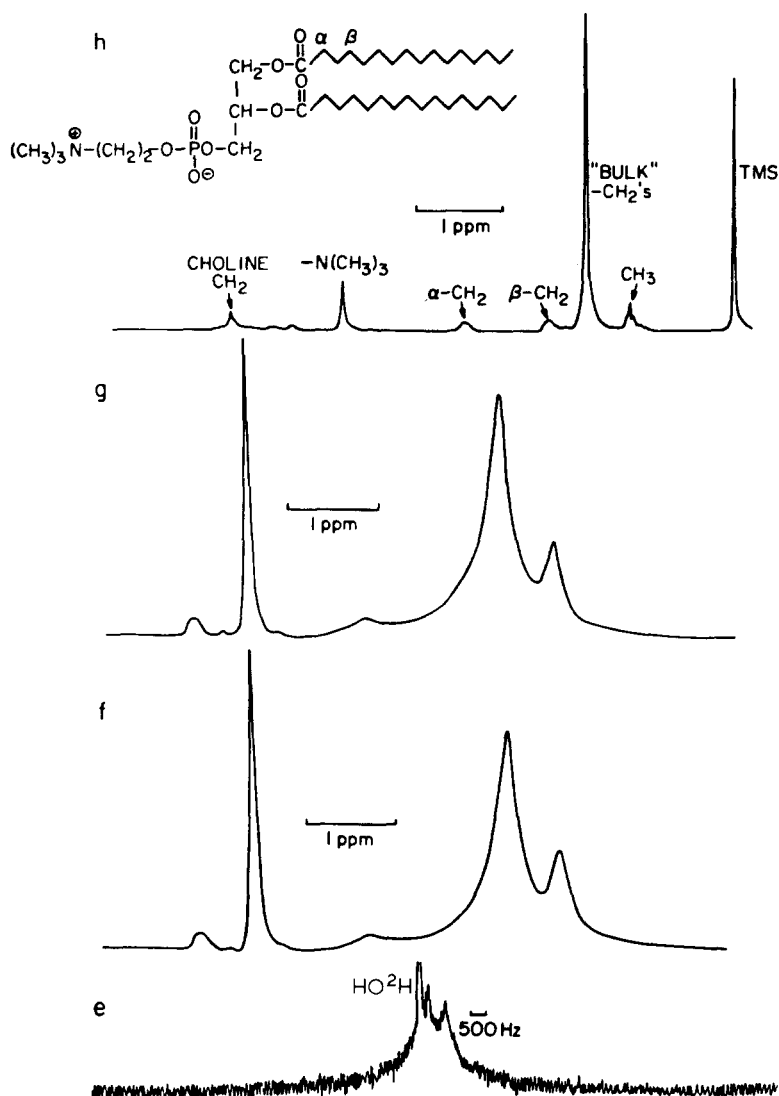


Fig. 3. PMR spectra. (a) 220 MHz spectrum of diphytanoyl phosphatidylcholine multilayer suspension in $^2\text{H}_2\text{O}$ /Ringer at 65°C (sweepwidth 20 KHz); (b) 100 MHz spectrum of sonicated diphytanoyl phosphatidylcholine vesicles in $^2\text{H}_2\text{O}$ /Ringer at 55°C (sweepwidth 1000 Hz); (c) 100 MHz spectrum of small sonicated diphytanoyl phosphatidylcholine bilayer vesicles in 5 mM $\text{La}(\text{NO}_3)_3$ at 55°C (sweepwidth 1000 Hz); (d) 220 MHz spectrum of diphytanoyl phosphatidylcholine in C^2HCl_3 at 20°C (sweepwidth 2500 Hz); (e) 220 MHz spectrum of dipalmitoyl phosphatidylcholine multilayer suspension in $^2\text{H}_2\text{O}$ /Ringer at 65°C (sweepwidth 20 kHz); (f) 100 MHz spectrum of sonicated dipalmitoyl phosphatidylcholine vesicles in $^2\text{H}_2\text{O}$ /Ringer at 55°C (sweepwidth 1000 Hz); (g) 100 MHz spectrum of small sonicated dipalmitoyl phosphatidylcholine vesicles in 5 mM $\text{La}(\text{NO}_3)_3$ at 55°C (sweepwidth 1000 Hz); (h) 220 MHz spectrum of dipalmitoyl phosphatidylcholine in C^2HCl_3 at 20°C (sweepwidth 2500 Hz).

[9], is approximately $75\ \mu\text{s}$ for this portion of the free induction decay. In the straight-chain lipids the rapidly decaying component accounts for about 70% of the signal in contrast to the present case where it constitutes roughly 40%. Since the methylene-methylene protons of the hydrocarbon chains of the branched

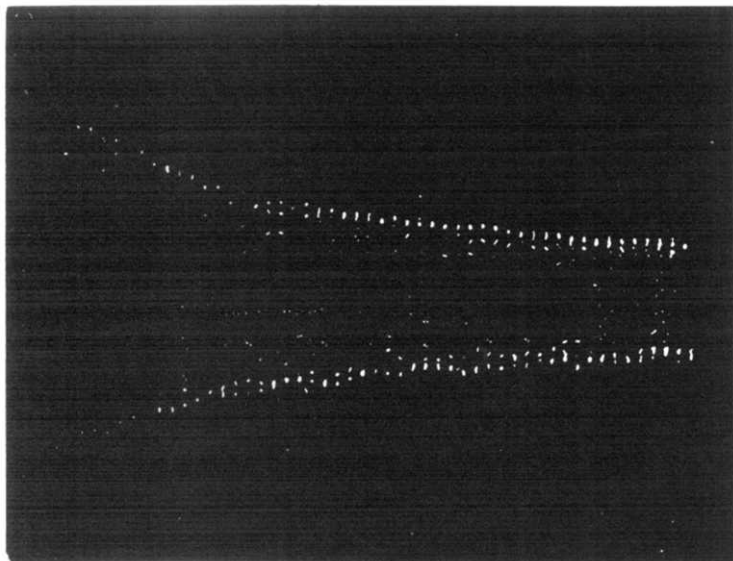


Fig. 4. A representative free induction decay of diphytanoyl phosphatidylcholine dispersed in $^2\text{H}_2\text{O}$ /Ringer taken at 57.4 MHz at 20°C . Total time elapsed is 500 μs , with each point corresponding to 0.5 μs .

lipids constitute about 40% of the total number of protons in the molecule, and since the very broad line (greater than 3000 Hz) in the frequency spectra of multilamellar samples of diphytanoyl phosphatidylcholine at 20°C are compatible with the 75 μs decay time, we assign the rapidly decaying component to these protons. The observed decay rate for the methylene groups is compatible with an effective order parameter $|S_{\text{eff}}| \approx 0.21$ for the methylene interproton vector in the hydrocarbon chain of diphytanoyl phosphatidylcholine [10]. This value is comparable to those found in many phospholipid systems [11].

The remaining more slowly decaying signal can be attributed to the choline and hydrocarbon methyl protons. These protons give rise to an order of magnitude narrower lines (approx. 400 Hz) in the frequency spectra of multilayers (Fig. 3a). As expected, the decay for this portion of the free induction decay is exponential with time (see Fig. 5b). The decay constant is approx. 570 μs .

Lipid/chloroform solutions. The frequency spectra taken at 220 MHz of diphytanoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine in deuteriochloroform (C^2HCl_3) together with their assignments are presented in Fig. 3d and h. Diphytanoyl phosphatidylcholine exhibits a series of overlapping doublets in the region of the methyl proton resonance which arise from the methyl branches on the diterpenoid hydrocarbon chain. This can be compared with the triplet which is observed for the terminal hydrocarbon methyl groups in dipalmitoyl phosphatidylcholine. The chemical shift range covered by the methyls in diphytanoyl phosphatidylcholine is larger than that found for the methyls in dipalmitoyl phosphatidylcholine due to the range of chemical environments experienced by these methyls. The chemical shift range for methyl protons in diphytanoyl phosphatidylcholine is approximately 0.11 ppm. The apparent linewidths observed for these hydrocarbon methyl

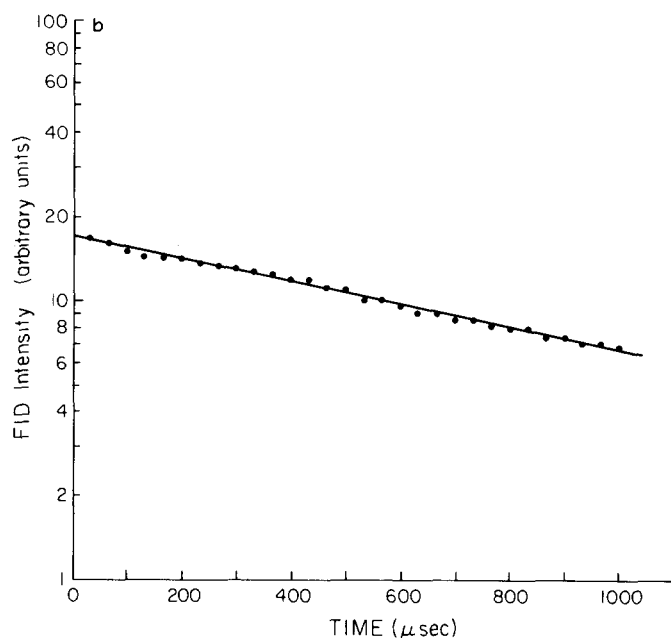
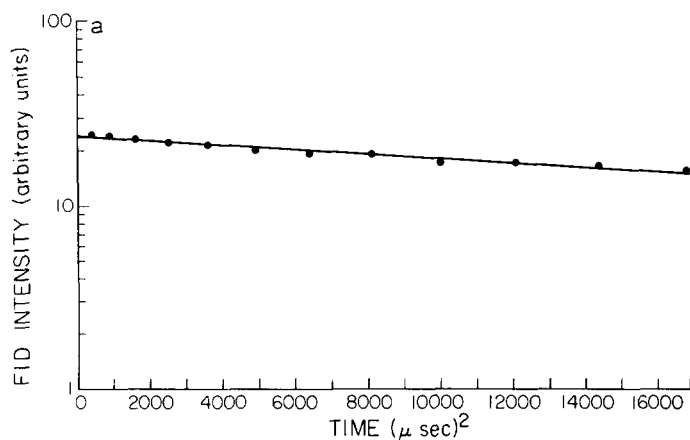


Fig. 5. Free induction decay components. (a) Plot of gaussian type decay of signal produced by hydrocarbon methylene protons of diphytanoyl phosphatidylcholine in multilayer sample. (b) Plot of exponential decay of portion of signal arising from hydrocarbon methyl protons of diphytanoyl phosphatidylcholine in multilayer sample.

signals in both lipids are governed partly by long-range spin-spin coupling and partly by molecular aggregation into inverse micelles.

The differences in chemical environments along the chain which produced the observed range of chemical shift values for the hydrocarbon methyl resonances has an even more pronounced effect on the spectral positions of the acyl methylene and methyne proton signals (see Fig. 3d). The range of chemical shift values observed for the acyl methylene-methyne protons in

diphytanoyl phosphatidylcholine extends from approx. 1.14 ppm to approx. 2.4 ppm ($\Delta\delta \approx 1.26$) in C^2HCl_3 . Due to complex spin-spin coupling and slight differences in chemical shifts, the homogeneous linewidth for the methylene protons in diphytanoyl phosphatidylcholine cannot be ascertained. By comparison all the methylene protons in dipalmitoyl phosphatidylcholine with the exception of those α and β to the carbonyl have very nearly the same chemical shift (see Fig. 3h).

The resonances observed for the methyl protons associated with the choline headgroup are sharp in both diphytanoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine. The choline methyl resonance occurs at $\delta = 3.35$ ppm in diphytanoyl phosphatidylcholine and $\delta = 3.31$ ppm in dipalmitoyl phosphatidylcholine. The slight broadening of these resonances may be attributable to inverse micelles in the sample. The remaining signals in the spectra arise from the methylene and methyne groups associated with the choline and glycerol moieties.

Vesicle suspensions. Fourier transform NMR spectra taken at 100 MHz at 55°C of sonicated suspensions of diphytanoyl and dipalmitoyl phosphatidylcholine prepared in 5 mM $\text{La}(\text{NO}_3)_3$ and $^2\text{H}_2\text{O}$ /Ringer are shown in Fig. 3b, c, f and g. In the diphytanoyl phosphatidylcholine vesicles the series of doublets observed for the acyl methyl protons in C^2HCl_3 have coalesced into one doublet at the temperature at which these spectra were taken. The doublet is a result of scalar spin-spin coupling to the methyne proton ($J \approx 5$ Hz) as this splitting was found to be independent of the magnetic field strength. At temperatures below 30°C the methyl signal broadens to the extent that the doublet is unresolved. The linewidth for the methyl resonances at high temperature are comparable to the chemical shift differences between various methyl signals observed in the spectrum of this lipid in C^2HCl_3 . In the dipalmitoyl phosphatidylcholine vesicles the hydrocarbon methyl triplet seen in C^2HCl_3 solution is broadened and poorly resolved.

The sharp methylene and methyne proton resonances observed for diphytanoyl phosphatidylcholine in C^2HCl_3 appear in sonicated suspensions as an envelope which spans the entire chemical shift range observed for these protons in C^2HCl_3 . No individual resonances are distinguishable. In fact, the lineshape for the methylene-methyne protons is distinctly asymmetric with an apparent linewidth (56–74 Hz at 100 MHz) partly determined by chemical shift dispersion. In contrast, for dipalmitoyl phosphatidylcholine vesicles at temperatures exceeding its gel to liquid crystalline phase transition temperature, it is possible to distinguish the signal arising from the α -methylene protons from that arising from the remaining hydrocarbon methylenes. Here the bulk of the methylene protons produce a sharp, reasonably symmetric signal at 100 MHz ($\Delta\nu < 20$ Hz).

The methyl protons associated with the choline headgroup in both lipids give rise to sharp signals in vesicle suspensions ($\Delta\nu \approx 6$ Hz at 100 MHz).

Vesicle suspensions of 10 mol% diphytanoyl phosphatidylcholine in diper-deuteropalmitoyl phosphatidylcholine yield NMR spectra with resonance positions and widths similar to those described above for the pure diphytanoyl phosphatidylcholine vesicles. However, at temperatures in excess of the gel to liquid crystalline phase transition temperature of the deuterated lipid (37.5°C)

there is a slight contribution to the peak height in the methylene region of these spectra from the residual methylene protons on the deuterated host lipid [6].

A four fold increase in the methylene proton signal linewidth is observed for the diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine on cooling from 60°C to 10°C (Fig. 6). This temperature range includes the gel to liquid crystalline phase transition temperature of the deuterated host lipid. The dependence of the methylene proton signal linewidth on temperature is most marked in the region of T_c for diperdeuteropalmitoyl phosphatidylcholine. This abrupt change in CH_2 linewidth is not observed over this temperature range for pure diphytanoyl phosphatidylcholine. For comparison the change in linewidth which has been observed for the dipalmitoyl phosphatidylcholine methylene proton signal in vesicle suspensions over the same temperature range is also included in Fig. 6. Here there is a 50 fold increase in linewidth on cooling the vesicle sample through the dipalmitoyl phosphatidylcholine phase transition [12]. These results taken together indicate that the temperature dependence of the methylene proton signal linewidth for diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine is a consequence of the gel to liquid crystalline phase transition in the deuterated lipid. The resonances observed from the methylene protons of diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine at temperatures above T_c for the host lipid are narrower than those observed in the pure lipid at comparable temperatures. This difference in linewidth is due in part to changes in average size of vesicles in the two samples as established by electron microscopy.

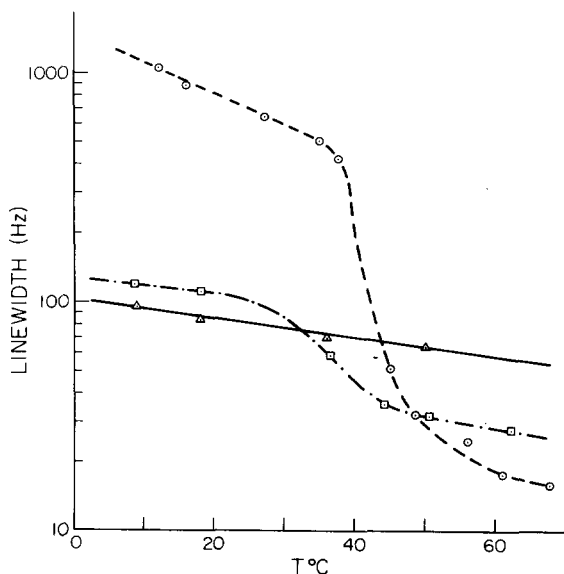


Fig. 6. Observed PMR linewidths of the hydrocarbon chain methylene protons at 220 MHz. $\circ$, small sonicated dipalmitoyl phosphatidylcholine vesicles (measurements below the thermal phase transition were obtained using 20 kHz sweepwidth); $\square$, vesicles prepared by sonicating a mixture of 10 mol% diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine; $\triangle$, sonicated diphytanoyl phosphatidylcholine vesicles as a function of temperature.

Methylene spin lattice relaxation rates. A comparison of the hydrocarbon chain methylene proton spin lattice relaxation rates for diphytanoyl phosphatidylcholine vesicles and for vesicles containing 10 mol% diphytanoyl phosphatidylcholine in a diperdeuteropalmitoyl phosphatidylcholine matrix at various temperatures and at two NMR frequencies (100 and 220 MHz) is shown in Fig. 7. For comparison we have included data [6] obtained on dipalmitoyl phosphatidylcholine under similar conditions. In the pure lipid the spin lattice relaxation rates ($1/T_1$) for the methylene protons decrease with increasing temperature at both NMR frequencies. In the mixture at temperatures well below the phase transition temperature for the deuterated host lipid the spin lattice relaxation rates resemble closely those found for the pure lipid at the same temperatures. This is especially evident at 220 MHz where the values are identical within experimental error. The relaxation rates in the mixture

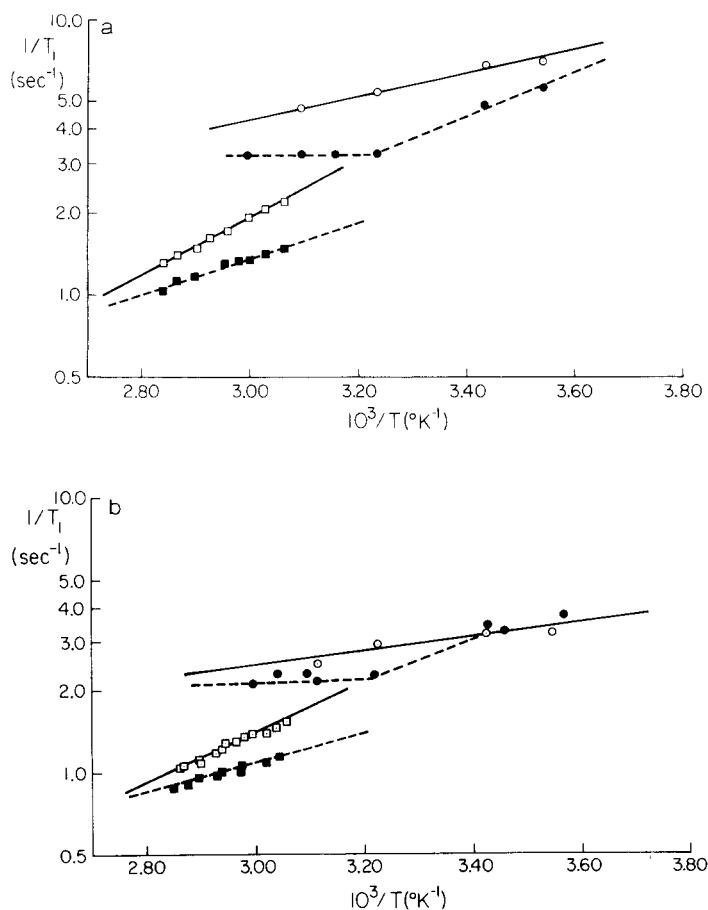


Fig. 7. Spin lattice relaxation rates ($1/T_1$) of the hydrocarbon methylene protons in sonicated bilayer vesicles as a function of reciprocal temperature and at two NMR frequencies: (a) 100 MHz; (b) 220 MHz. $\circ$, diphytanoyl phosphatidylcholine; $\bullet$, 10 mol% diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine; $\square$, dipalmitoyl phosphatidylcholine; $\blacksquare$, 10% dipalmitoyl phosphatidylcholine dispersed in 90% diperdeuteropalmitoyl phosphatidylcholine.

decrease with increasing temperature at both NMR frequencies up through the T_c for the host lipid, at which point the plots of $1/T_1$ versus the inverse of the temperature show a discontinuity.

At temperatures in excess of the phase transition temperature for the host lipid, it was noted that the plots of $\ln(h(\infty) - h(\tau))$ versus τ , from which the $1/T_1$ values are determined, become non-linear. The reason for this non-linearity, which is more pronounced at 100 MHz than at 220 MHz, is that the residual protons on the acyl moiety of the deuterated host lipid contribute to the observed peak height in the partially relaxed spectra, particularly for τ values around the 'null point'. To compensate for this the methylene peak areas of spectra taken at 100 MHz at identical temperatures and τ for a sample of 10 mol% diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine and for a sample containing an identical amount of diperdeuteropalmitoyl phosphatidylcholine alone were subtracted. From these data the corrected spin lattice relaxation rates plotted in Fig. 7 were obtained. This procedure proved to be unnecessary for the data taken at 220 MHz. The corrected spin lattice relaxation rates at 100 MHz for the methylene protons of diphytanoyl phosphatidylcholine show no temperature dependence at temperatures in excess of T_c for the host lipid, while the data at 220 MHz show only a very slight decrease with increasing temperature.

The signal from the methyne protons of the phytanic acid chain is masked by the hydrocarbon methylene signal envelope in the vesicle spectra. Accordingly, it is not possible to independently determine a spin lattice relaxation rate for the methyne protons. Also, in order to ensure that the T_1 values determined for the methylene protons were not influenced by the methyne relaxation, peak heights were sampled in the chemical shift region (1.15 ppm) well removed from that characteristic of the methyne protons (2.05 ppm).

Methyl spin lattice relaxation rates. The spin lattice relaxation rates for the hydrocarbon methyl protons in vesicle samples of pure diphytanoyl phosphatidylcholine and diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine host lipid at various temperatures and two NMR frequencies (100 and 220 MHz) are summarized in Fig. 8. It should be noted that all the hydrocarbon methyl protons appear to undergo spin lattice relaxation at the same rate. However, since the relaxation rate is rapid, the rate difference between any two methyls must be greater than 20% of the measured $1/T_1$ to permit observation of a relaxation rate difference exceeding the experimental error inherent in the measurement.

In the pure lipid, the spin lattice relaxation rates for the methyl protons are very similar to those found for the methylene protons. These relaxation rates decrease with increasing temperature at both NMR frequencies.

In the mixture of diphytanoyl phosphatidylcholine with diperdeuteropalmitoyl phosphatidylcholine at temperatures well below the phase transition temperature for the deuterated host lipid, the longitudinal relaxation rates of the methyls of the branched-chain lipid resemble closely those found for the pure lipid. Within experimental error at 220 MHz these rates for the pure lipid and mixture are indistinguishable. In the mixture, the relaxation rates decrease with increasing temperature at both NMR frequencies until a temperature of roughly 50°C is reached. At this temperature the plots of

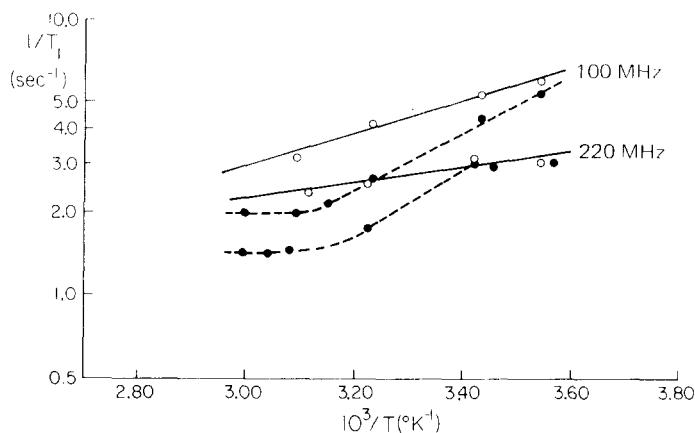


Fig. 8. Spin lattice relaxation rates ($1/T_1$) of the hydrocarbon methyl protons in sonicated bilayer vesicles as a function of reciprocal temperature and at two NMR frequencies (100 and 220 MHz). ○, diphytanoyl phosphatidylcholine; ●, 10 mol% diphytanoyl phosphatidylcholine dispersed in diperdeuteropalmitoyl phosphatidylcholine.

($1/T_1$) versus the inverse of the temperature show a discontinuity. This observation was noted both for the data obtained at 100 MHz and 220 MHz. At higher temperatures there appears to be no temperature dependence at 100 MHz and only a very slight one at 220 MHz.

Discussion

We have established that diphytanoyl phosphatidylcholine forms a lamellar bilayer phase in aqueous media and that many of the physicochemical properties of diphytanoyl phosphatidylcholine in the hydrated state are essentially those found for the more conventional straight-chain lipids. However, properties exhibited by diphytanoyl phosphatidylcholine bilayers are sufficiently different from those of straight-chain and unsaturated phosphatidylcholines that a comparison of these properties can provide a means of assessing the merits of various models which have been applied to lipid bilayers.

The absence of a thermal phase transition

We have obtained no evidence for a gel to liquid crystalline phase transition for diphytanoyl phosphatidylcholine bilayers over a temperature range extending from -120°C to $+120^\circ\text{C}$. In lipids the order-disorder phase transition is presumed to be associated with the onset of formation of kinks and other similar chain conformations involving *trans-gauche* conformers in the acyl chain [13–15]. Consequently, it is expected that the phase transition enthalpy and the temperature will be affected by the sum of the potential energies associated with the various rotamer states of the molecules in the bilayer. The overall potential energy is thought to be comprised of contributions from the intrachain [16] and interchain [17] interactions as well as the long range van der Waal's interactions [18]. In straight-chain lipids the relative stability of the *trans* vs. *gauche* rotamers in conjunction with the large lateral packing density

of the chains in the bilayer below the thermal phase transition are probably responsible for the comparatively large enthalpies (approx. 10 kcal/mol) and high temperatures (approx. 300 K) observed for their gel to liquid crystalline phase transitions. In contrast, the presence of methyl groups at regular intervals along the acyl chain, as with the phytanic acyl group, causes the *trans* and one of the *gauche* rotamers to be energetically more nearly equivalent. Moreover, the steric requirements of the methyl branches which prevent efficient lateral packing of the acyl chains should result in decreased short-range intermolecular interactions in the bilayer. Under these conditions, any order-disorder transition might, therefore, be expected to be a low-temperature and low-enthalpy transition, easily outside the temperature range studied here or beyond the detection limits of differential thermal analysis.

An expanded bilayer membrane

Bilayers formed from diphytanoyl phosphatidylcholine are expected to be 'expanded'. This expectation is corroborated by the observation that this lipid possesses a partial specific volume which is larger than any heretofore observed for other lipids [9,19]. This difference in the partial specific volume between diphytanoyl phosphatidylcholine and other lipids reflects, in part, the larger lateral close packing area in the branched-chain lipid. A recent monolayer study on lipids extracted from extreme halophilic bacteria yielded a close packing area of approx. 60 Å² for these lipids in comparison with the value of 45–50 Å² for egg yolk phosphatidylcholine [2].

Inasmuch as the close packing area in lipid bilayers is governed by the lipid chains, an implication of these data is that the area available to the choline head group in diphytanoyl phosphatidylcholine is abnormally large. The chemical shift of the choline methyl protons in dipalmitoyl phosphatidylcholine in vesicles has been shown to be dependent on the packing of the hydrocarbon chains. In particular, one observes a small chemical shift difference between the inner and outer choline groups in bilayer vesicles of 250–300 Å in diameter [9], presumably because of the packing differences of the molecules between the two monolayers of the bilayer specifically in the region of the polar head groups [20]. Such a chemical shift difference between the inner and outer choline signals is not observed in diphytanoyl phosphatidylcholine vesicles, even when these vesicles are of comparable sizes. This negative result suggests that here the chemical shift of the choline methyl protons is insensitive to the packing density of the molecules. The insensitivity most likely reflects the 'expanded' nature of the bilayer with the concomitant increased separation between contiguous choline moieties.

In spite of the expected smaller packing density for the diphytanoyl phosphatidylcholine, there appears to be no large effect on the order parameter associated with the chain methylene protons. This is in accord with a recently developed motional model for the hydrocarbon chain of phospholipids [21]. In this, it was shown that the measured NMR order parameter may be expressed by a product of two independent components so that

$$S_{\text{meas}} = S_{\alpha} \cdot S_{\gamma}$$

Here S_{γ} is a measure of intrachain order and is related to the fraction of *gauche*

rotamers present in the chain, while S_α reflects the amplitude of chain reorientation relative to a director along the bilayer normal. In qualitative terms, the value of about -0.21 estimated here for the order parameter of the interproton vector in the methylene group of the diphytanoyl phosphatidylcholine may be understood as follows. The smaller energy difference between the *trans* and *gauche* conformations suggest that one would expect less intrachain order and thus a smaller S_γ for this lipid than for, for example, dipalmitoyl phosphatidylcholine. However, the looser chain packing should decrease the cooperativity of the chain fluctuations causing the low frequency, high amplitude motions to diminish. The resulting decrease in range of amplitude of chain reorientation would cause an increase in S_α and compensate for the smaller S_γ , so that the product is comparable to that observed for other phospholipids.

Miscibility of diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine

No strong temperature dependence of the hydrocarbon methylene resonance is observed for diphytanoyl phosphatidylcholine vesicles. In contrast, when diphytanoyl phosphatidylcholine is incorporated in diperdeuteropalmitoyl phosphatidylcholine in vesicles, the hydrocarbon methylene linewidth becomes strongly temperature dependent especially in the range of the gel to liquid crystalline phase transition temperature (T_c) of the deuterated host lipid (see Fig. 6). It is interesting that in the temperature range below the T_c of the deuterated host lipid the hydrocarbon methylene linewidth of diphytanoyl phosphatidylcholine is similar to that observed for this resonance in the pure lipid at these temperatures. In turn, these linewidths are approx. ten times narrower than those observed for the same resonance arising from pure dipalmitoyl phosphatidylcholine vesicles over this temperature range. This may be taken as evidence for phase separation of diphytanoyl phosphatidylcholine from diperdeuteropalmitoyl phosphatidylcholine at a temperature near the T_c of the host lipid. In other words, diphytanoyl phosphatidylcholine does not appear to be soluble in or miscible with diperdeuteropalmitoyl phosphatidylcholine when the latter is not in the liquid crystalline state. This is the same conclusion which was drawn from the results of the differential thermograms.

This picture of immiscibility of diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine at temperatures below the T_c of the diperdeuteropalmitoyl phosphatidylcholine is corroborated by the observation of a discontinuity in the spin lattice relaxation rates of the hydrocarbon methylene protons of diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine over the temperature range which includes the T_c of the deuterated lipid. It has been shown for dipalmitoyl phosphatidylcholine that there is a small intermolecular contribution to the spin lattice relaxation rates of the hydrocarbon methylene protons [6]. We would expect, therefore, an intermolecular contribution to the hydrocarbon methylene proton spin lattice relaxation rates in pure diphytanoyl phosphatidylcholine. In the mixture of diphytanoyl phosphatidylcholine in diperdeuteropalmitoyl phosphatidylcholine at temperatures above the gel to liquid crystalline phase transition temperature for the deuterated lipid, we expect this intermolecular contribution to the hydrocarbon methylene spin lattice relaxation to be signifi-

cantly reduced. However, should phase separation occur when the lipid mixture is cooled to temperatures below the T_c of the deuterated host lipid, this intermolecular contribution to the methylene proton spin lattice relaxation rate would reappear. We feel that this is the origin of the discontinuity in the $(1/T_1)$ vs. temperature curves for the mixture of 10 mol% diphytanoyl phosphatidylcholine in dipalmitoyl phosphatidylcholine.

From these studies, we conclude that branching of the hydrocarbon chains of phosphatidylcholines maintains these lipids in the fluid state over a broad range of temperatures. This may be surprising since all indications are that the purple membrane of halobacterium is highly ordered [22]. However, we expect protein incorporation to lead to condensation ordering of the lipid matrix, particularly at the high concentration of protein found in the purple membrane. Moreover, if the lipid matrix itself were too ordered to begin with, it might not be thermodynamically feasible to insert the bacteriorhodopsin into the bilayer.

Motional state of the hydrocarbon chains

It is well known that the motional state of the hydrocarbon chains of phospholipid molecules in bilayers can be ascertained from their NMR spin lattice relaxation behavior. Proton spin lattice relaxation rates in lipid bilayer membranes have been found to decrease with increasing temperature and frequency of irradiation. This combination of temperature and magnetic field dependence can be accounted for if the relevant dipolar interactions are being modulated by at least two motions with different correlation times, $\tau_{\parallel}$ and $\tau_{\perp}$, such that $(\omega_0\tau_{\parallel})^2 \ll 1$ and $(\omega_0\tau_{\perp})^2 > 1$. In the presence of this anisotropic motion the spin lattice relaxation rate can be approximated by [6]

$$1/T_1 \simeq A\tau_{\parallel} + B/\omega_0^2\tau_{\perp}$$

The first term usually dominates the observed temperature dependence whereas the magnetic field dependence is contained in the second term. Petersen and Chan [21] have recently proposed that $\tau_{\parallel}$ is the correlation time associated with kink diffusion along the hydrocarbon chain, while $\tau_{\perp}$ is the correlation time connected with the reorientation of the hydrocarbon chain with respect to the bilayer normal. In the case of dipalmitoyl phosphatidylcholine bilayer vesicles the timescales of these two correlation times have been estimated to be $\tau_{\parallel} \approx 10^{-10}$ – 10^{-11} s and $\tau_{\perp} \approx 10^{-7}$ – 10^{-8} s [21] for the hydrocarbon methylene protons.

The spin lattice relaxation rates of the hydrocarbon protons in diphytanoyl phosphatidylcholine show a similar temperature and magnetic field dependence to that observed for other lipids. However, the spin lattice relaxation rates for the methylene hydrocarbon protons in diphytanoyl phosphatidylcholine are approximately two to three times larger than those observed for, for example, dipalmitoyl phosphatidylcholine. The frequency dependence of these spin lattice relaxation rates is also much more pronounced. These observations indicate that the two correlation times which are affecting the spin lattice relaxation rates of the hydrocarbon methylene protons in diphytanoyl phosphatidylcholine are approaching one another as well as the frequency of NMR observation. Specifically, calculations based on the anisotropic motional model pre-

sented above indicate that the frequency dependence observed for the diphytanoyl phosphatidylcholine spin lattice relaxation rates can be accounted for if $10 < \tau_{\perp}/\tau_{\parallel} < 100$. The activation energy for β -coupled *gauche* rotations involving methyl branch points in the phytanic acyl chain can be estimated to be approximately 4 kcal/mol [23]. According to the model of Petersen and Chan [21] this activation energy can be used to estimate the jump rate of a kink in the hydrocarbon chain and from hence $\tau_{\parallel}$. The predicted jump rate is 10^{-11} – 10^{-10} s and $\tau_{\parallel} \approx 10^{-9}$ – 10^{-8} s, a result which is in accord with experiments. Since it appears that $\tau_{\perp}$ and $\tau_{\parallel}$ are separated by about a factor of 10, this value for $\tau_{\parallel}$ would lead to a $\tau_{\perp} \approx 10^{-8}$ – 10^{-7} s.

The temperature and frequency dependence of the longitudinal relaxation rates of the hydrocarbon methyl protons in diphytanoyl phosphatidylcholine bilayer vesicles closely resemble that of the hydrocarbon methylene protons. However, this rate of relaxation appears abnormally rapid in comparison with the spin lattice relaxation rates customarily observed for hydrocarbon methyl protons in lipid bilayers. For example, it is approximately ten times more rapid than that observed for the terminal methyl groups of dipalmitoyl phosphatidylcholine bilayers. In straight-chain phosphatidylcholine bilayer vesicles the internal rotation of the terminal methyl top is very rapid leading to inefficient relaxation and small ($1/T_1$) values [24]. In diphytanoyl phosphatidylcholine steric factors may slow the spinning of the methyl top causing more efficient relaxation. Indeed, the data show that the methyl groups appear to be slowed to a rate approximating the rate of kink propagation in this system.

Conclusions

The introduction of an acyclic diterpenoid hydrocarbon chain into a phosphatidylcholine engenders major modifications to the lipid's behavior in comparison with straight-chain phosphatidylcholines in aqueous systems. These modifications undoubtedly arise from the steric effects of the methyl groups at the branch points along the hydrocarbon chain. The most interesting and significant consequence is that there is a loss of the gel to liquid crystalline phase transition which is characteristic of the straight-chain synthetic phosphatidylcholines and phosphatidylcholines extracted from biological sources. This study also indicates that the diphytanoyl bilayer is more expanded compared with more conventional bilayer systems. Despite the apparent loss of long-range order in the hydrocarbon region of the diphytanoyl phosphatidylcholine bilayer, the order parameter found for this lipid falls within the range of values obtained for other liquid crystalline systems.

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