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PHASE BEHAVIOR OF THE MAJOR LIPIDS OF *TETRAHYMENA* CILIARY MEMBRANES

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The major lipids of *Tetrahymena* membranes have been purified by thin-layer and high pressure liquid chromatography and the phosphatidylethanolamine and aminoethylphosphonate lipids were examined in detail. ^{31}P-NMR, X-ray diffraction and freeze-fracture electron microscopy were employed to describe the phase behavior of these lipids. The phosphatidylethanolamine was found to form a hexagonal phase above 10°C. The aminoethylphosphonate formed a lamellar phase up to 20°C, but converted to a hexagonal phase structure at 40°C. Small amounts of phosphatidylcholine stabilized the lamellar phase for the aminoethylphosphonate. ^{31}P-NMR spectra of the intact ciliary membranes were consistent with a phospholipid bilayer at 30°C, suggesting that phosphatidylcholine in the membrane stabilized the lamellar form, even though most of the lipid of that membrane prefers a hexagonal phase in pure form at 30°C. ^{31}P-NMR spectra also showed a distinctive difference in the chemical shift tensor of the aminoethylphosphonolipid, when compared to that of phosphatidylethanolamine, due to the difference in chemical structure of the polar headgroups of the two lipids.

Introduction

The membranes of *Tetrahymena* contain predominantly phosphatidylethanolamine, while phosphatidylcholine is a minor component (for review, see Refs. 1 and 2). The cilia of this protozoan are covered with a membrane which appears to be continuous with the plasma membrane, and the cilia themselves can be detached from the cell body by a relatively simple procedure to give a membrane preparation uncontaminated by internal cellular membranes; this preparation is comparable in purity to that of the erythrocyte ghost. Ciliary membranes from *Tetrahymena* are composed of approximately 60% phosphatidylethanolamine plus its analog, aminoethylphosphonolipid, together with 5–10% of a sphingolipid, ceramide aminoethylphosphonate. The remainder of the polar lipid of the ciliary membrane is 20% phosphatidylcholine plus minor components. It is of interest to ask how this membrane like that of *Escherichia coli*, achieves the bilayer structure while having such a high percentage of ethanolamine phospholipids, which may be expected to form hexagonal phases.

As has been shown previously [3], ^{31}P-NMR is a useful tool for exploring the phase behaviour of phospholipids. However, the importance of simultaneously studying the same systems with electron microscopy and X-ray diffraction for an accurate interpretation of the phase behavior has also been demonstrated [4]. Therefore, all three techniques

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PnE, aminoethylphosphonolipid.

are used here to study the phase behavior of the major individual lipids of *Tetrahymena* ciliary membranes. Only two of the three major lipids are studied in detail, because of the well-known propensity of the third major lipid, phosphatidylcholine, to form lamellar phases.

Materials

Egg phosphatidylcholine was purchased from Avanti Biochemicals. It exhibited a single spot when analyzed with two-dimensional thin-layer chromatography: First dimension: $CHCl_3$/CH_3OH/NH_4OH (65:25:5, v/v); second dimension: $CHCl_3$/$(CH_3)_2CO$/CH_3OH/CH_3COOH/H_2O (6:8:2:2:1, v/v). Phosphatidylethanolamine and aminoethylphosphonolipid were isolated from *Tetrahymena* as described below. Silica gel G plates (250 μm) were obtained from Fisher. ^2H_2O was purchased from Merck, Inc.

Methods

Preparation of cilia. *Tetrahymena pyriformis* W were grown as previously described [5], except that only 1% proteose-peptone and a growth temperature of 20°C were employed. Cells were recovered from the growth medium by centrifugation at 4°C at $4000 \times g$; the cell pellet was washed with cold buffer (25 mM Tris acetate, pH 7.5) and then deciliation was carried out by a modification of the method of Watson and Hopkins [6], employing glycerol rather than ethanol. Cells were removed from the deciliated suspension by two successive centrifugations at $800 \times g$, and cilia were pelleted by centrifugation at $10000 \times g$ for 10 min. The cilia were resuspended in distilled water containing sodium azide, to give a final protein concentration of 11.3 mg/ml, and were stored at 4°C until the ^{31}P-NMR spectrum was obtained. Protein concentration was determined by the biuret method [7].

Preparation of phospholipid fractions. Whole cell pellets were mixed with 4 volumes of chloroform/methanol (2:1, v/v) to give a Folch extraction. Lipids from the lower phase were subjected to thin-layer chromatography on Silica gel G plates with the solvent system chloroform/acetic acid/methanol/water (75:25:5:1.5, v/v) [8], which quantitatively separated the aminoethylphosphonolipid from phosphatidylethanolamine. Lipid zones were visualized with I_2 vapor by exposing each side of the plate to a stream of vapor, taking care to cover the center of the plate to protect it from exposure to I_2. Zones corresponding to PnE and PE were scraped from the plate, and the lipids were eluted with chloroform/methanol (2:1, v/v). Samples were dried by rotary evaporation until near dryness, and the last traces of acetic acid were removed by a gentle nitrogen stream. Rechromatography of purified lipid fractions in the same solvent system showed no significant contamination (< 1%) of either lipid by the other, as judged by intensity of ninhydrin staining. ^{31}P-NMR of the same samples in organic solvent confirmed this observation.

The phospholipids recovered from thin-layer chromatography were further purified by high-pressure liquid chromatography, using a Beckman Model 332 Liquid Chromatograph equipped with a Hitachi 100-10 spectrophotometer. The samples were run through an Ultrosphere-octadecyl precolumn, followed by an Ultrosphere-octyl column (Beckman Instruments); lipids were eluted by a programed gradient which began at 90% methanol/10% H_2O and decreased over a 10-min time period to 100% methanol. Lipids were detected by their absorbance at 210 nm. This procedure eliminated free fatty acids and oxidized phospholipids from the samples of phosphatidylethanolamine and aminoethylphosphonolipid to give a much purer preparation than that obtained directly from TLC.

Both the PE and phosphonolipid samples were divided into aliquots, and duplicate samples of about 1 mg each were transesterified by heating under nitrogen at 75°C for 30 min with 0.5 N HCl in methanol. Fatty acid methyl esters were extracted twice with two volumes of petroleum ether, dried under a nitrogen stream, and redissolved in 0.1 ml of benzene. Samples were subjected to gas-liquid chromatography on 15% HI-EFF-1BP (Applied Science Laboratories) as previously described [5].

Preparation of vesicles. Phosphatidylcholine and aminoethylphosphonolipid in the appropriate proportions were mixed in chloroform and evaporated to dryness under nitrogen, and then

under vacuum. The phospholipids were hydrated in 10 mM Tris buffer, 100 mM NaCl, pH 7.6, made from distilled, deionized water. Before hydrating the phospholipids, the oxygen content of the buffer was lowered by bubbling nitrogen through it. A dispersion of phosphatidylethanolamine together with aminoethylphosphonolipid was prepared in a similar manner.

The relative molar proportions of aminoethylphosphonolipid and phosphatidylcholine in the mixed vesicle preparation were estimated by solubilization of the lipids with deoxycholate to a detergent concentration of about 5%, followed by ^{31}P-NMR of the solubilized sample. The proportion of aminoethylphosphonolipid to phosphatidylcholine was approximately 5 : 1.

To prepare vesicles that would exhibit an isotropic ^{31}P-NMR resonance, hydrated egg phosphatidylcholine (50 mM) was sonicated with a Branson W350 probe type sonicator in an ice bath. The solution rapidly clarified (less than 5 min). Generally the vesicles were used the same day. However, when storage was necessary, they were stored under nitrogen at 4°C. The vesicles proved to be stable under these conditions for at least two days.

^{31}P-NMR. ^{31}P-NMR spectra of samples in 10-mm tubes were obtained at 81 MHz on a Bruker WP-200 Fourier transform spectrometer. Sample temperatures were as indicated on the figures and in the text, set by the temperature controller of the spectrometer within a range of ±1°C. Samples were incubated at the desired temperature for a minimum of 10 min before spectra were obtained. After each spectrum was taken, each sample was permitted to equilibrate at room temperature (23°C), so that the results presented do not represent a single heating curve. Spectra of 50 kHz width were obtained with 0.1 s between each scan. Continuous broadband proton decoupling (1 watt) was employed and 2000 data points collected. Line broadening of 100 Hz was introduced during signal enhancement. Experiments with smaller broadenings indicated no significant artifacts were introduced by this procedure. 10% $^{2}H_2O$ was included in the samples for spectrometer lock. High resolution samples were measured with 10-kHz width and 10 s between pulses, using 10 Hz line broadening.

X-ray and freeze-fracture. The methods for controlled temperature freeze-fracture and X-ray diffraction experiments have been described previously [9]. In short, the samples were divided again into two aliquots. One aliquot was placed in a Teflon-lined aluminum cell with mica windows for X-ray diffraction study. The diffraction patterns were recorded on a Frank-type camera mounted on a Jarrell-Ash microfocusing X-ray unit, and the exposure was recorded in eight hours while the sample temperature was kept constant during this time.

The other aliquot was used for freeze-fracture studies; 0.1 μl of the samples was sandwiched between two 75-μm thick copper foils, equilibrated at given temperatures for at least 10 min, and then rapidly quenched in liquid propane. All samples were fractured and replicated at −120°C in a Polaron E7500 unit at a vacuum of $5 \cdot 10^{-7}$ torr or better. The replicas were viewed in a Siemens 101 electron microscope.

Results

Characterization of purified lipid fractions

The purified phosphatidylethanolamine and aminoethylphosphonolipid fractions showed high percentages of polyunsaturated fatty acids, and the amount of 6,11-18:2 (cilienic acid) in the phosphonolipid was unusually high (Table I). These results are very similar to those reported by Pieringer and Conner [8], who analyzed lipids from the same strain of *Tetrahymena*, but the cells were grown at 25°C. This procedure does not detect glyceryl ether side chains, which are abundant in the phosphonolipid (see below). Our results are also similar to the fatty acid compositions of these two lipids reported by Nozawa and coworkers [10–12] despite the fact that their studies were performed with other strains of *Tetrahymena* (WH-14 and NT-1) maintained at different growth temperatures. In particular, Nozawa's group reports that NT-1 cells grown at 15°C have aminoethylphosphonolipid which contains 71% alkyl-acyl lipid (glyceryl ether lipid) and 29% diacyl lipid [12]. They further report that the purified alkyl aminoethylphosphonolipid has an unusually high proportion of 6,11-18:2 and 6,9,12-18:3, so that these two fatty acids together represent 87%

TABLE I

FATTY ACID COMPOSITIONS OF AMINOETHYLPHOSPHONOLIPID AND PHOSPHATIDYLETHANOLAMINE FRACTIONS PURIFIED FROM LIPID EXTRACTS OF TETRAHYMENA

Fatty acid	Percent composition [a] PE	Phosphonolipid PnE
12:0	1.7	0.2
14:0	8.0	4.0
16:0	3.4	4.2
9-16:1 + 17:0(i)	13.0	7.5
9-18:1	12.9	6.6
6,11-18:2	2.9	35.8
9,12-18:2	15.3	6.9
6,9,12-18:3	39.0	31.4
Other	3.8	3.4

[a] Methyl esters were prepared and quantitated as described in methods. This procedure measures only acyl groups; see Results for estimates of the glyceryl ether content of these lipids.

of the total acyl groups of the alkyl lipid. The purified phosphonolipid fraction from strain W grown at 25°C was reported to be composed of 62% alkyl-acyl and 38% diacyl species, while the purified phosphatidylethanolamine contained only 3% alkyl-acyl lipid [8]. While we did not determine the glyceryl ether content of our samples, we can infer that the phosphonolipid was predominantly the alkyl-acyl lipid (> 60%), and that the phosphatidylethanolamine sample contained virtually no alkyl-acyl lipid.

The phospholipid composition of cilia from *Tetrahymena* has been reported. Cilia from strain WH-14 grown at 25°C contained 33% PC, 11% PE and 49% aminoethylphosphonolipid [10]. Ciliary membranes from strain W grown at 29°C had a composition of 19% PC, 29% PE and 32% phosphonolipid (Kaneshiro, E.S., personal communication). The remaining lipids include aminoethylphosphonate, lysophospholipids and ceramide.

Phase behavior of Tetrahymena lipids; ^{31}P-NMR, X-ray and freeze-fracture electron microscope data

The shape of the ^{31}P-NMR resonance of phospholipids in aqueous dispersions is sensitive to the phase structure which they experience due to either partial or complete motional averaging of the ^{31}P chemical shift tensor (see Ref. 3, for additional information). In a phospholipid bilayer, motional averaging is largely confined to rotation about an axis perpendicular to the membrane surface, producing a spectrum of the shape seen in Fig. 1A. In a hexagonal phase additional motional averaging occurs about the cylinders composing that phase, producing a spectrum of the shape seen in fig. 1B. Fig. 1C shows the result of rapid isotropic rotation experienced in a small sonicated phospholipid vesicle resulting in the isotropic chemical shift associated with the particular phospholipid.

These characteristics of the ^{31}P-NMR spectrum are exploited to describe the phase behavior of the major phosphorus-containing lipids of the plasma membrane of *Tetrahymena*. Choline-containing phospholipids are well-known to exist in a lamellar phase near physiological temperature; therefore this study focuses on the ethanolamine-containing lipids.

^{31}P-NMR spectra of phosphatidylethanolamine isolated from *Tetrahymena* and characterized as described above are presented in Fig. 2. At 10°C and higher, spectra characteristic of hexagonal phase are observed.

^{31}P-NMR spectra of dispersions of the phosphonate derivative of phosphatidylethanolamine are presented in Fig. 3. At 10°C and 20°C, only

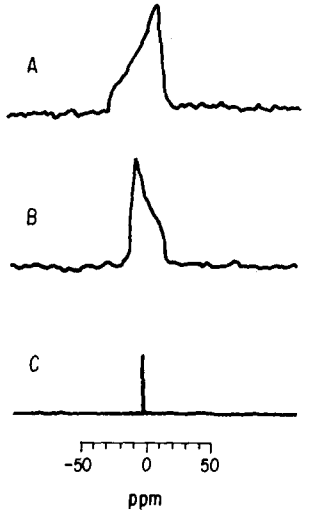

Fig. 1. 81 MHz ^{31}P-NMR spectra of phospholipids in 100 mM NaCl, 10 mM Tris, pH 7.6, at 30°C. (A) Unsonicated egg phosphatidylcholine; (B) unsonicated soy phosphatidylethanolamine; (C) sonicated egg phosphatidylcholine.

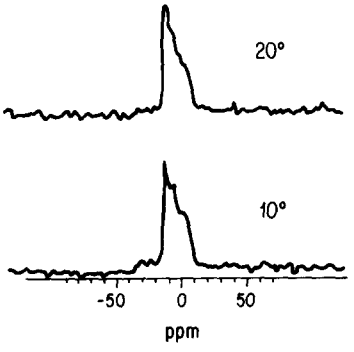

Fig. 2. 81 MHz ^{31}P-NMR spectra of phosphatidylethanolamine, isolated as described in the text from *Tetrahymena*, in 100 mM NaCl, 10 mM Tris, pH 7.6, at the temperatures indicated.

^{31}P-NMR spectra characteristic of lamellar phase are seen. At 30°C and higher, the spectra are complex and contain components resembling isotropic and hexagonal phase lipids.

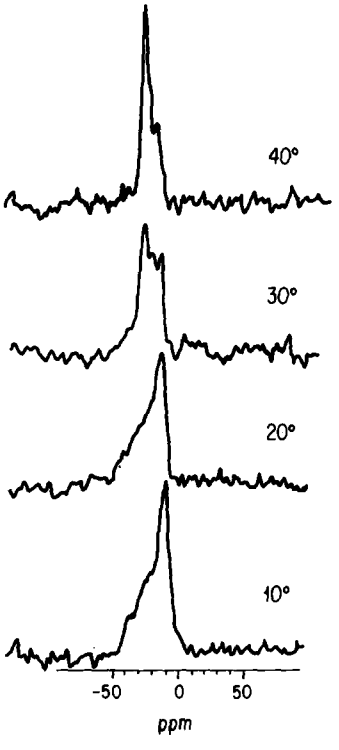

Fig. 3. 81 MHz ^{31}P-NMR spectra of aminoethylphosphonolipid, isolated as described in the text from *Tetrahymena*, in 100 mM NaCl, 100 mM Tris, pH 7.6, at the temperature indicated.

One factor which should be carefully noted is that the position of the ^{31}P-NMR resonances of the aminoethylphosphonolipid on the chemical shift scale is distinctly downfield from the position of normal phospholipids. This is emphasized in Fig. 4A in which the isotropic ^{31}P-NMR chemical shift of egg phosphatidylcholine is compared to that of aminoethylphosphonolipid. The approximately 20 ppm difference suggests that even with the broad resonances associated with aqueous phospholipid dispersions, some resolution of normal and phosphonate phospholipids should be possible. As anticipated, Fig. 4B shows a nearly complete resolution of two hexagonal phase resonances for a mixture of the normal and phosphono derivative of phosphatidylethanolamine from *Tetrahymena*.

Fig. 5 shows the effect of a small amount of egg phosphatidylcholine on the phase behavior of the phosphonolipid. As in the case of soy phos-

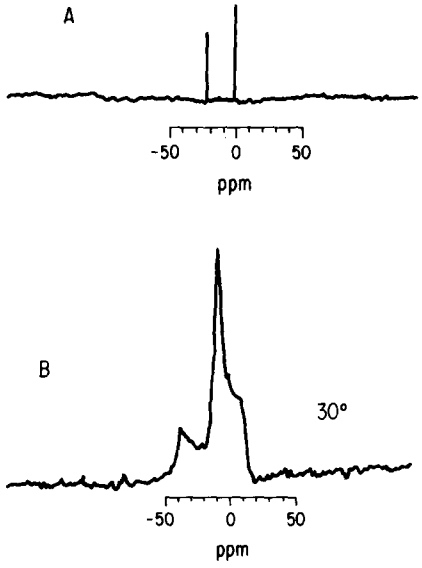

Fig. 4. 81 MHz ^{31}P-NMR spectra of lipids. (A) This spectrum arises from a mixture of egg phosphatidylcholine (the resonance on the right), and aminoethylphosphonolipid (the resonance on the left) in C^2HCl_3, which emphasizes the difference in the isotropic chemical shifts. (B) The aminoethylphosphonolipid and phosphatidylethanolamine were mixed initially in $CHCl_3$, dried, and hydrated in 100 mM NaCl, 10 mM Tris, pH 7.6 at 30°C. The observed spectrum consists of two slightly overlapping 'hexagonal' shaped spectra. The resonance on the left arises from the aminoethylphosphonolipid, while the resonance on the right arises from phosphatidylethanolamine.

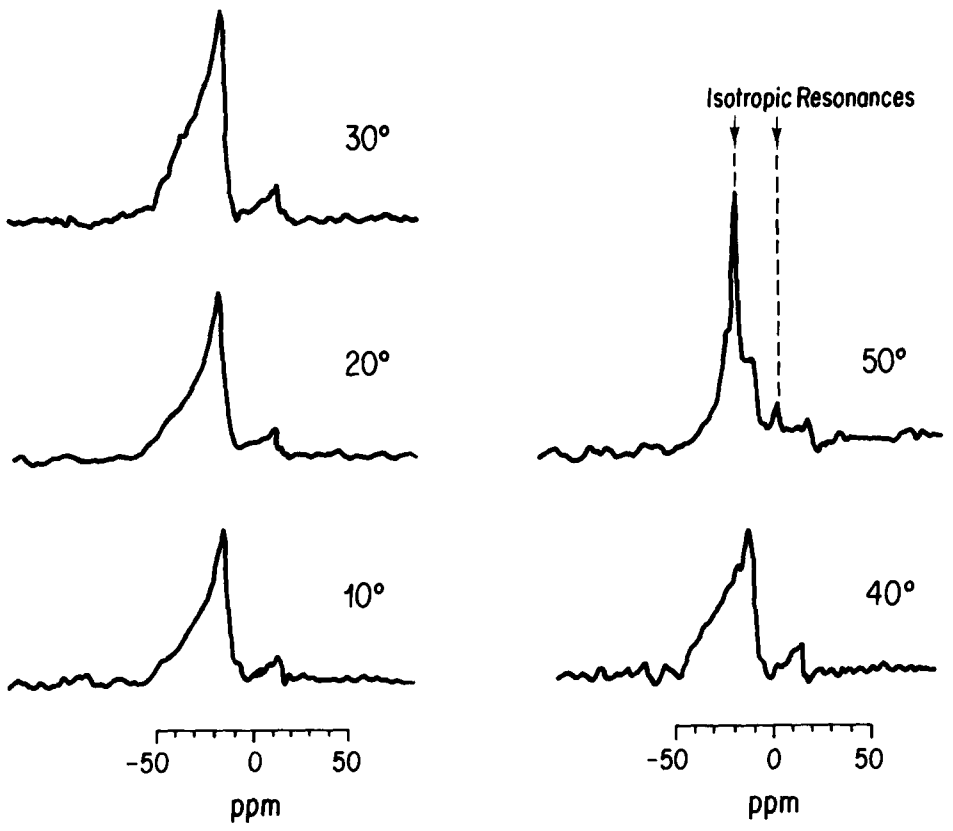

Fig. 5. 81 MHz ^{31}P-NMR spectra of a mixture of aminoethylphosphonolipid and egg phosphatidylcholine (5 : 1) in 100 mM NaCl, 10 mM Tris, pH 7.6, at the temperatures indicated. The spectra at most temperatures consist of overlapping 'bilayer' shaped resonances, the resonance on the right from phosphatidylcholine and the one on the left from aminoethylphosphonolipid. At 50°C an isotropic phase appears to which both phospholipids contribute and which consists of spontaneously generated small vesicles as demonstrated by freeze-fracture electron microscopy.

phatidylethanolamine, the lamellar phase of the aminoethylphosphonolipid is stabilized by the inclusion of small amounts of phosphatidylcholine [4]. At 50°C, isotropic resonances were observed. The spectra in Fig. 5 suggest the presence of two phases in the sample at 50°C, one exhibiting isotropic ^{31}P-NMR resonances and the other anisotropic ^{31}P-NMR resonances.

Finally, given these results, one could expect to see some resolution of the phosphate and phosphonate lipids in the ^{31}P-NMR spectrum of the intact ciliary membrane. Fig. 6 fulfills this expectation. Two overlapping lamellar resonances are observed at 30°C in these cilia. No isotropic or hexagonal shaped resonances are present. Thus, despite the high PE/PnE content of these membranes, the bilayer structure is clearly observed. The phosphonolipid content of our cilia preparation was estimated by simulation of a bilayer spectrum (an assumption) for the phospholipid component of Fig. 6, followed by subtraction of

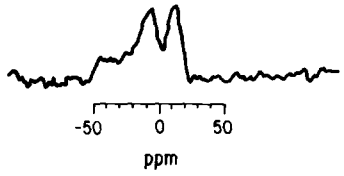

Fig. 6. 81 MHz ^{31}P-NMR spectra of *Tetrahymena* cilia, obtained as described in the text. The spectrum consists of two overlapping 'bilayer' shaped resonances; the one on the left arising from the phosphonolipids, and the one on the right arising from the phospholipids in the native intact cilia membranes.

this area from the total area of the spectrum. By this estimate, the phosphonolipid content was approx. 50%, which agrees reasonably well with the reported values [10].

As has been shown previously [4,13], ^{31}P-NMR spectra, while providing important clues to lipid phase behavior, are not diagnostic for the presence of certain phase structures. Therefore, X-ray and freeze-fracture electron microscopic data have been obtained on the same lipid samples used for ^{31}P-NMR measurements. These data then allow an interpretation of the ^{31}P-NMR data.

The small angle X-ray diffraction data of *Tetrahymena* PE at 10°C consist of a series of reflections having a spacing ratio of 1:1/3:1/2:1/7 superimposed on a weaker series of reflections having a spacing ratio of 1:1/2:1/3. The former series represents a hexagonal phase, the tube-to-tube distances being 74 Å, whereas the later represents a minor component of lamellar phase with a lamellar repeat of 51 Å. Freeze-fracture results also support this finding, showing a predominantly hexagonal structure. On the other hand, the *Tetrahymena* aminoethylphosphonolipid at 10°C is predominantly lamellar as judged by both freeze-fracture and X-ray diffraction, in complete agreement with ^{31}P-NMR results. At 40°C, freeze-fracture results show that the phosphonolipid is transformed to a predominantly hexagonal phase, although the hexagonal reflections are too weak to be determined by X-ray diffraction. In neither case were lipidic particles [4] observed by freeze-fracture. Small vesicles are seen, but do not represent the majority of the lipid sample.

Discussion

^{31}P-NMR has proven to be a useful probe of lipid behavior for lipids containing the phosphodiester structure. To fully exploit the information available from the ^{31}P-NMR membrane systems, an analysis of the ^{31}P chemical shift tensor was necessary because in membrane systems only a partially motionally averaged chemical shift tensor is observed in the ^{31}P-NMR spectra. Since the chemical shift tensor is dependent on the chemical bonding around the phosphorus, one might expect a different tensor for different chemical structures.

Phosphonolipids are common in some membrane systems, including the *Tetrahymena* membranes studied here. Because of the substitution of a carbon-phosphorus bond for an oxygen-phosphorus bond, a ^{31}P-NMR spectrum should reflect the difference in structure. As can be seen in Fig. 4A, the carbon-phosphorus bond moves the isotropic ^{31}P resonance downfield by approx. 20 ppm.

However, the aqueous dispersions of this lipid exhibited anisotropic resonance shapes completely analogous to the behavior of ordinary phospholipids. The 'bilayer' and 'hexagonal' resonances of the aminoethylphosphonolipid look like the 'bilayer' and 'hexagonal' resonances for phosphatidylethanolamine, but are displaced downfield. Instrumentation was not available to measure the complete ^{31}P chemical shift tensor, but the partially motionally averaged chemical shift tensors just referred to exhibit similar structure and pseudo-axial symmetry to those exhibited by phospholipids in the same phase structures.

An important caution about the interpretation of the ^{31}P-NMR spectrum of membranes in the absence of other information arises from Fig. 5. Isotropic ^{31}P-NMR resonances can be assigned to microscopic isotropic phases in membranes if corroborated by other techniques [4]. However, they can also be due to small vesicle formation as seen here and in the case of sphingomyelin [13]. Care should thus be taken in the interpretation of isotropic ^{31}P-NMR resonances from biological membranes, where small vesicles frequently contaminate membrane preparations.

It is evident from consideration of the temperature effects on the aminoethylphosphonolipid ^{31}P-NMR spectrum that this lipid is capable of lamellar phase formation at lower temperatures but shows a tendency toward hexagonal phase at 40°C. This is a different behavior from that of the phosphatidylethanolamine isolated from the same cells; *Tetrahymena* PE itself shows hexagonal phase behavior at all temperatures above 10°C. The difference in phase behavior of the two ethanolamine-containing lipids may be largely due to the presence in the aminoethylphosphonolipid of a high proportion (estimated to be greater than 60%) of glyceryl ether-containing molecules, while the phosphatidylethanolamine is almost entirely of the

diacyl variety. When a small amount of phosphatidylcholine is added to the aminoethylphosphonolipid, the ^{31}P-NMR spectrum taken at 30°C indicates that hexagonal phase behavior of the phosphonolipid has been suppressed by PC, which forms only lamellar phases. Each of these two lipids is seen to exhibit both anisotropic and isotropic resonances; thus, there is not a separation of the two lipids into different phases, but a mixture in each phase. The difference in the chemical shift of the two lipids allows them to be studied in a mixture such as that present in the membranes of *Tetrahymena*. The ^{31}P-NMR spectrum of cilia isolated from these cells clearly shows a bilayer profile for both phosphonolipid and phospholipid; there is no discernible hexagonal phase in either the phosphonolipid or phospholipid portion of the spectrum, taken at 30°C where the pure phosphonolipid shows some hexagonal phase. Thus, the aminoethylphosphonolipid may be a useful substitute for phosphatidylethanolamine in studies of lipid mixtures, since its behavior can be studied independently from that of the other lipids. Questions regarding the importance of phase separations in lipid mixtures could be resolved using this lipid as an NMR probe.

In summary, the phospholipids of the *Tetrahymena* ciliary membranes are found to be in a normal bilayer configuration, even though when purified, some of the components tend to form hexagonal phases. This behavior probably can be assigned to the presence in the membrane of phosphatidylcholine, a bilayer-stabilizing phospholipid, though an influence of tetrahymanol or the protein component of the membrane cannot be ruled out.

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