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CHARACTERIZATION OF THE PHASE BEHAVIOR OF PHOSPHONOLIPIDS IN MODEL AND BIOLOGICAL MEMBRANES BY ^{31}P-NMR *

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^{31}P-NMR is used to characterize the phase behavior of phosphonolipids in both model and biological membranes. (1′,2′-Dipalmitoyl-*sn*-glyceryl)-2-aminoethylphosphonate gives rise to static chemical shift tensor elements (−87, 5 and 63 ppm) which differ considerably from those reported for the analogous phospholipid, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (−81, −20 and 105 ppm). Phosphonolipid, as well as a mixture of phosphonolipid and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, in aqueous dispersion gives rise to ^{31}P spectra which may be interpreted in terms of lamellar structures. A mixture of phosphonolipid and egg phosphatidylethanolamine exhibits a bilayer-to-hexagonal phase transition with a concomitant decrease by one-half in the value of the ^{31}P chemical shift anisotropies of both the phosphonate and phosphate resonances. The chemical shift anisotropy associated with phosphonolipid has been found to be consistently smaller than that observed for the analogous phospholipid. ^{31}P-NMR spectra of total lipid extracts of *Tetrahymena* sp. indicate that both phospho- and phosphonolipids have a bilayer organization between −20 and 20°C.

Introduction

Derivatives of 2-aminoethylphosphonic acid (AEP) occur in certain bacteria, and in a wide variety of Protozoa, Colenterata and Mollusca [1]. In the ciliated protozoan, *Tetrahymena pyriformis*, AEP derivatives of ceramide [2] and acyl glycerol [3] occur along with phospholipids. ^{31}P-NMR spectra of *Tetrahymena* exhibit a broad resonance at about −22 ppm which is attributable to phosphonolipid (Deslauriers, Jarrell, Byrd and Smith, unpublished results). We have studied the phase behavior of a synthetic phosphonolipid in order to determine the effects of phosphonolipid on membrane structure.

It is well established that phospholipids in both model and biological membrane systems exhibit polymorphism which can be detected by ^{31}P-NMR. Both bilayer and nonbilayer structures give rise to characteristic ^{31}P-NMR lineshapes [4,5]. The interpretation of ^{31}P spectra with respect to lipid organization relies upon previous X-ray determinations of phospholipid organization in simple model systems. In the case of phosphonolipids such X-ray studies have not been reported. Furthermore, the lack of information on chemical shift tensors of phosphonate-containing lipids and their orientation with respect to the molecular frame of the phosphonate group makes the prediction of lineshapes for the various possible lipid phases impossible. Both phospho- and phosphonolipids occur in *Tetrahymena* [6], particularly in the cilia in which the phosphonate analog of phosphatidylethanolamine (PE) is the major lipid. In addition, these lipid classes contain unsaturated fatty acids [6] and raise the possibility of hexagonal phase formation [5].

In view of the latter observations, an analysis by ^{31}P-NMR of aqueous dispersions of phosphonate-containing lipids was undertaken to determine the

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phase characteristics and associated ^{31}P lineshapes of these major structural lipids of *Tetrahymena*. We report the static ^{31}P shielding tensor elements of AEP (powder) and (1′,2′-dipalmitoyl-*sn*-glyceryl)-2-aminoethylphosphonate (PL) (anhydrous). We demonstrate that phosphonolipids in bilayer and hexagonal structures give rise to ^{31}P-NMR lineshapes which are similar to those observed for phospholipids in the same structures. Examination of the ^{31}P spectra of lipids extracted from *Tetrahymena* sp. indicates that the phospho- and phosphonolipid are in a bilayer structure between –20 and 20°C.

Materials and Methods

AEP and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Sigma Chemical Co., St. Louis, MO. Egg PE was purchased from Lipid Products, South Nutfield, U.K. PL was prepared by the method of Baer and Stanacev [7]. Lipids were homogeneous to TLC on silica gel ($CHCl_3/CH_3OH/H_2O$, 65 : 25 : 4, v/v).

Hydrated lipid dispersions were prepared from 50 mg of pure phosphonolipid, 125 mg of phosphonolipid/DPPC (1 : 4, w/w) and 125 mg of phosphonolipid/PE (1 : 4, w/w) dissolved in $CHCl_3/CH_3OH$ (2 : 1, v/v). The solvent was removed by evaporation under nitrogen and the sample stored overnight under vacuum. The lipid was hydrated in 0.7 ml H_2O (2 mM EDTA/25 mM Tris-acetic acid, pH 7.0) by vortex mixing above the phase transition of the lipids, and NMR samples were sealed under vacuum in glass tubes (10 mm outer diameter).

Tetrahymena sp. No. 30202 was obtained from ATCC and grown in 2-l Fernbach flasks with 0.5% proteose peptone, 0.5% tryptone with 0.02% K_2HPO_4 at pH 7.2 (ATCC medium No. 357). Cells were harvested after 36 h growth at 25°C by centrifugation at 200 × *g* for 5 min at 5°C. Cells were washed in 0.4% NaCl and freeze-dried.

Total lipid was prepared by extracting cells with $CHCl_3/CH_3OH$ (2 : 1, v/v, 30 vol), and concentrating to dryness. Lipids were purified by Sephadex chromatography [8] and dispersed in water as described above.

^{31}P-NMR spectra were obtained on a Bruker CXP 300 spectrometer operating at 121.47 MHz using quadrature detection. All spectra were obtained in the presence of complete proton decoupling (about 6 G decoupling field for ^1H) which was gated on only during acquisition. Selective saturation of ^{31}P resonances was achieved using a DANTE pulse sequence [9,10]. The parameters used in performing the DANTE saturation sequence were chosen to ensure that (i) the sidebands displaced from the transmitter frequency [9], ν_0, are not in the region of any resonance intensity, and (ii) the duration of the saturation is empirically selected to be long relative to molecular reorientation, via tumbling or lateral diffusion, but less than or comparable to the T_1 of the saturated resonance. Failure to observe these conditions, in each lipid phase studied, can lead to distortion of the lineshape and/or incomplete saturation. Spectral subtractions were performed using the standard Bruker software.

Spectral simulation was performed according to the formulation reported by Seelig [4] for axially symmetric powder patterns. An orientation-dependent linewidth was used in the simulations to obtain the best fit to the experimental lineshape; however, a priori, this procedure is not unambiguous, since the true lineshape is a complex function of molecular motion [11]. A complete quantitative analysis of the lineshape is not pertinent to the present study, but it is the subject of further investigation.

Differential scanning calorimetric measurements were performed on 2 mg of lipid dispersed in 1 ml of H_2O using a Microcal MC-1 differential scanning calorimeter, with a temperature scanning rate of 1°C/min.

Results and Discussion

^{31}P-NMR spectra of solid AEP and phosphonolipid

The ^{31}P-NMR spectra of powdered AEP and PL are shown in Fig. 1. The principal elements of the shielding tensor of AEP have values of –63, –7 and 65 ppm * while the phosphonolipid gave corresponding values of –87, 5 and 63 ppm, respectively. ** Phosphoethanolamine has been reported to give rise to principal values of –63 (σ_{11}), –8 (σ_{22}) and 65 (σ_{33}) ppm *** [12], while anhydrous 1,2-dipalmi-

* Relative to AEP in H_2O at pH 7.0.

** Relative to the isotropic chemical shift of PL in H_2O, pH 7.0.

*** Relative to phosphoric acid.

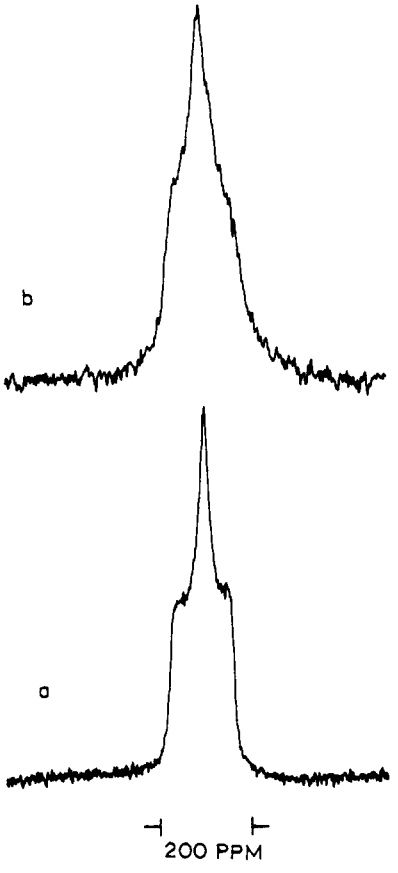

Fig. 1. ^{31}P-NMR spectra at 121.47 MHz, using 90° pulses and gated high-power ^{1}H decoupling during acquisition (5 ms) at a rate of 1 scan/10 s: (a) AEP (powder), 820 scans, and (b) PL (powder).

toyl-*sn*-glycero-3-phosphoethanolamine (DPPE) has values of −81, −20 and 105 ppm ***, respectively [13]. For the quantitative interpretation of phospholipid spectra it is assumed that the orientation of the shielding tensor with respect to the molecular frame is the same as that observed in phosphoethanolamine and barium diethyl phosphate. Although the principal values of the shielding tensor appear to be very similar for AEP and phosphoethanolamine, those of the phosphonolipid, PL, differ considerably from those of the analogous phospholipid. Therefore, in the case of PL the molecular orientation of the shielding tensor cannot be assumed to be the same as that of phospholipid, and as a result the ^{31}P-NMR lineshape for phospholipid in various phases cannot be predicted from the available data *.

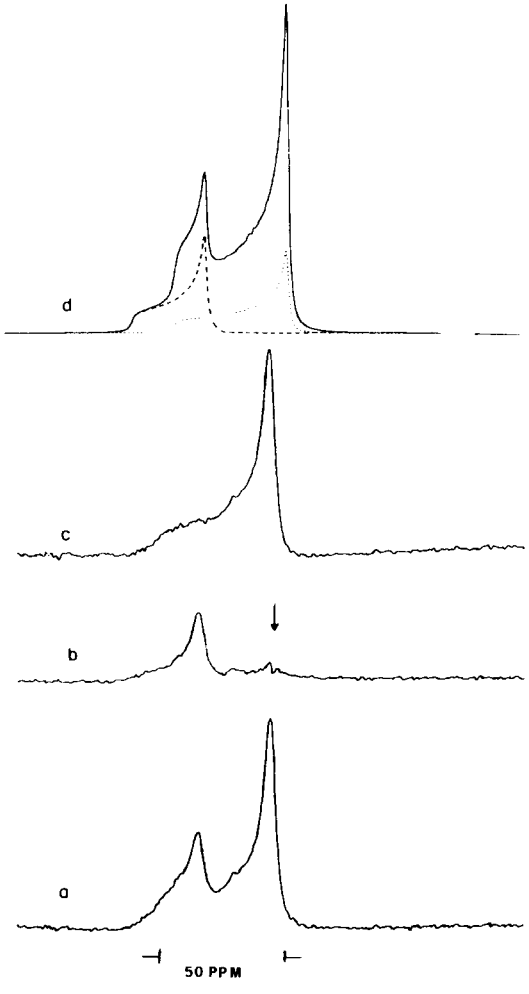

Fig. 2. ^{31}P-NMR spectra at 121.47 MHz of PL/DPPC mixture (1 : 4, w/w) in water at pH 7.0 at 50°C. (a) 90° pulses, 2 400 accumulations at 1 scan/s; (b) as in a but using a DANTE pulse sequence at the indicated frequency with 4° pulses separated by 50 μs and a total saturation time of 800 ms; (c) difference spectrum of a − b; (d) computer simulation of spectrum a in terms of two axially symmetric powder patterns characterized by a chemical shift difference of −28 ppm, angular-independent and angular-dependent linewidths of 70 Hz, chemical shift anisotropies of −46 and −30 ppm for phospho- and phosphonolipid, respectively.

^{31}*P-NMR spectra of lipid dispersions*

In order to obtain information on the ^{31}P-NMR

*** See footnote on p. 81.

* Single crystal studies on AEP are in progress.

lineshape of phosphonolipids in bilayer and hexagonal phases, PL was mixed with DPPC to form liposomes having a bilayer structure, and mixed with PE to give liposomes in which bilayer and/or hexagonal phases may be observed. The macroscopic lipid organization may be monitored via the phospholipid resonance, and, by use of selective saturation techniques, the lineshape of the phosphonolipid may be ascertained and correlated with the phase organization. ^{31}P spectra of the PL/DPPC system at 50°C are shown in Fig. 2. Since differential scanning calorimetry indicates that the lipid mixture undergoes a thermal phase transition which is centered at about 46°C, the spectra shown in Fig. 2 are for the system in the liquid crystalline phase. A bilayer structure may be concluded from the lineshape of the phospholipid resonance [4] (Fig. 2a and c). Due to the overlapping of resonances the true lineshape of the phosphonolipid is not clear. Saturation of the DPPC resonance using a DANTE pulse sequence [9,10] gave the spectrum of Fig. 2b in which the powder pattern of the phosphonate group is similar in shape to that observed for DPPC, but is considerably narrower, having a chemical shift anisotropy of about −28 ppm. Subtraction of the spectrum of Fig. 2b (normalized) from that of Fig. 2a gave the resultant spectrum in Fig. 2c in which a bilayer ^{31}P powder pattern for DPPC is readily seen and has a chemical shift anisotropy of about −47 ppm, in good agreement with the value of −48 ppm reported for DPPC at 50°C [14]. Using the values of the chemical shift anisotropies determined for the two lipids, the observed lineshape for the mixture may be easily simulated by the superposition of two powder patterns. Such a simulation is shown in Fig. 2d. Consequently, the lineshape which is characteristic of a bilayer structure for phospholipids is also characteristic of a bilayer structure for phosphonolipids.

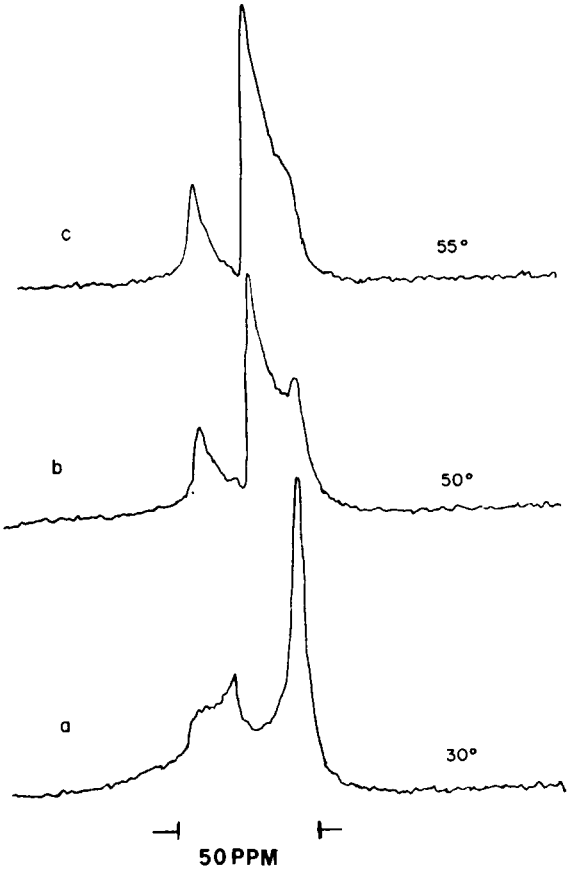

Fig. 3. Temperature dependence of ^{31}P-NMR spectra at 121.47 MHz of PL/PE (egg) mixture (1 : 4, w/w) in water at pH 7.0 using 90° pulses, 2 000 accumulations at 1 scan/s.

In order to exclude the possibility that the phosphonolipid may form a separate domain within a DPPC matrix and exhibit a structure which differs with that of DPPC in the mixture, we examined a mixture of PL and PE.

^{31}P-NMR spectra of the PL/PE system at several temperatures are shown in Fig. 3. Between 27 and 40°C the spectra are similar to those observed for the PL/DPPC system and are therefore due to overlapping bilayer spectra. Between 45 and 55°C the spectra contain two additional patterns (Fig. 3b) which are attributable to a hexagonal structure [5,15,16]. The onset and completion of the bilayer-hexagonal phase transition occurs at the same temperatures for both lipid components which indicates that the lipid mixture is behaving as a homogeneous mixture. At 55°C only one structure is present, as evidenced by the lineshape associated with PE which is characteristic of the hexagonal (H_{II}) phase (Fig. 3c), and has a chemical shift anisotropy of about 21 ppm. The lineshape of the phosphonolipid resonance is similar to that of PE but the associated chemical shift anisotropy is smaller, about 14–15 ppm, as was observed for PL in the PL/DPPC mixture. It is clear that in the PL/DPPC and PL/PE systems the phosphonolipid is exhibiting the same type of lipid organization as the phospholipids; consequently, its ^{31}P spectra may be inter-

preted unambiguously as bilayer for the PL/DPPC mixture and bilayer or hexagonal structures, depending on the temperature, for the PL/PE mixture.

PL dispersed in water undergoes a calorimetric phase transition which is centered at 63°C and which is almost identical in shape and temperature to that of DPPE dispersed in water. Liposomes of PL give rise to the ^{31}P spectra below (40°C) and at (63°C) the gel-liquid crystalline phase transition shown in Fig. 4. On the basis of the results with the lipid mixtures, the ^{31}P lineshapes indicate that PL gives rise to bilayer structures between 40 and 63°C. On going through the gel-liquid crystalline phase transition the chemical shift anisotropy of the PL resonance decreases from –49 (at 40°C) to –32 ppm (63°C) which is consistent with the decrease from –60 (at 41°C) to –40 ppm (at 61°C) observed for DPPE [14]. The relative change in the chemical shift anisotropy on going through the phase transition is the same for both lipid classes and therefore suggests that the motional changes occurring during the phase transition are essentially identical for both head-group classes.

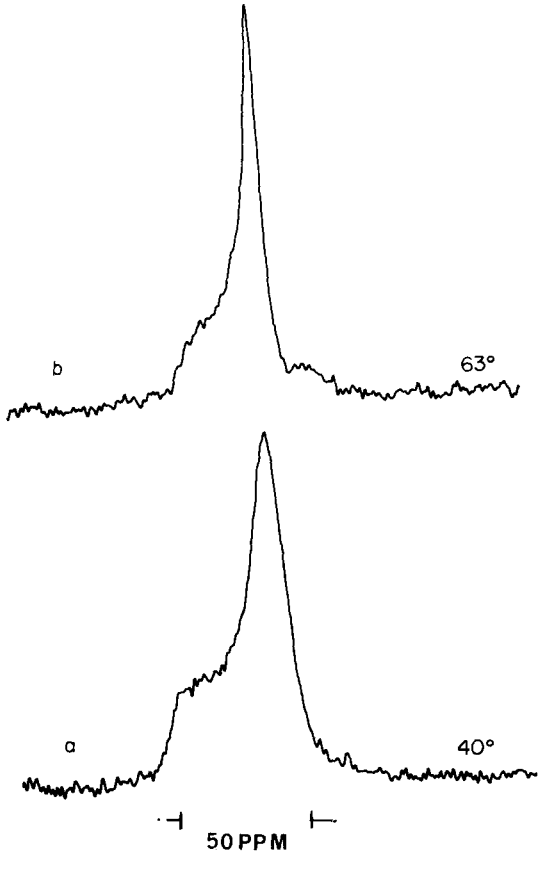

Fig. 4. ^{31}P-NMR spectra at 121.47 MHz of PL in water at pH 7.0 using 90° pulses and 2 400 accumulations at 1 scan/s.

The trends observed with the model systems indicate that the lineshape characteristics of phosphonolipid ^{31}P-NMR spectra are similar to those of phospholipids for the same phases, except that phosphonolipids exhibit a consistently smaller chemical shift anisotropy. Therefore, the interpretation of ^{31}P-NMR spectra of phospholipids with regard to lipid organization may be extended by direct analogy to phosphonolipids. Although lipid organization may be deduced from ^{31}P-NMR lineshapes, no insight into the associated order parameters of the phosphonate group can be obtained without additional information [4].

^{31}P-NMR spectra of extracted lipids from Tetrahymena sp.

In the preceding section the basis for interpreting ^{31}P spectra of phosphonolipids with regard to lipid organization was delineated.

As an example of how this information may be used to study the phase organization of a membrane with naturally occurring phosphonolipids, we have examined the extracted lipids of *Tetrahymena* sp. The ^{31}P spectra of an aqueous dispersion of the total lipids extracted from *Tetrahymena* sp. at several temperatures are shown in Fig. 5. Between –20 and 20°C the spectra consist of two overlapping patterns which are attributable to phospho- and phosphonolipids, and are reminiscent of the spectra of the PL/DPPC mixture (Fig. 2). The spectrum of the lipids at –10°C may be simulated using essentially the same parameters that were used in Fig. 2d, and a phospholipid/phosphonolipid ratio of 2.5 : 1, as shown in Fig. 5b. Raising the sample temperature to 20°C resulted in little change in lineshape other than a slight decrease in the overall width of the spectrum. Saturation of the phospholipid resonance using a DANTE pulse sequence gave the spectrum shown in Fig. 5e; due to incomplete saturation, a residual phospholipid resonance is present. The lineshape of the phosphonolipid resonance is indicative of a bilayer structure and has an associated shielding anisotropy of about –32 ±

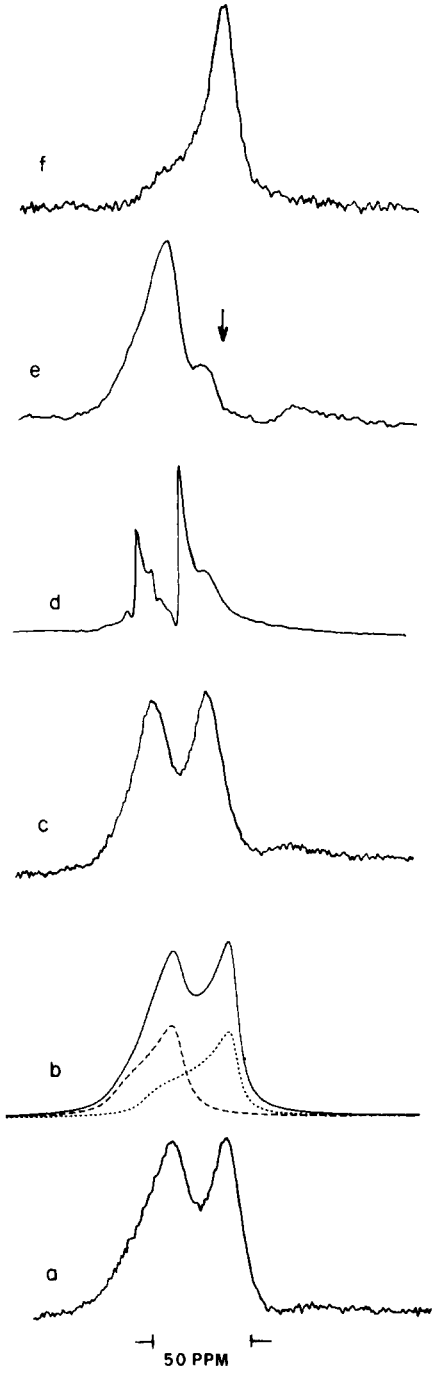

Fig. 5. Temperature dependence of ^{31}P-NMR spectra of the total lipid extract from *Tetrahymena* sp. in water using 90° pulses and 1 000 accumulations at 1 scan/s: (a) at −10°C; (b) computer simulation of spectrum a using: a chemical shift difference of −24 ppm; angular-independent linewidths of 450 and 640 Hz, and angular-dependent linewidths of 120 Hz, chemical shift anisotropies of −46 and −32 ppm for phospho- and phosphonolipid, at a ratio of 2.5 : 1, respectively; (c) at 20°C; (d) at 39°C; (e) as in c but using DANTE pulse sequence at the indicated frequency using 4° pulses separated by 50 μs and a total saturation time of 1 s; (e) difference spectrum of c − d.

2 ppm, a value comparable to that observed for PL (−32 ppm).

The phospholipid resonance lineshape, although a composite of all phospholipid head-group classes, is expected to be dominated by PE containing primarily unsaturated fatty acids, since this lipid class has been identified as one of the major phospholipids in *Tetrahymena* (50–70%) [6]. As a model of this phospholipid, the unsaturated lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine exhibits a bilayer ^{31}P spectrum at 5°C with a shielding anisotropy of −45 ppm [15]. A similar value may be expected for the phospholipid resonance of the *Tetrahymena* lipid extract. Spectral subtraction gave the lineshape of the phospholipid resonance (Fig. 5f) which is also consistent with a bilayer structure and has an apparent shielding anisotropy of about −40 ± 2 ppm. Therefore, between −20 and 20°C the lipid extract exhibits only a bilayer organization.

On raising the temperature further, relatively narrow resonances are observed which have frequencies corresponding to the respective isotropic chemical shifts (Fig. 5d). Similarly, whole cells of *Tetrahymena* exhibit narrow resonances between −20 and 25°C. The narrow resonances may reflect the effects of tumbling and lateral diffusion [17] or may arise from an 'intermediate phase' [5].

Conclusion

^{31}P-NMR has been used to determine the organization of phosphonolipids in aqueous dispersions and offers the potential of examining the organization of this lipid class in biological membranes. Since phospho- and phosphonolipids exhibit a relatively large difference in chemical shifts and a similar ^{31}P lineshape, the organization of the individual head groups in a mixture of the two classes may be readily deduced. The total lipid extract of *Tetrahymena* sp. exhibits a preference for lamellar structures in aqueous dispersions between −20 and 20°C with both phospho- and phosphonolipids assuming a bilayer

structure. The presence of phosphonolipid (diacylglycerol-containing) does not cause a significant perturbation of the phospholipid organization.

Although the total lipid extract of *Tetrahymena* gives rise to lamellar structures in water at ambient temperatures, a more complex organizational behavior appears to occur at higher temperatures (Fig. 5d). Further studies on the extracted lipids and on *Tetrahymena* are in progress to elucidate the nature of these structural changes.

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