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THE BILAYER PROPERTIES OF THE CILIARY MEMBRANES OF *TETRAHYMENA THERMOPHILA* AS REVEALED BY ^{31}P-NMR

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Cilia were isolated from *Tetrahymena thermophila* grown at 30°C in bacteriological peptone supplemented with yeast extract. ^{31}P-NMR spectra in the physiological range of temperatures were complex, consisting of two bilayer spectra, attributable to phosphono- and phospholipids, onto which were superimposed two isotropic peaks. Membrane vesicles isolated from cilia showed a similar spectrum, but at 25°C lipids extracted from cilia lacked both isotropic components, suggesting that the latter stem from a non-lipid component. On increasing the temperature from 5 to 50°C the spectra of the cilia preparations showed increasing amounts of the isotropic components, while the contribution of the powder patterns to the spectrum decreased. No hexagonal (H_{II}) phase was observed at any temperature in spite of the high membrane content of phosphatidylethanolamine and aminoethyl phospholipids; this could be due to the stabilising effect of sphingolipids or glyceryl ether lipids on the bilayer.

Introduction

The eukaryote ciliate *Tetrahymena*, due to its ease of culture and manipulation, has become an increasingly popular organism for various biochemical studies. Over the past few years, there have been numerous reports of the lipid composition of its different membranes, which appear to be broadly similar to those of other eukaryotes. However, certain constituents, in particular phosphonolipids and glyceryl ether phospholipids, are found in higher amounts in *Tetrahymena* than in, for example, mammalian cells [1,2]. Perhaps the most characteristic difference between *Tetrahymena* and other eukaryotes is that the external membranes of the former do not have a sterol component, but instead have a triterpenoic compound, tetrahymenol, which is functionally equivalent to a sterol. The various membranes of *Tetrahymena* also contain numerous proteins with molecular weights and functions similar to those found in their counterparts in other eukaryotic cells [3]. Recently, attention has turned to the compositional and physiological changes brought about by varying the culture temperature [1–4]. These studies have been important in elucidating the mechanisms whereby an eukaryote can modify its membranes in response to changes in the environment.

It has also emerged that the synthetic pathways to fatty acid components of *Tetrahymena* bear many similarities to the corresponding systems in rat liver, both with respect to the apparent preference of the desaturases for phospholipid-bound fatty acyl chains and the similar mode of action of the elongation and desaturation enzyme systems

Abbreviations: Mops, 4-morpholinepropanesulphonic acid.

[5,6]. These parallels between the lower and higher eukaryotes further demonstrate the potential of *Tetrahymena* as a convenient experimental system for the study of eukaryotic membranes.

Although both polarisation of fluorescence [7–9] and ESR [10,11] have been used to probe the state of the membranes in *Tetrahymena*, very little has been done using other potentially useful and non-perturbing techniques such as X-ray diffraction and NMR. ^{31}P-NMR was first utilised for the study of *Tetrahymena* membrane components by Jarrell et al. [12], who demonstrated in total lipid extracts that both phospho- and phosphonolipids gave bilayer structures in water between −20 and 20°C. This work was extended by Deslauriers et al. [13]. At the growth temperature (25°C), two non-isotropic components were observed in each of the phospho- and phosphonolipid subspectra. One component of each subspectrum was characteristic of lamellar phase lipid while the other was suggestive of lipid in a hexagonal phase. At 45°C, the lipid extracts showed 'isotropic' and 'hexagonal' phase resonances for each of the phosphono- and phospholipid components. In order to extend these studies to naturally occurring membranes, we decided to record ^{31}P-NMR spectra of pure membranes isolated from *Tetrahymena thermophila*.

Materials and Methods

Tetrahymena thermophila Chx-2/Chx-2 (cysen, IV), hereafter referred to as *Tetrahymena*, were grown at 30°C in a medium consisting of 1% bacteriological peptone (L37 Oxoid) and 0.1% yeast extract (L21 Oxoid), supplemented with 36 μM $FeCl_3$. Small cultures, set up in 2-l Fernbach flasks, were used to inoculate cultures in 12-l fermentors to which 150 μl of Dow Corning antifoam A was added. Cell densities were determined by counting formaldehyde-fixed cells in a cytometer (Sedgwick rafter).

25-l batches of cells in mid-logarithmic phase of growth were cooled on ice and then harvested at 0.5 l/min using a Sorval continuous flow rotor running at $1700 \times g_{av}$. The cell pellets were resuspended in cold buffer (Mops/NaCl/EDTA: 0.15 M Mops/0.45 M NaCl/10 mM $Na_2 \cdot$ EDTA (pH 7.15)), cooled for 10 min on ice, and the cells spun down in oil tubes at $380 \times g_{av}$. The cells were carefully resuspended in Mops/NaCl/EDTA, and allowed to shrink for 20 min before shearing off the cilia with a loose Dounce homogeniser. As the cilia tend to shed spontaneously in this buffer, only a few strokes are required. The homogenate was then centrifuged at $1130 \times g_{av}$ for 8 min and the supernatant recentrifuged for 5 min at the same speed. The supernatant was removed carefully, and the pure cilia pelleted at $14400 \times g_{av}$ for 8 min. The cilia were finally washed once in 0.15 M Mops (pH 7.15) and pelleted at $18100 \times g_{av}$ before resuspending in 0.15 M Mops for ^{31}P-NMR or 1 mM Tris buffer, pH 8.0 for the membrane vesicle preparation.

Membrane vesicles were prepared according to Adoutte et al. [14]. The band of membranes was diluted in 0.15 M Mops and centrifuged at $95400 \times g_{av}$ for 15 min before resuspending in the same buffer for ESR or ^{31}P-NMR measurements.

For ESR measurements, 0.1 ml of membrane vesicles were incubated with 10 nM probe (5- or 12-doxylstearate) for 10 min at 25°C, and spectra run in a Varian E-9 spectrometer.

The total phosphorus content of the membrane samples was determined according to (Ref. 15 and Johns, J., unpublished results). A factor of 20 was used for converting mg P to mg phospholipid.

Fresh membrane preparations were fixed overnight at 4°C in freshly diluted 6% glutaraldehyde, and post-fixed in 2% OsO_4 prior to dehydration and embedding in Epon for sectioning.

Total lipids were extracted from washed, freeze-dried cilia with chloroform/methanol (2:1, v/v) [16]. In some experiments, the total lipids were subsequently passed through a Sephadex column to eliminate proteolipids [17]. In all cases the lipids were then brough to dryness on a rotary evaporator, solvent traces removed by freeze-drying for several hours, and lipid vesicles formed by addition of about 1 ml of buffer, followed by cycles of vortexing and freeze-thawing until a stable emulsion was formed. The efficiency of the lipid extraction was determined by methanolysis of aliquots of the cilia before extraction, and of the total lipids obtained after extraction. The amount of lipid was measured by gas-liquid chromatography [3].

^{31}P-NMR spectra of the membrane and vesicle

preparations were recorded on a Bruker CXP-300 spectrometer operating at 121.47 MHz in the Fourier transform mode using quadrature detection. Samples were run as aqueous dispersions in 10-mm sample tubes. Spectra were acquired using the chemical shift echo technique [18] with phase alternation. Selective saturation of ^{31}P resonances was achieved using a DANTE pulse sequence [19]. The duration of the saturation was chosen 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. Chemical shifts are reported in parts per million (ppm) from a reference of 85% H_3PO_4 contained in a sealed melting point tube. The methodology and interpretation of the ^{31}P-NMR spectroscopy are discussed in detail in a recent review [20].

Results

Sufficient quantities of ciliary membrane can be obtained from 25-l cultures of *Tetrahymena* to give reasonable ^{31}P-NMR spectra within 30–60 min. One such spectrum, shown in Fig. 1, demonstrates the presence of both phospho- and phosphonolipid components, each subspectrum showing two well-separated resonances (20 and 10; 0 and −14 ppm, respectively). These spectra were both very reproducible and stable. Initial experiments demonstrated that, if held at 4°C, the cilia preparations gave identical spectra over a 16 h period. This was marked contrast to pellicle (outer membrane) fractions, whose spectra under the same experimental conditions changed noticeably within an hour.

In order to establish that the four resonances arose from the ciliary membrane and not, for example, from the axonemal components, we isolated ciliary membrane vesicles and examined their ^{31}P NMR spectra (Fig. 2). A comparison of Figs. 1 and 2 makes it clear that the resonances occur at identical frequencies, the main difference between the two samples being in the relative intensities of the resonance near 0 ppm. Thus, in intact cilia, the ^{31}P-NMR spectra can be attributed to membrane-associated phosphorus components.

Since the shape of the bilayer powder spectrum is markedly influenced by the dimensions of the membrane structures being examined [21], samples

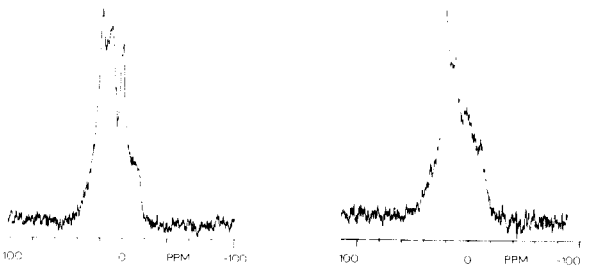

Fig. 1. ^{31}P-NMR spectra (121.47 MHz) of cilia from *T. thermophila*, sweep width 125 kHz, 4 K data points, 1800 scans, 5°C. Spectra were acquired with a phase cycled echo, ^{1}H-decoupling, a 90° pulse of 7 μs, and a recycle delay of 2 s.

Fig. 2. ^{31}P-NMR spectrum of vesicles obtained from cilia of *T. thermophila*, with a 90° pulse of 9 μs, 2500 scans, at 8°C. Other conditions are as in Fig. 1.

of both the cilia and ciliary membrane vesicles were fixed for examination in the electron microscope. Initially, prior to measuring their ^{31}P-NMR spectrum, we resuspended the isolated cilia in 0.15 M Mops buffer containing 4 M glycerol. This was done in an attempt to preserve the axonemal microtubules, and thereby enhance the structural stability of the entire cilium. However, this strategy was not successful, inasmuch as the lumen of the cilium was seen to contain a disorganised meshwork of repolymerised tubulin. However, the ciliary plasma membrane remained intact, and only very few small vesicles were seen. Subsequently, we repeated the ^{31}P-NMR experiments using cilia resuspended in 0.15 M Mops, and obtained identical spectra, which is in accord with the fact that axonemal components do not contribute to the spectra (vide supra). Fig. 3a shows a portion of an intact cilium in longitudinal section, while Figs. 3b and 3c show a typical ciliary membrane vesicle preparation. The vesicles are relatively large, with diameters ranging from 400 to 650 nm. A large proportion of the vesicles are multilamellar.

The spectra in Figs. 1 and 2 are reminiscent of those previously reported for an aqueous dispersion of a *Tetrahymena* total lipid extract [13]. In order to characterise the constituent parts of the spectra, we employed DANTE pulse sequences [19] to saturate either the high or the low-field resonances of the phospho- and phosphonolipid subspectra (Fig. 4). These saturation experiments enabled us to demonstrate that the total ^{31}P-NMR

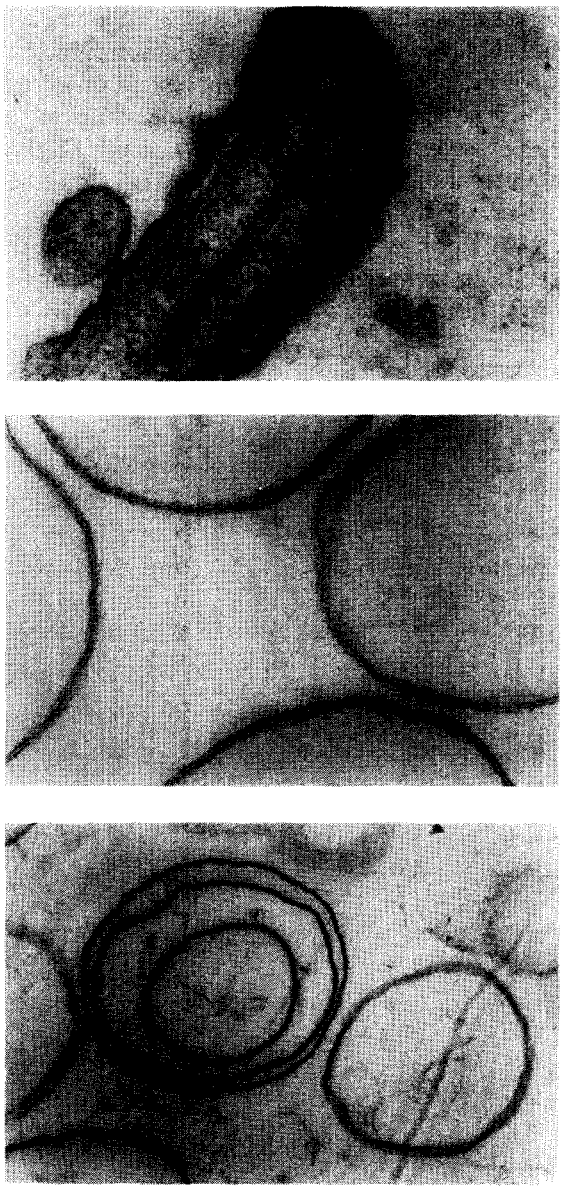

Fig. 3. Electron micrographs of cilia isolated from *T. thermophila*. Magnification: a and b, 100000×; c, 50000×.

spectrum of cilia membranes is made up of a phosphonolipid bilayer-type spectrum and a phospholipid bilayer-type spectrum, onto each of which is superimposed an isotropic component containing a phosphono- and a phospho-group, respectively. Both the chemical shift anisotropies and the shift differences are in good agreement with those expected for a membrane containing these components [12,13].

A number of reports in the literature show that a wide range of membrane-associated enzymatic and physiological properties of *Tetrahymena* are temperature-dependent (see Ref. 4 and references cited therein), with abrupt changes occurring in many of the properties around 16 and 25 °C. To date there has been no satisfactory explanation for

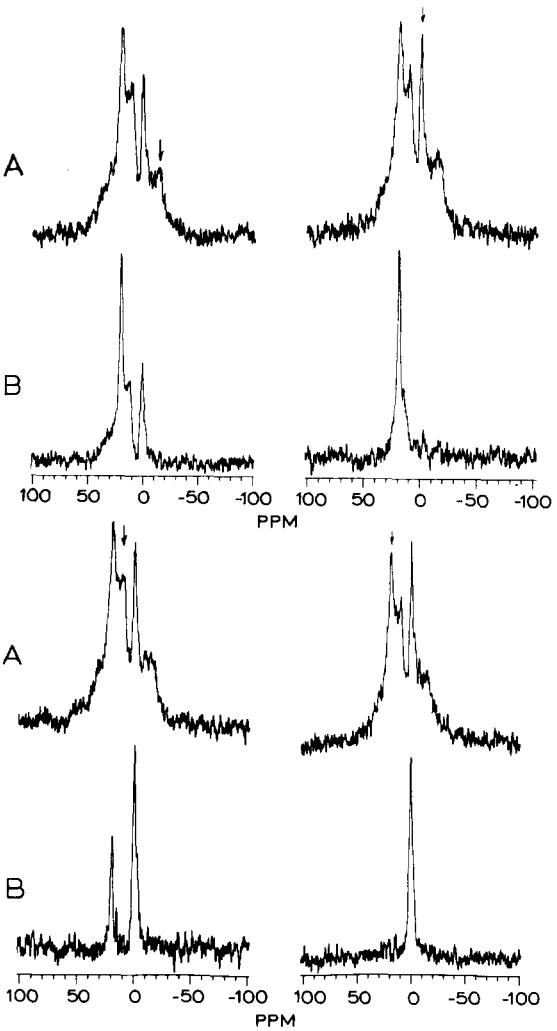

Fig. 4. ^{31}P-NMR spectra of cilia from *T. thermophila*; acquired using a phase-cycled echo; selective saturation was achieved using a DANTE pulse sequence. The 90° pulse was 5.5 μs. Saturation pulses of 0.5 μs were followed by a 50 μs delay. The number of saturation pulses in (A) was 4 and in (B) 4000. Saturation pulses were applied at −2100, 0, 1400, 2300 Hz from H_3PO_4 (i.e., centered successively on each major peak, as indicated by vertical arrows). Other conditions as in Fig. 1.

these changes. We therefore recorded spectra of intact cilia at various temperatures between 4 and 50 °C (Fig. 5). It can be seen that while the bilayer structure is predominant at low temperatures (5–15 °C), above 15 °C the intensities of the isotropic (non-bilayer) components become progressively greater. At 40 °C and above, the spectra consist essentially of three isotropic resonances which appear at about 20, 16 and 0 ppm, two of which (20 and 0 ppm) occur close to the isotropic chemical shift values for the phosphono- and phospholipids. However, the third isotropic resonance at 16 ppm cannot readily be accounted for. At no temperature was there any indication of the lipids assuming a hexagonal (H_{II}) phase. Cooling the preparation from 50 °C to 25 °C and then further to 15 °C and 10 °C before freezing at −25 °C for 6 h, did not result in reversion of the spectra to their original form. We believe that this irreversible increase in the isotropic ^{31}P-NMR sig-

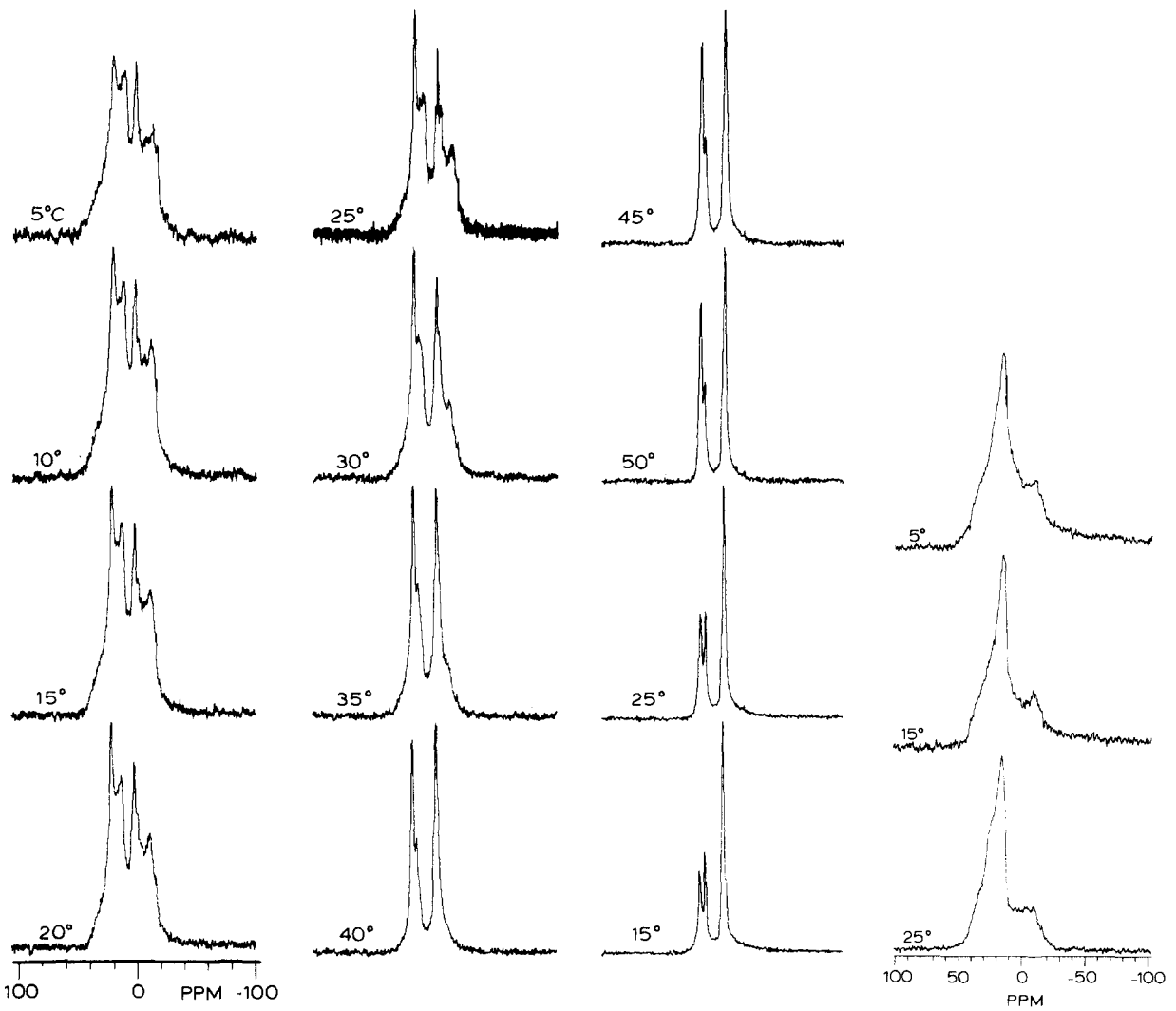

Fig. 5. ^{31}P-NMR spectra of cilia from *T. thermophila* obtained from 5 to 45 °C in 5 deg. C steps. After acquisition at 45 °C, the sample was cooled to 25 °C and 15 °C, and spectra were recorded again. Other conditions are as described in Fig. 1.

Fig. 6. ^{31}P-NMR spectra of lipids extracted from cilia of *T. thermophila*. The 90 ° pulse was 15 μs, and the number of scans 8000. Other conditions are described in Fig. 1.

nal is due to a change in membrane morphology, because examination of the sample in the phase-contrast microscope, after all the spectra had been recorded, showed an extensive breakdown of the cilia, with both membrane fusion and vesiculation having occurred.

In order to determine whether the isotropic resonances seen in the ciliary membranes could be due to non-lipid membrane components, total lipid extracts of cilia were made, dispersed in Mops buffer, and their ^{31}P-NMR spectra recorded at 5, 15 and 25 °C (Fig. 6). In contrast to the intact cilia and cilia membrane vesicle preparations, no isotropic resonances were observed at these temperatures, the spectra exhibiting only the powder patterns of phosphono- and phospholipid in the bilayer configuration. We checked the efficacy of the lipid extraction method by determining the fatty acid content of aliquots of a freeze-dried cilia preparation before and after chloroform/methanol extraction. More than 94% of the hydrolysable fatty acids were removed by the solvent, indicating that the ^{31}P-NMR spectra of the lipids are representative of the bulk of the lipid components, and that the absence of isotropic resonances in the total lipid spectrum is not merely due to incomplete extraction. The absence of isotropic components, such as seen in the spectra of intact cilia, Figs. 1 and 2, was a strong indication that some other membrane constituents, probably protein in nature, were responsible. To establish this, the cilia residue remaining after the extensive extraction of lipid was freeze-dried to remove solvent traces, and the proteins subsequently extracted with 2% SDS + 0.5% Triton for 2 h at room temperature. The sample was thereafter centrifuged at 4000 × g for 10 min, and the supernatant placed in an NMR tube for recording its ^{31}P-NMR spectrum at 5 °C (Fig. 7). Two isotropic peaks were observed corresponding to phosphono- and phospho-groups (possibly attached to proteins or in phosphonoglycans) (Fig. 7).

In another experiment, total lipids were extracted from cilia, and the proteolipids removed by passage through Sephadex. This lipid fraction, in contrast to the total lipids, proved extremely difficult to disperse in aqueous buffer, but once dispersed, yielded ^{31}P-NMR spectra that were identical to those shown in Fig. 6.

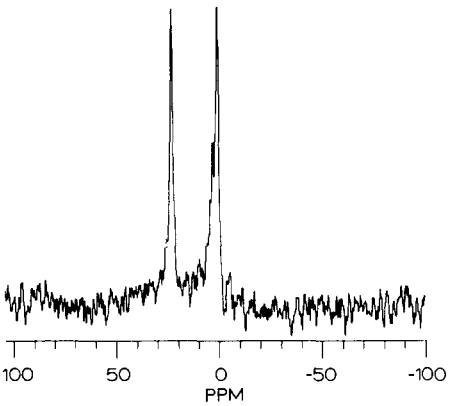

Fig. 7. ^{31}P-NMR spectra of SDS-denatured, delipidated cilia. The 90 ° pulse was 20 μs, and the number of scans 724. Other conditions are described in Fig. 1.

In addition to the ^{31}P-NMR studies reported, we examined the cilia membrane preparations using the electron spin resonance technique. Fig. 8 shows the results obtained with two different spin probes, when the half-width at half-line height of the low field resonance is plotted as a function of temperature. This type of plot has been shown [22] to be a sensitive measure of the onset of a phase transition. In ciliary membrane vesicles there is a break at around 21 °C, which is more pronounced with the fatty acid probe labelled at the 12-position than with that labelled at the 5-position. These data are consistent with the ^{31}P-NMR spectral behaviour, Fig. 5, indicating a structural change around 20 °C.

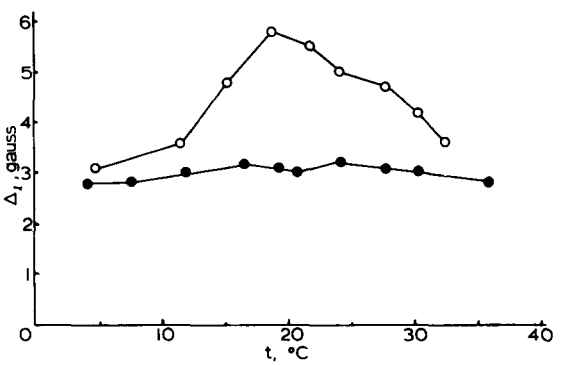

Fig. 8. The half-widths at half-line height of the low field lines in the electron spin resonance spectra of 5-doxylstearate (closed circles) and 12-doxylstearate (open circles) in vesicles from *T. thermophila* cilia.

Discussion

By isolating cilia in sufficiently large quantities, we have been able to obtain the ^{31}P-NMR spectra at a much higher degree of resolution than has been previously possible [23]. Furthermore, we have demonstrated that the spectra obtained from cilia reflect resonances from bilayer components, and not for example from axonemal guanine nucleotides [24,25]. The cilia spectra have been resolved by DANTE pulse sequences into subspectra which are consistent with the existence of two bilayer powder patterns and two isotropic resonances.

The two isotropic components are absent in the spectra of total lipid extract vesicles, but are present in an SDS extract of the residue remaining after the cilia have been completely delipidated. This indicates that the isotropic resonances existing at or below 25 °C originate almost entirely from the non-lipid components of the ciliary membrane. *Tetrahymena* cilia membranes have a relatively high protein to lipid ratio (2 : 1 on a weight basis [26,27]), and recent studies have shown that amongst the proteins present there is a single membrane-bound phosphonoglycoprotein [28]. It therefore seems likely that the isotropic resonances seen below physiological temperatures stem from the less restricted motion of the phosphono- or phospho-groups linked to a protein, rather than from some more mobile lipid component, as has been suggested for other membrane systems [29,30]. At the growth temperature and above, it is clear that the isotropic components at around 0 and 20 ppm, which correspond to the known isotropic chemical shifts for the phospho- and phosphonolipids [12], are due to changes in the state of the membrane. The isotropic resonance at 16–17 ppm, seen most clearly above 35 °C, does not correspond to any known lipid resonance, and we presume that it arises from a membrane constituent whose phosphonate group movement has become modified as a result of denaturation and changes in the property of the bilayer. Confirmation of these suggestions will require model studies on phosphonoprotein, both in aqueous solution and in reconstituted membranes.

The two powder patterns seen in the spectra of cilia and of total lipid extracts are due to phosphono- and phospholipids within the membrane. DANTE pulse sequence and difference techniques yield spectra characteristic of lipid bilayers. Even at 40 °C, which is above the highest temperature tolerated by *Tetrahymena thermophila* [31], the membrane remains partly in the bilayer form. At none of the temperatures studied is there any evidence for a hexagonal [32] lipid phase, despite the tendency of ethanolamine-containing lipids from *Tetrahymena* to form hexagonal phases [23], and the high content of phosphatidylethanolamine and its phosphono analogue [31]. This suggests that some component of the membrane stabilises the bilayer phase. It has been suggested previously [23] that the small amount of phosphatidylcholine in *Tetrahymena* membranes could be responsible for such a stabilisation. However, in the light of recent observations that *Tetrahymena* ciliary membranes have a high sphingolipid content [33], and that sphingolipids exert a stabilising effect on the bilayer [34], it is more likely the sphingolipids which prevent hexagonal phase from forming. Another candidate for bilayer stabilisation could be the glyceryl ether lipids, which constitute about 33% of the *Tetrahymena* plasma membrane lipids [35], and have been shown to promote closer bilayer packing [36].

The experiments carried out with lipid or protein extracts of cilia should be interpreted with caution, as the properties of vesicles, in which the original bilayer assymetry and packing pattern have been destroyed, may well be different from those of the intact membrane. In addition, in membranes with a high protein content, such as cilia, there are probably appreciable interactions between the lipid and protein components [37]. Indeed, it has been demonstrated that the mere isolation of a membrane can be seen to change the temperature-dependent phase properties [38]. For this reason we were particularly careful to check the stability of the cilia membranes with time, and have been reassured by the finding that the membrane vesicles give ^{31}P-NMR spectra essentially identical to those of the intact cilia.

The spectra shown in Fig. 5 demonstrate a gradual loss of bilayer structure and a concomitant increase in a lipid phase exhibiting rapid isotropic motion as the temperature is raised. Although it is difficult to quantify the gradual change from bi-

layer to non-bilayer as the temperature is increased, it is obvious that the bilayer contribution to the ^{31}P-NMR spectrum dominates until 15–20 °C. Thereafter there is a more gradual change as the percentage of the lipid phase exhibiting isotropic motion increases. Cooling the sample from 50 °C to 15 °C, and thereafter freezing at −25 °C for several hours did not lead to a reversal of the spectra to their original (5 °C) form. This irreversible change in the membrane structure could be due to either lipid degradation or changes in the membrane morphology. Lipid degradation can probably be ruled out since (a) we have obtained identical spectra of the same cilia preparations over long periods (up to 28 h at 5–10 °C); and (b) it is known that lipid breakdown, due to endogeneous lipases, take place relatively slowly in cilia [39], probably due to the high content of glyceryl ethers and phosphono-compounds in these membranes. Examination under the phase-contrast microscope of samples exposed to elevated temperatures shows that the membranes both fuse and vesiculate, so that the preparation finally bears little resemblance to the cilia originally isolated.

The conclusions drawn from the ^{31}P-NMR experiments were supported by the ESR data of Fig. 8. Although the changes are small, it would seem that around 20 °C, some change in membrane organization occurs in the ciliary membrane. The higher temperature phase has properties that lead to ^{31}P spectra indicative of complete isotropy, whereas the ESR spectra are typical of anisotropic motion. Thus, the dynamical process leading to averaging of NMR parameters must occur more rapidly than 5 kHz, and less rapidly than 80 MHz. Although various models exist for this type of phase [40,41], it is not possible to choose between them based on these data alone. Whether or not the membrane structural alteration is correlated with the regulation of swimming, which undergoes a sharp change at 17 °C [4], is at present under investigation. Such studies are relatively simple in *Tetrahymena*, since a fatty acid auxotrophe is available whose membrane composition can be modulated with ease [7]. Thus, the phase properties of the modified membranes can be directly related to the rate of swimming.

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