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PHOSPHORUS NUCLEAR MAGNETIC RESONANCE OF *ACHOLEPLASMA LAIDLAWII* CELL MEMBRANES AND DERIVED LIPOSOMES

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SUMMARY

1. The 129 MHz ^{31}P-NMR spectrum of *Acholeplasma laidlawii* membranes is very similar to the spectrum of the derived liposomes and is a typical "solid state" spectrum in which the major contribution to the linewidth is made by the chemical shift anisotropy. From the value of the chemical shift anisotropy an order parameter of 0.15 is estimated for the lipid phosphates in both membranes.

2. The ^{31}P-NMR spectrum of the *A. laidlawii* membrane is insensitive to pronase digestion of 40–60 % of the membrane proteins and subsequent cytochrome C binding. These results indicate that either no strong lipid polar headgroup – protein interactions occur in the membrane or that the lipid-protein "complexes" in the membrane have a fast rotation (T_c shorter than 10^{-6} s) along an axis perpendicular to the plane of the membrane.

3. Phospholipase A_2 degrades all the phosphatidylglycerol in the membrane. The resulting membrane contains a phosphoglycolipid as the sole phosphorus-containing compound. The ^{31}P-NMR spectrum of these membranes is identical to the spectrum of the native membranes suggesting a similar motion for the phosphate groups in both lipids.

4. Ca^{2+} binding to liposomes prepared from either the total polar lipids or the total phosphorus-containing lipids isolated from the *A. laidlawii* membrane does not affect the ^{31}P-NMR spectrum.

5. The ^{31}P-NMR spectrum of the membranes and derived liposomes, however, is sensitive to lipid phase transitions. When the membrane lipids are in the gel state a broadening of the ^{31}P resonance occurs demonstrating that the polar head group motion in a biological membrane is more restricted below the lipid-phase transition temperature.

INTRODUCTION

Phosphorus nuclear magnetic resonance has been shown to give valuable information about the motion of the polar headgroup of phospholipids in model

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membrane systems. For lipid dispersions (liposomes) [1, 2], sonicated lipid dispersions (vesicles) [1, 3, 4] and oriented multilayers of phosphatidylcholine [5] it was found that the allowed motion of the phosphate group is strongly dependent upon the gel $\rightarrow$ liquid crystalline phase transition and the incorporation of cholesterol in the bilayer [1–5]. On the other hand, in the liquid crystalline state the ^{31}P-NMR linewidth was found to be independent of the chain length and unsaturation of the fatty acids chains present in the phosphatidylcholine molecule [2].

Little is known about the motional characteristics of the lipid phosphates in biological membranes. ^{31}P-NMR spectra of *Escherichia coli* membranes [6] and sarcoplasmic reticuluum membrane fragments [7] have been reported but the information derived from these spectra was rather limited due to the complex lipid composition of the membranes and poor signal to noise ratios of the ^{31}P resonances. We have therefore systematically investigated the membrane of *Acholeplasma laidlawii* using ^{31}P-NMR. This microorganism is very suitable for such a study because (1) no cell wall is present, (2) pure membranes can be easily isolated, (3) the fatty acid and sterol composition of the membrane, and therefore the gel $\rightarrow$ liquid crystalline phase transition temperatures, can be varied substantially [8–11], and (4) the lipid composition is rather simple. The total lipids consists of approximately 50 % glycolipids (e.g. mono and diglucosyl diacylglycerol) and about 50 % phosphorus-containing lipids in the form of phosphatidylglycerol and a phosphoglycolipid (for structure see Fig. 1) [12, 13]. This lipid composition can be further simplified using phospholipase A_2, which completely removes phosphatidylglycerol from the membrane. In this work we have studied the effect of phospholipase A_2 degradation of phosphatidylglycerol, pronase digestion of the membrane proteins, cytochrome C and Ca^{2+} binding, phase transitions and cholesterol incorporation on the NMR spectra of the phosphate groups in both *A. laidlawii* membranes and derived liposomes.
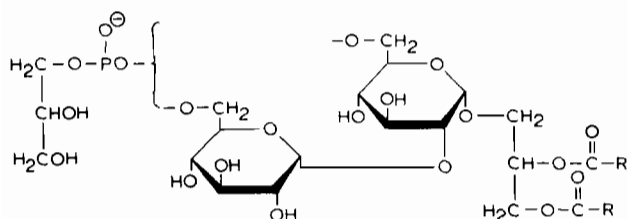

Fig. 1. Chemical structure of the *A. laidlawii* phosphoglycolipid 3-(*sn*-glycerol-3-phosphoryl-6′-[*O*-α-D-glucopyranosyl-(1 → 2)-*O*-α-D-glucopyranosyl]-*sn*-1,2-diacylgycerol.

MATERIALS AND METHODS

Materials and analytical methods

Cholesterol was purchased from Fluka (Buchs, Switzerland) and was recrystallised twice from ethanol. Pronase was obtained from Calbiochem (Hereford, UK) and horse heart cytochrome *c* from Sigma (St. Louis, USA). Pure phospholipase A_2 from pancreas was generously supplied by Dr. J. A. F. Op den Kamp. Protein was determined according to the method of Lowry (14) and lipid phosphorus via the Fiske-SubbaRow procedure [15].

Organism

A. *laidlawii* strain B cells were grown in a lipid-poor tryptose medium supplemented with 0.12 mM fatty acid or 0.06 mM fatty acid and 0.06 mM cholesterol, as described previously [10, 11]. Cells were harvested in the logarithmic phase, and were either washed and suspended in 150 mM NaCl, 25 mM Tris/acetic acid (pH 7.5) or used for the preparation of membranes.

Preparation of membranes

Membranes were prepared by the osmotic lysis method as described by van Golde et al. [16]. Subsequently the membranes were suspended in 10 mM Tris/acetic acid (pH 7.5) and centrifuged at 0 °C for 15 min at 37 000 × g. The membrane preparation was then frozen and stored at −20 °C for not more than 2 weeks. Samples for NMR experiments were prepared by adding 10 % ^2H_2O to the thawed membrane pellet and then centrifuging (at 0 °C) for 15 min at 37 500 × g. A fluffy layer on top of the tight pellet, which consists of very small membrane fragments, was discarded and the remainder of the pellet was used for the NMR experiments.

Pronase digestion of membranes

Membranes were digested with pronase as described by Rottem et al. [17]. Typically, 300 μg pronase were added to 2.5 ml of membrane solution (containing about 15 mg protein) in 25 mM Tris/acetic acid buffer (pH 7.5) and the mixture was incubated at 37 °C for 1 h. The reaction was stopped by chilling to 0 °C. The membranes were the washed twice in the above buffer. For the last washing 10 % ^2H_2O was added to the buffer. Under these conditions 40–60 % of the protein was removed from the membrane.

Phospholipase A_2 action on membranes

Phosphatidylglycerol was degraded in the A. *laidlawii* membrane by pancreatic phospholipase A_2 (Bevers, E. M. and Op den Kamp, J. A. F., unpublished observations). 5 μl phospholipase A_2 (1 mg/ml) was added to 5 ml of membrane suspension (15 mg protein) in 25 mM Tris · HCl (pH 7.5) containing 20 mM $CaCl_2$. After a 30 min incubation at 37 °C, the membranes were washed three times in 25 mM Tris · HCl (pH 7.5) with or without 5 mM EDTA. The fluffy layer on top of the final membrane pellet (after centrifugation at 37 500 × g for 15 min) was discarded and the remaining sample was used for further experiments. After extraction of the membrane lipids according to Bligh and Dyer [18], thin-layer chromatography on silicagel G in the system chloroform/methanol/water/acetic acid (65 : 25 : 4 : 1) showed that no detectable amounts of phosphatidylglycerol were present.

A substantial amount of fatty acids was detected, but no lysophosphatidylglycerol was present. This effect is attributed to the active endogenous lysophospholipase, which immediately converts the lysophosphatidylglycerol into free fatty acids and water-soluble glycerol phosphorylglycerol [16]. No degradation of the phosphoglycolipid (Fig. 1) and the glycolipids occurred, since these are not substrates for the pancreatic phospholipase A_2.

Cytochrome c binding to the membrane

The binding of cytochrome C to native membranes and membranes treated

with pronase or phospholipase A_2 was studied as described by Rottem et al. [17]. 20 ml of membrane suspension in 25 mM Tris · HCl (pH 7.5) containing 15 mg protein (7 mg in case of the pronase-digested membranes) were incubated for 1 h at 37 °C with 20 ml of 25 mM Tris · HCl (pH 7.5) containing 15 mg cytochrome c. The membranes were then washed once in a 25 mM Tris · HCl buffer (pH 7.5) containing 5 mM EDTA. The final pellet was dark brown rather than yellow because of the binding of cytochrome c, which is reported as 0.47 and 1.35 mg/mg membrane protein for native and pronase-treated membranes, respectively [17].

Lipid extraction and preparation of lipid dispersions

Lipids were isolated from the membranes via the Bligh and Dyer procedure [18] with 100 mM NaCl and 50 mM disodium EDTA present in the aqueous phase. The residual protein was removed by column chromatography over silica gel. Neutral lipids (mainly carotenoids) were eluted with chloroform and total polar lipids (glyco and phospholipids) with chloroform/methanol (1 : 1, v/v). The phosphorus-containing lipids were separated from the two glycolipids by column chromatography over silicagel, eluted with chloroform containing increasing amounts of methanol. Mono- and diglucosyl diacylglycerol were eluted from the column with 5–10 %, methanol and phosphatidylglycerol and the phosphoglycolipid with 10–20 % methanol. After evaporation of the solvent, the lipids were dissolved in diethyl ether and centrifuged for 10 min at $3000 \times g$ to remove residual silicagel. Lipids were finally dissolved in chloroform and stored under N_2 at -20 °C.

To prepare liposomes a chloroform solution containing 15 mg lipid (total polar lipids or total phosphorus-containing lipids) was dried down under a stream of N_2 such that a thin film of lipid formed on the bottom of the glass vial. After overnight storage under high vacuum, 1.0 ml of ^2H_2O containing 25 mM Tris/acetic acid (p^2H 7.0) and 0.2 mM EDTA was added. Liposomes were formed by agitating the vial on a vortex mixer for 10 min at room temperature. Vesicles were prepared by sonicating the liposome dispersion at 0 °C under N_2 until complete clearness, employing a Dawe sonifier at power setting 2. Titanium particles from the probe and any residual liposomes were removed by centrifugation.

Nuclear magnetic resonance

Most measurements were done on a 129 MHz ^{31}P-NMR spectrometer built in this laboratory [19] which was interfaced with a Nicolet B-NC-12 computer and equipped with quadrature detection, temperature control and field stabilisation via a deuterium lock. For some measurements a Brucker WH90 spectrometer operating at 36.4 MHz was used. This spectrometer was similarly equipped (except for quadrature detection) and in addition had a broad band proton decoupling facility. Accumulated free induction decays were obtained from 15 000–100 000 transients with a 0.4 s interpulse time. The estimated error in the determination in the width at half height in the ^{31}P NMR spectra of *A. laidlawii* membranes and derived liposomes is estimated as 0.3 KHz. 85 % phosphoric acid was used as an external reference.

RESULTS

³¹P NMR spectra of whole cells and isolated membranes of A. laidlawii and derived liposomes and vesicles

     A. laidlawii cells grown on oleic acid have membrane lipids composed of up to 60 % of this fatty acid [9–11]. These cell membranes are in the liquid crystalline state above 5 °C [20]. The 129 MHz ³¹P-NMR spectra of whole cells, isolated membranes and derived liposomes at 18 °C are compared in Figs 2a, 2b and 2c. In the whole cells (Fig. 2A) three major ³¹P resonances can be observed. The resonances marked with an asterisk arise from phosphorus-containing compounds in the cell cytoplasm since (a) they are absent in the ³¹P-NMR spectrum of isolated membranes (Fig. 2B) and (b) they are observed in the ³¹P-NMR spectrum of the supernatant of a cell suspension treated with 10 % trichloroacetic acid. These resonances are relatively broad and therefore must come either from bound phosphates or from very large phosphorus-containing macromolecules. Another possibility is that the cytoplasm of the *A. laidlawii* cell is very viscous. The other major resonance in the ³¹P-NMR spectrum would appear to be due to the cell membrane as it has a similar chemical shift and linewidth as the membrane spectrum (Fig. 2B). The complexity and lack of resolution in the whole cell spectrum makes it difficult to extract quantitative information concerning motion in the polar head group in these systems.
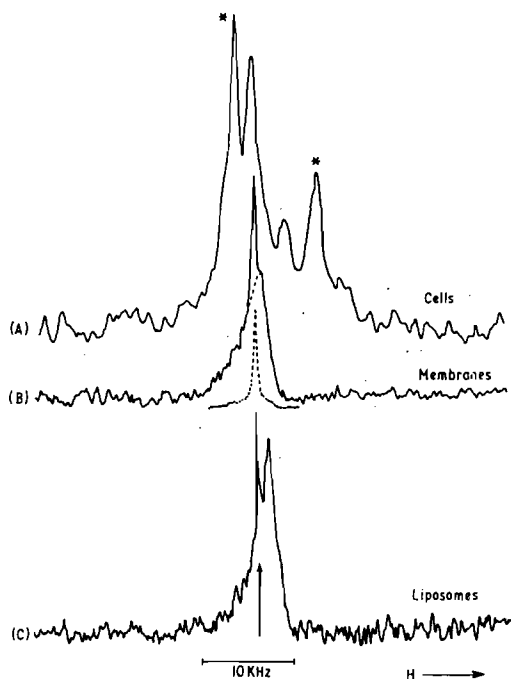

Fig. 2. 129 MHz ³¹P-NMR spectra of *A. laidlawii* cells (A), membranes (B) and derived liposomes (C) at 18 °C. The cells were grown on 0.12 mM oleic acid. The dotted curve underneath the spectrum (B) shows the 129 MHz ³¹P-NMR spectrum of 30-s sonicated membranes. The arrow denotes the resonance frequency of 85 % phosphoric acid.

The ^{31}P-NMR spectrum of the isolated membranes consists of a broad reso-
nance (width at half height 2.7 kHz) on which a much narrower resonance (half
width approx. 0.5 kHz) is superimposed. Both resonances must arise from phospho-
lipids as they are the only major phosphorus-containing compounds in the membrane
[21]. We have assigned the narrow line as the ^{31}P-NMR signal from small membrane
fragments formed by the osmotic lysis procedure because (a) brief (30-s) sonication
(which breaks the membranes into small fragments) causes significant line narrowing
(see Fig. 2B, dotted curve) due to the faster isotropic tumbling rate of the small
membrane fragments, (b) addition of 50 % (v/v) glycerol to the membrane suspension
which increases the viscosity and decreases the tumbling rate of the small membrane
fragments causes a marked broadening of the narrow line in the ^{31}P-NMR spectrum,
and (c) the relative intensity of the narrow line varies between different preparations.
If after centrifugation the fluffy layer on top of the membrane pellet is taken together
with the more tightly packed pellet the relative intensity of the narrow line increases.
As it is impossible to separate completely the fluffy layer from the tight pellet a minor
(10–20 %) percentage of small membrane fragments is always present in the membrane
preparation.

The broad ^{31}P signal in the membrane preparation (which accounts for
80–90 % of the total intensity) comes from the two phosphorus-containing lipids in the
large membrane fragments. These large (0.5–1.0 μm diameter) membrane fragments
have correspondingly slow isotropic tumbling rates. In such systems only the aniso-
tropic restricted motions available to the phospholipid in the membrane influence
the observed ^{31}P-NMR spectra. At the high magnetic field strength employed in this
work it has been shown that the chemical shift anisotropy of the phosphate phos-
phorus provides the dominant contribution to the observed ^{31}P-NMR line width
[1, 3]. In the absence of a fast isotropic averaging mechanism (which occurs for small
membrane fragments and sonicated liposomes) the ^{31}P-NMR signals have a charac-
teristic "solid state" shape which is typical of unsonicated liposomes [1–3].

Liposomes prepared from the total polar lipids isolated from the A. laidlawii
cell membrane have a ^{31}P-NMR spectrum (Fig. 2C) which is remarkably similar to
the spectrum of the membranes (Fig. 2B). About 5 % of the total intensity of the ^{31}P
signal from the liposomes is present in a narrow line, which we attribute to a small
fraction of the lipids in the liposome preparation being present in the form of either
small vesicles or micelles. The ^{31}P-NMR spectrum of liposomes prepared from the
total phospholipids was identical to the spectrum of the total polar lipids (containing
50 % glycolipids) shown in Fig. 2C.

Sonication of phospholipid liposomes produces small vesicles which give high
resolution ^{31}P NMR spectra [3]. The effect of chemical shift anisotropy in these
systems is such that better resolution of the ^{31}P NMR spectra is obtained at 36.4 MHz
than at 129 MHz [3]. The 36.4 MHz ^{31}P NMR spectrum of vesicles prepared from
the total phospholipids shows two major resonances (Fig. 3) which may be assigned
to the two phosphate-containing lipids occuring in the A. laidlawii membrane.
Resonance 1 is assigned to phosphatidylglycerol, as it has an identical chemical shift
to that observed for synthetic phosphatidylglycerol vesicles. Resonance 2 must there-
fore originate from the phosphoglycolipid (Fig. 2). The ratio of the intensities of
resonances 2 and 1 is 0.6, in agreement with the known lipid composition [12]. The
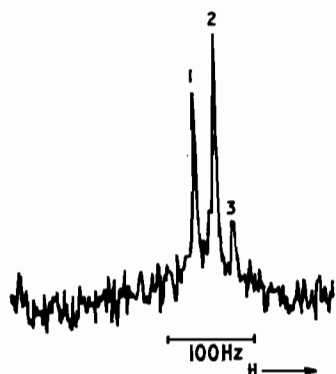origin of the minor resonance 3 is unknown.

Fig. 3. 36.4 MHz ^{31}P-NMR spectrum at 18 °C of sonicated vesicles prepared from the total polar lipids isolated from membranes of 0.12 mM oleic acid-grown *A. laidlawii* cells. Chemical shifts down field of the ^{31}P resonance of triphenyl phosphine in chloroform are 6.5, 5.8 and 5.3 ppm for reso-. nances 1, 2 and 3, respectively.

Effect of pronase digestion and cytochrome c binding

Pronase digestion of 40–60 % of the membrane proteins did not significantly affect the ^{31}P-NMR spectrum of the *A. laidlawii* membrane. The width at half height of the spectrum of pronase-digested membranes was 2.8 KHz. The relative intensity of the narrow signal increased to about 30 %, demonstrating that pronase treatment tends to break the membrane into smaller fragments. Cytochrome *c* binding to either native or pronase-digested membranes did not significantly affect the ^{31}P-NMR spectrum of these membranes. It has to be noted that, under our experimental conditions, lines as broad as the signals from anhydrous dipalmitoyl phosphatidylcholine (35 KHz) can be observed [2]. Any significant fraction of immobilised phosphorus atoms should therefore be observable. From a comparison of the signal to noise ratio of the phospholipid signals in the ^{31}P spectra of phosphatidylcholine liposomes and *A. laidlawii* membranes and by measuring the intensity of the *A. laidlawii* membrane signals against an internal reference, we estimate that we observe at least 75 % of the phosphorus atoms in the sample. Because of the fast pulse rate and relatively long T_1 values, which can differ for the various phospholipids [22], an accurate determination of the fraction of the lipid phosphates showing up in the spectrum is very difficult.

Phospholipase A$_2$ treatment of the A. laidlawii cell membrane

Phospholipase A$_2$ degrades all the phosphatidylglycerol in the *A. laidlawii* membrane. The resulting membrane thus contains only one phosphorus-containing lipid, i.e. the phosphoglycolipid (Fig. 1). The ^{31}P-NMR spectrum of the phospholipase A$_2$-treated membranes (Fig. 4) is very similar to the non-treated membranes. The half width is 3.0 KHz indicating that in the membrane the motion of the phosphate groups of phosphatidylglycerol and the phosphoglycolipid are approximately the same. This is a surprising result in view of the very different structures of the two lipids.

Effect of Ca^{2+}

As both phosphorus-containing lipids in the *A. laidlawii* membrane are acidic phospholipids, a strong interaction with divalent cations may be expected. Addition
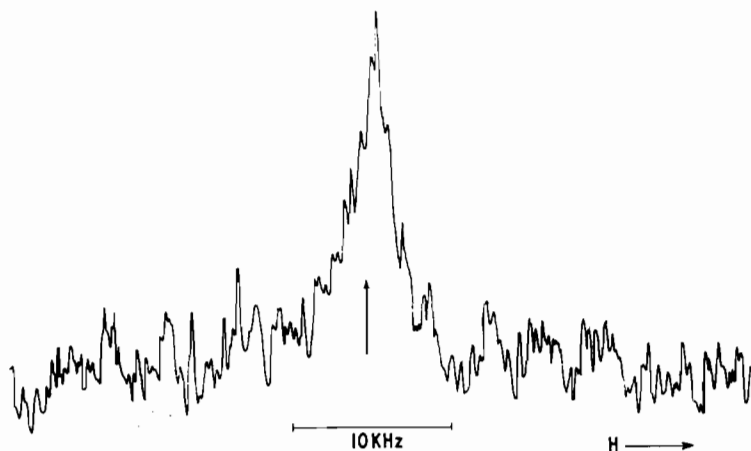
Fig. 4. 129 MHz ^{31}P-NMR spectrum at 18 °C of phospholipase A$_2$-treated membranes of *A. laidlawii* cells grown on 0.12 mM oleic acid. Phospholipase A$_2$ degradation was carried out as described in the Materials and Methods section and were finally washed with 5 mM EDTA-containing buffer. The arrow denotes the resonance frequency of 85 % phosphoric acid.

of up to 30 mM Ca^{2+} to either native, pronase-digested or phospholipase A$_2$-treated membranes did not affect significantly the 2.7–3.0 KHz broad resonance in the ^{31}P spectrum. The intensity of the narrow component did, however, decrease with increasing concentration of Ca^{2+}. This is interpreted as a result of Ca^{2+}-induced aggregation of small membrane fragments as the ^{31}P-NMR spectrum of 30-s sonicated membranes to which 25 mM Ca^{2+} was added was similar to the spectrum of
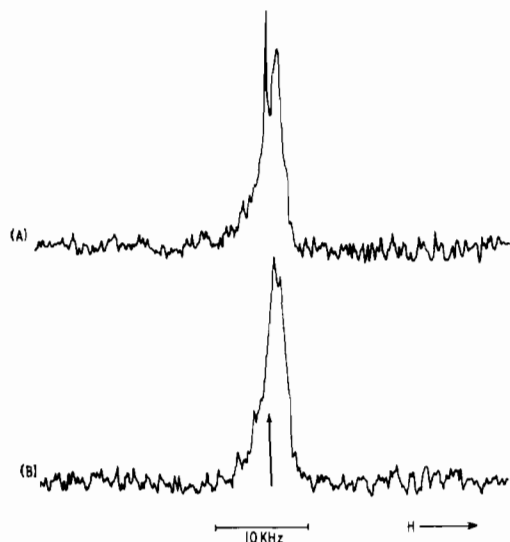

Fig. 5. 129 MHz ^{31}P-NMR spectra at 18 °C of liposomes prepared from the total polar lipids isolated from membranes of 0.12 mM oleic acid-grown *A. laidlawii* cells (A) without Ca^{2+} and (B) in the presence of 25 mM CaCl$_2$. The arrow denotes the resonance frequency of phosphoric acid.
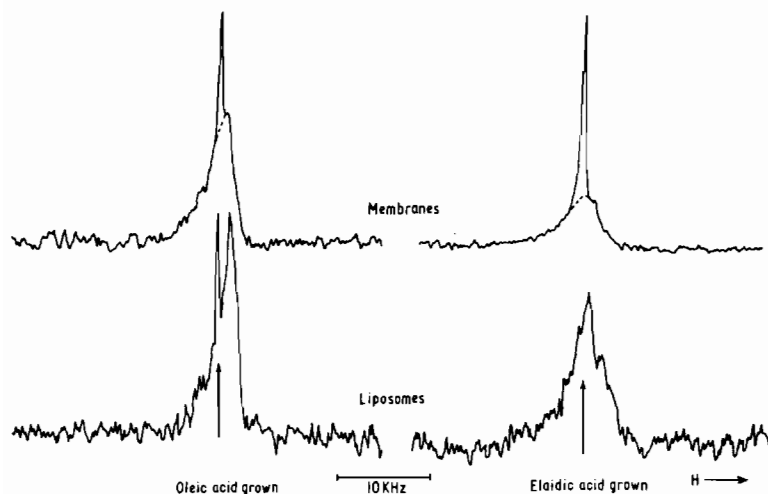
Fig. 6. 129 MHz ^{31}P-NMR spectrum at 4 °C of *A. laidlawii* membranes and derived liposomes. The *A. laidlawii* cells were grown on either 0.12 mM oleic acid or 0.12 mM elaidic acid. The arrow denotes the resonance frequency of 85 % phosphoric acid.

native membranes. Ca^{2+} addition to liposomes prepared from the total polar lipids causes only the elimination of the narrow line in the ^{31}P-NMR spectrum (Fig. 5). Brief sonication to enable Ca^{2+} to enter the inner aqueous compartment of the liposomes had no effect on the ^{31}P-NMR spectrum of the liposomes. In these liposomes
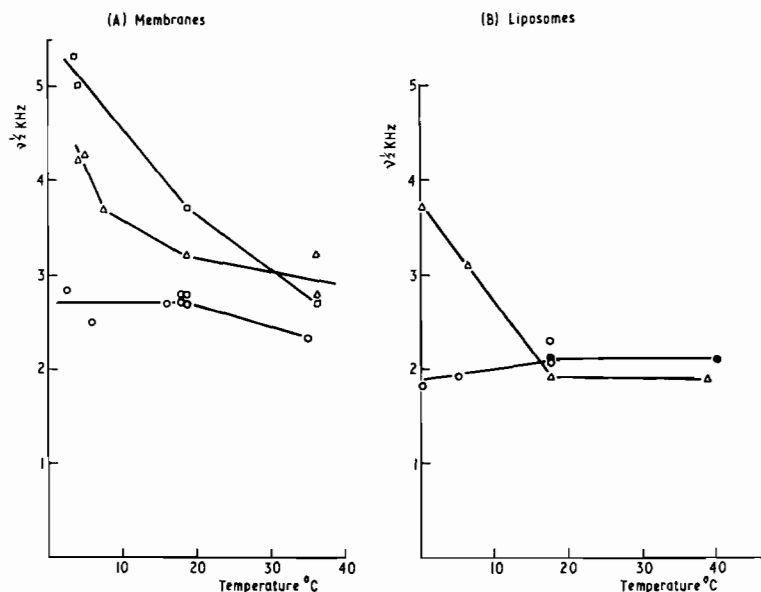

Fig. 7. Temperature dependence of the width at half height of the 129 MHz ^{31}P-NMR spectra of *A. laidlawii* membranes (A) and derived liposomes (B). Cells were grown on 0.12 mM of stearic acid (□), elaidic acid (△) or oleic acid (○). (●) Liposomes prepared from the total phospholipids isolated from oleic acid grown cells.

the presence of the glycolipids interferes with the formation of the specific acidic lipid Ca^{2+} complexes [23–27], since no precipitation of lipid occured after the addition of Ca^{2+}. Liposomes prepared of the total phosphorus-containing lipids, however, immediately precipitated after the addition of 30 mM Ca^{2+}, demonstrating the complex formation with Ca^{2+}. The ^{31}P-NMR spectrum of this complex was indistinguishable from the spectrum shown in Fig. 5B.

Effect of lipid phase transitions and cholesterol incorporation

In membranes of *A. laidlawii* cells grown on elaidic and stearic acid gel → liquid crystalline phase transitions occur in the temperature ranges 15–37 °C and 25–55 °C, respectively [10, 20]. Below 15 °C all the membrane lipids of the elaidic and stearic acid-grown cells are in gel state, as detected by differential scanning calorimetry [10, 20]. Fig. 6 compares the ^{31}P-NMR spectra of membranes of oleic and elaidic acid-grown cells at 4 °C. The spectra of the membranes of the elaidic acid-grown cells and the derived liposomes are significantly broader than the spectra of the membranes and derived liposomes of the oleic acid-grown cells. In Fig. 7 the
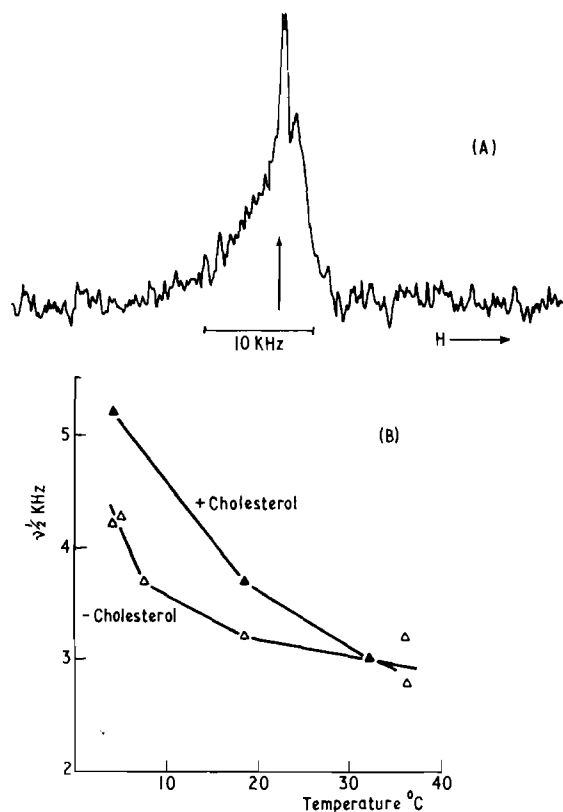

Fig. 8. (A) 129 MHz ^{31}P-NMR spectrum at 4 °C of membranes obtained from *A. laidlawii* cells grown on 0.06 mM elaidic acid and 0.06 mM cholesterol. The arrow denotes the resonance position of 85 % phosphoric acid. (B) Temperature dependence of the width at half height of the 129 MHz ^{31}P-NMR spectra of membranes obtained from *A. laidlawii* cells grown on 0.12 mM elaidic acid (Δ) or 0.06 mM elaidic acid and 0.06 mM cholesterol (▲).

temperature dependence of the line width is shown. It may be observed that for both membranes and liposomes the ^{31}P-NMR linewidth increases significantly when the lipids enter the gel state.

A. *laidlawii* cells grown in the presence of both elaidic acid and cholesterol incorporate up to 15 mol% cholesterol in the membrane without changing the membrane fatty acid composition [10]. The heat content of the phase transition is significantly reduced, demonstrating the lipid-cholesterol interaction in the membrane [10]. Cholesterol incorporation does not significantly influence the ^{31}P-NMR line width of the membranes above 20 °C (Fig. 8). Below this temperature the line width is slightly increased by the incorporation of cholesterol.

DISCUSSION

The 129 MHz ^{31}P-NMR spectra of native, phospholipase A$_2$-treated and pronase-digested A. *laidlawii* cell membranes and derived liposomes in the liquid-crystalline state closely resemble the 129 MHz ^{31}P-NMR spectra of phosphatidylcholine liposomes above the phase transition temperature [2]. The width at half height of the spectra of the A. *laidlawii* membranes and derived liposomes is only slightly (about 0.5 kHz) larger than the width of the spectra of the phosphatidylcholine liposomes.

The predominant contribution to the ^{31}P-NMR linewidth in these systems is made by the chemical shift anisotropy of the phosphate phosphorus [1–3, 5]. From the resulting spectra it is possible to estimate an order parameter $S_{CSA} = \Delta v_{EFF}/\Delta v_{CSA}$ which is characteristic of the motion in the polar headgroup region [1]. A measure of Δv_{EFF} (the frequency separation between the low field shoulder and the high field peak [1]) is difficult to obtain for such spectra as depicted in Figs. 2B, 2C and 4. However, very similar lineshape and linewidths are observed for non-oriented partially hydrated egg phosphatidylglycerol, which may be subsequently oriented to obtain a measure of Δv_{EFF} as $\Delta v_{EFF} = 5.0 \pm$ kHz [5]. It may thus be inferred that $\Delta v_{EFF} = 5 \pm 1$ kHz for the spectra shown in Figs. 2B, 2C and 4. The order parameter thus obtained ($S_{CSA} = 0.15$ for $\Delta v_{CSA} = 35$ kHz [1]) is similar to those obtained for a variety of oriented phospholipids; phosphatidylcholine, phosphatidylinositol, phosphatidylserine, and phosphatidylethanolamine in the liquid-crystalline state [5]. It should be noted that a measure of S_{CSA} does not allow an unambiguous determination of the motion in the phosphate region of the polar headgroup. This is because S_{CSA} is sensitive to two classes of motion which may be expected to occur, namely rotation of the entire polar headgroup about the long axis of the phospholipid molecule and vibrational motion about an axis perpendicular to the axis of rotation, both of which may produce similar effects (Cullis, P. R., McLaughlin, A. C. and Hemminga, M. A., in preparation). However, similar values of S_{CSA} in different membrane systems definitely indicates that the allowed motion of the phosphate group in these systems is also similar.

The phosphate group in the phosphoglycolipid is at a far greater distance from the glycerol backbone than for phosphatidylglycerol and might therefore be expected to have a greater motional freedom. A correspondingly narrower resonance is not observed, however, (the narrow lines observed in Figs. 2, 6 and 7 are, as pointed out in the results section, due to small membrane fragments). Our results are in agreement

with preliminary observations in oriented phospholipid systems in the liquid-crystalline state for which the primary motion experienced in the polar headgroup region is likely to be the rapid rotation about the long axis of the phospholipid molecule (Cullis, P. R., McLaughlin, A. C. and Hemminga, M. A., in preparation).

In our Ca^{2+} experiments on membranes and liposomes, we noted that the ^{31}P-NMR spectra of membranes and liposomes are not much affected by the presence of Ca^{2+}. It is known, however, that Ca^{2+} does interact with the phosphate groups of negatively charged phospholipids and brings about a strong reduction in molecular area and a corresponding increase in the hydrophobic interaction of the hydrocarbon chains [23–27].

The ^{31}P-NMR spectra arising from A. laidlawii membranes and derived liposomes are similar, furthermore the ^{31}P-NMR spectra of membranes are insensitive to 40–60 % removal of the membrane proteins by pronase. This could be explained if the protein lipid "complexes" themselves experienced fast rotation about an axis perpendicular to the plane of the membrane (with a rotational correlation time of less than 10^{-6} s) and if the orientation of the polar headgroup was not much affected by its association with protein. In this regard it is interesting to note that dichroism data imply that rhodopsin molecules in a membrane have (isotropic) rotational correlation times faster than 10^{-9} s [28]. An alternative explanation would be that the phosphorus-containing lipids in A. laidlawii membranes are not closely associated with the membrane proteins. It has been noted that phospholipase C from Bacillus cereus can degrade all the phosphatidylglycerol in isolated A. laidlawii cell membranes (Bevers, E. M. and Op den Kamp, J. A. F., unpublished observations). Apparently the phosphate groups of all phosphatidylglycerol molecules are available to the enzyme and are not in strong interaction with membrane proteins. This is not a general phenomenon as in Mycoplasma mycoides membranes it was demonstrated that a significant fraction of the membrane proteins had to be degraded in the membrane before the polar headgroups of the phospholipids could be attacked by phospholipase C [29].

The heat content of the gel → liquid-crystalline phase transition in lipid bilayers is gradually decreased by the incorporation of increasing amounts of cholesterol. At 50 mol% cholesterol no phase transition can be detected [10, 31]. Cholesterol incorporation in phosphatidylcholine liposomes brings about a strong reduction in the 129 MHz ^{31}P-NMR linewidth below the phase transition temperature [2]. Above this temperature cholesterol causes only a small decrease in linewidth. Our data on the 15 mol% cholesterol-containing A. laidlawii membranes are in contrast with these findings. The ^{31}P-NMR spectra of the phospholipids in the A. laidlawii membrane are not much affected by the incorporation of cholesterol. In fact a slight increase in the ^{31}P-NMR linewidths occurs below the temperature where all the lipids would be in the gel state in the absence of cholesterol. Further investigations are necessary to explain this seeming contradiction.

The gel → liquid-crystalline phase transition occurring in membranes of A. laidlawii cells grown on elaidic and stearic acid strongly affects the ^{31}P-NMR spectrum of the phosphate groups of the membrane lipids. When the lipids are in the gel state the motion is more restricted, as was reported for phosphatidylcholine liposomes [1, 2, 5], vesicles [1, 3, 4] and oriented multilayers [5]. Thus in a biological membrane the polar headgroup mobility is strongly affected by lipid phase transitions. Many important membrane functions require the existence of specific lipids in the membrane

and are influenced by lipid phase transitions. For instance, the $(Na^+ + K^+)$-ATPase from kidney medulla tissue depends upon the presence of negatively charged lipids and is affected by the lipid phase transition [30]. It would appear that polar interactions between the protein and lipid head group must play an important role in determining the enzymatic activity. A change in polar interaction due to decreased polar headgroup motion might be a possible explanation for the observed decreased enzymatic activity when the lipids enter the gel state.

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424

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