Biochimica et Biophysica Acta, 375 (1975) 186–208
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BBA 76836

NMR STUDIES ON PHOSPHOLIPID BILAYERS
SOME FACTORS AFFECTING LIPID DISTRIBUTION

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(Received July 1st, 1974)

SUMMARY

1. ^{1}H-NMR and ^{31}P-NMR are used to measure the outside/inside distribution of phospholipids in mixed vesicles.

2. Ferricyanide is a suitable shift reagent for measuring the outside/inside ratio of lecithin using ^{1}H-NMR even when the phospholipid mixture contains negative lipids.

3. ^{31}P-NMR can be used to measure the distribution of all phospholipids present provided the resonances are separated.

4. At 36.4 MHz the inside and outside phosphorus in lecithin vesicles have different chemical shifts. The separation at room temperature is 4–5 Hz and the individual linewidths are about 4Hz.

5. In a mixture of lecithin with phosphatidylethanolamine the latter has preference for the inside layer of the bilayer. The same holds for mixtures of lecithin with phosphatidylserine, phosphatidylinositol and phosphatidic acid.

6. In mixtures of lecithin and phosphatidylserine the preference of the latter for the inside is increased at lower pH under which conditions the negative charge of the phosphatidylserine is decreased.

7. In mixtures of lecithin with sphingomyelin the lecithin has a higher concentration at the inside.

8. The effect of vesicle size on the ^{31}P-NMR linewidth and the temperature dependence of this linewidth is in agreement with the conclusion of Berden et al. (FEBS Lett. (1974), 46, 55–58) that the chemical shift anisotropy, modulated by the isotropic tumbling of the vesicles, makes a contribution to the linewidth. The chemical shift difference between outside and inside phosphorus can be used as a parameter for the measurement of the packing density at the inside and of the size of the vesicles.

9. It is concluded that both charge and the packing properties of the headgroup are major factors in determining the distribution of phospholipids in mixed vesicles.

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INTRODUCTION

The asymmetry of biological membranes has been known for some time. Recently it has been demonstrated that not only the protein components have a unique distribution across the membrane but also the phospholipids are distributed asymmetrically [1, 2]. Litman has reported an asymmetric distribution of the phospholipids in vesicles comprised of phosphatidylcholine (lecithin) and phosphatidylethanolamine [3]. The experiments of Michaelson et al. showed that in vesicles containing lecithin and phosphatidylglycerol the charged component has a preference for the outside of the bilayer [4]. This, together with some theoretical calculations [5], leads to the suggestion that the charge in the phospholipid headgroup is of primary importance in determining lipid distribution in mixed bilayers. We have already reported that in certain situations this is not necessarily so [6]. Here we report detailed studies which demonstrate that many negative lipids behave differently from phosphatidylglycerol and therefore the charge is only one of the factors that have to be taken into account. Furthermore our ^{31}P-NMR linewidths data show that ^{31}P-NMR is a more useful technique in studying phospholipid bilayers than could be expected previously.

MATERIALS AND METHODS

Egg yolk lecithin, egg yolk phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were purchased from Lipid Products, Nutfield, U.K. Sphingomyelin, phosphatidic acid and dipalmitoyllecithin were obtained from Koch-Light Laboratories, Colnbrook, U.K. All lipids were used without further purification.

Cobaltous chloride, EDTA and potassium ferricyanide were obtained from Fisons, Loughborough, U.K.; praseodymium oxide (used for preparing praseodymium chloride) from Koch-Light, Trizma Base from Sigma, St. Louis, U.S.A.; ^2H_2O from Ryvan, Southampton, U.K. and Sepharose 4B from Pharmacia, Uppsala, Sweden. All chemicals were analar grade.

Preparations of vesicles were made by sonicating phospholipid suspensions in ^2H_2O (containing acetate or Tris–^{2}HCl) for 20–30 min with a Dawe soniprobe, power level 3, under a stream of nitrogen. The sample was kept in an ice–water bath to prevent the temperature rising above 30–40 °C except in the case of the mixture of synthetic β,γ-dipalmitoyl L-(3)lecithin and sphingomyelin. Thin-layer chromatography was used to check the absence of significant amounts of degradation products after sonication.

The use of ferricyanide as shift reagent in concentrations up to 300 mM did not affect the chemical properties of the phospholipids. The number of double bounds in the fatty acid chains was not affected, the Klein oxidation index [7] did not change and also thin-layer chromatography did not reveal any new components.

^{1}H-NMR was done at 270 MHz, using a Bruker spectrometer operating in the Fourier Transform mode. The spectrometer was interfaced with a Nicolet 1085 computer and equipped with a temperature control device. ^2H_2O was used for an internal lock.

^{31}P-NMR was done at 36.43 MHz, using a Bruker HX 90 spectrometer, equipped with a Nicolet B-NC 12 computer, a temperature control device, broad

band proton noise decoupling and deuterium lock. The instrument was operated exclusively in the Fourier Transform mode.

All NMR experiments on vesicles were done on samples in ^2H_2O. The given p^2H values are derived from the measured pH values by the addition of 0.4. For the ^{31}P-NMR measurements of lipid extracts the dried extract was suspended in ^2H_2O medium containing 1 mM EDTA, sonicated and then dissolved in a one phase solvent (water, methanol and chloroform). From this solution the lipid was extracted in chloroform. For the measurement the sample tube was inserted in a wider tube containing ^2H_2O for the internal lock system.

Except for the synthetic β,γ-dipalmitoyl L-(3)lecithin and sphingomyelin mixture, all vesicle preparations were kept at 4–5 °C, also during the chromatography over Sepharose 4B.

RESULTS

The use of ferricyanide as shift reagent

When egg yolk lecithin is sonicated small vesicles are formed which can be fractionated on a Sepharose 4B column according to the method of Huang [8]. A typical ^1H-NMR spectrum of lecithin vesicles is shown in Fig. 1. The resonance of the $-N^+(CH_3)_3$ group is slightly asymmetric, supposedly due to the different chemical

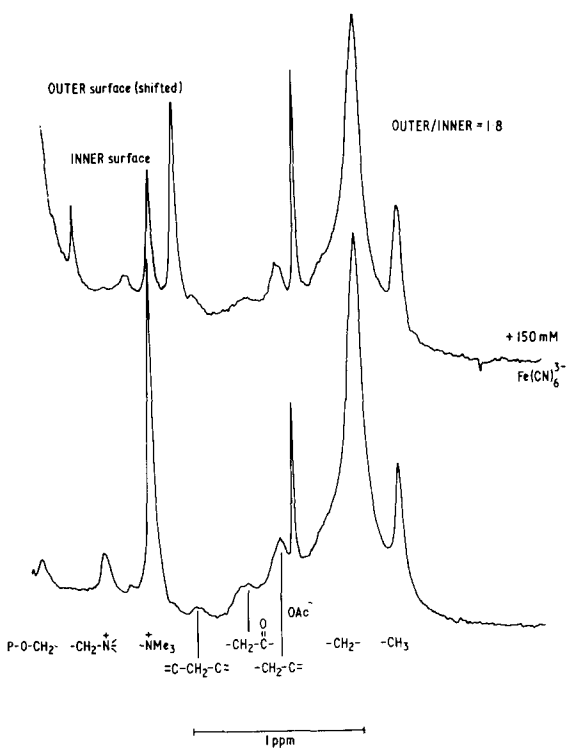

Fig. 1. ^1H-NMR spectra of egg lecithin vesicles (p^2H 7.4) in the absence and presence of 150 mM potassium ferricyanide. The low-field part of the spectrum, containing the H^2HO and the $-HC=C$ resonances, has been left out.

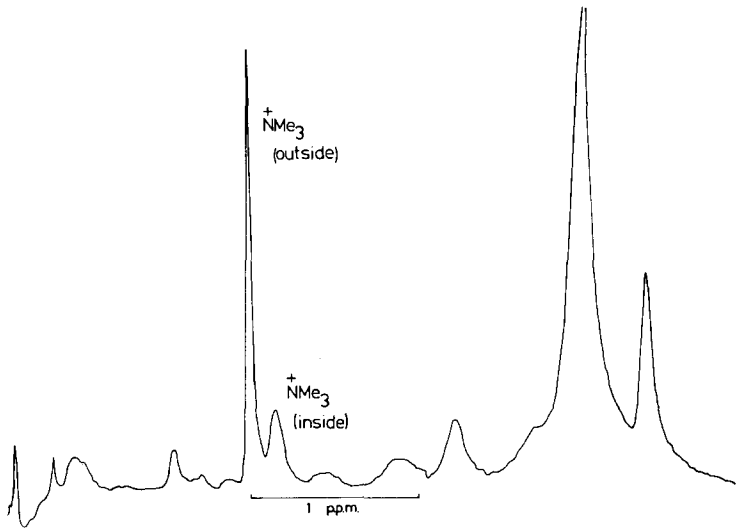

Fig. 2. ^{1}H-NMR spectrum of egg lecithin vesicles containing 150 mM potassium ferricyanide in the trapped volume. After sonication in the presence of ferricyanide the sample was fractionated over a Sepharose 4B column, free of ferricyanide. Fraction 19 (cf. Figs. 3 and 4) was used for the given spectrum.

shifts of these groups on the outside and inside of the bilayer. (In the spectrum shown this shift difference is less clearly visible than in the spectra reported by Kostelnik and Castellano [9] because of artificial broadening due to exponential multiplication of the free induction decay to improve signal/noise.) That the two types of signal arise out of the positions of the $-N^+(CH_3)_3$ groups is confirmed by the observation that on addition of an anionic "shift reagent", potassium ferricyanide [10], to the outside medium, the position of only the larger resonance is shifted (Fig. 1). To assign the shifted resonance as being due to groups on the outside of the vesicles we have to substantiate two assumptions. The first is that the reagent does not produce

TABLE I

t_1 VALUES OF PROTONS IN LECITHIN VESICLES IN THE PRESENCE OF 150 mM POTASSIUM FERRICYANIDE AT 270 MHz AND 20 °C

Group	t_1 Value (msec)	
	Unfractionated dispersion	Fraction 16
$-N^+(CH_3)_3$ outside	250	240
$-N^+(CH_3)_3$ inside	300	270
$-CH_2-N$ outside	280	—
$-CH_2-N$ inside	300	—
$-CH_2-COO$	370	—
$-CH_2-C=$	400	420
$-(CH_2)_n-$	460	440
$-CH_3$	650	650

any chemical change in the lipid. This is demonstrated by the fact that the effect is immediate after the addition of the ferricyanide and has no time dependence up to several days. Also, analysis of the lipid failed to detect any new components after the completion of the experiments. The second assumption, that the reagent does not penetrate the bilayer, is borne out by the following observation: when a lecithin suspension is sonicated in the presence of ferricyanide and the untrapped reagent is removed by fractionation on Sepharose 4B only the "inside" resonance is shifted (Fig. 2) (and somewhat broadened). Even after two days no leakage is observed. Moreover, the ratio of the two resonances (shifted/non-shifted) is 1/1.9–2.0 in Fig. 2 in comparison with the value of 1.9 in a similar fraction with ferricyanide added at the outside (Fraction 19 in Fig. 4).

The advantage of ferricyanide as a shift reagent is that it causes only slight broadening and little effect (10–20 %) on the spin lattice relaxation time t_1 [10]. (The broadening observed when the reagent was sonicated with the lipids (Fig. 2) may be due to a change in the coordination of the Fe^{3+} on sonication as evidenced by a slight colour change in the reagent.) Since the t_1 relaxation times of the inside and outside $-N^+(CH_3)_3$ resonances are nearly identical (see t_1 values given in Table I), the areas under the respective peaks in the presence of ferricyanide can be used as a measure of the proportions of the $-N^+(CH_3)_3$ groups in the two positions even under conditions where the delay time between subsequent pulses in the NMR experiment is somewhat less than 5 times t_1. Comparison of the $-N^+(CH_3)_3$ peaks with the area under the end $-CH_3$ resonances, however, is not valid under these conditions since the $-CH_3$ protons have a longer t_1 (see Table I) and their magnetization is less fully recovered at the start of the subsequent pulse.

Size distribution of lecithin bilayer vesicles

When egg yolk lecithin vesicles are fractionated on a Sepharose 4B column the data obtained from the elution profile, phosphate analysis and electron microscopy are similar to those reported [11] and show that under the given sonication conditions no multilayer structures or very large vesicles survive. The high resolution ^{1}H-NMR spectra of the various fractions are similar and the linewidths of the most intense resonances are identical over the whole range of vesicle sizes. Only the linewidth of the $-(CH_2)_n-$resonance deviates significantly in the two extreme fractions, Fractions 10 and 23 (Fig. 3). Since these two fractions together contain very little phospholipid ($\approx$ 2 %) the result shows that the spectrum of the unfractionated sample is the same as that of one of the main fractions containing a homogeneous population of vesicles.

Using the peak areas under the $-N^+(CH_3)_3$ resonances in the presence of ferricyanide the ratio of outside/inside headgroups for the different fractions can be obtained (Fig. 4). The indicated error limits are inversely related to the concentration of lipid in each fraction. In the same figure is plotted the calculated average vesicle size for each fraction. This size is calculated from the outside/inside ratio assuming that the packing density is the same on both sides of the bilayer and that the thickness of the bilayer is 37 Å [12]. It can be seen that beyond Fraction 16 the size does not decrease very much and in this region the mean outside/inside ratio is 1.9, a ratio that is only slightly higher than the value observed for an unfractionated sample (1.8–1.85). Although the diameters calculated are in reasonable agreement with those derived from gel filtration, sedimentation or trapped volume experiments, and with those

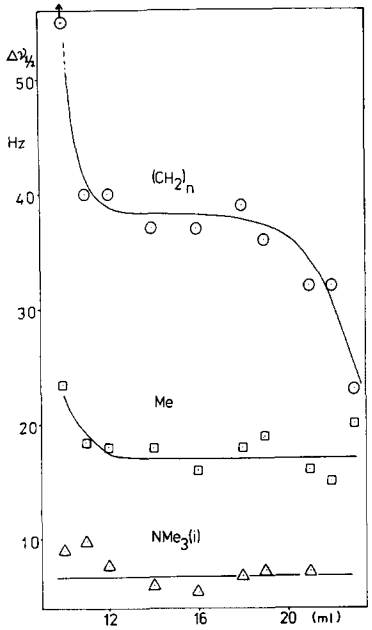

Fig. 3. Linewidth of proton NMR resonances of egg lecithin vesicles in the presence of 150 mM potassium ferricyanide. The sonicated sample was fractionated using a Sepharose 4B column in 20 mM acetate, p^{2}H 7.4. Fraction 10 represents the void volume.

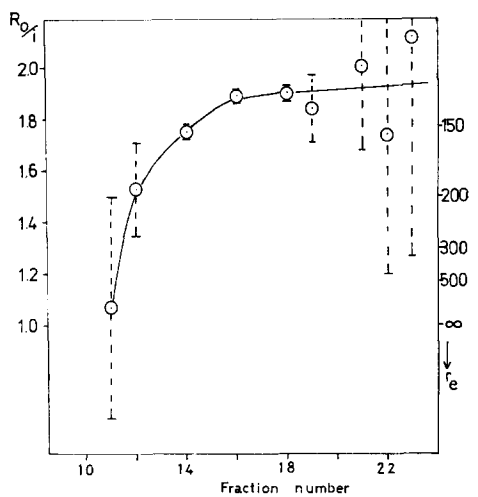

Fig. 4. Distribution of lecithin among the outside and inside layers of egg lecithin vesicles. For Fraction 10 (void volume) the ratio outside/inside was immeasurable. For the other fractions the ratio outside/inside was obtained from the ^{1}H-NMR spectrum in the presence of 150 mM ferricyanide. The percentage error limits were calculated as twice the inverse of the signal/noise ratio. The r_e scale was calculated from the outside/inside scale, assuming a bilayer thickness or 37 Å and equal packing density at inside and outside surface.

seen in the electron microscope [11, 12], there is a tendency for the shift-reagent method to give larger values for the size of the smaller liposomes.

Since for the seven main fractions (Fractions 13–19), containing about 85 % of the total lipid, the ratio outside/inside varies only from 1.65–1.9, while for the unfractionated sample this ratio is 1.8–1.85, the total distribution appears to be very homogeneous with only a minor amount of the phospholipid present in smaller or larger vesicles.

This relative homogeneity of the vesicle sizes, even in the absence of fractionation, is further demonstrated by examining the effect of sonication time. Although the number of phospholipid molecules in vesicles which are small enough to give sharp resonances in the ^{1}H-NMR spectrum, increases progressively until about 15 min, the ratio of outside/inside $-N^+(CH_3)_3$ resonances does not increase significantly after the first minute of sonication (Table II). Thus it appears, in agreement with earlier conclusions [13], that the sonication process produces small vesicles of a very specified size in a discrete manner, rather than by progressive reduction in the vesicle size.

TABLE II

EFFECT OF SONICATION TIME ON THE $-N^+(CH_3)_3$ RESONANCES OF LECITHIN VESICLES AT 270 MHz

Sonication time (min.)	Relative intensity	Outside/inside
1	6.4	1.8
4	9.9	1.6
7	12.0	1.92
10	12.4	1.88
15	12.9	2.04
25	13.4	1.86
40	12.9	1.86
95	12.7	1.90

^{31}P-NMR studies on lecithin bilayer vesicles

The relative homogeneity of vesicle sizes is very fortunate in view of using ^{31}P-NMR since with this technique the concentration of the samples must be higher than in proton NMR for a similar signal/noise ratio in a similar time and thus the dilution effects from size separation would not be tolerable. In all ^{31}P-NMR experiments described here we used unfractionated samples.

Fig. 5A shows that the ^{31}P-NMR spectrum of egg lecithin vesicles consists of two peaks, 4–5 Hz ($\approx$ 0.13 ppm) apart at 25 °C. The spectrum is very similar to the spectrum of dipalmitoyllecithin vesicles above the transition temperature [14]. At increasing temperature the two lines become slightly narrower but since the separation decreases at higher temperature (not shown) the resolution does not change very much. On addition of Mn^{2+} ions (cf. ref. 15) the low-field peak broadens and finally disappears while in the presence of praseodymium chloride or europium chloride the same peak is shifted (downfield or upfield respectively) and broadened as well. Fig. 5B shows that a small amount of Co^{2+} broadens the peak at lower

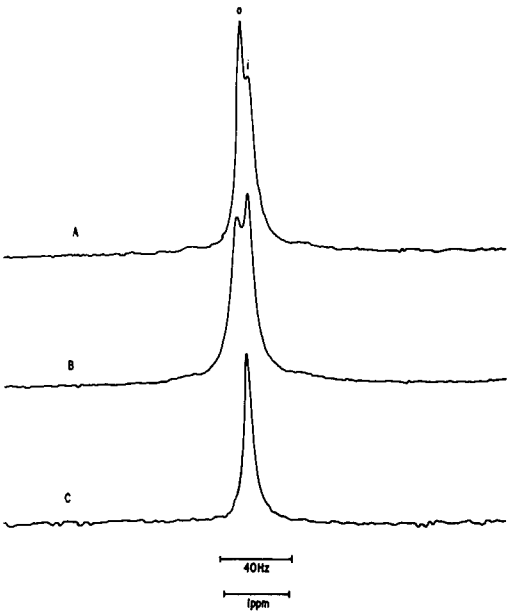

Fig. 5. ^{31}P-NMR spectra of egg lecithin vesicles (50 mg/ml lipid) in 25 mM Tris-^{2}HCl ($p^{2}H$ 7.6) and 1 mM EDTA at 27 °C. A, no additions; B, 1 mM $CoCl_2$ added; C, 4 mM $CoCl_2$ added.

field, concomitant with a slight shift downfield. At higher concentrations of Co^{2+} the affected resonance cannot be seen any more on the scale used in Fig. 5C because of the large broadening. These experiments identify the low field resonance with the outside and the high field resonance with the inside phosphate groups.

The remaining inside resonance in the presence of paramagnetic ions has a proton decoupled linewidth of 4–5 Hz. In the absence of proton decoupling a quintet can be seen, showing a coupling constant of 6.1 Hz between the phosphorus nucleus and the 4 -CH_2- protons next to the phosphate group. The found value for the linewidth is even less than the value reported for a solution of phospholipids in organic solvent (15–20 Hz [16]). Since this seemed unusual, considering that the proton linewidths are smaller in solutions than in vesicles [13], we measured some lipid extracts from various sources, taking care that no metal ions were present. One example is shown in Fig. 6. The lipid extract of chromaffin granule membranes, treated and measured as described in Materials and Methods, gave rise to a spectrum of very narrow resonances (1.5 and 3 Hz wide). The resonances due to the main components (phosphatidylethanolamine, lecithin and lysolecithin) are furthermore well separated.

Since in lecithin vesicles the outside and inside resonances are partly separated, the spin lattice relaxation time t_1 of each of them can be measured (Fig. 7). The t_1 value for the outside (3.0 s) is longer than that for the inside (2.4 s) and the overlap between the two resonances makes it likely that the real difference in t_1 values is even slightly larger than measured.

Measurements at 129 MHz (not shown) confirmed for egg lecithin the conclusion of Berden et al. [14] for dipalmitoyllecithin vesicles and synthetic β,γ-dipalmitoyl L-(3)lecithin and sphingomyelin mixtures that the ^{31}P-NMR linewidths are strongly frequency dependent, indicating a contribution of chemical shift anisotropy,

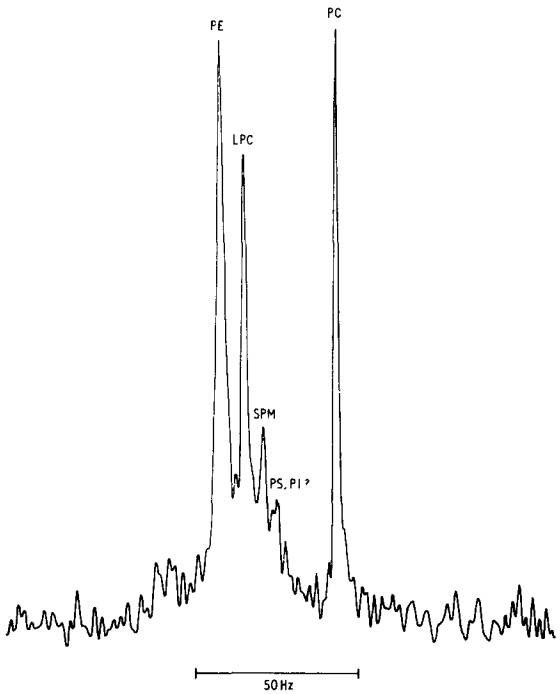

Fig. 6. ^{31}P-NMR spectrum of a lipid extract from chromaffin granule membranes at 25 °C. The lipid extract had been dried, sonicated in 1 mM EDTA–25 mM Tris–^{2}HCl (p^2H 7.4) buffer, dissolved in chloroform–methanol–water (1 : 2.2 : 1) and extracted into the chloroform layer after addition of more chloroform and water. The sample tube was inserted in a larger tube containing ^2H_2O for the locking. PE, phosphatidylethanolamine; SPM, sphingomyelin; PC, lecithin; PS, phosphatidylserine; PI, phosphatidylinositol; LPC, lysolecithin.

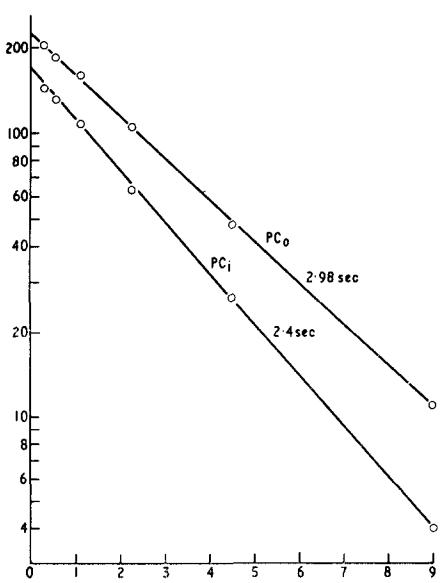

Fig. 7. ^{31}P-NMR t_1 measurement for the two resonances of egg lecithin vesicles (50 mg/ml lipids) at 27 °C. The medium contained 20 mM sodium acetate, p^2H 6.6. The 180–90° pulse sequence was used, the delay time between subsequent sequences being 15 s. PC$_i$, lecithin (inside); PC$_o$, lecithin (outside).

modulated by the isotropic tumbling of the vesicles, to the linewidth.

Distribution of zwitterionic phospholipids in mixed vesicles

TABLE III

DISTRIBUTION OF PHOSPHATIDYLCHOLINE IN PHOSPHATIDYLCHOLINE–PHOSPHATIDYLETHANOLAMINE (1 : 1) VESICLES ($p^2H = 6.5$)

Fraction number	$-N^+(CH_3)_3/-CH_3$	Outside/inside of $-N^+(CH_3)_3$
9 (void volume)	0.7	1.7
11	0.8	1.8
13	0.9	1.8
15	0.8	1.8
17	0.8	1.9
19	0.9	1.9
21	0.8	1.7

When a 1 : 1 mixture of egg lecithin and egg phosphatidylethanolamine is sonicated, vesicles are formed, but not when the relative amount of phosphatidylethanolamine is much further increased (cf. ref. 3). The elution pattern on Sepha-

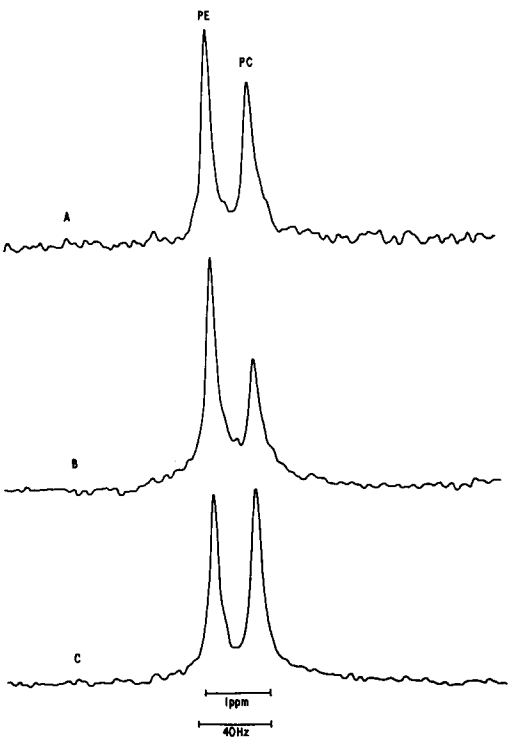

Fig. 8. ^{31}P-NMR spectra of phosphatidylethanolamine/lecithin vesicles (50 mg/ml lipid) in 25 mM Tris–^2HCl (p^2H 7.2), 1 mM EDTA at 50 °C. A, no additions; B, 3 mM $CoCl_2$ added (only the inside resonances show up); C, after resonication in 3 mM $CoCl_2$, 5 mM EDTA was added (only the outside resonances show up in the spectrum). PE, phosphatidylethanolamine; PC, lecithin.

rose 4B indicates that the size of the vesicles is larger than that of lecithin vesicles. For various fractions of a 1 : 1 mixture (original concentration 96 mM lipid) the ^{1}H-NMR spectrum was measured in both the absence and presence of 150 mM potassium ferricyanide. The results for the lecithin distribution are given in Table III. The ratio $-N^+(CH_3)_3/-CH_3$ is different from the expected 0.75 because of the difference in t_1 for the two groups of protons (see above). However, the constancy of this ratio (measured in the absence of ferricyanide) shows that the composition of the vesicles in all fractions is uniform as is the outside/inside ratio of the $-N^+(CH_3)_3$ resonances measured after the addition of the shift reagent. This latter indicates that the vesicle population is very homogeneous, but also, if the vesicles are really larger than lecithin vesicles, that the distribution of the phospholipids is asymmetrical (preference of lecithin for the outside). To verify this we measured a similar sample by ^{31}P-NMR. The relevant spectra, measured at 50 °C, are shown in Fig. 8 and the calculated distribution in Table IV. These data clearly confirm that the vesicles are larger than lecithin vesicles (outside/inside of total lipid is 1.4) and the distribution asymmetrical.

TABLE IV

DISTRIBUTION OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE IN PHOSPHATIDYLCHOLINE/PHOSPHATIDYLETHANOLAMINE VESICLES AS DERIVED FROM ^{31}P-NMR SPECTRA (p^2H = 7.2)

Total phosphatidylethanolamine/ phosphatidylcholine	1.08	total lipid outside/inside	1.41
Inside phosphatidylethanolamine/ phosphatidylcholine	1.38	phosphatidylethanolamine outside/inside	1.17
Outside phosphatidylethanolamine/ phosphatidylcholine	0.92	phosphatidylcholine outside/inside	1.76

Apart from telling us the distribution of the phospholipids over the inside and outside of the vesicles, the spectra of Fig. 8 give more information. First, there seems to be very little chemical shift difference between the outside and inside resonances; the positions are within the error limits identical (B and C respectively) and the combined resonances are not broader than the individual ones. Second, to get separation of the phosphatidylethanolamine and lecithin resonances, the measurement had to be done at 50 °C, the lines being too broad at room temperature. Third, the temperature dependence of the width of both lines (Fig. 9) is much larger than is the case for lecithin vesicles. The implications of these characteristics will be discussed later.

Another example of a zwitterionic mixture is a lecithin–sphingomyelin mixture. Since both phospholipids contain choline the ^{1}H-NMR spectrum does not give information about the distribution. Some ^{31}P-NMR spectra of a dipalmitoyllecithin–sphingomyelin mixture at 50 °C are shown in Fig. 10. The linewidths and the chemical shift differences between outside and inside are similar to those in dipalmitoyllecithin vesicles at 50 °C [14]. Fig. 10B shows that both phospholipids are identically affected by low amounts of Co^{2+} and that for both the outside resonance is at low field from the inside resonance. Comparison of Fig. 10A with 10C indicates the presence of a slight asymmetry. Outside dipalmitoyllecithin–sphingomyelin is smaller than inside dipalmitoyllecithin–sphingomyelin (1.2 and 1.52 respectively, while the overall dipalmitoyllecithin–sphingomyelin equals 1.3).

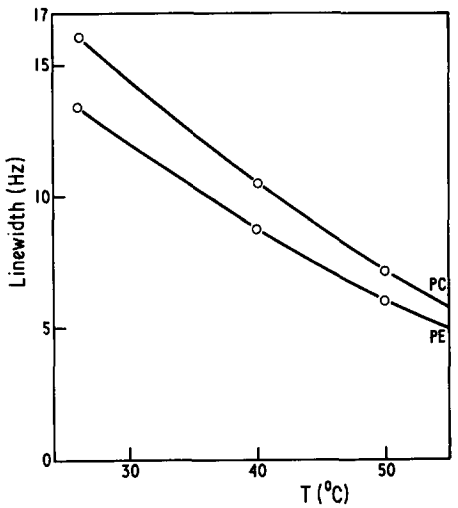

Fig. 9. Temperature dependence of the linewidths of the phosphatidylethanolamine and lecithin ^{31}P-NMR resonances of phosphatidylethanolamine–lecithin vesicles (see legend to Fig. 8). PC, lecithin; PE, phosphatidylethanolamine.

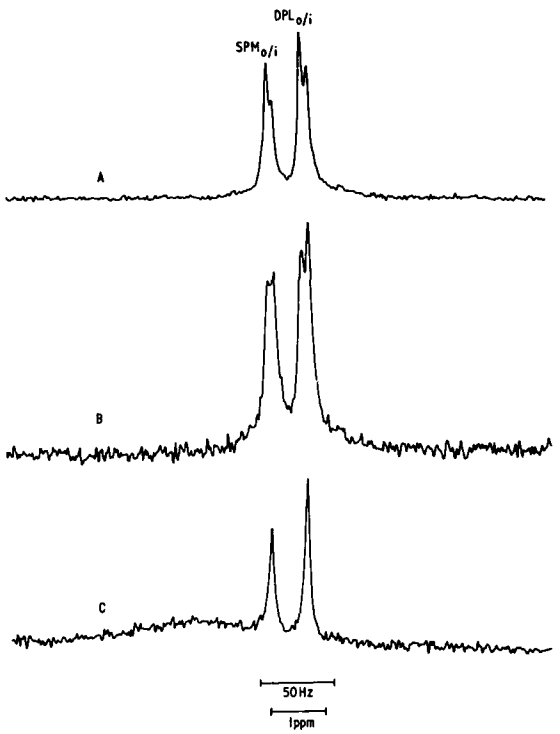

Fig. 10. ^{31}P-NMR spectra at 50 °C of dipalmitoyllecithin/sphingomyelin vesicles (50 mg/ml lipid) in 25 mM Tris–^{2}HCl (p^{2}H 7.2)–5 mM EDTA. A, no additions; B, 5 mM $CoCl_2$ added; C, 7 mM $CoCl_2$ added. $DPL_{o/i}$, dipalmitoyllecithin (outside/inside); $SPM_{o/i}$, sphingomyelin (outside/inside).

TABLE V

DISTRIBUTION OF LECITHIN IN LECITHIN/PHOSPHATIDIC ACID VESICLES (p^2H 6)
PC, lecithin; PA, phosphatidic acid.

Fraction	$-N^+(CH_3)_3/-CH_3$	Outside/inside of $-N^+(CH_3)_3$
A: PC–PA = 1 : 1, Expected $-N^+(CH_3)_3/-CH_3$ is 0.75		
Total	0.8	
17	0.9	2.0
20	0.9	2.4
23	0.9	2.8
B: PC–PA = 3 : 1 Expected $-N^+(CH_3)_3/-CH_3$ is 1.13		
Total	1.3	2.1
17	1.4	2.0
20	1.3	2.0
23	1.3	2.4

From consideration of charge repulsion one might expect that in mixtures of a negatively charged phospholipid and lecithin the former occupies preferentially the outside of the bilayer [5]. For a lecithin–phosphatidylglycerol mixture Michaelson et al. [4] have demonstrated this phenomenon. Yet in mixtures of lecithin with phosphatidic acid, phosphatidylserine or phosphatidylinositol, we find a preference of the negative lipid for the inside of the vesicle. Table V summarizes the results of experiments with lecithin–phosphatidic acid mixtures, using potassium ferricyanide as a shift reagent. Using Mn^{2+} as a broadening probe [15] we get similar results. Again the ratio $-N^+(CH_3)_3/-CH_3$ is higher than predicted by the composition of the vesicles because of the difference in t_1 for the two proton groups. The values for the outside/inside distribution of the lecithin in the mixtures are equal or higher than those in equivalent fractions of lecithin and the elution profile on Sepharose 4B indicated that the vesicles are larger than lecithin vesicles, as is also reported by Johnson [12]. This means that the overall outside/inside is smaller than the lecithin outside/inside and the phospholipid distribution asymmetric, the phosphatidic acid preferring the inside.

In the case of lecithin–phosphatidylserine and lecithin–phosphatidylinositol mixtures we observed remarkably high outside/inside ratios for lecithin, especially in the fractions containing the smallest vesicles. Fig. 11 shows the ^1H-NMR spectrum of one of the fractions of a 3 : 2 mixture of lecithin and phosphatidylserine, both in the absence and presence of 250 mM potassium ferricyanide. The sonication was done in 20 mM acetate at p^2H 4.6. The elution profile and the measured optical density indicated that the vesicles were significantly smaller than lecithin vesicles, but of similar size as phosphatidylserine vesicles (cf. ref. 17).

Since at increased p^2H values during sonication the outside/inside ratio of the lecithin was smaller, we did the experiment shown in Fig. 12 to see whether solely the difference in p^2H was responsible for this different outside/inside of the lecithin (a difference in size could be responsible as well). Five samples were prepared (lecithin–phosphatidylserine = 3/2) at different p^2H values and the distribution of lecithin was measured in the presence of ferricyanide. Of the remainder of each sample the p^2H was changed by addition of ^2HCl or NaO^2H to the outside medium. The samples

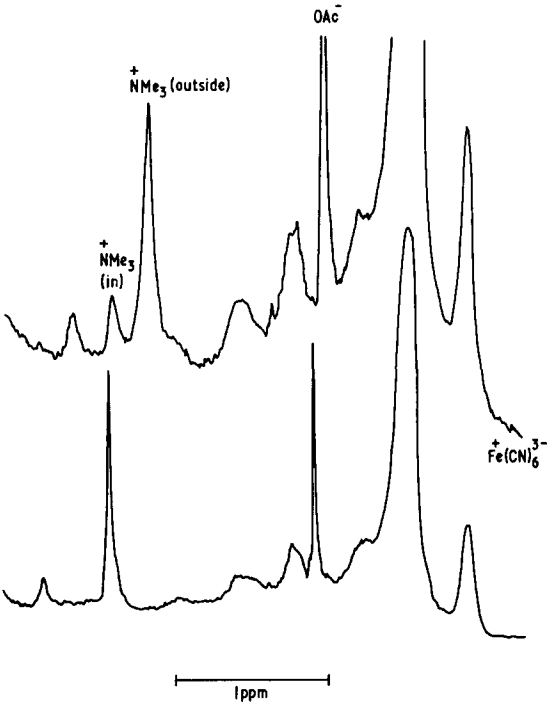

Fig. 11. ^{1}H-NMR spectrum of Fraction 21 of lecithin–phosphatidylserine vesicles fractionated over Sepharose 4B. The original sample contained 60 mM lecithin and 40 mM phosphatidylserine in 20 mM sodium acetate, p^{2}H 4.6. The column was equilibrated in 20 mM acetate p^{2}H 7.9 and the p^{2}H of the fractions was between 6.5 and 7.5. The samples and the column were kept at 4–5 °C.

were kept at 25 °C and after 2 h the p^{2}H values reached a constant level slightly above, respectively below, the adjusted values, suggesting an equilibration of the p^{2}H over the outside medium and the trapped volume. Then the ratio outside/inside of the lecithin was again determined and the result plotted against the measured pH. In Fig. 12 the abscissa shows the measured pH values, not the assumed p^{2}H values. If we may assume that the size of the vesicles does not change on standing, the experiment shows that the variation in outside/inside for lecithin is solely a function of p^{2}H and the size of the vesicles is not dependent on the p^{2}H.

Although the data of Fig. 12 clearly show that at decreasing p^{2}H (i.e. decreasing negative charge in the phosphatidylserine headgroup) the phosphatidylserine shows more preference for the inside layer of the bilayer, it is not clear how the size of the vesicles affects the distribution and how large the actual asymmetry is. Fig. 13 shows the outside/inside ratios of lecithin in the fractions of a 1 : 1 mixture of lecithin and phosphatidylserine. Since the lecithin–phosphatidylserine vesicles appear to be smaller than the lecithin vesicles, the results suggest that in the Fractions 10–15 (containing about 25 % of the lipid) the distribution is fairly symmetrical, while in the higher fractions, containing the bulk of the lipid and the smaller vesicles, the distribution is asymmetrical, lecithin preferring the outside layer. The second question, the actual extent of the asymmetry, was tackled by ^{31}P-NMR, which method, as used by us, does not distinguish between the smaller and larger vesicles in one preparation.

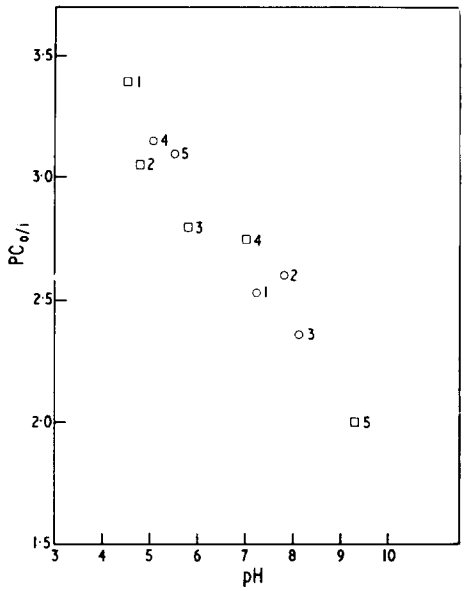

Fig. 12. pH dependence of lecithin distribution in lecithin–phosphatidylserine vesicles (20 mg/ml lipid) in 20 mM sodium acetate. The lecithin distribution was measured from the ^{1}H-NMR spectrum in the presence of 250 mM ferricyanide. □, lecithin-phosphatidylserine vesicles, sonicated at various pH values; ○, the p^{2}H of the original samples was changed by addition of ^{2}HCl or NaO^{2}H and after 2 h at 25 °C 250 mM ferricyanide was added and the spectra measured. The numbers indicate the corresponding samples. The measured pH values are given. PC$_{o/i}$, lecithin (outside/inside).

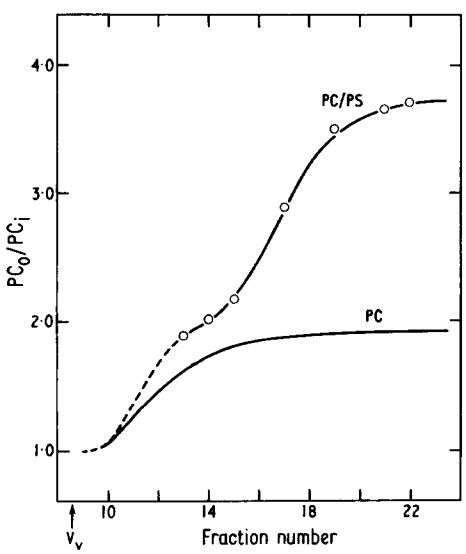

Fig. 13. Lecithin distribution in lecithin–phosphatidylserine PC/PS, vesicles after fractionation of a 1 : 1 mixture over Sepharose 4B. The sonication was done at p^{2}H 7.2 and the column was equilibrated in the same medium (20 mM sodium acetate). The ratio lecithin (outside/inside) (PC$_o$/PC$_i$) was determined from the ^{1}H-NMR spectrum in the presence of 250 mM ferricyanide. For several samples this ratio was also measured using Mn^{2+} to broaden out the outside resonances (especially the $-N^+-(CH_3)_3$ resonance [15]). The results with this method were essentially identical with the results obtained with ferricyanide. The curve marked PC shows the corresponding values for lecithin vesicles.

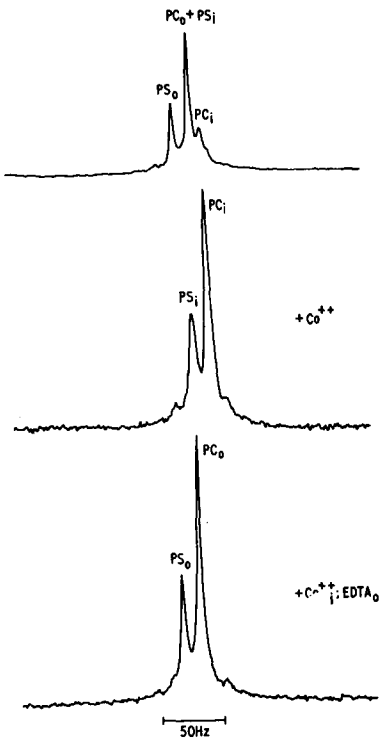

Fig. 14. ^{31}P-NMR spectra of lecithin/phosphatidylserine vesicles (50 mg/ml lipid) in 20 mM sodium acetate–1 mM EDTA (p^2H 7.2) at 26 °C. The delay time between subsequent pulses was 15 s. Upper spectrum, no additions; middle spectrum, 3 mM $CoCl_2$ added; lower spectrum, after resonication in the presence of 3 mM $CoCl_2$, 5 mM EDTA was added to the outside medium. PC_o, lecithin (outside); PC_i lecithin (inside); PS_o, phosphatidylserine (outside); PS_i, phosphatidylserine (inside).

Doing ^{31}P-NMR it became clear that for experiments with negatively charged phospholipids the presence of EDTA was necessary to obtain maximal resolution, although it had no effect on the ^{1}H-NMR spectra. Fig. 14A shows the spectrum of a lecithin–phosphatidylserine mixture at neutral p^2H. Three resonances can easily be distinguished and identified as lecithin (outside), lecithin (inside) and phosphatidylserine (outside). The fourth resonance, phosphatidylserine (inside), could be identified when the spectrum was measured in the presence of Co^{2+} under which condition the outside resonances were broadened beyond detection. A spectrum of only the outside resonances (Fig. 14C) was obtained via resonication of the sample in the presence of Co^{2+}, followed by addition of EDTA to bind the Co^{2+} ions in the outside medium. Since the ^{1}H-NMR data suggested that at neutral p^2H in a 3 : 2 mixture the distribution is not far from symmetrical, we measured all the spectra using a delay time of 15 s (5 times longest t_1, see later) to reach maximal accuracy. The areas of all the resonances were compared and the distribution of both lecithin and phosphatidylserine over the outside and inside calculated (Table VI). The resulting value for lecithin(outside)–lecithin(inside) could be verified via the ^{1}H-NMR spectrum in the presence of potassium ferricyanide. The measured value (2.5) agrees very well with the value of 2.45 calculated from the ^{31}P spectra. The data of Table VI show

TABLE VI

DISTRIBUTION OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLSERINE IN PHOSPHATIDYLCHOLINE/PHOSPHATIDYLSERINE VESICLES AS DERIVED FROM ^{31}P-NMR SPECTRA AT 36.4 MHz

PC, lecithin; PS, phosphatidylserine

A: p^2H = 7.2					
Total	PC–PS	1.8	total	outside/inside	2.3
Inside	PC–PS	1.6	PC	outside/inside	2.45
Outside	PC–PS	1.9	PS	outside/inside	2.06
B: p^2H = 4.9					
Total	PC–PS	1.8	total	outside/inside	2.3
Inside	PC–PS	0.83	PC	outside/inside	3.7★
Outside	PC–PS	2.64	PS	outside/inside	1.15

★ derived from ^{1}H-NMR spectrum in the presence of ferricyanide.

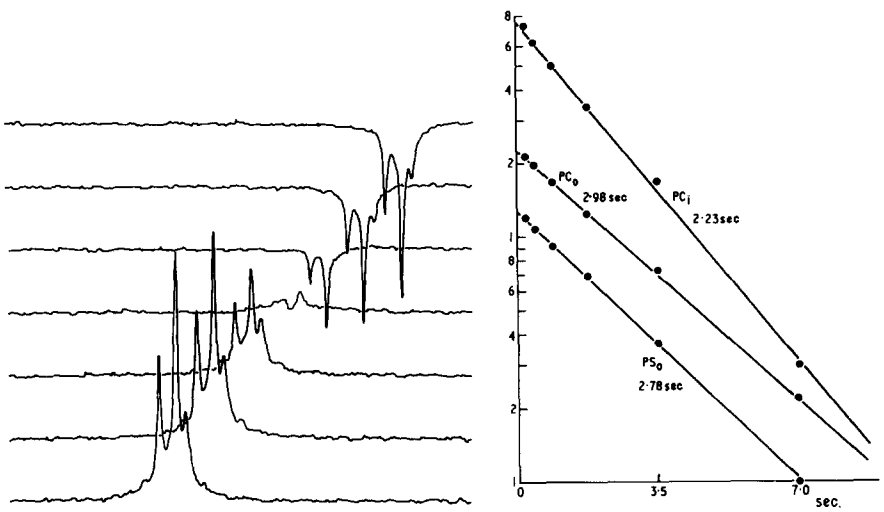

Fig. 15. ^{31}P-NMR t_1 measurement on lecithin/phosphatidylserine vesicles (sample as used for Fig. 14, upper curve) at 27 °C. The 180°–90° pulse sequence was used, the delay-time between subsequent sequences being 15 s and the number of transients 100. The lower trace shows the 90 ° spectrum, for the other spectra the delay-time between the 180° and 90 ° pulse is 7.0, 3.5, 1.75, 0.875, 0.437 and 0.218 s respectively. PS_0, phosphatidylserine (outside); PC_0, lecithin (outside); PC_i, lecithin (inside).

that the vesicles are indeed significantly smaller than lecithin vesicles (outside/inside of total lipid is 2.3 compared with 1.8 for lecithin vesicles) but that there still is some asymmetry at this p^2H, opposite to the asymmetry expected from the charge effect. The spectra of Fig. 14 show some further characteristics: the chemical shift difference between inside and outside is larger than in lecithin vesicles and the linewidths of the outside resonances are narrower (lecithin(outside) is 3.5 Hz wide and phosphatidylserine (outside) 3.6 Hz). On the other hand the inside resonances are broader, lecithin (inside) is 6 Hz wide and phosphatidylserine (inside) nearly 8. Fig. 15 shows a t_1 measurement for the same sample. The difference between lecithin (outside) (3.0 sec) and phosphatidylserine (outside) (2.8 s) is very small, but the t_1 relaxation time of the

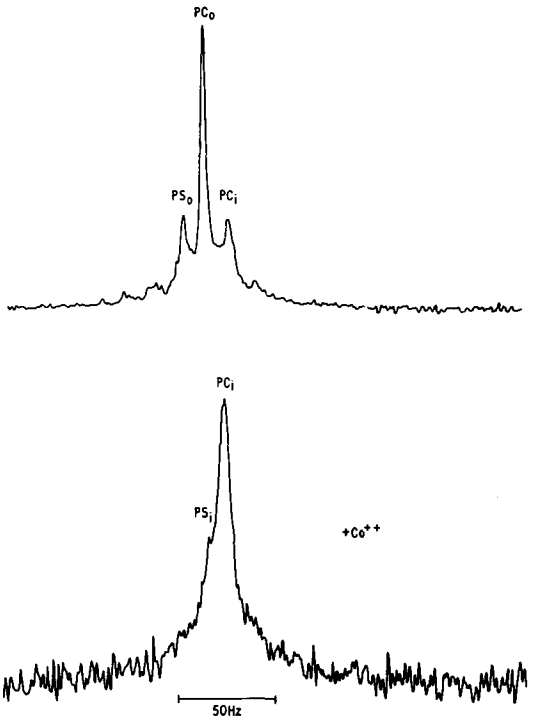

Fig. 16. ^{31}P-NMR spectra of lecithin/phosphatidylserine vesicles (50 mg/ml lipid) in 20 mM sodium acetate–1 mM EDTA (p^{2}H 4.9) at 27 °C. The delay time between subsequent pulses was 2.1 s. Upper spectrum, no further additions; lower spectrum, 3 mM $CoCl_2$ added. PC_o, lecithin (outside); PC_i, lecithin (inside); PS_i, phosphatidylserine (inside); PS_o, phosphatidylserine (outside).

lecithin (inside) resonance (2.2 s) is clearly shorter than that of the outside resonances. The t_1 for phosphatidylserine (inside) could not be measured in this experiment but the middle spectrum in Fig. 15 shows that the phosphatidylserine (inside) resonance has about the same t_1 as the lecithin (inside) resonance (both resonances are positive).

To verify the earlier conclusion from ^{1}H-NMR data that at lower p^{2}H the asymmetry is substantially higher we prepared an exactly similar sample as used for the experiment of Fig. 14, but at p^{2}H 4.9. The upper spectrum of Fig. 16 shows that the ratio lecithin (outside)–phosphatidylserine (outside) is now much larger than at neutral p^{2}H. Unfortunately, the lower spectrum, in the presence of Co^{2+}, shows that the resonances of lecithin (inside) and phosphatidylserine (inside) now overlap with the consequence that their ratio cannot be calculated. Since we know that lecithin–phosphatidylserine equals 1.8 and the ratio lecithin (outside)–lecithin (inside) is 3.7 (from the ^{1}H-NMR spectrum in the presence of ferricyanide), we still can calculate the complete distribution if we assume that the total outside/inside ratio is the same as at neutral p^{2}H (cf. data of Fig. 12). The results of these calculations are given in Table VI and the value for lecithin (outside)–phosphatidylserine (outside) agrees very well with the ^{31}P-NMR spectrum, indicating that the assumptions did not introduce a large error.

DISCUSSION

From the ^{1}H-NMR spectra shown it is clear that ferricyanide, although highly negatively charged, is a suitable shift reagent not only for uncharged vesicles but also for vesicles containing negatively charged lipids. But ^{1}H-NMR gives limited information about distribution of phospholipids in mixed vesicles since only lecithin or sphingomyelin, containing a choline headgroup, can easily be measured separately. Although the headgroups of other phospholipids have one or more specific resonances, these cannot easily be used for inside/outside measurements because of their low intensity or their position in the spectrum. The distribution of lecithin or sphingomyelin in mixed vesicles over the outside and inside layer provides a measure of asymmetry only when the overall outside/inside distribution is known, and ^{1}H-NMR cannot easily provide this measurement.

^{31}P-NMR, on the other hand, can give all the measurements needed: each class of phospholipids has one specific resonance and, provided sufficient separation of the resonances is obtained, the distribution of each component in a mixture can be measured using a shift reagent or a broadening probe. It is shown in this paper that in simple mixtures complete separation is obtained. The resonances of phosphatidylethanolamine, phosphatidylserine and sphingomyelin can be well separated from that of lecithin and the same holds for the resonances of phosphatidylinositol, phosphatidic acid and cardiolipin, although all these resonances overlap partly among themselves. In solution in organic solvents, even much better resolution can be obtained, partly because the resonances are narrower and partly because the chemical shift differences are larger. As a consequence of these results the use of ^{31}P-NMR is a simpler and quicker way for determining phospholipid composition and purity than thin-layer chromatography provided enough material is available and paramagnetic cations are absent.

^{31}P-NMR, however, also has some disadvantages compared to ^{1}H-NMR. Since the sensitivity is less, the measurements on fractionated samples are highly time consuming unless the original sample is extremely concentrated or the fractions obtained are reconcentrated. Fortunately, the ^{1}H-NMR experiments showed that the distribution of vesicle sizes in an unfractionated sample is very homogeneous so that the use of fractionated samples for the ^{31}P measurements was not necessary. A second disadvantage of ^{31}P-NMR is the long spin lattice relaxation time for ^{31}P nuclei. Taking lecithin–phosphatidylserine vesicles (p^{2}H 7.2) as an example we can see that the t_1 for lecithin (outside) (3 s) is slightly longer than that for phosphatidylserine (outside) (2.8 s) and significantly longer than that for the lecithin (inside) resonance. This has the consequence that maximal accuracy in comparing the areas under the various peaks as a measure for the relative amounts of the various components is reached when a delay time between subsequent pulses of not less than 15 s is used. In the chosen example the resonances are fairly narrow and 100 scans (25 min) give a reasonable signal/noise, but when the resonances are broader the accumulation time becomes very long*.

^{31}P-NMR of phospholipid vesicles

Berden et al. [14] have shown that the use of higher magnetic fields to increase the chemical shift differences between the various resonances is not useful since part

of the linewidth is due to chemical shift anisotropy which increases as the square of the field. Because of this, the frequency at which optimal resolution is obtained depends on the contribution of the chemical shift anisotropy to the linewidth. In lecithin vesicles where this contribution at 36.4 MHz is about 30 % (see ref. 14 and for a more detailed examination and discussion, ref. 18), the optimal frequency for resolution is about 50 MHz, but in larger vesicles, so as lecithin–phosphatidylethanolamine vesicles, which have a slower tumbling and therefore a larger contribution of chemical shift anisotropy, this optimal frequency is lower. Since vesicles prepared from lipid extracts from chromaffin granule membranes or sub-mitochondrial particles appeared to be fairly large, possibly due to the high content of phosphatidyl ethanolamine and gave broad resonances at 36.4 MHz (not shown), the optimal frequency for studying this type of vesicles is probably in the order of 24 MHz so that a 60 MHz proton spectrometer, operating at 24 MHz for ^{31}P, may be more useful than a high frequency spectrometer.

We have seen that the ^{31}P-NMR spectra of different vesicles show different characteristics, and if we are able to interpret these characteristics, the ^{31}P-NMR spectra can give useful information about the vesicles. Four characteristics can be listed: linewidth, temperature dependence of the linewidth, chemical shift difference between outside and inside, and difference in t_1 between outside and inside resonances.

Considering the contribution of chemical shift anisotropy to the linewidth we may expect that the linewidth increases with the size of the vesicles because of an increase in tumbling time. For the vesicles studied so far, this relation seems appropriate since lecithin–phosphatidylserine vesicles (linewidth lecithin (outside) 3.5 Hz) are found to be smaller than lecithin vesicles (linewidth 4–5 Hz) which in turn are smaller than lecithin/phosphatidylethanolamine vesicles (linewidth lecithin (outside) 15 Hz at 27 °C). Furthermore, the temperature dependence of the part of the linewidth that is due to chemical shift anisotropy will be increased by the additional dependence on the viscosity. The finding that for lecithin–phosphatidylethanolamine vesicles the

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* The ^{31}P-NMR spectra of the phosphatidylethanolamine–lecithin, dipalmitoyllecithin–sphingomyelin and lecithin–phosphatidylserine (p^2H 4.9) vesicles were obtained using a pulse rate of 1 per 2.1 s. Since the difference in t_1 for the outside resonances in Fig. 15 is only 7 % and also the two inside resonance have about equal t_1 values (difference within 10 %), the relatively short delay time between subsequent pulses will not introduce an error larger than about 5 % in the ratio of the outside resonances or the ratio of the inside resonances. Since, however, the difference in t_1 between the outside resonances at the one hand and the inside resonances at the other hand, is larger (25–30 % for lecithin–phosphatidylserine vesicles, p^2H 7.2) a more substantial error can be made in measuring the ratio of the two lipid components when these components have a very different outside/inside distribution.

If it is true that the difference in t_1 between the outside and inside resonances reflects the same vesicle properties as the chemical shift difference between inside and outside (see below) we may expect that for the phosphatidylethanolamine–lecithin vesicles the difference in t_1 for the outside and inside resonance is less than 10 % and the error made in the calculation of the ratio phosphatidylethanolamine: lecithin will not exceed 5 %. For the dipalmitoyllecithin–sphingomyelin mixture the difference in t_1 for outside and inside resonances will be equal to that in lecithin vesicles (20 %), but since the outside/inside ratios for the two components are not highly different (1.7 and 2.15 respectively) the actual error in dipalmitoyllecithin–sphingomyelin appears to be only 5 %.

For the lecithin–phosphatidylserine vesicles (p^2H 4.9) the error is substantially more and therefore we did not use the ^{31}P-NMR spectrum for the calculation of total lecithin–phosphatidylserine.

temperature dependence of the linewidth is larger than for lecithin vesicles supports the proposal that the relative broadness of the resonances of lecithin–phosphatidylethanolamine vesicles is due to a large contribution of chemical shift anisotropy. Also the third characteristic seems to be related to the size of the vesicles. The chemical shift difference between lecithin (outside) and lecithin (inside) is largest in lecithin–phosphatidylserine vesicles and smallest in lecithin/phosphatidylethanolamine vesicles while for lecithin and lecithin–sphingomyelin vesicles this value is intermediate. We have seen before that the size of lecithin vesicles as estimated from the outside/inside distribution tends to be greater than the size measured via other methods. This can be explained by the assumption that the packing density in the inside layer is higher than in the outside layer, which appears logical on the basis of the necessity of hydrophobic interactions between the fatty acid chains. From this assumption we can derive further that the difference in packing density will be highly dependent on the size of the vesicles (the curvature of the inside layer). The chemical shift difference between outside and inside phosphorus may therefore well be a useful parameter for the difference in packing density of the headgroups in the two layers. That the inside resonances are at high field from the outside resonances is in agreement with this proposal if we may assume that a higher density of the negative phosphate groups causes a more effective shielding of the phosphorus nucleus from the applied magnetic field resulting in a requirement for a higher field strength at resonance position. The additional finding (not shown) that the discussed chemical shift difference decreases at increasing temperature can be interpreted as an indication that an increase in motion of the headgroup in the headgroup layer decreases the effect of high curvature on the packing density. The fourth listed characteristic of the ^{31}P-NMR resonances, the difference in t_1 between outside and inside phosphorus resonances, can be explained on the same basis as the chemical shift difference but more t_1 measurements have to be done for certainty. It appears then that all listed characteristics are interrelated and together they form a solid base for comparison of various vesicles.

Role of charge and size of headgroup in determining phospholipid distribution over the outside and inside of vesicles

The presented data give evidence that in mixtures of lecithin with phosphatidic acid, phosphatidylethanolamine and phosphatidylserine the latter prefers location at the inside of the bilayer. Experiments with phosphatidylinositol showed that phosphatidylinositol behaved very similar to phosphatidylserine. In mixtures of lecithin with sphingomyelin the latter is preferentially located at the outside of the bilayer. The effect of increased charge, extensively studied only in the case of phosphatidylserine, is to decrease the affinity of the charged phospholipid for the inside of the bilayer, as might be expected [5]. Combining the results shown in Fig. 12 and the found outside/inside distribution of total lipid in lecithin/phosphatidylserine (3 : 2) vesicles, we may conclude that the distribution becomes symmetrical at pH values between 8 and 9 and asymmetrical again (phosphatidylserine more at the outside) at still higher pH values where the charge of the phosphatidylserine headgroup is between 1 and 2. This pH dependence of the phosphatidylserine distribution in lecithin/phosphatidylserine vesicles can be compared with the pH dependence of the phase-transition temperature of phosphatidylserine [18].

The preference of sphingomyelin for the outside layer in mixtures with lecithin

may well be due to a difference in geometry of the headgroup relative to that of lecithin, requiring a slightly larger area. The suggestion of Henderson et al. [16] that in sphingomyelin the phosphate group forms a hydrogen bound with the –NH of the sphingosine could well be the reason for this.

The four lipids shown to prefer the inside layer of the vesicles all have a smaller headgroup than lecithin and this seems to be the most important factor in determining the observed asymmetry. In the case of phosphatidylserine we may infer from the effect of pH that in the absence of any charge difference between lecithin and phosphatidylserine the asymmetry would be extremely large. The fact that the asymmetry is not great in phosphatidylethanolamine/lecithin vesicles, although phosphatidylethanolamine is uncharged and has an even smaller headgroup than phosphatidylserine, is not contradictory. The size of the phosphatidylethanolamine–lecithin vesicles studied was much larger than that of the lecithin–phosphatidylserine vesicles so that the difference in curvature between the outside and inside is much less pronounced and so the driving force for asymmetrical distribution weaker. We have initiated studies on phosphatidylethanolamine–lecithin vesicles containing lower amounts of phosphatidylethanolamine to see whether under these conditions the vesicles are smaller and the observed asymmetry greater (which would be in contrast to the observations of Litman [3]).

The distribution in phosphatidylglycerol/lecithin vesicles as reported by Michaelson et al. [4] has been interpreted [4, 5] as being due to the difference in charge between the two classes of phospholipids. Since, however, we have no clear information about the effect of the differences in headgroup and about the size of the vesicles, this interpretation needs further investigation.

A different problem is of why lecithin–phosphatidic acid, lecithin–phosphatidylethanolamine and possibly lecithin/phosphatidylglycerol vesicles are so much larger than lecithin–phosphatidylserine or lecithin–phosphatidylinositol vesicles. This difference is clearly not caused just by differences in the size of the headgroup. There seems to be, however, a relationship between the size of vesicles of the individual lipids and the size of vesicles containing mixtures with lecithin. As mentioned before (see also ref. 17) phosphatidylserine and phosphatidylinositol form very small vesicles (indicating that the electrostatic repulsion between neighbouring headgroups is less important than the hydrophobic interactions between the fatty acid chains), whereas phosphatidylethanolamine and phosphatidic acid do not form vesicles at all under our conditions of sonication.

We do not know at present to what extent the various asymmetries observed are solely the result of the nature of the headgroup, since the fatty acid chain composition of the different lipids used is also a variable. The contribution of this factor to lipid asymmetry remains to be elucidated.

It may appear that most biological membranes do not contain regions of high curvature, considering the radii of the various particles. It is striking, however, that in erythrocytes, lecithin and sphingomyelin are mainly localized in the outside surface and phosphatidylethanolamine and phosphatidylserine in the inside surface [2]. This suggests to us that biological membranes in vivo could possess large areas of high curvature as is known for the mitochondrial inner membrane. The use of small vesicles as model systems for biological membranes may thus be more justified than has previously been thought [19]. On the other hand, we believe that ^{31}P-NMR,

providing the resolution required to study lipid distribution in model vesicles, can also be used to study some aspects of lipid distribution in intact biological membranes.

ACKNOWLEDGEMENTS

We thank the Oxford Enzyme Group for the use of the 270 MHz spectrometer and Mrs Dr E. Richards for the use of 36.4 MHz ^{31}P-NMR facilities. We also thank Dr A. C. McLaughlin for helpful suggestions in preparing the manuscript. J.A.B. is recipient of a stipend from the Netherlands Organisation for the advancement of Pure Research (Z.W.O.).

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