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INTERVESICULAR PHOSPHOLIPID EXCHANGE. AN NMR STUDY

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Phospholipid exchange between phosphatidylinositol and phosphatidylcholine vesicles has been studied by NMR spectroscopy with use of hydrophilic paramagnetic lanthanide probes (Pr^{3+} and Eu^{3+} ions). The dependence of the lanthanide induced shifts in the ^{1}H and ^{31}P NMR spectra on the phospholipid composition of the vesicles could be used for its quantitative evaluation. The method has been proved to be applicable for studying phospholipid exchange stimulated by soluble proteins (postmicrosomal supernatant fraction) from rat liver. Furthermore it has been shown that the phospholipid molecules newly introduced by protein-stimulated exchange are predominantly incorporated into the outer monolayer of the vesicular bilayer membrane. This makes it possible to produce liposomes with asymmetric distribution of the phospholipids across the bilayer.

I. Introduction

Phospholipids have been shown to undergo exchange between different cellular membrane fractions during in vitro incubation (for a review see ref. [1]). Since the sites of cellular phospholipid biosynthesis frequently differ from those of membrane development such an exchange process is believed to play a certain role in the transfer of phospholipids from the former sites to the latter.

The in vitro exchange of phospholipids between mitochondria and microsomes from rat liver [2–7] and hepatoma [8] as well as between mitochondria and artificial phospholipid aggregates [9] is enhanced by a soluble protein fraction obtained from rat liver. The two lipids which are exchanged most rapidly between mitochondria and microsomes are phosphatidylcholine (PC) and phosphatidylinositol (PI) [2, 4, 5, 8]. A protein which specifically stimulates PC exchange between mitochondria and microsomes has been isolated in purified form from beef liver [10-12].

The procedure generally used for studying the phospholipid exchange activity of proteins is based on the transfer of radioactive labelled phospholipids from labelled to unlabelled subcellular particles or liposomes [9–12]. This method has various disadvantages. It requires labelled subcellular fractions or phospholipids, it is very cumbersome, especially if one desires to investigate the kinetics of the exchange process and the determination can be performed only on particle populations differing sufficiently in their sedimentation characteristics. Furthermore it is difficult

to evaluate the contribution of each step of the procedure to the overall observed exchange process.

For studying intermembrane phospholipid exchange two methods using artificial systems have also been suggested. In one of them the phospholipid exchange between immunosensitized and non-sensitized liposomes is investigated using immunoprecipitation for their separation [13]. According to the second method phospholipid exchange between two monolayers or between a monolayer and liposomes is followed by measuring changes in the surface radioactivity of the monolayer [14]. The first method includes a time consuming procedure of antiserum preparation, while the second one requires labelled synthetic phospholipids.

The present paper describes a completely different approach to phospholipid exchange studies based on following the changes in the NMR spectra of liposomes with different lipid composition during the exchange process.

Recently we have proposed the use of NMR shift and broadening reagents for studying artificial phospholipid membranes [15–17]. This method has been used successfully by other authors (see, for example, ref. [18–23]). We have found that the interaction of paramagnetic Mn^{2+} ions with sonicated PC dispersions broadens the $N^+(CH_3)_3$ proton resonance signal [15] and that Eu^{3+} and Pr^{3+} ions shift the signal upfield and downfield, respectively [16, 17]. Since the effects of paramagnetic ions on the NMR signal are determined not only by the nature of the ion and the structure of the molecule with which it interacts, but also by the environment of the interacting group, it could be anticipated that the $N^+(CH_3)_3$ signal of pure PC-vesicles and of vesicles containing other phospholipids as well should be affected to a different extent by paramagnetic ions. The differential shifting and broadening of the signal should thus permit one to follow the alterations in the phospholipid composition of different liposomes during the exchange process.

The present study which has been undertaken to investigate such a possibility, has been carried out in three stages. First we compared the effects of paramagnetic ions on the NMR spectra of sonicated dispersions of two individual phospholipids (PI and PC) and on mixed liposomes obtained by co-sonication of the two lipids. Then we studied a mixture of the separately sonicated PI and PC, i.e. a dispersion containing a heterogeneous population of phospholipid particles. Finally, we examined the time-dependent changes of the ^1H and ^{31}P signals during incubation of this mixture in the absence and in the presence of a phospholipid-exchanging protein fraction (PEPF) from rat liver.

II. Experimental

A. Materials

Phosphatidylcholine (PC) and phosphatidylinositol (PI) were isolated from egg yolk [24] and bakers yeast [25], respecticely. Just before use the phospholipids

were further purified by column chromatography on silica gel (KSK, 100–150 mesh) and the purity was checked by thin-layer chromatography. PEPF (a postmicrosomal supernatant fraction) was isolated from rat liver as described by Wirtz and Zilversmit [3]. The content of lipid phosphorus in PEPF was 0.84 μg per mg of protein. Chromatographic estimation of the lipid admixtures showed the presence of neutral lipids, fatty acids, phosphatidylethanolamine, PC, sphingomyeline, lyso-PC and PI (the latter two in traces only). For investigation of phospholipid exchange stimulated by PEPF an aqueous (D_2O) 50 mg/ml PEPF solution in 15 mM Tris–HCl (pH 5.1) containing 135 mM KCl and 1 mM EDTA was used. In the samples containing PEPF and phospholipid dispersions the EDTA content was 0.1 mM. A control experiment has shown that even higher EDTA concentrations (1 mM) practically do not influence the splitting between "outer" and "inner" $N^+(CH_3)_3$ signals of the PC liposomes in the presence of Pr^{3+} ions.

B. Methods

Phospholipid dispersions from pure PC and PI (K^+-salt) and from mixtures of these lipids in various ratios were prepared in D_2O by ultrasonication as described earlier [16]. The concentration of PC in all the samples was 30 μM/ml. The vesicular structure of the PI liposomes was confirmed by ^{31}P NMR spectroscopy with $MnSO_4$ as relaxation reagent (see below). In the studies of phospholipid exchange PC and PI liposomes (3 : 1 by weight) were incubated together at 50°C in the presence of PEPF (5 mg of PEPF per ml of incubation mixture). After incubation solutions of $Pr(NO_3)_3$ or $Eu(NO_3)_3$ in D_2O were added and the ^1H NMR spectrum was recorded. Control experiments were carried out in all cases by incubation of corresponding samples without PEPF.

^1H NMR spectra were obtained on a Varian XL-100-15 spectrometer at 100 MHz and ^{31}P NMR at 40.5 MHz with proton noise decoupling and internal ^2D lock. Errors in chemical shift and halfwidth measurements were ± 0.005 p.p.m. and ± 0.1 Hz, respectively, for ^1H nuclei and ± 0.01 p.p.m. and ± 0.3 Hz, respectively, for ^{31}P nuclei. Chemical shifts for protons were measured relative to the signal of $(CH_2)_n$ groups which is not influenced by paramagnetic ions [26]. The chemical shift of the $(CH_2)_n$ signal relative to the external standard (tetramethylsilane) is 1.35 p.p.m. for sonicated PC dispersions (without paramagnetic salt). The phosphorus chemical shifts were measured relative to external 0.5% H_3PO_4 in D_2O. ^1H NMR spectra were recorded in continuous wave with use of 5 mm tubes. ^{31}P NMR spectra were obtained in 12 mm tubes by the Fourier transform technique with a pulse interval of 9 sec (900 scans).

III. Results

A. Phosphatidylcholine liposomes

The effects of Mn^{2+} [15, 16, 18, 19], Eu^{3+} [16, 18, 20], Nd^{3+} [20] and Pr^{3+} [21] on the ^1H NMR spectra and of Pr^{3+} [17], Eu^{3+} [18] and Mn^{2+} [18] on the ^{31}P NMR spectra of PC liposomes have been described previously. The use of both Eu^{3+} and Pr^{3+} ions in the present study has made it possible to shift the signals either upfield or down. Thus when PC (30 μM/ml) was sonicated in the presence of $Pr(NO_3)_3$ the $N^+(CH_3)_3$-signal appeared at low field whereas in the presence of Eu^{3+} it appeared at high field, the shift induced by Pr^{3+} ($\Delta\delta_{ind}$) being greater than that by Eu^{3+} (the $\Delta\delta_{ind}$ values at 10^{-2} concentrations of the shift salts were 0.318 p.p.m. and 0.156 p.p.m. respectively).

Addition of $Pr(NO_3)_3$ or $Eu(NO_3)_3$ to pre-sonicated PC liposomes splits the $N^+(CH_3)_3$ signal into two components: a stationary signal (from the inwardly facing PC molecules) and a signal shifted to lower or higher field due to outwardly facing molecules that are capable of interacting with paramagnetic ions (fig. 1). The position of the unshifted $N^+(CH_3)_3$ signal was independent of the Pr^{3+} and Eu^{3+} concentrations, but that of the "outer" N-methyl groups moved increasingly with increasing salt concentration. Measurement of the relative areas under both signals showed that about $\frac{1}{3}$ of the PC molecules were inward-facing. This figure is in good agreement with previously obtained values [14, 15] which correspond to an average particle diameter of about 250 Å, assuming that the bilayer thickness is about 50 Å.

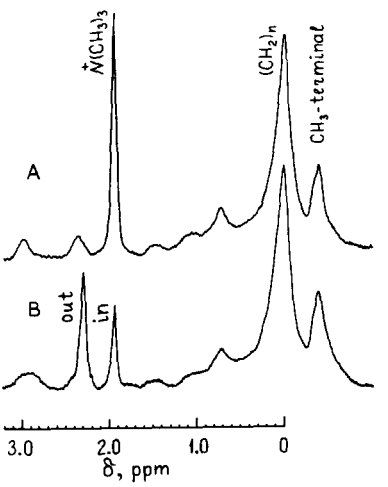

Fig. 1. ^1H NMR spectra of a sonicated 65 μM/ml dispersion of egg yolk PC in D_2O: (A) without $Pr(NO_3)_3$; (B) after addition of 0.01 M $Pr(NO_3)_3$.

B. Phosphatidylinositol liposomes

Earlier it had been shown by electron microscopy that PI dispersed in water behaves similarly to PC in forming bilayer aggregates [27]. However the morphology and dimensions of the latter depend largely upon the conditions used in preparing the PI dispersions. Because of this it was necessary to control the size and vesicularity of the sonicated PI-liposomes. The absence of prominent narrow signals from the polar head groups of the PI molecules precludes using ^1H NMR spectroscopy for this purpose; phosphorus resonance turned out to be more suitable. Mn^{2+} ions were chosen as the hydrophilic paramagnetic probe because the Pr^{3+} and Eu^{3+} salts induced rapid flocculation of the sonicated PI dispersions. Using ^{31}P NMR with Mn^{2+} additives we were able to demonstrate the vesicular nature of the PI liposomes obtained in our experiments.

As can be seen from the spectra shown in fig. 2 the addition of $MnSO_4$ (10^{-6} M) to a sonicated PI dispersion splits the phosphorus signal into two components, one

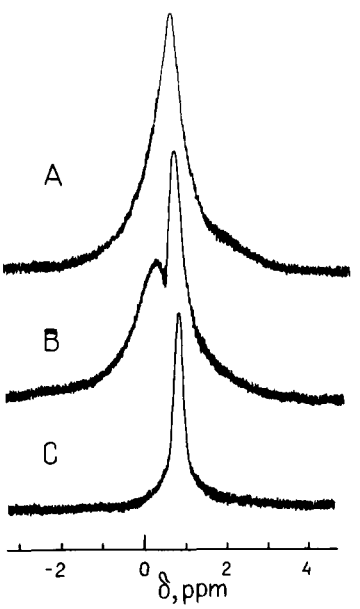

Fig. 2. ^{31}P NMR spectra of a sonicated 79 μM/ml dispersion of bakers yeast PI (K^+-salt) in D_2O: (A) without paramagnetic salts; (B) after addition of $2 \cdot 10^{-6}$ M $MnSO_4$. (C) after addition of 10^{-5} M $MnSO_4$.

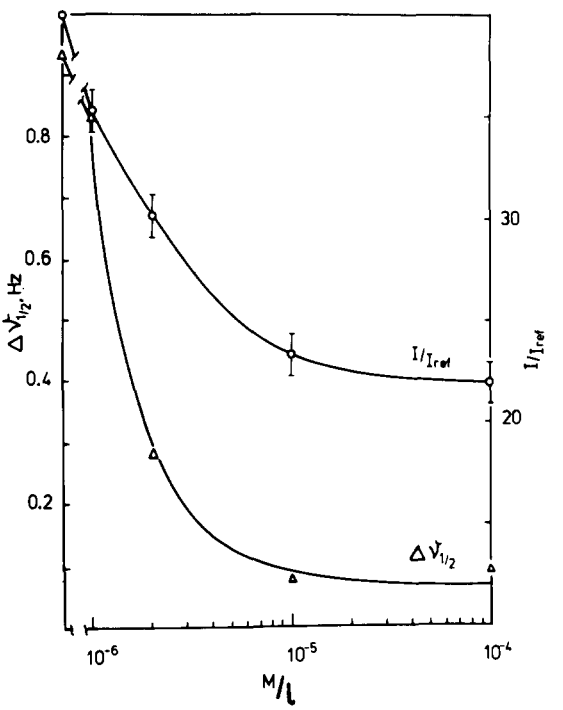

Fig. 3. Line-width ($\Delta\nu_{1/2}$) and relative intensity (I/I_{ref}) of ^{31}P NMR signal at various $MnSO_4$ concentrations in a sonicated 79 μM/ml dispersion of PI in D_2O.

narrow and the other broadened. The addition of increasing amounts of Mn^{2+} causes further broadening of the latter signal until at 10^{-5} M Mn^{2+} it is broadened beyond detection (and hence does not contribute to the intensity of the remaining component). The narrow component remains unaltered and persists even at higher Mn^{2+} concentrations. Fig. 3 shows the dependence of the integral intensity (I) of the ^{31}P signal related to the external reference (0.5% w/w H_3PO_4 in D_2O) peak intensity (I_{ref}) and the total ^{31}P signal width at half height ($\Delta\nu_{1/2}$) on the concentration of $MnSO_4$ introduced into the PI sample after sonication. The width at half height of the narrow signal did not change on letting the sample stand for at least 12 h implying that the paramagnetic ions did not penetrate into the internal cavity of the PI vesicles.

In agreement with the interpretation given earlier for the effects of paramagnetic ions on the NMR spectra of PC liposomes [15–17] we assign the narrow signal to the phosphate groups located on the inner membrane surface which does not come in contact with Mn^{2+}, whereas the broadened signal arises from the outwardly facing nuclei which are in contact with the Mn^{2+} ions. Hence it follows that the sonicated PI dispersions consist of sealed vesicles which are impermeable or of low permeability to Mn^{2+} ions.

The size of the PI vesicles can be evaluated from the integral intensity ratios of the phosphorus signals obtained in the absence of Mn^{2+} and at Mn^{2+} concentrations higher than 10^{-5}M, when the intensity of the "inner" signal becomes constant (fig. 3). At these concentrations the narrow component retains approximately 25–30% of the original peak area. This value is close to the relative intensity of the "inner" signal in the case of sonicated PC liposomes (see above). The similarity in the relative intensities of the "outer" and "inner" signals from the PI and PC liposomes suggests these liposomes to have comparable sizes and shapes. Since sonicated PC liposomes of such size are known to be mostly single shelled [28] the sonicated PI dispersion probably also contains predominantly single-bilayer vesicles.

C. Co-sonicated phosphatidylcholine-phosphatidylinositol liposomes

In the absence of paramagnetic ions the ^1H NMR spectrum of a sonicated PC–PI co-dispersion resembles that of PC alone, with increased intensity in resonances from proton groups which are duplicated in the two molecules. Addition of $Pr(NO_3)_3$ to the PC–PI co-dispersion splits the $N^+(CH_3)_3$ signal into the expected two components from outwardly and inwardly facing PC molecules (fig. 4B). As one can judge from the integral intensity ratios of the $N^+(CH_3)_3$ signals from the outer and inner surfaces the average size of the co-sonicated PI–PC liposomes does not differ much from that of sonicated PC liposomes.

The "outer" $N^+(CH_3)_3$ signal was found to be more downfield shifted and wider than the corresponding one in the spectrum of pure PC liposomes (cf. figs. 4A and B). The magnitude of the Pr^{3+} induced shift depended on the PC/PI ratio increasing with increase in PI concentration (table 1). Evidently both the increase of the in-

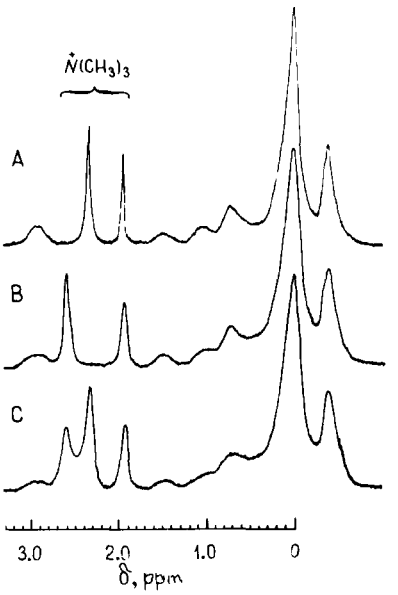

Fig. 4. ^1H NMR spectra of phospholipid dispersions in D_2O after addition 10^{-2} M $Pr(NO_3)_3$; (A) a sonicated 30 μM/ml PC dispersion; (B) a co-sonicated PC (30 μM/ml) and PI (10 μM/ml) dispersion; (C) a 1 : 1 mixture of samples A and B.

Table 1
Pr^{3+} induced shifts ($\Delta\delta_{ind}$) of the $N^+(CH_3)_3$ signal in ^1H NMR spectra of co-sonicated PI–PC dispersions in D_2O. PC concentration, 60 μM/ml; $Pr(NO_3)_3$ concentration, 0.01 M.

Exp. No.	PI content (moles % of PC)	$\Delta\delta_{ind}$ (p.p.m.)
1	0	0.41 [a]
2	5	0.49
3	10	0.53
4	30	0.67

[a] The chemical shift of the $N^+(CH_3)_3$ groups located on the internal surface of vesicles is 1.98 p.p.m. relative to the $(CH_2)_n$ signal.

duced shift and the broadening of the "outer" signal in the spectra of the PC–PI liposomes are caused by stronger binding of the paramagnetic ions to the liposome surface in the presence of negatively charged PI.

Recently Litman [29] arrived at the conclusion that co-sonicated PC–PI dispersions consist of a rather homogeneous liposome population. The PI–PC co-dispersions prepared in our experiments contained no individual PI vesicles as confirmed by the observation that $Pr(NO_3)_3$ caused intense flocculation of freshly

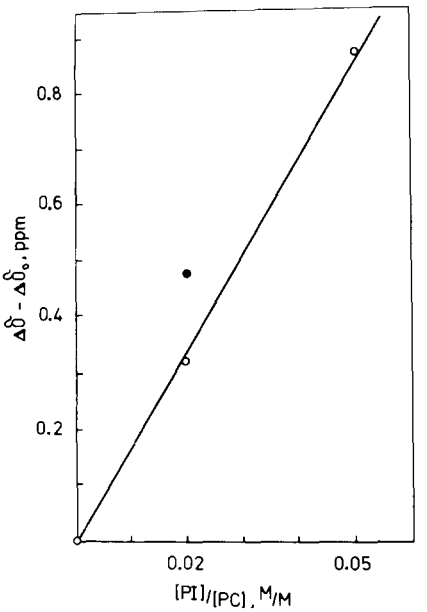

Fig. 5. Pr^{3+} induced shifts ($\Delta\delta_{ind}$) of the "outer" signal in the ^{31}P NMR spectra of co-sonicated PC/PI dispersions at various PI content. PC: 65 μM/ml; $Pr(NO_3)_3$: 10^{-3} M; $\Delta\delta_0$ = 0.28 ppm.

prepared mixtures of separately sonicated PI and PC vesicles but not of co-sonicated PI–PC vesicles.

Evidence suggesting the absence of pure PC liposomes in the co-sonicated dispersion was obtained by an NMR study of a mixture of sonicated PC liposomes with co-sonicated PI–PC liposomes. When $Pr(NO_3)_3$ was added to this mixture three $N^+(CH_3)_3$ signals appeared (fig. 4C). The most highfield (stationary) signal is due to inwardly facing PC molecules of both vesicle types which do not interact with Pr^{3+}. The most downfield shifted signal corresponds to outwardly facing lecithins that are in contact with both PI molecules and Pr^{3+} ions, while the intermediate signal is due to the outwardly facing molecules of the pure PC vesicles, whose phosphate groups contact with Pr^{3+} but not with PI.

Summarizing, it can be said that the co-sonicated PC–PI liposomes appear to be homogeneous and that the paramagnetic ion induced shift of the $N^+(CH_3)_3$ resonance for such liposomes depends on their composition.

The quantitative analysis of such liposomes may be accomplished by ^{31}P NMR. The magnitude of the Pr^{3+} induced shift of the ^{31}P signal strongly depends on the PI content even at low PI/PC ratios (fig. 5). Thus the praseodymium induced shift of the "outer" signal amounted to 1.15 p.p.m. in the case of co-sonicated 10% PC/PI (95 : 5) dispersion against 0.28 p.p.m. for a 10% PC dispersion at the same $Pr(NO_3)_3$ concentration (10^{-3} M). This permits to use ^{31}P NMR in the presence of paramagnetic shift reagents for in situ determination of the phospholipid composition of PC–PI liposomes containing rather small amounts (e.g. 1%) of PI.

Michaelson et al. [18] stated that liposomes with an asymmetric phospholipid distribution across the bilayer were formed spontaneously on co-sonication of 1 : 1 mixtures of PC and phosphatidylglycerol. We found that the integral intensity ratio of the $N^+(CH_3)_3$ signals from the outer and inner surfaces of co-sonicated PC/PI liposomes was about 2 : 1 and did not depend on the PI content at least up to 30 mole % of PI. Similar concentration independent ratios of the integral intensities of the "inner" and "outer" ^{31}P signals were also obtained from the ^{31}P NMR spectra of the same PC/PI dispersions. These data show that at the concentrations tested PI does not tend to distribute asymmetrically across the liposomal bilayer. However it is possible that noticeable trans-bilayer asymmetry arises spontaneously when the content of negatively charged phospholipid is sufficiently high. Alternatively the ability to form asymmetric bilayers spontaneously may depend upon the structure of the negatively charged phospholipid.

D. Spontaneous and protein stimulated phospholipid exchange between PC and PI liposomes

The dependence of the lanthanide induced shifts in the ^1H and ^{31}P NMR spectra of liposomes on their composition has been employed to follow the intervesicular phospholipid exchange stimulated by a protein factor present in the postmicrosomal fraction of rat liver (PEPF). Table 2 gives the "outer" $N^+(CH_3)_3$ proton shifts induced by Pr^{3+} and Eu^{3+} salts for the mixtures of separately sonicated PC and PI liposomes after incubation with or without PEPF. It can be seen that the induced shifts were much larger when the mixtures were incubated with PEPF (table 2, experiments No. 1–4). At the same time, PEPF did not affect the position of the "outer" $N^+(CH_3)_3$ signal on incubation with PC liposomes or co-sonicated PC–PI liposomes (table 2, experiment No. 5). We conclude that the increase of the induced shifts observed in experiments No. 1–4 (table 2) is due to incorporation of PI molecules into the PC liposomes and that this process is strongly enhanced by PEPF.

The incubation experiments carried out without PEPF (experiments No. 1–4, table 2) reveal a spontaneous exchange which usually proceeds much slower than the PEPF stimulated transfer (cf. experiments 1 and 2 in table 2) although incidentally it was found to be comparatively rapid. We suppose that this "spontaneous" exchange is caused by formation of lyso-PC during sonication. Evidence in favour of this idea will be presented in a forthcoming communication [30]. In preliminary experiments it was found that the lyso-PC stimulated exchange is nonspecific and that it requires amounts of lyso-PC which greatly exceeds those present in PEPF.

The amount of PI introduced into the PC liposomes by PEPF can be evaluated from the data of table 2. Thus the $\Delta\delta_{ind}$ value obtained in experiment No. 2 (table 2) corresponds to incorporation of about 43% of the starting PI in the presence of PEPF whereas only 3% are incorporated without PEPF. These figures were obtained presuming that PI is incorporated symmetrically into both monolayers.

Table 2
Lanthanide induced shifts ($\Delta\delta_{ind}$) of the $N^+(CH_3)_3$ signal in ^1H NMR spectra of mixtures of separately sonicated PC (30 μM/ml) and PI (10 μM/ml) liposomes after incubation (at 50°C) with PEPF (5 mg/ml) and in the absence of PEPF.

Exp.	Incubation time (hr)	Shift reagent	$\Delta\delta_{ind}$ (p.p.m.) after incubation Without PEPF	With PEPF
1	3	5×10^{-3} M/l, $Pr(NO_3)_3$	0.31 [a]	0.42
2	3	10^{-2} M/l, $Pr(NO_3)_3$	0.44	0.56
3	2	2×10^{-2} M/l, $Eu(NO_3)_3$	−0.30 [b]	−0.42 [b]
4	4	2×10^{-2} M/l, $Eu(NO_3)_3$	−0.31 [b]	−0.44 [b]
5	3 [c]	2×10^{-2} M/l, $Eu(NO_3)_3$	−0.29 [b]	−0.30 [b,d]

[a] For pure PC liposomes $\Delta\delta_{ind}$ = 0.27 p.p.m.
[b] Minus corresponds to a highfield shift.
[c] Control experiment: incubation of PC liposomes was carried out without PI.
[d] Concentration of PEPF 10 mg/ml.

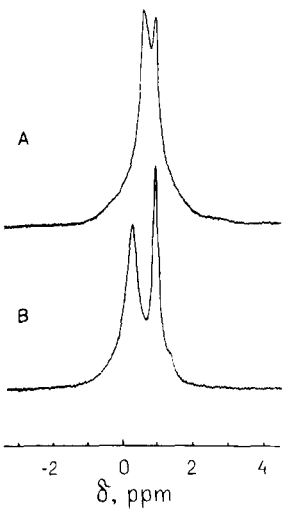

Fig. 6. ^{31}P NMR spectra of 98 : 2 PC–PI liposomes: (A) obtained from PC and PI by co-sonication; (B) obtained by incubation of separately sonicated PC and PI liposomes with PEPF (5 mg/ml). $Pr(NO_3)_3$ (10^{-3} M) was added to samples A and B before recording the spectra. Sample A also contained PEPF (5 mg/ml) added after sonication.

However the following experiments demonstrate that an asymmetric bilayer structure is formed under the conditions of PEPF stimulated phospholipid exchange. We have found that at low starting PI/PC ratios the PI incorporation is practically complete after 4–5 hr. Fig. 6 shows ^{31}P NMR spectra in the presence of Pr^{3+} ions for PC and PI liposomes sonicated separately, mixed in a 98 : 2 ratio and then incubated for 5 hr at 50°C with 5 mg/ml of PEPF (fig. 6B) and for a co-sonicated 98 : 2 PC–PI dispersion in the presence of the same amount of PEPF (fig. 6A). The induced shift is evidently much larger for the former sample. Thus the apparent PI concentration is essentially higher in PC–PI liposomes obtained by PEPF exchange than in the co-sonicated sample. A simple calculation permits to estimate the portion of PI molecules located on the outer surface of the mixed PC–PI bilayer. As can be seen from fig. 5 at low PI concentrations the induced shift ($\Delta\delta_{ind}$) of the "outer" ^{31}P signal depends linearly on the PI content in the co-sonicated PC-PI dispersion. Since Pr^{3+} ions interact only with external surface of the liposomes the $\Delta\delta_{ind}$ value is defined by the PI/PC ratio of the outer monolayer:

$$\Delta\delta_{ind} = \boldsymbol{a}[PI/PC]_{out} + \Delta\delta_0 \qquad (1)$$

where $\boldsymbol{a}$ is a proportionality coefficient and $\Delta\delta_0$ the distance between the "outer" and "inner" signals in the absence of PI, i.e. for pure PC liposomes. As we have found the PI distribution between the outer and inner monolayers of co-sonicated PC–PI liposomes to be symmetrical (at least at PI contents not exceeding 30 mole %) the coefficient $\boldsymbol{a}$ can be calculated from the data presented on fig. 5 by using the equation:

$$\Delta\delta_{ind} = \boldsymbol{a}C + \Delta\delta_0$$

where C is the PI concentration relatively to the PC content in the dispersion. The value for $\boldsymbol{a}$ thus obtained was 17.7 p.p.m./mole % PI. Designating the PI_{inn}/PI_{out} ratio for the cases of trans-bilayer symmetrical and asymmetrical distribution as R and X, respectively, we obtain:

$$[PI/PC]_{out} = C(1 + R)/(1 + X)\,. \qquad (2)$$

Combining eqs. (1) and (2) we find:

$$X = PI_{inn}/PI_{out} = [\boldsymbol{a}C(1 + R)/(\Delta\delta_{ind} - \Delta\delta_0)] - 1\,.$$

Assuming $R = 0.50 \pm 0.03$ in accordance with the ratios of the integral intensities of the "inner" and "outer" signal in the ^1H and ^{31}P NMR spectra of pure PC and co-sonicated PC–PI liposomes we found $X = 0.13$ at $C = 2 : 98$ ($\Delta\delta_{ind} = 0.63$ p.p.m.) for the PEPF stimulated incorporation of PI molecules in PC liposomes. The X value obtained corresponds to a relative PI content of 2.8 mole % in the outer monolayer and only 0.6 mole % in the inner one. In other words nearly 89% of PI turned out to be located at the outer surface of the liposomal bilayer.

Thus the protein induced intermembrane phospholipid exchange appears to be a highly efficient way of producing trans-bilayer asymmetric liposomes from various phospholipids at any component ratios.

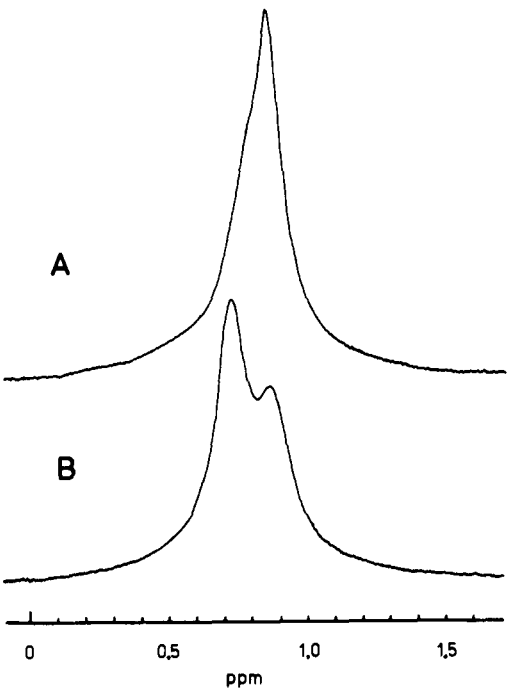

Fig. 7. ^{31}P NMR spectra of a 10% PC dispersion in D_2O: (A) before and (B) after addition of 5 mg/ml PEPF.

E. Interaction of the phospholipid exchange protein with liposomes

As was mentioned in the preceeding section PEPF causes no changes in the paramagnetic ion induced shift of the $N^+(CH_3)_3$ signal in the ^1H NMR spectrum of PC liposomes. The signal was also not shifted when PEPF was added to PC liposomes in the absence of paramagnetic ions or in the presence of diamagnetic La^{3+}, however in all cases both the $N^+(CH_3)_3$ and $(CH_2)_n$ signals were broadened (table 3). A pronounced effect of PEPF was observed in the ^{31}P NMR spectrum of PC liposomes. As can be seen from fig. 7 in the presence of PEPF the phosphorus resonance splits into two components – one shifted and the other unaltered – presumably due to differences in the magnetic environment of the outer and inner surfaces of the membrane. Addition of $Pr(NO_3)_3$ induces further shifting only of the latter signal while the former remains unaltered (table 4). It follows that the signal shifted by PEPF must belong to the outward facing phosphate groups, i.e. that binding of the proteins present in PEPF occurs only at the outer surface of the liposomes.

Stronger effects were observed with PI liposomes. On addition of PEPF (5 mg/ml)

Table 3
Effects of PEPF and diamagnetic La^{3+} on the line width ($\Delta\nu_{1/2}$) of the $(CH_2)_n$ and $N^+(CH_3)_3$ proton signals of PC liposomes. [a]

Exp.	Additives	$\Delta\nu_{1/2}$(Hz)	
		$(CH_2)_n$	$N^+(CH_3)_3$
1	–	12.0	4.2
2	PEPF	14.1	4.3
3	$La(NO_3)_3$	15.1	4.8
4	PEPF + $La(NO_3)_3$	18.2	4.7

[a] Data are given for 30 μM/ml PC dispersions in D_2O, concentration of PEPF is 5 mg/ml, concentration of $La(NO_3)_3$ is 10^{-2} M.

Table 4
Chemical shifts in the ^{31}P NMR spectra of 9% PC dispersions with PEPF and $Pr(NO_3)_3$

Sample	^{31}P signals, p.p.m.	
	"outer"	"inner"
PC dispersion	0.81 (shoulder)	0.88
After addition of PEPF (5 mg/ml)	0.75	0.86
After addition of PEPF (5 mg/ml) and $Pr(NO_3)_3$ (10^{-3} M)	0.59	0.86

to a sonicated PI dispersion (30 mg/ml) the latter became turbid and the intensity of the phosphorus resonance decreased sharply pointing to partial aggregation and decreased diffusional mobility of the vesicles (no sedimentation occurred). The effects of PEPF on co-sonicated PC–PI liposomes were of intermediate character and magnitude. Of partial importance is the fact that the distance between the "outer" and "inner" signals in the ^1H and ^{31}P NMR spectra of PC–PI liposomes and of pure PC liposomes did not change during incubation with PEPF for 8–10 h ($Pr(NO_3)_3$ was added to the sample just before recording the spectrum). This implies that the liposomes do not become permeable for Pr^{3+} ions during incubation (cf. [31]). Accordingly it may be concluded that the protein has no detergent activity and apparently does not disturb the integrity of the bilayer of the PC and PC–PI liposomes.

IV. Discussion

Since the crude lipid exchange protein of rat liver is known to stimulate almost equally the exchange of both PC and PI [2, 4, 8, 12], incubation of separately sonicated PC and PI liposomes with PEPF could be expected to result not only in PI transfer but also in incorporation of PC molecules into the PI liposomes. This process should lead to the appearance of a third highly shifted component of the $N^+(CH_3)_3$ signal in the ^1H NMR spectrum of the incubation mixture due to formation of liposomes with a high PI/PC ratio.

Such signals were never observed in our experiments with PEPF which may be explained either by the low PC content in the resulting liposomes or by the assumption that the protein induced process is a one way traffic involving only PI transfer to PC liposomes. This suggestion is sustained by the fact that PC exchange between mitochondria and liposomes is inhibited as the negative charge of the latter increases [32].

Generally speaking the transfer of phospholipid molecules from one liposome species to another can proceed by two possible mechanisms. In the first phospholipids could partly go into solution and then be captured by the liposomes. Alternatively it is possible that the phospholipid passes from one liposome to the other on impact. Because of the very low critical micelle concentrations of phospholipids and the coalescence preventing electrical and structure-mechanical properties of the bilayer both possibilities normally are realized only to a minor extent and no appreciable phospholipid exchange or transfer takes place in the absence of stimulating agents [33].

The stimulating effect of PEPF on PI incorporation into the PC liposomes could be due to its solubilizing properties, to its facilitation of collisions by lowering the surface charge of the liposome and/or to its enhancing the efficiency of collisions by reducing the structural viscosity of the bilayer. Data of the present work (broadening of both the $N^+(CH_3)_3$ and $(CH_2)_n$ signals in the presence of PEPF; see § III.E) demonstrate that PEPF is not increasing the fluidity of the bilayer but is rather exerting the opposite effect.

Liposome coalescence (fusion) as the cause of PEPF induced PI transfer also seems unlikely because this mechanism should lead to PC–PI liposome with a symmetrical distribution of PI between the outer and inner monolayers. Such liposomes arise when PC and PI liposomes are incubated with lysolecithin [30] but are not formed when the phospholipid transfer is stimulated by PEPF. Thus the most likely possibility is that PEPF acts as a solubilizing factor although it displays no detectable disintegrating action on the bilayer. A possible explanation is that PEPF may be adsorbed on the outer surface of the liposome (which is manifested by changes in the NMR spectra; see tables 3 and 4) but does not cause gross disorganisation of the lipid molecules. If so, the possible mechanism of the PI transferring action of PEPF may be imagined as consisting of the following steps: adsorption of a PEPF component on the outer surface of a PI liposome; insertion of the protein into the

PI bilayer; detachment of the lipoprotein complex from the PI liposomes; adsorption of the lipoprotein on the outer surface of a PC liposome and incorporation of the PI molecules into its outer monolayer. Although this mechanism remains partly hypothetical it may form a basis for further experiments.

V. Conclusion

As a result of the present study the conclusion may be drawn that NMR shift and relaxation reagents are useful tools for the study of phospholipid exchange between different liposome species. The method has a number of merits. It is a one step procedure and requires no radioactive label, no separation of the membrane fractions or phospholipids after incubation. It is fast, accurate and should therefore be a useful supplement to the radioactive determinations of the phospholipid exchange activity of proteins.

We as yet do not know whether the phospholipid exchange activity of the soluble liver protein fraction observed in an artificial system is actually relevant to intracellular phospholipid transport. It may well be that the exchange enhancing proteins carry out other functions in the cell and that any protein capable of forming soluble lipoprotein complexes without denaturation or of partly neutralizing charged phospholipids would act in a similar way. In order to answer this question much still remains to be elucidated concerning the specificity of phospholipid-protein interaction including the kinetics of the process. In any case however, a new procedure has been elaborated for mechanistic studies of intermembrane phospholipid exchange which probably will provide additional information on this biologically important process.

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