STUDY OF INTERVESICULAR PHOSPHOLIPID EXCHANGE BY NMR.

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Summary: NMR spectroscopy with the use of non-penetrating paramagnetic probes permits in situ determination of the composition of the outer surface of phospholipid vesicles. The method was employed to follow the phospholipid exchange between phosphatidylinositol and phosphatidylcholine vesicles induced by a postmicrosomal protein fraction from rat liver. The effects of these proteins on the lipid bilayer and the structure of the vesicles produced by exchange were studied.

Phospholipids have been shown to undergo exchange between different membrane fractions during incubation (1). The in vitro transfer of phospholipids from mitochondria to microsomes and vice versa is enhanced by cell supernatant fractions obtained from various tissues of rats and beefs (2-8). Such protein stimulated phospholipid transfer is believed to play an important role in the biosynthesis, repair and modification of cell membranes.

The procedure generally used for studying the phospholipid

ABBREVIATIONS USED: PC, phosphatidylcholine; PI, phosphatidylinositol; PEPF, phospholipid exchanging protein fraction.

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exchange activity of proteins is very cumbersome including not less then 5 different steps (incubation of the protein under investigation with labelled and non-labelled subcellular particles and/or liposomes, their separation by centrifugation, extraction and chromatography of the lipids and determination of their specific radio-activity). For studying PC exchange two artificial systems have also been proposed. In one of them the exchange between sensitized and non-sensitized liposomes is investigated using immunoprecipitation for their separation (9). According to the second method PC exchange between two monolayers or between a monolayer and liposomes is followed by measuring changes in the surface radioactivity of the monolayer (10). The first method includes a time consuming procedure of antiserum preparation, the second one requires labelled phospholipids.

In the present paper we suggest a completely different approach to phospholipid exchange studies which is based on the dependence of the lanthanide induced shifts in the ^{1}H and ^{31}P NMR spectra on the phospholipid composition of the liposomes. By this method the phospholipid exchange between two populations of liposomes can be investigated in situ without their preliminary separation.

MATERIALS AND METHODS. PEPF was isolated from rat liver as described by Wirtz and Zilversmit (3). Phospholipid dispersions from pure egg PC and yeast PI (K^{+} -salt) and from mixtures of these lipids in various ratios were prepared in D_2O by ultrasonication as described earlier (11). In the study of phospholipid exchange PC and PI liposomes were incubated together at 50°C in the presence of PEPF (5 mg per ml of incubation mixture). After incubation solutions of $Pr(NO_3)_3$ or $Eu(NO_3)_3$ in D_2O were added and ^{1}H or ^{31}P NMR spectra were recorded. Control experiments were carried out in all cases by incubation of corresponding samples without PEPF.

^{1}H NMR spectra were obtained on a Varian XL-100-15 spectrometer at 100 MHz and ^{31}P NMR spectra at 40.5 MHz with proton noise decoupling and internal ^{2}D lock. Chemical shifts for protons were measured relatively the signal of $(CH_2)_n$ groups and for phosphorus nuclei relatively external 0.5% H_3PO_4 in D_2O.

RESULTS AND DISCUSSION. A comparison of the ^{1}H NMR spectra of sonicated PC liposomes and co-sonicated (7:3) PC-PI liposomes showed them to be quite similar. Addition of $Pr(NO_3)_3$ to both liposome preparations splits the $\overset{+}{N}(CH_3)_3$ signal into two components - a lowfield one from outwardly facing PC molecules which interact with Pr^{3+} ions and a highfield component from inwardly facing PC molecules which do not interact with Pr^{3+} ions (11-13). In the spectrum of the co-sonicated PC-PI liposomes the "outer" $\overset{+}{N}(CH_3)_3$ signal was more downfield shifted and wider than the corresponding signal in the spectrum of pure PC liposomes (Fig.1A and Fig.1B). The magnitude of the Pr^{3+} induced shift depended on the PC/PI ratio increasing with the increase of PI content (Table 1). When $Pr(NO_3)_3$ was added to a mixture of sonicated PC liposomes and co-sonicated PC-PI liposomes three $\overset{+}{N}(CH_3)_3$ signals appeared (Fig.1C). The most higfield (stationary) signal is due to inwardly facing PC molecules of both vesicle types. The most downfield shifted signal corresponds to outwardly facing PC molecules that are in contact with both PI and Pr^{3+}, while the intermediate signal is due to the outwardly facing molecules of pure PC liposomes whose phosphate groups contact with Pr^{3+} but not with PI. From these data it is evident that the Pr^{3+} induced shift of the $\overset{+}{N}(CH_3)_3$ resonance in the NMR spectra of mixtures of PC liposomes and co-sonicated PC-PI liposomes can be used for the evaluation of their composition.

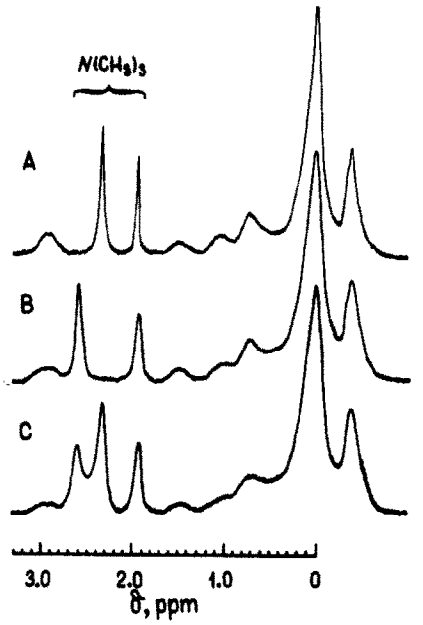

Fig. 1. ^{1}H NMR spectra of phospholipid dispersions in D_2O after addition of 10^{-2} M/l $Pr(NO_3)_3$: A - a sonicated 30 μM/ml PC dispersion; B - a co-sonicated PC (30 μM/ml) and PI (10 μM/ml) dispersion; C - a 1:1 mixture of samples A and B.

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

Exp. No.	PI content (% of PC)	$\Delta\delta$ ind. (p.p.m.)
1	0	0.41
2	5	0.49
3	10	0.53
4	30	0.67

The dependence of the lanthanide induced shifts in the ^{1}H and ^{31}P NMR spectra of PC-PI liposomes on their composition was employed to follow the intermembrane PI transfer stimulated by PEPF. It was found that PEPF did not influence the

shift of the $\overset{+}{N}(CH_3)_3$ signal in the ^1H NMR spectrum of pure PC liposomes in the presence or absence of Eu^{3+}, whereas the phosphorus resonance splits into two components - one shifted and the other unaltered. Addition of $Pr(NO_3)_3$ induces further shifting only of the latter signal while the former remains again unaltered. It follows that the signal shifted by PEPF must belong to the outward facing phosphate groups, i.e. that binding of PEPF occurs only at the outer surface of the PC liposomes. The effects of PEPF on co-sonicated PC-PI liposomes were similar, the "inner" $\overset{+}{N}(CH_3)_3$ signals again remaining unshifted.

As can be seen from Table 2 the induced shifts of the "outer" $\overset{+}{N}(CH_3)_3$ signal increased on incubation of separately

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

Exp. No.	Incubation time (hrs)	Shift reagent	$\Delta\delta_{ind.}$ (p.p.m.) after incubation	
			without PEPF	with PEPF
1	3	$5\cdot10^{-3}$ 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	$2\cdot10^{-2}$ M/l, $Eu(NO_3)_3$	-0.30[b]	-0.42[b]
4	4	- " -	-0.31[b]	-0.44[b]
5	3[c]	- " -	-0.29[b]	-0.30[b,d]

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

sonicated PC and PI liposomes both in the absence and in the presence of PEPF, however in the latter case the shift was larger (see Table 2, experiments 1-4). Obviously the increase of the induced shifts observed in these experiments is due to incorporation of PI molecules into the PC liposomes this process being strongly enhanced by PEPF. We suppose the "spontaneous" exchange in the absence of PEPF (experiments 1 and 2 in table 2) to be caused by lyso-PC formed during sonication. In preliminary experiments it was found that the lyso-PC stimulated exchange was nonspecific and that it required amounts of lyso-PC which greatly exceeded those present in PEPF.

Of partial importance was the fact that the distance between the "outer" and "inner" signals in the ^{1}H and ^{31}P NMR spectra of co-sonicated PC-PI liposomes did not change during incubation with PEPF for 8-10 hrs. This implies that the liposomes do not become permeable during incubation. Accordingly it may be concluded that the protein does not disturb the integrity of the bilayer of PC and PC-PI liposomes. Since both the $\overset{+}{N}(CH_3)_3$ and $(CH_2)_n$ signals are broadened in the presence of PEPF it seems that PEPF is not increasing the fluidity of the bilayer. From these data it appears that the stimulating effect of PEPF on PI incorporation into the PC liposomes is not due to its detergent action or to reduction of the structural viscosity of the bilayer. Liposome coalescence (fusion) as the cause of the protein induced PI transfer also seems unlikely because this mechanism should lead to PC-PI liposomes with a more symmetrical distribution of PI between the outer and inner monolayers. Such "symmetrical" liposomes arise on co-sonication of PI and PC (at least

at comparatively low PI/PC ratios*) or when PC and PI liposomes are incubated with lyso-PC. On the contrary the protein stimulated PI transfer leads to liposomes in which about 90% of PI is located at the outer surface of the liposomes even when their total PI content is as low as 2% relatively to the PC content (14). Thus the most likely possibility is that PEPF acts as a solubilizing factor although it displays no detectable desintegrating action on the PC and PI-PC liposomes and does not cause gross desorganization of the lipid molecules in the bilayer.

We are at present trying to employ the above procedure in assaying the activity of lipid exchange proteins, in studying the mechanism of protein induced lipid transfer and in investigating the trans-bilayer asymmetry of the liposomes produced.

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*The integral intensity ratio of the $\overset{+}{N}(CH_3)_3$ 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 (up to 30 mole % of PI). Constant ratios of the integral intensities of the "inner" and "outer signals were obtained also from the ^{31}P spectra of co-sonicated PC-PI dispersions at various component ratios.

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