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INVESTIGATION OF THE INSIDE-OUTSIDE DISTRIBUTION, INTERMEMBRANE EXCHANGE AND TRANSBILAYER MOVEMENT OF PHOSPHOLIPIDS IN SONICATED VESICLES BY SHIFT REAGENT NMR

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Summary

1. A new NMR approach is described for the investigation of transbilayer asymmetry in phospholipid vesicles consisting of phosphatidylcholine and negatively charged phospholipids. The method makes use of the dependence of the pseudocontact shift of the N-methyl proton resonance induced by paramagnetic ions on the surface concentration of negatively charged phospholipids. When two differently shifting paramagnetic probes are applied from the outside and the inside of a vesicular membrane the transbilayer phospholipid distribution can be estimated without knowledge of the inner and outer radii of the vesicles and the packing density of the phospholipid molecules.

2. The method was employed to study the transbilayer asymmetry in vesicles obtained by cosonication of phosphatidylcholine with phosphatidylserine, phosphatidylglycerol or phosphatidylinositol. The three negative phospholipids were found to distribute with a higher surface concentration in the inner vesicular shell than in the outer one when their total content did not exceed 25 mol%. However, as the amount of negatively charged phospholipids increases the ratio of their inside to outside surface concentrations, i.e., the transbilayer asymmetry of the vesicles, decreases. Prolonged incubation (for several days) does not change the compositional asymmetry of the cosonicated vesicles.

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Abbreviations: PC, phosphatidylcholine; NCP, negatively charged phospholipids.

3. By the 'double-probe' technique it was established that spontaneous exchange between separately sonicated phosphatidylcholine and phosphatidylinositol vesicles results in formation of highly asymmetric mixed vesicles with phosphatidylinositol residing only in the outer monolayer. In the presence of antioxidant (α-tocopherol) the bilayer asymmetry is preserved for days. However lipid peroxidation induces rapid transbilayer movement (flip-flop) of phospholipids leading to an 'inverted' asymmetry resembling that of cosonicated vesicles. It is suggested that lipid peroxidation promotes phospholipid flip-flop by partially converting the bilayer structure into a non-bilayer configuration. Moderate quantities of lysophosphatidylcholine (up to 15 mol%) induce neither detectable perturbations of the bilayer nor rapid phospholipid flip-flop.

Introduction

NMR spectroscopy with the use of hydrophilic paramagnetic probes (lanthanide ions) has been shown [1] to permit discrimination between phospholipid molecules located on the outer and inner surfaces of vesicular bilayers. This technique has successfully been used for the elucidation of factors determining the transbilayer asymmetry of sonicated multicomponent liposomes (for a review see Ref. 2).

The usual approach is to compare the integral intensities of the 'inner' and 'outer' signals for a give phospholipid component separated by lanthanide ions added from the outside or the inside of the vesicles. However this approach suffers from certain drawbacks. Thus, accurate integral intensity measurements are possible only for phospholipids present in fairly high concentrations. For calculations of phospholipid asymmetry based on integral intensities the exact inner and outer radii of vesicles must be known. Besides, intensities taken from ^{31}P- and ^{13}C-NMR spectra ought to be corrected considering possibly different Overhauser effects inside and outside the vesicles [3,4].

The present paper describes another approach to the estimation of the transbilayer phospholipid distribution in vesicles consisting of phosphatidylcholine and a negatively charged phospholipid. For such estimation we use the dependence of the pseudocontact shift of the N-methyl resonance induced by lanthanides on the surface concentration of the charged phospholipids [5,6]. This allows the elimination of the difficulties encountered in measurements of intensity ratios and the study of the bilayer asymmetry even at rather low concentrations of the negatively charged phospholipid.

The method was employed to determine the transbilayer phospholipid distribution in mixed vesicles obtained either by cosonication of phosphatidylcholine with negatively charged phospholipids (phosphatidylserine, phosphatidylglycerol and phosphatidylinositol) or by intervesicular phospholipid exchange between separately sonicated phosphatidylcholine and phosphatidylinositol vesicles.

Preliminary reports of this work have been published [7—10].

Materials and Methods

Egg yolk phosphatidylcholine [11], ox brain phosphatidylserine [12] and baker's yeast phosphatidylinositol [13] were isolated as described before.

Phosphatidylglycerol was prepared from egg yolk phosphatidylcholine by phospholipase D treatment [14]. Lysophosphatidylcholine was prepared by enzymatic hydrolysis of egg yolk phosphatidylcholine with phospholipase A_2 (*Naja naja oxiana*) [15]. Phospholipids were purified by column chromatography on silica gel KSK (100—150 mesh). Purified phosphatidylserine, phosphatidylglycerol and phosphatidylinositol were converted into sodium salts as described earlier [16]. α-Tocopherol was purified by vacuum distillation under nitrogen.

Phospholipid vesicles were prepared by sonication (22 kHz) until complete clearness (10—15 min, +5°C, under argon) with subsequent centrifugation (10 000 × *g*, 20 min) to remove residual multilamellar liposomes. The final phosphatidylcholine concentration was 5 mg/ml in all vesicle preparations. All experiments were performed in ^2H_2O without buffer at p^{2}H 4.9—5.5. Over this p^{2}H range the change of the $\Delta\delta$ * values calculated from ^{1}H-NMR spectra of the vesicles was in most cases less than 0.4 Hz.

For determination of the transbilayer distribution of phospholipids with the 'double-probe' technique, vesicles were prepared by sonication in 10 mM $Pr(NO_3)_3$ with subsequent 10-fold dilution by 10 mM $Eu(NO_3)_3$. Hence, 10 mM Pr^{3+} was used as the internal probe whereas the extravesicular medium contained 10 mM Eu^{3+} and 1 mM Pr^{3+}.

For study of intervesicular phospholipid exchange, phosphatidylcholine vesicles were prepared in 10 mM $Pr(NO_3)_3$ or 30 mM KCl. Non-entrapped Pr^{3+} outside the vesicles was removed by dialysis against 30 mM KCl. The final concentration of Pr^{3+} after dialysis did not exceed 0.01 mM. For exchange, phosphatidylcholine vesicles were incubated at 23°C with sonicated phosphatidylinositol vesicles prepared in 30 mM KCl (in some experiments non-sonicated phosphatidylinositol liposomes were used for exchange). After incubation the shift reagent (10 mM Eu^{3+} or 1 mM Yb^{3+}) was added. In some cases addition of the shift reagent resulted in partial precipitation of vesicles (presumably due to flocculation of phosphatidylinositol vesicles or mixed vesicles with high phosphatidylinositol content). ^{1}H-NMR spectra were recorded both in the presence of the precipitate and after its removal by centrifugation. In the former case all resonances were somewhat broadened due to inhomogeneity of samples. However no significant differences of chemical shifts and integral intensities of the N-methyl signals were observed between the two types of samples.

For studying the transbilayer movement of phospholipids, mixed phosphatidylinositol-phosphatidylcholine vesicles were obtained after 20 h incubation of a mixture (3 : 1) of separately sonicated phosphatidylcholine and phosphatidylinositol vesicles. At that time intervesicular exchange was practically completed. To follow phospholipid flip-flop the outside probe (1 mM Yb^{3+}) was added and incubation was continued with periodical recording of ^{1}H-NMR spectra and measuring the degree of lipid peroxidation. The peroxidation index (A_{233}/A_{215}) was estimated as the ratio of absorbances at 233 and 215 nm [17].

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* $\Delta\delta$ is the difference between the induced paramagnetic shift of the N-methyl proton resonance of mixed phosphatidylcholine-negatively charged phospholipid vesicles (δ_{PC+NCP}) and that of pure phosphatidylcholine vesicles (δ_{PC}) observed in the presence of the same paramagnetic ion, i.e., $\Delta\delta = \delta_{PC+NCP} - \delta_{PC}$.

^{1}H-NMR spectra were recorded at 60 MHz with a Bruker WP-60 spectrometer operating in the Fourier transform mode. Chemical shifts of the N-methyl resonances were measured relative to the $(CH_2)_n$ signal of the fatty acid chains. The error of chemical shift measurements was ±0.2 Hz. The relative error of integral intensity measurements did not exceed ±10%. ^{31}P-NMR measurements were carried out at 24.3 MHz employing a Bruker WP-60 spectrometer in the presence of a broad band proton decoupling. Accumulated free induction decays were obtained from up to 20 000 transitions employing a 15% (w/v) phosphatidylcholine dispersion.

Ultraviolet measurements were performed with a Hitachi EPS-3T spectrometer.

The computer analysis of experimental data was carried out with a Hewlett-Packard minicomputer 9825A using a program for the minimization technique written by Dr. A.M. Shkrob.

Results and Discussion

1. Determination of negative phospholipid surface concentrations from chemical shift data

As has been shown [2] two N-methyl signals, one shifted and the other remaining unshifted, are observed in ^{1}H-NMR spectra of sonicated phosphatidylcholine vesicles after addition of paramagnetic ions (see Fig. 1A, B). The unchanged signal is due to inward facing phosphatidylcholine molecules while the shifted one arises from phosphatidylcholine molecules located in the outer monolayer which is in contact with the impermeant shift reagent. The shift of the N-methyl signal induced by paramagnetic ions increases when negatively charged phospholipids, e.g. phosphatidylinositol or phosphatidylserine, are present in the vesicular bilayer [5,6]. Presumably this additional shift is caused by a higher cation binding capacity of the vesicles in the presence of negatively charged phospholipids.

The shift increment ($\Delta\delta$) due to the presence of a charged phospholipid can be defined as

$$\Delta\delta = \delta_{PC+NCP} - \delta_{PC} \quad (1)$$

where δ_{PC+NCP} and δ_{PC} are the induced shifts of the N-methyl signal in the presence and in the absence of the charged phospholipids respectively.

When the shift reagent is introduced inside or outside the vesicles the shift increments of the inner and outer N-methyl signals ($\Delta\delta_{in}$ and $\Delta\delta_{out}$ * defined according to Eqn. 1; see also Fig. 1C, D) can be used for determination of the surface concentration of the charged phospholipid in each monolayer provided that the exact relation between $\Delta\delta$ and the surface concentration of the charged phospholipids is known. NMR measurements performed on micelles consisting of lysophosphatidylcholine mixed with phosphatidylserine or phosphatidylglycerol demonstrated a linear relationship between these two variables (Fig. 2) at charged phospholipid to lysophosphatidylcholine ratios up to 0.3. In

* $\Delta\delta_{in}$ and $\Delta\delta_{out}$ refer to N-methyl signals from inward and outward facing phosphatidylcholine molecules respectively.

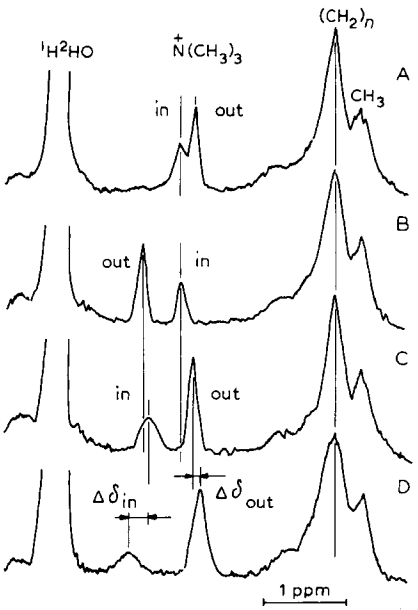

Fig. 1. ^{1}H-NMR spectra of sonicated phospholipid dispersions in ^2H_2O: A, phosphatidylcholine vesicles containing 10 mM $Eu(NO_3)_3$ outside; B, phosphatidylcholine vesicles containing 10 mM $Pr(NO_3)_3$ outside; C, phosphatidylcholine vesicles containing 10 mM $Pr(NO_3)_3$ inside and 10 mM $Eu(NO_3)_3$ outside; D, vesicles composed of phosphatidylcholine and phosphatidylserine in molar ratio 5 : 1 containing 10 mM $Pr(NO_3)_3$ inside and 10 mM $Eu(NO_3)_3$ outside.

the case of phosphatidylinositol the plot was linear if the ratio of phosphatidylinositol to lysophosphatidylcholine did not exceed 0.2.

Chemical shift data obtained for mixed lysophosphatidylcholine micelles can not be directly applied to vesicular structures formed by phosphatidylcholine because structural differences between these lipids might influence their interaction with negatively charged phospholipids and hence the binding of paramagnetic ions to the interface. Besides, experiments with phosphatidylcholine

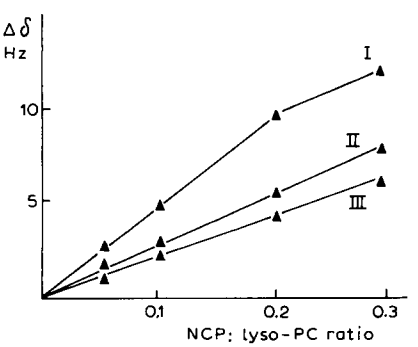

Fig. 2. Dependence of Δδ on the content of negatively charged phospholipids (NCP) in mixed lysophosphatidylcholine (lyso-PC) micelles. Shift reagent, 10 mM $Eu(NO_3)_3$. I, phosphatidylinositol; II, phosphatidylglycerol; III, phosphatidylserine.

vesicles showed the shifting efficiences of a given paramagnetic ion inside and outside the vesicle to be different (Table I), the shifting effects of inside ions always being lower than those of outside ions. (These differences may be explained by different ion binding due to different curvature and hence different packing densities of the outer and inner shells of the vesicular membrane.) This implies that different calibrations should be used for determination of the charged phospholipid concentrations in the outer and inner monolayer.

If we assume by analogy with the mixed lysophosphatidylcholine micelles that $\Delta\delta_{in}$ and $\Delta\delta_{out}$ for mixed vesicles are directly proportional to the surface concentration of the charged phospholipid in the corresponding monolayer, then we can write down that

$$\Delta\delta_{in} = k_{in} \cdot [NCP]_{in} \tag{2}$$

and

$$\Delta\delta_{out} = k_{out} \cdot [NCP]_{out} \tag{3}$$

where k_{in} and k_{out} are constants characteristic of the shifting efficiency of the paramagnetic probe inside and outside the vesicle, $[NCP]_{in}$ and $[NCP]_{out}$ are the molar ratios of the negatively charged phosholipid to phosphatidylcholine at the inner and outer surface, respectively. The constants, k_{in} and k_{out}, are difficult to obtain by direct calibration but one may derive them when considering theoretically the dependence of the measured spectral parameters on the total content of negatively charged phospholipids in the vesicles.

The total ratio of the negative phospholipid to phosphatidylcholine in a vesicle is given by

$$[NCP] = \frac{[NCP]}{[PC]} = \frac{[NCP]_{out} \cdot [PC]_{out} + [NCP]_{in} \cdot [PC]_{in}}{[PC]} \tag{4}$$

where [NCP] and [PC] are the total amounts (in moles) of the negative phospholipid and phosphatidylcholine, $[PC]_{out}$ and $[PC]_{in}$ are the amounts of phosphatidylcholine (in moles) on the outside and inside of the vesicle.

The integral intensities of the inner and outer N-methyl signals, I_{out} and I_{in}, are proportional to the numbers of phosphatidylcholine molecules in the inner and outer monolayer of the vesicle shell. Therefore $[PC]_{out}$ and $[PC]_{in}$ can be calculated as

TABLE I

SHIFTING EFFECTS OF Eu^{3+} AND Pr^{3+} IN ^{1}H-NMR SPECTRA OF SONICATED PHOSPHATIDYLCHOLINE VESICLES

Lanthanide ion *	Site of application	Separation between the inner and outer N-methyl signals (Hz)
Eu^{3+}	outside	11.0
Eu^{3+}	inside **	8.0
Pr^{3+}	outside	25.5
Pr^{3+}	inside **	19.0

* 10 mM $Eu(NO_3)_3$ and $Pr(NO_3)_3$ were used.

** Paramagnetic ions were removed from the outside of the vesicles by dialysis against isotonic KCl.

$$[PC]_{out} = \frac{[PC] \cdot n}{1+n} \text{ and } [PC]_{in} = \frac{[PC]}{1+n}$$

where $n = I_{out}/I_{in}$.

According to Eqns. 2 and 3 the concentrations of the charged phospholipid in each monolayer are given by $[NCP]_{out} = \Delta\delta_{out}/k_{out}$ and $[NCP]_{in} = \Delta\delta_{in}/k_{in}$.

By substituting $[PC]_{out}$, $[PC]_{in}$, $[NCP]_{out}$ and $[NCP]_{in}$ in Eqn. 4 we obtain

$$[NCP] = \frac{\Delta\delta_{out}}{k_{out}} \cdot \frac{n}{1+n} + \frac{\Delta\delta_{in}}{k_{in}} \cdot \frac{1}{1+n} \tag{5}$$

If we put $k_{out} = m \cdot k_{in}$, Eqn. 5 may be rewritten as

$$\frac{n}{m(n+1)} \cdot \Delta\delta_{out} + \frac{1}{n+1} \cdot \Delta\delta_{in} = k_{in} \cdot [NCP] \tag{6}$$

Thus a linear relationship should exist between the total concentration of the negative phospholipid in the vesicles and the left part of Eqn. 6 including the spectral parameters $\Delta\delta_{out}$, $\Delta\delta_{in}$ and n. Therefore the values of m and k_{in} may be calculated by fitting the experimentally determined values of $\Delta\delta_{out}$, $\Delta\delta_{in}$ and n with the theoretically derived Eqn. 6.

In principle $\Delta\delta_{in}$ and $\Delta\delta_{out}$ could be measured with a single shift reagent present both inside and outside the vesicles but in this case it proved difficult to discriminate between overlapping inner and outer N-methyl resonances. In order to improve the separation of these resonances we applied simultaneously two different shift reagents, one of them being placed into the intravesicular space and the other added to the extravesicular solution. The most appropriate combination turned out to be a pair of reagents shifting the resonances in opposite directions, such as Eu^{3+} and Pr^{3+} which shift the N-methyl signal up field and down field, respectively (Fig. 1A, B). Accordingly, in our experiments Eu^{3+} was applied from the outside and Pr^{3+} from the inside of the vesicle membrane.

The ^{1}H-NMR data for mixed vesicles prepared by cosonication of phosphatidylcholine with phosphatidylserine, phosphatidylglycerol and phosphatidylinositol are presented in Table II. The values of $\Delta\delta_{out}$, $\Delta\delta_{in}$ and n of the three sets of experiments were fitted with the theoretically derived equation (Eqn. 6). Computer analysis of the data of Table II showed that the best fit to a linear relationship according to Eqn. 6 was observed at the values of k_{in} and m presented in Table III. Thus our supposition about a linear relationship between $\Delta\delta_{in}$ and $\Delta\delta_{out}$ and the content of the negatively charged phospholipid in the corresponding monolayer proved to be valid. It is of interest that in the case of phosphatidylinositol a linear dependence was observed only at phosphatidylinositol to phosphatidylcholine ratios not exceeding 0.2 (exactly in the same concentration range as with mixed phosphatidylinositol-lysophosphatidylcholine micelles).

TABLE II

$\Delta\delta_{out}$, $\Delta\delta_{in}$ AND n VALUES MEASURED FROM ^{1}H-NMR SPECTRA OF MIXED VESICLES OBTAINED BY COSONICATION OF PHOSPHATIDYLCHOLINE (PC) WITH NEGATIVELY CHARGED PHOSPHOLIPIDS (NCP)

Shifting system, Eu^{3+} outside and Pr^{3+} inside. Mean values of 5—10 experiments are presented. For low NCP/PC ratios the relative error was determined by the absolute error of measurement of $\Delta\delta$ values (±0.4 Hz); for high NCP/PC ratios the relative error was approx. 10%.

Type of vesicle	Total NCP/PC ratio in the vesicles	$\Delta\delta^*_{out}$ * (Hz)	$\Delta\delta_{out}$ (Hz)	$\Delta\delta_{in}$ (Hz)	$n = I_{out}/I_{in}$
Phosphatidylcholine-phosphatidylserine	0.05	0.3	0.4	4.0	2.3
	0.1	0.7	0.9	7.5	2.7
	0.2	2.5	3.2	10.5	3.2
	0.3	5.0	4.8	15.5	3.8
Phosphatidylcholine-phosphatidylglycerol	0.05	0.7	0.8	4.0	2.2
	0.1	1.4	1.7	7.0	2.4
	0.2	3.3	4.0	14.5	2.9
	0.3	5.5	6.7	22.5	3.9
Phosphatidylcholine-phosphatidylinositol	0.05	1.0	1.5	6.0	2.2
	0.1	1.9	3.0	11.0	2.6
	0.2	5.0	7.5	20.0	3.0
	0.3	7.0	8.5	27.5	3.8

* The $\Delta\delta^*_{out}$ values were obtained using a single probe (10 mM Eu^{3+} outside).

2. Transbilayer asymmetry in cosonicated vesicles

Table III summarizes the estimated parameters, k_{in} and k_{out}, for the phospholipid mixtures studied. These parameters are expressed in Hz and correspond to the $\Delta\delta$ values at a 1 : 1 molar ratio of negative phospholipid to phosphatidylcholine. By using k_{in} and k_{out} the $\Delta\delta_{in}$ and $\Delta\delta_{out}$ values from Table II can be easily transformed into the surface concentrations of negatively charged phospholipids in each monolayer (Table IV).

It can be seen from Table IV that for all lipid mixtures studied the concentrations of the negative phospholipid in the outer monolayer are always lower and those in the inner monolayer always higher than the total content of the

TABLE III

PARAMETERS m, k_{in}, k_{out} AND r (THE COEFFICIENT OF LINEAR REGRESSION) DERIVED BY FITTING THE DATA OF TABLE II WITH EQUATION 6

Phospholipid mixture	m	k_{in} (Hz)	k_{out} (Hz)	r^2
Phosphatidylcholine-phosphatidylserine	0.614	31.2	19.5	0.9991
Phosphatidylcholine-phosphatidylglycerol	0.927	34.5	32.0	0.9995
Phosphatidylcholine-phosphatidylinositol *	0.934	55.1	51.3	0.9985

* Data obtained for the phosphatidylinositol-phosphatidylcholine ratio of 0.3 were not used in calculations.

TABLE IV

SURFACE CONCENTRATIONS, $(NCP)_{out}$ AND $(NCP)_{in}$, AND ASYMMETRY COEFFICIENTS, K_{as}

Calculated from the data of Table II using the k_{in} and k_{out} values from Table III.

Type of vesicle	Total NCP/PC ratio in the vesicles	$(NCP)^{*}_{out}$ *	$(NCP)_{out}$	$(NCP)_{in}$	K_{as}
Phosphatidylcholine-phosphatidylserine	0.05	0.01	0.02	0.12	6
	0.1	0.04	0.05	0.24	5
	0.2	0.13	0.16	0.34	2.1
	0.3	0.25	0.24	0.48	2.0
Phosphatidylcholine-phosphatidylglycerol	0.05	0.02	0.03	0.12	4
	0.1	0.04	0.05	0.20	4.0
	0.2	0.10	0.12	0.42	3.5
	0.3	0.17	0.21	0.65	3.1
Phosphatidylcholine-phosphatidylinositol	0.05	0.02	0.03	0.11	3.7
	0.1	0.04	0.06	0.20	3.3
	0.2	0.10	0.15	0.36	2.4
	0.3	0.14	0.17	—	—

* Calculated for data obtained using a single probe (10 mM Eu^{3+}, outside).

negative phospholipid in the vesicles. In order to characterize the bilayer asymmetry we introduce the asymmetry coefficient, K_{as}, representing the ratio of the negative phospholipid concentration in the inner monolayer to that in the outer monolayer, i.e.,

$$K_{as} = [NCP]_{in}/[NCP]_{out}$$

As can be seen from Table IV the asymmetry coefficients at all negative phospholipid to phosphatidylcholine ratios employed are much higher than 1, pointing to a preferential localization of the negative phospholipids in the inner monolayer. However as the content of the negative phospholipid in the cosonicated mixture increases the asymmetry coefficients decrease.

Thus at low negative phospholipid to phosphatidylcholine ratios (up to 23 mol% of the negative phospholipid) phosphatidylserine, phosphatidylglycerol and phosphatidylinositol settle in the inner shell with higher surface concentrations than in the outer one. This finding is in agreement with previous data obtained for negative phospholipids by shift reagent NMR [18] and by chemical modification with impermeant reagents [19,20]. However recently Massari et al. [21] using a technique based on interaction of negative phospholipids with positive metachromic dyes arrived at an opposite conclusion that negative phospholipids prefer the external layer when their content in the vesicles is low and tend to distribute in the internal layer at high negative lipid to phosphatidylcholine ratios.

The reason for this discrepancy is not clear. One possibility is that the experiments with metachromic dyes were done at pH values which were 2—2.5 pH units higher than in our experiments. (Earlier the acidity of the medium has been shown to affect the distribution of negatively charged phospholipids [18].) Another possibility is that cationic dyes bind differently to the outer

and inner surfaces of vesicle bilayer. Although Massari et al. discussed such a possibility they did not compare the binding capacities of the two surfaces. A priori it is also possible that the presence of polyvalent metal cations or positive dyes may influence the transverse distribution of the negative phospholipids in a different way. It should be noted that when Eu^{3+} was applied from the outside to mixed vesicles obtained by cosonication in the absence of paramagnetic ions the $\Delta\delta_{out}$ values were somewhat lower than those found for vesicles obtained by cosonication in the presence of Pr^{3+} (Table II). This implies that cations may diminish the asymmetric distribution of negative phospholipids across the bilayer presumably by charge neutralization. Furthermore in the case of highly asymmetrical phosphatidylcholine-phosphatidylinositol vesicles no rapid flip-flop was induced by paramagnetic ions (see below) but such a possibility can not be excluded for the metachromic dyes.

3. Transbilayer asymmetry in vesicles formed by intervesicular phospholipid exchange

After co-incubation of separately sonicated phosphatidylinositol vesicles with phosphatidylcholine vesicles enclosing Pr^{3+} and subsequent flocculation of the highly charged vesicle fraction by addition of Eu^{3+}, the inside N-methyl signal in the ^{1}H-NMR spectra of the remaining vesicles was not altered, whereas the outside signal was progessively shifted up field as the amount of phosphatidylinositol in the incubation mixture increased (Table V). At the same time the intensity ratio of the signals from the outside and inside facing N-methyl groups (I_{out}/I_{in}) decreased. This decrease could be caused either by an increase of the vesicle size or by partial substitution of the outside facing phosphatidylcholine molecules for phosphatidylinositol or by both factors. Since we have no independent data on the average vesicle radii, the first explanation cannot be ruled out at the moment. But, whatever the sizes of the vesicles are, the marked increase of $\Delta\delta_{out}$ after coincubation (Table V and Fig. 3) shows unequivocally that appreciable amounts of phosphatidylinositol are spontaneously incorporated into phosphatidylcholine vesicles, while the

TABLE V

^{1}H-NMR DATA, $\Delta\delta_{out}$, $\Delta\delta_{in}$ AND I_{out}/I_{in}, FOR MIXED PHOSPHATIDYLINOSITOL—PHOSPHATIDYLCHOLINE (PI-PC) VESICLES OBTAINED BY CO-INCUBATION (20 h, 23°C) OF SEPARATELY SONICATED PHOSPHATIDYLINOSITOL AND PHOSPHATIDYLCHOLINE VESICLES

Phosphatidylcholine vesicles contained 10 mM Pr^{3+} inside. Outer probe added after incubation, 10 mM Eu^{3+}.

PI/PC ratio in the incubation mixture	$\Delta\delta_{out}$ (Hz)	$\Delta\delta_{in}$ (Hz)	I_{out}/I_{in}	PI/PC ratio in the outer monolayer of mixed vesicles *
0.1	4.0	0.0	2.0	0.01
0.2	7.5	0.0	2.0	0.15
0.3	9.5	0.0	1.9	0.19
0.4	11.0	0.0	1.8	0.21
0.5	12.0	0.0	1.8	0.23
1.0	13.0	0.0	1.7	0.25

* Calculated using k_{out} = 51.3 Hz from Table III.

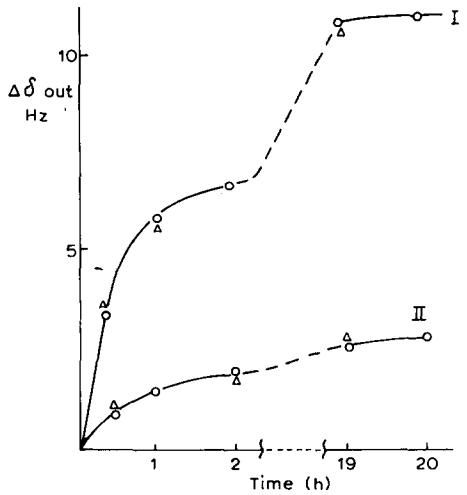

Fig. 3. Changes of $\Delta\delta_{out}$ for the outer N-methyl signal from phosphatidylcholine vesicles during incubation at 23°C with sonicated (I) and non-sonicated (II) phosphatidylinositol liposomes. Molar phosphatidylinositol: phosphatidylcholine ratio, 0.3. △, phosphatidylcholine vesicles enclosing 10 mM Pr^{3+}; ○, vesicles not containing paramagnetic ions in the inner aqueous space.

persistence of the position of the inner signal demonstrates that all phosphatidylinositol molecules incorporated, reside in the outer shell of the vesicle membrane. Thus, spontaneous exchange between phosphatidylcholine and phosphatidylinositol vesicles yields highly asymmetric mixed vesicles with a non-equilibrium transmembrane distribution of the two lipid components.

Fig. 3 shows the time dependence of $\Delta\delta_{out}$ observed on incubation of a 10 : 3 mixture of separately sonicated phosphatidylcholine vesicles with phosphatidylinositol vesicles and with non-sonicated multilayer liposomes. In both cases biphasic curves were obtained and the time course of $\Delta\delta_{out}$ was the same for phosphatidylcholine vesicles enclosing Pr^{3+} and for vesicles not containing paramagnetic ions.

The initial rapid increase of $\Delta\delta_{out}$ reflects the increase of the surface concentration of phosphatidylinositol in the outer monolayer of the phosphatidylcholine vesicles.

The driving force of the exchange could be sought in the higher electrostatic repulsion and the smaller size [5] (and consequently higher strain) of the phosphatidylinositol vesicles. Spontaneous intervesicular exchange has already been reported for phospholipid vesicles of different radii [22—24]. The mechanism underlying the intermembrane phospholipid exchange remains obscure. Possible mechanisms involve fusion of vesicles, interversicular transfer of phospholipids during collision or exchange via soluted phospholipid molecules or micelles. The selection between these possibilities requires accurate kinetic measurements. At the moment a collision dependent mechanism seems more probable on the grounds of the different results obtained when phosphatidylcholine vesicles were incubated with non-sonicated or with sonicated phosphatidylinositol. As can be seen from Fig. 3 with unilamellar phosphatidyl-

inositol vesicles the exchange occurs much faster than with multilamellar liposomes.

4. Transbilayer movement of phospholipids in phosphatidylcholine-phosphatidylinositol vesicles

The $\Delta\delta_{out}$ values of the mixed vesicles obtained by cosonication of phosphatidylcholine with phosphatidylinositol did not change during incubation of the vesicles for 7.5 days indicating that the transbilayer distribution of the phospholipids was not altered. In the presence of antioxidant (0.8% α-tocopherol) the $\Delta\delta_{out}$ of phosphatidylcholine-phosphatidylinositol vesicles obtained by spontaneous exchange were also remarkably stable even in the presence of 15 mol% of lysophosphatidylcholine (Fig. 4). The fact that the inside and outside signals did not merge shows that the integrity of the membrane was maintained. The relative stability of the asymmetric phospholipid distribution in the 'non-equilibrated' phosphatidylcholine-phosphatidylinositol vesicles formed by intervesicular exchange is in marked contrast to the reported short half-time of phospholipid flip-flop in analogous 'non-equilibrated' phosphatidylcholine-phosphatidic acid vesicles obtained by restricted phospholipase D hydrolysis of sonicated phosphatidylcholine [25]. The different behavior of

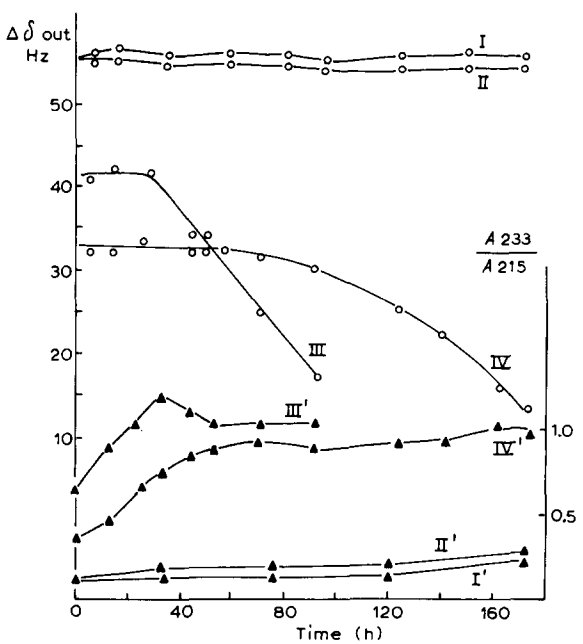

Fig. 4. Time dependent changes of the peroxidation index, A_{233}/A_{215} (▲) and of $\Delta\delta_{out}$ (○) for phosphatidylcholine-phosphatidylinositol vesicles obtained by coincubation (20 h, 23° C) of separately sonicated phosphatidylcholine and phosphatidylinositol vesicles (3 : 1); samples with antioxidant were additionally incubated for 2 h at 50° C in order to increase the amount of the incorporated phosphatidylinositol. I and I', vesicles containing 0.8 mol% of α-tocopherol; II and II', vesicles containing 0.8 mol% of α-tocopherol and 15 mol% of lysophosphatidylcholine; III and III', vesicles without antioxidant (initial, i.e. after incorporation of phosphatidylinositol, $A_{233}/A_{215} = 0.65$); IV and IV', vesicles without antioxidant (initial $A_{233}/A_{215} = 0.36$). Approximate concentration of phosphatidylinositol in the outer monolayer of phosphatidylcholine vesicles: I and II, 0.25; III, 0.13; IV, 0.17.

the two vesicle types may be related to differences in size and charge of the polar head groups of the negatively charged components. Since phosphatidic acid differs in those respects much more from phosphatidylcholine than does phosphatidylinositol, introduction of phosphatidic acid into the outer phosphatidylcholine monolayer may be expected to cause larger stress as compared to introduction of phosphatidylinositol.

In the absence of antioxidant the vesicles obtained by spontaneous exchange were much less stable. After an initial period of 1—2 days the $\Delta\delta_{out}$ value began to decrease (Fig. 4). When the initial content of peroxidation products was higher, the induction period became shorter and the decline of $\Delta\delta_{out}$ was much faster. It is noteworthy that for all samples studied the onset of rapid $\Delta\delta_{out}$ reduction coincided with the time when the peroxidation index, A_{233}/A_{215} [17], reached the value of 1.0—1.2. Initially the rapid decrease of $\Delta\delta_{out}$ induced by lipid peroxidation was not accompanied by breakdown of the permeability barrier as revealed by the persistance of the inner N-methyl signal during 90 h incubation in the presence of Yb^{3+} added from the outside (Fig. 4). However after longer incubation times (170 h and 90 h for vesicles with initial A_{233}/A_{215} values of 0.36 and 0.65, respectively) the vesicles became leaky, presumably due to accumulation of secondary oxidation products.

When the incubated intact vesicles contained outside Yb^{3+} and inside Pr^{3+} the decrease of $\Delta\delta_{out}$ was accompanied by a simultaneous increase of $\Delta\delta_{in}$. Since the permeability barrier of the vesicles was maintained during this period the observed changes are indicative of a gradual translocation of outward facing phosphatidylinositol into the inner shell. The peroxide induced decrease of $\Delta\delta_{out}$ was accompanied by a simultaneous increase of the I_{out}/I_{in} ratio. Thus the '$A_{233}/A_{215} = 0.65$' vesicles showed an initial I_{out}/I_{in} ratio of 1.7 which reached a maximal value of 2.3 after incubation for 80—90 h (i.e., just before breakdown of the permeability barrier). This maximal I_{out}/I_{in} ratio is close to the value shown by cosonicated vesicles of similar phospholipid composition.

Sensitivity of flip-flop to lipid oxidation was already suggested by Kornberg and McConnel [26] and by Johnson et al. [27] in order to explain large variations in the flip-flop rates shown by phosphatidylcholine preparations of different age. Kornberg and McConnell [26] proposed a direct involvement of oxidized lipids in the translocation process when their content is very low. In our experiments considerable amounts of primary oxidation products accumulated in the vesicle membrane without disturbance of the permeability barrier. For such cases we consider another possibility, namely that peroxidation promotes transbilayer movement of phospholipids by partially transforming the bilayer structure into a non-bilayer one. According to Cullis and de Kruijff [28] such inhomogeneity of the membrane may be the mechanism underlying flip-flop. In the present work this suggestion is supported by changes observed in the ^{31}P-NMR spectra of multilayer phosphatidylcholine liposomes during peroxidation (Fig. 5).

Recently it has been shown that the type of packing of phospholipid molecules can be evaluated from the 'effective chemical shift anisotropy', $\Delta\sigma_{CSA}^{EFF}$, and the general shape of the ^{31}P-NMR signal [28]. The ^{31}P-NMR signal of freshly prepared non-oxidized multilayer liposomes (Fig. 5A) had a $\Delta\sigma_{CSA}^{EFF}$ value of approx. 40 ppm and a low-field shoulder characteristic of phospholipids

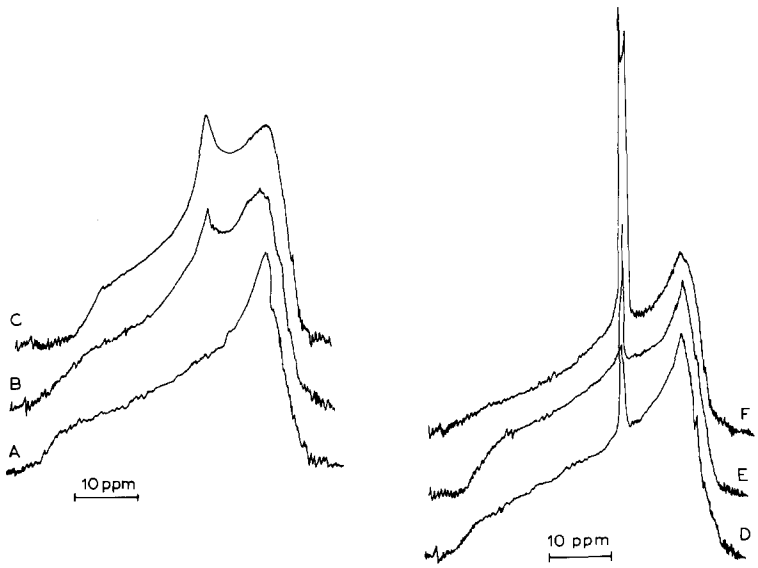

Fig. 5. ^{31}P-NMR spectra of multilayer phosphatidylcholine liposomes of different degree of peroxidation A, A_{233}/A_{215} = 0.05; B, A_{233}/A_{215} = 0.60; C, A_{233}/A_{215} = 1.10; D, A_{233}/A_{215} = 0.06 in the presence of 7.5 mol% of lysophosphatidylcholine; E, A_{233}/A_{215} = 0.07 in the presence of 15 mol% of lysophosphatidylcholine; F, A_{233}/A_{215} = 0.06 liposomes were preincubated (2 h, 21°C) with 15 mol% of lysophosphatidylcholine.

experiencing restricted anisotropic motion in bilayers. As the amount of lipid peroxides increases a second (low-field) maximum appears (Fig. 5B, C). This rather narrow and symmetrical signal corresponds to another type of packing in which the lipid molecules undergo more isotropic motions than in the lamellar or hexagonal H_{II} phases. The amount of such isotropically moving molecules increases with the degree of oxidation. However even in oxidized liposomes with a peroxidation index A_{233}/A_{215} as high as 1.10 their content does not exceed 6% of the total phosphatidylcholine.

Introduction into non-oxidized phosphatidylcholine liposomes of lysophosphatidylcholine (up to 15 mol%) does not induce significant changes of the ^{31}P resonance (Fig. 5D, E). The minor (less than 3% of the total lipids) and very narrow signal observed in the NMR spectra arises most likely from small lysophosphatidylcholine micelles although the bulk of lysophosphatidylcholine is incorporated into the multilayer liposomes (for comparison, see Fig. 5E and F). Thus, in marked contrast to lipid peroxidation, introduction of moderate amounts of lysophosphatidylcholine seems not to induce structural changes in the phosphatidylcholine bilayer. This difference may account for the different influence of lysophosphatidylcholine and lipid peroxides on the phospholipid flip-flop rates.

General conclusions

The results of the present studies may have several biological implications. Firstly, it becomes clear that when considering various causes of topographical

phospholipid asymmetry [29] one must take into account the possible contribution of spontaneous intermembrane lipid exchange. Secondly, our results show that non-equilibrium transverse phospholipid distributions may be stable and persist for days to high-potential barriers for flip-flop. On the other hand rapid phospholipid flip-flop without loss of barrier properties of the bilayer may be induced by lipid peroxidation. In some biological membranes lipid peroxidation is absent or very low due to the presence of natural antioxidants. Thus the presence of tocopherol in red blood cells [30] may account for the low peroxidation index and relatively long flip-flop half-times in erythrocyte membranes. On the contrary, microsomes are apparently less protected against peroxidation than other biological membranes [31]. This may be one of the causes of the rapid phospholipid flip-flop in rat liver microsomes [32,33]. The dependence of transbilayer phospholipid movement in microsomes and other biological membranes on their peroxide content is at present under investigation.

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References

1 Bergelson, L.D., Barsukov, L.I., Dubrovina, N.I. and Bystrov, V.F. (1970) Dokl. Akad. Nauk SSSR 194, 708—711
2 Bergelson, L.D. (1977) Meth. Membrane Biol. 9, 275—335
3 De Kruijff, B., Cullis, P.R. and Radda, G.K. (1975) Biochim. Biophys. Acta 406, 6—14
4 Yeagle, P.L., Hutton, W.C., Huang, C.H. and Martin, R.B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3477—3481
5 Barsukov, L.I., Shapiro, Yu.E., Victorov, A.V., Volkova, V.I., Bystrov, V.F. and Bergelson, L.D. (1975) Chem. Phys. Lipids 14, 211—266
6 Barsukov, L.I., Shapiro, Yu.E., Victorov, A.V., Volkova, V.I., Bystrov, V.F. and Bergelson, L.D. (1977) Bioorg. Chem. (USSR) 3, 1355—1361
7 Victorov, A.V., Vasilenko, I.A., Barsukov, L.I., Evstigneeva, R.P. and Bergelson, L.D. (1977) Dokl. Akad. Nauk SSSR 234, 207—210
8 Victorov, A.V., Vasilenko, I.A., Barsukov, L.I., Evstigneeva, R.P. and Bergelson, L.D. (1979) Dokl. Akad. Nauk SSSR 245, 1243—1246
9 Victorov, A.V., Vasilenko, I.A., Barsukov, L.I., Evstigneeva, R.P. and Bergelson, L.D. (1979) Dokl. Akad. Nauk SSSR 246, 479—482
10 Victorov, A.V., Vasilenko, I.A. and Evstigneeva, R.P. (1979) Bioorg. Chem. (USSR) 5, 1584—1586
11 Dawson, R.M. (1965) Biochem. J. 88, 414—423
12 Ansell, G.B. and Hawthorne, J.N. (1964) in Phospholipids, Chemistry, Metabolism and Function, Vol. 3, pp. 102—104, BBA Library, Elsevier, Amsterdam
13 Trevelyan, W.E. (1966) J. Lipid Res. 7, 445—450
14 Yang, S.F. (1969) Meth. Enzymol. 14, 208—212
15 Moore, J.H. and Williams, D.L. (1964) Biochim. Biophys. Acta 84, 41—54
16 Colacicco, G. and Rapport, M.M. (1967) J. Lipid Res. 8, 513—515
17 Klein, R.A. (1970) Biochim. Biophys. Acta 210, 485—489
18 Berden, J.A., Barker, R.W. and Radda, G.K. (1975) Biochim. Biophys. Acta 375, 186—208
19 Litman, B.J. (1973) Biochemistry 12, 2545—2554
20 Litman, B.J. (1974) Biochemistry 13, 2844—2848
21 Massari, S., Pascolini, D. and Gradenigo, G. (1978) Biochemistry 17, 4465—4469
22 Papahadjopoulos, D., Hui, S., Vail, W.J. and Poste, L. (1976) Biochim. Biophys. Acta 448, 245—283
23 Martin, F.J. and MacDonald, R.C. (1976) Biochemistry 15, 321—327
24 Kremer, J.M.H., Kops-Werkhoven, M.M., Pathamanoharan, C., Gijzeman, O.L.J. and Wierseman, P.H. (1977) Biochim. Biophys. Acta 471, 177—188
25 De Kruijff, B. and Baken, P. (1978) Biochim. Biophys. Acta 507, 38—47

26 Kornberg, R.D. and McConnell, H.M. (1971) Biochemistry 10, 1111—1120
27 Johnson, L.W., Hughes, M.E. and Zilversmit, D.B. (1975) Biochim. Biophys. Acta 375, 176—185
28 Cullis, P.R. and de Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207—218
29 Bergelson, L.D. and Barsukov, L.I. (1977) Science 197, 224—230
30 Silver, R., Winter, R. and Hayden, H.J. (1969) J. Clin. Invest. 48, 2089—2095
31 Wills, E.D. and Wilkinson, A.E. (1967) Radiat. Res. 31, 732—747
32 Zilversmit, D.B. and Hughes, M.E. (1977) Biochim. Biophys. Acta 469, 99—110
33 Van den Besselaar, A.M.H.P., de Kruijff, B., van den Bosch, H. and van Deenen, L.L.M. (1978) Bio chim. Biophys. Acta 510, 242—255