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A HIGH-RESOLUTION NMR STUDY (^1H, ^{13}C, ^{31}P) OF THE INTERACTION OF PARAMAGNETIC IONS WITH PHOSPHOLIPIDS IN AQUEOUS DISPERSIONS

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^1H-, ^{13}C- and ^{31}P-NMR spectra of egg-yolk phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidic acid (PA) and cosonicated mixtures of these phospholipids were obtained from ultrasonicated dispersions containing Pr^{3+}, Eu^{3+}, Gd^{3+} and Mn^{2+} ions.

The differences in chemical shift values, Δ_N, between the "inner" and "outer" resonance signals for the different nuclei of the polar head group of egg-yolk phosphatidyl choline provide information about the average distances of the paramagnetic ion within the polar groups of the phospholipid molecules. In the $Pr(^2H_2O)_n^{3+}$/egg-yolk phosphatidylcholine system the ions are nearest to the phosphate and $-CH_2CH_2$ group, respectively but relatively far from the $N(CH_3)_3$ group of the polar head group of the lipid.

The integral analysis of the ^1H-NMR spectra obtained from dispersions containing Pr^{3+} and Mn^{2+} ions enables us to calculate the number of the polar groups in both sides of the egg-yolk phosphatidylcholine bilayer, the size of the lipid vesicle and to give some features of the arrangement of the phospholipid molecules in cosonicated egg-yolk phosphatidylcholine/phosphatidylserine vesicles. At p^{2}H 8.3 in PC/PS mixtures an extreme asymmetry is observed with PS preferentially in the outer side of the membrane. This side contains approximately three times more PS than PC molecules.

Some comments are made concerning the quantitative integral analysis of proton-noise decoupled ^{31}P-NMR spectra as obtained from similar phospholipid mixtures by Michaelson et al. and Berden et al..

1. Introduction

As discussed previously [1] an interaction of dipalmitoylphosphatidylcholine and phosphatidylserine with manganous ions has been investigated by means of the proton-relaxation enhancement (PRE) method. These PRE measurements seize the polar head groups of the phospholipids as a whole and lead to a formal description of the "ion-lipid complex" by introducing interacting sites and association constants. Besides this limitation the PRE method is in its most direct application only useful for certain paramagnetic ions. These results, however, reveal some features of the bound Mn^{2+}–aquo complex and its molecular dynamics.

Further information about the interaction of paramagnetic ions with phospholipids should be expected from high-resolution NMR measurements. Each resonance of the different nuclei of the polar group can be analysed and the application of "relaxation probes" and "shift probes" [2] provides a wide variety of independent experiments.

As reported by Bystrov et al. [3] the addition of $EuCl_3$ to a lecithin dispersion gives rise to a splitting of the trimethylammonium signal from the phospholipid in the ^1H-NMR spectrum, and, upon the addition of $MnCl_2$ the high-field peak (from the external polar groups) disappears. The synthetic bilayer membrane is impermeable to the ions and only the phospholipid molecules in the outer monolayer are interacting with the ions.

The integral analysis of the relative peak areas can be used for evaluating the number of the polar groups in the inner and outer layer, the size of the phospholipid vesicle and the distribution of the phospholipid molecules in cosonicated vesicles [4–6].

Furthermore, the use of "shift probes" provides the possibility to study the orientation of the hydrated paramagnetic ions relative to the different nuclei of the polar group.

In this paper, firstly we report a more detailed study of the interaction of Pr^{3+}, Gd^{3+} and Mn^{2+} ions with egg-yolk phosphatidylcholine and phosphatidylserine

by means of ^{1}H-, ^{13}C- and ^{31}P-NMR measurements. Secondly, we report our observations concerning the distribution of the phospholipid molecules in an equimolar mixture of co-sonicated PC/PS vesicles.

The results presented here demonstrate that high-resolution NMR measurements can be used for a more exact localization of the sites of interaction with the ions and for demonstrating an asymmetrical partition of the molecules between the inner and outer faces of the vesicles in the PC/PS mixture.

2. Materials and methods

Egg-yolk phosphatidylcholine was extracted from egg-yolk and purified by column chromatography employing the well-known procedures of Singleton et al. [7] and Dawson et al. [8].

Phosphatidylserine ex bovine brain was purchased from Koch-Light Lab., Colnbrook, and was purified as well by chromatography.

Phosphatidic acid was obtained from PC by eliminating enzymatically (phospholipase D in the presence of Ca^{2+} ions) the choline group following the procedures of Kates et al. [9] and Papahadjopoulos et al. [10]. After extraction of the reaction products with a solution of $CHCl_3/CH_3OH$ (1 : 1) and a subsequent extraction with the same volume of pure $CHCl_3$ – thin-layer chromatography indicates complete yield – the phosphatidic acid was converted to the sodium salt by some extractions with 1 M aqueous NaCl solution.

For obtaining ^{1}H spectra 200 mg of the lipid were dissolved in $CHCl_3$ and treated as described previously [1] except that the dispersions were made in $^{2}H_2O$ (99.75%, E. Merck, Darmstadt).

To get well resolved ^{13}C and ^{31}P spectra 800 mg and 400 mg of the lipid, respectively were used for preparing the sonicated vesicles. In addition the dispersions for the ^{31}P measurements contained 10^{-6} M ethylenediamintetraacetic acid to avoid interferences from paramagnetic traces.

Cosonicated PC/PS, PC/PA and PS/PA vesicles were prepared in the same way from equimolar amounts of PC, PS and PA.

All experiments were performed in $^{2}H_2O$ without buffer at about $p^{2}H$ 6.4 – if there is no additional remark. Pr^{3+}, Eu^{3+}, Gd^{3+} and Mn^{2+} ions were added as small volumes of aqueous solutions of $Pr(NO_3)_3 \cdot 5H_2O$, $Eu(NO_3)_3 \cdot 5H_2O$, $Gd(NO_3)_3 \cdot 5H_2O$ (Koch-Light Lab., Colnbrook) and $MnCl_2 \cdot 4H_2O$ (E. Merck, Darmstadt) to the phospholipid dispersions after sonication.

^{1}H-NMR spectra were recorded at 30°C on a Varian HA-100 spectrometer with a 2% solution of C_6H_6 in tetramethylsilan (TMS) as external standard. The C_6H_6 signal at 7.23 ppm was used as reference signal for integrating the signal of the $\dot{N}(CH_3)_3$ group.

^{13}C- and ^{31}P-NMR spectra were obtained at 26°C by using the Fourier-transform technique on a Bruker HX-90 spectrometer with heteronuclear ^{2}H-lock system operating at 22.63 MHz for ^{13}C, and 36.43 MHz for ^{31}P resonance, respectively. The data were accumulated in a 8k-Nicolet 1080 data system (model B-NC 12). Typically, 10000 pulse repetitions for ^{13}C and 70000 pulse repetitions for ^{31}P measurements were accumulated. To obtain simplified spectra and to improve sensitivity the proton-noise decoupling technique was used (symbols: ^{13}C-{^{1}H}, ^{31}P–{^{1}H}). The shortcomings in such a decoupling are that one has to know the nuclear Overhauser enhancement (NOE, [11]) for exact integration (see sections 3.3 and 4).

3. Results

3.1. ^{1}H-, ^{13}C- and ^{31}P-NMR spectra of egg-yolk phosphatidylcholine as obtained from dispersions containing Pr^{3+}, Eu^{3+} and Gd^{3+} ions

As mentioned above the addition of Eu^{3+} ions to egg-yolk lecithin in aqueous dispersion causes the splitting of the signal of the $\dot{N}(CH_3)_3$ protons of the phospholipid. The further addition of Mn^{2+} ions gives rise to a disappearing of the high-field component [signal from the $\dot{N}(CH_3)_3$ groups in the external face of the bilayer]. Fig. 1 shows the 100 MHz ^{1}H-NMR spectrum of PC obtained after addition of 6.4 mM Pr^{3+} ions. The magnitude of the chemical shift produced by these ions was approximately 11.0 Hz larger than that one caused by Eu^{3+} ions at the same concentration.

The integral analysis of the relative peak areas leads to the result that – corresponding to an intensity ratio $I_{f'}/I_f = I_{outer}/I_{inner}$ of 1.80 – 35.7% ± 1.5% of PC molecules are in the inner layer of the vesicle under the experimental conditions of preparation mentioned above (compare [3,4]). We interprete the observation that the intensity ratio $I_{f'}/I_f$ remains unaltered with increas-

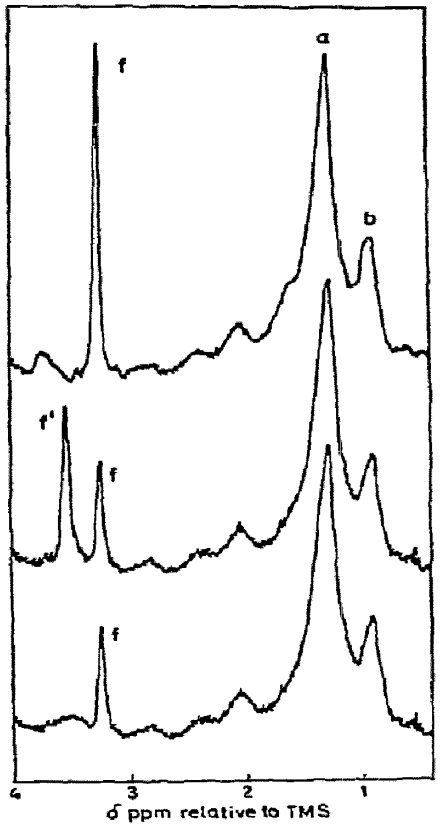

Fig. 1. 100 MHz ^{1}H-NMR spectra of egg-yolk phosphatidylcholine (0.05 M) in ultrasonicated dispersion at a temperature of 30°C. Above(1): in ^2H_2O. Middle(2): after addition of Pr^{3+} ions (6.4 mM). Below(3): after addition of Gd^{3+} ions (0.78 mM) to sample (2). Peak f represents the signal of the $\overset{+}{N}(CH_3)_3$ groups in the inner side of the bilayer and peak f' the one in the outer side.

ing Pr^{3+}-ion concentration in such a manner that all external polar groups are equivalent, i.e., there is a rapid exchange of metal ions between the interacting sites which are fairly uniformly distributed over the surface of the vesicle.

Fig. 2 shows the $^{13}C-\{^1H\}$ NMR spectrum of PC in ultrasonicated dispersion in the region of 40–80 ppm relative to TMS obtained after addition of 29 mM Pr^{3+} ions. The signal of the $\overset{+}{N}(CH_3)_3$ group also splits into two components. The difference in chemical shift is 22.63 Hz at nearly the same ratio of PC molecules to Pr^{3+} ions as in the dispersions used for recording

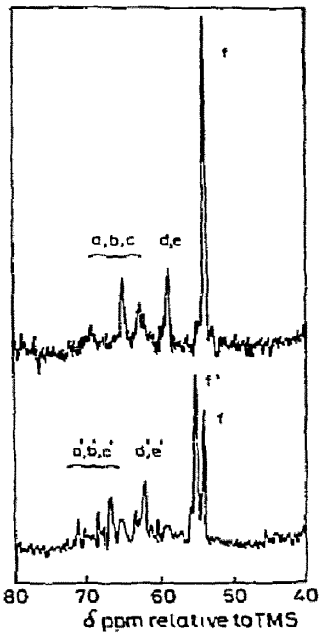

Fig. 2. 22.63 MHz $^{13}C-\{^1H\}$ NMR spectra of egg-yolk phosphatidylcholine (0.2 M) in ultrasonicated dispersion in the region of 40–80 ppm relative to TMS at a temperature of 26°C. Above(1): in ^2H_2O. Below(2): after addition of Pr^{3+} ions (29 mM). The assignment of peak f and f' is the same as in fig. 1.

the ^{1}H spectra. Furthermore, the signals of the glycerol backbone (a,b,c) and the $-CH_2CH_2$ group (d,e) are shifted downfield. The values of the chemical shift differences produced by the interaction of the ions with these groups are 38.48 Hz (a,b,c) and 65.63 Hz (d,e). One can suppose from these observations that the interaction is not restricted to the protons of the $\overset{+}{N}(CH_3)_3$ group but also effects the magnetic properties of the neighbouring atoms or groups.

Fig. 3 shows the $^{31}P-\{^1H\}$ NMR spectrum of PC obtained after addition of 15 mM Pr^{3+} ions. The phosphorus signal splits into two components of which the down-field one is considerably more broadened than the signals in the ^{1}H and ^{13}C spectra (see figs. 1 and 2). This observation indicates that there is a strong interaction which leads to a strong increase in the relaxation rate of the phosphorus. The value of the chemical shift difference is 356 Hz at nearly the same ratio of PC molecules to Pr^{3+} ions as in the dispersions used for obtaining the ^{1}H and ^{13}C spectra. It shows that the interaction of the polar groups with the ions especially

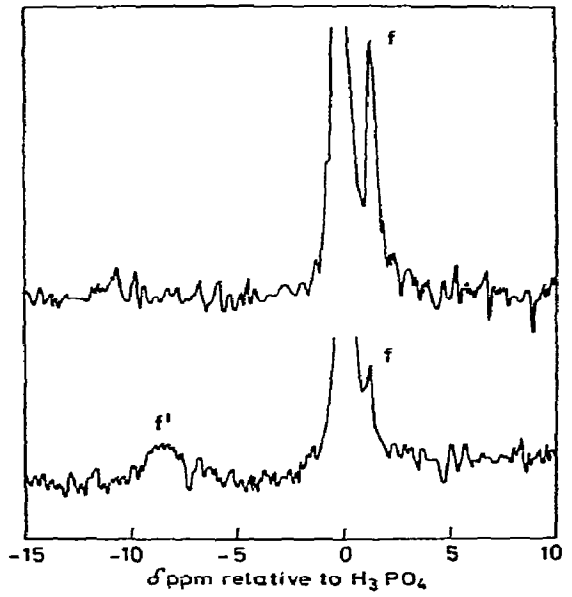

Fig. 3. 36.43 MHz $^{31}P-\{^1H\}$NMR spectra of egg-yolk phosphatidylcholine (0.1 M) in ultrasonicated dispersion at a temperature of 26°C. Above(1): in ^2H_2O. Below(2): after addition of Pr^{3+}-ions (15 mM). The assignment of peak f and f' is the same as in fig. 1. The peak at 0 ppm is the resonance of the phosphorus reference of H_3PO_4.

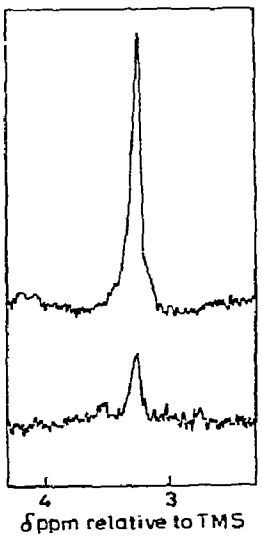

Fig. 4. $\dot{N}(CH_3)_3$-resonance signals of ^{1}H-NMR spectra of egg-yolk phosphatidylcholine (0.05 M). Above(1): in ^2H_2O. Below(2): after addition of Mn^{2+} ions (0.55 mM).

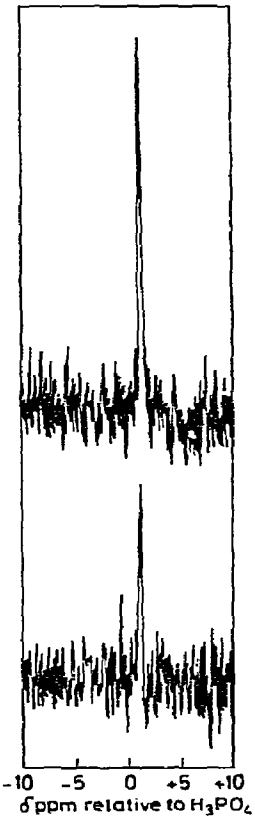

Fig. 5. 36.43 MHz $^{31}P-\{^1H\}$NMR spectra of egg-yolk phosphatidylcholine (0.1 M). Above(1): in ^2H_2O. Below(2): after addition of Mn^{2+} ions (0.9 mM).

influences the magnetic properties of the phosphorus atoms.

3.2. ^1H- and ^{31}P-NMR spectra of egg-yolk phosphatidylcholine and phosphatidylserine as obtained from dispersions containing Mn^{2+} ions

Figs. 4 and 5 show the $\dot{N}(CH_3)_3$ peak of a ^{1}H-NMR spectrum of PC and the $^{31}P-\{^1H\}$NMR spectrum of PC obtained after addition of Mn^{2+} ions (0.55 mM and 0.9 mM, respectively). The signal height and integral intensity decrease with increasing Mn^{2+}-ion concentration. After the addition of 0.1 mM $MnCl_2$ and higher salt concentrations the resonance signals remain unaltered in its structure. The peak area is eventually only 35.3%

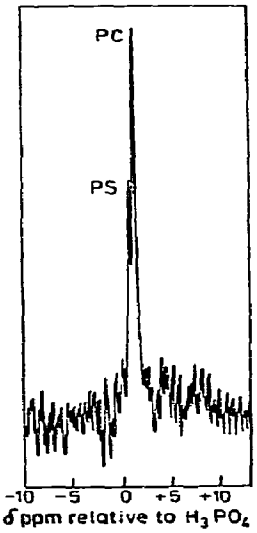

Fig. 6. 36.43 MHz $^{31}P-\{^1H\}$ NMR spectrum of a cosonicated mixture (1:1) of egg-yolk phosphatidylcholine (PC) and phosphatidylserine (PS) at p^2H 8.3.

± 1.1% of the original intensity. This value agrees very well with the value obtained by integral analysis of the ^1H spectra of a PC dispersion in the presence of Pr^{3+} ions (see section 3.1).

The paramagnetic ions cause a decrease of the phosphorus peak area (see fig. 5) such that eventually 41.6% of the original intensity is left in the spectrum. This value agrees only in the order of magnitude with the value obtained from the ^1H measurements. Two explanations may be considered:

(1) The average vesicle size is larger, since an increased lipid concentration has been used for preparing the dispersion.

(2) The nuclear Overhauser enhancement has been altered in the presence of Mn^{2+} ions (cf. section 3.3). For our present purpose we therefore may confine to the ^1H spectra by analysing the integrated peak areas.

On the assumption that there is a spherical double layer of 47 Å thickness [12] and an equal packing density at both sides of the membrane we obtain an average vesicle size of 364 Å in diameter which is the characteristic mean size of vesicles in ultrasonicated dispersions which have not undergone a gel permeation chromatography [12,6].

3.3. ^1H- and ^{31}P-NMR spectra of cosonicated mixtures of egg-yolk phosphatidylcholine and phosphatidylserine as obtained from dispersions containing Mn^{2+} ions

Before elucidating the spectra as obtained from mixtures in the presence of Mn^{2+} ions we may consider some features of the ^{31}P spectra in the absence of those ions.

Fig. 6 shows the $^{31}P-\{^1H\}$ NMR spectrum of a cosonicated mixture of PS and PC at p^2H 8.3.

The line widths of the PC and PS phosphorus signals in cosonicated dispersions are equal to those for pure PC and PS vesicles, respectively and have values of 10 Hz ± 1.2 Hz. Our interpretation of these observations agrees with that of Michaelson et al. [4] who analysed mixtures of egg-yolk phosphatidylcholine and phosphatidylglycerol:

(1) Each cosonicated vesicle contains PC- and PS-molecules.

(2) The molecules are not present in separated blocks but rather are dispersed at least within the outer monolayer.

The two phosphorus signal heights of the PC/PS mixture (see fig. 6) differ greatly. This observation is in contrast to the results of measurements on mixtures investigated by the authors mentioned above although we got optically clear dispersions of a molar ratio of 1 : 1. We assume that the nuclear Overhauser contribution to the phosphorus resonances of PC and PS, respectively are different and one has to be cautious in interpreting the integrated peak areas of proton-noise decoupled spectra without knowing the exact Overhauser enhancement of the signal.

Table 1
Chemical shift values of the phosphorus signals of egg-yolk phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidic acid (PA) in cosonicated vesicles as a function of p^2H

Mixture	Chemical shift (δ ppm relative to H_3PO_4)	p^2H Value
PC/PS	+0.96/+0.46	8.3
PC/PA	+0.87/−1.26	8.3
PC/PA	+0.81/−3.98	12.4
PS/PA	+0.40/−1.92	8.3
PS/PA	+0.23/−3.94	12.3

The chemical shift values of the phosphorus signal of PS slightly depend on the p^2H value, whereas the PA signal shows a strong dependence upon ionic strength. Table I summarizes the chemical shift data of PC/PS, PC/PA and PS/PA mixtures as a function of some p^2H values.

In the phospholipid mixtures the signal height and integral intensity of the proton resonance of the $\dot{N}(CH_3)_3$ group decrease with increasing Mn^{2+} ion concentration in similar way as in pure PC dispersions. The integrated peak area, however, eventually is 67.4% ± 2.5% of the original intensity in contrast to 35.3% in pure PC dispersions. On the assumption of similar size and shape of the mixed vesicle there are also 35.3% of the total PC and PS molecules in the inner layer of the vesicle. Since the mixed vesicle contains 32.6% of the PC molecules in the outer monolayer – the intensity of the $\dot{N}(CH_3)_3$ resonance originates only from the PC molecules – the number of PC and PS molecules in both sides of the membrane can be evaluated if the cross-sectional area, F, per lipid molecule is known. On the assumption of similar molecular area of both lipids ($F = 58$ Å^2 [13]) the outer side of the mixed vesicle contains approximately three times more phosphatidylserine than egg-yolk phosphatidylcholine molecules while there are only approximately 5% of the total PS molecules in the inner layer. The PC and PS molecules seem to be characteristically partitioned between both sides of the membrane and leaving it asymmetrically [14,4,5].

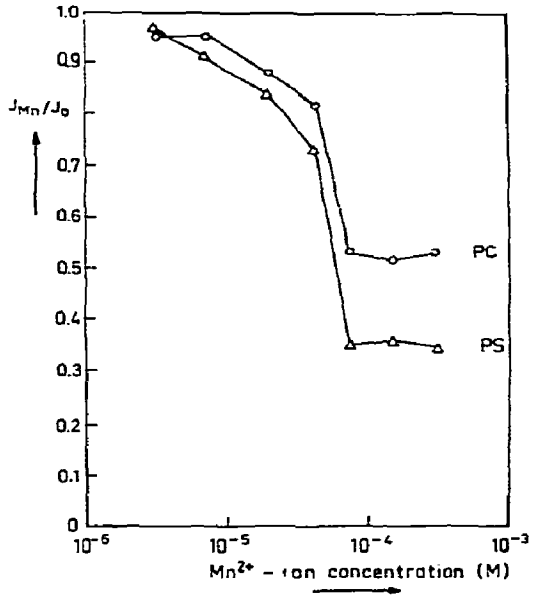

Fig. 7. The values of the relative signal heights, I_{Mn}/I_0, of the phosphorus resonances in a cosonicated mixture (1 : 1) of egg-yolk phosphatidylcholine (PC) and phosphatidylserine (PS) as a function of Mn^{2+}-ion concentration.

Fig. 7 shows the values of the relative signal heights I_{Mn}/I_0 of the phosphorus resonances * of a cosonicated PC/PS mixture as a function of the Mn^{2+}-ion concentration. The values of the intensities decrease with increasing Mn^{2+}-ion concentration in a similar way for both lipids but a different intensity ratio is obtained after reaching the "plateau". Working on the basis of the results of the ^1H measurements one has to conclude that the phosphorus resonance of PS has nearly to disappear while the signal of PC should decrease to a value of again approximately 67.4% of its maximum value. This is, however, not observed! Since the ^1H measurements are unambiguously to interpret and well to reproduce we conclude that the nuclear Overhauser enhancement has been changed by the addition of the Mn^{2+} ions. For this reason we think that it is not possible in the present state of work to calculate the distribution of, e.g., PA/PS mixtures because no one of these two lipids has an appropriate polar head group feasible for investigations by normal cw ^1H-NMR measurements.

4. Discussion

While the PRE measurements [1] see the polar head group of the phospholipids as an only interacting site and lead to a formal description of the "ion-lipid complex" the analysis of the high-resolution NMR experiments permits us to inform about some particulars concerning the sites of the interaction with the paramagnetic ions. As you can see from table 2, the difference in chemical shift, Δ_N, between the "inner" and "outer" resonance signals of PC dispersions in the presence of Pr^{3+} ions is the largest for the phosphorus resonance.

The chemical shift value for a nucleus N depends upon the reciprocal cube of the distance r_N from the paramagnetic ion (see e.g., [15]). This fact involves that

* The signal heights were corrected with regard to equal signal/noise ratio.

Table 2
Differences in chemical shift values, Δ_N, between the "inner" and "outer" resonance signals of the polar head group of egg-yolk phosphatidylcholine in the presence of Pr^{3+} ions and the chemical shift differences, $\Delta^{31}P/\Delta_N$, (see discussion) for the different nuclei relative to phosphorus as unity

Polar group	Observed resonance (see figs. 1–3)	Δ_N (Hz)	$\Delta^{31}P/\Delta_N$
	$\overset{+}{N}(C\underline{H}_3)_3$	29.0	12.3
CH_3	$\overset{+}{N}(\underline{C}H_3)_3$	22.63	15.7
$CH_3-\overset{+}{N}-CH_3$			
CH_2	$\underline{C}H_2$	65.63	5.4
CH_2	$\underline{C}H_2$		
O			
$O=P-O^-$	O	365.0	1.0
O	$O=P-O^-$		
CH_2	O		
CH	$-\underline{C}H_2$	38.47	9.3
CH_2	$-\underline{C}H$		
	$-\underline{C}H_2$		

the shift values induced by the Pr^{3+} ions can be interpreted in terms of molecular geometry. A reasonable qualitative interpretation of the data summarized in table 2 is that in the "ion-lipid complex" the Pr^{3+} ion is localized closer to the phosphate group than to the $\overset{+}{N}(CH_3)_3$ and the other groups. To emphasize this interpretation the ratios of the chemical shift differences for the different nuclei relative to phosphorus as unity are also summarized in table 2.

Since we do not know the exact stereochemistry of the metal ion interaction with the phospholipid head group it is not possible in the present state of work to give information about the absolute distances of the paramagnetic ion within the polar groups of the phospholipid molecules.

The negatively charged phosphate groups appear to cause an increased probability of finding the paramagnetic ion in the favoured positions mentioned above and hence effect the magnetic properties of the Mn^{2+}-aquo complex [1] as well as the properties of the neighbouring nuclei of the phosphorus even if in a smaller extent.

A quantitative integral analysis of the $^{31}P-\{^1H\}$ NMR spectra is not possible without the knowledge of the exact nuclear Overhauser enhancement (see sections 3.2 and 3.3)!

It should be noted that such a ^{31}P analysis for mixtures of egg-yolk phosphatidylcholine/phosphatidylglycerol and phosphatidylethanolamine, respectively has been made by Michaelson et al. [4,5]. They have found that 60–65% of the total lipids take the outside surface of these cosonicated vesicles. This conclusion, however, cannot be frawn from the ^{31}P measurements until one has obtained a clear information about the Overhauser enhancement in phospholipid dispersions.

The statement (cf. point 2, section 3.3) that the molecules are not present in separated blocks but rather are dispersed at least within the outer monolayer can be explained by the negative charge of the phosphatidylserine head group. Because of the electrostatic repulsion between the PS molecules a block formation seems to be energetically unfavourable (compare [4]). A separation of the PS molecules by the PC molecules can reduce the repulsion between the PS molecules to a minimum. Furthermore, the repulsion between the negatively charged head groups of the PS molecules will be minimized in an asymmetrical distribution – where nearly all PS molecules are in the outer layer – since the polar groups of the mixed vesicle are less tightly packed in the outer monolayer than those in the inner one.

In a recently published paper, Berden et al. [6] report – among other things – a ^{31}P-NMR study on the arrangement of egg-yolk lecithin and phosphatidylserine (3:2) in cosonicated vesicles. The authors come to the result that in such mixtures phosphatidylserine has a preference for the inside monolayer in the p^2H range of 4.6–7.2! They conclude that the distribution becomes symmetrically at p^2H values between 8 and 9 and asymmetrically again – now phosphatidylserine more at the outside – at still higher p^2H values. These results were interpreted in such a manner that not the difference in the charge of both lipids is the most important factor in determining the distribution but the relative smallness of the polar head group of phosphatidylserine. Our result that in PC/PS mixtures at p^2H

8.3 where PS exists in the form of its disodium salt [16] an extreme asymmetry is observed with PS preferentially in the outer side of the membrane is, however, not necessarily in contradiction to the findings of the authors cited above. Since the average diameter of our vesicles is somewhat larger than the diameter of the vesicles investigated by Berden et al., the difference in the radius of curvature between the inner and outer layer is not so markedly pronounced in our vesicles. For this reason phosphatidylserine in our case seems to have a preference for the outside at lower p^2H values so that the difference in charge of the polar groups should be really the dominant factor for the observed asymmetry.

Therefore it is necessary to take the charge and structure of the polar groups into account as well as the size and shape of the mixed vesicle when interpreting the results of measurements concerning the arrangement of phospholipids in cosonicated vesicles. Furthermore, the comments as noted above for the integral analysis of $^{31}P-\{^1H\}$ NMR spectra are also relevant to the work of Berden et al..

The simultaneous use of "relaxation and shift probes" have shown the possibility of obtaining some essential structural parameters in model systems for membranes. As seen in the discussion above the treatment of some important problems like integral analysis of proton-noise decoupled spectra and quantitative evaluation of the chemical shift data is still in a preliminary state of development, but we think further relaxation- and shift-NMR measurements to be very useful in providing information about the orientation of the paramagnetic ions relative to the different nuclei of the polar head group as well as about the asymmetrical distribution in phospholipid mixtures.

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