# Head Group Modulation of Membrane Fluidity in Sonicated Phospholipid Dispersions†

D. M. Michaelson,‡ A. F. Horwitz,§ and M. P. Klein*

ABSTRACT: The mobility of the polar head group, as well as of the hydrocarbon region, of phosphatidylethanolamine (PE) molecules arranged in sonicated dispersions is markedly dependent on the charge of the ethanolamine head group: there is more molecular motion when PE is negatively charged than at pD levels where it is zwitterionic. At physiological values of pD, the molecular motions of PE are more restricted than are those of phosphatidylcholine (PC). These results were obtained by preparing, at various pD values, sonicated dispersions of PE, of PC, and of equimolar mixtures of PC and PE, and by studying the proton and phosphorus magnetic resonance spectra of these dispersions. At pD levels below the p$K$ of the head group amine of PE the spectra are broader and of a lower intensity than at pD levels above the p$K$. In addition, we found that the PE and PC molecules are symmetrically distributed between the inner and outer surface of the single-shelled cosonicated PC:PE vesicles buffered at pD 11.0. This finding was obtained by exploiting the effects of the paramagnetic ions $Fe(CN)_6^{3-}$ and $Mn^{2+}$ on the proton and phosphorus magnetic resonance spectra. At the concentrations employed, these ions do not permeate the vesicles, thus permitting a distinction between the signals of PE and PC which originate on the outer and on the inner surfaces. The observed bilayer symmetry and head group modulation of the fluidity of sonicated PE and cosonicated PC:PE dispersions are discussed in the context of the structure and fluidity of biological membranes.

The wealth of studies on the composition of animal cell membranes has shown that phosphatidylethanolamine (PE)[1] and phosphatidylcholine (PC) are generally the most abundant phospholipids in any particular membrane (Answell and Hawthorne, 1964; Rouser *et al.*, 1968). The zwitterionic nature of these molecules distinguishes them from the other phospholipid classes. They differ from each other only by the nature of their head group amines; PE has a primary amine while PC has a quaternary trimethylamine. Although a great deal of information is accumulating on the structure and characteristics of PC in bilayers and membranes, relatively less is known about PE.

Model membrane studies have shown that PE differs from PC in several physical properties. Luzzati (1968) and Papahadjopoulus and Miller (1967) have demonstrated that PE and PC exhibit different phase behavior, Steim (1968) has shown that PE has a higher endothermic transition temperature than has PC, and Phillips *et al.* (1972) have shown that PE is packed more tightly than is PC, suggesting a difference in molecular mobility. Recently evidence from work on erythrocyte membranes indicates that the PE and PC molecules are distributed asymmetrically between the inner and outer surfaces of the membranes (Bretscher, 1972, 1973; Verkleij *et al.*, 1973). All of these observations suggest that the chemical differences between these two zwitterionic phosphatides are of structural and physiological importance.

To characterize further some of the factors by which PE and PC affect the structure of biological membranes, we studied the dynamics and spatial arrangement of these molecules in a model system, *i.e.*, sonicated phospholipid dispersions (Attwood and Saunders, 1965). We extend our previous work on phospholipid head groups (Horwitz *et al.*, 1973; Michaelson *et al.*, 1973) and present our observations regarding the effects of the head groups and of pD on the molecular motions of PE and PC in pure sonicated dispersions, the effects of pD on cosonicated PC:PE dispersions, and the distribution, at high pD, of these molecules between the inner and outer surfaces of the cosonicated dispersions. We have employed the phosphorus and proton magnetic resonance spectra of the sonicated dispersions and the effects thereon of pD and paramagnetic ions (*e.g.*, $Mn^{2+}$, $Fe(CN)_6^{3-}$) to study the mobility and the transbilayer distribution of the phospholipids in the sonicated dispersions.

## Materials and Methods

Bacterial phosphatidylethanolamine (*Escherichia coli* PE) was extracted and purified from a variant of a $\beta$ oxidation$^-$ unsaturated fatty acid auxotroph of *E. coli* (30E$\beta$ox$^-$, which was a gift from C. F. Fox). The bacteria were grown at 15° in medium A (Davis and Mingioli, 1950), supplemented with 1% casamino acids (Difco), 5 mg/l. of vitamin $B_1$, 0.5% Triton X-100 and 0.1 g/l. of oleate. The cells were harvested by centrifugation at midlog phase, and the phospholipids were extracted by the Bligh and Dyer method as described by Ames (1968). PE was purified from the crude extract by DEAE-cellulose column chromatography employing an elution scheme similar to that described by Rouser *et al.* (sequence 3 in Rouser *et al.*, 1969). Egg yolk phosphatidylcholine (egg PC) was extracted and purified following the procedure of Singelton *et al.* (1965). The purified phospholipids were shown by thin-layer chromatography (tlc), employing precoated silica plates (Adsorbosil 5, obtained from

† From the Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. *Received December 17, 1973.* This work was supported by the U. S. Atomic Energy Commission. This is the seventh paper in the series "Magnetic Resonance Studies of Membrane and Model Systems."

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§ Present address: Departments of Biophysics and Physical Biochemistry, Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104.

[1] Abbreviations used are: PE, phosphatidylethanolamine; PC, phosphatidylcholine; egg PC, egg yolk phosphatidylcholine.

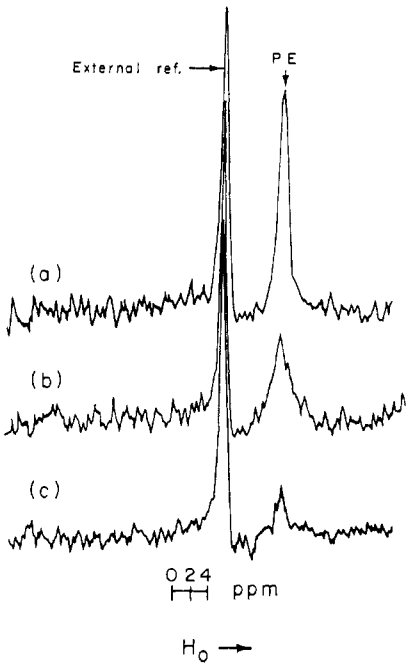


FIGURE 1: $^{31}P$ nmr spectra of sonicated egg PE dispersions buffered at pD 11.0 (a), 9.3 (b), and 8.0 (c). The external reference is $K_3PO_4$ at pD 14.0.

Applied Science), each to give a single spot. The plates were developed in chloroform–methanol–water (65:25:4, by volume) and phospholipid spots were visualized with iodine vapor. Egg PE was purchased from Supelco, Inc.; it was slightly oxidized, as revealed by gas chromatography, and was shown by tlc to give a single spot. The lipids were stored in chloroform under argon at −20°, and their concentration was determined by the phosphate procedure (scaled down by 10) of McClaire (1971).

Sonicated PE dispersions and cosonicated PC:PE dispersions, containing equal concentrations of PC and PE, were prepared as previously published (Horwitz and Klein, 1972) in 50 mM deuterated buffer containing 150 mM NaCl and $10^{-6}$ M EDTA, except that boric acid–borax buffer was used when the sonicated dispersions were buffered at pD 8.0 and 9.3, and borax–NaOH buffer was used when the dispersion was buffered at pD 11.0. In the experiments employing $Mn^{2+}$ and $Fe(CN)_6^{3-}$, small volumes of concentrated solutions of $MnCl_2$ (in $D_2O$) and $K_3Fe(CN)_6$ (in borax–NaOH buffer at pD 11.0) were added to the phospholipid dispersions after sonication. The sample was vortexed gently during the addition of the paramagnetic ion in order to obtain rapid mixing. After every experiment the lipids were extracted and shown by tlc each to give a single spot. Experiments were performed with freshly prepared samples, not more than 12 hr after sonication, since after more than 24 hr at high pD some of the phospholipids were, as shown by tlc, degraded to lysophospholipids.

Electron microscopy was performed on ammonium molybdate negatively stained sonicated phospholipid dispersions (Papahadjopoulus and Miller, 1967). The technique was as follows: about 5 mg of PE or PE plus PC (1:1) were sonicated in 2 ml of $D_2O$ solution containing 0.2 M ammonium acetate and adjusted to the appropriate pD with ammonium hydroxide. Immediately before application to the grid, the sonicated dispersions were negatively stained by the addition of 1 volume of 2% ammonium molybdate solution of equal pD and osmolarity. Ammonium hydroxide and ammonium acetate instead of NaCl and the borax buffers employed in the magnetic resonance studies were used to avoid crystallization of inorganic salts on the microscope grid. We used ammonium molybdate rather than phosphotungstic acid as a negative stain (Papahadjopoulus and Miller, 1967), because of the low solubility of the latter at high pD.

Proton magnetic resonance (pmr) spectra were recorded at specific temperatures, between 20 and 45°, on a Varian HR-220 spectrometer with $Me_4Si$ as an external reference. The spectra were integrated using an external standard of acetone. Phosphorus magnetic resonance ($^{31}P$ nmr) measurements were made at an ambient probe temperature of 31° in a pulsed Fourier mode at 24.3 MHz as discussed elsewhere (Horwitz and Klein, 1972) with an external standard of $K_3PO_4$ at pD 14.0.

## Results

I. *Sonicated PE Dispersions.* It has been reported that sonication of egg PE at neutral and slightly acidic pH values results in the formation of large precipitable aggregates (Papahadjopoulus and Miller, 1967). We find that sonication of egg PE at basic pD, *i.e.*, 8.0, 9.3, and 11.0, results in optically clear dispersions. Sonication of *E. coli* PE at pD 11.0 similarly produces a clear dispersion, whereas sonication at pD 9.3 results in a clear dispersion which contains only about 25% (7.5 mM) of the PE molecules; the remainder of the material forms an aggregate, similar to that found at neutral pD values, which is pelleted by centrifugation after sonication. Electron microscopy of all of these optically clear dispersions reveals that the PE molecules are organized in "onion-like" multilamellar structures. The sonicated PE dispersions buffered at any of the above basic pD values are heterogeneous in shape and size: their shape varies between spherical and elongated, and their sizes range from about 0.05 to 0.5 $\mu$. We were unable to discern differences between the electron micrographs of the dispersions buffered at the various pD values.

By contrast, the phosphorus and proton magnetic resonance spectra of the sonicated egg PE dispersions were markedly affected by the pD at which they were buffered. The $^{31}P$ nmr spectrum of sonicated PE dispersions buffered at pD 11.0 contains a single resonance which is shifted 138 Hz (5.7 ppm) to higher field from the phosphorus resonance of an external standard of $K_3PO_4$ at pD 14.0.[2] It is 24 ± 1 Hz wide for sonicated egg PE dispersions (Figure 1a) and 27 ± 2 Hz wide for sonicated *E. coli* PE dispersions. Integration, using the external reference as an intensity standard, revealed that for both samples, 100 ± 10% of the PE molecules contribute to the resonance.

When buffered at pD 9.3, the sonicated PE dispersions yield phosphorus magnetic resonances of decreased intensity; the egg PE dispersions yield a resonance (Figure 1b) whose integrated area corresponds to only 70% of the PE nuclei and whose residual line is broadened to 43 ± 2 Hz, whereas the phosphorus resonance of the *E. coli* PE sample buffered at pD 9.3 is broadened beyond detection. A further decrease of

[2] The chemical shifts of the phosphorus resonance of sonicated egg PC buffered at pD 8.0 and at 11.0 are identical: 160 Hz (6.6 ppm) to higher field than an external standard of $K_3PO_4$ at pD 14.0. Thus the phosphorus resonance of sonicated PE is shifted 22 Hz (0.9 ppm) to lower field than that of sonicated egg PC. This shift is partly due to differences in susceptibilities and partly to an intrinsic difference in the chemical shifts of PE and PC, since for a cosonicated dispersion of PC and PE the PE phosphorus resonance is shifted only 11.0 Hz (0.45 ppm) to lower field than the PC phosphorus resonance.

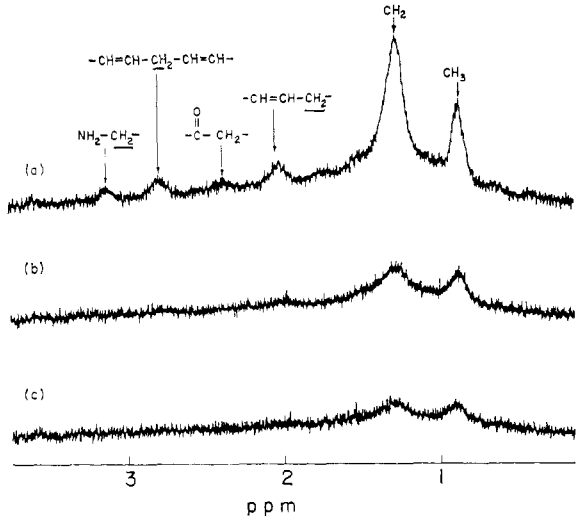


FIGURE 2: Proton magnetic resonance spectra of sonicated egg PE dispersions buffered at pD 11.0 (a), 9.3 (b), and 8.0 (c). The spectra were recorded at 22°; the abscissa scale is ppm downfield from $Me_4Si$.

the pD to 8.0 results in an additional decrease of the phosphorus resonance intensity of sonicated egg PE, which accounts for only about 20% of the phospholipid nuclei (Figure 1c). The residual line width of this resonance is 28 ± 2 Hz. The chemical shift of the phosphorus resonance does not, within the range investigated, show a pD dependence.

The pmr spectra of sonicated PE dispersions also show a marked pD dependence. The pmr spectrum of sonicated PE buffered at pD 11.0 contains several distinguishable resonances which are assigned by comparison to the spectrum of egg PC and of head group model compounds (Chapman and Morrison, 1966; Dufourq and Lussan, 1972). The spectrum of sonicated egg PE (Figure 2a) and *E. coli* PE are similar, except that in the latter the diallylic proton resonance is missing. This is expected since *E. coli* PE contains no polyunsaturated fatty acids. Integration of the fatty acid terminal methyl proton resonance of sonicated egg PE revealed that at 22° about 55% of the phospholipids contribute to this resonance, and that at 45° a larger fraction of the molecules, 75%, contribute to this peak (Figure 3). The ratio of the area of the terminal methyl proton resonance to those of the methylene plus terminal methyl resonances at 22° as well as at 45° is, within experimental error, similar to the ratio expected from the fatty acid analysis (Figure 4). Analogous results were obtained with sonicated *E. coli* PE dispersions. Thus we may conclude that at 22°, 55% of the PE fatty acid protons contribute to the spectral intensity and at 45°, 75% so contribute. In addition to the increase in intensity, raising the temperature to 45° causes a narrowing of the detectable resonances, the

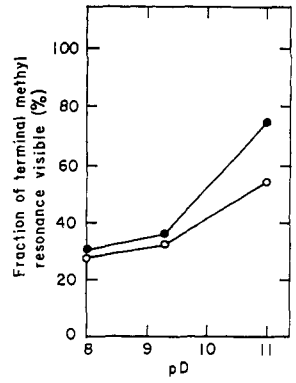


FIGURE 3: The effect of pD on the fraction of PE molecules contributing to the intensity of the high-resolution terminal methyl proton resonance of sonicated egg PE dispersons: (○) spectra recorded at 22°; (●) spectra recorded at 45°.

fatty acid methylene resonances exhibiting the greatest narrowing (Table I). Data from the literature (Linden *et al.*, 1973), as well as the nmr data reported here, imply that all of our measurements were performed at temperatures above the endothermic transition.

Sonication of egg PE at lower pD values, pD 9.3 and 8.0, results in a marked decrease of the proton spectral intensity

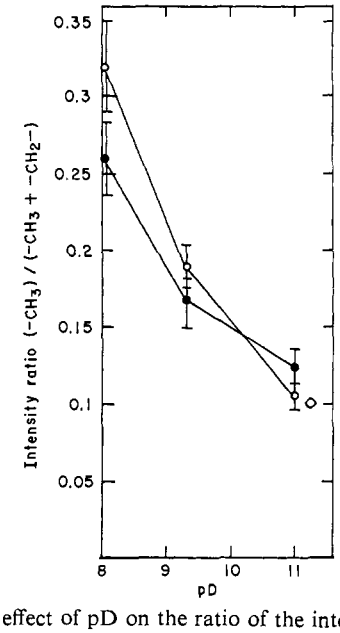


FIGURE 4: The effect of pD on the ratio of the intensity of the terminal methyl proton resonance to the sum of the intensities of the fatty acid methylene plus terminal methyl proton resonances of sonicated egg PE dispersions. The expected ratio, calculated from the fatty acid composition is indicated by ◊. The graph calculated from spectra recorded at 22° is denoted by ○, and from data obtained at 45° by ●.

TABLE I: Effect of pD and Temperature on Pmr Line Widths (Hz) of Sonicated PE Dispersions.

| | | $-CH_2-$ | | $-CH_3$ | | $-CH_2-NH_2$ | |
|---|---|---|---|---|---|---|---|
| PE | pD | 22° | 45° | 22° | 45° | 22° | 45° |
| Egg | 11.0 | 38 ± 2 | 28 ± 2 | 17 ± 1 | 16 ± 1 | 23 ± 2 | 23 ± 2 |
| | 9.3 | 85 ± 5 | 60 ± 4 | 27 ± 2 | 21 ± 2 | | |
| | 8.0 | 100 ± 5 | 75 ± 5 | 28 ± 2 | 28 ± 2 | | |
| *E. coli* 30Eβox⁻ | 11.0 | 37 ± 2 | 29 ± 1 | 18 ± 1 | 16 ± 1 | 24 ± 2 | 22 ± 2 |
| | 9.3 | 75 ± 2 | 60 ± 5 | 48 ± 2 | 36 ± 2 | | |

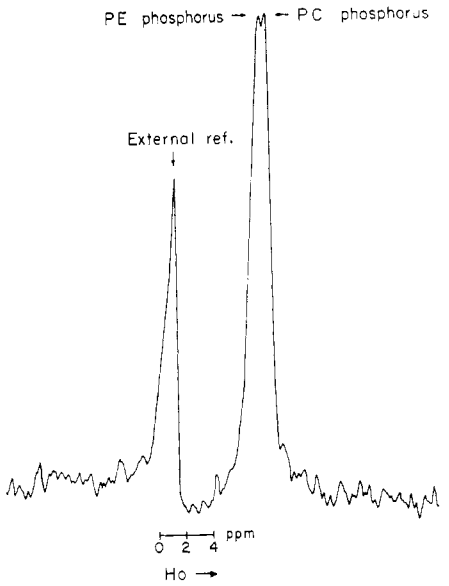


FIGURE 5: $^{31}P$ nmr spectrum of cosonicated PC:PE dispersions buffered at pD 11.0. The external reference is $K_3PO_4$ at pH 14.0.

(Figure 2b,c); however, the fatty acid methylene and terminal methyl proton resonances are affected to different extents. The fraction of detectable terminal methyl protons is reduced to approximately 35% at pD 9.3 and to about 30% at pD 8.0 (Figure 3), with the methylene proton resonances exhibiting a more pronounced decrease. This is demonstrated in Figure 4, where the pD dependence of the ratio of the intensities of the terminal methyl to the fatty acid methylene plus terminal methyl resonances is plotted. Raising the temperature from 22 to 45° results in a narrowing of the fatty acid proton resonances (Table I) as well as a small increase in the spectral intensity (Figures 3 and 4). Thus, at lower pD a relatively smaller fraction of the methylene proton resonances is detectable, *i.e.*, the effect of pD is more pronounced on those protons along the fatty acid chains than those at the ends.

A similar trend was observed in the pmr spectra of sonicated *E. coli* PE dispersions. About 20% of the terminal methyl protons are accounted for at 22° and 50% at 45°. The ratio of the intensity of the fatty acid terminal methyl proton resonance to the sum of the intensities of the fatty acid methylene and terminal methyl proton resonance is increased more than twofold in the spectrum of the sonicated dispersions buffered at pD 9.3 as compared to those buffered at pD 11.0 at 22°, and approximately fourfold at 45°. This trend is further reflected by the observation (Table I) that the visible methylene resonance is broadened more by the pD decrease than is the visible terminal methyl resonance. Furthermore, for both samples the allylic proton resonances and the head group $\alpha$-carbon proton resonance are broadened beyond detection at pD 9.3. In contrast to the proton and phosphorus magnetic resonance spectra of sonicated PE, those of sonicated PC dispersions accounted for virtually all the nuclei and showed no pD dependence within the range of pD 8.0 to pD 11.0.

The pmr spectra of both sonicated egg PE and *E. coli* PE dispersions broaden and decrease in intensity as the samples age at room temperature. The effect is more pronounced for the spectra of the samples buffered at pD 11.0 than for those buffered at pD 9.3. The spectra lose about half their intensity within 12 hr after sonication at pD 11.0. The rate of spectral deterioration is slightly faster for sonicated egg PE dispersions than for those of *E. coli* PE; however, for both samples and at either pD value the decay is not uniform: it is more pronounced along the fatty acid chains than at their terminal methyl ends. The spectral decay is reversible, *i.e.*, resonication restored the original spectrum. Again by contrast, the pmr spectra of sonicated PC were stable, at the pD values investigated, for at least 24 hr.

The decrease of the acuity and intensity of the spectra of both sonicated *E. coli* PE and egg PE dispersions as the pD is lowered from 11.0 to 8.0, concurring with the p$K$ of the head group primary amine, is suggestive. (The precise value of this p$K$ is not known; however, the value for a PE head group analog, *o*-phosphorylethanolamine, is 10.2.) Surface potential studies of PE monolayers also reflect the titration, at these pH values, of the head group primary amine (Papahadjopoulos, 1968). Since rapid isotropic molecular motion is a prerequisite for obtaining high resolution spectra (Abragam, 1961; Horwitz *et al.*, 1973), the decrease of spectral intensity is a manifestation of reduced motional isotropy, increased correlation times, or both. These in turn may be a result of the change in the interactions between the head groups of the PE molecules as they pass from the negatively charged state at high pD values to the zwitterionic at lower values of pD. The observed differences between the pD dependence of the spectra of the two PE samples may be due to their different fatty acid composition, to the partial oxidation of the fatty acids of egg PE, or to both.

II. *Cosonicated PC:PE Dispersions.* Cosonication of equimolar concentrations of egg PC and *E. coli* PE (cosonicated PC:PE) at pD 11.0 results in optically clear dispersions which, due to light scattering, have a bluish hue, and yield high resolution $^{31}P$ nmr and pmr spectra. Electron microscopy of these dispersions revealed single-shelled spherical vesicles which on the average measure 200–500 Å in diameter. The $^{31}P$ nmr spectrum of these vesicles contains two closely spaced resonances which are shifted 151 Hz (6.2 ppm) and 162 Hz (6.7 ppm) to higher field than an external reference of $K_3PO_4$ at pD 14.0. A comparison of these shifts to those of the resonances of sonicated dispersions of pure PC and pure PE as well as to the phosphorus chemical shifts observed from PE and PC dissolved in chloroform permitted the assignment of the lower field resonance to PE and that residing at the higher field to PC (Figure 5). Integration using the external reference as an intensity standard revealed that 95 ± 5% of the PE and PC molecules contribute to these resonances.

The pmr spectrum of cosonicated PC:PE dispersions buffered at pD 11.0 is shown in Figure 6a. Some of the resonances in this spectrum originate from PC, some from PE, and some arise from both classes of molecules. The intensity of the choline $N^+(CH_3)_3$ proton resonance, which originates from the PC head group, is 95 ± 10% of that expected; the intensity of the $\alpha$-carbon proton resonance of the ethanolamine head group is 70 ± 10% of that expected; the intensity of the terminal methyl proton resonance, which originates from both PE and PC, is 80 ± 10% of that expected for the total lipid concentration. We have not measured directly the respective contributions of PC and PE to the latter resonance. However, the relative intensities of the head group and terminal methyl proton resonances are consistent with the assumption that virtually all the PC and about 70% of the PE terminal methyl protons contribute to this resonance. This assertion is supported by the observation that the intensity of the pmr spectra of sonicated egg PC dispersions buffered at pD 11.0 accounts for all the PC protons, whereas less than all the PE protons contribute to the intensity of the pmr spectra of sonicated PE buffered at pD 11.0. We have recorded the pmr spectra at 25 and 45°. The resonances, in particular the

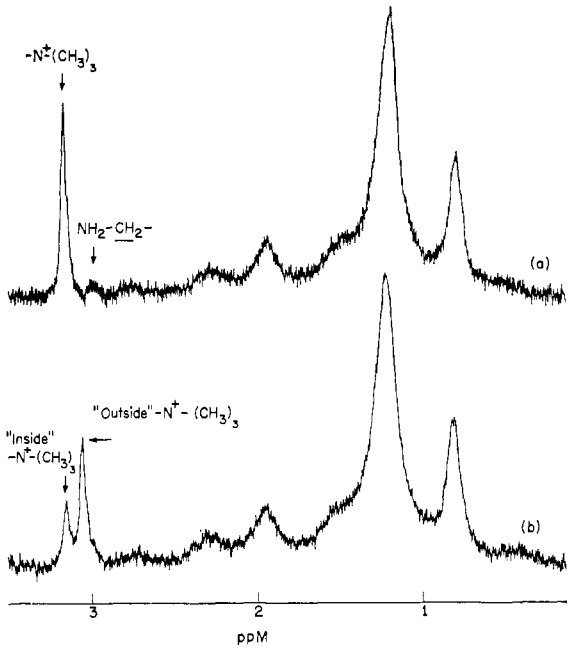


FIGURE 6: Proton magnetic resonance spectra of 25 mM cosonicated PC:PE dispersions, before (a) and after (b) the addition of 0.1 M $K_3Fe(CN)_6$. "Outside" $N^+$–$(CH_3)_3$ and "inside" $N^+$–$(CH_3)_3$ correspond respectively to the resonances originating from PC molecules in the outer and inner monolayers.

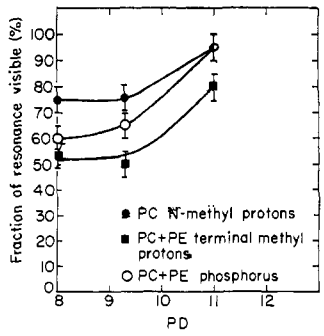


FIGURE 7: The effect of pD on the fraction of the phospholipid molecules contributing to the $^{31}P$ nmr and pmr spectra of cosonicated PC:PE dispersions: (○) phosphorus resonances of PC plus PE; (■) terminal methyl proton resonance; (●) choline $N^+$–$(CH_3)_3$ proton resonance.

methylene proton resonances, were narrower at the elevated temperature. Nevertheless there was no detectable change in spectral intensity, *i.e.*, the fraction of the protons contributing to the respective resonances did not increase upon raising the temperature to 45°. The pmr spectrum was stable at room temperature for at least 12 hr after sonication.

Cosonication of equimolar concentrations of egg PC and *E. coli* PE buffered at pD 9.3 and at 8.0 results in optically clear dispersions. Electron microscopy of these dispersions revealed that, contrary to what we observed at pD 11.0, PE and PC are arranged in heterogeneous structures which are either multilamellar or single shelled. Furthermore, the intensity of the phosphorus resonances, which at pD 11.0 accounts for virtually all the PE and PC molecules, accounts for only 65% of the phospholipid molecules at pD 9.3 and 60% of them at pD 8.0 (Figure 7). The intensity of the pmr spectra is also reduced at these pD values; the choline $N^+$–$(CH_3)_3$ proton resonance is reduced to about 75%, and that of the terminal methyl protons accounts for only 50% of the phospholipid molecules (Figure 7). The nmr and pmr spectra of sonicated egg PC dispersions are pD independent within the range of pD 11.0 to pD 8.0; however, as has been shown in the previous section, the intensity of the corresponding spectra of sonicated PE dispersions decreases as the pD is lowered from 11.0 to 8.0. These results suggests that the relative contributions of PC and PE to the spectra of cosonicated PC:PE may be pD dependent, *i.e.*, that at the lower pD value relatively fewer PE than PC molecules contribute to the spectra. This suggestion is supported by the observation that the ratio of the intensity of the choline $N^+(CH_3)_3$ resonance, which originates only from PC molecules, to that of the terminal methyl proton resonance, which originates from both PE and PC, decreases from about 1.25 at pD 8.0 to about 0.95 at pD 11.0. Since the $^{31}P$ nmr and pmr spectra of sonicated PC dispersions are independent of pD, within the range investigated, while the corresponding spectra of PC molecules organized in cosonicated PC:PE dispersions are pD dependent, we may conclude that in the cosonicated dispersions PE influences the molecular motions of PC.

III. *Inside–Outside Distribution of PC and PE in Cosonicated Dispersions.* In a recent study (Michaelson *et al.*, 1973) we exploited the effects of paramagnetic ions on nmr spectra to study the distribution of phosphatidylglycerol and PC molecules between the inner and outer surfaces of cosonicated vesicles. We now report our findings from a similar study performed with cosonicated PC:PE dispersions. Only dispersions buffered at pD 11.0 are presently suitable for this study. That is so because cosonication of PC and PE at pD 11.0 results in single-shelled vesicles which yield a $^{31}P$ nmr spectrum which accounts for all the phospholipid molecules and a pmr spectrum which accounts for virtually all the PC molecules and about 70% of the PE molecules, whereas cosonication at pD 8.0 and 9.3 results in a heterogeneous population of multilamellar structures and single-shelled vesicles, which yield proton and phosphorus magnetic resonance spectra of decreased intensity.

Figure 6b shows the pmr spectrum of cosonicated PC:PE (25 mM) to which 0.1 M $K_3Fe(CN)_6$ was added. These ions shift part of the choline $N^+(CH_3)_3$ resonance 33 Hz (0.15 ppm) to higher field and do not affect the resonances which originate from the hydrophobic region of the phospholipids. Resonication of the dispersion in the presence of $K_3Fe(CN)_6$ results in a coalescence of the choline $N^+(CH_3)_3$ resonances to a single peak which is shifted upfield. When 0.2 M $K_3Fe(CN)_6$ was added the upfield shift of the choline $N^+$–$(CH_3)_3$ proton resonance increased to 42 Hz (0.19 ppm); however, the relative intensities of the shifted and unshifted components of the choline $N^+$–$(CH_3)_3$ resonance were unaltered. Similar observations were previously obtained with sonicated dispersions of pure egg PC (Kostelnik and Castellano, 1972). Our interpretation of these results is that the sonicated vesicles are impermeable to the $Fe(CN)_6^{3-}$ ions and hence only those resonances which originate from nuclei facing the "outside" of the vesicles and are present at the polar region are shifted by these ions. Thus resonication in the presence of the paramagnetic anions results in their introduction into the internal aqueous phase of the vesicles and the consequent shifting of the "inside" as well as the "outside" choline $N^+$–$(CH_3)_3$ resonances. We did not do the parallel $^{31}P$ nmr experiment since $K_3Fe(CN)_6$ was shown to have no effect on the $^{31}P$ nmr spectra of sonicated PC. This behavior is probably due to the bulkiness and large negative charge of the ferricyanide anion. Since the choline $N^+$–$(CH_3)_3$ resonance represents virtually all the PC molecules, we employed the relative intensities of the "inside" and "outside" resonances to

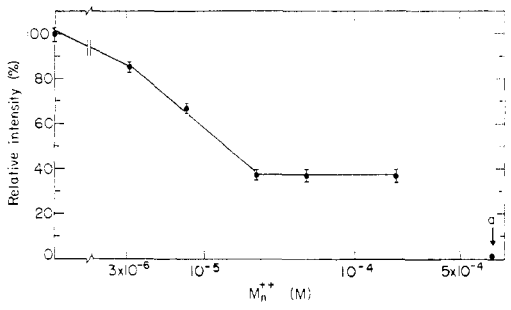


FIGURE 8: The change in intensity of the $^{31}P$ nmr spectra relative to the original spectra *vs.* $MnCl_2$ addition to cosonicated PC:PE dispersions buffered at pD 11.0. The signal intensity after resonication in the presence of $Mn^{2+}$ is indicated by (a).

calculate the distribution of the PC molecules between these two surfaces. The apparent signal intensities from the "inside" and "outside" were unsuitable for direct entry into the calculations but needed correction because the "outside" choline $N^+$–$(CH_3)_3$ resonance is shifted upfield and thus overlaps the ethanolamine $\alpha$-carbon proton resonance (compare Figures 6a and b). We took this fact into consideration and therefore compared the intensities of the total choline $N^+$–$(CH_3)_3$ resonance and the "inside" unshifted resonance (both resonances are sufficiently shifted from the PE $\alpha$-carbon proton resonance) to calculate the distribution of PC between the surfaces of the vesicles. We thus obtained that 35–40% of the PC molecules face the "inside" and 60–65% face the "outside."

In order to establish the symmetry properties of the vesicles we need to know, in addition to the distribution of PC, the *total* phospholipid distribution between the inner and outer surfaces of the vesicles. To answer this question, we studied the effects of the addition of the paramagnetic cation, $Mn^{2+}$, on the $^{31}P$ nmr spectra of the cosonicated dispersions. $Mn^{2+}$ is one member of a class of paramagnetic ions which broaden, rather than shift, the magnetic resonance of nuclei with which they interact. Prior to the addition of $Mn^{2+}$ the intensity of the phosphorus resonances corresponds to virtually all the PE plus PC molecules; upon addition of increasing concentrations of $Mn^{2+}$ the phosphorus resonances broaden and hence decrease in amplitude. At $Mn^{2+}$ concentrations below $10^{-5}$ M, the resonances contain broadened as well as unaltered components. At higher $Mn^{2+}$ concentrations (approximately $2 \times 10^{-5}$ M) a plateau is reached whence further $Mn^{2+}$ addition (up to $2 \times 10^{-4}$ M) causes no further decrease in the intensity of the resonances (Figure 8).[3] Resonication at plateau levels of $Mn^{2+}$ results in a broadening beyond detection of all the phosphorus resonances. The intensity of the resonances at the plateau is 35–40% that of the initial intensity and, within experimental error, contains equal contributions from PE and PC. We similarly interpret these results to show that, at the concentration studied, the cosonicated vesicles are impermeable to $Mn^{2+}$ and therefore $Mn^{2+}$ broadens only the phosphorus resonances which originate from the outer surface; the inner surface phosphorus resonances are unaffected except after resonication which introduces the $Mn^{2+}$ ions into the internal aqueous phase of the vesicles. We thus obtain that 60–65% of the *total* lipids and of PC face the "outside" surface and 35–40% of the *total* lipid and of PC face the "inside" surface of the cosonicated PC:PE vesicles. This ratio of total "inside" to total "outside" phospholipid is that expected for vesicles of this size.

In summary, our data from the experiments employing the paramagnetic ions show that, on the average, PC and PE are distributed symmetrically between the inner and outer surfaces of the cosonicated vesicles buffered at pD 11.0.

## Discussion

Our experiments show that at physiological pH values the proton and phosphorus magnetic resonance spectra of sonicated *E. coli* PE and of egg PE dispersions are broad and unresolved. By contrast, sonicated egg PC and sonicated PE dispersions buffered at pH values above the p*K* of the ethanolamine yield high-resolution spectra whose integrated intensities correspond to most of the lipid molecules.

These data suggest to us that the charge of the primary amine has a marked effect on the packing and mobility of the head group and of the hydrophilic region of the PE molecule. We propose, in agreement with Phillips *et al.* (1972), that when PE is zwitterionic the head groups interact strongly and tightly with those of neighboring PE molecules; the positively charged amine group of one molecule may attract electrostatically the negatively charged phosphate group of an adjacent molecule, thus restricting molecular motion. At higher pD levels where PE is negatively charged this attraction is reduced and may become repulsive, thus permitting greater molecular motion. This interpretation is further fortified by our observation that the pD-dependent broadening and decrease in spectral intensities are more pronounced for resonances originating from nuclei situated at or close to the head group than at the fatty acid termini.

The nature of the distribution of the protons and phosphorus nuclei which yield either broad undetected resonances or high-resolution spectra is not established at present. A possible distribution is one where only a specific fraction of the PE molecules, either within each bilayer or in a specific subpopulation of the heterogeneous dispersions, yield high-resolution spectra. Another possibility is that within each PE molecule only a fraction of the nuclei, possibly those furthest removed from the head group and/or those oriented at the magic angle, yield the high-resolution spectra (Chan *et al.*, 1972). An alternative interpretation is that the pD dependences of the spectra are due to gross structural variations between the PE dispersions buffered at the different pD values. The observation, by electron microscopy, that the sizes and shapes of the optically clear sonicated dispersions are not significantly altered by the pD at which they are buffered renders this interpretation less likely but does not exclude it.

The mobility differences suggested by the nuclear magnetic resonance (nmr) results are strengthened by previous experimental observations. Shimshick and McConnell (1973) have

[3] We have found that addition of $MnCl_2$ to cosonicated PC:PE dispersions buffered at pD 11.0 results in a visible precipitate at about $10^{-4}$ M $MnCl_2$. This precipitate is probably $Mn(OH)_2$ which has a low solubility at basic pD. Thus the $Mn^{2+}$ concentrations noted in the abscissa of Figure 8 are probably inexact. The precipitate did not contain any phospholipid vesicles, as revealed by the observation that the intensity of the fatty acid methylene and terminal methyl proton resonances were unaffected by the addition of $Mn^{2+}$ at the entire concentration range investigated. The choline-$N^+$–$(CH_3)_3$ proton resonance was broadened by increasing $Mn^{2+}$ concentration; however, the maximum broadening was only threefold and thus there was no change in the observed intensity of this resonance. Furthermore, resonication in the presence of $Mn^{2+}$ did not result in a decrease in its amplitude. The observation that, at pD 11.0, $Mn^{2+}$ significantly broadens the $^{31}P$ nmr resonances and only slightly that of the choline-$N^+$–$(CH_3)_3$ protons, as well as the low solubility of $Mn^{2+}$ at high pD values, suggest to us that there is little free $Mn^{2+}$ in solution. That is, most of the $Mn^{2+}$ is either bound to the negatively charged phosphate groups, thus broadening their resonances, or precipitated out of solution.

employed a spin-labeled fluidity probe to study phospholipid dispersions of PE and PC. From the partition coefficient equations stated in their paper one calculates that their probe is more soluble in PC than in PE dispersions, thus implying that PE is less fluid. Steim (1968) and Chapman and Wallach (1968) have shown that for a given fatty acid composition the endothermic transition of PE is at a higher temperature than that of PC, and Phillips *et al.* (1972) have employed X-ray diffraction to show that, at neutral pH, PE molecules are more closely packed than are those of PC. Thus it seems that in addition to the generally accepted role of the fatty acids in affecting the fluidity of model as well as biological membranes, the phospholipid head groups, their charge and structure, may also affect the fluidity of the membrane. Consequently, parameters such as pH and ionic strength may significantly affect membrane fluidity. This supposition has been examined recently by Overath and Träuble (1973), who showed the transition temperature of acidic phospholipids to be inversely proportional to ionic strength.

Our experiments show that cosonication of equimolar concentrations of PE and PC at pD values below the p$K$ of the ethanolamine results in single-shelled vesicles as well as multilamellar heterogeneous structures; these preparations yield magnetic resonance spectra of reduced intensity. By contrast, cosonication of these molecules at pD 11.0 results in single-shelled vesicles which yield a $^{31}P$ nmr spectrum which accounts for all the phospholipid molecules and a pmr spectrum which accounts for almost all the PC and about 70% of the PE. The decrease in spectral intensity as the pD is reduced is qualitatively similar to that observed with pure PE dispersions. By contrast, however, we have shown that the magnetic resonance spectra of sonicated PC show no pD dependence, in this range; consequently, we suggest that the pD effect on the spectra of these cosonicated dispersions results from the net negative charge on most of the PE molecules at pD 11.0 and their zwitterionic nature at the lower pD values investigated (recall that the pD for the ethanolamine is about 10.2). The factors contributing to the reduced spectral intensities are, at present, difficult to assess, as noted above in the discussion of the PE dispersions.

We have studied the effects of paramagnetic ions (*i.e.*, $Mn^{2+}$, $Fe(CN)_6^{3-}$) on the magnetic resonance spectra of cosonicated PC:PE vesicles buffered at pD 11.0. Our data suggest that the PE and PC, at this pD, are distributed symmetrically between the inner and outer surfaces of the vesicles. Litman (1973) has used selective chemical labeling to study the "inside–outside" distribution of PE and PC in mixed phospholipid vesicles, buffered at pH 5.0. His data show that cosonicated dispersions composed of equimolar concentrations of PC and PE are asymmetric; the inner surface contains more PE molecules and the outer surface contains more PC molecules. The difference between our findings and those of Litman (1973) is most likely a result of the different pH values we employed, as well as the smaller radii of his vesicles. The small sizes of the cosonicated vesicles suggest that the head groups of the molecules on the inner surfaces are packed more tightly than are those on the outer monolayer. The head group of PE is less bulky than that of PC and it is reasonable to conjecture that this parameter would permit tighter packing of PE than of PC. Also, at neutral and acidic values of pH where both molecules are zwitterionic, the primary amine on the PE head group permits stronger electrostatic intermolecular attraction and hence tighter binding than does the quaternary amine of PC. Both of these considerations can account for Litman's observation (1973) of asymmetric vesicles with the greater fraction of the PE molecules residing on the inner surfaces at acidic values of pD. A similar distribution at higher pD values, *i.e.*, above the p$K$ of the ethanolamine, where most of the PE molecules bear a net negative charge, would result in an unfavorably large electrostatic repulsive energy. A more uniform partitioning of PE molecules between the surfaces of the vesicles, as we observed, must reflect an energetically more favorable distribution.

With reservations, the observed effects of the ethanolamine head group on the mobility and "inside"–"outside" distribution of these molecules in membrane model systems may be applicable to biological membranes. Many biological membranes contain relatively large amounts of PE and PC and in some membranes, *e.g.*, human erythrocytes (Bretscher, 1972; 1973; Verkleij *et al.*, 1973), these molecules are believed to be asymmetrically distributed between the inner and outer layers of the membrane.

Our results and those of Litman (1973) imply that the structure and charge of the phospholipid head groups affect their spontaneous partitioning between the surfaces of model membranes. In biological membranes, however, the spatial arrangement of these molecules is undoubtedly also determined biosynthetically as, for example, by the "Flipase" conjectured by Bretscher (1973) and asymmetrically situated biosynthetic or other enzymes.

The observed differences in the mobilities of PE and PC suggest that PE may alter membrane fluidity either locally or in general, and that in membranes asymmetric in PE and PC, proteins or other components embedded in different sides of the membrane would experience different viscosities. However, it has been observed that PE (and also phosphatidylserine) in a wide variety of membranes (but not all) has esterified to it the majority of the polyunsaturated fatty acids (Answell and Hawthorne, 1964; Rouser *et al.*, 1968; Stancliff *et al.*, 1969; Breckenridge *et al.*, 1972; Mason *et al.*, 1973). The physiological significance of such a distribution may be to compensate for the decrease in mobility that the PE molecules experience, due to their head group, by providing them with more fluid-like fatty acids. But this argument can also be permuted; perhaps one of the roles of PE is to carry these polyunsaturated fatty acids, whose primary function is not to alter the membrane fluidity, in general, but rather to provide a proper local environment or to serve as a store of biosynthetic precursors for molecules derived from polyunsaturated fatty acids, *e.g.*, the prostaglandins (Hinman, 1972). This latter possibility is made attractive by the recent demonstration of a phospholipase that shows high specificity for PE over PC (Victoria *et al.*, 1971).

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# Isolation and Properties of the Principal Liver Protein Conjugate of a Hepatic Carcinogen†

Sam Sorof,* Brahma P. Sani, Valerie M. Kish,‡ and H. Paul Meloche

ABSTRACT: Certain cellular proteins of unknown function are preferred targets of chemical carcinogens during cell transformation to malignancy *in vivo* and in culture. In the present study, the principal species of liver protein–carcinogen conjugate ($h_2$-5*S* azoprotein) was reproducibly isolated 88–91% pure in 50-mg amounts from livers of rats fed the hepatic azocarcinogen, 3′-methyl-4-dimethylaminoazobenzene. The molecular weight of the azoprotein subunit was determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and by amino acid analysis to be 44,000. The azoprotein consists of two indistinguishable subunits that are not disulfide linked in a molecule of 88,000 molecular weight. The azoprotein molecule contains an average of two bound azocarcinogen residues per two subunits. The limiting amino acids in the protein are tyrosine, tryptophan, and methionine, which are present to the extent of two, two, and five residues per subunit, respectively. This characterization of the principal azoprotein of rat liver provides the basis of its ultimate identification, and for the subsequent determination of the possible importance of the carcinogen–protein interaction in liver carcinogenesis by aminoazo dyes.

The alteration of certain unknown cellular macromolecules by chemical carcinogens is presumed to be essential in carcinogenesis. Particular cellular proteins are preferred targets of chemical carcinogens *in vivo* and in cell culture. During transformation to malignancy caused by aminoazo dyes and by *N*-2-fluorenylacetamide (2-acetylaminofluorene) in rat liver (Sorof *et al.*, 1963, 1969, 1970), and by polycyclic hydrocarbons in mouse skin and cell culture (Tasseron *et al.*, 1970; Kuroki and Heidelberger, 1972), carcinogen derivatives covalently interact mainly with few protein species. The resulting conjugates, which are present in largest amount, are in the cytosols of these organs and cells, and belong to the electrophoretic classes of relatively basic proteins, termed "*h*."

The principal species of conjugate found in liver after the

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‡ Present address: Worcester Foundation for Experimental Biology, Schrewsbury, Mass. 05145.