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MOLECULAR ARCHITECTURE AND DYNAMICS OF PHOSPHOLIPID DISPERSIONS

Daniel Morris Michaelson
(Ph. D. thesis)

November 1973

Prepared for the U. S. Atomic Energy Commission
under Contract W-7405-ENG-48

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MOLECULAR ARCHITECTURE AND DYNAMICS OF PHOSPHOLIPID DISPERSIONS

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The spatial arrangement of the phospholipids in the co-sonicated vesicles was obtained by exploiting the effects of paramagnetic ions on their proton and phosphorus nuclear magnetic resonances. The ^{31}P and ^{1}H resonances originating from the head groups of PG and PC molecules, sonicated separately, have different chemical shifts and broaden differentially upon addition of Mn^{+2}. At Mn^{+2} concentrations less than $10^{-4}M$, these ions premeate none of the vesicles, permitting a distinction of the signals originating on the outer surface from those on the inner surface. PE and PC also yield distinctive ^{31}P and ^{1}H resonances. The paramagnetic ions $Fe(CM)_6^{-3}$ and Mn^{+2} were used to study the distribution of PE and PC molecules between the inner and outer monolayers of bilayered co-sonicated vesicles.

In sonicated dispersions of PE, the NMR spectral features reflected a marked decrease in mobility as the molecules passed from the negatively charged to the zwitterionic form. At pH levels below the pk of the head group amine the spectra are broader and of a lower intensity than at pH levels above this pk. Similar characteristics were observed with co-sonicated PC:PE dispersions whereas within the same pH range, the corresponding spectra of sonicated PC dispersions showed no pH dependence.

The observed head group modulation of membrane fluidity and the effect of the chemical structure and electrostatic charge of the head group on the spatial organization of the lipids in the co-sonicated vesicles, is discussed in the context of the structure and fluidity of biological membranes.

ACKNOWLEDGMENTS

I would like to thank Professor Melvin Calvin for his advice and support of this project. I am especially indebted to Dr. Melvin Klein for his guidance, encouragement and helpful criticism and to Dr. Alan F. Horwitz for his friendship and for sharing with me his enthisiasm, knowledge and original ideas--working with them has been very rewarding.

I am grateful to Sue Kohler, Ora Canaani and Dr. Micha Tokiewicz for their support and for many productive discussions.

To Dr. William Horsley and Dr. David Ellett for overcoming the often-occurring spectrometer failures.

To the technical staff of the Laboratory of Chemical Biodynamics: Paul Hayes, Bill Hart, Bill McAllister, Dick O' Brien, J. Onffroy, and, especially Evie Litton, for having typed this thesis.

This work was done under the auspices of the U. S. Atomic Energy Commission.

ABBREVIATIONS

DEAE cellulose	Diethylaminoethyl cellulose
EDTA	Ethylenediaminetetraacetic acid
NMR	Nuclear magnetic resonance
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PMR	Proton magnetic resonance
ϕMR	Phosphorus magnetic resonance
TLC	Thin layer chromatography
TMS	Tetramethylsilane

DEDICATION

To my parents.

I. INTRODUCTION

A. The Structure of Biological Membranes

During the last century, permeability studies have led to the hypothesis that cells are encompassed by a thin membrane which serves as a barrier between the cell and its environment. The indirect evidence for this hypothesis was so compelling that the direct visualization, in the electron microscope, of the plasma membrane and other, newly discovered, intracellular membranes as well as their more recent isolation and purification, only ratified an already existant theory. In addition to being a permeability barrier, membranes play a major role in a multitude of biological phenomena including transport, energy metabolism, macromolecular synthesis, cell-cell recognition, and cellular recognition of specific chemical agents. The apparent importance of membranes, as well as recent advances in biochemical and biophysical techniques, have brought their study to a central position in modern biology. In this introduction we wish to review briefly what is known about the composition and architecture of the membranes.

The major constituents of biological membranes are proteins, lipids and carbohydrates. The proteins commonly comprise more than half the weight of the membrane, the lipids constitute about 40 percent and the carbohydrates 10 percent or less. Much of contemporary membrane research is directed towards furthering our under-

standing of the structural and functional properties of membrane proteins, in particular those characteristics which distinguish them from the cytoplasmic proteins. These proteins can be divided into two classes: peripheral proteins which are eluted from the membrane by mild treatments, such as alteration of pH and ionic strength, and integral proteins, the isolation of which requires more drastic procedures, such as the application of detergents. The amino acid composition of the peripheral proteins is similar to that of cytoplasmic proteins, whereas the integral proteins possess significantly more hydrophobic amino acids than do the water soluble proteins. The structural compatibility of the integral proteins and the lipid constituent of the membrane is further exemplified by the findings that some of these proteins have discrete regions which are respectively enriched in either hydrophilic or hydrophobic amino acids; the regions enriched in hydrophobic side chains are buried within the membrane and the regions endowed with hydrophilic amino acids are on the surface of the membrane (for review, Guidotti, 1972; Steck and Fox, 1972).

Enumeration of the membrane proteins by functional classification (e.g., enzymes, transport systems, antigens) will suggest the presence of several dozens of different membrane polypeptides. This approach may be misleading since many of these proteins exist only in trace amounts, for example the Na^+ K^+ dependent ATPase constitutes only 0.005 percent of the polypeptide chains of the human erythrocyte membrane (Dunham and Hoffman), 1970; Fairbanks et al., 1971). It has been suggested that biological

membranes contain fundamental, non-enzymatic, polypeptide units - "structural proteins" which dominate their protein composition. This unifying theory which was proposed and developed by Green and associates (Green et al., 1967) is presently being reevaluated. Recent investigations have shown a multitude of membranes to contain 10 to 20 major different polypeptide chains; furthermore no consistent similarities were observed between the polypeptide chains derived from different membranes. Thus it seems that most biological membranes are composed primarily of a few polypeptides plus a complement of other functional proteins at the trace level.

A second major constituents of biological membranes are the lipids. Membrane lipids are amphipathic, i.e. they contain polar and apolar regions; their structure thus renders them ideally suited for the formation of an interface between a polar solvent and a hydrophobic region. Common lipid constituents of membranes are phospholipids and glycolipids in prokaryotic cells and phospholipids, sphingolipids, glycolipids, and sterols in eukaryotic cells (for review, Korn, 1969; Low and Snyder, 1972). The phospholipids, which are a constituent of all biological membranes, have been studied extensively. These molecules are composed of a glycerol phosphate backbone to which two fatty acids and a substituted alcohol are esterified; the fatty acids are esterified to the hydroxyl groups of carbons 2 and 3 of the glycerol moity and the alcohol is esterified to the phosphate group. The phospholipids are present in several classes, each defined according to its polar head group. (Figure 1).

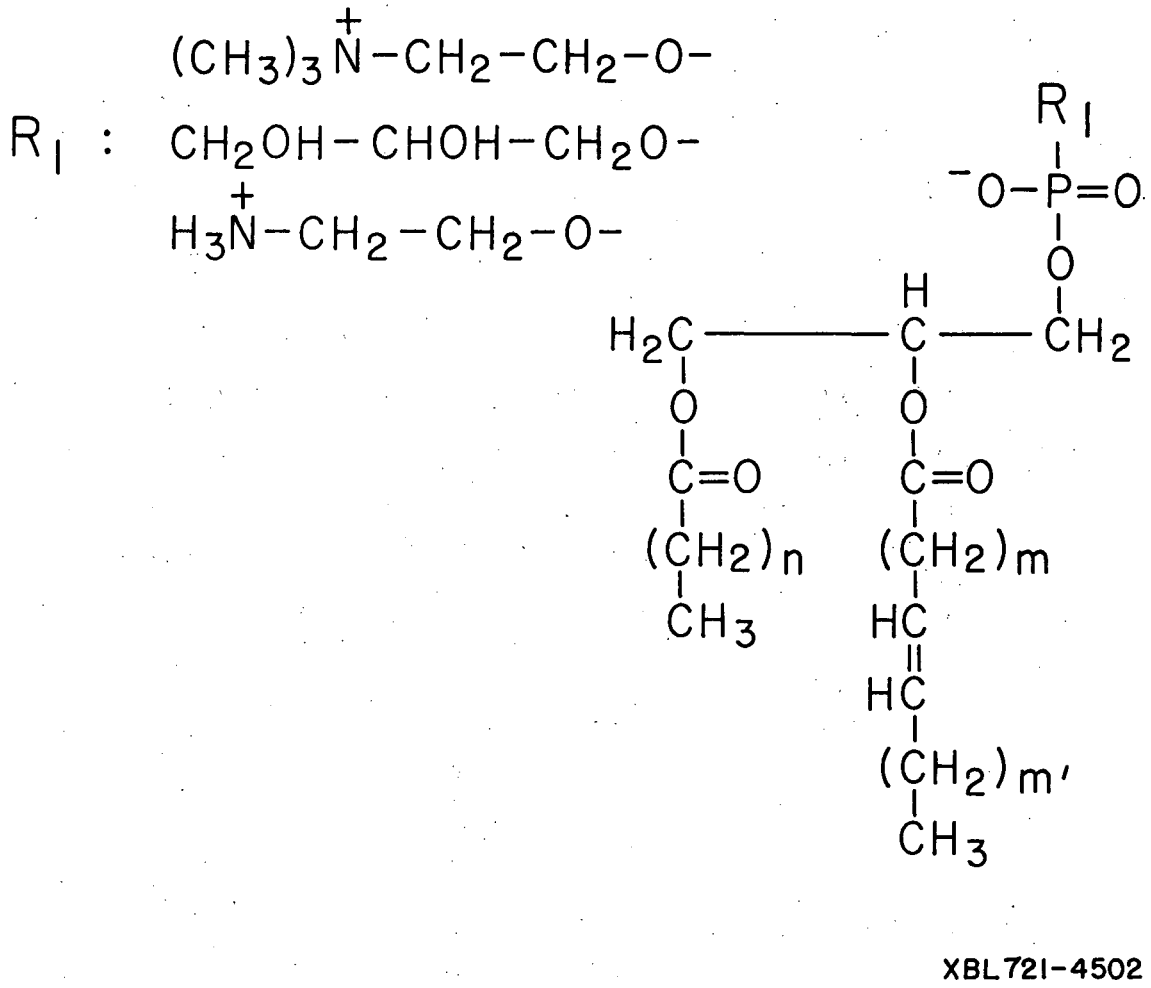

Figure 1. A phospholipid molecule with a saturated and an unsaturated fatty acid. The polar R_1 groups shown are only a few of many possibilities; they are, from top to bottom, choline, glycerol and ethanolamine.

Different membranes often vary in their phospholipid content and in the fatty acid composition of the latter. The fatty acids vary in length, common lengths being between 10 and 20 carbon atoms; they vary in the degree of unsaturation; and in some cases, e.g., Gram positive bacteria, they are branched (for review, Rouser et al., 1968; Korn, 1969; Kates and Wassef, 1970). The phospholipid composition of a variety of cell membranes can be altered experimentally, both the polar head groups and the fatty acids may vary under different physiological conditions. For example, the fatty acid composition of bacterial membranes changes as a function of the temperature of growth (Cronan and Vagelos, 1972) and in some bacteria the phospholipid classes present in the membrane depend on the pH at which the cells were grown (Op den Kamp et al., 1969; Op den Kamp et al., 1971; Haest et al., 1972). In yeast mitochondria both the polar head group and the fatty acids are altered upon changing the growth condition from anaerobic to aerobic. The variability in fatty acid composition has been studied extensively and its physiological significance is beginning to be understood and will be discussed later; however, little is known about the function of the variability in the head group composition.

In addition, to proteins and lipids, many biological membranes contain carbohydrate moities. Their amount is usually small; from zero to less than 10 percent of the mass of the membrane. When present, they are usually covalently bound to the protein or the lipid constituents of the membrane. The segments of the glycoproteins and glycolipids which contain the carbohydrates are presumed to extend

within the membrane decreases and consequently, in order to maintain the necessary fluidity, a change in the fatty acid composition to more fluid-like is required.

We have mentioned earlier that membrane proteins can be classified as either integral or peripheral. It is assumed that only integral proteins are critical for the structural integrity of the membrane and that the peripheral proteins, though interesting in their own right, are only loosely bound to the membrane and therefore their properties and interactions may not be directly pertinent to the central problems of membrane structure. Presently, the structure of a few integral proteins has been partially elucidated; the available data have been generalized to all integral proteins, which are postulated to be globular and amphipathic. The hydrophobic region is presumed to be embedded in the lipid bilayer and the hydrophilic region to extend to the aqueous phase. The membrane thus has the form of a fluid-mosaic (for an extensive review of this model, Singer and Nicolson, 1972) which consists of a fluid bilayer in which the proteins are embedded. The proteins may be partially embedded in the membrane or they may traverse it and extend on both sides (Figure 2).

The "fluid-mosaic" model leads to several predictions which may be examined experimentally. From a structural point of view, the model predicts that the proteins may be tightly associated with only the fraction of the lipids which are in immediate contact with it and more loosely associated with the rest of the lipids, a prediction which has been verified experimentally. Furthermore this model

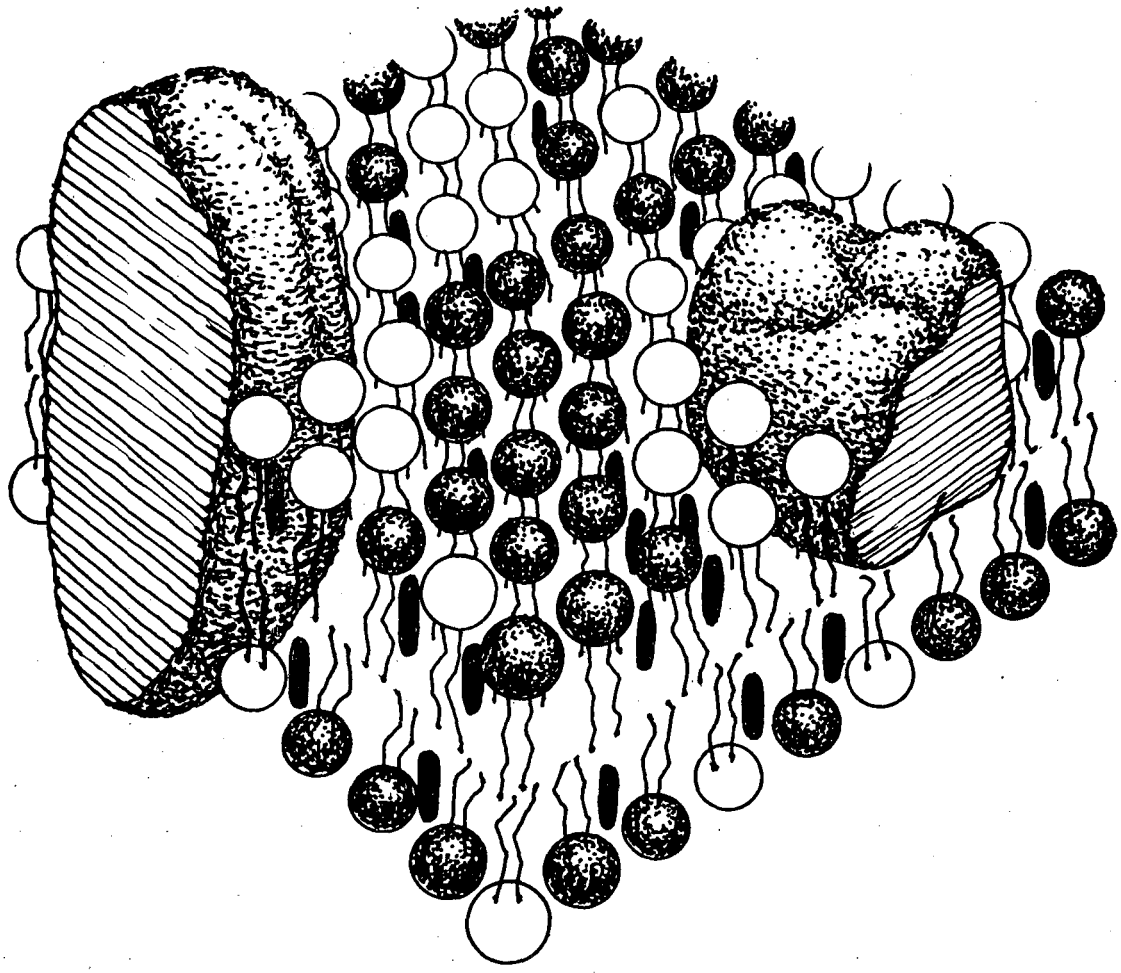

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Figure 2. A schematic three-dimensional and cross-sectional view of a biological membrane. The solid bodies represent the integral proteins, the spheres and tails represent respectively the head groups and fatty acids of the phospholipids and the solid bars represent sterol molecules. As illustrated, some of the proteins are presumed to be partially embedded in the lipid bilayer and some to protrude from it. The arrangement of different classes of phospholipids (represented schematically by filled and empty spheres) and of the sterols within and between the surfaces of the membrane is drawn arbitrarily since it is not known in detail.

accounts for the variability in lipid to protein ratio which exists among different membranes by asserting that in membranes with high lipid content the bilayer is more extended than in those which contain relatively less lipid. The development of freeze fracture electron microscopy has rendered it possible to visualize directly the interior of the membrane as well as its surfaces. This technique has revealed the presence of a mosaic of particles which are embedded in the bilayer and protrude to the surface (Pinto da Silva and Branton, 1970). These particles, in accordance with the present model, have been identified as protein molecules.

The fluid mosaic model further predicts that the membrane proteins which are embedded in the fluid lipid bilayer undergo rapid molecular motion and that consequently their arrangement within the membrane is irregular and non-periodic. This prediction has been verified by very elegant experiments (Frye and Edidin, 1970; Cone, 1972; Brown, 1972) which have shown that some membrane proteins may diffuse laterally in the plane of the membrane and that they may also undergo rapid rotational motion. Furthermore, freeze fracture electron microscopy has shown that the proteins are usually arranged in irregular non-periodic patterns. The molecular motion of the lipids and the proteins is presumed to occur primarily in the planes of the membranes rather than across them, consequently specific molecules may preferentially face the inner or the outer surface of the membrane; thus in principle both lipids and proteins may be asymmetrically distributed between the surfaces of the membrane. Only a few membranes

have been investigated with regard to this question; the extensively studied erythrocyte membrane seems to be asymmetric with regard to the inside outside distribution of both the proteins and the lipids (Bretscher, 1973).

B. Outline of Research

The observation that different membranes often contain different phospholipid classes and that some membranes alter their phospholipid composition in response to changes in physiological conditions (see Section a) suggests that the variability in head group composition is of physiological importance; however, little is known about the function of this variability. The spatial organization of the phospholipids within the bilayer is not known and the factors affecting it are not understood; neither is it known if the fluidity of the membrane is determined solely by the hydrocarbon constituents or whether the head groups also affect it. Previous work by Bretscher (1972, 1973) and by Casper and Kirshner (1971) suggests that different lipids may be partitioned preferentially between the inner and outer faces of the membrane, thus rendering it asymmetric. The demonstration that specific phospholipids are required for the activation of certain membrane proteins (Steck and Fox, 1972) may suggest that within the plane of the membrane these molecules are arranged in a non-random heterogeneous pattern.

In order to gain further understanding of some of the factors which affect the arrangement of phospholipids in cellular membranes

and of the effects of the head group on the fluidity of the membranes, we studied their organization and molecular motion in a model system, i.e., sonicated phospholipid dispersions (Atwood and Saunders, 1965). We have examined the phospholipid arrangement within and between the monolayers of bilayered sonicated dispersions composed of two classes of phospholipids as well as the effect of head group on the mobility of these molecules. We have employed phosphorus and proton magnetic resonance spectroscopies to study molecular motion, and the effects thereon of paramagnetic ions (e.g., Mn^{+2}, En^{+2}, $Fe(CN)_6^{-3}$) to study differentially the spatial organization of the phospholipids.

C. NMR Spectroscopy and the Effects Thereon of Paramagnetic Ions

When nuclei such as those of phosphorus and hydrogen atoms, which possess a spin $I = 1/2$, are placed in a static magnetic field $\vec{H}_o$ they distribute themselves, according to the Boltzman distribution, between two energy levels corresponding to orientations of the spin parallel and antiparallel to the magnetic field. The energy gap between these levels depends on the magnetic field $\vec{H}_o$; it is peculiar to each nucleus and is determined by the atomic electronic structure encompassing the nucleus as well as by the chemical environment of the atoms. It is convenient to describe a system of spins in terms of a magnetization $\vec{M}$ which is the vectorial sum of the spins of the individual nuclei. Thus at equilibrium $\vec{M} = M_{o_z}$ where M_{o_z} is the net nuclear spin magnetization in the direction of the static magnetic field $\vec{H}_o$. Upon application of a perturbing magnetic field the magnetization vector may

change its direction and magnitude; after terminating the perturbation, the magnetization will relax back to its equilibrium value M_{o_z}. The relaxation rate at which M_z relaxes to M_{o_z} is called spin-lattice relaxation and is denoted by T_1, whereas the rate at which M_{xy} relaxes to its equilibrium value, i.e., to zero, is called spin-spin relaxation and is denoted by T_2.

Nuclear magnetic resonances can be observed in several ways which are generally classified as continuous wave and pulsed techniques. The continuous wave experiment consists of slowly sweeping the magnetic field with a fixed radio frequency (rf) radiation applied perpendicular to it, or alternatively slowly sweeping the rf frequency, which is applied to a sample in a fixed magnetic field. A resonance is detected when the rf energy is equal to the energy gap between the nuclear spin states oriented parallel and antiparallel to the magnetic field. In the pulsed experiment the sample is placed in a fixed magnetic field and a rf pulse perpendicular to the magnetic field is applied to it. The observation of the nuclear spin system is made after the rf is turned off. It is possible to obtain the same spectral information from a pulsed experiment and subsequent mathematical analysis (Fourier transformation) as can be obtained from continuous wave experiments. However, the pulsed methods are more efficient, thus yielding a significant savings in time; moreover by employing the appropriate pulsed experiments T_1 and T_2 may be measured directly (Farrar and Becker, 1971).

Significant information regarding molecular motion may be obtained from the magnetic resonance relaxation times. Nuclei in a

static magnetic field $\vec{H}_o$ precess about its axis with a frequency ω_o which is proportional to $\vec{H}_o$, the proportionality constant, γ_N, being the gyromagnetic ratio. If in addition to an external field $\vec{H}_o$ which is applied in the Z direction, the nuclei sense other fields which may, for example, arise from dipole-dipole interaction with neighboring nuclei, they will precess at different frequencies which are proportional to the effective fields sensed by the nuclei. Thus the loss of magnetization in the xy plane may be due to "fanning-out" of the individual spins, due to the different precession rates. Since magnetization in the xy plane can be lost by "fanning-out" as well as by reorienting towards the z direction, the latter being the T_1 process, we obtain that always $T_2 \leq T_1$. The "fanning-out" will be faster the greater the difference is in the precession frequencies of the individual nuclei. Therefore nuclei which are held in a solid and thereby interacting strongly with neighboring nuclei will have very short T_2's. An equivalent view is that the different effective fields felt by nuclei in the solid will result in the magnetic resonances being very broad; thus we see that the line width is inversely proportional to T_2. When the solid is melted the magnetic interactions, such as the dipole-dipole interactions, which are orientation and distance dependent, are averaged by molecular motion, and consequently T_2 will become longer, i.e., the resonances will sharpen. In general T_2 becomes longer the faster molecular motion is; T_1 however, has a different dependence on motion. It can be shown that T_1 has a minimum when molecular motion is at a frequency similar to that of the nuclear precession.

The exact dependence of T_1 and T_2 on molecular motion is a function of molecular geometry and the mechanism of magnetic interaction. A review of these mechanisms (Abragam, 1961; Carrington and McLachlan, 1967) is beyond the scope of this introduction. However, since the effect of paramagnetic ions on nuclear magnetic resonances has been extensively employed by us, we shall discuss briefly the physical basis of their effects.

The magnitude of the magnetic moment of an unpaired electron is about 10^3 times that of a nucleus. Consequently randomly fluctuating magnetic fields due to an unpaired electron usually dominate the magnetic resonance relaxation times of nuclei in its neighborhood. In addition unpaired electrons may, under the appropriate conditions, induce a shift in the frequency of the nuclear resonance. We wish to illustrate the origin of these effects by the following example. Let us assume that a nucleus can exist in two magnetic environments, a and b, where ω_a and ω_b are the resonance frequencies of the nuclei at these environments, and that the nuclei can exchange between these environments at a rate ω_e. It can be shown that when $\omega_e << |\omega_a - \omega_b|$, the nuclei will yield two resonances which gradually coalesce as ω_e is increased relative to $|\omega_a - \omega_b|$ (Chapter 12, Carrington and McLachlan, 1967). The frequency of the coalesced resonance is the weighted average of ω_a and ω_b, the weighting factor being the residence time of the nuclei at the environments, whereas the line width of the coalesced resonance depends on the relative magnitude of ω_e and $\omega_a - \omega_b$; it is broad for $\omega_e \sim |\omega_a - \omega_b|$ and narrow for $\omega_e >> |\omega_a - \omega_b|$.

In contrast to our simple two-state example, a nucleus which interacts with an unpaired electron is usually exchanging randomly among a continuum of magnetic environments the characteristics of which depend on the exact nature of the interaction. For example, the electron spin-nuclear spin magnetic dipole-dipole interaction of a given electron and nucleus depends on the relative orientation of the external magnetic field and the vector connecting the nucleus and the electron as well as on the length of the latter, whereas the Fermi contact interaction depends instead on the scalar product and proximity of the electron and nuclear spins. The rate of exchange of these environments usually depends on the tumbling rate of the unpaired electron-nucleus complex, the electronic relaxation time and the chemical exchange time of a nucleus between the coordination sphere of a paramagnetic ion and the bulk medium. The dominant effect of a given paramagnetic ion on a nucleus depends on the mechanism of interaction and on the rate of exchange. The major interactions of one class of paramagnetic ions, a member of which is Eu^{+3}, are: electron spin-orbit, electron orbit-nuclear spin, electron spin-nuclear spin dipolar coupling and Fermi contact interaction. The weighted average of these interactions is usually not zero and the rate of exchange, which is determined by the fastest rate process, is usually very fast since the electronic relaxation times of paramagnetic ions such as Eu^{+3} is very short. Consequently we obtain that the major effect of such paramagnetic ions is to shift the nuclear magnetic resonances.

Other paramagnetic ions, such as Mn^{+2}, have much slower electronic relaxation times and therefore their interaction with the nuclei is modulated by molecular tumbling as well as by chemical exchange rates. These rates are usually in the intermediate range, thus yielding broadened nuclear resonances. In addition, the weighted average of the magnetic interactions of these ions with the nuclei is usually zero and consequently the broadened resonance is unshifted. We thus see that paramagnetic ions can be classified as shifters and broadeners depending on whether their major effect is to shift the frequency of the nuclear magnetic resonances or to shorten their relaxation time. The theoretical expressions which relate both the shift and the relaxation times to structural and dynamic parameters of the paramagnetic ion-nucleus complex have been developed (Swift and Connick, 1962; Eaton and Phillips, 1965), thus rendering it possible to employ these ions as a probe for investigating molecular structure and dynamics. We have used these ions and their effects on nuclear magnetic resonance spectra, to study the molecular arrangement of different phospholipid classes within and between the inner and outer surfaces of mixed cosonicated phospholipid vesicles.

II. PHOSPHOLIPID SYNTHESIS AND PURIFICATION

A. Purification of Egg Phosphatidylcholine.

Due to the high phosphatidylcholine (PC) content and ease of extraction, egg yolk was chosen as our PC source. Egg PC was extracted and purified following the procedure of Singelton et al. (1965). This procedure involves two steps: preparation of crude phospholipid extract and isolation from it of egg PC by aluminum oxide column chromatography. Fresh egg yolks were blended with acetone and the acetone extract, which contained most of the neutral fat and pigment, was discarded. The solids were suspended in 95% ethanol and the mixture then filtered. The ethanol extract was concentrated to dryness on a rotatory evaporator and the crude phosphatides were further extracted with petroleum-ether. Finally, the crude phosphatides were precipitated with excess acetone, filtered and dried. The average yield of crude phosphatides was 70% (by weight); they were stored in chloroform under argon at -20° C.

Egg PC was purified from the crude extract on an aluminum oxide (Merck) column. The column measured 4 cm I.D. by 90 cm in length and contained 625 gr of aluminum oxide, which were slurred in chloroform prior to packing the column (the absorbent bed was about 50 cm in length). The crude phosphatides (25 gr) were washed on the column with chloroform, and fractions were eluted with chloroform: methanol (8:1). Phospholipid concentrations in the eluted fractions

were determined by the phosphate procedure (scaled down by 10) of McClaire (1971) and lipid purity was assessed by thin layer chromatography (TLC), employing precoated Adsorbosil plates (Applied Science). The plates were developed in chloroform:methanol:H_2O (65:25:4) by volume) and lipid spots were visualized with iodine vapors. The aluminum oxide column yielded 9.5 gr of pure egg PC, which were stored in ethanol under argon at -20° C. Egg PC had the following fatty acid composition, as determined by gas chromatography:palmitic (16:0), 34% by weight, palmitoleic (16:1), 1.2%; stearic (18:0), 10.5%; oleic (18:1), 25.8%; linoleic (18:2), 10.6% .

B. Synthesis and Purification of Phosphatidylglycerol.

Phosphatidylglycerol (PG) was synthesized enzymatically from purified egg PC, employing the phosphatidyltransferase activity of Savoy cabbage phospholipase D. This enzyme, which catalyzes the hydrolytic cleavage of the terminal diester bond of glycerophosphatides containing choline, ethanol amine, serine and glycerol with the formation of phoshatidic acid (PA), also mediates transphosphatidylation, by which the phosphatidyl group of the phospholipid is transferred to one of the following alcohols: methanol, ethanol, glycerol, ethanol amine and serine. Thus PC in the presence of Savoy phospholipase D and glycerol will undergo the following reactions:

$$PC + H_2O \rightarrow PA + \text{choline} \qquad (a)$$

$$PC + \text{glycerol} \rightarrow PG + \text{choline} \qquad (b)$$

PG which is a product of reaction (b) will also undergo hydrolytic cleavage:

$$PG + H_2O \rightarrow PA + \text{glycerol.} \qquad (c)$$

Since we are interested in obtaining a high yield of PG, assay conditions were varied with the intention of establishing the conditions which result in highest PG yields.

1. Methods

Purification of Savoy cabbage phospholipase D. Savoy cabbage purchased from local markets was used as enzyme source. Enzyme purification was done following the procedure of Yang (1969), except that only the first three steps, the preparation of crude extract, heat coagulation, and acetone precipitation, were employed. Steps 4 and 5 were omitted. The enzyme preparation lost approximately half of its activity within one week.

Phospholipase D enzyme assay. A typical incubation mixture contained 1-10 μmoles of egg PC, 80 μmole acetate buffer (pH 5.6), 40 μmole $CaCl_2$, between 0.1 and 1.0 units of phospholipase D preparation (1 unit activity = the amount of enzyme which catalyzes the hydrolysis of 1 μmole PC to PA, at room temperature, in 10 minutes), and a suitable amount of vacuum distilled glycerol, all in a total volume of 1 ml. The reaction was initiated with 0.5 ml of freshly distilled ethyl-ether; then, under argon atmosphere, the mixture was shaken and incubated at room temperature for the desired time. The reaction was terminated with 0.1 ml of 1 M citric acid, and the

phospholipids were extracted with 2 ml of ethyl-methanol (4:1 by volume). The TLC system described in the following section was employed for separating the phospholipids. Spots were visualized with iodine vapor, then scraped and analyzed for phosphate content.

Thin layer chromatography. Phospholipids were separated by thin layer chromatography (TLC) using silica gel G plates, prepared by coating glass plates with a suspension of silica gel in a solution of methanol-H_2O (1:1 by volume) containing 0.5 N oxalic acid. Oxalic acid complexes with divalent ions and thus prevents the streaking of acidic phospholipids. Plates were developed in chloroform-methanol-concentrated HCl (87:13:0.5 by volume) and phospholipid spots were visualized with iodine vapors.

Phosphate determination. Two methods were used. When the amount of phosphate in a TLC spot was sought, the method of Yang et al. (1967) was used. When the phosphate content of a solution was sought, we used the method of McClaire (1971) scaled down by a factor of ten.

Conversion of PA and PG to Na salt. PA and PG were dissolved in chloroform and the solution was extracted three times with an 0.5 N aqueous solution of Na_2SO_4.

2. Synthesis of PG

Two sets of experiments were performed: one with 3 μmole PC, and the other with 1 μmole PC. Within each set, the time course

of the reaction was followed for several assay mixtures containing different amounts of glycerol. The reaction was followed until all the PC was converted to PG and PA. Figure 3 illustrates the levels of PG achieved for different glycerol concentrations with 1 μmole PC as starting material. In Table I the maximum level of PG obtained in two series of experiments is summarized. As can be seen from Table I the highest percent of PG was obtained when 1 μmole PC and 4% glycerol (v/v) were present in the reaction mixture. For large scale PG synthesis the assay mixture was scaled up one thousand fold, thus obtaining approximately 0.4 gr PG from 0.75 gr PC. The products of the reaction--i.e., PG and PA--were converted to the Na salt and stored under argon at -20° C.

Table I
The effect of glycerol and PC concentration on the level of maximum PG synthesized.

% glycerol in reaction mixture	A	B
0	0%	0%
0.4	17%	18%
1	32%	30%
4	52%	30%
9	30%	25%

Column A = 1 μmole PC in reaction mixture.
Column B = 3 μmole PC in reaction mixture.

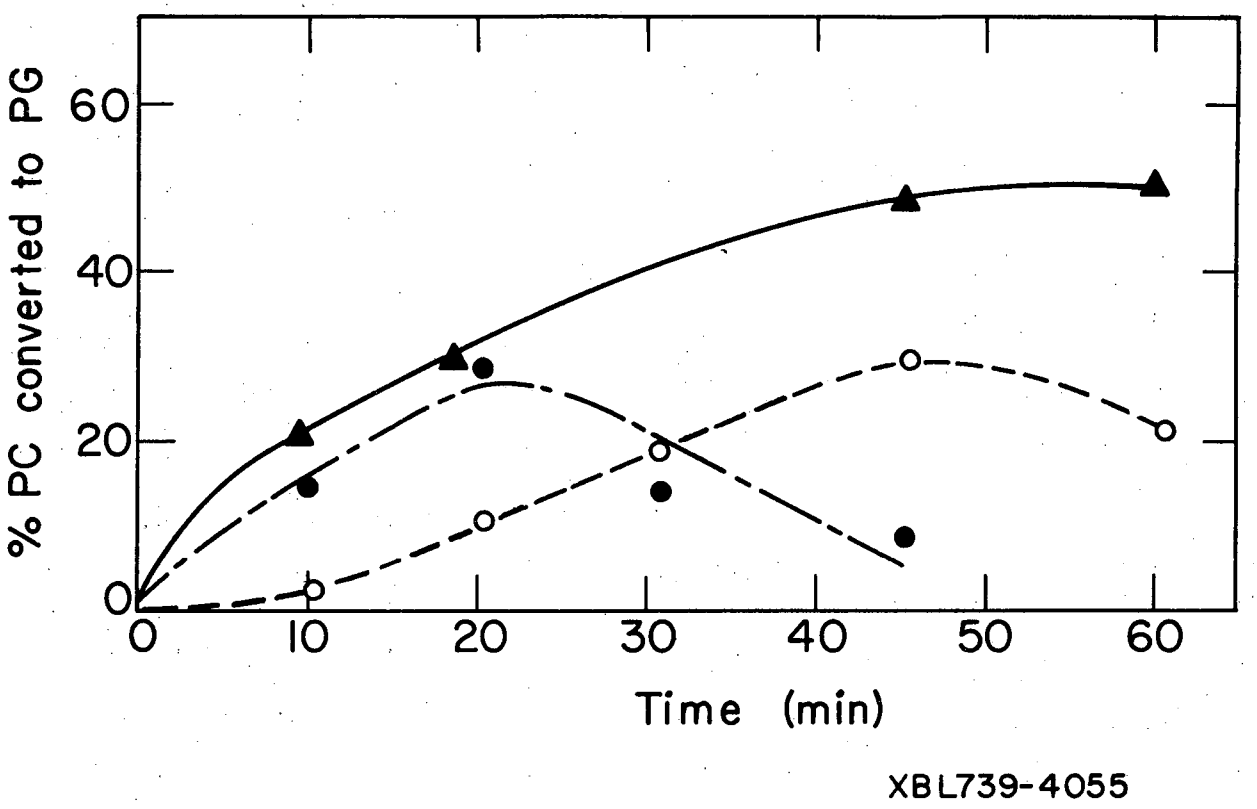

Figure 3. Effect of glycerol concentration on the transferase activity of phospholipase D. ●: 9% glycerol, ▲: 4% glycerol, O: 1% glycerol. All assays were done at room temperature.

3. Purification of PG

Under the conditions established above, the conversion of PC mediated by phospholipase D results in a mixture of PG and PA and trace amounts of PC. We first attempted a separation of these phospholipids by means of a silica column (Lennorz et al., 1967), using mixtures of chloroform and methanol as eluents. This procedure was found unsatisfactory. Even though we used very small increments of methanol in our eluting solvents and redistilled the chloroform and methanol used, there was a significant overlap between the two phospholipids as they came off the column. Thus we decided to employ DEAE-cellulose column chromatography.

A column measuring 7 cm I.D. and 75 cm in height was packed with 250 gr of DEAE cellulose which were prewashed and slurred in acetic acid. The packed column was then washed with reagent grade methanol, mixtures of chloroform in methanol, and chloroform (Rouser et al., 1967). The column was loaded with the phospholipid mixture (0.5 gr in 200 ml chloroform) and fractions were eluted through an elution scheme, Table II, which is a modification of a procedure described by Rouser et al., (1967). The separation was satisfactory: PC was eluted with chloroform:methanol (7:3) and PG and PA came off the column separately with the linear gradient of ammonium acetate in chloroform:methanol (4:1).

After elution, the purified PG and PA fractions were concentrated and the lipids were converted to the NA salt and stored in chloroform under argon at -20° C. The purified PG had a fatty

Table II

Elution scheme of DEAE column.

Eluting Solvent	Elution Volume*
Chloroform	6
Chloroform/methanol (7:3)	3
Chloroform/methanol (1:1)	3
Methanol	3
Chloroform/acetic acid (3:1)	3
Methanol	4
Linear gradient of ammonium acetate in chloroform/methanol (4:1)	**

*Elution volumes are specified in column volumes.

**0-30 mM ammonium acetate linear gradient over a volume of 4 column volumes.

acid composition similar to that of egg PC: palmitic (16:0); 36%; by weight; palmitolic (16:1), 2.9%; stearic (18:0), 13.5%; oleic (18:1), 30%; linoleic (18:2), 10.1%.

C. Purification of Bacterial Phosphatidylethanolamine.

Bacterial phosphatidylethanolamine (PE) was extracted and purified from a variant of a β oxidation$^-$ unsaturated fatty acid auxotroph of E. coli (30E β ox$^-$, which was a gift from C. F. Fox). The bacteria were grown at 15° C in medium A (Davis and Mingioli, 1950), supplemented with 1% casamino acids (Difco), 5 mg/liter vitamin B_1, 0.5% Triton X-100 and 0.1 g/liter oleate. The cells were harvested

by centrifugation at mid-log phase, and the phospholipids were extracted by the Bligh and Dyer method as described by Ames (1968). The column used was that described in Section B of this chapter. It was recycled prior to use (Rouser et al., 1969) and the elution scheme used was similar to that shown in Table II, except that prior to elution with chloroform:methanol (7:3) the column was eluted with an equal volume of an additional solvent mixture-chloroform:methanol (9:1). This addition was found necessary since without it, PE which elutes with chloroform:methanl (7:3) was shown by TLC (employing the system described in Section A) to contain impurities. The purified PE had the following fatty acid composition:myristic (14:0), 8.7%; palmitic (16:0), 35%; palmitoleic (16:1), 15.4%; stearic (18:0), 0.9%; oleic (18:1), 40%. PE was stored in chloroform under argon at -20° C.

III. TRANSBILAYER ASYMMETRY AND SURFACE HOMOGENEITY OF CO-SONICATED PHOSPHATIDYLCHOLINE: PHOSPHATIDYLGLYCEROL VESICLES

In this chapter we present our observations regarding the arrangement of a zwitterionic phospholipid--phosphatidylcholine, and an anionic one--phosphatidylglycerol, within and between the monlayers of the bilayered sonicated vesicles. We have employed the phosphorus and proton magnetic resonance spectra of the sonicated dispersions and the effects thereon of paramagnetic ions (e.g., Mn^{+2}, Eu^{+3}) to study differentially the organization of the phospholipids.

A. Materials and Methods.

Phosphotidylglycerol (PG) and phosphotidylcholine (PC), were synthesized and purified following the procedures described in Chapter II. Sonicated PG and PC vesicles were prepared by sonication of an unsonicated dispersion, which was prepared by the following methods: 150 to 75 μmoles of pure lipid were dried onto the walls of a round bottom flask, then 2 ml of a buffer containing 0.15 M NaCl and 50 mM Na Borate at pD 8.0 were added and the sample was agitated with 2 glass beads for 5 minutes on a vortex mixer. The sonication was performed for 15 minutes on 2 ml samples, on ice and under argon, using the 1/2-inch tip on a Branson 105 E Sonicator at a power setting of 4-5 (68 watts). The sonicated dispersions were centrifuged at 17,000 x g for 30 minutes at 4° C and EDTA added, to a final concentration of 10^{-6}M. Both phospholipids yielded clear dispersions on

sonication (Papahadjopoulus and Ohki, 1970; Atwood and Saunders, 1965). Co-sonicated PG:PC vesicles were prepared similarly from equal amounts of PG and PC. In the experiments employing Mn^{+2} and Eu^{+3}, small volumes of the above buffer containing $MnCl_2$ or $EuCl_3$ were added to the phospholipid dispersions after sonication. The sample was vortexed gently during the addition of the paramagnetic ions in order to obtain rapid mixing. After every experiment the phospholipids were extracted by the Bligh and Dyer method, as described by Ames (1968), and were shown by TLC to each give a single spot.

Proton magnetic resonance (PMR) were recorded at 20° C on a Varian HR-220 spectrometer with TMS as an external standard. Phosphorus magnetic resonance (ϕMR) measurements were made at an ambient probe temperature of 31° C in a pulsed Fourier mode at 24.3 MH_z, as described elsewhere (Horwitz and Klein, 1972), with an external standard of a saturated pyrophosphate solution at pH 9.0.

B. Results.

1. Aqueous Sonicated Dispersions of PG and PC are Distinguishable

a) Comparison of PG and PC ϕMR and PMR spectra

Sonicated PC and sonicated PG dispersions have characteristic and distinguishable phosphorus and proton magnetic resonance spectra which are sensitive to the dynamics and environment of the phospholipids (Horwitz, 1972; Horwitz et al., 1973). The ϕMR spectra of sonicated PC and of sonicated PG, shown in Figure 4a, each contain a single peak. The PC phosphorus line width (19±1 Hz) is narrower

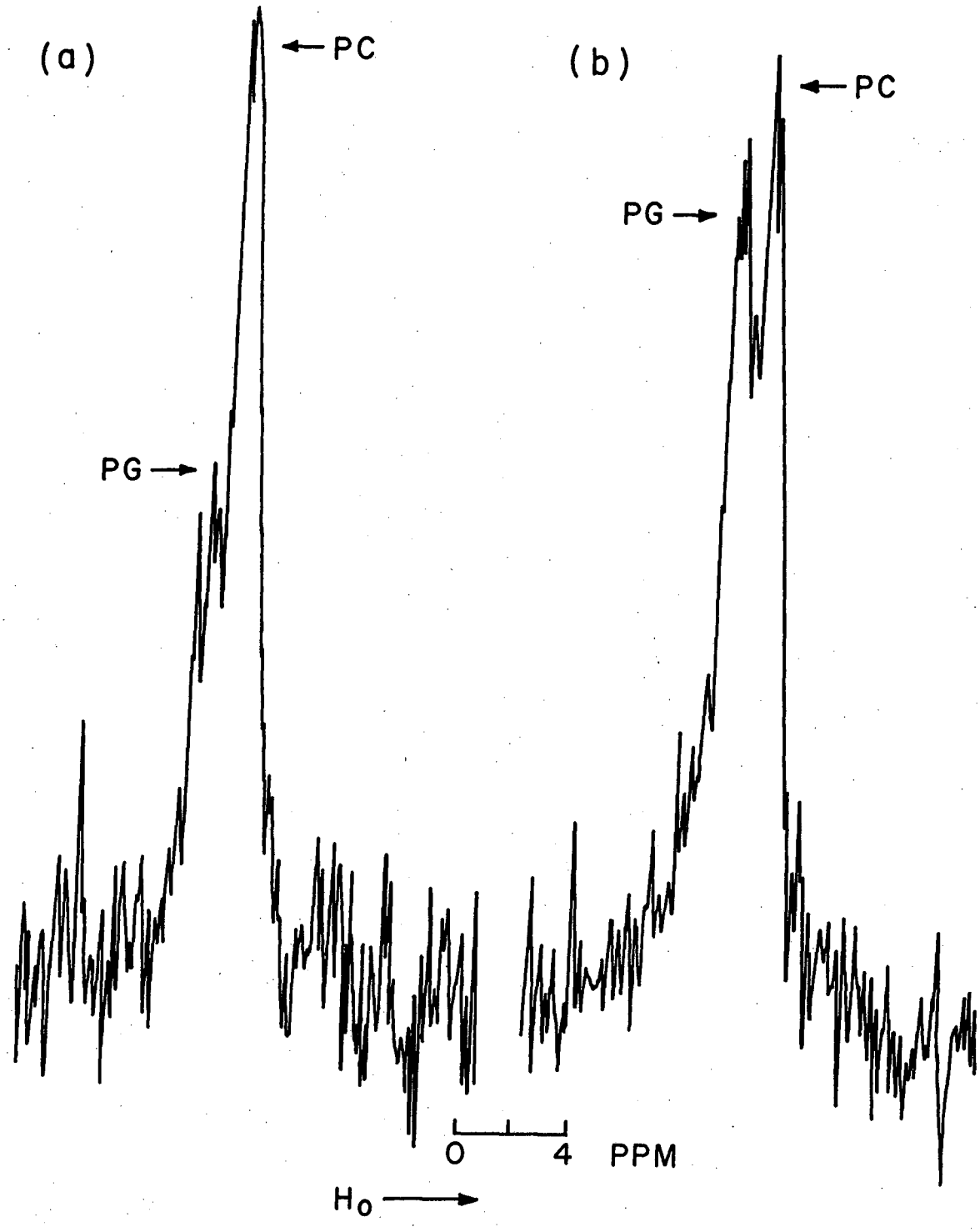

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Figure 4. Comparison of the ϕMR spectra of PG and PC in sonicated dispersions.

a) ^{31}P spectrum of a mixture of 30 mM sonicated PG and 30 mM sonicated PC.

b) ^{31}P spectrum of the same sample after co-sonication.

than the corresponding PG line width (30± 2 Hz), and the two resonances have different chemical shifts: PC is shifted 0.93 ppm (22.5 Hz) to higher field from PG. The PMR spectra of sonicated PC and sonicated PG are likewise distinguishable (Figure 5). Sonicated PC dispersions have an intense choline N^{+}-$(CH_3)_3$ proton peak which is absent in the corresponding PG spectra, while PG dispersions have an additional peak which is absent in the PC dispersions. This peak is shifted 3.7 ppm to lower field from TMS and is 27± 2 Hz wide: its relative area and chemical shift are consistent with the assignment that it arises from the non-exchangeable protons of carbons 2 and 3 of the head group glycerol.

b) Paramagnetic ions interact differently with sonicated dispersions of PC and PG

The effects of paramagnetic ions, e.g., Mn^{+2} and Eu^{+3}, on nuclear magnetic resonances are determined by the nature of the ion and the structure and environment of the molecule with which the ion interacts (Appendix 1). The effects of Mn^{+2} on the phosphorus and proton magnetic resonance spectra of sonicated PC and sonicated PG are different. The extent of broadening of the resonances of sonicated PC by Mn^{+2} is shown in Figure 6. The addition of increasing concentrations of Mn^{+2} to sonicated PC dispersions broadens and hence decreases the amplitude of the phosphorus and choline N^{+}-$(CH_3)_3$ proton resonances. At 10^{-5} M Mn^{+2} each of these resonances contains two components, one broadened and one unaltered. At higher Mn^{+2} concentrations (approximately 10^{-4} M) a plateau is reached where

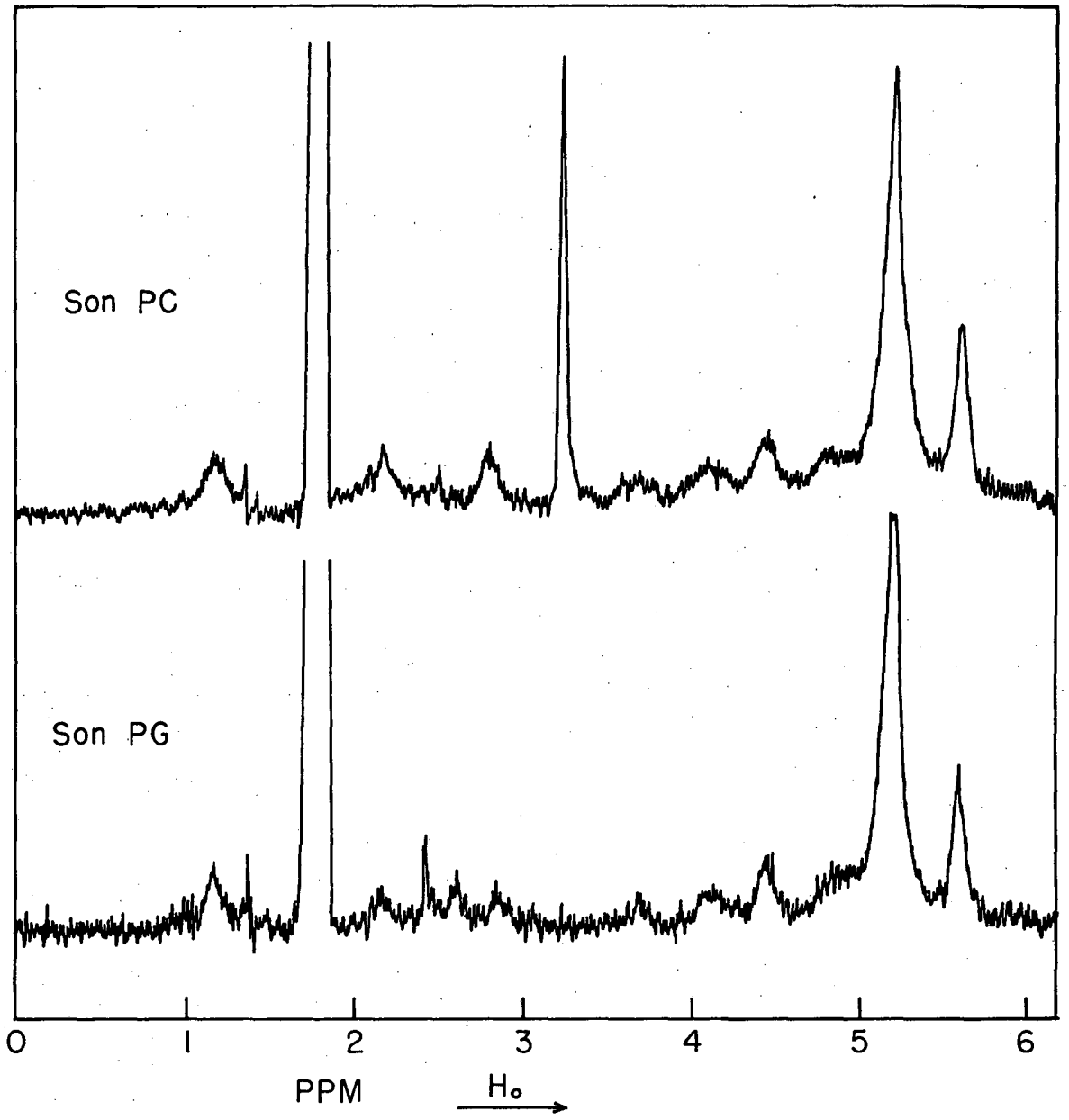

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Figure 5. Comparison of the proton magnetic resonance spectra of sonicated PC dispersions (son PC) and of sonicated PG dispersions (son PG).

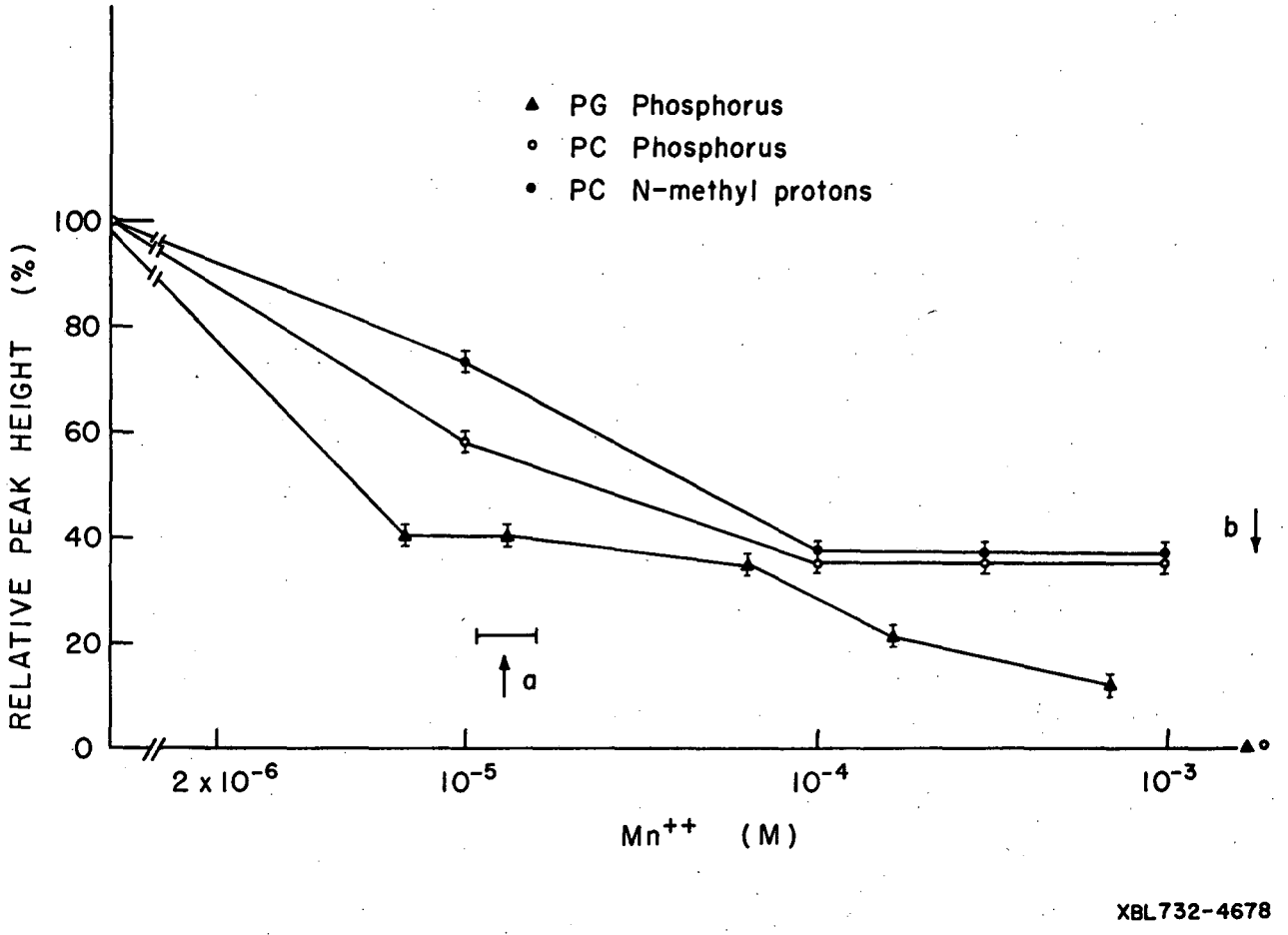

Figure 6. The change in peak height relative to the original resonance vs. $MnCl_2$ addition to sonicated PC and to sonicated PG dispersions. For PG the ϕMR linewidth at the plateau was narrower than the initial resonance. This difference was accounted for by normalization of the ϕMR peak height (see text). Thus, for all the graphs the percent peak height at the plateau is equal to the detectable fraction of the original intensities. The concentration of the vesicles, assuming their molecular weight to be be 2.1×10^{-5} (Huang, 1969), is indicated by (a), and the signal intensities after resonication in the presence of Mn^{+2} by (b).

additional increase in Mn^{+2} concentrations causes no further decrease in peak height. The line width and chemical shift of the residual phosphorus and choline $N^{+}-(CH_3)_3$ proton resonances are equal to those of the original peaks, whereas their areas are 37% of the initial peak areas. Resonication of the vesicles in the plateau regime (e.g., in the presence of 10^{-3} M Mn^{+2}) results in broadening, beyond detection, of all of the phosphorus and choline $N^{+}-(CH_3)_3$ proton resonances. At these concentrations there is no detectable effect of Mn^{+2} on the intensity and line widths of the fatty acid proton resonances.

Our interpretation of the data agrees with that of Bystrov et al. (1971): Sonicated PC vesicles are impermeable to Mn^{+2} ions, and hence the Mn^{+2} broadens only the resonances of nuclei which face the "outside" of the vesicle and are present at or very near the polar region. The plateau therefore arises when sufficient Mn^{+2} is added to broaden, beyond detection, the resonances which arise from the "outside" nuclei. The magnitude and equality of the plateaus is in accord with the size of the single shelled sonicated PC vesicles and the bilayer's thickness (Huang, 1969). Thus resonication in the presence of Mn^{+2} results in the introduction of the ion into the internal aqueous phase of the vesicles, and the consequent broadening of the "inside" as well as "outside" phosphorus and choline $N^{+}-(CH_3)_3$ proton resonances.

The effect of Mn^{+2} on the phosphorus magnetic resonance spectra of sonicated PG is shown in Figure 6. The addition of 7×10^{-6} M Mn^{+2} (less than one ion per vesicle), which is the lowest concentration

examined thus far, to sonicated PG dispersions broadens beyond detection approximately 60% of the original phosphorus resonance; no further change is observed on increasing the Mn^{+2} concentration up to approximately 10^{-4} M. The similarity in the intensities at the plateaus of the PC and PG ^{31}P resonances suggests that both types of vesicles have a similar ratio of "outside" to "inside" molecules, that sonicated PG vesicles are mostly single shelled, and that their size and shape are similar to those of sonicated PC vesicles. The ϕMR line width of sonicated PG in the plateau regime (14 Hz) is narrower than that in the absence of the metal ion (30 Hz). By contrast, as noted above, the width of the ϕMR of PC vesicles is the same at the plateau of $[Mn^{+2}]$ as it is prior to the addition of the ion. We have observed that the position of the PG phosphorus resonance at the plateau is shifted by 0.32 ppm (8 Hz) to higher field than the initial resonance. A plausible explanation of this observation is that the resonance position of the "inside" and "outside" PG phosphorus resonances differ by ~0.5 ppm. (The ϕMR chemical shifts of phosphates and phosphate esters are dominated by the state of protonation, which suggests that the separation between the "inside" and "outside" ϕMR may be enhanced by a pH gradient across the vesicle as well as observation at higher magnetic fields.)

Mn^{+2} concentrations higher than 10^{-4} M result in a gradual decrease in the PG phosphorus peak height (Figure 6); however, the width of the residual line is unaltered. At these Mn^{+2} concentrations the sample becomes turbid and a decrease in the gross intensity of the

PMR spectrum is observed, suggesting that the decrease in the intensity of the phosphorus resonance is due to ion leakage and vesicle precipitation. This interpretation is supported by the observations (Papahadjopoulus and Ohki, 1970; Ohki, 1972) that asymmetric addition of bivalent ions to sonicated phospholipid vesicles and to black membranes composed of anionic phospholipids induces their leakage and breakage.

A comparison of the effects of Mn^{+2} on sonicated PG and sonicated PC suggests that the intereaction of Mn^{+2} with PG is stronger than that with PC. This conclusion is based on the observations that, unlike with PC, Mn^{+2} detectably broadens the fatty acid methylene proton resonances of PG, and that the phosphorus peak of PG reaches its plateau at significantly lower Mn^{+2} concentrations than does the PC phosphorus resonance. These differences are not unexpected, since Mn^{+2} broadening depends on the binding constant and the nature of the complexes formed between Mn^{+2} and the phospholipids (see Appendix 1). As is shown schematically in Figure 7, we can expect that the structural and organizational differences between PG and PC could cause their resonances to be differentially broadened by Mn^{+2}.

Another useful paramagnetic ion is Eu^{+3} which, unlike Mn^{+2}, primarily shifts nuclear magnetic resonances when the chemical exchange between bound and free earth ion is rapid (Eaton and Phillips, 1965). This ion also has been employed by Bystrov et al., (1971), who showed that when added to sonicated PC it shifts to higher fields the choline N^{+}-$(CH_3)_3$ proton resonance which originates from PC

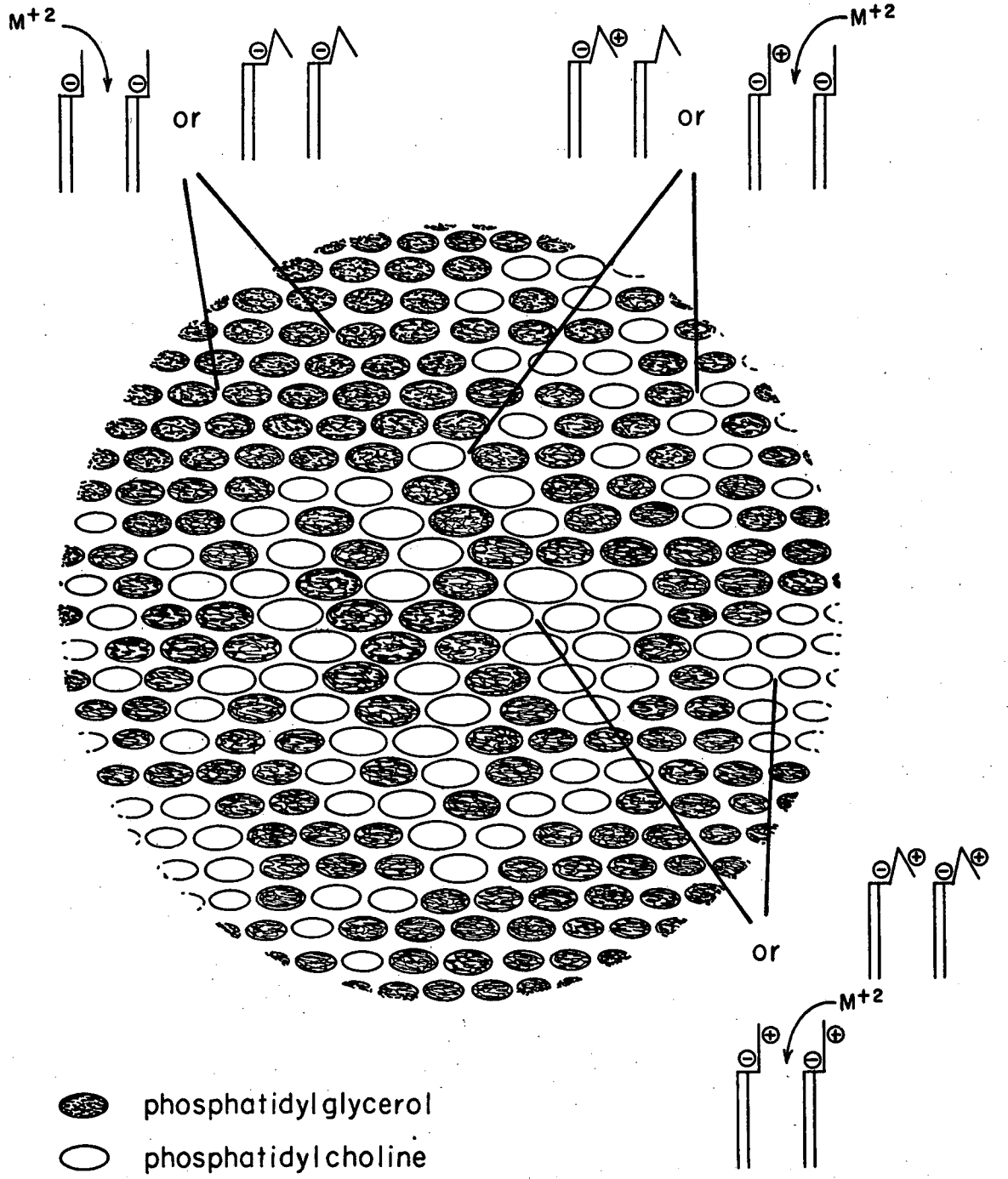

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Figure 7. A perspective view of a bilayer illustrating possible spatial arrangements of the two different classes of phospholipids and some different possible interactions between neighboring phospholipids in the presence and absence of metal ions.

molecules present on the "outside" of the vesicle. We have extended these studies and investigated the effect of Eu^{+3} on the PMR and also on the ϕMR spectra of sonicated PC and PG dispersions. With sonicated PC we found that Eu^{+3} shifts part of the phosphorus resonance (Figure 8) and part of the choline N^{+}-$(CH_3)_3$ proton resonance to higher fields (Figure 9). In both instances the unshifted component has the same line width as the original peak, and it retains approximately 37% of the area of the original peak, whereas the shifted component is broadened and contains approximately 63% of the total area. The extent of the shifts, shown in Figure 10, was much greater for the phosphorus than for the choline N^{+}-$(CH_3)_3$ peaks. Resonication in the presence of Eu^{+3} results in the introduction of Eu^{+3} into the vesicle's inner aqueous phase (Bystrov et al., 1971), and therefore the resonicated vesicles have single phosphorus and choline N^{+}-$(CH_3)_3$ proton peaks which have the same line width and shifts as those of the shifted resonances prior to resonication.

The interaction of Eu^{+3} with sonicated PG was quite different from its interaction with sonicated PC. By contrast with PC, the addition of Eu^{+3} to sonicated PG dispersions did not significantly shift but rather broadened the phosphorus peak. We attribute this broadening to a lowered rate of exchange between bound and free rare earth, thus lending further support to the conclusion that the metals bind more strongly to PG than to PC. The extent of broadening paralleled the Eu^{+3} concentration and resulted in broadening beyond detection of the total phosphorus peak at 1 mM Eu^{+3}; at approximately 5×10^{-4} M Eu^{+3},

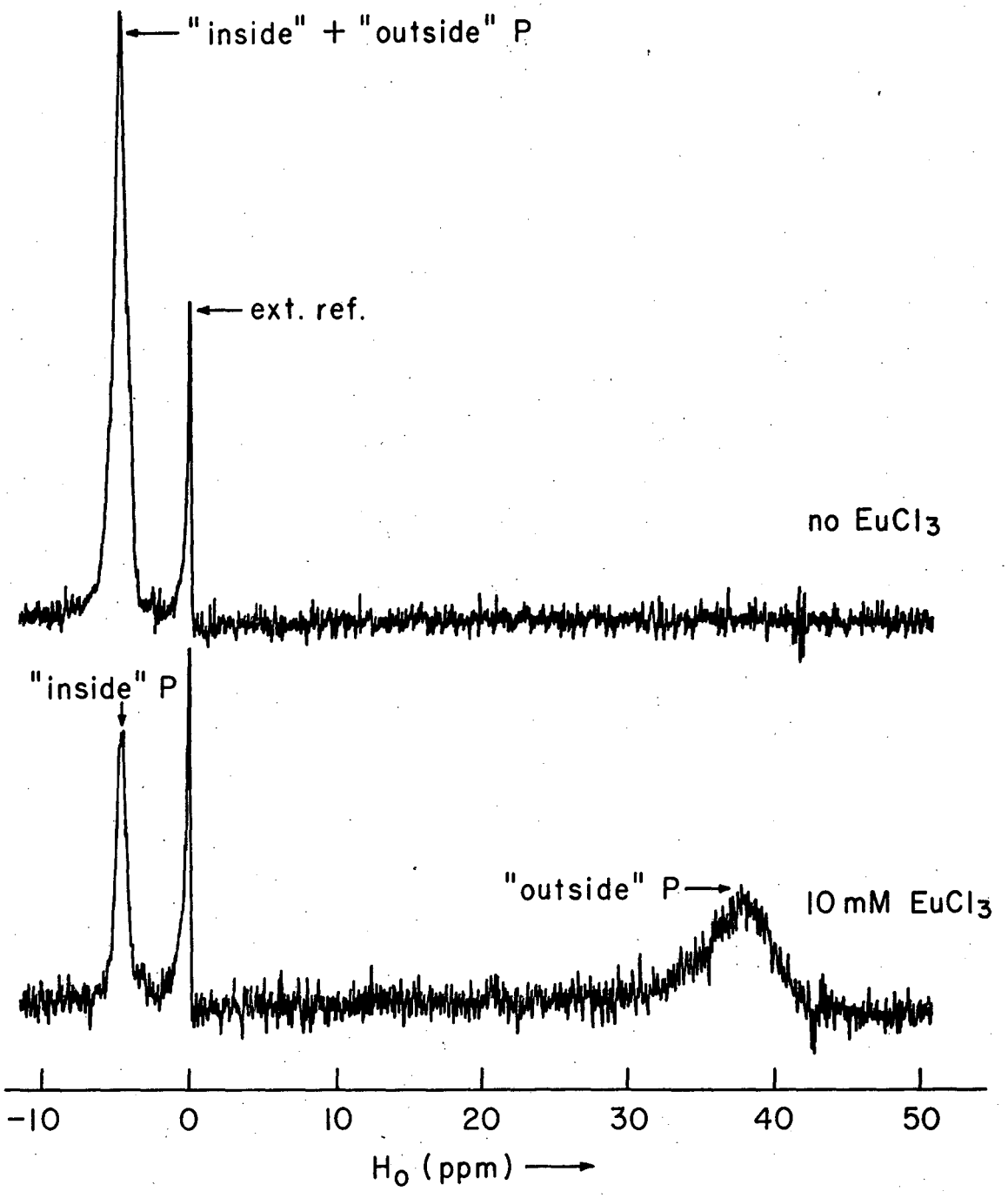

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Figure 8. ^{31}P spectrum of 50 mM sonicated PC, before and after the addition of 10 mM $EuCl_3$. The external reference is pyrophosphate at pH = 9.0. "Outside" P corresponds to those phosphorus atoms in the outer monolayer, while "inside" P corresponds to those in the inner monolayer.

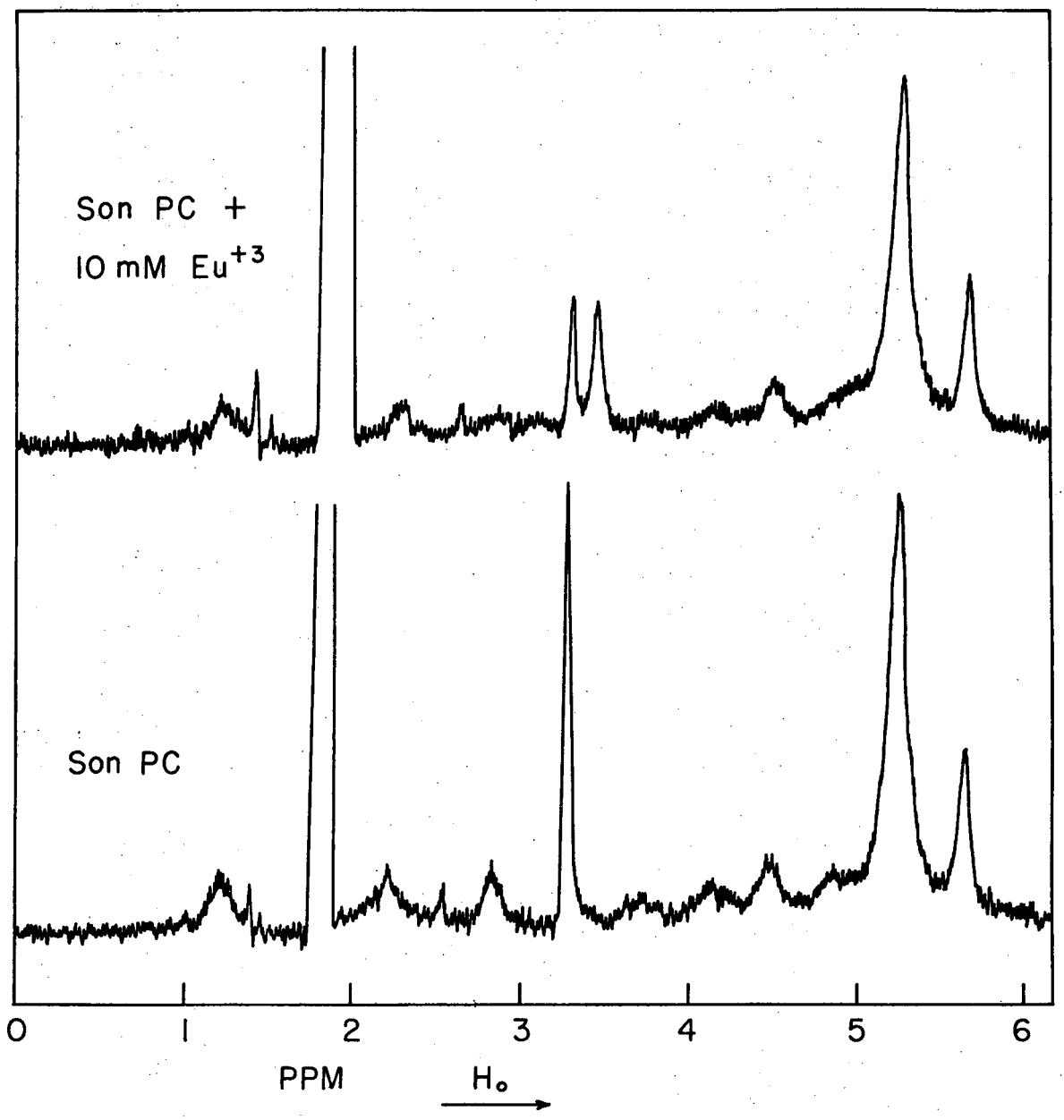

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Figure 9. Proton magnetic resonance spectra of 50 mM sonicated PC, before and after the addition of 10 mM $EuCl_3$.

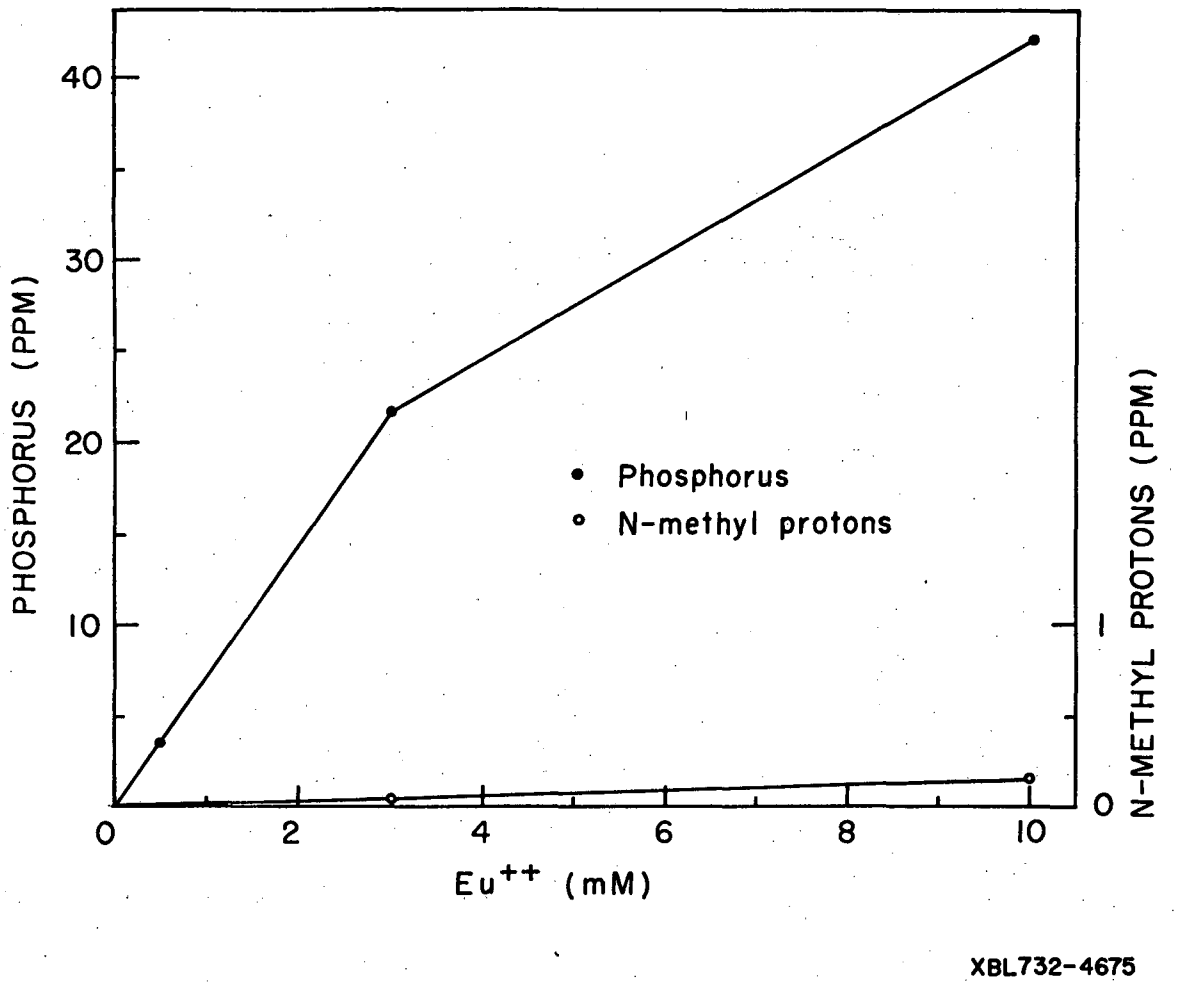

Figure 10. Eu^{+3} induced chemical shift of ^{31}P and choline-N-methyl proton resonances of sonicated PC (40 mM).

the vesicles began to leak and precipitate. In view of these observations, we chose to employ Mn^{+2} as our initial probe in investigating the organization of PG and PC in co-sonicated vesicles.

2. PG and PC within the Outer Monolayer of a Co-sonicated PG:PC Vesicle are not Segregated

In co-sonicated PG:PC vesicles, the PG and PC phosphorus resonances have similar line widths (approximately 21 Hz). This contrasts with what is observed with pure PC and pure PG vesicles mixed together, as is shown in Figure 4. The narrowing of the PG phosphorus resonances upon co-sonication indicates that our co-sonicated vesicles each contain PC and PG molecules. This assertion is in accord with previous experiments (Bangham, 1968) which have shown that co-sonication of a mixture of phospholipids results in mixed vesicles. Further, the narrowing of the PG phosphorus resonance line to a width similar to that of PC suggests that in the co-sonicated vesicles PG and PC do not reside in segregated environments, but rather are dispersed within the vesicle's monolayers.

A second line of evidence suggesting a non-"patched" arrangement within the outer monolayer, is the nature of the broadening by Mn^{+2} of the phosphorus and choline N^{+}-$(CH_3)_3$ proton resonances. From the slopes shown in Figure 11 we observe that in co-sonicated vesicles the peak heights of the PG and PC phosphorus resonances decrease similarly with increasing Mn^{+2} concentrations, and that they are intermediate to those observed in the respective pure PC and pure PG experiments (Figure 6). This finding is incompatible with the

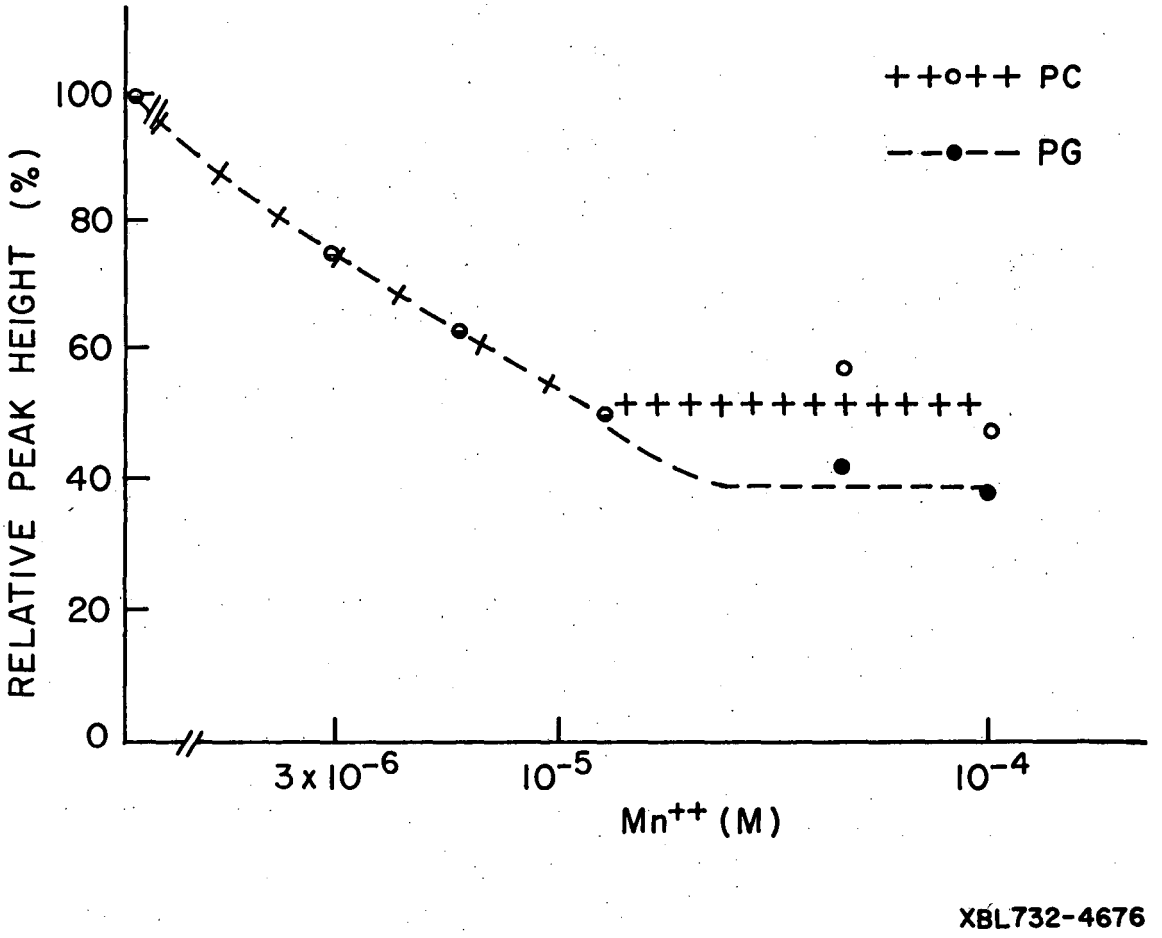

Figure 11. The change in the peak height relative to the original ^{31}P resonance vs. $MnCl_2$ addition to 60 mM co-sonicated PG:PC (1:1) dispersions.

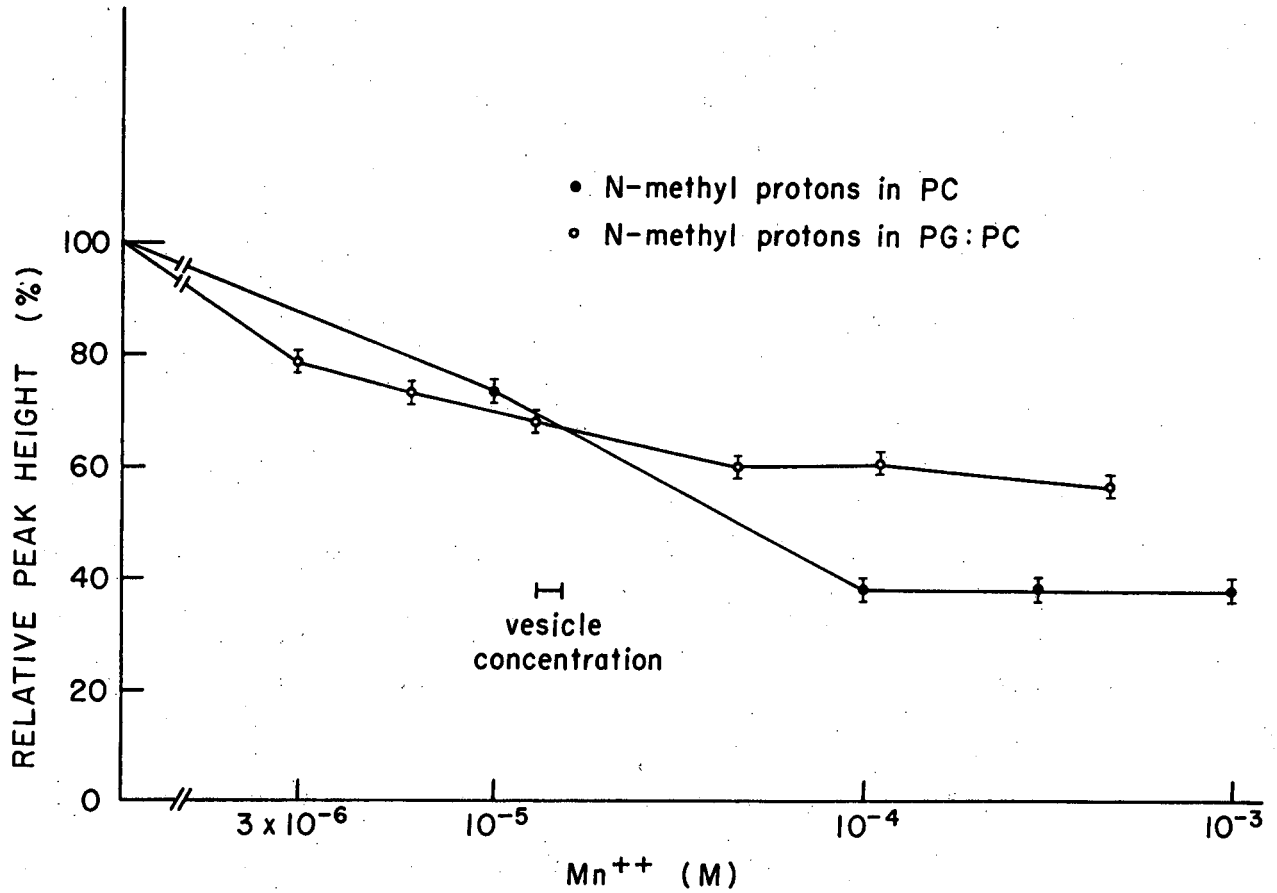

Figure 12. The change in the relative peak height of the PC N-methyl proton resonance vs. $MnCl_2$ addition to sonicated PC and to co-sonicated PG:PC (1:1) vesicles. For the co-sonicated vesicles the PC N-methyl peak at the plateau (Mn^{+2} concentrations higher than 4×10^{-4} M) was broader (14 Hz) than the original peak (9 Hz). The data were normalized to account for this difference; thus for both plots the percent peak height at the plateau is equal to the detectable fraction of the original intensity.

existence, within the outer monolayer of large aggregates of PG and PC (see Appendix 2). The data shown in Figure 12, that the choline N^{+}-$(CH_3)_3$ proton peak reaches its plateau at lower Mn^{+2} concentrations in co-sonicated vesicles than in pure PC vesicles, further supports this conclusion.

3. In Co-sonicated PG:PC Dispersions PG and PC are Distributed Asymmetrically Between the Inner and Outer Monolayers

Analogously to what is observed with pure dispersions (Figure 6), the gradual broadening of the phosphorus resonances of PG and PC, by increasing Mn^{+2} concentrations, is followed by a plateau which extends from 10^{-5} M to approximately 10^{-4} M Mn^{+2}. (At higher concentrations there is further decrease in the intensity of the phosphorus resonances, probably resulting from vesicle leakage and precipitation.) Figure 11 shows that the amplitude of the PC peak at the plateau is higher than that of PG. Since these residual resonances have similar line widths which are the same as those of the original peaks, and because the sum of their areas is 40% that of the unbroadened PG and PC resonances, we can conclude that the "inner" monolayer contains more PC than PG. The error in the intensity of the PG phosphorus resonance at the plateau is relatively large, due to the smaller amount of PG present on the "inside"; and consequently only a qualitative statement can be made concerning the asymmetry of the vesicles.

A more quantitative measure of the degree of asymmetry is provided by the PMR results shown in Figure 12. The plateau levels

of the choline N^+-$(CH_3)_3$ proton resonances in co-sonicated PG:PC and in pure PC vesicles are substantially different. For the co-sonicated vesicles, at the plateau approximately 40% of the original choline N^+-$(CH_3)_3$ proton resonance is broadened while in pure PC dispersions about 63% is affected. The "outside" monolayer in the co-sonicated vesicles contains 60% of the total PG plus PC molecules; because only 40% of the PC is on the "outside", we thus obtain that in co-sonicated vesicles there are twice as many PG as PC molecules facing the "outside". These results cannot be accounted for by the possibility that co-sonication resulted in a heterogeneous population of symmetric vesicles. We thus conclude that the individual vesicles are asymmetric, and that on the average their outer monolayer contains twice as many PG as PC molecules.

C. Discussion

Our data suggest that PG and PC are distributed asymmetrically between the inner and outer halves of the bilayered co-sonicated dispersions, and that within the outer monolayer (and possibly the inner monolayer) these molecules are not arranged in large aggregates which contain significantly more molecules in their center than on their boundaries. We may further infer from our Mn^{+2} experiments that at the concentrations employed, the ion does not induce the formation of such "patches". The absence of large segregates may be explained by the fact that PG is negatively charged while PC is zwitterionic; due to the electrostatic repulsion between the PG molecules, their aggregation is energetically unfavorable. Although a conclusion

regarding the precise arrangement of these phospholipids in the bilayer cannot as yet be made, a plausible arrangement is one in which the PG molecules are separated by the PC molecules, thus minimizing the electrostatic repulsion between the negatively charged PG. A similar pattern has been suggested for the arrangement of acidic and zwitterionic phospholipids in monolayers (Dawson, 1968). However, under suitable conditions different phospholipids may segregate. For example, those with fatty acids of different lengths and/or degrees of unsaturation may be separated at the appropriate temperatures into different phases (Ladbrooke and Chapman, 1969; Horwitz, unpublished results) and, further, appropriate concentrations of polyvalent ions may induce the segregation of differently charged phospholipids.

The observed asymmetry of the co-sonicated vesicles is most likely due to the small radius of curvature of the vesicles and to the difference in the charge of the PG and the PC head groups. That is, since the co-sonicated vesicles are of a size similar to that of the PC vesicles, i.e., 200-300 Å (see Results section), the head groups of the molecules in the inner monolayer are packed more tightly than those in the outer monolayer. As a consequence, the electrostatic repulsion between the negatively charged PG molecules will be reduced in an asymmetric arrangement with more of the PG present on the "outside". The asymmetry of the vesicles is not induced by Mn^{+2}, since the distribution of PG and PC across the bilayer was unaltered by increasing Mn^{+2} concentrations (see Figures 11 and 12).

It is likely that the phospholipids are partitioned asymmetrically between the inner and outer faces of some biological membranes

and that they are in part segregated within the plane of the membranes. Bretscher (1972) has proposed that in red blood cells the phospholipids are distributed asymmetrically between the faces of the membrane, and the observation that specific phospholipids are required for restoring the activity of several isolated membrane proteins (Steck and Fox, 1972) may be interpreted to suggest that in the membrane some of the phospholipids are segregated. The presumed asymmetric organization of the phospholipids may be of physiological importance; in particular, it may provide the proper matrices for enzymes which are located only on one side of the membrane (Douce et al., 1973; Fressenden-Raden and Racker, 1971). The assumed asymmetric and "patched" arrangement of the phospholipids may be determined by several processes. It might arise thermodynamically by the interactions between the membrane components, particularly those between the phospholipids and the proteins and between phospholipids differing in fatty acids and head groups, it might arise biosynthetically, or it might arise by a combination of both.

With reservations, the conclusions obtained from our experiments may be applicable to the problem of the organization of biological membranes. Some membranes such as the highly convoluted mitochondrial inner membrane and the membranes of neurotransmitter vesicles have radii of curvature similar to those of the sonicated vesicles. The size and shape of such membranes may affect the distribution of differently charged phospholipids between the two faces of the membrane. Also, the electrostatic potentials present across many

cellular membranes may modify the distribution of disimilar phospholipids across the membrane. (Alternatively, a spontaneous transmembrane potential can arise from an asymmetric arrangement of phospholipids with differently charged head groups.) We further infer that to the extent that acidic and zwitterionic phospholipids with similar fatty acids are segregated in biological membranes, such an arrangement does not arise spontaneously, but rather it is induced by other factors such as the membrane proteins and polyvalent cations.

A useful approach for studying the problem of the organization of phospholipids in biological membranes may be to investigate the effect of paramagnetic ions on their phosphorus and proton magnetic resonance spectra. ϕMR is particularly suitable because in membranes the major phosphorus containing molecules are the phospholipids, and in addition, the observation that PG and PC phosphorus resonances have different chemical shifts and the likely possibility that other head groups also have different shifts, presents the potentiality of differentially studying the phospholipids in intact cellular membranes. Since the biological membranes examined thus far have broad phosphorus resonances (Davis, 1972), line narrowing techniques (Sarles and Cotts, 1958; Mehring et al., 1971) and shift reagents including lanthinide and transition groups ions might be useful for these studies.

IV. HEAD GROUP MODULATION OF MEMBRANE FLUIDITY AND TRANSBILAYER SYMMETRY OF CO-SONICATED PHOSPHATIDYLCHOLINE:PHOSPHATIDYLETHANOLAMINE DISPERSIONS

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are two major phospholipid classes (Ansell and Hawthorne, 1964); these molecules are zwitterionic at neutral pH and thus differ from other phospholipid classes which possess a net charge at physiological pH values. The structural and functional role of the zwitterionic head groups, as well as of the other head groups, is not understood. The observation that membrane model systems composed of PE and PC respectively, possess very different structural properties (Papahajopoulus and Miller, 1967) suggests that the chemical differences between the PE and PC head groups are of importance. Previous work by Steim (1968) and by Phillips et al., (1972) indicates that in membrane model systems composed of PE and PC respectively, the PE molecules are less fluid and more closely packed than those of PC. In addition, it has been proposed that the erythrocyte membrane is asymmetric with regard to the distribution of PE and PC between the inner and outer surfaces of the membrane: the PC molecules are presumed to predominantly face the outside, and those of PE the inside, of the membrane (Bretscher, 1972; 1973).

We have investigated the spatial arrangement and dynamics of PC and PE in sonicated phospholipid dispersions. In this chapter our observations are presented regarding the effects of the ethanolamine

head group and of pH on the molecular motion of PE in sonicated dispersions, as well as our findings regarding the effect of pH on co-sonicated PC:PE dispersions and the distribution, at high pH, of these molecules between the inner and outer surfaces of the co-sonicated dispersions. We have employed the phosphorus and proton magnetic resonance spectra of the sonicated dispersions and the effects thereon of pH 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.

A. Materials and Methods.

Bacterial phosphatidylethanolamine (E. coli PE) was extracted and purified from a variant of a β oxidation$^-$ unsaturated fatty acid auxotroph of E. coli (30E β ox$^-$) and egg PC was purified from egg yolk, as described in chapter II. 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° C, and their concentration was determined by the phosphate procedure (scaled down by 10) of McClaire (1971).

Sonicated PE dispersions and co-sonicated PC:PE dispersions, containing equal concentrations of PC and PE, were prepared, as described in Chapter III, 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 lyso-phospholipids.

Electron microscopy was performed on ammonium molybdate negatively stained sonicated phospholipid dispersions (Papahadjopoulus and Miller, 1967). The technique was as follows: about 5 mg PE or PE plus PC (1:1) were sonicated in 2 ml 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. The use of ammonium hydroxide and ammonium acetate, which are volatile materials, instead of NaCl and the borax buffers used in the magnetic resonance studies, avoided crystallization of inorganic salts on the microscope grid. We employed 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° C and 45° C, on a Varian HR-220 spectrometer with TMS as an external reference. The spectra were

integrated using an external standard of acetone. Phosphorus magnetic resonance (φMR) measurements were made at an ambient probe temperature of 31° C 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.

B. Results

1. Sonicated Egg 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 (Papahajopoulus 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 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 microns (Figure 13). 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 φMR spectrum of sonicated PE dispersions buffered at pD 11.0 contains a single resonance which is shifted

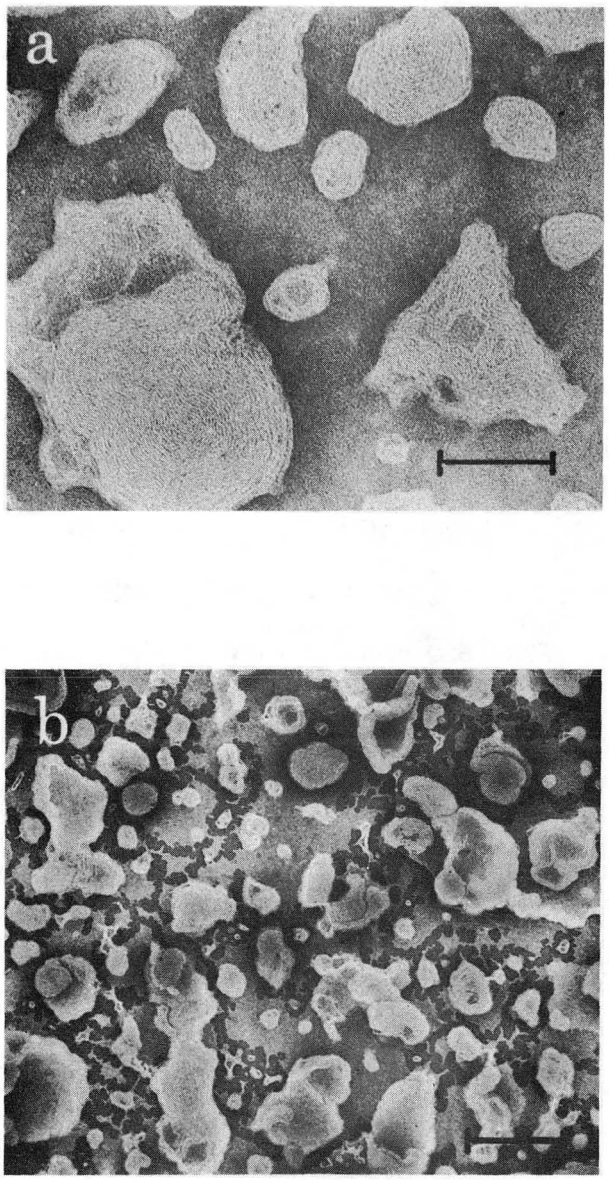

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Figure 13. Electron microscopy, at two magnifications, of sonicated egg PE dispersions buffered at pD 11.0 and negatively stained with ammonium molybdate. Magnification: A, scale = 0.1 micron and B, scale = 0.5 micron.

138 Hz (5.7 ppm) to higher field from the phosphorus resonance of an external standard of K_3PO_4 at pD 14.0.* It is 24 ± 1 Hz wide for sonicated egg PE dispersions (Fig. 14a) 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 (Fig. 14b) whose integrated area corresponds to only 70% of the PE molecules and whose residual line width 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 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 molecules (Fig. 14c). 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.

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*The chemical shifts of the phosphorus resonance of sonicated egg PC buffered at pD 8.0 and 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 co-sonicated 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.

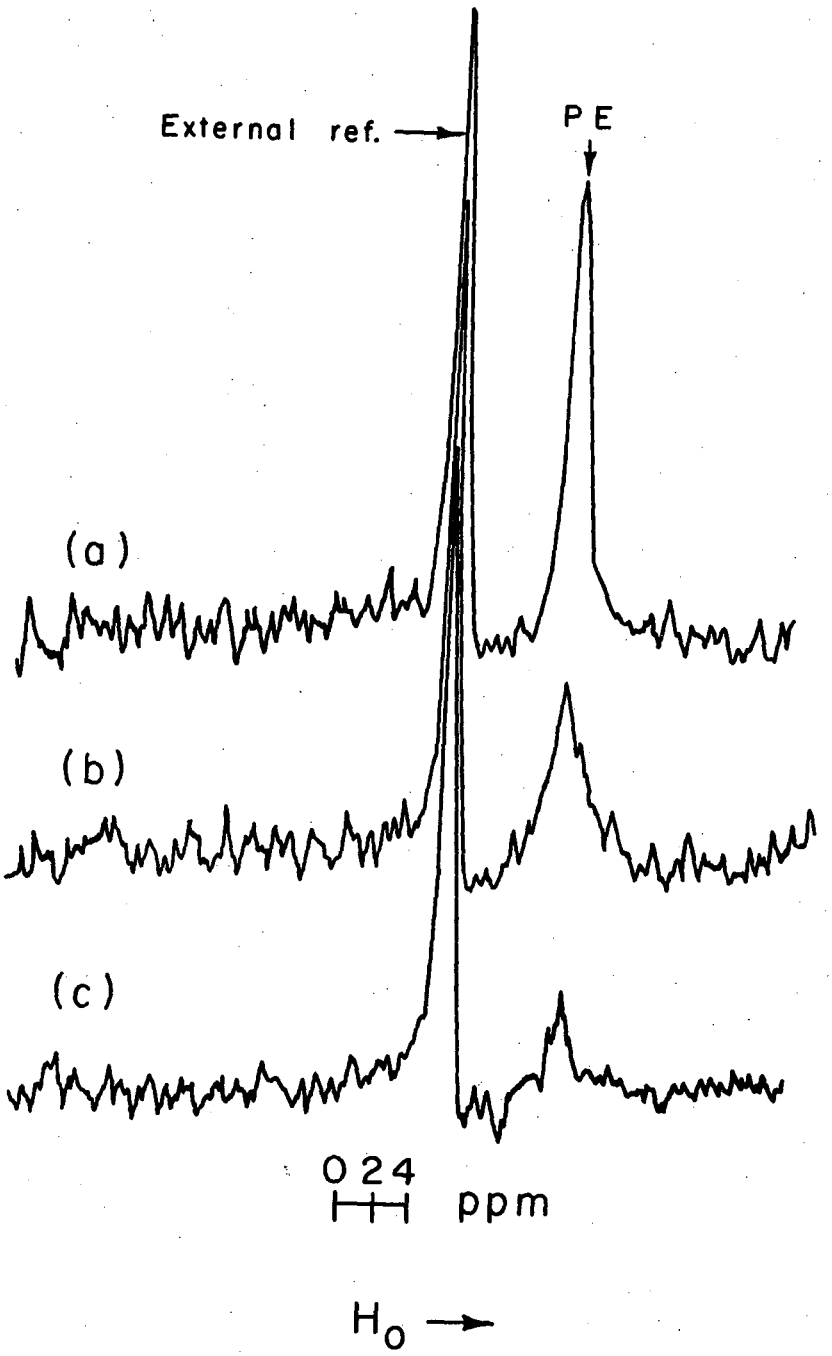

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Figure 14. ^{31}P NMR spectra of sonicated egg PE dispersions buffered at pD 11.0 (a), 9.3 (b), and 8.0 (c). The external reference is K_3PO_4 at pD 14.0.

The PMR spectra of sonicated PE dispersions similarly 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 phosphatidylcholine (PC) and of head group model compounds (Chapman and Morrison, 1966; Dufourq and Lussan, 1972). The spectrum of sonicated egg PE (Figure 15a) 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° C about 55% of the phospholipids contribute to this resonance, and that at 45° C a larger fraction of the molecules, 75%, contribute to this peak (Fig. 16). The ratio of the area of the terminal methyl proton resonance to those of the methylene plus terminal methyl resonances at 22° C as well as at 45° C is, within experimental error, similar to the ratio expected from the fatty acid analysis (Fig. 17). Analogous results were obtained with sonicated E. coli PE dispersions. Thus we may conclude that at 22° C, 55% of the PE fatty acid protons contribute to the spectral intensity and at 45° C, 75% so contribute. In addition to the increase in intensity, raising the temperature to 45° C causes a narrowing of the detectable resonances, the fatty acid methylene resonances exhibiting the greatest narrowing (Table III).

Sonication of egg PE at lower pD values, pD 9.3 and 8.0, results in a marked decrease of the proton spectral intensity (Fig. 15b, c); however, the fatty acid methylene and terminal methyl proton resonances are affected to different extents. The fraction of detectable

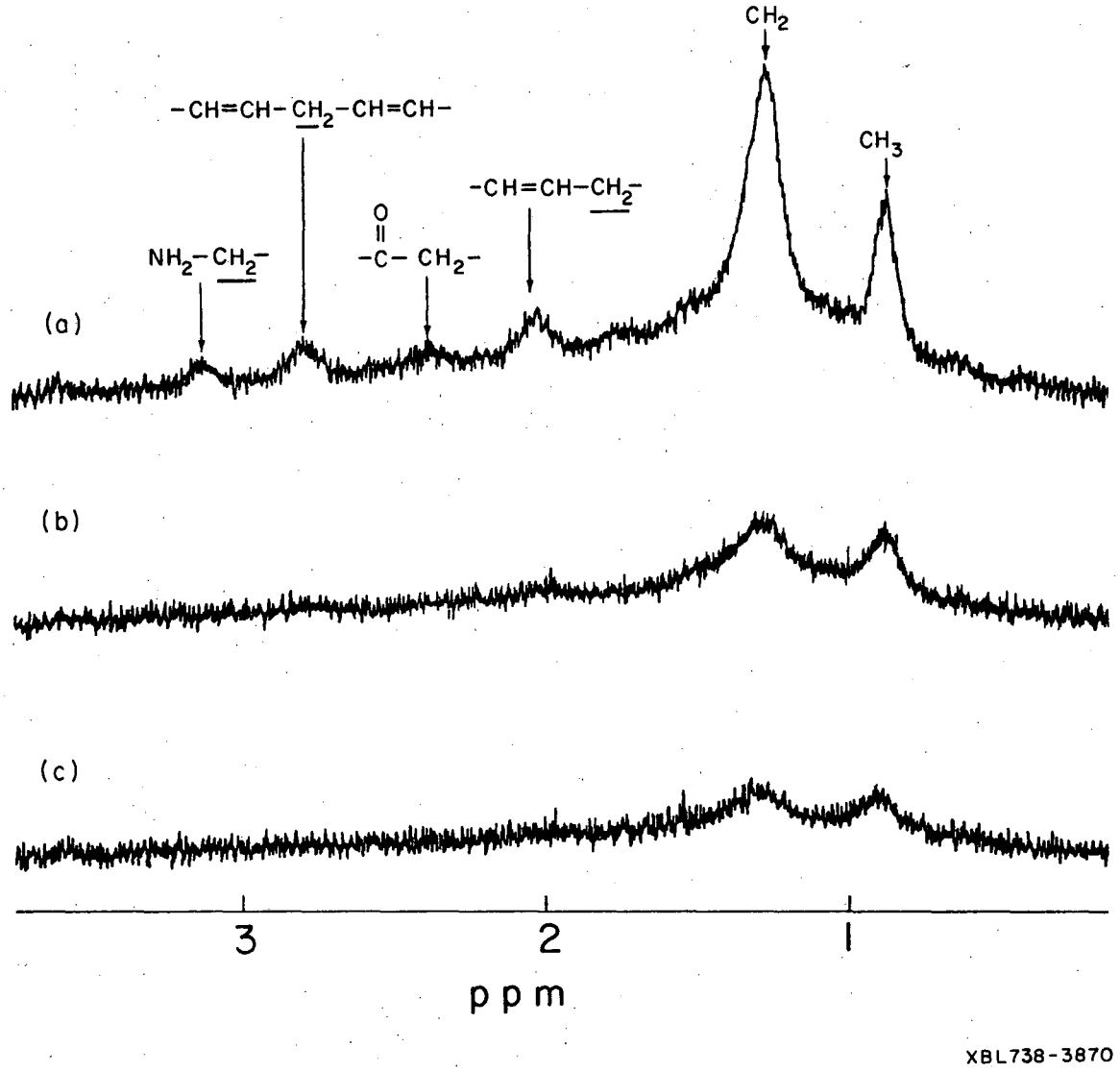

Figure 15. Proton magnetic resonance spectra of sonicated egg PE dispersions buffered at Pd 11.0 (a), 9.3 (b), and 8.0 (c). The spectra were recorded at 22° C, the abscissa scale is ppm downfield from TMS.

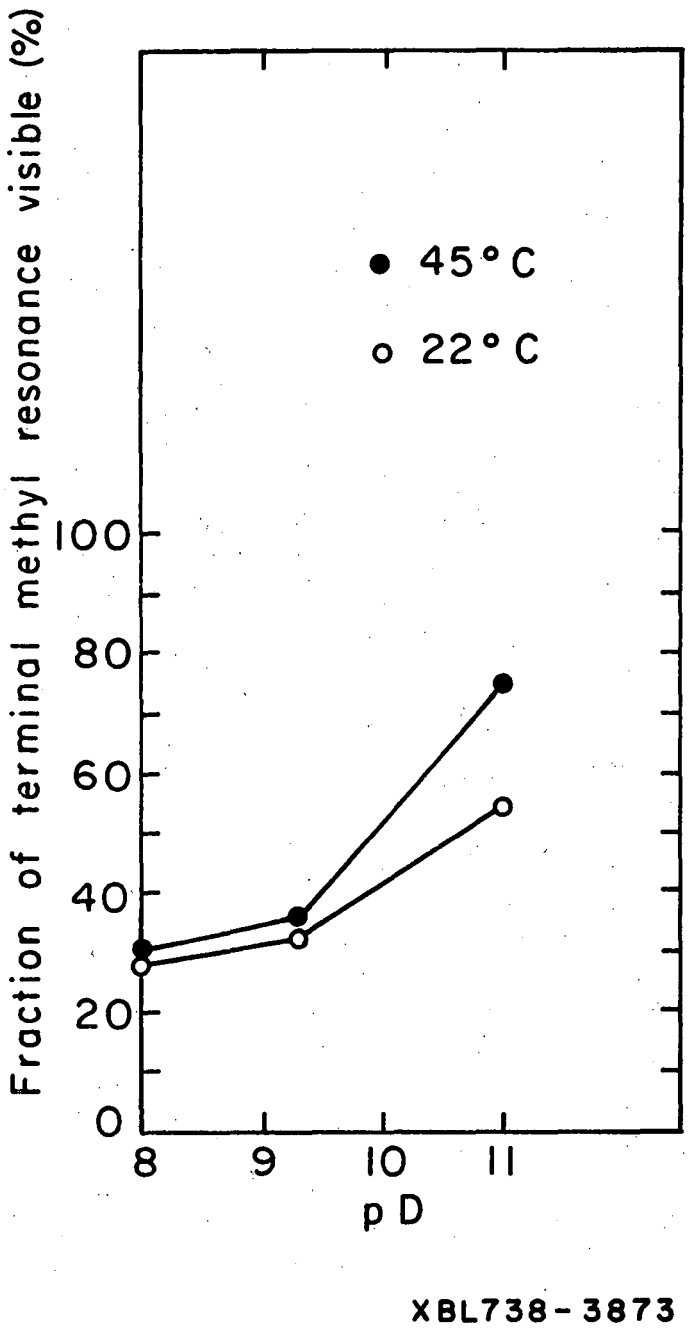

Figure 16. The effect of PD on the fraction of PE molecules contributing to the intensity of the high resolution terminal methyl proton resonance of sonicated egg PE dispersions; 0- spectra recorded at 22° C and ●- spectra recorded at 45° C.

Table III. Effect of pD and Temperature on PMR Linewidths (Hz) of Sonicated PE Dispersions.

PE	pD	$-CH_2-$		$-CH_3$		$-\underline{CH_2}-NH_2$	
		22° C	45° C	22° C	45° C	22° C	45° C
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 30E ox⁻	11.0	37±2	29±1	18±1	16±1	24±2	22±2
	9.3	75±2	60±5	48±2	36±2	---	---

terminal methyl protons is reduced to approximately 35% at pD 9.3 and to about 30% at pD 8.0 (Fig. 16), with the methylene proton resonances exhibiting a more pronounced decrease. This is demonstrated in Figure 17, 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° C to 45° C results in a narrowing of the fatty acid proton resonances (Table III) as well as a small increase in the spectral intensity (Figs. 16 and 17). 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

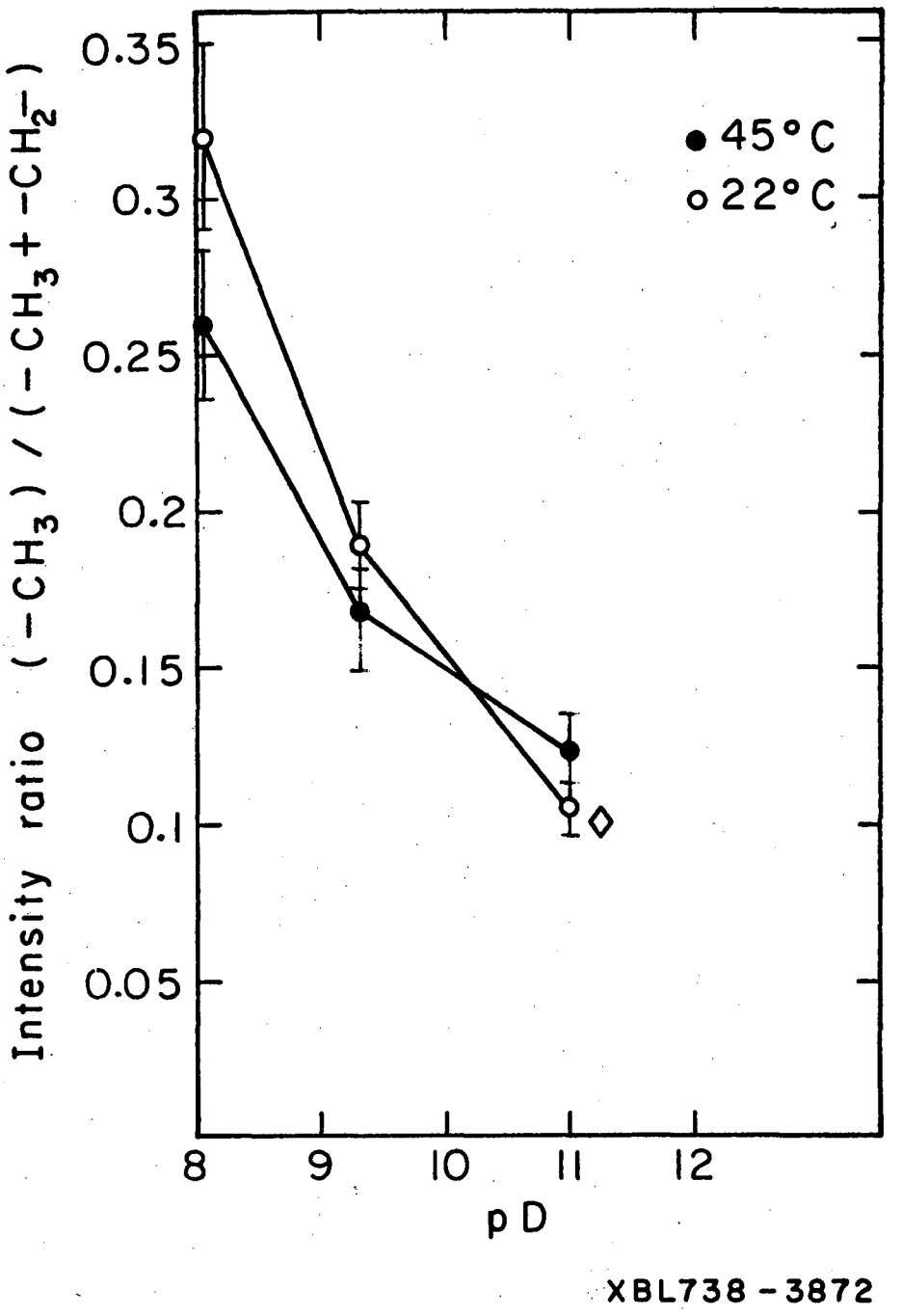

Figure 17. The effect of pD on the ratio of the intensity of the terminal methyl proton resonance to the sum of the intensities of the fatty acid methyl proton resonances of sonicated egg PE dispersions. The expected ratio, calculated from the fatty acid composition, is indicated by ◇. The graph calculated from spectra recorded at 22°C is denoted by 0, and from data obtained at 45°C by ●.

accounted for at 22° C and 50% at 45° C. 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° C, and approximately fourfold at 45° C. This trend is further reflected by the observation (Table III) 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 α-carbon proton resonance are broadened beyond detection at pD 9.3.

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, where the spectra lose about half their intensity within 12 hr after sonication, then for those buffered at pD 9.3. 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 decay of the spectra is reversible, i. e., resonication restored the original spectrum.

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 pK of the head group primary amine at approximately pD 10.2, is suggestive (the pK value cited here is that of

the amine group of a PE head group analog, o-phosphorylethanolamine). Since rapid molecular motion is a prerequisite for obtaining a high resolution spectrum (Abragam, 1961), the decrease of spectral intensity is a manifestation of reduced motion or an increased correlation time. This, 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 both 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.

2. Co-sonicated PC:PE Dispersions

Co-sonication of equimolar concentrations of egg PC and E. coli PE (co-sonicated PC:PE) at pD 11.0 results in an optically clear dispersion which, due to light scattering, has a bluish hue and which yields high resolution phosphorus and proton magnetic resonance spectra. Electron microscopy of these dispersions revealed that the PE and PC molecules are arranged in single shelled spherical vesicles which on the average measure 200-500 Å in diameter (Fig. 18a). The ϕMR 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 assigning the lower field resonance to PE and that residing at the higher field to PC (Fig. 19).

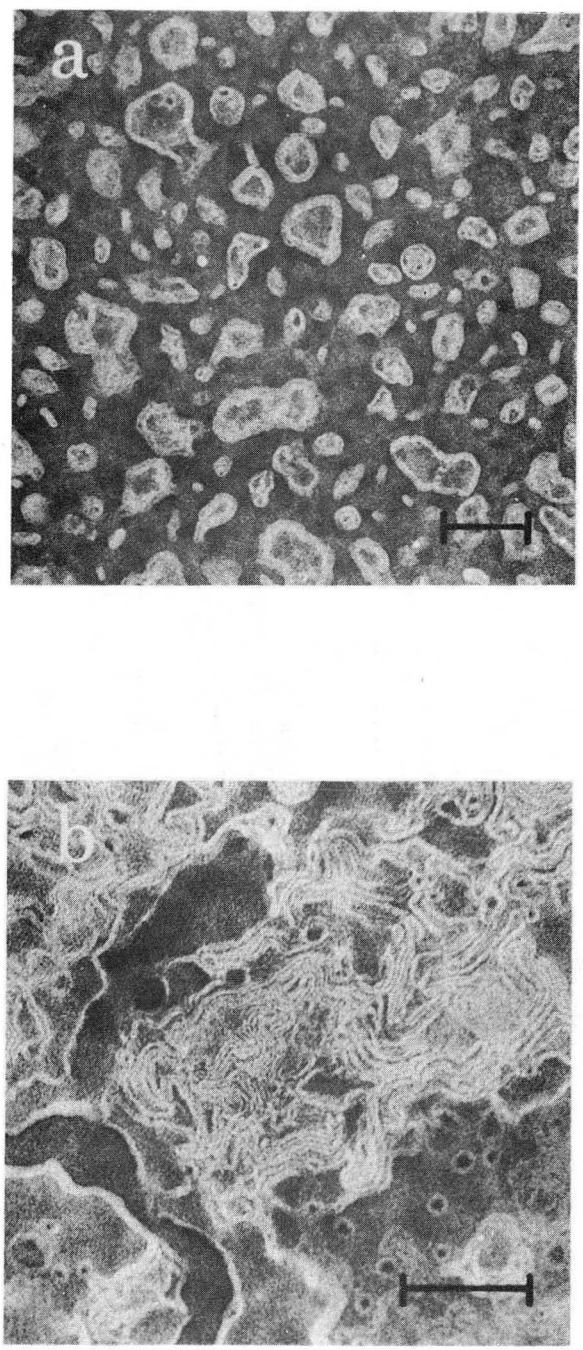

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Figure 18. Electron microscopy of co-sonicated PC:PE dispersions buffered at pD 11.0 (a), pD 8.0 (b), and negatively stained with ammonium molybdate. Scale = 0.1 micron.

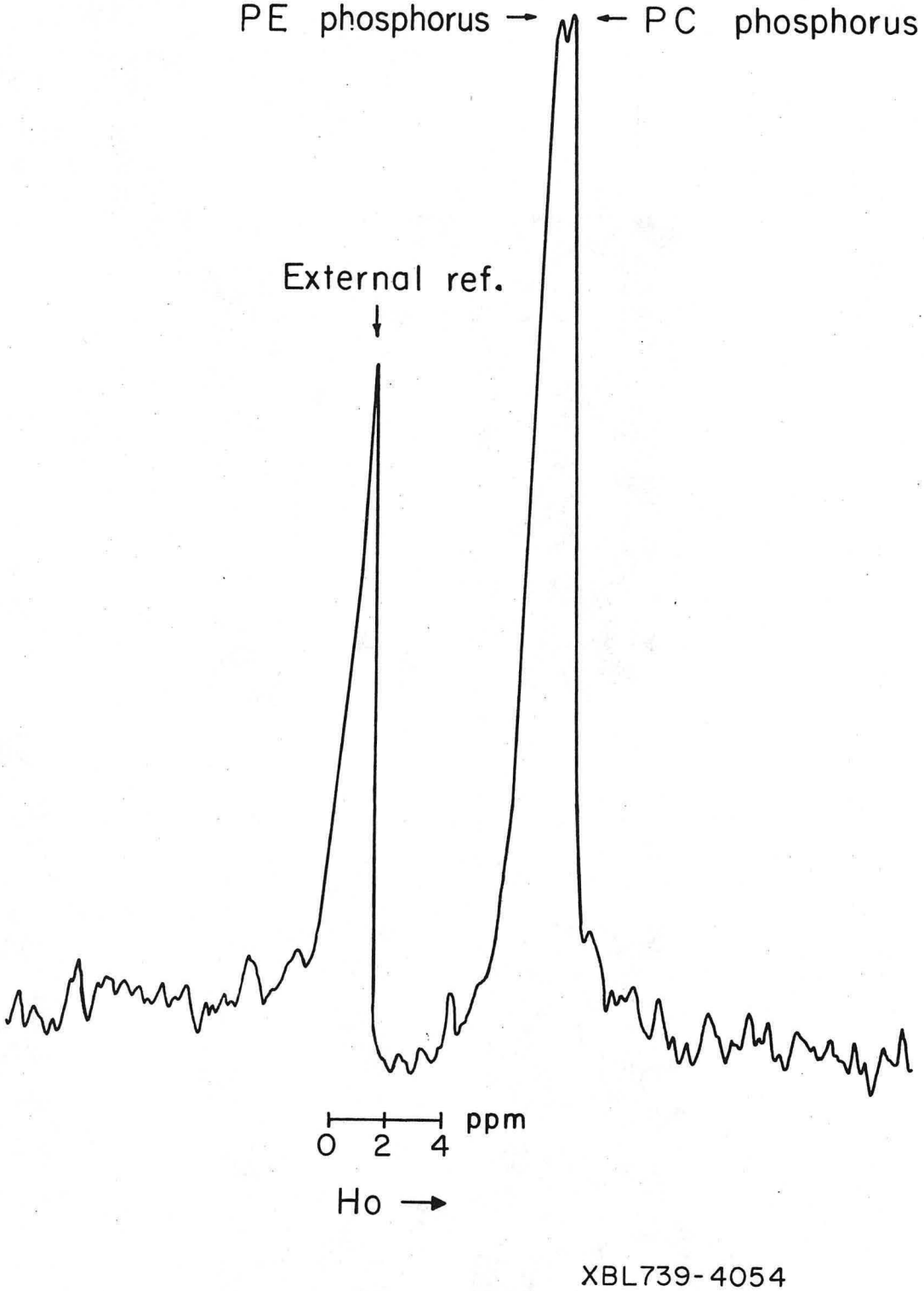

Figure 19. ^{31}P NMR spectrum of co-sonicated PC:PE dispersions buffered at pD 11.0. The external reference is K_3PO_4 at pD 14.0.

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 co-sonicated PC:PE dispersions buffered at pD 11.0 is shown in Figure 20a. 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 α-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 molecules contribute to this resonance. This assertion is supported by our observation that the intensity of the PMR spectrum of sonicated egg PC dispersions buffered at pD 11.0, accounts for all the PC molecules, whereas less than all the PE molecules contribute to the intensity of the PMR spectrum of sonicated PE buffered at pD 11.0. We have recorded the PMR spectrum at 25° C and 45° C. The resonances, in particular the methylene proton resonances, were narrower at the elevated temperature. Nevertheless there was no change in spectral intensity, i.e., the fraction of the molecules contributing to the respective resonances did not increase upon raising the temperature to

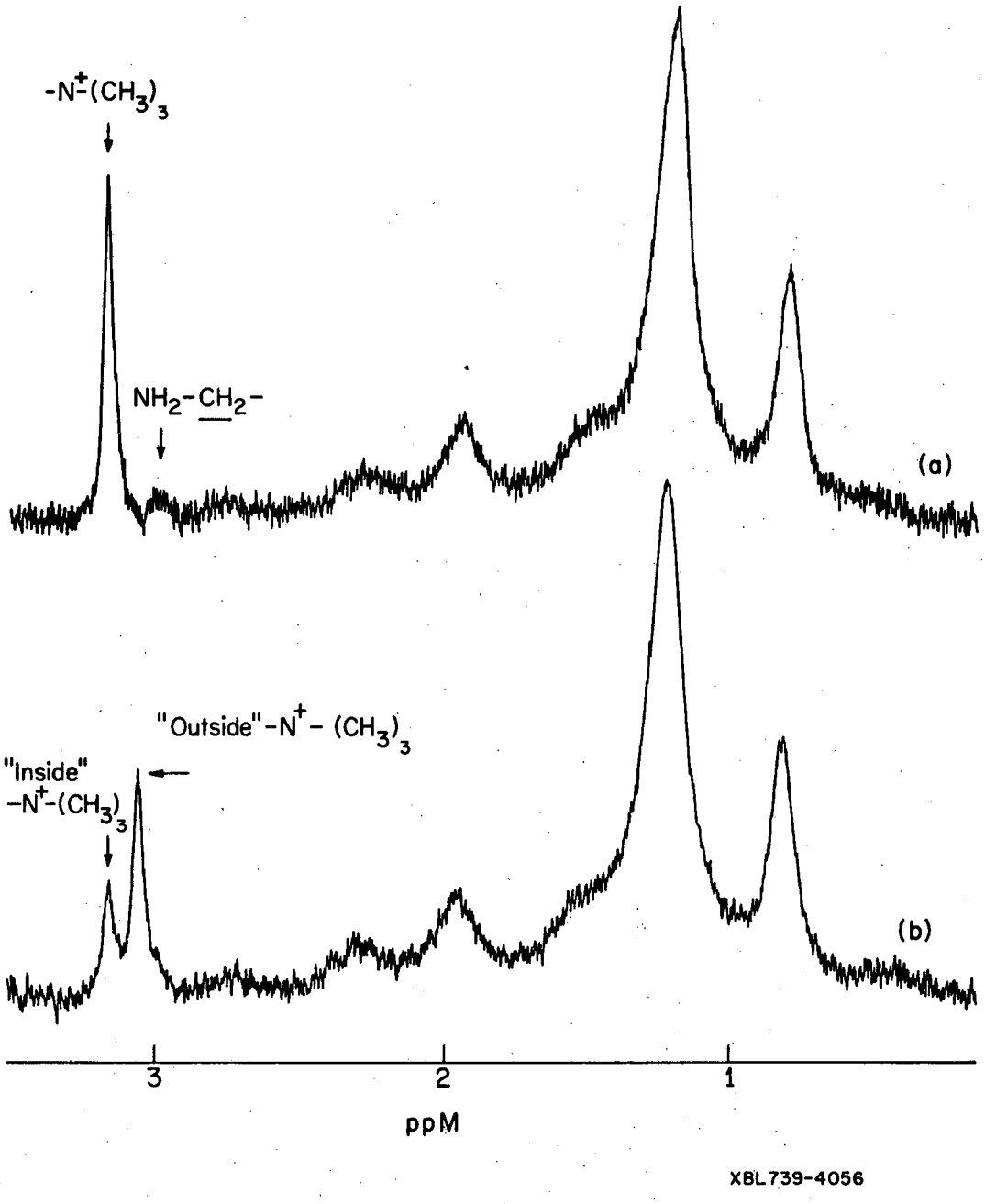

Figure 20. Proton magnetic resonance spectra of 25 mM co-sonicated PC:PE dispersions, before (a) and after (b) the addition of 0.1 M $K_3Fe(CN_6)$. "Outside" N^+-$(CH_3)_3$ and "inside" N^+-$(CH_3)_3$ correspond respectively to the resonances originating from PC molecules in the outer and inner monolayers.

45° C. The PMR spectrum was stable at room temperature for at least 12 h after sonication.

Co-sonication 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 single-shelled vesicles as well as in heterogenous structures which contain more than a single bilayer (Fig. 18b). 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 (Fig. 21). 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 (Fig. 21). The phosphorus and proton magnetic resonance 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 suggest that the relative contributions of PC and PE to the spectra of co-sonicated 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

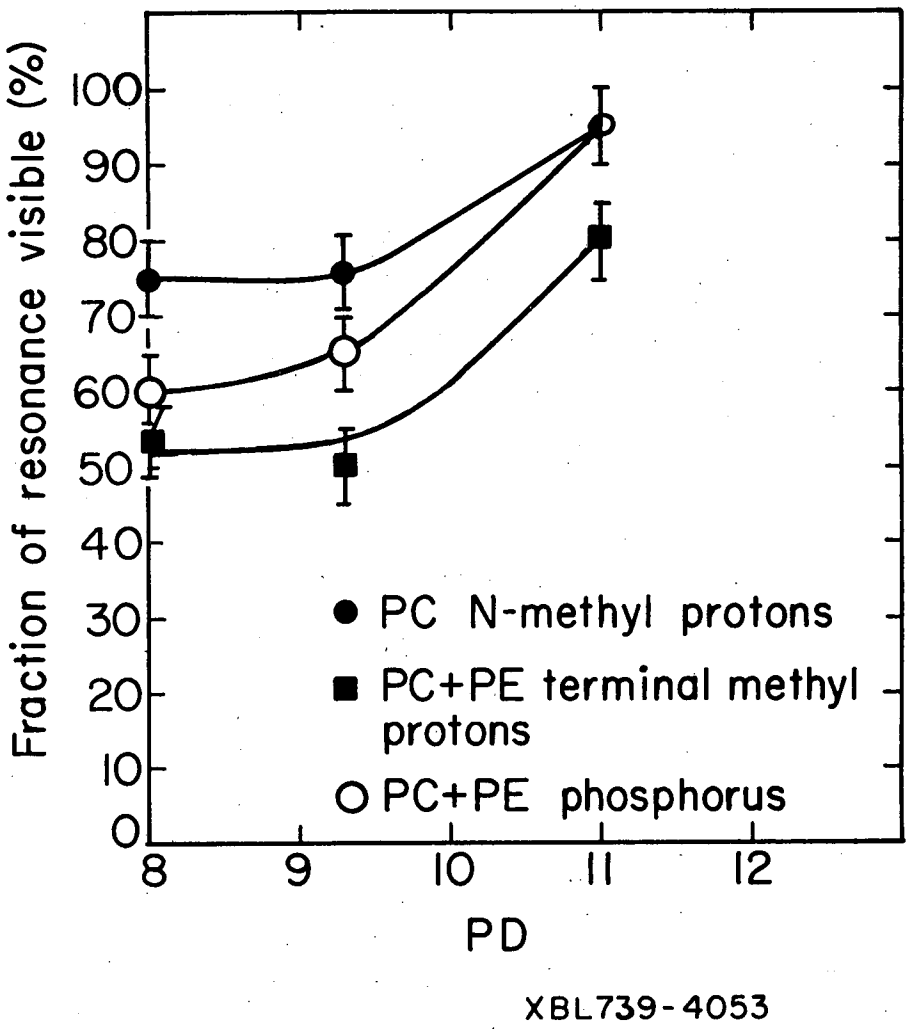

Figure 21. The effect of pD on the fraction of the phospholipid molecules contributing to the ϕMR and PMR spectra of co-sonicated PC:PE dispersions. o = phosphorus resonances of PC plus PE. ■ = terminal methyl proton resonance. ● = choline N^{+}-$(CH_3)_3$ proton resonance.

about 1.25 at pD 8.0 to about 0.95 at pD 11.0.

3. Inside-outside Distribution of PC and PE in Co-sonicated Dispersions

In the previous chapter we reported the use of paramagnetic ions to study the distribution of phosphatidylglycerol (PG) and PC molecules between the inner and outer surfaces of co-sonicated vesicles. We now report our findings from a similar study performed with co-sonicated PC:PE dispersions. Only the dispersions buffered at pD 11.0 are presently suitable for this study. That is so because co-sonication of PC and PE at pD 11.0 results in single shelled vesicles which yield a ϕMR 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 co-sonication at pD 8.0 and 9.3 results in a heterogenous population of multilamellar structures and single-shelled vesicles, which yield proton and phosphorus magnetic resonance spectra of decreased intensity.

Figure 20b shows the PMR spectrum of co-sonicated 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 $K_3Fe(CN)_6$ are added the upfield shift of the choline-N^+-$(CH_3)_3$ proton resonance increases to 42 Hz (0.19 ppm); however, the relative

intensities of the shifted and unshifted components of the choline N^+ $-(CH_3)_3$ resonance is unaltered. Similar observation 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 they shift only those resonances which originate from nuclei facing the "outside" of the vesicles and are present at the polar region. Thus resonication in the presence of this paramagnetic anion results in its 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 ϕMR experiment since $K_3Fe(CN)_6$ was shown, by us, to have no effect on the ϕMR 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 calculate the distribution of the PC molecules between these two surfaces. However, a correction must be made, because the "outside" choline-N^+-$(CH_3)_3$ resonance is shifted upfield and thus overlaps the ethanolamine α-carbon proton resonance (compare Figs. 20a and 20b). 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 α-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 ϕMR spectra of the co-sonicated dispersions. 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 and unaltered components. At higher Mn^{+2} concentrations (approximately $2\ 10^{-5}$M) a plateau is reached when further Mn^{+2} addition (up to $2\ 10^{-4}$ M) causes no further decrease in the intensity of the resonances (Fig. 22).* Resonication at the plateau results in a broadening

*We have found that addition of $MnCl_2$ to co-sonicated 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 22 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 ϕMR 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.

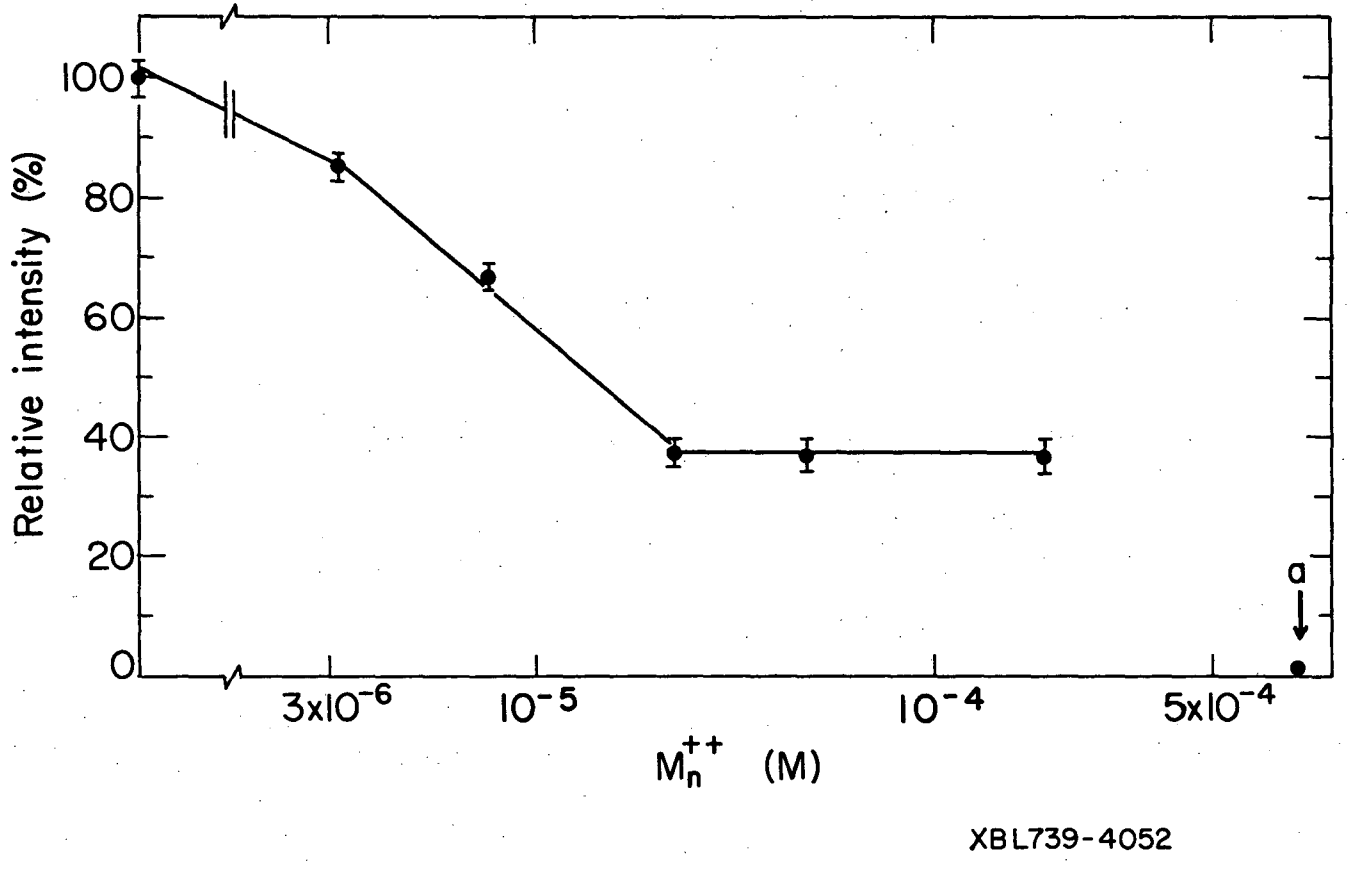

Figure 22. The change in intensity of the ϕMR spectra relative to the original spectra vs. $MnCl_2$ addition to co-sonicated PC:PE dispersions buffered at pD 11.0.

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, it contains equal contributions from PE and PC. We similarly interpret these results to show that, at the concentration studied, the co-sonicated 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 face the "outside" surface and 35-40% face the "inside" surface of the co-sonicated PC:PE vesicles; this distribution is in accordance with the size of the vesicles and the bilayer's thickness.

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 co-sonicated vesicles buffered at pD 11.0.

C. Discussion

Our experiments show that the phosphorus and proton magnetic resonance spectra of sonicated aqueous dispersions of E. coli PE and egg PE, have a marked pD dependence. At pD levels above the pK of ethanolamine the integrated intensities of the high resolution spectra correspond to most of the phospholipid molecules, whereas at pD levels lower than the pK of ethanolamine, the phosphorus and proton magnetic resonances contain narrow high resolution, as well as broad and unresolved components, and therefore the intensity and

resolution of the spectra are greatly reduced.

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 the hydrophilic region of the PE molecule. We propose 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 terminals. An alternative interpretation is that the pD dependence of the spectra is due to gross structural variations between the PE dispersions buffered at the different pD values investigated. 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 unlikely.

The nature of the distribution of the protons and the phosphorus nuclei to those which do and do not yield high resolution spectra is not established at present. A possible distribution is one where only a specific fraction of the PE molecules, which are either within each bilayer or in a specific sub population of the heterogenous 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, yield the high resolution spectra. In addition, our data do not warrant a conclusion regarding the exact nature of the pD effect on molecular motion. It may affect the lateral diffusion rate in the planes of the bilayered lamellae (Kornberg and McConnell, 1971), it may affect the extent and rate of flexing of the head group and the fatty acids (Horwitz et al., 1973), or it may affect both. The decay of the spectra with time suggests that as the sample ages an aggregation process occurs whereby the PE molecules reach a more stable state of restricted mobility.

Our interpretation of the data is consistent with previous experimental observations and their interpretations. Shimshick and McConnell (1973) have employed a spin-labeled fluidity probe to study phospholipid dispersions composed of PE and PC mixtures; they interpret their data similarly to suggest that, at neutral pH, the ethanolamine head group of PE solidifies the hydrocarbon region of the phospholipid dispersions. In addition, 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, where both types of molecules are composed of identical fatty acids, and Phillips et al., (1972) have employed X-ray crystallography to show that, at neutral pH, PE molecules are more closely packed than 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 (Stoeckenius and Engelman, 1969; Singer and Nicolson, 1972), the phospholipid head groups, their charge and

structure, may also affect the fluidity of the membranes. Consequently, parameters such as pH and ionic strength may significantly influence membrane fluidity. This supposition has been recently examined by Overath and Träuble (1973), who showed the phase transition temperature of dispersions composed of acidic phospholipids to be inversely proportional to ionic strength.

Our experiments with co-sonicated PC:PE dispersions show that at pD 11.0 co-sonication results in single-shelled vesicles which yield a ϕMR 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 molecules, whereas co-sonication of these molecules at pD 9.3 and 8.0 results in single-shelled vesicles as well as multilamellar heterogenous structures, which yield magnetic resonance spectra of reduced intensity. The decrease in spectral intensity with pD 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 the co-sonicated dispersions results from the negative charge of most of the PE molecules at pD 11.0 and their zwitterionic nature at lower pD values investigated. The spatial arrangement of the PC and PE molecules which do and do not contribute to the magnetic resonance spectra is, at present, difficult to assess. Litman (1973a) has suggested that co-sonicated dispersions containing equal concentrations of PC and PE and buffered at neutral pH, are heterogenous with regard to the distribution of PC and PE. Thus, it is possible that the reduced spectra observed at pD 8.0 and pD 9.3

arise preferentially from a sub population of the heterogenous dispersions.

We have studied the effects of paramagnetic ions (i.e., Mn^{+2}, $Fe(CN)_6^{+3}$) on the magnetic resonance spectra of co-sonicated PC:PE vesicles buffered at pD 11.0. Our data suggests the PE and PC, at this pD, are distributed symmetrically between the inner and outer surfaces of the vesicles. Litman (1973b) has used selective chemical labeling to study the spatial arrangement of PE and PC in mixed phospholipid vesicles, buffered at pH 5.0. His data show that co-sonicated 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 thos of Litman (1973b) is most likely a result of the different pH values we employed, as well as the small radius of curvature of the vesicles. That is, due to the small size of the co-sonicated vesicles, the head groups of the molecules in the inner monolayer are packed more tightly than those in the outer monolayer. As a consequence, the electrostatic repulsion between the negatively charged PE molecules, at pD 11.0, will be reduced in an arrangement where relative to their arrangement at neutral pH more PE molecules will face the outside. Furthermore, the vesicles prepared at pH 5.0 have a smaller diameter, of about 105 A° (Litman, 1973b), than those formed at pD 11.0. Therefore the difference between the tightness of the packing of the head groups present on the inner and outer monolayers of the bilayered vesicles may be reduced at pD 11.0 as compared to the neutral pH and consequently the vesicles prepared at pD 11.0 will be more

symmetric than those buffered at natural pH.

With reservations, the observed effects of the charge and structure 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), these molecules are believed to be asymmetrically distributed between the inner and outer layers of the membrane. Since the fluidity of the membrane depends on the molecular motion of the phospholipids, it is possible that membrane components embedded in different sides of the membranes experience different viscosities. This suggestion is based on the assumption that both phospholipid classes have the same fatty acid composition. However, it has been observed that PE in human erythrocytes (Rouser et al., 1968) as well as in many other cellular membranes (Answell and Hawthorne, 1964; Stancliff et al., 1969; Ostwald et al., 1970) has more unsaturated fatty acids, in particular more polyunsaturated fatty acids, than PC. 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. Thus we propose that the spatial distribution of different phospholipid classes across the membrane, coupled with their respective fatty acid composition, are important factors in determining the fluidity of the inner half and the outer half of biological membranes.

V. CONCLUSIONS

The spatial arrangements and molecular dynamics of different classes of phospholipids have been studied in a membrane model system: sonicated phospholipid dispersions. The data show that the dynamics of these molecules may be modulated by the polar head groups and that the extent of the modulation depends on the electrostatic charge and the chemical structure of this polar moiety. Also, we have shown that the chemical composition and electrostatic charge of the polar head group affect the relative phospholipid distribution between the inner and outer surfaces of the mixed co-sonicated vesicles.

These findings were obtained by studying the proton and phosphorus magnetic resonance spectra of the sonicated dispersions. The spectral changes accompanying pH changes were interpreted to reflect head group modulation of the membrane fluidity and the effects, on these spectra, of paramagnetic ions were utilized to study differentially the arrangement of the different lipid classes within and between the monolayers of the bilayered co-sonicated vesicles. Since only three classes of phospholipids, PC, PG and PE, were investigated thus far, further studies employing additional molecules are required to deepen our understanding of the structural and dynamic roles of the different head groups.

The approach taken in this study is applicable to the investigation of biological membranes. The proton, phosphorus and Carbon-13 magnetic resonance spectra of several cellular membranes have been published recently (Metcalfe et al., 1972; Davis and Inesi, 1972; Sheetz and Chan, 1972; Robinson et al., 1972; Keough et al., 1972). In general only a fraction of the lipids yield high resolution spectra, whereas the membrane proteins and the remainder of the lipids yield broad and barely detectable resonances. Consequently line narrowing techniques (Sarles and Cotts, 1958; Mehring et al., 1971) will be required in order to employ the above spectra to investigate the mobility and spatial arrangement of the lipids in biological membranes. In addition, a variety of other spectroscopic methods may be applied to probe the structures and dynamics of membranes, each according to its particular specificity and time range. Such studies alone and when combined with chemical investigations employing specific chemical reagents will no doubt provide interesting results.

APPENDIX 1

The broadening by Mn^{+2} of the resonances of nuclei present on the outer monolayer of sonicated phospholipid disperions can be treated similarly to the case of aqueous solutions of Mn^{+2}. Equation 1 derived by Swift and Connick (1962) describes the broadening of the water proton resonance in a dilute Mn^{+2} solution.

$$1/T_2 - 1/T_{2_o} = pq/(T_{2m} + \tau) \qquad (1)$$

where T_2 and T_{2_o} are respectively the proton transverse relaxation times in the presence and absence of Mn^{+2}, τ is the lifetime of the water -Mn^{+2} complex, q is the solvation number of Mn^{+2} in the complex, and p is the molar ratio of Mn^{+2} to water protons. For the derivation of this equation it was assumed that the protons are in two environments, bound to Mn^{+2} and unbound in the bulk solvent, and that the Mn^{+2} concentration is much less than the proton concentration. In applying this equation to the nuclear resonances of phospholipids arranged in sonicated dispersions, two additional points should be noted. First, the Mn^{+2} can be bound either to the phospholipids or free in the bulk solution, and second, the phospholipids are arranged in a two-dimensional array which may affect the value or values of q.

Mn^{+2} may form several types of complexes with the sonicated dispersions: each complex is characterized by the number of phospholipid molecules which are bound to the ion, j, and the number

of water molecules, i, which are displaced by the phospholipids from the first coordination sphere of Mn^{+2}. We designate $[Mn^{+2}PL_i^j]$ as the concentration of complexes where j phospholipid molecules are bound to Mn^{+2}, and a fraction i of these molecules is complexed in the first coordination sphere of the ion. In addition, the total concentrations of Mn^{+2} and the "outside" phospholipids are represented by $[Mn^{+2}]_o$ and $[PL]_o$. Let us assume that a given phospholipid does not exchange directly between two Mn^{+2} complexes (this assumption is justified since in our experiments $[Mn^{+2}]_o << [PL]_o$), then Eq. 1 takes the form

$$\Delta \equiv 1/T_2 - 1/T_{2_o} = \sum_{i,j} \frac{[Mn^{+2}PL_i^j]}{[PL]_o} \left(\frac{i}{T_{2m}^j + \tau_i^j} + \frac{j-i}{T_{2n_i}^j + \tau_i^j} \right) \qquad (2)$$

where τ_i^j is the lifetime of the $Mn^{+2}PL_i^j$ complex, $T_{2m_i}^j$ is the transverse relaxation time of the resonances which arise from the i molecules which are bound to the first coordination sphere of Mn^{+2}, and $T_{2n_j}^j$ is the transverse relaxation time of the resonances which arise from the j-i molecules in the complex which are not bound in the first coordination sphere of the ion. The summation extends over all possible complexes. We define apparent equilibrium constants

$$K_i^j = \frac{[MnPL_i^j]}{[Mn^{+2}]_f [PL]_f} \qquad (3)$$

where $[Mn^{+2}]_f$ and $[PL]_f$ are respectively the concentrations of the free Mn^{+2} and the free "outside" phospholipids. By combining Eqs. 2

and 3 we get Eq. 4.

$$\Delta \equiv 1/T_2 - 1/T_{2_o} = \sum_{i,j} \frac{K_i^j [MN^{+2}]_o}{1+[PL]_o (\sum_{i,j} K_j^i)} \left(\frac{i}{T_{2m_i}^j + \tau_i^j} + \frac{j-i}{T_{2n_i}^j + \tau_i^j} \right). \quad (4)$$

Let us assume that all the Mn^{+2} complexes contain q phospholipid molecules, that the complexes have similar lifetimes, τ, and that in the complexes the transverse relaxation times of similar nuclei are equal and designated by T_{2m}. If now we define an effective equilibrium constant

$$K_{eff} = \frac{[Mn^{+2}PL]}{[PL]_f [Mn^{+2}]_f}, \quad (5)$$

where $[Mn^{+2}PL]$ is the concentration of bound Mn^{+2}, Eqs. 4 and 5 may be combined to give Eq. 6a.

$$\Delta \equiv 1/T_2 - 1/T_{2_o} = \frac{K_{eff}[Mn^{+2}]_o}{(1+K_{eff}[PL]_o)} \quad \frac{q}{(T_{2m}+\tau)}. \quad (6a)$$

In cases where the effective equilibrium constant is not known, an upper limit of Δ is given by Eq. 6b.

$$\Delta \equiv 1/T_2 - 1/T_{2_o} \leq \frac{[Mn^{+2}]_o q}{[PL]_o (T_{2m}+\tau)}. \quad (6b)$$

Employing Eqs. 6a and 6b and the data from Figure 2, some parameters describing the interaction between Mn^{+2} and sonicated PC and sonicated PG dispersions can be estimated. A comparison of Δ for the "outside" phosphorus and choline-N^{+}-$(CH_3)_3$ proton resonances of sonicated PC reveals that their ratio is 8. Assuming that K_{eff} is the same for both nuclei and that the denominator in equation 6a is dominated by T_{2m}, we obtain that the ratio of the T_{2m} of the ^{31}P resonance to that of the choline-N^{+}-$(CH_3)_3$ proton resonance is 8. Since T_{2m} depends on the distance between the Mn^{+2} ion and the nuclei, the correlation times of the complex and the magnitude of the hyperfine interaction (Solomon, 1955; Bloembergen, 1951), additional experiments are required before we can employ the ratio of the T_{2m} of the phosphorus and the choline-N^{+}-$(CH_3)_3$ proton resonances for calculating the ratio of their distances from the Mn^{+2} ion. However, a reasonable interpretation of this ratio is that in the Mn^{+2}-PC complex, the ion is located closer to the phosphorus than to the choline-N^{+}-$(CH_3)_3$ protons. This interpretation is in accord with the data shown in Figure 5, which suggests that when Eu^{+3} is bound to the "outside" PC it is located closer to the phosphorus than to the choline-N^{+}-$(CH_3)_3$ protons (McConnell and Robertson, 1958; Eaton and Phillips, 1965). Our conclusion regarding the location of the bound Mn^{+2} and Eu^{+3} agrees with studies of PC monolayers (Shah and Schulman, 1967) which indicate that Ca^{+2} bound to PC monolayers is in closer proximity to the phosphorus than to the choline-N^{+}-$(CH_3)_3$ protons.

The determination of $T_{2m} + \tau$ for the phosphorus and the choline-N^+-$(CH_3)_3$ protons is not yet possible since the equilibrium constant and q are not known. However, assuming q = 4, we calculated (using Eq. 6b) an upper limit of $T_{2m} + \tau$ for the phosphorus and the choline-N^+-$(CH_3)_3$ resonances to be 1.2×10^{-5} and 9.6×10^{-5} sec, respectively. Since the "outside" phosphorus resonance of sonicated PG was broadened beyond detection by the lowest Mn^{+2} concentration employed, only a lower limit for Δ can be obtained for PG. Thus applying Eq. 6b, we calculated an upper limit of $T_{2m} + \tau$ to be 7×10^{-6} sec. It is of interest to note that the "outside" phosphorus resonance was broadened beyond detection by less than one ion per vesicle. This observation raises the possibility that a given ion broadens most of the "outside" resonances of a given PG vesicle before it diffuses to another vesicle. However, several diffusion processes can determine τ:diffusion of Mn^{+2} from the complex into the bulk solvent, diffusion of Mn^{+2} to other PG molecules, and the exchange of PG molecules into and out of the Mn^{+2} complex; therefore we cannot as yet conclude which process is rate limiting. In any case, 7×10^{-6} sec is an upper limit for the fastest of these processes.

From Eq. 6a we see that the difference in the value of several parameters could explain the observation that Mn^{+2} broadens the "outside" PG phosphorus resonance more than that of PC. This difference might arise from stronger binding of Mn^{+2} to the negatively charged PG than to the zwitterionic PC, a smaller T_{2m} for the PG ^{31}P resonance than for the PC ^{31}P resonance, or both.

APPENDIX 2

The Mn^{+2} broadening of the resonances of the nuclei present on the "outside" of co-sonicated PG:PC vesicles can be used to elucidate the spatial arrangement of PG and PC within the outer monolayer. We assume that Mn^{+2} is in equilibrium between being free in the bulk solution and being bound to the vesicles, that Mn^{+2} can form complexes, designated $Mn^{+2}PL_{ij}$, with i molecules of PC and j molecules of PG, that all the complexes have the same total number of phospholipid molecules q (i.e., $q = i + j$), and that in a given complex the same nuclei of the bound PC and PG each have a single transverse relaxation time which we designate respectively as $T_{2PC_{ij}}$, and $T_{2PG_{ij}}$. We further assume that the nature of the $Mn^{+2}PL_{ij}$ complex is independent of the neighbors of the PG and PC molecules in the complex (this assumption may not always be valid, see last section of this appendix). Since in our experiments the total Mn^{+2} concentration, $[Mn^{+2}]_o$, is much smaller than the total concentration of the "outside" PC, $[PC]_o$, and of the "outside" PG, $[PG]_o$, we may use the Swift and Connick equation (1962). Equation 1 is the expression for the Mn^{+2} broadening of the "outside" PC resonances.

$$\Delta_{PC} \equiv 1/T_2 - 1/T_{2_o} = \sum_{\substack{i=0 \\ i+j=q}}^{q} \frac{[Mn^{+2}PL_{ij}]}{[PC]_o} \frac{i}{(T_{2PC_{ij}} + \tau_{ij})} \qquad (1)$$

where τ_{ij} is the lifetime of each complex. A similar equation can be written for the PG resonances. If we assume that the free Mn^{+2} is in equilibrium between the bulk solution and the $Mn^{+2}PL_{ij}$ complexes, we may define apparent equilibrium constants K_{ij}:

$$K_{ij} = \frac{[Mn^{+2}PL_{ij}]}{[PL_{ij}]_f [Mn^{+2}]_f} \tag{2}$$

where $[Mn^{+2}]_f$ is the concentration of Mn^{+2} in the bulk solution and $[PL_{ij}]_f$ is the concentration of sites where Mn^{+2} binds to i PC and to j PG molecules. Since $[PL_{ij}]_f$ is a function of the total phospholipid concentration, we may define (Eq. 3) a proportionality constant, a_{ij}, between these quantities.

$$[PL_{ij}]_f = a_{ij} ([PC]_o + [PG]_o). \tag{3}$$

The proportionality constants may be affected by the ratio $[PG]_o/[PC]_o$, and may also be a function of the Mn^{+2} concentration. This coefficient is of great significance, since knowing its value for all the possible PL_{ij} is equivalent to knowing the spatial arrangement of PG and PC within the surface. Equations 1, 2, and 3 may be combined to give Eq. 4. A similar expression may be written for the PG resonances.

$$\Delta_{PC} \equiv 1/T_2 - 1/T_{2_o} = \sum_{\substack{i=0 \\ i+j=q}}^{q} \frac{i K_{ij} a_{ij} ([PC]_o + [PG]_o)}{\{1 + ([PC]_o + [PG]_o)(\sum_{\substack{i=0 \\ i+j=q}}^{q} K_{ij} a_{ij})\}} \frac{1}{(T_{2PC_{ij}} + \tau_{ij})} \tag{4}$$

If we assume that the phospholipids are arranged in large patches which contain significantly more molecules in their center than on their boundaries, then Eq. 4 can be simplified to Eq. 5,

$$\Delta_{PC} \equiv \frac{1}{T_2} - \frac{1}{T_{2_o}} = \frac{K_{PC}[MN^{+2}]_o q}{(1+K_{PC}[PC]_o+K_{PG}[PG]_o)} \quad \frac{1}{(T_{2PC}+\tau)} \qquad (5)$$

where K_{PC} and K_{PG} are the equilibrium constants of Mn^{+2} with PC and PG as defined in Appendix 1. A comparison of Eq. 5 of this appendix and Eq. 6a of Appendix 1 reveals that under this assumption the "outside" resonances of both PC and PG should be broadened less when these molecules are in the co-sonicated vesicles than when they are in the respective pure vesicles. However, using the data shown in Figures 2 and 5, we find that the "outside" PC ^{31}P resonance is broadened more when PC is in the co-sonicated vesicles than when it is in the PC vesicles, whereas for PG the opposite holds. If we assume more elaborate arrangements, then Eq. 4 rather than Eq. 5 has to be applied to our experimental results. Since Eq. 4 contains more variables, the analysis becomes very complicated. However, with reasonable estimates of the relative magnitude of the unknown parameters our data suggest that PG and PC are not arranged in small patches even if the boundaries are included. Thus we may conclude that within the NMR experiment time scale PG and PC are not segregated in the outer monolayer of the co-sonicated vesicles; a more precise statement about the arrangement of these molecules in the bilayer cannot as yet be given.

We made two simplifying assumptions in the derivation of Eq. 4: that the Mn^{+2} forms only one type of complex with i PC molecules and j PG molecules, where always $i+j=q$, and that the nature of the complex is independent of the nearest neighbors of the phospholipids in the complex. The first assumption is not a necessary requirement for showing that our data is inconsistent with a spatial arrangement where PG and PC are segregated into large patches. The second assumption may not always be justified, but if most of the PG and PC molecules were segregated, then most of the Mn^{+2} complexes would have been in either a PG or a PC environment and therefore this assumption would not have affected Eq. 4.

REFERENCES

Abragam, A. (1961), The Principles of Nuclear Magnetism, Oxford University Press, Clarendon, London.

Anes, G. F. (1968), J. Bact. 95, 833.

Answell, G. B., and Hawthorne, J. N. (1964), "Phospholipids", BBA library, Vol. 3.

Atwood, D., and Saunders, L. (1965), Biochim. Biophys. Acta 98, 344.

Bangham, A. D. (1968), Progress in Biophys. and Mol. Biol. 17, 31.

Bloembergen, N. J. (1957), J. Chem. Phys. 27, 572.

Bretscher, M. S. (1972), Nature New Biology 236, 46.

Bretscher, M. S. (1973), Science 181, 622.

Brown, P. K, (1972), Nature New Biol. 236, 35.

Bystrov, V. B., Dubrovina, N. I., Barsukov, L. I., and Bergelson, L. D. (1971), Chem. Phys. Lipids 6, 343.

Carrington, A., and McCachlan, A. D. (1967), Introduction to Magnetic Resonance, Harper and Row, New York.

Caspar, D. L. D., and Kirshner, D. A. (1971), Nature New Biology 231, 46.

Chapman, D., and Morrison, A. (1966), J. Biol. Chem. 241, 5044.

Chapman, D., and Wallach, D. F. H. (1968), in Biological Membranes, Chapman, D., Ed., London and New York, Academic Press, p. 125.

Cone, R. A. (1972), Nature New Biol. 236, 39.

Cronan, J. E., and Vegelos, P. R. (1972), Biochim. Biophys. Acta 265, 25.

Davis, B. D., and Mingioli, E. S. (1950), J. Bact. 60, 17.

Davis, D. G. (1972), Biochem. Biophys. Res. Commun. 49, 1492.

Davis, D. G., and Inesi, G. (1972), Biochim. Biophys. Acta 282, 180.

Dawson, R. M. C. (1968), in Biological Membranes, Chapman, D., Ed., London and New York, Academic Press, p. 203.

Douce, R., Mannella, C. A., and Bonner, W. D. (1973), in press.

Dufourq, J., and Lussan, C. (1972), FEBS Letters 26, 35.

Dunham, P. B., and Hoffman, J. F. (1970), Proc. Natl. Acad. Sci. 66, 939.

Eaton, D. R., and Phillips, W. D. (1965), in Advances in Magnetic Resonance, Vol. 1, Waugh, J. S., Ed., New York and London, Academic Press.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), Biochemistry 10, 2606.

Farrar, T. C., and Becker, E. D. (1971), Pulse and Fourier Transform NMR, Academic Press, New York and London.

Fressenden-Raden, J. M., and Racker, E. (1971), in Structure and Function of Biological Membranes, Rothfield, L. I., Ed., New York and London, Academic Press, p. 401.

Frye, L. D., and Edidin, M. (1970), J. Cell. Sci. 7, 319.

Ginsburg, V., and Kobata (1971) in Structure and Function of Biological Membranes, Rothfield, L. I., Ed., New York and London, Academic Press, p. 439.

Green, D. E., Allmann, D. W., Bachman, E., Baum, H., Kopaczyk, K., Korman, E. F., Lipton, S., McLennan, D. H., McConnell, D. G., Perdue, J. F., Rieske, J. S., and Tzagoloff, A. (1967), Biochemistry 119, 312.

Guidotti, G. (1972), Annu. Rev. Biochem. 41, 731.

Haest, C. W. M., De Gier, J., Op den Kamp, J. A. F., Bartels, P., and Van Deenen, L. L. M. (1972), Biochim. Biophys. Acta 255, 720.

Horwitz, A. F. (1972), in Membrane Molecular Biology, Fox, C. E., and Keith, A. D., Eds., Stanford California, Sinauer Associates, p. 164.

Horwitz, A. F., and Klein, M. P. (1972), J. Supramolecular Structure 1, 19.

Horwitz, A. F., Klein, M. P., Michaelson, D. M., and Kohler, S. J. (1973), presented in the International Conference on Electron Spin Resonance and Nuclear Magnetic Resonance in Biology and Medicine, and the Fifth International Conference on Magnetic Resonance in Biological Systems. Ann. N.Y. Sci. (in press).

Huang, C. (1969), Biochemistry 8, 344.

Kates, M., and Wassef, M. K. (1970), Ann. Rev. Biochem. 39, 323.

Keogh, K. M., Oldfield, E. and Chapman, D. (1973), Chem. and Phys. of Lipids 10, 37.

Korn, E. D. (1969), Annu. Rev. Biochem. 38, 263.

Kornberg, R. D., and McConnell, H. M. (1971), Proc. Nat. Acad. Sci. U. S. 68, 2564.

Kostelnik, R. J., and Castellano, S. M. (1972), J. Magnetic Resonance 7, 219.

Ladbrooke, B. D., and Chapman, D. (1969), Chem. Phys. Lipids 3, 304.

Law, J. H., and Snyder, W. R. (1971) in Membrane Molecular Biology, Fox., C. F., and Keith, A. D., Eds., Stanford, California, Sinauer Associates, p. 3.

Lennarz, W. J., Bonsen, P. P. M., and Van Deenen, L. L. M. (1967), Biochemistry 6, 2307.

Litman, B. J. (1973a), Biochemistry 12, 2545.

Litman, B. J. (1973b) (in press).

McClaire, C. W. F. (1971), Anal. Biochem. 39, 527.

McConnell, H. M., and Robertson, R. E. (1958), J. Chem. Phys. 29, 1361.

Mehring, M., Pines, A., Rhim, W.-K., and Waugh, J. S. (1971), J. Chem. Phys. 54, 3239.

Metcalfe, J. C., Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K., and Partington, P. (1971), Nature 233, 199.

Mueller, P., Rudin, D. O., Tien, H. T., and Wescott, W. C. (1964), Recent Progr. Surface Sci. 1, 379.

Ohki, S. (1972), Biochim. Biophys. Acta 255, 57.

Op den Kamp, J. A. F., Redai, I., and Van Deenen, L. L. M. (1969), J. Bact. 99, 298.

Op den Kamp, J. A. F., Verheij, H. M., and Van Deenen, L. L. M. (1971), Bioorganic Chemistry 1, 174.

Ostwald, R., Yamanaka, W., and Light, M. (1970), Proc. Soc. Exp. Biol. and Med. 134, 814.

Overath, P., and Träuble, H. (1973), Biochemistry 12, 2625.

Papahadjopoulus, D., and Miller, N. (1967), Biochim. Biophys. Acta 135, 624.

Papahadjopoulus, D., and Ohki, S. (1970) in Liquid Crystals and Ordered Fluids, Johnson, J. F., and Porter, R. S., Eds. New York and London, Plenum Press, p. 13.

Phillips, M. C., Finer, E. G., and Hauser, H. (1972), Biochim. Biophys. Acta 290, 397.

Pinto da Silva, P., and Branton, D. (1970), J. Cell Biol. 45, 598.

Robinson, J. D., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1972), Biochemistry 11, 2903.

Rouser, G., Kritchevsky, G., Yamamoto, A., Simon, G., Galli, C., and Bauman, A. J., (1969), Methods in Enzymol. 14, 272.

Rouser, G., Nelson, G. J., Fleischer, S., and Simon, G. (1968), in Biological Membranes, Chapman, D., Ed., London and New York, Academic Press, p. 5.

Sarles, L. R., and Cotts, R. M. (1958), Phys. Rev. 111, 853.

Shah, D. O., and Schulman, J. H. (1967), J. Lipid Res. 8, 227.

Sheetz, M. P., and Chan, S. I. (1972), Biochemistry, 11, 548.

Shimshick, E. J., and McConnell, H. M. (p973), Biochemistry 12, 2351.

Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1965), J. Amer. Oil Chem. Soc. 42, 53.

Singer, S. J., and Nicolson, G. L. (1972), Science 175, 720.

Solomon, I. (1955), Phys. Rev. 99, 5599.

Spiro, R. G. (1970), Annu. Rev. Biochem. 39, 599.

Stancliff, R. C., Williams, M. A., Utsumi, K., and Packer, L. (1969), Arch. Biochem. Biophys. 131, 629.

Steck, T. L., and Fox, C. F. (1972), in Membrane Molecular Biology, Fox., C. F., and Keith, A. D., Eds., Stanford, California, Sinauer Associates, p. 27.

Steim, J. M. (1968), Adv. Chem. Series 84, 259.

Stoeckenius, W., and Engelman, D. M. (1969), J. Cell Biol. 42, 613.

Swift, T. J., and Connick, R. E. (1962), J. Chem. Phys. 37, 307.

Yang, S. F., Freer, S., and Benson, A. A. (1967), J. Biol. Chem. 424, 477.

Yang, S. F. (1969), Methods in Enzymol. 14, 208.

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