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ABSOLUTE ASYMMETRIC SYNTHESIS. III. HINDERED ROTATION ABOUT ARYL—ETHYLENE BONDS IN THE EXCITED STATES OF DIARYL ETHYLENES. STRUCTURAL EFFECTS ON THE ASYMMETRIC SYNTHESIS OF 2 - AND 4-SUBSTITUTED HEXAHELICENES

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The phospholipid regions in cellular membranes are involved in a variety of functions, including the maintenance of permeability barriers and associations with some proteins to form functional entities.[1,2] The evidence available from several physical techniques suggests that in nearly all of the membranes studied thus far, there are regions in which the phospholipids are arranged in bilayers which exhibit varying degrees of mobility (or fluidity).[3-7] The bilayer arrangement of phospholipids in cellular membranes is not accidental; it is a manifestation of their amphiphilic nature, and it occurs when they are isolated and dispersed into water.[8] Since the phospholipids spontaneously form bilayer structures in water that are similar to those found in cellular membranes, and since phospholipids in lipid-water model systems are more easily studied than those in complicated membranes, it has been assumed that bilayers composed of aqueous phospholipid dispersions provide a reasonable model and convenient point of departure for studying the phospholipids in membranes.

The spontaneous formation of phospholipids into fluid bilayers explains neither the diversity of fatty acids nor the variety of polar headgroups found in natural membranes. That is, phosphatidylcholine (lecithin or PC) with a specific ratio of only two fatty acids will form bilayers that are fluid throughout the entire physiological range of temperatures.[9] It is likely, therefore, that the variety of fatty acids and headgroups are required for differing structural and functional roles which, in turn, may derive from the altered bilayer properties or from associations with other membrane components.

Partial solutions to questions concerning the nature of phospholipid bilayers are beginning to emerge from the results of several physical techniques. Of these techniques, NMR has been of great use in elucidating the motion of the fluid fatty acids of lecithin.[10] In particular, T_1 measurements have provided information concerning "fast" processes, and T_2 measurements have provided information about relatively slower processes. In this paper we discuss briefly some relevant topics in relaxation theory, review our previous work on both sonicated and unsonicated lecithins and the dynamic model we proposed to explain the relaxation data,[10-12] report some effects of altered fatty acid composition on the NMR parameters,[13] report results concerning the interactions of cations with two phospholipids; phosphatidylcholine which is zwitterionic and phosphatidylglycerol (PG) which is anionic,[14] and, finally, discuss the behavior of aqueous dispersions of PC-PG mixtures.

Nuclear Relaxation

Relaxation theory has been presented at varying levels of sophistication in specialized lecii,[15-17] and we limit our discussion here to a brief recapitulation and emphasis of some basic and important features that are particularly relevant to the topics in this paper.

The spin-lattice relaxation time, T_1, characterizes the rate at which the spin populations of the energy levels approach a Boltzman distribution. This thermal equilibrium is achieved by transitions between energy levels induced by components of dipolar motion at the resonance frequency, ω_o, and at $2\omega_o$. The transverse relaxation time, T_2 (whose reciprocal is proportional to the linewidth), characterizes the rate of loss of phase coherence in the x-y plane. This process has contributions from spin-lattice relaxation as well as from components of

dipolar motion near zero frequency.

Thus the relaxation rates are proportional to the spectral density function, $J(\omega)$, evaluated at frequencies near zero, ω_o, and $2\omega_o$. The spectral density function, in turn, is the Fourier transform of the correlation function which is used to describe stochastic processes, e.g., molecular motion in liquids. In general, the correlation function is assumed to decay exponentially with a time constant, τ_c, the correlation time. The reciprocal of the correlation time, $1/\tau_c$, is a measure of the maximum frequency components of molecular motion. Figure 1-a shows three sets of spectral density functions for collections of isotropically tumbling molecules characterized by single correlation times, τ_{c_1}, τ_{c_2}, or τ_{c_3}. Figure 1-b shows a function characterized by two different correlation times and represents, e.g., a collection of anisotropic rotators. For isotropic motion the transverse relaxation rate, $1/T_2$, which is proportional to the weighted sum of $J(o)$, $J(\omega_o)$ and $J(2\omega_o)$, will increase linearly with increasing correlation time, except when the motion is very slow. The spin-lattice relaxation time, T_1, will decrease with increasing τ_c until $\omega_o\tau_c \cong 1$, at which point it will increase as τ_c increases further. When the motion is fast, i.e., $\omega_o\tau_c << 1$, $T_1 = T_2$.

A description of anisotropic motions is considerably more complicated.[18] As a relatively simple model, consider a collection of sticks undergoing rapid, random axial motion, and a slower random tumbling of the stick. One correlation time can be assigned to each process, τ_{c_b} and τ_{c_a}, respectively. A spectral density plot for such complex motion is shown in Figure 1-b. The functional dependence of T_1 on correlation time is no longer simple and will exhibit two minima; additionally, when $\omega_o\tau_{c_b} <<1$, $T_1 = T_2$. In such a situation T_2 reflects the slower components of

motion, the tumbling of the stick, and T_1 reflects the rapid axial components of motion. In the case of protons, the relative areas under the spectral density functions for the two types of motion depends on the angle between the inter-proton vector and the rotation axis. When the angle is 90° the two areas are equal.

Consider the type of motion described above for protons when the angle is 90°, the tumbling motion is very slow, and the axial motion is very fast. In this example, the value of T_1 will be <u>within</u> a factor of two of its maximum for the particular value of correlation time; the linewidth, by contrast, will be <u>reduced</u> by only a factor of two. Thus in this example changes in motion may be most directly evident in the values of T_2.

Experimental Procedures

Theoretical prescriptions relating the relaxation rates to correlation times were presented several years ago,[17,19] but experimental methods for determining the values of these relaxation times for molecules yielding complex NMR spectra at low concentrations are of more recent origin.[20-22] Fourier transform NMR, renowned for its sensitivity enhancement or time conservation as compared with the traditional continuous wave methods,[20,21] provides the method of choice for determining relaxation times in complex spectra.[11,22-23] The Fourier transform of the free induction decay following a series of 180° - 90° pulse pairs gives rise to partially relaxed spectra from which the T_1 values can be determined.[22] Similarly, Fourier transformation of the last echo elicited by a series of Carr-Purcell 90° - 180°- - -180° sequences[25] can yield the T_2 values.[11]

(Spectra with resolved J-coupling are not simply amenable to this technique.[24] Measurements of $T_{1\rho}$ will yield the required data for such molecules.) The T_2 values determined by these methods will not be dominated by contributions from the magnetic field inhomogeneities or sample susceptibility factors.[25]

I. The Molecular Dynamics of the Fluid Fatty Acids

Egg Lecithin

Spin-lattice relaxation times for the resolved resonances of sonicated egg yolk lecithin (EYL) are shown in Figure 2. The T_1 values for the vinyl, allyl, and α-carbonyl resonances, as well as those of the choline and terminal methyl protons, are not identical; this observation, together with the narrow linewidths and high resolution nature of the spectra, argue against the proposal that spin-diffusion to a heat sink is a major source of relaxation in sonicated lecithin.[11] That the methylene proton relaxation data could be fit by a single exponential therefore reflects the roughly uniform nature of a component of their motion. It is not intended to imply that all of the methylene protons relax with the same value of T_1, but rather, that any distribution of T_1 values over the entire length of the methylene chain is relatively shallow, and any large departures are limited to short segments. The proton T_1 values have intermolecular contributions in addition to the intramolecular contribution from protons on neighboring carbons as well as those from the protons on the same carbon atom. Studies of anhydrous soaps and n-alkanes suggest that the former contributions may be substantial very near the methyl terminus,[26-29] where the relatively high activation energy has been attributed to intermolecular effects. However, methylene C^{13} T_1 values[30] and their distribution for the majority of the methylene carbons are similar to those for protons and support the suggested interpretation.

All of the proton T_1 values increased with increasing temperature and Arrhenius plots of such data yield activation energies of about 3 kcal/mole, also shown in Figure 2. The fact that the T_1 values increase with temperature (decreasing correlation time), for experiments performed at 220 MHz, shows that the correlation time is less than 10^{-9} sec; calculations which assume axial or isotropic motion [18] suggest that the true value is probably $\sim 10^{-10}$ sec. (The correlation time is not the inverse of a resonant frequency but is related to some average time between reorientational or diffusive jumps.) The value of the activation energy is very similar to that reported for the barrier to internal rotations (trans-gauche isomerizations) in n-alkanes. This evidence, together with the distribution of T_1 values and activation energies for the individually resolved fatty acid protons, argues against rotations of the entire lecithin molecule or rotations of the individual fatty acid chains as the principal sources of thermal relaxation and suggests that trans-gauche isomerizations are largely responsbile for thermal relaxation. This interpretation is supported by the observations that the value of the activation nergy does not substantially differ for fatty acids in micelles or in organic solvents. In addition, estimates of the interconversion rate, $1/2\pi\tau_c$, using an activation energy of 3.0 kcal/mole in the Eyring absolute rate equation[31] are similar to those obtained from the relaxation times.

The implication is that the methylene groups interconvert between trans and gauche configurations at a rate ($1/\tau_c \sim 10^{10}$ sec^{-1}) that increases only slightly over much of the fatty acid chain. This fact is interesting and significant in the light of simple statistical models of fatty acid chain motion. Such models predict that if the probability of a trans configuration at any point along the chain were uniform or increased,

and if the configuration of a given C-C bond were independent of that of any other, the correlation would decrease considerably along the fatty acid chain.[32-35] Since the relaxation time, T_1, is inversely related to the correlation time, τ_c, such a model would predict a pronounced increase of T_1 along the chain (in this region where $\tau_c << 1/\omega_o$), a prediction contrary to observation. There is neither experimental evidence nor obvious physical reason to deduce that the probability of a gauche configuration decreases along the fatty acid chain; and therefore, the configuration of each methylene pair is not independent.

Consider a type of coupled motion in which pairs of gauche configurations of opposite polarities occur on sites separated by one (β-coupled) or a few carbon atoms. Since the conformations resulting from these configurations are roughly straight, as shown in Figure 3, they minimize (or result from) the collisional encounters with neighboring chains that would result from a single gauche configuration, Figure 3. It is physically plausible to anticipate that these configurations have a roughly uniform or slightly increasing probability of occurring at any given position along most of the length of the fatty acid chain, and thus provide a reasonable basis for the T_1 data. Very near the methyl terminus single gauche configurations would not result in collisional interactions with neighboring chains, and the value of T_1 could be expected to increase, as seen in the ^{13}C T_1 values.[30]

Thus a physically reasonable model for the fatty acid motion that accounts for the observed spin-lattice relaxation is one in which gauche pairs separated by one or a few carbon atoms form frequently with a probability that increases relatively little along the fatty acid chain.[10,11]

The values of T_2 determined by spin-echo experiments[11] are shown in Figure 4. Recall that T_1 increases with decreasing τ_c while for each resonance $T_1 \neq T_2$. Therefore for each resonance there are at least two values of the correlation time and, implicitly, two components of motion; one fast which determines T_1, and one slower which makes an additional contribution to T_2. Unlike the spin-lattice relaxation rates which could be fit by a single exponential, the T_2 values increase by about a factor of 2-4 on progressing from the polar end to positions near the methyl terminus where they exhibit a further abrupt increase by another factor of 3-4. This trend in transverse relaxation rates is similar to that reported by others.[36]

Allowance for occasional coupled configurations in which a larger segment of the chain could be displaced (i.e., more than one carbon atom between each gauche-gauche pair, or single gauche configurations, Figure 3) permits a simple modification of the model presented for thermal relaxation to extend it to account for the transverse relaxation.[10-12] No information is currently available on the relative probability of the β-coupled to these other configurations which may vary with position along the chain. It is important to note that relative to T_1, T_2 is sensitive to the angle through which the methylene pair rotates. Thus, T_2, but not T_1, would be affected substantially. Since the details of the motion strongly affect T_2, it is difficult to assign a value of τ_c to the motions underlying the T_2 processes. If the motion were assumed to be either axial or isotropic, the correlation time is estimated to be $\sim 10^{-8}$ sec.[11,12] The ratio of correlation times for the T_1 and T_2 processes can be estimated by calculating an activation energy for the T_2 processes from temperature

data for a specific methylene resonance, assume that the preexponential factors in the Arrhenius equation are equal, and using Equation 1. The ratio so determined is 10^{-2} which agrees with the ratio of correlation times derived from the relaxation data.

$$\frac{\tau_c(T_1)}{\tau_c(T_2)} = e^{-(\Delta E_{a_1} - \Delta E_{a_2})/RT} \qquad (1)$$

Summarizing the foregoing discussion concerning EYL, the relaxation data suggest a model of fatty acid chain motion in which the fatty acid is configurationally mobile yet conformationally relatively ordered. This concept is illustrated in Figure 5. The data presented and the model proposed have been discussed elsewhere and are supported by the results from several other investigators using a variety of different techniques.[12]

As for the T_1 processes, those for T_2 contain contributions from intermolecular interactions as well as interactions between protons on different carbon atoms.

Non-bilayer Systems

NMR studies of fatty acids in non-bilayer structures can help to elucidate those features which characterize bilayers. Free fatty acids and lysolecithin both readily form micelles. For myristate and monopalmitoyllysolecithin the values of T_1 and $\Delta\nu$ at 20°C are 0.4 sec and 6 Hz, and 0.3 sec and 14 Hz, respectively. These values of T_1, which increase with increasing temperature, differ only slightly from those obtained from EYL. The transverse relaxation rates are considerably smaller than those exhibited by EYL indicating that the motion is significantly less anisotropic. A similar conclusion can

be drawn from the T_1 and linewidth data from lecithins in $COCl_3$ and in MeOD. At this time we have no pulsed T_2 data for these systems.

In view of the model proposed for fatty acid chain motion in the bilayers, the less anisotropic character exhibited by the micelles and these molecules in organic solvents reflects the increased probability of motions involving segments of the fatty acids larger than those envisioned for example by the β-coupled isomerizations. Such dynamics would lead to a more disordered system as is observed with spin- labels in similar systems.

Other Lecithins[37]

EYL, dipalmitoyl, dimyristoyl, distearoly, and dioleoyl lecithins have been used as starting points for most work since they are easily obtained in large quantities either from egg yolks or from commercial sources. To determine the role of different fatty acids on bilayer structure, it is necessary to use these and other synthetic lecithins or those from other biological sources. We have studied sonicated dispersions from two commercially available lecithins, dipalmitoyl[10] and dimyristoyl,[11] and reported that their relaxation data are similar to those of EYL and that as the dispersions are heated through their endothermic transition temperatures there is a dramatic increase in motion of all of the nuclei on the molecule, especially those of the fatty acid chain.

More recently we have studied the lecithin from R. pilimanae, a yeast.[13] Relative to EYL this lecithin contains a higher percentage of unsaturated fatty acids, and a higher proportion of its saturated fatty acids are short chains. The dramatic effect of this fatty acid composi-

tion on the NMR linewidths is shown in Figure 6. This figure shows the PMR spectra of unsonicated yeast lecithin which exhibits resonances far narrower than those of unsonicated EYL and thus reflects the much shorter correlation times in the former as compared to the latter. Hence, the primary effect of short chain fatty acids or of monounsaturated fatty acids, or both, is to decrease the correlation time for transverse relaxation.

For unsonicated dispersions of EYL, spin-lattice relaxation is reported to occur by spin-diffusion to a heat sink, presumably the terminal methyl group.[38] That such a mechanism is inoperative in the yeast lecithin is demonstrated by using unsonicated dispersions of this material in which 70% of the hydrogen was replaced with deuterium. Were it to occur, the spin diffusion would be interrupted by the deuterium. In the deuterated samples, both longitudinal and transverse relaxation rates were smaller by a factor of two to three than those in the protonated material. Such a decrease probably reflects the pattern of incorporation of the deuterium, which has a smaller gyromagnetic ratio than that of the proton. These samples also exhibited a distribution of transverse relaxation rates along the chain and values of T_1 which increased with increasing temperature.

Summary of Interpretation

1. Several gauche configurations are present at any instant of time.

2. The methylene groups frequently interconvert between trans and gauche forms; the gauche configurations very often occur in pairs (e.g., β-coupled). These configurations avoid or are imposed by collisional encounters with neighboring chains and result in conformations which are roughly linear.

3. Configurations resulting in "non-linear" conformations occur less frequently than those possessing linear conformations.

4. The probability of occurence of β-coupled configurations relative to other configurations is a characteristic of the particular bilayer.

5. Non-linear conformations and/or increased probability of β-coupled configurations along the fatty acid produce more disorder and result in a flexibility gradient which increases from the carbonyl to near the methyl terminus, where there is an abrupt increase.

6. The flexibility gradient leads to a potential packing problem which can be eliminated by a "statistical bend" in the fatty acid chains.[36]

7. The effect of molecules such as cholesterol is to increase the relative probability of β-coupled to the other configurations and thus increase the order of the chains. Agents which are fluidizers apparently decrease this ratio, decrease the anisotropy, and result in chains which are more disordered.

Biophysical Conclusions

The role of phospholipid fluidity in membrane processes, such as transport and diffusion of membrane antigens, etc., has been discussed by others.[6,7,39] In general, however, it appears that one function of the fluid phospholipids is to provide a two-dimensional quasi-liquid that is relatively impermeable and in which molecular processes requiring the motions of molecules can function; i.e., they provide a pliable, yet relatively impermeable matrix in which conformational alterations and motions of proteins can occur. A more rigid molecular support would either preclude such motions or structural alterations or could be ruptured by such processes, or both. This fluid and flexible character is plausibly the

reason that membranes contain a collection of a large number of small mobile molecules rather than an aggregation of a smaller number of large molecules as are found in cell walls. One may also postulate an evolutionary role for membrane formation from phospholipids. Since they spontaneously form bilayers, it is conceivable that they might have formed the primitive membranes.

Since most membranes contain regions which are flexible in the manner discussed above, it is probable that many membrane processes involve motion or significant conformational alterations. The motional or conformational requirements of transport is one example, and the interpretation that the protein rhodopsin sinks deeper into the membrane upon bleaching is another.[40]

The fatty acid conformations discussed for the fluid phospholipid molecules result in regions of free volume. The migration of such regions provide a mechanism for the transport of small molecules. Träuble[41] has discussed this possibility in terms of kink migration. The proposed configurations are consistent with this concept and can provide larger free volumes than can the kinks. Such a consideration suggests a role for cholesterol. Cholesterol decreases the frequency of formation of regions of free volume, decreases the sizes of these regions, and, as is observed,[42] should decrease the rate of transport of small molecules.

II. The Polar Region of Phospholipids

Phospholipids are generally classed according to their polar moiety;[1,43] some common classes are shown in Figure 7. The more frequently encountered R_1 groups can be arranged as follows: Zwitterionic-choline and ethanolamine; anionic-serine, inositol, glycerol, and phosphatidylglycerol. Additionally, there are sugar containing phospholipids, the glycolipids, and a cationic form, o-lysylphosphatidylglycerol.[44]

Little is known about the structure or function of the polar region of phospholipids. Their importance, however, is inferred from the variety of polar headgroups found in different cells,[43] from the change in headgroup distribution in response to new conditions of growth, and from the specificity of certain classes of phospholipids in the activation of some membranous enzymes.[2] The available information concerning the conformation of the polar region, its role in determining bilayer properties, the function of the different classes, and the spatial arrangement is summarized briefly.

Efforts to determi .e the conformation of the polar regions of model systems have relied on several techniques. Surface pressure and potential measurements on phosphatidylethanolamine (PE) at an air water i-terface have been interpreted as showing that the axis of the zwitterion is parallel to the plane of the surface.[47-48] X-ray diffraction data from multilayers of PC have been interpreted similarly.[49] X-ray analysis of single crystals of glycerophosphorylcholine and similar other headgroup molecules,[50] and NMR conformational analysis of these molecules in D_2O and of lecithin in organic solvents show that the choline methylenes assume a gauche configuration. For ethanolamine the X-ray data differ from those in solution where the methylenes rotate freely.[51]

Several techniques have been employed to study the role of the headgroups in the determination of model bilayer properties. Studies of sonicated and unsonicated aqueous phospholipid dispersions, of monolayers at air-water interfaces, and of black films have shown that the headgroup determines, in part, the permeability of small molecules through these model membranes.[52,53] The effects of divalent cations were important parameters in such experiments. These cations drastically modified the permeability properties of anionic phospholipids and are believed to induce conformational changes in diphosphatidylglycerol,[54] but they have relatively little or no effect on PC.[55] The effects of divalent ions are suppressed in bilayers containing both anionic phospholipids and PC. And finally, Steim has shown that the calorimetric endothermic transition temperature of dimyristoyl PC is 30° lower than that of dimyristoyl PE.[56] Thus, the measured and measurable properties of bilayers are sensitive to the nature of the headgroups, to their heterogeneity in dispersions, and to their interactions with cations.

The functions of the different classes of phospholipids in cellular membranes are not well understood. It is probable, however, that their interactions with divalent ions and their tendencies to form either random mixtures or to segregate can have profound effects.[57] The role of the phospholipid class in a wide variety of cellular functions has been discussed by others.[53,55] In particular, a significant effort has been expended in demonstrating their role in the activation of membranous enzymes[2] and determining the permeability of cellular membranes.[52]

The spatial arrangement of phospholipids has been studied by Bretcher[58] and by Caspar and Kirshner[59] who suggest that different lipids may be segregated between the interior and exterior of membranes, thus rendering them asymmetric.

NMR Studies of Phosphatidyl Choline and Phosphatidylglycerol

The sensitivity of NMR relaxation rates and chemical shifts to the dynamics and environment of the nuclei under investigation has been used to study the dynamics of fatty acid chain motion.[10] It can be exploited similarly for studying the dynamics and environment of the polar region, the interactions of ions with the various headgroups, and the effects of both headgroups and their interactions with ions on the fatty acid chain motions. Some phospholipids, e.g., PC, exhibit well resolved proton resonances, but most dispersions are not so accommodating. By definition, however, all phospholipids contain one phosphorus atom occupying an identical molecular position, affording an excellent NMR probe for this region. Thus we have supplemented our proton data, initially, with phosphorus NMR (ØMR) measurements of sonicated aqueous dispersions of zwitterionic PC[11] and anionic PG.[60]

Lecithin

The choline resonances of lecithin in both sonicated and unsonicated dispersions has been studied by others as well as ourselves.[10] The N-methyl resonance is relatively sharp, $\Delta\nu \cong 3$ Hz, intense, and well resolved from the other resonances.

The ØMR spectra and relaxation rates for EYL and dimyristoyl lecithin have been published earlier,[60] and we review some relevant points. The ØMR of solid L-α-glycerolphosphorylcholine is a gaussian line 4.6×10^3 Hz in width. This width agrees with that calculated to arise

from dipolar interactions with the four nearest neighbor protons whose relative positions were taken from the X-ray data of Sundaralingam.[61] The ØMR spectra of unsonicated vesicles contain two components: 73.5% is 590 Hz in width while the remaining 26.5% is 72 Hz wide. There is some slight indication that these two components exhibit different chemical shifts. Upon sonication for 3 min the line appears single with a width of 20 Hz. The ØMR corresponds to 90 $\pm$ 10% of the total phosphorus and exhibits no further change upon continued sonication. The value of T_2 obtained by a Carr-Purcell sequence was 0.11 sec, which corresponds to a linewidth of 2.8 Hz. This difference between the values of inverse linewidth and transverse relaxation time indicated that there are non-dipolar contributions to the ØMR linewidth in the sonicated materials as is also reported for the PMR.[11] The phosphorus T_1 value is about 1.4 sec. The chemical shift is 5.3 ppm to higher frequency than internal pyrophosphate at pH = 8.9. The phosphorus relaxation mechanisms for these molecules are not yet completely determined and we do not, therefore, assign a correlation time.

The phosphorus resonances of sonicated lecithins are simple and relatively narrow. It is well known that the chemical shifts of simple water soluble phosphates are sensitive to pH, to neighboring atoms, to the solvent, and to metal ions. Hence, it is likely that different phospholipids in differing environments may be distinguishable.

Phosphatidylglycerol

In the initial experiments we were interested in studying various headgroups while maintaining a constant fatty acid composition. To this

end, PG was synthesized enzymatically from PC using phospholipase D and was purified on DEAE.[14, 62-63]

The ØMR spectra and relaxation rates of PG are similar to those of PC, but exhibit some differences. The ØMR of sonicated PG is 10 Hz wider than PC, the chemical shift of PG is 1.8 ppm to lower field of PC, and the PMR linewidth of unsonicated PG is somewhat narrower than that of PC. The origins of these differences are being studied. That the methylene PMR linewidth in the unsonicated PG dispersions is relatively narrower may reflect a less dense packing near the polar end of the molecule resulting from the coulombic repulsion between the charged headgroups. The chemical shift difference may arise from the different chemical nature of the headgroups, from their environments in the bilayers, or from both. Experiments on isolated headgroups are underway to determine the pertinent factors.

The different chemical shifts of these two classes are potentially of importance for it may permit us to distinguish these two headgroups when they are present simultaneously in the same bilayer, and provide information on their re pective environments and dynamics. It is possible that the other classes of headgroups and will exhibit different chemical shifts, and we will examine them and seek to elucidate the origins of the differences.

The effects of the paramagnetic ions Mn^{++} and Eu^{+++} on the NMR spectra of PG and PC are very different. Figure 8 shows the effect of Mn^{++} ions on the intensity of the ØMR. As Mn^{++} is added to sonicated dispersions of PC and PG, the ØMR signal intensities decrease to an aymptotic value of about 38% of the untreated samples. The plateau is reached at substantially lower Mn^{++} concentrations for PG than for PC and the

asymptotic concentrations correspond to the unaltered phospholipids in the inside of the vesicles; the vesicles are largely impermeable to the ions and only those phospholipids on the outside are broadened. This interpretation is substantiated by the calculated ratio of the numbers of phospholipids on the inside and outside and by the fact that additional sonication in the presence of the Mn^{++} completely obliterates the ØMR.[64] Upon the addition of Eu^{+++} ions to PC, 67% of the choline and phosphorus resonances were shifted, as shown in Figure 9. This shifting effect was not to be observed with PG.

In the PG experiments, all of the "external" signal was broadened beyond detectability at concentrations of one Mn^{++} per vesicle, or less. This effect may arise either by a given Mn^{++} visiting a small number of vesicles, and relaxing all exterior nuclei while diffusing around them, or that the ion visits a small number of headgroups for a time sufficient to relax them and then leaves to bind to another site on the same or another vesicle, or by a combination of both. The first process is completed in less than 10^{-3} sec where the residence time of the Mn^{++} at any particular site is less than 10^{-4} sec. In the second process the lifetime at any particular site is less than 1.4×10^{-6} sec.[65]

The interactions of Mn with sonicated PC differ from those with PG. In PC, the ØMR consisted of a relatively sharp peak superposed on a broad signal which broadened further upon the addition of Mn^{++} until a plateau was reached. For this system one calculates a lower limit of 5.3×10^{-6} sec for the residence time at any site.[65] These Mn^{++} interactions are being explored further by measurements of the water relaxation enhancement and by EPR studies.

The NMR literature pertaining to the structural use of rare earth "shift reagents" has virtually exploded in recent years.[66] In many instances the binding of rare earth ions can give detailed information on the structure of the metal complex. To localize more specifically the binding site of the cations and to obtain information concerning the conformation of the headgroup in the presence of cations, we compared the chemical shift of the phosphorus to that of the choline N-methyl protons. A representative ØMR spectrum is shown in Figure 10 and the shifts of the protons and phosphorus lines are shown in Figure 9.

The ØMR shift, which increases with Eu^{+++} concentration, results from rapid exchange between bound and unbound ions, where the exchange rate is greater than 10^{+3} sec^{-1}. One component of the ØMR, corresponding to 63% of the initial peak area, is shifted. We again interpret this as corresponding to the phospholipids on the outside of the vesicles. The N-methyl resonance behaves similarly. The effect of Eu^{+++} on the N-methyl PMR has also been observed by others.[64] For both the PMR and ØMR, there are line broadenings proportional to the magnitude of th shifts.

Additional experiments are in progress to determine the origins of the broadenings, to measure the relative contributions of the ions to T_1 and T_2 processes, to determine the exchange times and activation energies, and determine the fractional contact and pseudo-contact interactions to the shifts. In any event, the observations offer strong evidence that the headgroup conformation is such that the Eu^{+++} is very near the phosphodiester but distant from the N-methyl.

In summary, the simple, relatively narrow resonances, the different chemical shifts and the differential binding of paramagnetic ions observed using phosphorus NMR can be exploited to measure lateral diffusion rates, demonstrate asymmetric distributions of phospholipid classes between the two bilayer surfaces, and to explore the spatial distribution of different classes of phospholipids on a single bilayer surface. The problem of spatial distribution is illustrated in Figure 11 which shows a schematic view of a bilayer from the top. The question of interest is what is the arrangement of two or more different classes of phospholipids, i.e., patched or random, in model and cellular membranes. The differential effects of ions on PG and PG phosphorus and proton resonance and possibly chemical shift differences can be exploited to answer questions like these. The importance of lipid arrangements is suggested by experiments which show a specific phospholipid requirement, PG, for a PEP phosphotransferase dependent glucose transport system in E. coli.[2]

PC-PG Mixtures

In our initial experiments we found that Mn^{++} decreased the choline N-methyl proton peak height more rapidly for PC-PG mixtures than it did for pure PC vesicles. Further, the plateau was at 60% of the original peak area. These data suggest a partial randomization and an asymmetric distribution between the two bilayer surfaces with PC preferring the inner monolayer. These preliminary data are encouraging and more complete. Data and interpretations will appear elsewhere.[14]

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Figure Legends

Fig. 1. a) Spectral density functions, $J(\omega)$, for collections of molecules undergoing isotropic, random motion, described by the correlation times τ_{c_1}, τ_{c_2}, and τ_{c_3}. b) A spectral density function, $J(\omega)$ for a collection of molecules undergoing rapid, random, axial motion, described by the correlation time τ_{c_b}; and a slower random tumbling described by the correlation time τ_{c_a}. ω_o is the resonant frequency.

Fig. 2. The spin-lattice relaxation times, T_1, and activation energies, ΔE_a, for the ^{31}P and the resolved proton resonances in egg yolk lecithin. The 220 MHz PMR measurements were made at 40°. The ^{31}P data were obtained at 24.3 MHz.

Fig. 3. Palmitic acid with a) a single gauche configuration, b) two gauche configurations separated by six carbon atoms, and c) two gauche configurations separated by one carbon atom (β-coupled).

Fig. 4. The transverse relaxation times, T_2, for the ^{31}P and the resolved proton resonances of sonicated egg yolk lecithin. The values are estimated by a spin-echo Fourier transform method at 20°.

Fig. 5. A lifelike illustration of the configurationally mobile, yet relatively ordered, fatty acids of lecithin.

Fig. 6. The 220 MHz PMR spectra of yeast and egg lecithin dispersions. The spectrum of unsonicated DYL was recorded using a larger sample than was used for the other spectra. The changes in relative peak intensities between the PYL and DYL samples reflect variations in the amount of deuterium incorporated in the different positions of DYL. With the instrument settings used, the HOD peaks were off-scale and therefore were not scanned. The chemical shifts are relative to TMS and the resonances are assigned

according to Chapman[21] and Dea.[22] SSB = spinning sideband of the HOD.

Fig. 7. A phospholipid molecule with a saturated and an unsaturated fatty acid. In general n = 12-16, and m = n' = 7. The R_1 groups shown are only a few of many possibilities; they are, from top to bottom, choline, glycerol, and ethanolamine.

Fig. 8. The relative change in area of the original resonance as $MnCl_2$ is added to sonicated egg yolk lecithin and phosphatidylglycerol derivatives from this lecithin. The concentration of vesicles is indicated by (a), and the signal intensities after sonication by (b).

Fig. 9. Eu^{+++} induced chemical shift of ^{31}P and choline N-methyl protons of sonicated egg yolk lecithin (30 mM).

Fig. 10. ^{31}P NMR spectrum of 30 mM sonicated egg yolk lecithin. The external reference is pyrophosphate at pH = 9.0. "Outside P" corresponds to those phosphorus atoms on lipids in the outer bilayer, while "inside P" corresponds to those in the inner bilayer.

Fig. 11. A bilayer viewed from above. This illustrates the possible spatial arrangements of two different classes of phospholipids and the different possible interactions between neighboring phospholipids in the presence and absence of metal ions.

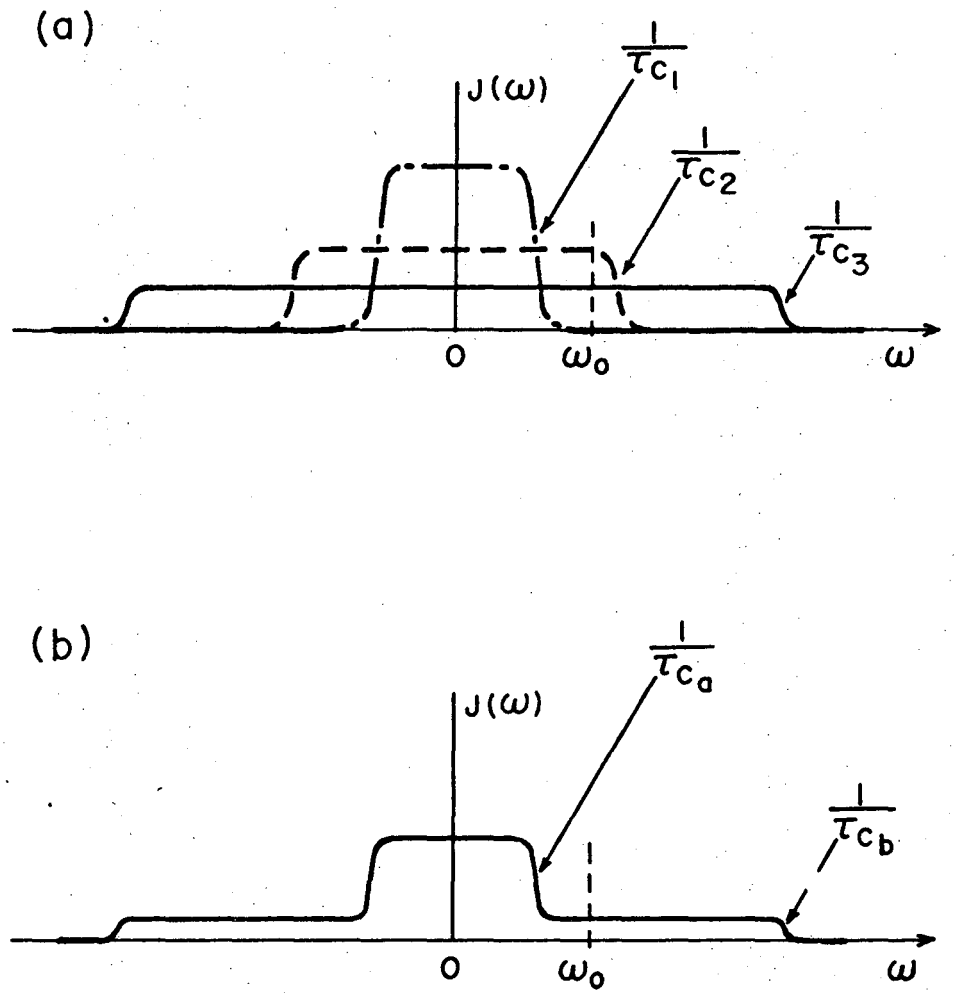

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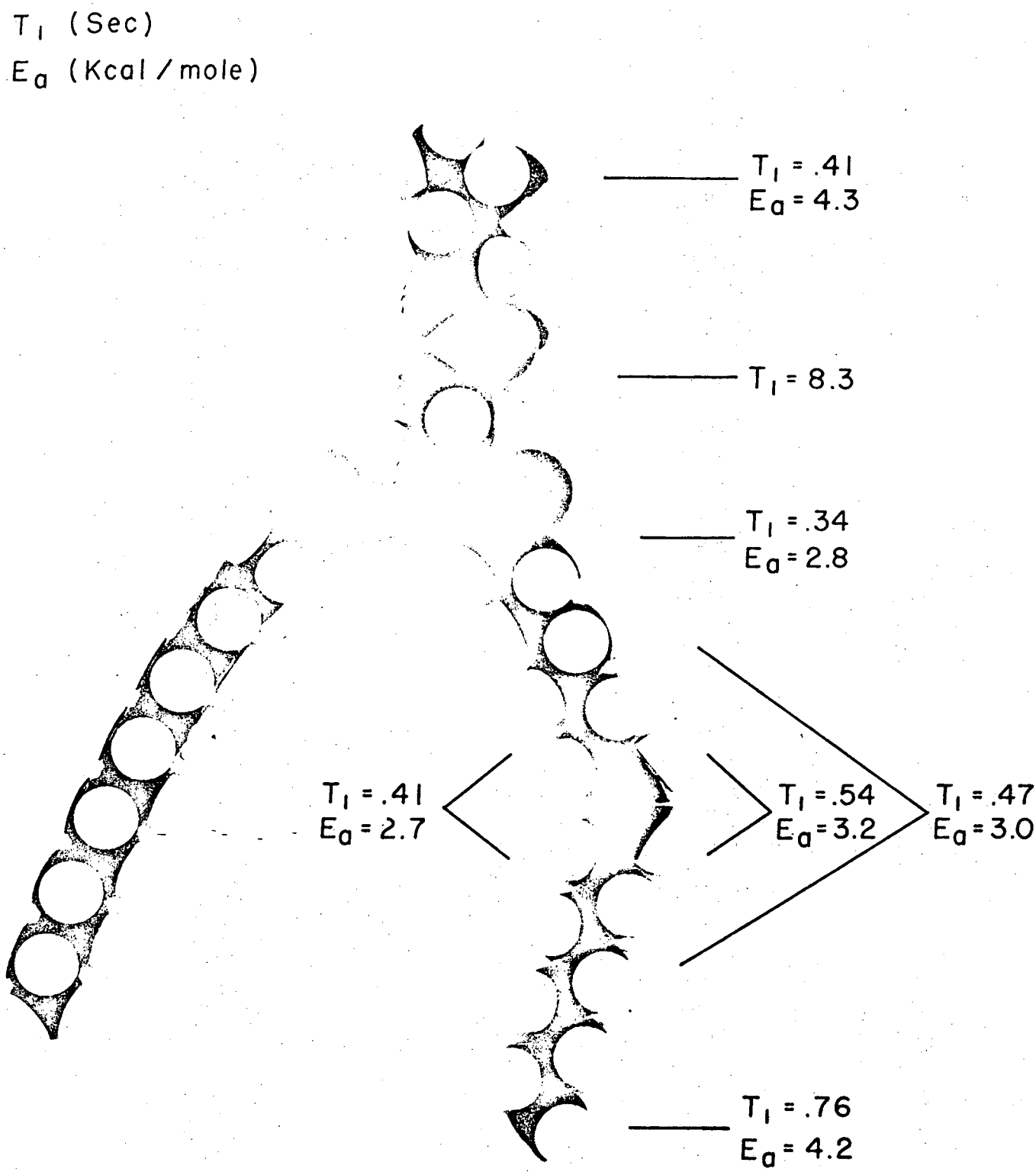
Fig. 1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T_1 = .41
E_a = 2.7
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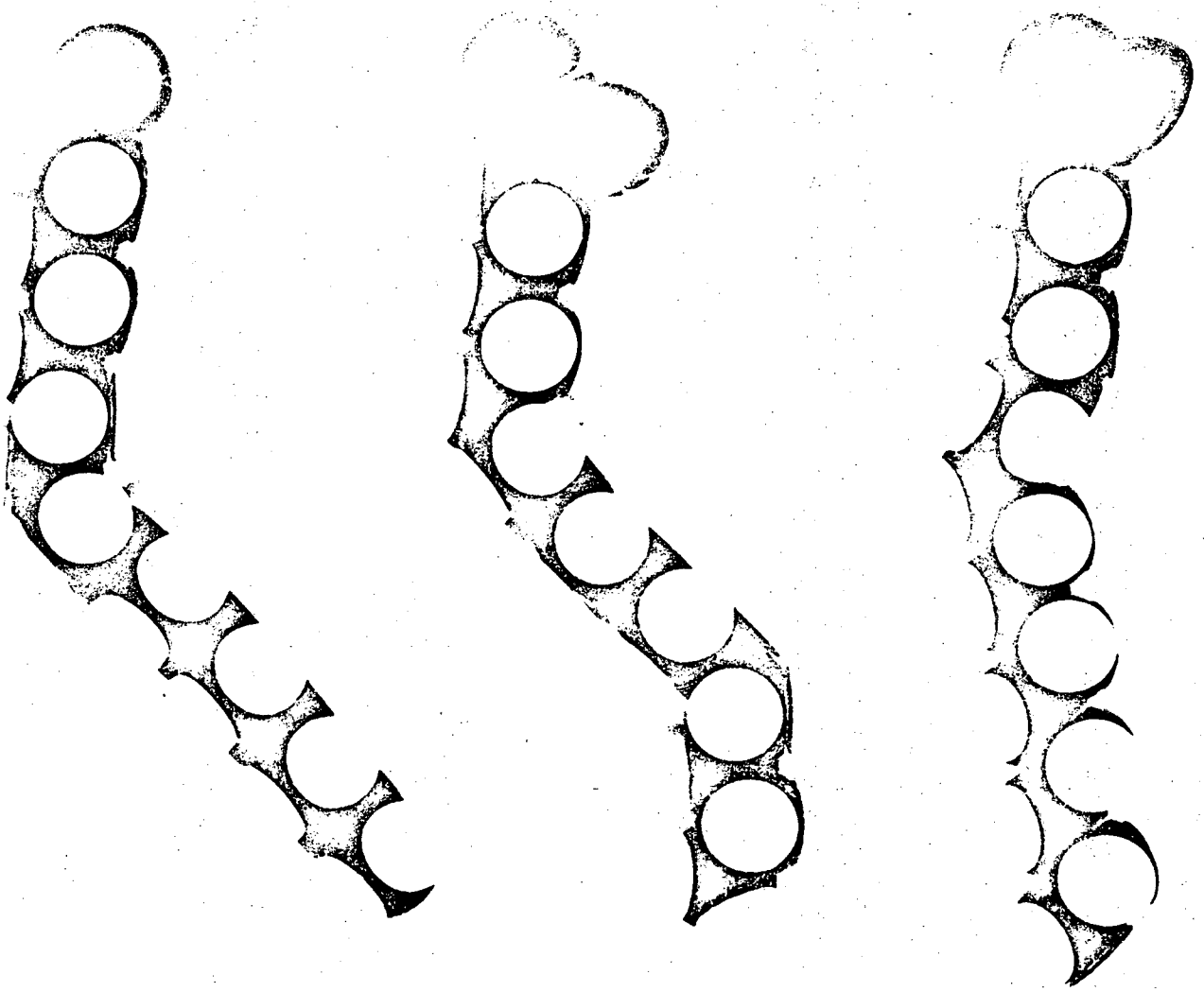
E_a = 4.2

Fig. 2



(a) (b) (c)

Fig. 3

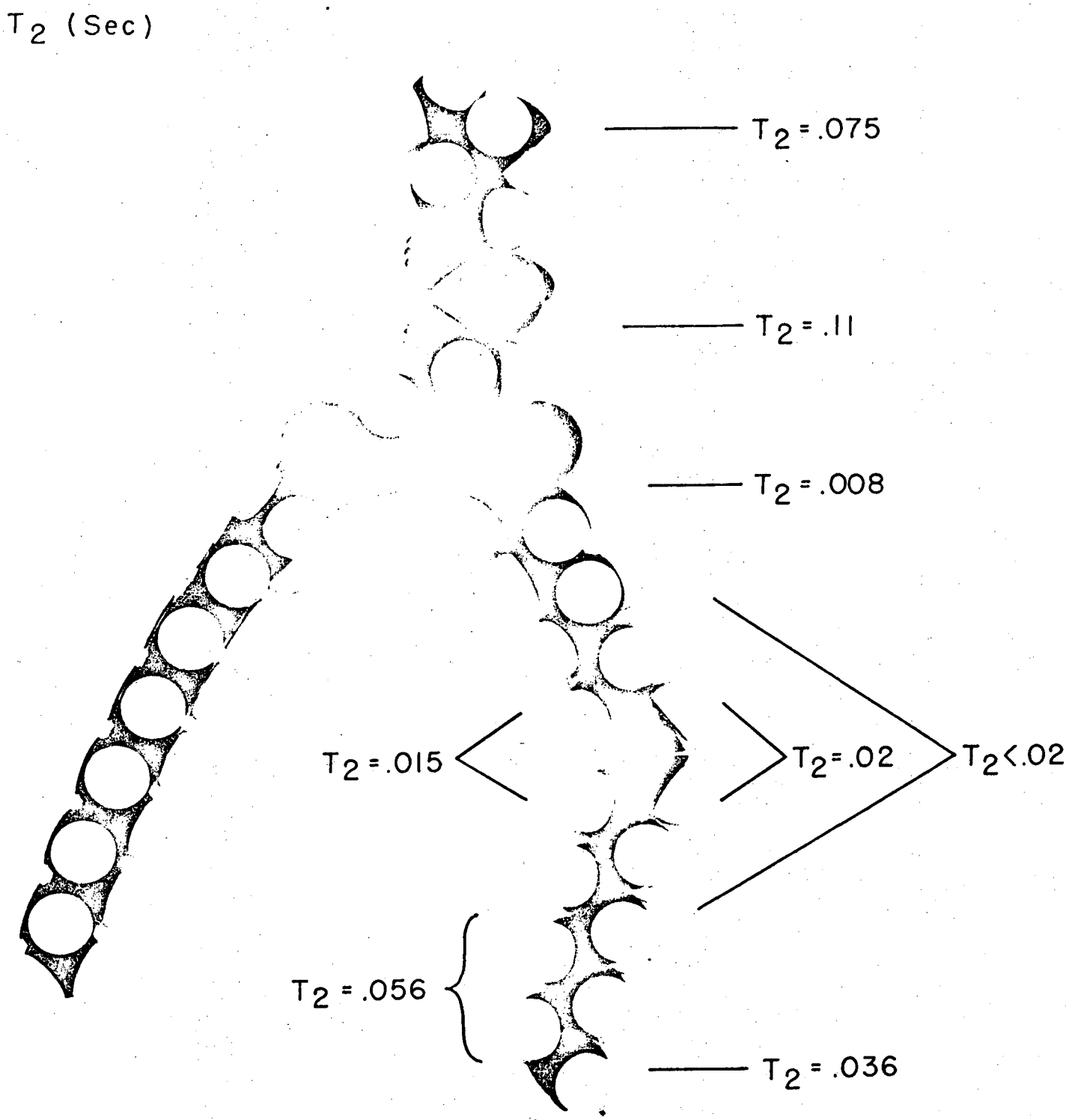


Fig. 4

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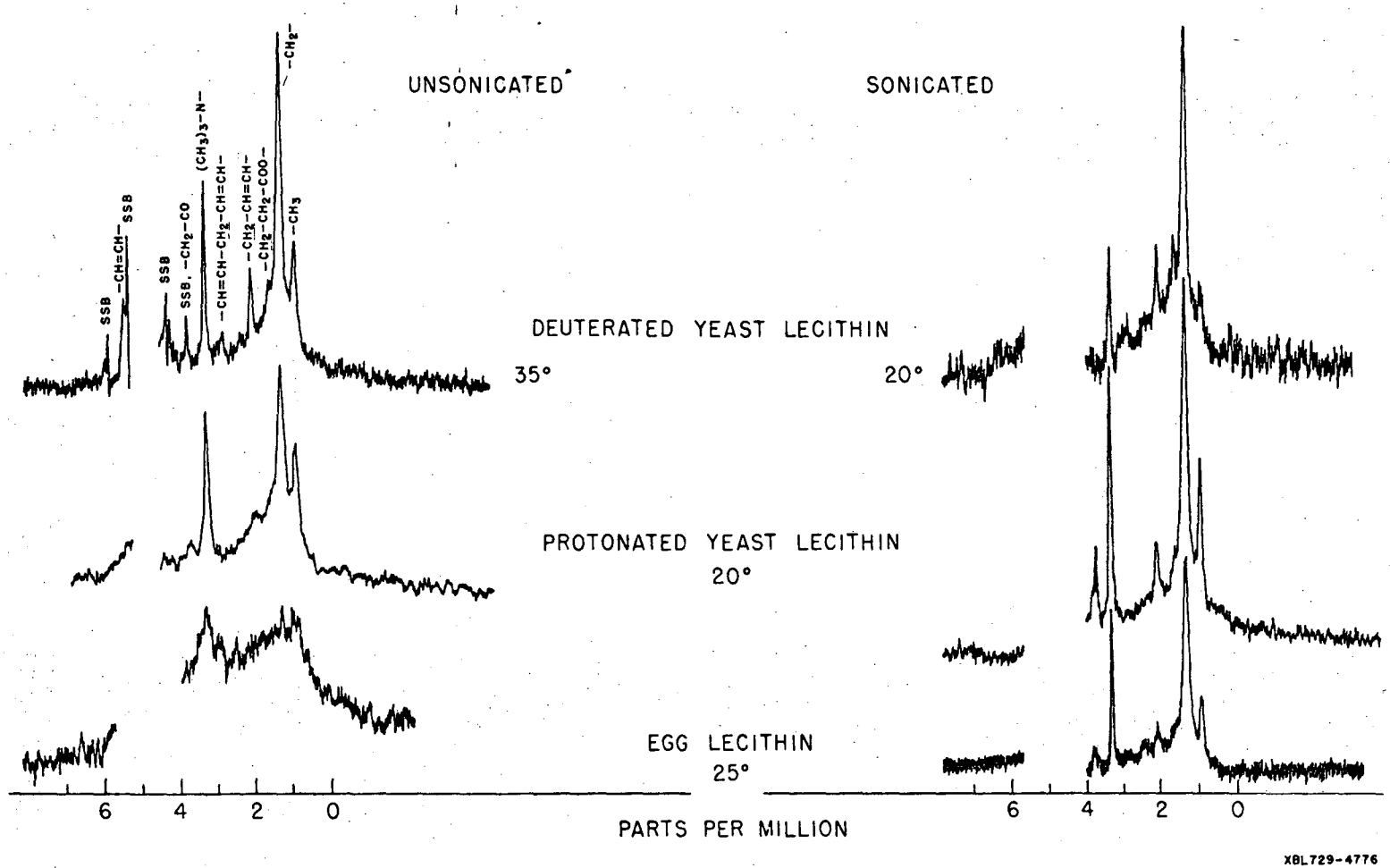
Fig. 5


UNSONICATED
SONICATED
SSB
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SSB
SSB
SSB, -CH2-CO
(CH3)3-N-
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-CH2-CH=CH-
-CH2-CH2-COO-
-CH2-
-CH3
DEUTERATED YEAST LECITHIN
35°
20°
PROTONATED YEAST LECITHIN
20°
EGG LECITHIN
25°
6
4
2
0
PARTS PER MILLION
XBL729-4776

Fig. 6

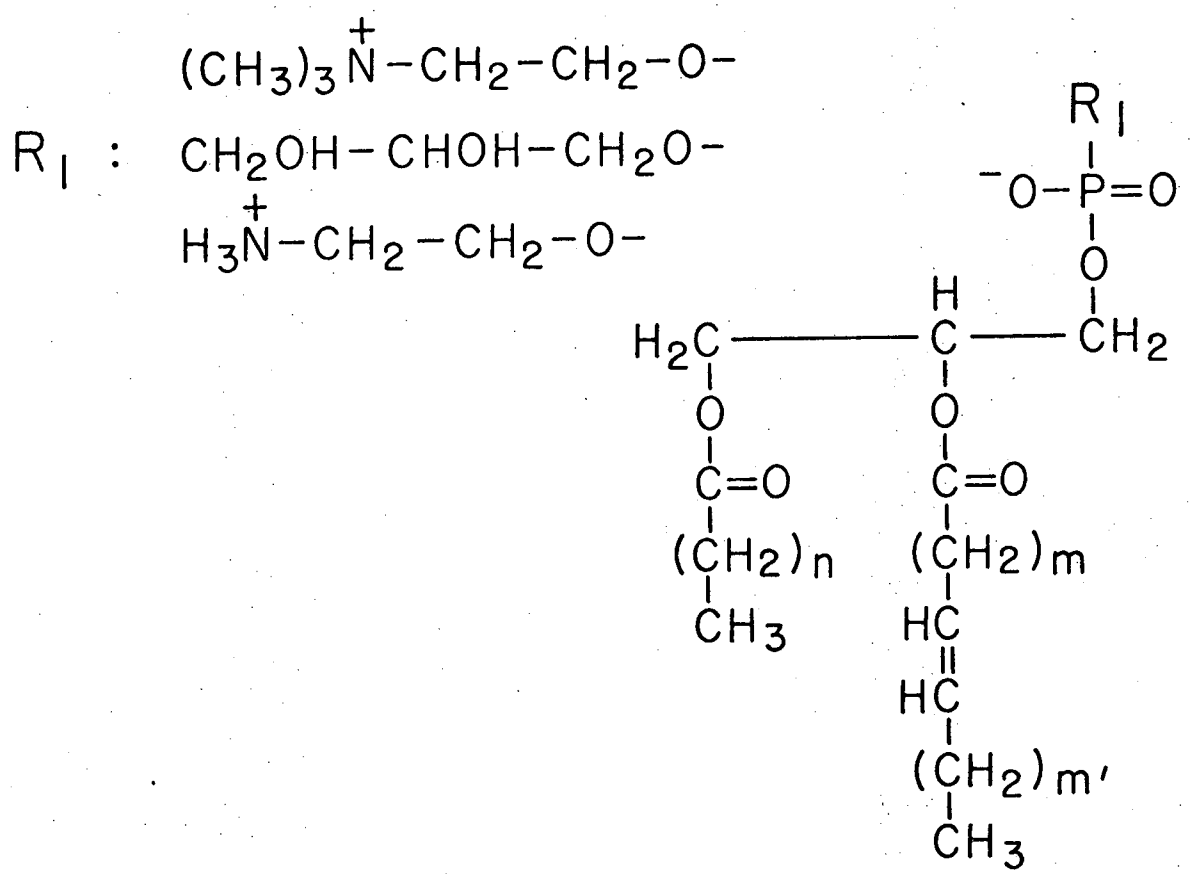

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Fig. 7

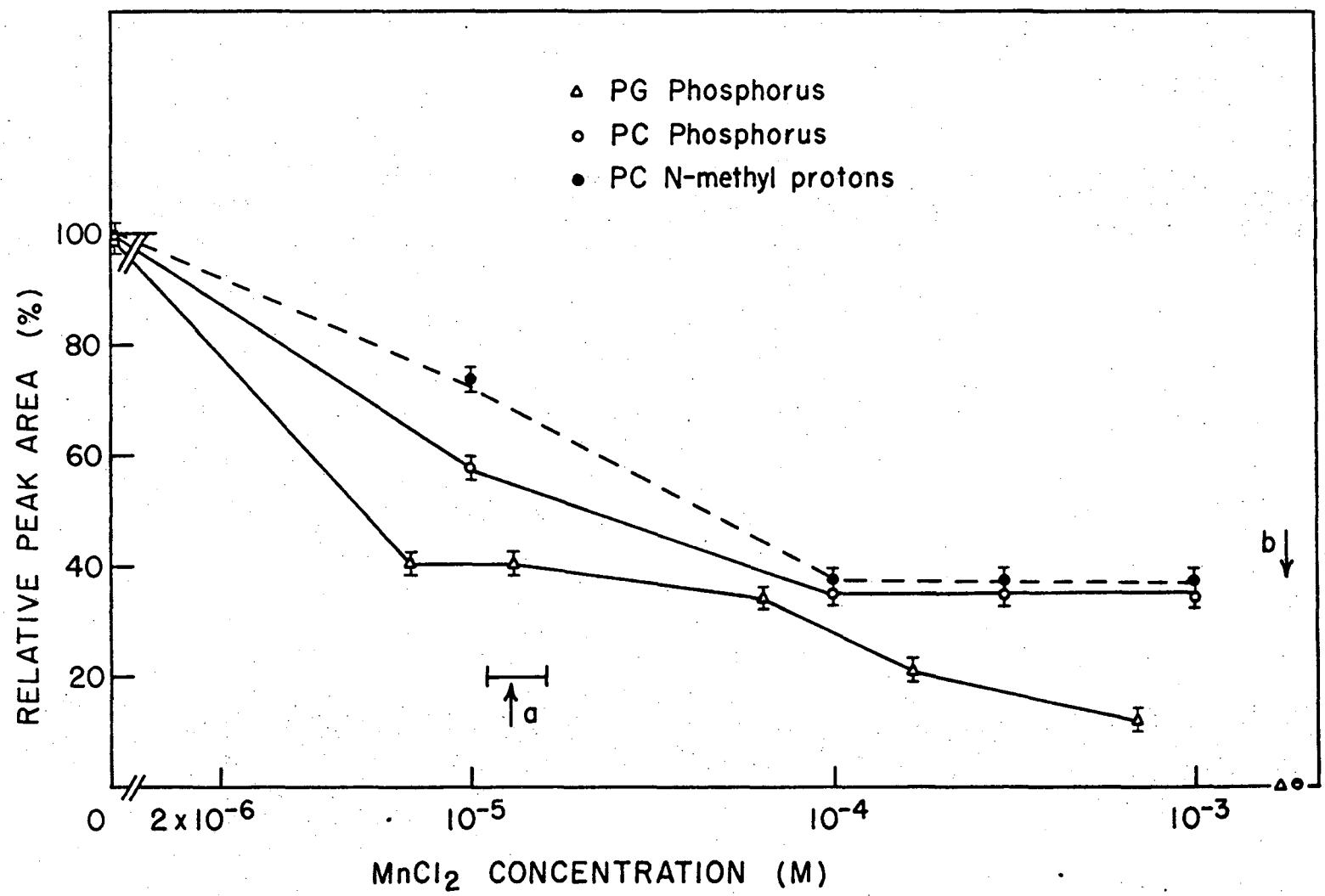

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Fig. 8

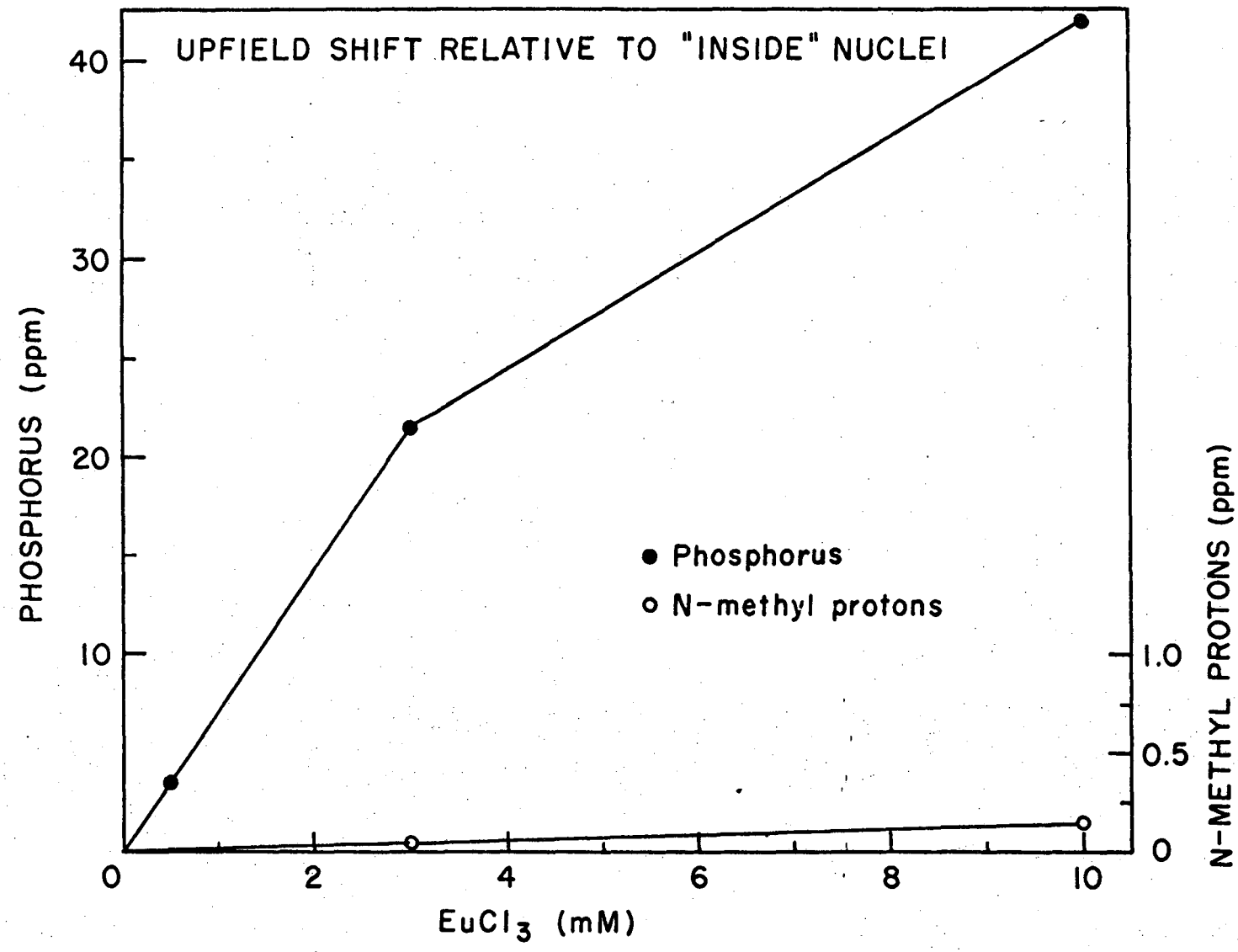

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Fig. 9

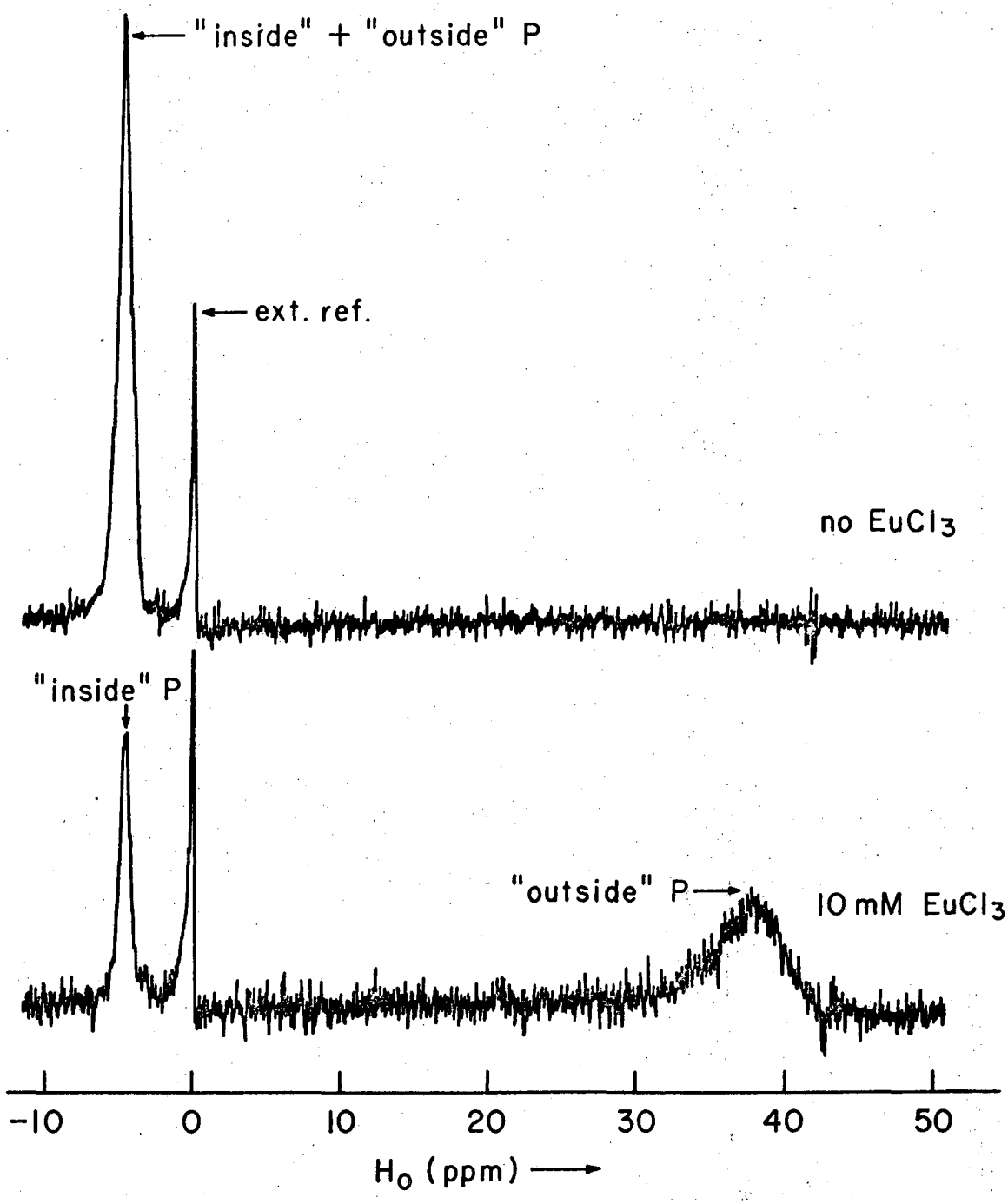

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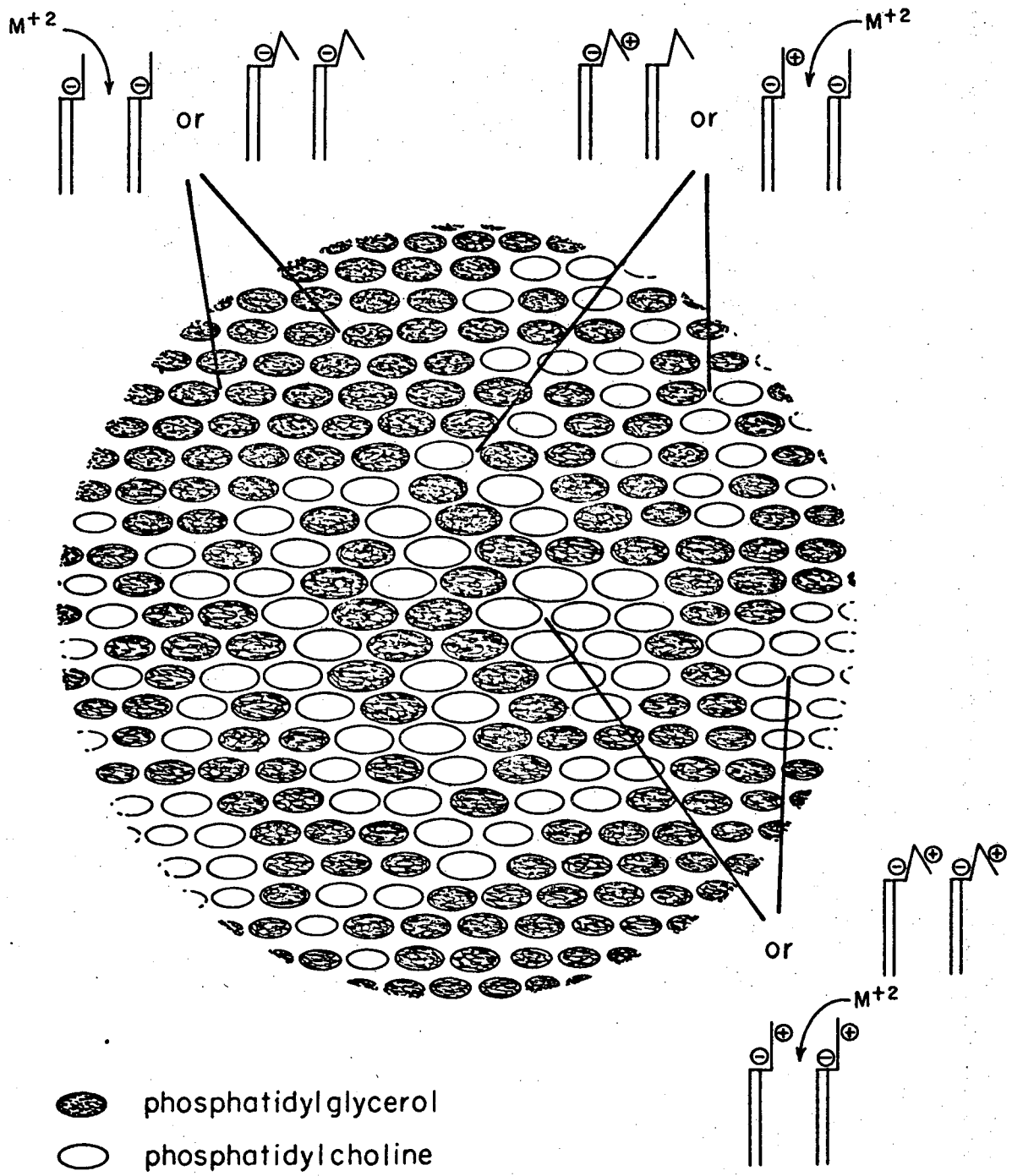
Fig. 10



XBL 7211-4797

Fig. 11

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