PROTON MAGNETIC RESONANCE STUDIES OF MEMBRANE SYSTEMS

I. EFFECT OF SONICATION AND OSMOTIC PRESSURE ON THE STRUCTURE OF THE LECITHIN BILAYER

II. PMR STUDY OF THE HUMAN ERYTHROCYTE MEMBRANE UPON PERTURBATION

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To Noni, Nenni and Kack

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ABSTRACT

I. Effect of Sonication and Osmotic Pressure on the Structure of the Lecithin Bilayer

The properties of dipalmitoyl lecithin vesicles of varying sizes have been compared employing proton and ^{31}P nmr spectroscopy and dilatometry. These studies indicate that small vesicles of about 250 Å in diameter are inherently disordered and that the well-known spectral differences generally observed for vesicles of different curvatures, such as lecithin multilayers and sonicated bilayers, arise from variations in the molecular packing of phospholipid molecules in the bilayer phase. Upon further perturbation of the membrane by an external osmotic pressure spectral changes were noted which were consistent with the expected changes in molecular packing. It was also noted that the structural rigidity of the bilayer appeared to change upon melting.

II. PMR Study of the Human Erythrocyte Membrane upon Perturbation

An analysis of the pmr spectra of human erythrocyte membranes has been made in terms of the membrane components. The spectrum at 75° was assigned to 20% of the membrane proteins, 20% of the choline methyl groups of the phospholipids, and the acetamide groups of the sugars. Changes in the pmr spectra were noted when the membranes were altered by either protein solubilization or membrane fragmentation. The choline methyl signal was found to be

particularly sensitive to the state of the membranes. Certain proteins were found to be released from the membranes at high temperatures and this phenomenon was dependent upon the pH and the solvent, as well as the presence of certain ions in solution. The amount of proteins solubilized, however, was significantly less than that observed by the high-resolution pmr method. The effect of certain divalent ions was particularly striking. The presence of more than 5×10^{-4} M Mg^{2+}, for example, was sufficient to stabilize the proteins in the membrane and eliminate the high-resolution pmr signal.

B. cereus and Cl. perfringens phospholipase C treatments were found to have quite different effects on human erythrocyte membranes from the pmr spectra of the treated membranes. These differences are explained in terms of the properties of the unreacted lipids left by the two enzymes.

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PART I

EFFECT OF SONICATION AND OSMOTIC PRESSURE ON THE STRUCTURE OF THE LECITHIN BILAYER

1. INTRODUCTION

Biological membranes have come under careful scrutiny recently in an attempt to understand the mechanisms of active transport, nerve transmission, and other membrane functions. Most biological membranes, however, are complex structures of lipids, proteins and sometimes carbohydrates in lamellar arrays. In the past they have confounded efforts to characterize them exactly in vivo or in vitro. Early investigators, however, did notice that the membrane barrier was most permeable to lipid soluble materials and good correlations were found between the olive oil-water partition coefficients and the permeability coefficients for different compounds[1]. Other ions and molecules did move up a concentration gradient in crossing the membrane and were not lipid soluble. These compounds were presumed to be actively transported across the membrane with energy from ATP hydrolysis by an unknown mechanism. With this and other information from known surface chemistry and electric impedance studies, Davson and Danielli[2] formulated the hypothesis that a lipid bilayer was the basis of the biological membrane and proteins coated the bilayer surfaces. That hypothesis lived for many years and only recent data have caused it to be modified. The lipids are still believed to be largely in the bilayer phase (also liquid crystalline or smectic mesophase). Evidence for this has come from freeze-etch microscopy[3], differential scanning calorimetry[4, 5], x-ray diffraction[6], as well as electron spin resonance spin-label studies[7-10]. Other phases such as the cubic or hexagonal phase have been commonly found in

lipid-water mixtures of low water content[11], but none of these phases have been shown to exist in biological membranes. There has been, however, much speculation about other phases[12] to explain ion transport and nerve propagation phenomena. The active and facilitated transport of molecules does appear to be carried out by the membrane proteins and not the lipids although the proteins are dependent on surrounding lipids for activity[13]. These transport proteins function to carry ions or molecules across the barrier of the lipid bilayer and their effects are added to the basic leakage fluxes of a bilayer. In terms of structure also it is felt that the lipid bilayer is the basic unit to which proteins are added to form a functioning membrane[14]. For these reasons lipid bilayers are the model systems of choice in membrane research. Further, it is important to the analysis of data from biological membranes to be able to separate the contribution of the lipid bilayer from that of the protein. We have, therefore, attempted to characterize further the structural phenomena of lipid bilayers alone. It is felt that some of the findings point up features of these model systems which are also important in the structure of biological membranes.

1.1. Types of Bilayers

The next question is what type of lipid should be used for model system work. There is a vast variety of biological lipids which will form a bilayer phase since the different types of lipids can also have a wide range of fatty acid components. The major group of lipids in

nearly all natural membranes is the phospholipid, and as a consequence other lipids such as steroids, carotenoids, etc. have received much less attention. Generally, the phospholipid bilayers are characterized by a charged group at the water interface connected through glycerin ester linkages to two fatty acids which are aligned perpendicular to the bilayer plane in the hydrocarbon interior. The charged group contains a phosphate diester linked to both the glycerin backbone and a neutral or positively charged group (choline, ethanolamine, serine, inositol, etc.). Phospholipids are normally zwitterionic or are negatively charged although gram positive bacteria do contain a positively charged phospholipid (phosphatidyl lysine). The actual phospholipid composition of many different biological membranes has been extensively studied and catalogued[15]. These data show a wide variety in the phospholipid compositions of different membranes even from the same organism. This suggests that phospholipid charge groups play an important role in membrane function which is further borne out by the fact that certain enzymes require a specific phospholipid for activity[16]. This is not to say that the phospholipid composition of any membrane is rigidly fixed since certain lipids fall into substitution groups such as the choline lipids or the charged lipids[17]. Still there seems to be limits to the variations in phospholipid concentrations which indicate that no single model system can be considered general. Many investigators have, however, settled on lecithin, a zwitterionic choline phospholipid, since it is the most common lipid in animal systems. The work to be reported here is largely on lecithin for that reason.

There are many kinds of lecithin, bovine, egg, soybean, etc. which have different fatty acid compositions. Because the fatty acids are not strongly correlated with certain charge groups, their composition is generally treated separately from the charge group distribution. The fatty acids found in biological membranes normally range from 14 to 24 carbons in length with only an even number of carbon atoms and have from 0 to 6 cis double bonds along the chain. The first double bond is normally found at the 9th carbon and subsequent double bonds at the 12th, 15th, etc. such that the double bonds are separated by one CH_2- group. In a phospholipid molecule the two fatty acids are most often different and the fatty acid in the 2 position of the glycerol is normally unsaturated[18]. In biological membranes the fatty acid composition can vary widely even with simple changes in diet; however, there are limits to the variability[19]. In general, these limits are to be related to the disorder or mobility of the fatty acids since lipid motion is felt to be an important factor in membrane function. Mobility of the fatty acid is in turn related to the length of the fatty acid chain and the number of unsaturated groups such that shorter and more unsaturated fats tend to disorder the bilayer. These trends are noted in the crystalline-liquid crystalline phase transition of the various lecithins. For example, this transition occurs at 20°C, 40°C and 63°C for C_{14}, C_{16} and C_{18} saturated fatty acid lecithins and -7°C and 63°C for a C_{18} fatty acid lecithin with and without a double bond[20]. Greater disorder is manifested in greater permeability of liposomes, natural membranes, and black lipid films for carrier mechanisms[21-23]. Pore diffusion rates very obviously should not be affected by the fatty

acid mobility; however, the rate of forming and breaking of pores might well be affected also. The mobility of the fatty acids seems to be a very important parameter in describing any bilayer and an increasing number of applications of this principle are being found in biology.

1.2. Physical Methods

The description above of the lipid composition of biological membranes leads us to choose lecithin for model lipid system studies. As a model system lecithin has another important property in that a vast amount of work has been done on it. For a number of years investigators have been trying to understand the physical aspects of lipid bilayer structure by studying two-phase lecithin and water systems. A number of different techniques have been applied successfully to lecithin bilayers and have provided a general understanding of the bilayer structure. Building on this basis we have attempted to understand certain of the structural restraints on the lecithin bilayer. In the following section we will summarize the present understanding of lecithin bilayer structure by experimental method used.

1.2.1. Differential Scanning Calorimetry. Phospholipids were found to undergo a highly endothermic transition in the solid phase well below the capillary melting temperature[20]. This crystalline to liquid crystalline phase transition of lecithin was also observed when the lipid was dispersed in water although hydration to form an α-gel lowered the transition temperature. The magnitude of the entropy increase in the transition was directly related to the length of the two

saturated fatty acids in the lecithin as is the case for n-alkanes, saturated triglycerides and saturated fatty acids upon fusion, which means that the entropy change is configurational in nature[24]. Other thermodynamic variables are also related to alkane length but do not exhibit the lack of temperature dependence of entropy or the strong correlation to mobility. Phillips et al. compared, therefore, the configurational entropy change per methylene group of lecithin in the α-gel to smectic mesophase transition (1.25 e.u.) with that of an alkane in the crystal to isotropic liquid transition (2.6 e.u.). The lower entropy change for lecithin methylenes was interpreted as a reflection of lower fluidity in the lecithin bilayer as opposed to the molten alkane. This bilayer state of intermediate fluidity is a vexation to many investigators because of the difficulties in theoretically treating states of intermediate mobility.

In addition to the entropy of the transition the temperature of the transition has been fruitfully studied for different phospholipids and their mixtures. Since the transition is endothermic, an increase in the transition temperature means a lower enthalpy for the system. In this regard it is interesting to note that dipalmitoyl phosphatidyl ethanolamine has a transition temperature 30° higher than that for dipalmitoyl lecithin thus indicating that the ethanolamine charge group undergoes a greater enthalpy change upon melting than a choline charge group in the bilayer[25]. Perhaps as a consequence of this difference in bilayer stability when phosphatidyl ethanolamine was substituted for phosphatidyl choline in pneumococcus, the cells remained associated after cell division and were not lysed as easily[26].

If the fatty acids were unsaturated, the transition temperature was drastically lowered as in dioleoyl lecithin (-22°) as opposed to distearoyl lecithin (58°)[24]. The presence of the cis double bond apparently disrupted the Van der Waals interactions between neighboring chains raising the enthalpy and entropy of the system. Although it is not necessarily obvious, the presence of unsaturated fatty acids also raises the passive permeability of model bilayers[21].

In the α-gel as in other crystalline organics the addition of a foreign type of molecule lowers the transition temperature. A case in point is cholesterol which, when added to lecithin in the α-gel phase, lowers the transition temperature and in a 1:1 mixture wipes out the phase transition completely[27]. These important thermodynamic data about the lecithin bilayer phase are valuable products of differential scanning calorimetry studies of bilayers.

1.2.2. X-Ray Diffraction. It is difficult to understand any system without accurate knowledge of its overall dimensions and x-ray diffraction has provided those data for lipid bilayers. Because of the regularity of the bilayer thickness observers were able to measure that distance under a variety of conditions and also the lateral area. The other spacing which they were able to follow was a relatively broad band at ~4.6 Å from the hydrocarbon chains. If the lecithins were in the α-gel or crystalline state this spacing was at 4.2 Å and sharp[27]. From measurements of the angular dependence of these spacings in oriented bilayers it is known that the 4.6 Å reflection is generally oriented perpendicular to the bilayer reflection or parallel to the

fatty acid chain axes. Exact measurements of the angular distribution of the 4.6 Å spacing in egg lecithin bilayers indicates that the spacing is not rigidly oriented at 90° to the bilayer plane and even has a significant value for angles parallel to the plane[28]. Levine and Wilkins interpret this in terms of domains of segments of chains in which the segments are oriented parallel to the bilayer plane. They also add that there is no evidence for regular domains from the long spacings as were observed for other systems where domains were known to occur. They use a model of segments of a rubber molecule with fixed ends to predict the observed angular distribution rather than the gaussian model for the configuration of chains suggested by Luzzati[29]. They also observe that the presence of cholesterol causes a higher asymmetry or orientation of the fatty acid chains as measured by the stronger orientation of the 4.6 Å spacing.

From the amplitudes of the lamellar diffractions an electron density distribution of the bilayer can be calculated and these distributions show a lower electron density in the center of the bilayer. This lower electron density is possibly a reflection of the greater mobility at the methyl ends of the fatty acids, the differences in chain lengths, the presence of bent fatty acid chains[30] or the presence of kinks further up the chains[31].

The thickness of the bilayer and the area of the lecithin molecule were found to be reflective of changes in the bilayer state. For example, upon the transition from α-gel to liquid crystalline phase there is a decrease in the thickness of the bilayer from 46 to 41 Å and an increase in lateral area from 48 to 58 Å^2[32]. Also the addition of

cholesterol to dipalmitoyl lecithin causes an increase in bilayer thickness presumably because it orients the fatty acids perpendicular to the bilayer whereas they could orient at other angles without cholesterol[33].

1.2.3. Electron Microscopy. The application of electron microscopy to the study of lecithin bilayers has produced a number of interesting and exciting artifacts[34, 35]. More importantly, however, it has given us pictures of the gross morphology of lipids in water under a variety of conditions. For example, it gives us an excellent means of sizing sonicated vesicles[36] or visualizing liposomes[37]. Papahadjopoulus and Miller[38] have also used it to understand the morphology of different lipids in water.

1.2.4. Dilatometry. Changes in the specific volume of fatty acids and their glycerides have been noted at the transition from the solid to liquid phase[39]. Since the α-gel to smectic mesophase transition of lecithin was similar to a solid to liquid phase transition, it might be expected to cause a significant specific volume change. Träuble and Haynes did observe a sharp volume increase at the transition temperature in a lecithin suspension[40]. The percentage increase they observed, however, was only 1.4% versus 20% for the corresponding fatty acid or triglyceride. X-ray evidence also indicated an increase in specific volume at the transition but the percentage increase observed was $5\% \pm 5\%$[41]. Träuble and Haynes had no accurate measure of their lipid concentration which leaves their data suspect, and further experiments reported here will give more precise measurements of the apparent molal volume change. In any case the actual

volume increase is much less than in the case of the free fatty acids or glycerides which follows from the fact that the α-gel to smectic mesophase transition is a solid to solid phase transition. Perhaps the transition should be compared with the rotational phase transition of paraffins which is accompanied by a 2 to 4% volume change as determined by x-ray studies[41].

1.2.5. Black Lipid Membranes. We have limited our discussion until this time to studies of pure phospholipid bilayers, but there is another type of bilayer called the black lipid membrane which has received considerable attention. Several recent extensive reviews have been written on the topic and cover many more aspects of black lipid film work than we will attempt to discuss here[42-44]. These membranes, although they contain phospholipids and are the thickness of a lipid bilayer, also contain a considerable amount of solvent such as n-octane or decane since they are formed from only a 5-10% solution of the lipid. Once this solution is painted across a hole in a teflon sheet it appears brilliantly colored by reflected light and suddenly a black spot, indicating a very thin membrane, will appear and spread to cover most of the area. At the edge of the black lipid membrane is a torus of excess lipid and solvent.

Although these bilayers are not 100% phospholipid, their electrical properties do appear to be nearly the same as those of phospholipids in biological membranes[45]. The important facts are that black lipid membranes are relatively easy to prepare and a wide variety of experiments can be performed on them. Of current interest are the

effects of various compounds on bilayer electrical properties. The purpose of these studies is to better understand the action of nerve membranes and biological ion transport in general. Small peptide antibiotics have been helpful in such studies since certain ones have been known to alter radically the ion permeability properties of black lipid membranes. Some of these antibiotics, such as valinomycin and nonactin, have been shown to be ion selective and to increase ion conductance continuously in an almost linear relation with antibiotic concentration. Others, such as alamethicin[46], gramicidin A[47] and EIM[48], cause time-dependent jumps in the conductance with the total conductance equal to an integral number of these jumps. The former type of antibiotic is classed as a carrier and the latter as a pore former.

The carrier antibiotics have been used in a number of studies of the surface charge of the bilayer and its effect on the distribution of ions at the surface[49, 50]. The results were consistent with the predictions of the Gouy-Chapman theory of the diffuse double layer. This means that the apparent membrane charge is highly dependent on the ion concentrations in the surrounding medium. Local anesthetics and other small amphiphilic charged molecules were found to strongly affect the charge density on the membrane and consequently the ion transport properties[51]. Most significantly, these studies follow a predictable behavior in line with the existing theory of the diffuse double layer, indicating that the charge properties of at least black lipid membranes and probably lipid bilayers are well behaved.

Using black lipid films of glyceryl distearate and glyceryl

dipalmitate in decane which underwent a phase transition at about 40°C, Krasne et al.[23] found that the membrane conductance with carriers present was a thousand-fold lower when the lipids were in the "frozen" rather than the "melted" state. On the other hand the channel-former, gramicidin A, had the same effect on the membrane above and below the melting temperature. The authors further feel that the difference with the carriers was not due to a difference in solubility but was an effect of the fatty acid mobility.

Several investigators have observed that the capacitance of a black lipid membrane increases when a larger potential is applied across the membrane[52-54]. Although this phenomenon could be caused by a variety of factors, the most generally accepted hypothesis is that the membrane is thinning. With the measurement of small changes in membrane thickness under a known force investigators have attempted to analyze some of the forces important in structuring the bilayer.

There is normally a high flux of water across biological membranes which has been measured by tracer atom diffusion and osmotic swelling[55]. A number of investigators have also used these techniques to follow the water flux across black lipid membranes[56-58]. For a while, however, radioactive tracer measurements were giving erroneously low values for the water diffusion rates because of the presence of unstirred layers of water near the membrane[59]. Once this phenomenon was noted the majority of the measurements were made by osmotic flow, in which experiments the black lipid film separates an open chamber from one connected to a microliter syringe.

Characteristic values for the water flux across a biological membrane are from 0.4 to 400 μ/sec. and the rates appear to be related in part to the disorder in the bilayer[60]. The temperature dependence of the water flux has been studied in many cases to obtain an activation energy for the water diffusion process. From this activation energy for lecithin black lipid films (~12 kcal/mole) many investigators have favored the solubility-diffusion model for water permeation of the membrane as opposed to the large or small pore hypotheses. According to the solubility-diffusion model, the total activation energy is the sum of the activation energy of diffusion (3-4.5 kcal/mole) and the enthalpy of water partition coefficient for the membrane-solution system (8-10 kcal/mole).[57] Graziani and Livne support the feeling that this activation energy is primarily a measure of the nature of the membrane surface since they observed that the water permeability of lecithin is greater with unsaturated fatty acids although the activation energy is unaffected. On the other hand, monogalactosyl diglyceride exhibits a higher activation energy for water diffusion and a higher permeability coefficient.

1.2.6. Spectrophotometric Methods. The use of spectrophotometric methods in the study of bilayers has been somewhat limited. There are no strong uv or visible transitions from lecithin and IR is complicated by the strong water interference. One interesting internal-reflectance IR study was performed on the dry lecithin-cholesterol complex by following changes in the OH stretching absorption of cholesterol[61]. The change in the cholesterol absorption indicated some

hydrogen bonding to the lecithin which was presumably through one of the ester oxygens.

Other light studies of lecithin bilayers have involved probe molecules whose absorption or fluorescence characteristics change as a result of the interaction of the molecule with the bilayer presumably because of the lower dielectric constant. Träuble[62] has used (1-anilino-8 napthalene sulfonate) ANS and bromthymol blue to follow the α-gel to smectic mesophase transition of dipalmitoyl lecithin. The important advantage of these probe molecules is that stop-flow and temperature-jump methods can be used to gain kinetic information about the transition. Also, Träuble was able to observe hysteresis in the transition only in the presence of acetylcholine, divalent, or trivalent cations, which had interesting implications for nerve processes.

Finally, light scattering from lipid vesicles has been used to study their size[63] as well as the Chapman transition[62]. Aside from the normal vesicle sizing, light scattering has been used in the study of changes in internal volume of large vesicles[64]. Reeves and Dowben have treated the turbidity changes of large vesicles to obtain diffusion rates for water across the bilayer in the presence of salt gradients. Light scattering will also be a valuable tool in the study of vesicle fusion since it is readily measured and highly dependent on the vesicle size.

1.2.7. ESR Spin Labels. The use of nitroxide esr spin labels has been extensive in the study of lipid mobility in the bilayer. The spin label, however, is a large probe molecule (larger in area than

a fatty acid chain) and when added onto a molecule in the bilayer can cause a significant perturbation of the environment it is supposed to monitor[65]. This can obviously be a problem in measuring absolute mobilities of the system as opposed to relative mobilities with the same probe under different conditions.

The possible motions of a lecithin molecule in a bilayer are from the bilayer to the water phase, from one side of the bilayer to the other, two-dimensional lateral motion in the bilayer, rotary motion about the molecule's long axis and segmental motion. All of these motions have been studied by spin labels.

The basic motions of the lecithin molecule perpendicular to the bilayer have been studied by Kornberg and McConnell. Their results using a lecithin spin label with the label of the choline portion of the molecule indicate that the rate this spin-labelled molecule crosses the bilayer is on the order of $\sim(24 \text{ hr.})^{-1}$ [66]. In another study they found that the labelled molecule did not transfer from bilayer to bilayer through the aqueous phase at a rate faster than $(24 \text{ hr.})^{-1}$ [67].

The only fast translational motion of lecithin is in the two dimensional plane of the bilayer and that has been analyzed only recently by spin labels. Earlier work by Kornberg and McConnell[67] on the nmr of a spin-labelled lecithin and normal lecithin mixture showed that the frequency of the translational step for lateral diffusion is greater than $3 \times 10^3 \text{ sec}^{-1}$ at 0°C. In a more recent study, Devaux and McConnell[68] estimate the diffusion constant for a spin-labelled dipalmitoyl lecithin in dihydrosterculoyl lecithin to be $1.8 \pm 0.6 \times 10^{-8}$ cm^2/sec. They use summations of empirical spectra of different spin

label concentrations in lecithin to compute the time course of the outward spread of labelled lipid from a spot containing only spin labelled lipid into unlabelled lipid. The value for the diffusion constant determined in this manner agrees well with that calculated earlier by Träuble and Sackmann[69] from the esr spectra of a spin labelled steroid in dipalmitoyl lecithin bilayers. They have determined values for the exchange interaction between the androstan spin labels within the bilayer by computer analysis of the esr spectra. Träuble and Sackmann emphasize that this diffusion coefficient is a thousand-fold slower than typical values in liquids but it does predict an average lateral movement of 10,000 Å in 1 sec. or a hopping frequency of $10^{6.5-7.0}$ sec^{-1}. Devaux and McConnell, on the other hand, emphasize that surface diffusion can be important for biological processes. They point out that if the ratio of the two dimensional to three dimensional diffusion coefficients is greater than 10^{-3}, then surface diffusion is more efficient over a distance of 10 μ or more in reaching a specific target.

In addition to translational motion the whole lecithin molecule can undergo rotational motion about its long axis. In practice this motion is hard to analyze separately from segmental motions of the fatty acids; however, steroid spin labels have been used in the determination of their rotation frequency. For an androstan spin label this rotation frequency is 10^{8} sec^{-1} which should be faster than for the phospholipids[70].

Numerous studies have been made of the segmental motion of the fatty acids using spin labels, and although investigators maintain that the inside of the bilayer is fluid[71], they also agree that it is

considerably less fluid than a soap micelle[72]. In attempting to understand the motion of the fatty acid chains, investigators have synthesized a series of fatty acids and their corresponding lecithins with the oxazolidine ring at different carbons along the fatty acid chain. From oriented bilayer studies they have found an increase in the anisotropy of the ring motion in going toward the lipid charge group. McConnell and coworkers maintain that the local methylene chain motion near the center of the bilayer can approach that of an isotropic liquid hydrocarbon[10,71], whereas in the same bilayer, the local methylene chain motion near the polar head group region can approach that of a solid, crystalline hydrocarbon.[73] These conclusions come from the analysis of the order parameter of the oxazolidine ring esr spectrum in oriented bilayers. By carrying out a more complete analysis of these esr spectra, McFarland and McConnell[74] have calculated that on the time scale of $> 10^{-8}$ sec. there is a preferential orientation of the first 6-9 carbons of the lecithin fatty acid chain at about 30° with the perpendicular to the bilayer surface. From this and other physical evidence they have postulated that lipid fatty acids are slanted near the bilayer surface. They maintain further that this bend can account for many biological phenomena of cooperativity or signal propagation in the lipid bilayer. We should point out that a kink model[31] can be proposed to account for the same phenomena such as the esr spectra and the lower electron density at the center of the bilayer. There are at present no data to prove one or the other model correct.

1.2.8. Proton Magnetic Resonance Spectroscopy. Pmr spectroscopy was chosen for this study of lipid bilayers for a number of reasons. Primarily, the difference between sonicated and unsonicated liposomes was first noted by pmr and thus it seemed that pmr should be a sensitive detector of changes in the bilayer structure. Also, this method made use of unaltered compounds so that there were no problems of perturbing the local environment by foreign probe molecules.

There were originally many problems to be overcome in the interpretation of the pmr results because intermediate and anisotropic mobilities had not been treated extensively in pmr theory. The bilayer was also another phase than water and phase boundary considerations of serious magnitude could be imagined. Furthermore, because some of the resonances were extremely broad in some cases and others were sharp, it was difficult to analyze all of the proton resonances from a single spectrum.

Much of the early work on the pmr of lipids was done in Chapman's group in England. Chapman's motivation was to understand the state of lipids in different phases. Several paramount problems in the data analysis soon arose which cast serious doubts on most earlier interpretations. Originally, the data[75] were treated by applying the theory of Kubo and Tomita[76] for the analysis of the T_1 and the standard T_2 formulation[77] for small molecules for analysis of the linewidth data which were both formulated on the assumption that the motion was fast and isotropic. A serious discrepancy was noted in that the observed spin-lattice relaxation time increased with increasing temperature[75] and it was quite different than the transverse relaxation time as

measured from the linewidth of the proton resonances from the lecithin bilayers. Penkett et al. did not resolve this problem but did note that the methylene proton resonance linewidth was field dependent. Hansen and Lawson[78] and Kaufman et al.[79] carried this further and from Carr-Purcell type measurements of the spin-spin relaxation time concluded that the observed linewidth was the result of diffusion of the molecules through microscopic inhomogeneities in the samples or internal magnetic field inhomogeneity respectively. These hypotheses were shown to be wrong by several recent papers which clarify the picture greatly. Chan et al.[80] and Oldfield et al.[81] have observed that the transverse relaxation time of the fatty acid protons (T_2, which is equal to $1/\pi$ (linewidth at half height)) is field independent at least to magnetic field strengths of 25 Kgauss, and have discounted the Carr-Purcell experiments as artifactual. They have further postulated that diffusion of spin energy down the fatty acid chains to a methyl heat sink accounts for the single T_1 observed for most protons and is possible because of the strong dipole-dipole interactions. In addition, Finer et al.[82] have found that in oriented bilayers the linewidth is markedly reduced when the perpendicular to the bilayer is oriented at 54°44' with respect to the magnetic field. Since this is the angle at which dipole-dipole line broadening is removed, the positive result shows that dipole-dipole interactions and not field gradients must be the cause of the broad proton resonances. A rigorous theoretical treatment of the proton resonances from lecithin bilayers is being completed in our laboratory, from which it is hoped that we can obtain an orientation parameter for motion of the fatty acid chains. It is

important to note that these lecithin molecules have not been altered in any way. For this reason it is not surprising that the results indicate that the motion is much more restricted than spin label studies predict.

Throughout the controversy over the cause of line broadening in multilayer lecithin samples, the line sharpening caused by sonication was attributed to the faster rotational averaging of the small vesicles themselves[75]. Sheard[83] challenged that interpretation but his work was not widely accepted and several recent papers attempt to refute it[82, 84, 85]. Our feeling, like Sheard's, was that the nature of the bilayer was altered by sonication.

1.3. Question of Sonication

This problem of the interpretation of the pmr spectra of the sonicated lecithin vesicles was chosen as a starting point for this work because the questions it raised about the nature of the bilayer in sonicated vesicles were fundamental to many important model lipid studies. Multilamellar lipid structures were not suited for physical or transport studies[86] whereas sonicated vesicles of a homogeneous size had a single lamella separating two water spaces[87] and thus were ideal for such studies[88]. Most investigators, however, considered the bilayer in a small vesicle to be completely normal, and many studies were carried out on that system under the premise that the bilayer was like that in a multilayer[88-91]. We have probed this problem in detail in an attempt to understand if the bilayer properties were changed and how were they changed. Further we pursued the effects of other

mechanical perturbations on the bilayer. For this work we have chosen to use dipalmitoyl lecithin and to study it with pmr spectroscopy and other physical methods. In this study we exploited not only the ability of pmr to monitor mobility in the bilayer but also the sensitivity of pmr to exchange processes on the order of 10^{-1} to 10^{-4} seconds, the ability of pmr to quantitate the concentration of specific protons, and the sensitivity of the pmr resonance linewidth to the presence of paramagnetic species.

2. EXPERIMENTAL

2.1. Preparation of Dipalmitoyl Lecithin Samples.

L-α-dipalmitoyl phosphatidyl choline from General Biochemicals was checked for purity by thin layer chromatography and found to contain less than 1% impurity. It was also shown to have no effect on the conductivity of deionized and distilled water. This lecithin was used without further purification in the preparation of the following types of bilayers.

2.1.1. Unsonicated Bilayers. Dipalmitoyl lecithin samples in the range of 100-300 mg/ml lecithin in H_2O were suspended by repeated passage at 60°C through a 6" long 20 gauge needle.

2.1.2. Vesicles Containing High Salt. A suspension of about 50 mg/ml lecithin in D_2O (0.1 M NaCl, 2 mM PO_4 at pD 7.8) was sonicated for five minutes at power level 6 with a Bronson sonifier model S-75. Initially the temperature was 20°C but during sonication

the temperature of the sample rose above 50°C. The sample was then centrifuged at 20,000 × G in a Sorvall RC-2 centrifuge for 30 minutes and the lower layer was drawn off since the larger particles centrifuged to the surface. The lower layer, containing the smaller vesicles, was then applied to a sepharose 4B column (2.5 × 80 cm), which had been calibrated with bacteriophage φx 174, a nearly spherical virus of about 250 Å in diameter (donated by Anthony Zuccarelli, Division of Biology, Caltech). The phospholipid concentration in each fraction was monitored by measuring the amount of light scattered at 300 mμ with a Beckman D. U. spectrophotometer. From each major peak in the elution curve several of the six milliliter fractions were pooled and were then concentrated on an Amicon pressure dialysis apparatus with a UM-10 membrane. The concentrated small vesicle fraction was centrifuged again at 20,000 × G for 30 minutes and the supernatant, which contained the smaller vesicles, was withdrawn. All concentrated samples were then dialyzed in D_2O containing 2 mM PO_4 at pD 7.2. The purity of the lecithin in the vesicles was rechecked by TLC and the lipid was found to give a single spot. Divalent ions were added to the vesicles in the form of microliter amounts of a 0.1 M solution of the sulfate salts in D_2O. In the viscosity experiments the sample viscosity was increased by the addition of 1.5% (w/v) of native calf thymus DNA (Worthington Biochemicals), and the solution was made homogeneous by passage through a 22 gauge syringe needle.

2.1.3. Vesicles with Little or No Salt. Suspensions of 50-100 mg/ml lecithin in water or in D_2O (2 mM PO_4 at pD 7.8) were sonicated

for ten minutes at 60°C with a Bronson ultrasonicator using a microtip probe at power level 5. Samples were then centrifuged at 20, 000 × G for 30 minutes. Samples of small vesicles were obtained directly from the supernatant in this procedure and used for nmr and dilatometry experiments. Larger vesicles were prepared by passage of a sample sonicated in 2 mM PO_4 at pD 7. 8 through a sepharose 4B column equilibrated in the same buffer. Considerable aggregation occurred on the column under these conditions and no small vesicle fraction could be obtained. These larger vesicles from the column were then concentrated by pressure dialysis and centrifuged at 20, 000 × G for ten minutes. For pmr studies the supernatant was dialyzed into D_2O (2 mM PO_4 at pD 7. 8).

2. 2. Electron Microscopy

Portions of the vesicle solutions were diluted to 1-5 mg/ml lecithin and a drop of the solution was applied to a 400 mesh copper grid coated with parlodion sprayed with a thin film of carbon. After 30 seconds the majority of the liquid was drawn off with a blotter and a drop of 2% phosphotungstic acid solution (pH 7. 4) was applied. After another 30 seconds the grid was blotted to remove excess liquid and allowed to dry. The grids were observed on a Phillips 300 electron microscope operating at 60 kilovolts and pictures were recorded on a 35 mm camera at 10-50, 000 × magnification. Polystyrene beads with a diameter of 1000 Å ± 30 Å were used to calibrate the electron micrographs and size distributions were obtained by sizing several hundred vesicles from the electron micrographs. Samples for

electron microscopy were taken both prior to and after proton magnetic resonance measurements.

2.3. Dilatometry and Molal Volume Experiments

A simple volumetric dilatometer was used to measure the temperature dependence of the volume of the lipid-water system. The dilatometer had a capacity of 5.0 ml and was calibrated with distilled, deionized water over the temperature range of 25-45 °C. The temperature dependence of the sonicated and unsonicated systems were studied over the range of 26° to 45 °C by placing the dilatometer in a temperature controlled water bath and by following the meniscus in the capillary tube (1.1 mm in diameter) with a cathetometer. The full range of temperature was covered in 3-4 hours.

In these experiments only suspensions of sonicated and unsonicated dipalmitoyl lecithin containing no salt were studied and the phospholipid concentration was varied from 4 to 16%. Two independent methods were used to determine the apparent molal volume of the lecithin at 27 °C. First, the volume of the sample in the dilatometer was measured at several different lecithin concentrations and the apparent molal volume of the lecithin was obtained using the following formula:

$$\overline{V} = x_{H_2O}\,\overline{V}_{H_2O} + x_L\,\overline{V}_L \quad ,$$

where $\overline{V}$ is the apparent molal volume of the lecithin suspension as measured; and $\overline{V}_{H_2O}$ and $\overline{V}_L$ denote respectively the apparent molal volumes of the H_2O and lecithin in the two-component system. x_{H_2O}

and x_L denote the mole fractions of the components. The phospholipid concentration was determined by two methods: by drying and weighing, and by colorimetric analysis for total phosphorus using the method of Bartlett.[92] Secondly, the apparent molal volume of the lecithin was obtained from the buoyant density of the lecithin in mixtures of D_2O and H_2O as measured by centrifugation to within ± 0.0005 mg/ml. Ideal mixing of D_2O and H_2O was assumed in these experiments and the following formula was used to calculate the density of the lecithin at neutral buoyancy from which the apparent molal volume was determined.

$$\rho_{sample} = \frac{\text{Vol. \% } D_2O}{100} (\rho_{D_2O} - \rho_{H_2O}) + \rho_{H_2O}$$

2.4. Osmotic Pressure Experiments

In these experiments low concentrations of lecithin were used (5-10 mg/ml) to insure that the kinetics of vesicle fusion would be slow. Samples were sonicated for about 10 minutes and power level 5 in the appropriate salt solution, and after centrifugation at 20,000 × G for one-half hour, they were dialyzed in fresh D_2O solutions to remove H_2O. In one case these experiments were carried out in conjunction with a study of the volume of water inside the vesicle performed by Annie Yau.

2.5. Water Diffusion

Isoosmotic small and larger vesicles in 0.1 M NaCl at pH 7.6 from the sepharose 4B column or freshly sonicated vesicles in 2 mM

PO_4 at pH 7.6 were used in the water diffusion experiments at concentrations of 30-100 mg/ml dipalmitoyl lecithin. To each sample was added from 0.01 to 0.03 M $MnSO_4$. In one case the sample was diluted 1:1 with D_2O (isoosmotic) and a spectrum was recorded immediately afterwards.

2.6. Proton Magnetic Resonance Measurements

All pmr spectra were taken on a modified Varian HR-220 superconducting nmr spectrometer equipped with frequency sweep and multinuclear capabilities, and a C-1024 time-averaging computer was used to enhance the signal-to-noise ratio. Probe temperature was measured with an ethylene glycol sample using the calibration curve supplied by Varian. Chemical shifts were measured relative to an external acetone standard in D_2O doped with $MnSO_4$ which was in turn calibrated versus temperature with DSS (3-(trimethylsilyl) propane-sulfonic acid sodium salt). The area of the acetone resonance was used as an area standard and was calibrated using a 0.003 M solution of tetrabutyl ammonium bromide.

2.7. ^{31}P NMR Measurements

^{31}P nmr spectra of sonicated and unsonicated dipalmitoyl lecithin bilayers were recorded at a magnetic field of 22 Kg and the resonance frequency of 36.4 MHz on a Bruker H-90 spectrometer equipped with a Nicolet 1074-PDP-8e computer system operating in the Fourier transform mode, and at 53 Kg and 89 MHz on a modified Varian HR-220 superconducting nmr spectrometer equipped with frequency sweep

and multinuclear capabilities. Proton noise decoupling was used to remove any effects arising from phosphorus-proton spin-spin couplings, and either a C-1024 time-averaging computer or a Varian 620i computer operating in the accumulation mode was used to enhance the signal-to-noise ratio.

3. RESULTS

3.1. Size Distribution of Vesicles

The particle size distributions of vesicle samples were found to vary with the length of sonication and whether the vesicles were passed through a sepharose 4B column in the presence or absence of salt. For this reason all of the vesicle samples were visualized by negative staining in the electron microscope. Representative micrographs are shown in Fig. 1. From such micrographs size distributions and the average vesicle diameter were obtained for the various vesicle samples. These data are summarized in Table I. The estimate of 300 Å for the average size of small vesicles containing high salt correlates well with the estimate of about 250 Å from the elution curve of the sepharose 4B column as shown in Fig. 2. The major fractions marked I and II, which will henceforth be labelled "larger vesicles" and "small vesicles", had average diameters of 900 Å and 300 Å respectively. A similar elution curve was not obtained for vesicles in low salt since aggregation occurred in the column and thus only larger vesicles were obtained from the column under these conditions. These larger vesicles had an average diameter of 1000 Å. Small

FIGURE 1

a. Small vesicles containing 0.1 M NaCl before heating;

b. After heating to 45°C for one-half hour.

c. Larger vesicles containing 0.1 M NaCl before heating;

d. After heating to 45°C for one-half hour.

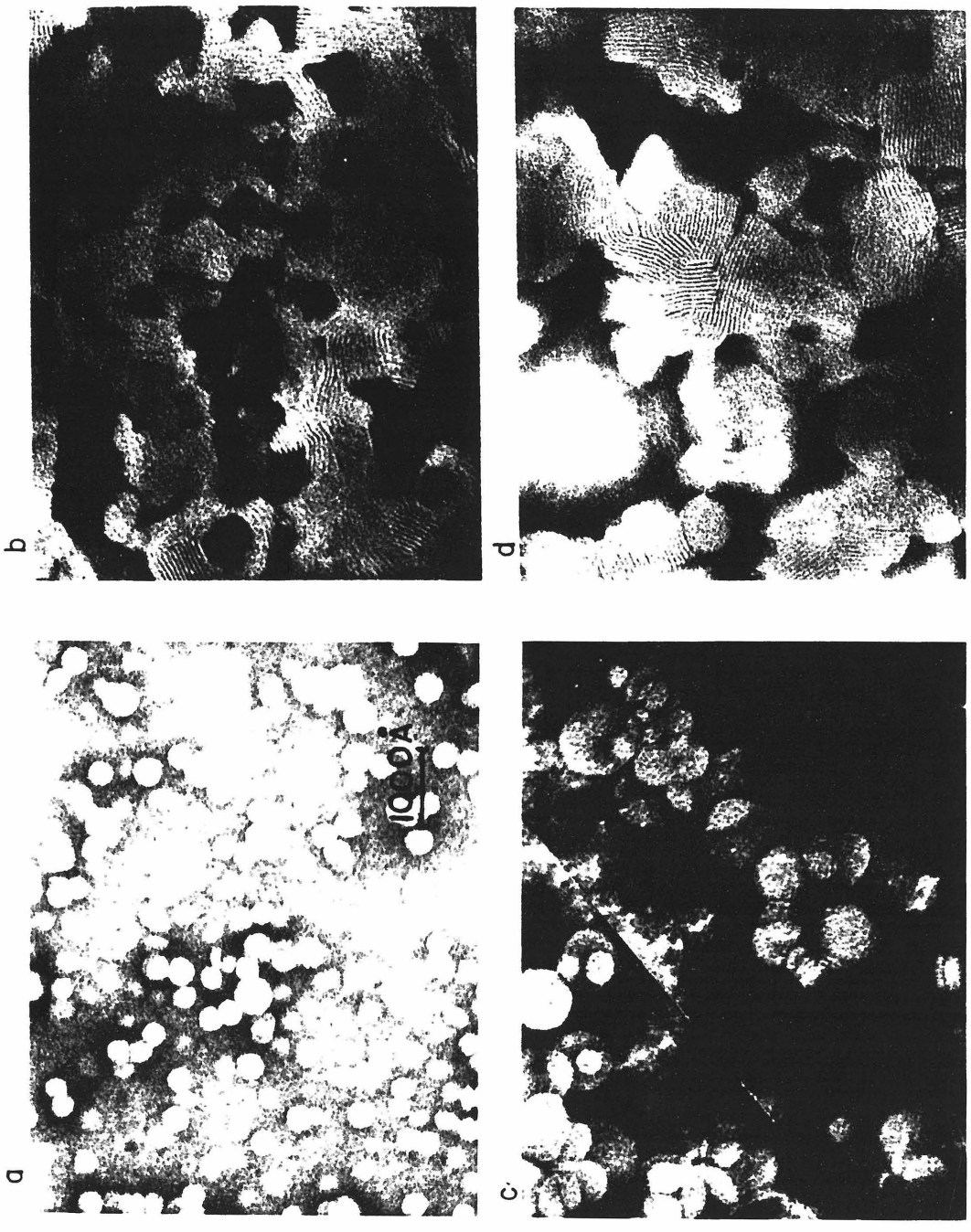
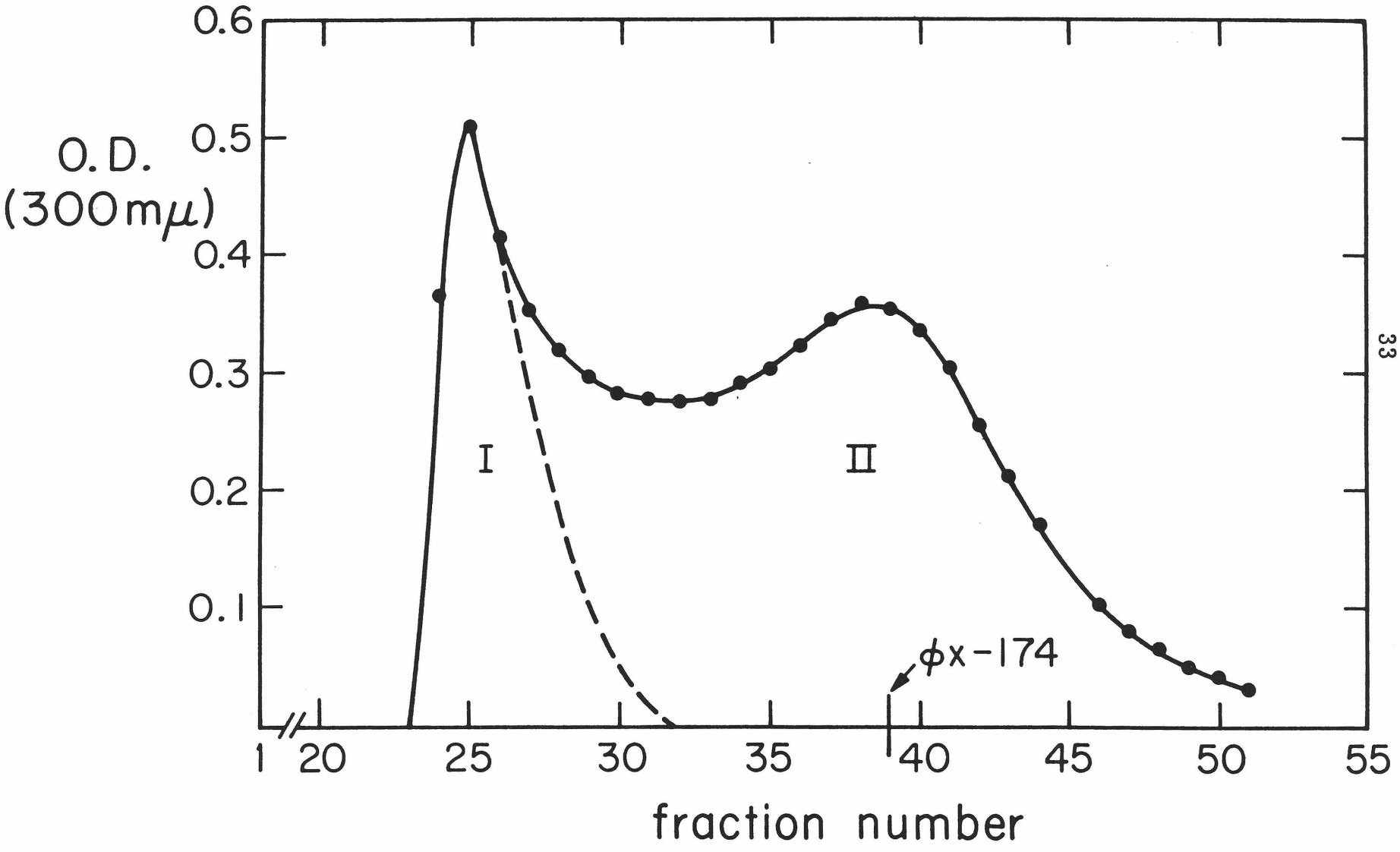

a
10 0Å
b
c
d

TABLE I. Percent by Weight of Lecithin in Different Size Vesicles and the Average Vesicle Size.

Sample	150-350 Å	350-450 Å	450-600 Å	600-800 Å	800-1000 Å	1000-1400 Å	Average
Small vesicles containing high salt	69%	22%	9%				300 Å
Larger vesicles containing high salt		4%	12%	14%	41%	29%	900 Å
Small vesicles containing low salt	33%	44%	9%	10%	4%		350 Å
Larger vesicles containing low salt				16%	43%	41%	1000 Å
Sonicated dilatometry sample after 16 hrs.	7%	21%	22%	17%	16%	17%	600 Å

FIGURE 2

Optical density at 300 m μ of 6 ml eluant fractions from a sepharose 4B column (2.5 × 80 cm) in 0.1 M NaCl and 2 mM PO_4 at pH 7.8 for vesicles containing 0.1 M NaCl.


0.6
0.5
0.4
0.3
0.2
0.1
O.D.
(300mμ)
I
II
φx-174
1
20
25
30
35
40
45
50
55
fraction number

vesicles in low salt were therefore taken directly from the freshly sonicated samples after centrifugation. Electron microscopy pictures indicate an average diameter of 350 Å for these vesicles.

The size distribution of all vesicle fractions changed gradually over the period of several days. In particular, vesicles prepared in high salt aggregated rapidly when heated above 40°C as shown in Fig. 1b and d. Since these vesicles have been dialyzed into D_2O containing only 2 mM PO_4, this aggregation appears to be the result of an osmotic pressure gradient of about 4 atmospheres across the bilayer[93]. Whereas vesicles containing high salt inside (0.1 M NaCl and 2 mM PO_4) and low salt outside (2 mM PO_4) were found to burst within about 20 minutes above 40°C, the vesicles in isotonic medium were stable for at least several hours at high temperatures. However, the dilatometry measurements indicate a decrease in the molal volume of lecithin when these vesicles are allowed to stand for a period of 16 hours just after sonication.

3.2. Effect of Temperature on the PMR Spectra

We have studied the crystalline ⇌ liquid-crystalline phase transition of dipalmitoyl lecithin[20] by pmr for both the larger and small vesicles containing high and low salt. The pmr spectrum of dipalmitoyl lecithin bilayers includes resonances from the choline methyl groups at -3.25 ppm, from the fatty acid methylene protons at -1.25 ppm and from the fatty acid methyl protons at -0.90 ppm from DSS[75]. Glycerol and certain methylene group proton resonances are too broad to be observed under these conditions.

The effect of temperature on the spectrum of small and larger vesicles containing high salt in the spectral region 0 to -3.5 ppm is depicted in Figs. 3 and 4. As expected the pmr spectrum of both vesicle samples showed marked changes in intensity and linewidth near 40°C as a result of the first order phase change in the lecithin bilayer. However, striking differences in the spectral behavior of the two vesicle samples were noted.

Below the phase (Chapman) transition the larger vesicles exhibited only a very broad (~150 Hz) choline resonance of less than half of the expected intensity. As shown in Fig. 5, only at temperatures near the phase transition were most of the choline protons detected. In addition, the transition from few to many cholines observed and from a broad to a narrow choline resonance occurred abruptly in the temperature range 37-42°C. By contrast all of the choline protons were detected in the case of the small vesicles even at 19°C. In both cases the choline methyl resonance exhibited appreciable narrowing at the Chapman transition.

There was no evidence of the fatty acid proton resonances in the spectra of the small and larger vesicles at low temperatures. In the case of the small vesicles these fatty acid resonances became apparent at 31°C and in the case of the larger vesicles at 39°C. Although both samples underwent a transition from the crystalline to liquid-crystalline phase near 40°C, the actual transition temperature for the fatty acids in the small vesicles was about 2°C lower than for the larger vesicles and the transition was broader for the small vesicles covering 3-4°C, as shown in Fig. 6. In addition a higher

FIGURE 3

PMR spectra (220 MHz) of small vesicles (~300 Å) containing 0.1 M NaCl in the region from 0 to -3.5 ppm from DSS.

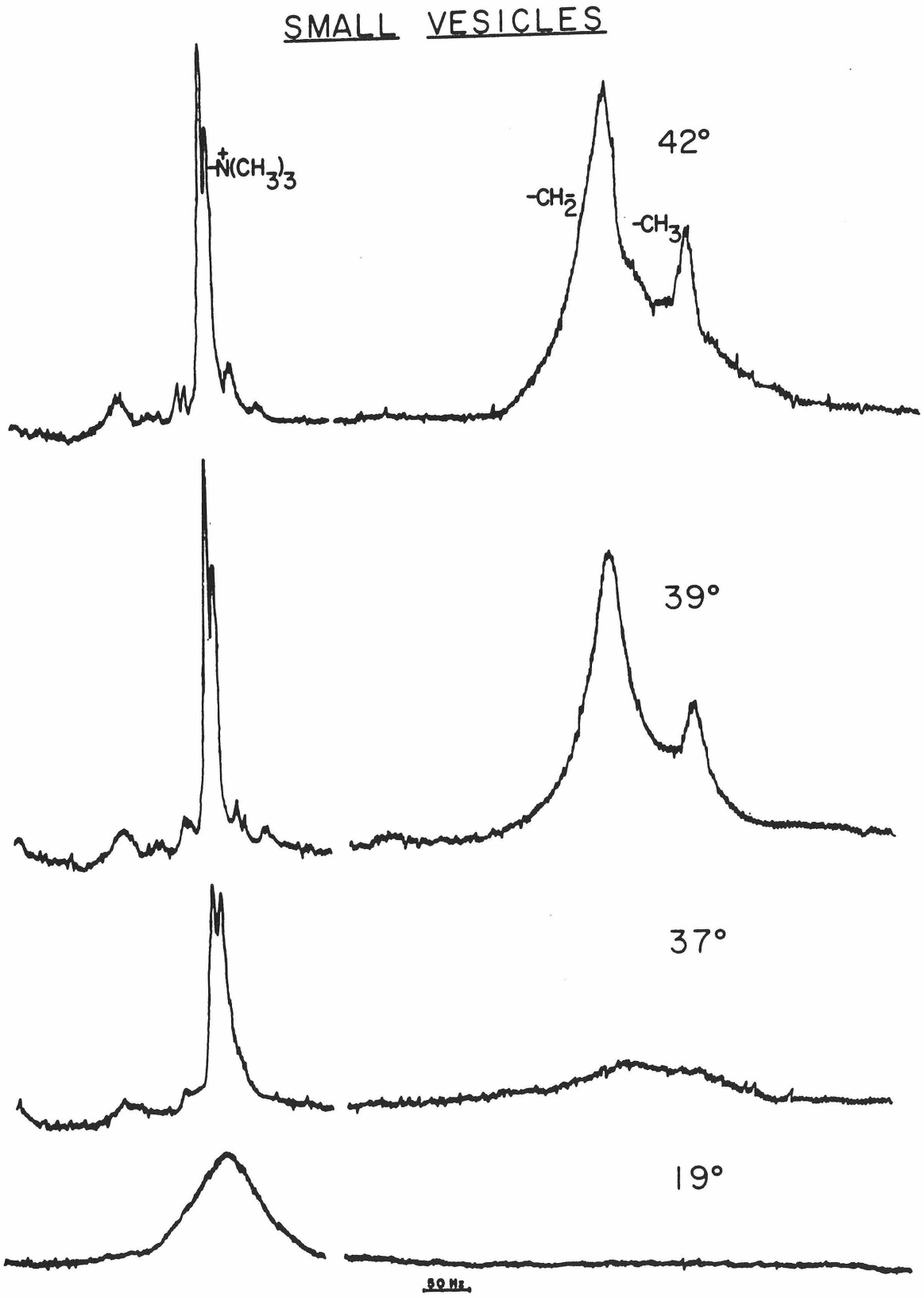

SMALL VESICLES
$-\overset{+}{N}(CH_3)_3$
$-CH_2$
$-CH_3$
42°
39°
37°
19°
50 Hz.

FIGURE 4

PMR spectra (220 MHz) of larger vesicles (~900 Å) containing 0.1 M NaCl in the region from 0 to -3.5 ppm from DSS.

LARGER VESICLES

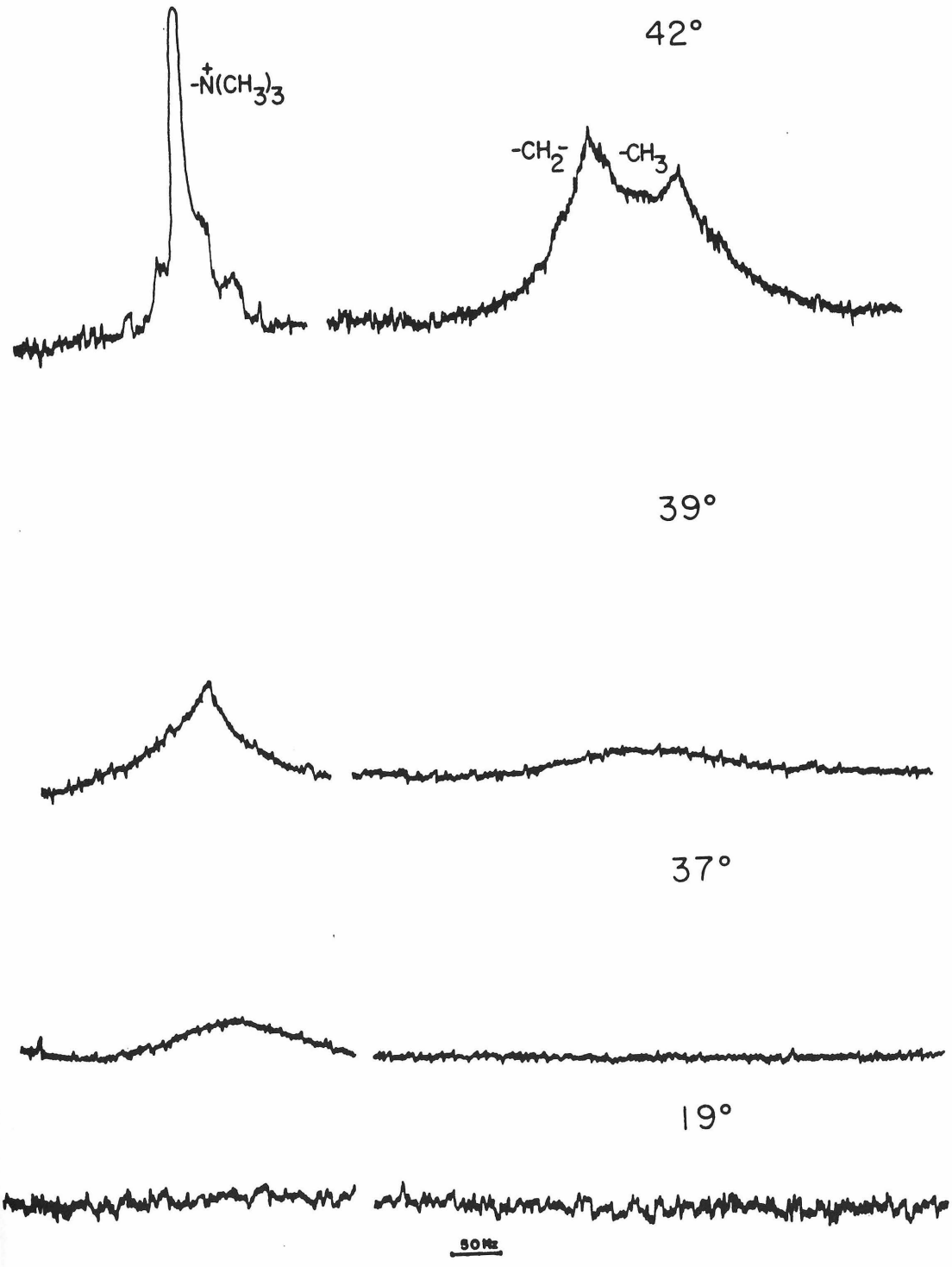


FIGURE 5

Percentage of the choline methyl proton resonance detected versus temperature for (○) small vesicles containing 0.1 M NaCl and (△) larger vesicles containing 0.1 M NaCl.

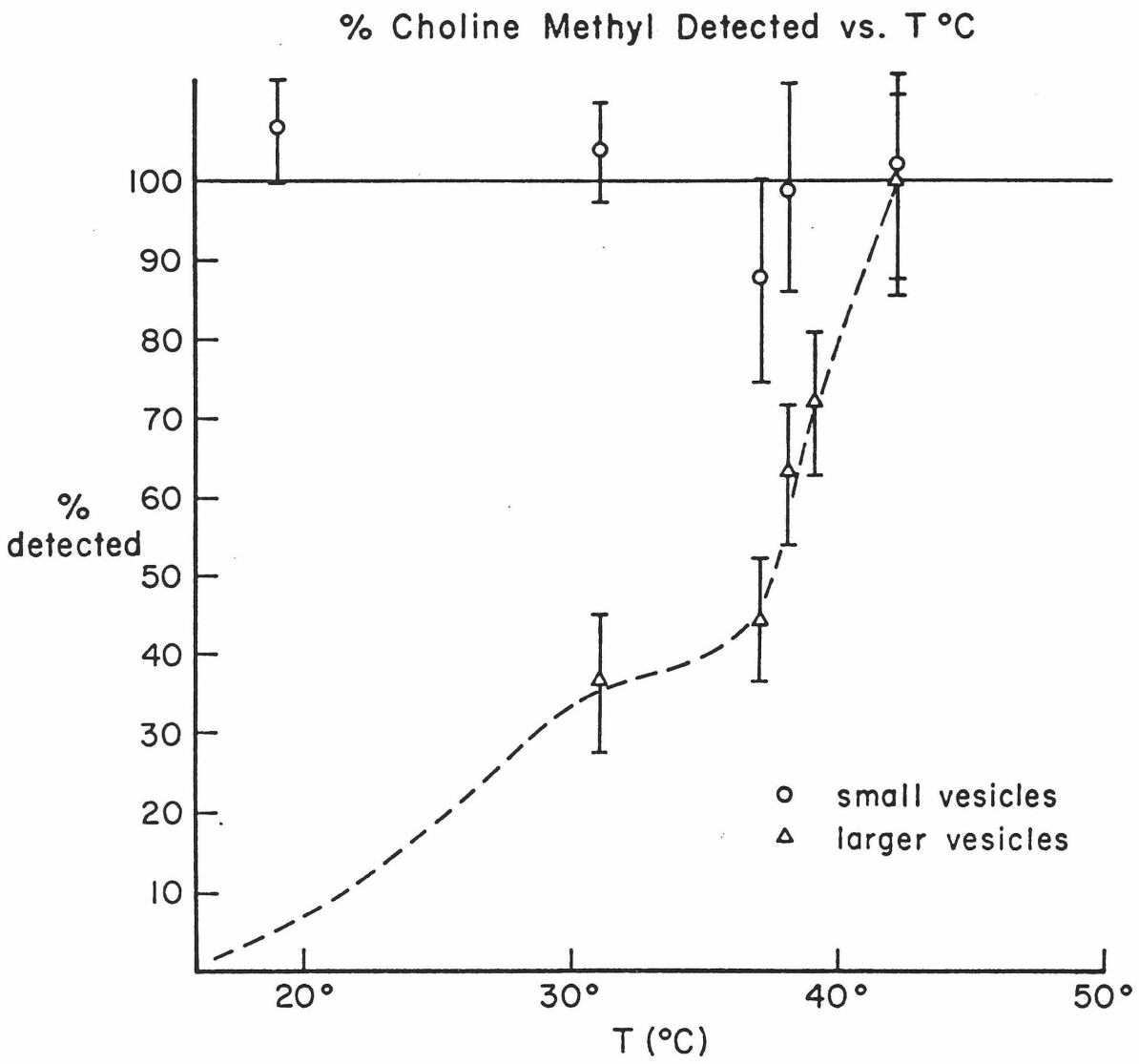
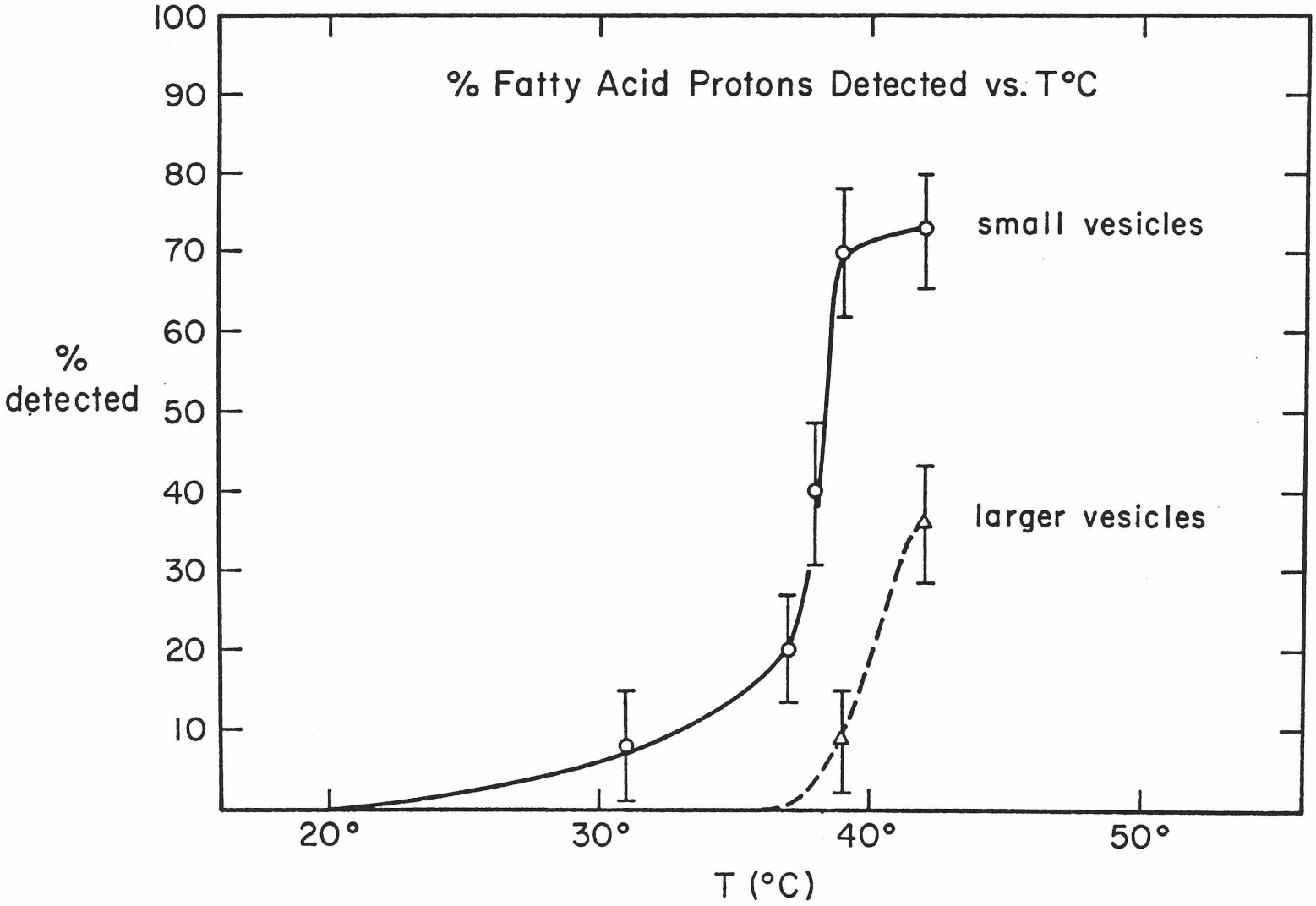

% Choline Methyl Detected vs. T °C
100
90
80
70
60
50
40
30
20
10
% detected
20°
30°
40°
50°
T (°C)
small vesicles
larger vesicles

FIGURE 6

Percentage of the fatty acid proton resonances detected versus temperature for (○) small vesicles containing 0.1 M NaCl and (Δ) larger vesicles containing 0.1 M NaCl.


% Fatty Acid Protons Detected vs. T°C
100
90
80
70
60
50
40
30
20
10
% detected
small vesicles
larger vesicles
20°
30°
40°
50°
T (°C)

percentage of the protons of these resonances were melted in the smaller vesicles. On the basis of these intensities at 42°C it was estimated that along with the terminal methyl group, 9-11 of the 13 methylene groups became sufficiently mobile to give a high resolution spectrum in the small vesicles whereas only 3-5 methylene groups were melted in the larger vesicles.

Near the phase transition the small vesicles also exhibited two choline resonances of unequal intensity separated by ~0.04 ppm. The high field resonance was less intense and like the choline resonance in the larger vesicles, was chemically shifted -514 Hz from the aliphatic methyl resonance. The more intense resonance appeared at -522 Hz from the aliphatic methyl absorption. These choline resonances appeared to coalesce at temperatures above 70°C.

Other than the greater tendency of the vesicles containing high salt to aggregate above the phase transitions, there were only minor differences between the spectra of vesicles with the high salt concentration inside and those which were isoosmotic with the 2 mM PO_4 outside the vesicles. Because of the aggregation tendencies of the high salt vesicles at the phase transition they were not studied at temperatures above 42°C.

3.3. Effect of Mn^{++} on PMR Spectrum

As an aid in the assignment of the lecithin resonances, Mn^{++} ion was added to the solution of intact vesicles in order to cause extreme broadening of the proton resonances from groups contacting the extravesicular water space. The result was a radical decrease

in the intensity of the choline resonance while the fatty acid resonances were unaffected. In the case of the small vesicles addition of Mn^{++} caused a decrease of the choline resonance area to 30-40% of the total at temperatures below the phase transition while the linewidth of the residual signal remained constant. This implies that 60-70% of the lipid is on the outside of the vesicle, in rough agreement with calculations of the relative areas of the inside and outside surfaces assuming a thickness of 46 Å for the bilayer. The percentage of the cholines observed with Mn^{++} present decreased at the transition temperature in the samples in high salt. Because the loss of choline intensity was not accompanied by any change in linewidth, the change probably reflects the breaking open of vesicles.

For both samples the observed choline resonances in the presence of Mn^{++} were chemically shifted -514 Hz from the aliphatic methyl peak. This chemical shift may be compared with the chemical shift of choline methyl protons in the absence of Mn^{++}, which was -514 Hz for the larger vesicles and the less intense resonance from the small vesicles, and -522 Hz for the more intense resonance from the small vesicles. Thus the chemical shift of the choline methyl resonance from the inside of the small vesicles was the same as that from the inside and outside choline groups of the larger vesicles, whereas the outside choline resonance in the small vesicles was chemically shifted to lower field with respect to the other choline resonances.

3.4. Effect of Viscosity on the PMR Spectrum

In order to ascertain whether the pmr linewidths were

controlled by the vesicle tumbling rate, 1.5% of calf thymus DNA was added to a sample of small vesicles to create a gel of extremely high solution viscosity. In these experiments the water resonance was broadened to a width of ~3 Hz. This indicates a decrease in the rotational tumbling rate of the water molecules by 20-30 fold although from estimates of the viscosity there has been a much greater increase in the viscosity of the medium.

In Fig. 7 it is seen that the addition of DNA to the small vesicle sample caused only a slight increase in linewidth and slight decrease in intensity (<10%) in the pmr spectrum. It is interesting to note that these small vesicles in DNA which are now rotating at least as slowly as the larger vesicles in aqueous solution exhibit quite a different pmr spectrum from the larger vesicles at the same temperatures.

3.5. Dilatometry and Molal Volume Experiments

In order to confirm our pmr observations that the Chapman transition appeared at a lower temperature and was broader for lecithin in the small vesicles, we have also made dilatometry measurements. Träuble and Haynes[40] have shown that the transition of a lecithin bilayer from the crystalline to liquid-crystalline phase leads to an abrupt increase of ~1.4% in the apparent molal volume of lecithin. We have exploited this method with more precision and accuracy in the hope that these measurements might shed further light on the pmr observations.

In these experiments we recorded the volume changes from 26C of lecithin suspensions as shown in Figs. 8 and 9. The volume change of the water alone in each sample is plotted on the same graphs.

FIGURE 7

PMR spectra (220 MHz) at 38°C of (A) small vesicles containing 0.1 M NaCl, (B) small vesicles containing 0.1 M NaCl in a 1.5% DNA solution, and (C) larger vesicles containing 0.1 M NaCl.

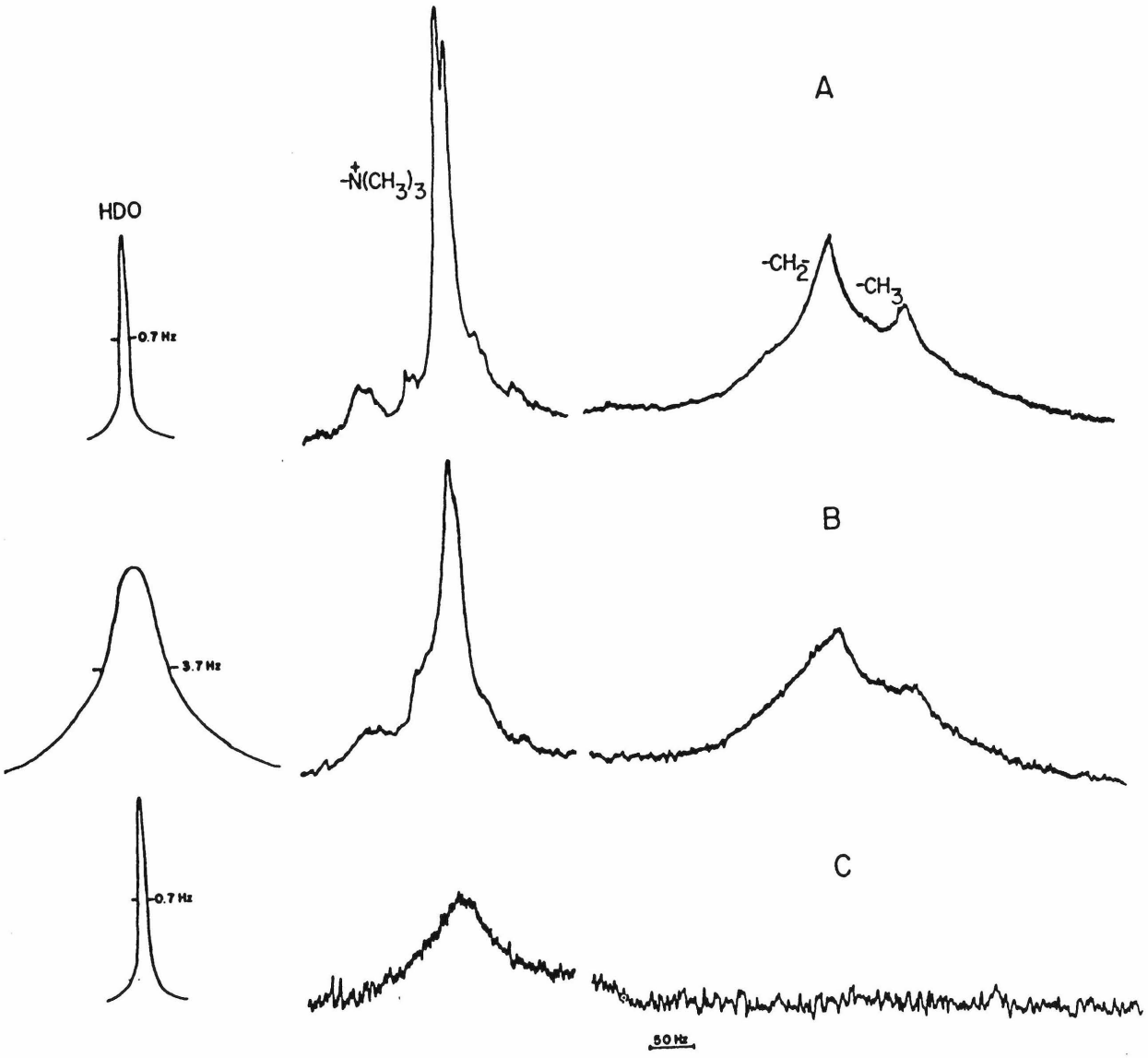

HDO
0.7 Hz
$-\overset{+}{N}(CH_3)_3$
A
$-CH_2-$
$-CH_3$
3.7 Hz
B
0.7 Hz
C
50 Hz

FIGURE 8

The actual volume change (ΔV) from the volume at 26°C of an unsonicated lecithin and water mixture upon heating (●), upon cooling (◎) and of the water in the mixture alone (■).

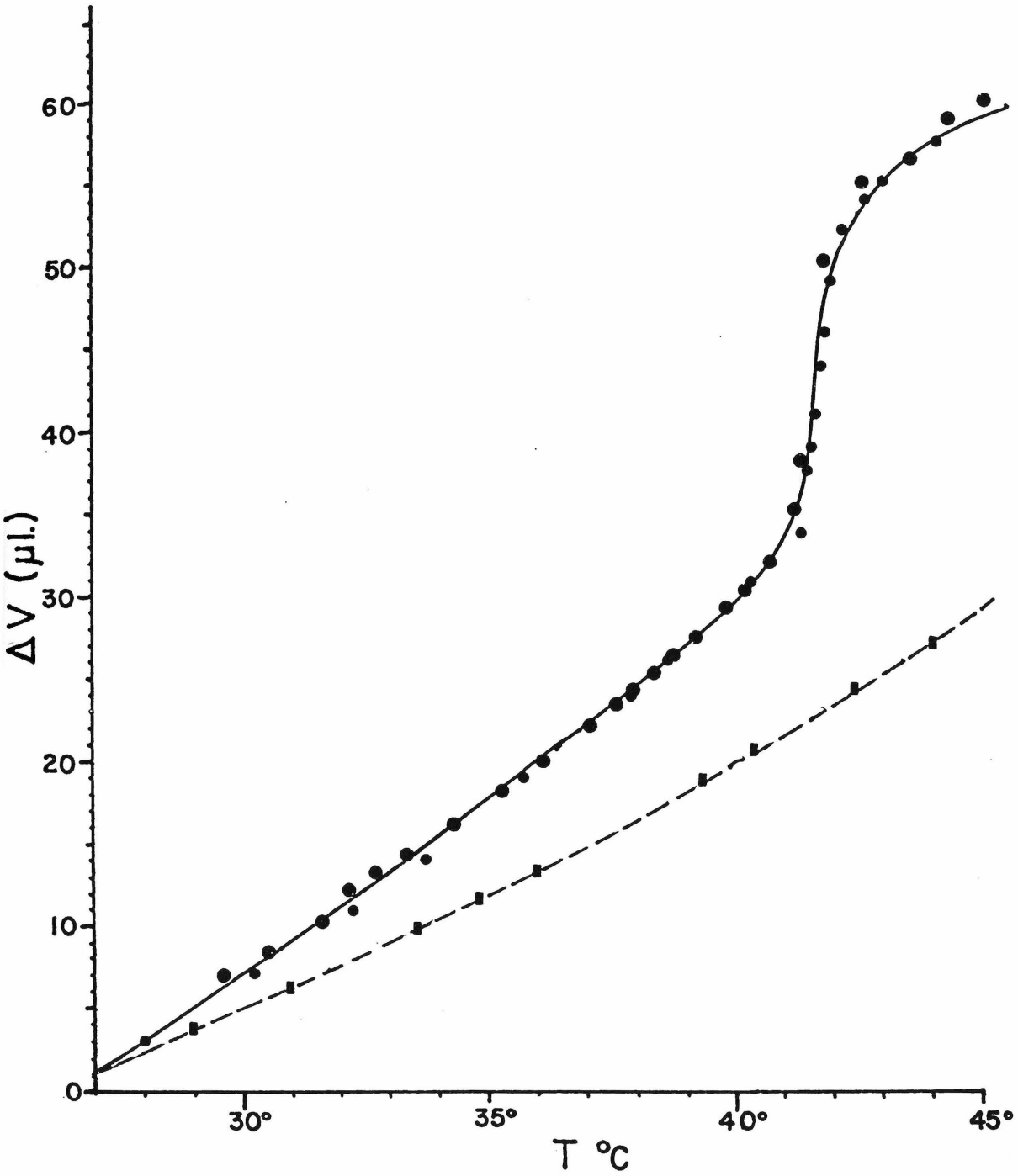
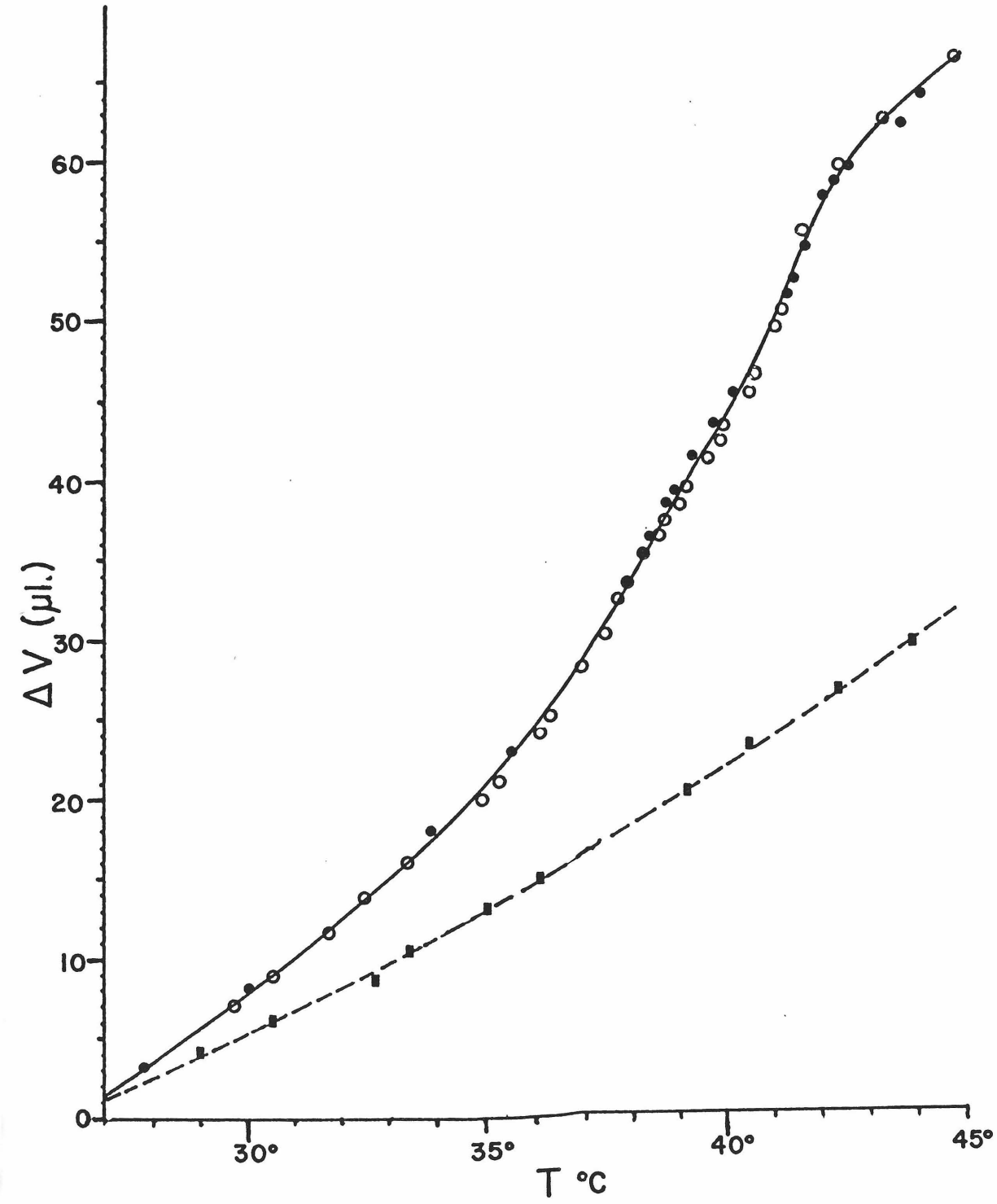

60
50
40
30
20
10
0
ΔV (μl.)
30°
35°
40°
45°
T °C

FIGURE 9

The actual volume change (ΔV) from the volume at 26°C of a sonicated lecithin and water mixture upon heating (●), upon cooling (O) and of the water in the mixture alone (■).


60
50
40
30
20
10
0
ΔV (μl.)
30°
35°
40°
45°
T °C

The water expansion curve was obtained by multiplying the values obtained in a calibration run with pure water by the ratio of the mass of water in the suspension to that in the calibration run. The lecithin expansion was obtained from the difference in the two curves and this along with the mass and volume of lecithin present was used to calculate the apparent molal volume of lecithin.

Fig. 10 summarizes the change in the apparent molal volumes of both lecithins in the region of the Chapman transition. It is evident from these apparent molal volumes that the melting behavior of sonicated and unsonicated lecithins is different. As in the nmr observations, the dilatometry measurements indicate that the fatty acids in the small vesicles melt at a lower temperature and have a broader Chapman transition. Moreover, these experiments reveal that the apparent molal volume of sonicated lecithin is nearly 1% greater than that of unsonicated lecithin below the Chapman transition (27°C), although above the phase transition temperature the apparent molal volumes of both lecithins are equal within experimental error. Table II lists the densities and apparent molal volumes of sonicated and unsonicated lecithin at 27°C in the absence of salt. Upon the melting of the fatty acid chains the apparent molal volume increased by 3.1% in the case of sonicated lecithin and about 4.0% for the unsonicated.

In the absence of salt, heating the vesicle samples through the phase transition and then cooling to below the phase transition revealed no evidence of hysteresis over the time course of the experiments (~8 hours), as shown in Fig. 10. Under these conditions the vesicle size distribution also remained relatively stable over a period of weeks.

FIGURE 10

The apparent molal volume of dipalmitoyl lecithin ($\overline{V}$) versus temperature for (×) a sonicated sample being heated, (■) being cooled, and (○) an unsonicated sample being heated, (●) being cooled.

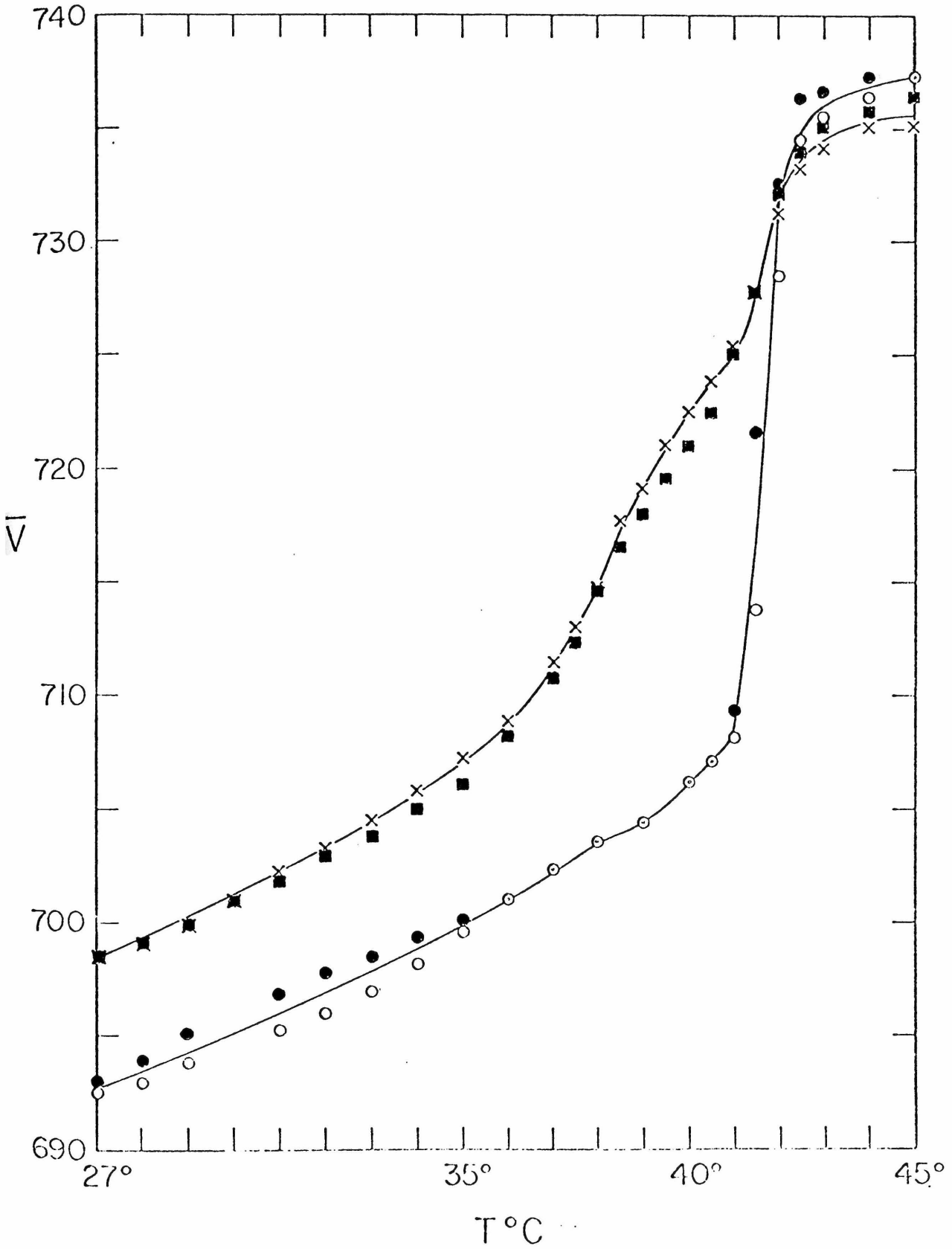

740
730
720
710
700
690
$\bar{V}$
27°
35°
40°
45°
T °C

TABLE II. Densities and Partial Molal Volume of Sonicated and Unsonicated Lecithin Bilayers at 27°C.

Type of Bilayer	Method of Concentration Determination	No. of Determinations	$\rho_{27°}$	$\overline{V}_{27°}$
Sonicated	Bartlett	12	1.056 ± 0.003	695 ± 1
	Drying and weighing	3	1.0506 ± 0.0005	698.5 ± 0.3
	Drying and weighafter 16 hr.	3	1.0536 ± 0.0005	696.6 ± 0.3
Unsonicated	Bartlett	12	1.063 ± 0.003	690 ± 1
	Drying and weighing	4	1.0600 ± 0.0005	691.6 ± 0.3
	Buoyant density	1	1.0585 ± 0.0005	693.4 ± 0.3

This was not true for vesicles in 0.03 M NaCl. In that case the lecithin vesicles tended to aggregate to form larger vesicles even below the Chapman transition. In fact, after two weeks, their melting behavior approached that of the unsonicated bilayers as shown in Fig. 11. If, in addition, there was a salt concentration gradient, as when the vesicles had been dialyzed into low salt D_2O so that an osmotic pressure existed across the bilayer, the vesicles became unstable and above the Chapman transition aggregated in a matter of minutes.

3.6. ^{31}P NMR Studies

In order to confirm the pmr results and to obtain an additional handle on their interpretation, we have examined the ^{31}P nmr spectrum of lecithin bilayers in both the sonicated and unsonicated states. In these experiments we have exploited the fact that the magnetic shielding of the phosphorus nucleus in the glycerophosphocholine moiety is anisotropic (Fig. 12). Consequently, to obtain a rotationally averaged spectrum the motion of this group must be sufficiently isotropic and quick so that the motional modulation of this chemical shift anisotropy is fast compared with the chemical shift anisotropy expressed in Hz. Since the chemical shift anisotropy is proportional to the magnetic field strength, these experiments must be undertaken at sufficiently high fields so that the effect is manifested in the presence of dipolar effects.

The ^{31}P spectra of unsonicated multilayers of phosphatidyl choline and phosphatidyl ethanolamine at 89 MHz clearly reveal an asymmetric resonance (Fig. 12). This resonance, which is spread over 5000 Hz, is reminiscent of a powder spectrum of nuclei with a

FIGURE 11

The change in the volume of lecithin (ΔV_{lec}) from the volume at 26° in a 0.03 M NaCl solution for unsonicated lecithin (⊗), for freshly sonicated lecithin (●) and for sonicated lecithin after one week (◉).

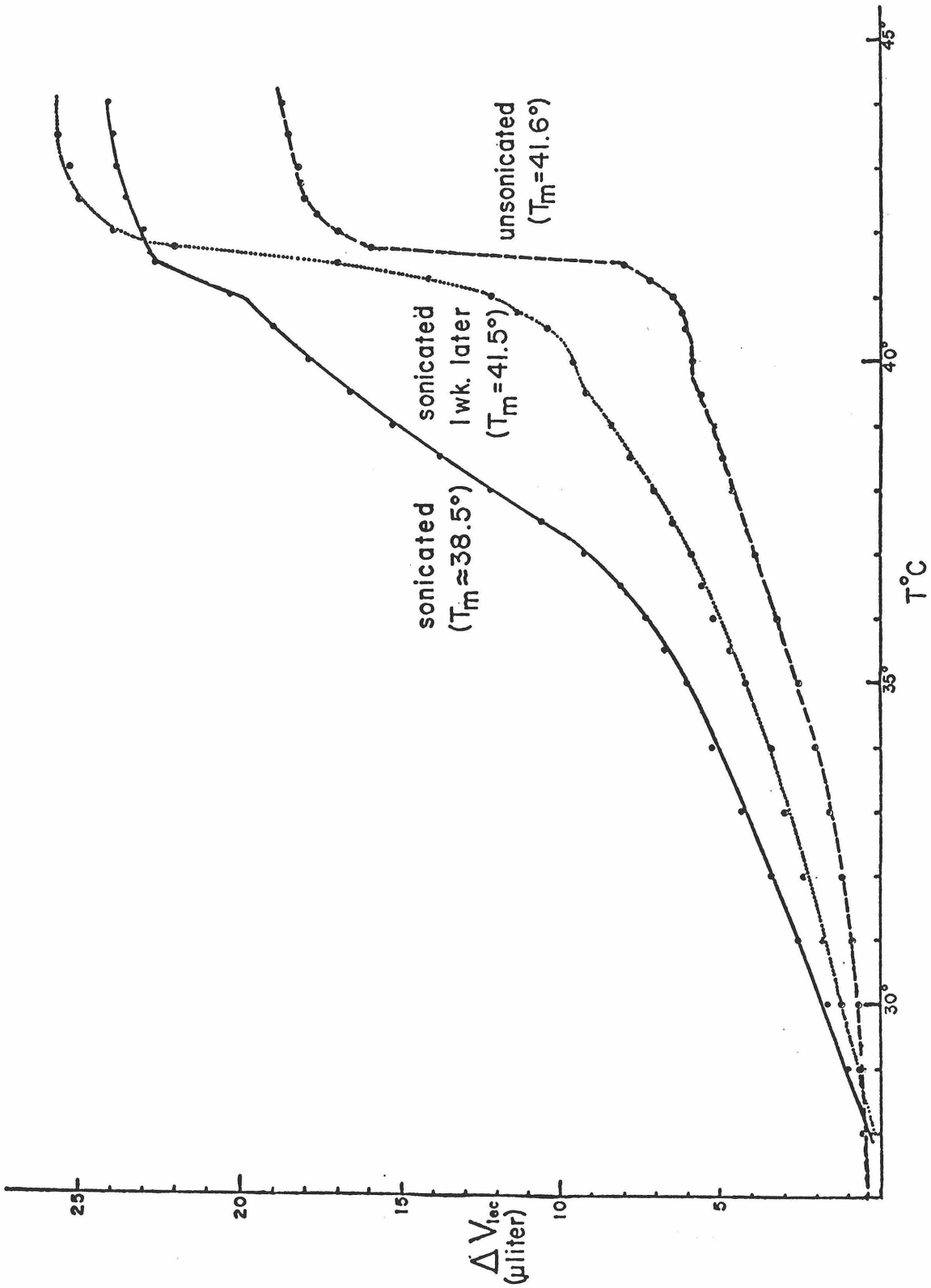
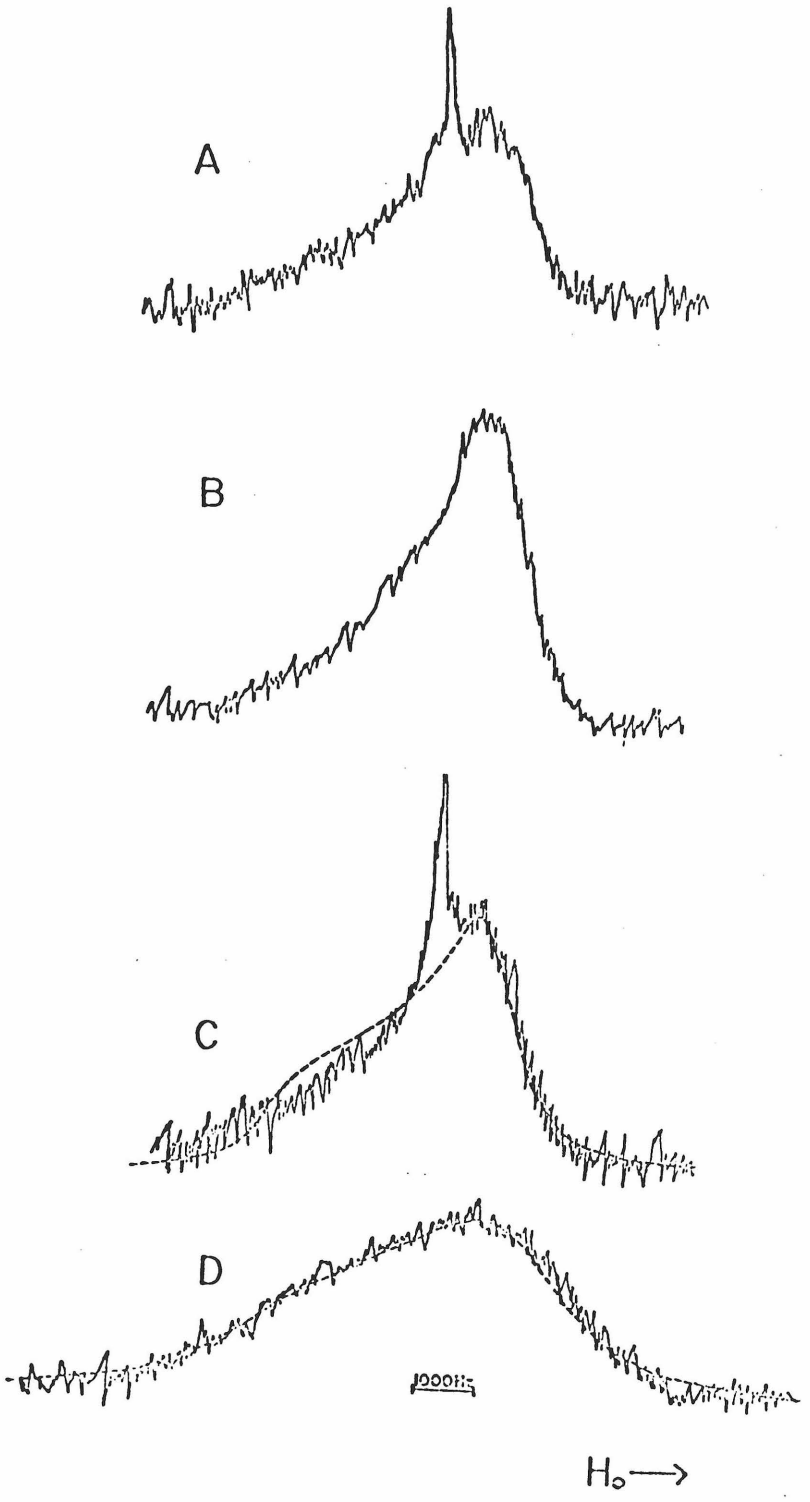

sonicated
($T_m \approx 38.5°$)
sonicated
1 wk. later
($T_m = 41.5°$)
unsonicated
($T_m = 41.6°$)
ΔV_{lec}
(μliter)
25
20
15
10
5
30°
35°
40°
45°
T°C

FIGURE 12

^{31}P nmr spectra (89 MHz) of unsonicated (A) dimyristoyl lecithin at 38°C, (B) dipalmitoyl phosphatidyl ethanolamine at 85°C, (C) dipalmitoyl lecithin at 55°C with the computed spectrum (---), and (D) dipalmitoyl lecithin at 18°C with the computed spectrum (---). The symmetrical resonance in A and C is presumably from lecithin in a different configuration since the intensity of this resonance varies from sample to sample and the resonance was only found in saturated lecithins above the phase transition.


A
B
C
D
1000H
$H_o \longrightarrow$

chemical shift anisotropy, and in fact it was possible to obtain a theoretical fit to these spectra assuming a chemical shift anisotropy $\delta_{\perp} - \delta_{\parallel} \approx 5000$ Hz ± 1000 Hz below and 4000 ± 1000 Hz above the phase transition plus the appropriate dipolar linewidth. For the spectrum below the Chapman transition, the dipolar linewidth giving the best fit is 2800 ± 400 Hz. This is reduced to 1100 ± 200 Hz above the transition.

The ^{31}P nmr spectra of unsonicated lipids were analyzed by comparison with computed spectra of anisotropic resonances as shown in Fig. 12. The anisotropic resonances were computed by summing Lorentzian lines of a given dipolar linewidth whose position and intensity were determined by the angle of the parallel shielding tensor with the applied field according to relationships formulated by F. Tsay for all angles on a sphere. The relationship between the angle and the chemical shift was

$$\delta_{\theta} = \sqrt{(\delta_{\perp} \sin \theta)^2 + (\delta_{\parallel} \cos \theta)^2}$$

to give the vectorial sum of the perpendicular and parallel components of the shielding. The relative intensity of the resonance at an angle θ was given by the following relation

$$I_{\theta} \alpha \sin \theta$$

assuming no preferential orientations.

Sonicated lecithin gave a single symmetrical resonance with a linewidth of ~70 Hz at 18°C which sharpened gradually to ~35 Hz at 55°C. This linewidth was field independent as determined by

measurements at 22 and 53 Kgauss (Fig. 13). When 2% (w:v) DNA was added to a concentrated sonicated suspension (~ 10% lecithin w:v), the linewidth of the ^{31}P resonance remained ~ 70 Hz at 18°C even though some aggregation occurred in addition to the viscosity increase.

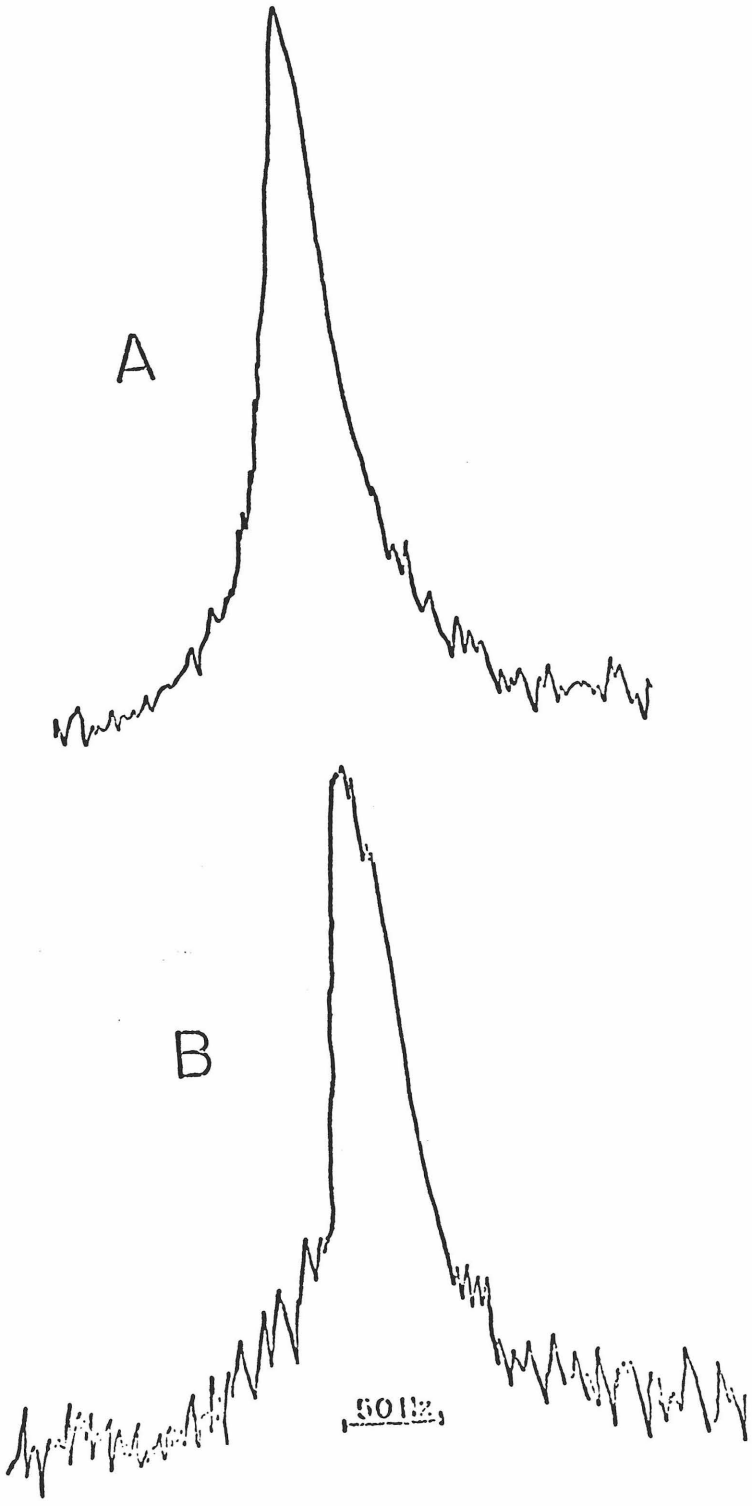
3.7. Water Diffusion Experiments

A slightly broadened proton resonance was observed with the approximate chemical shift of water when 0.01 M Mn^{++} ion was added to the suspension to broaden the resonance from water outside the vesicles. This resonance was believed to be from water inside the vesicles and was believed to be broadened by exchange with the external water since the linewidth increased with increasing temperature. To test that the broadening was not the result of some other mechanism such as the internal exchange with bound water at the bilayer surface, a sample was diluted 1:1 by D_2O and the resonance intensity was found to decrease by a factor of four in less than a minute which is the time required to record the spectrum. Also the linewidth was found to be independent of field as expected for exchange broadening in the slow exchange limit.

Provided the water exchange rate is in the slow exchange limit we can obtain the permeability coefficient for water from the mean lifetime of water in the vesicles and average size of the vesicles. The mean lifetime of the water in the vesicles is measured by the exchange contribution to the linewidth at half height ($\Delta \nu_{\frac{1}{2}}$). The linewidth at half height (exclusive of the magnetic contribution) is equal to the sum of two quantities

FIGURE 13

^{31}P nmr spectra at 35 °C of sonicated dipalmitoyl lecithin at (A) 36.4 MHz and (B) 89 MHz.


A
B
50 Hz

$$\pi \Delta \nu_{\frac{1}{2}} = \frac{1}{T_2} + K_{IO}$$

where T_2 is the spin-spin relaxation time for water inside the vesicle and K_{IO} is the pseudo-first order rate constant for exchange of protons from the inside to the outside of the vesicle. We have been able to separate the exchange contribution from the T_2 by studying $\Delta \nu_{\frac{1}{2}}$ as a function of temperature. At low temperatures $1/T_2$ is large and it determines the observed linewidth but at high temperatures the linewidth is essentially determined by K_{IO}. In Fig. 14 we plot log $\Delta \nu_{\frac{1}{2}}$ versus $1/T°K$ and we see that the contribution of K_{IO} to $\Delta \nu_{\frac{1}{2}}$ predominates above 10°C. From the slope of this plot we can obtain the activation energy for the exchange process described by K_{IO}, and it is 13 ± 1 kcal for the small and 20 ± 1 kcal for the larger vesicles. This difference was reproducible for several samples of both types of vesicles.

Provided we know the mean lifetime of water in the small vesicle, K_{IO}, we can determine the permeability coefficient (ρ) of the bilayer from the vesicle size by the following relation

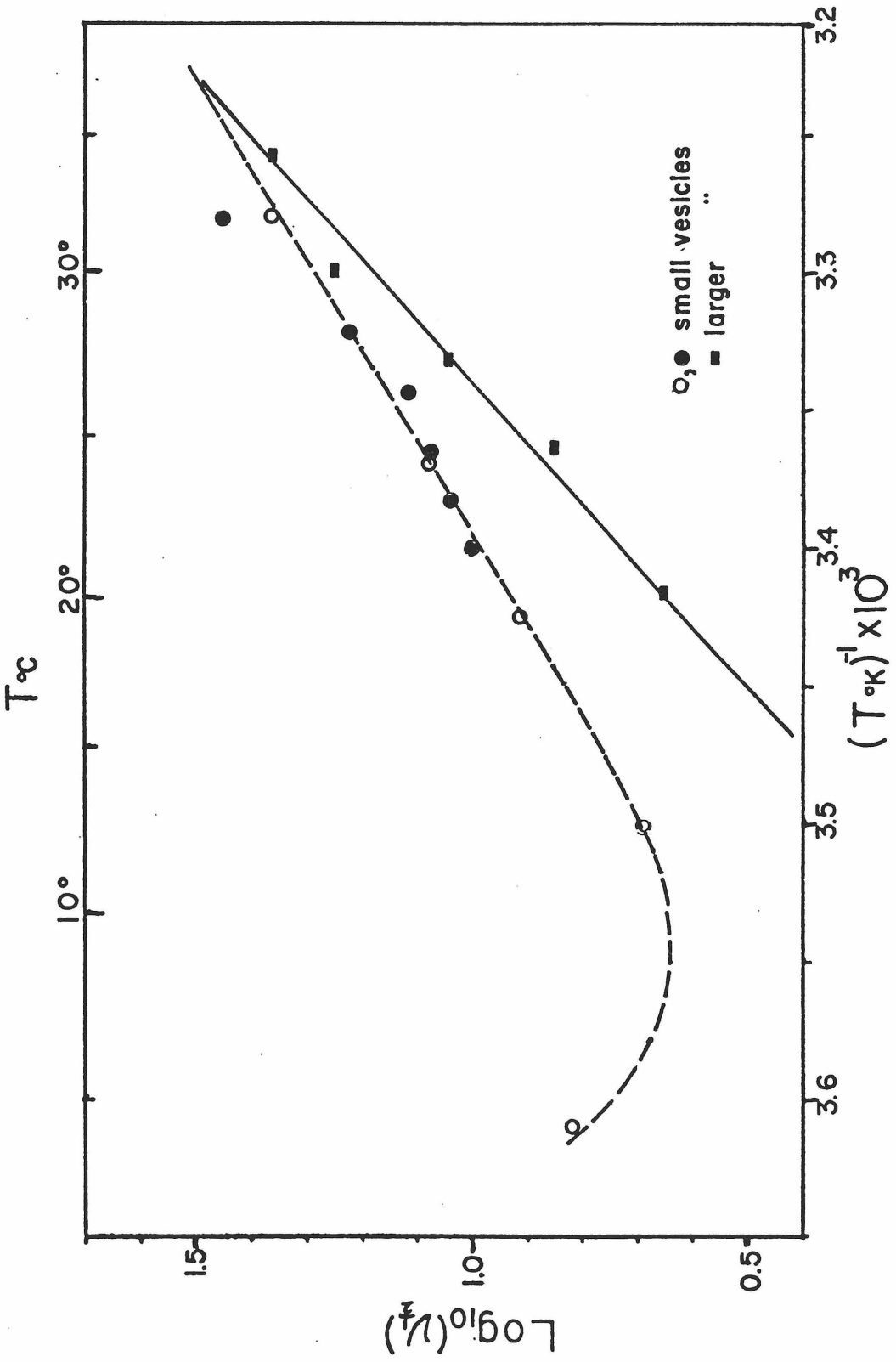
$$\rho = K_{IO} (V/S)$$

where V is the inside volume of the vesicle and S is the surface area inside the vesicle. We compute V and S from the average vesicle radius (r) by the following formula

$$V = \frac{4}{3} \pi (r - 50 \text{ Å})^3$$

FIGURE 14

The $\log_{10}$ of the linewidth at half height of the inside water resonance (minus the magnetic contribution) from small vesicles (◎ and ⊙) and from large vesicles (■).


Log10(μ)
1.5
1.0
0.5
T°c
10°
20°
30°
3.2
3.3
3.4
3.5
3.6
(T°K)⁻¹ x10³
o,● small vesicles
■ larger "

$$S = 4\pi (r - 50\ \text{Å})^2$$

$$\text{or } V/S = \frac{1}{3}(r - 50\ \text{Å})$$

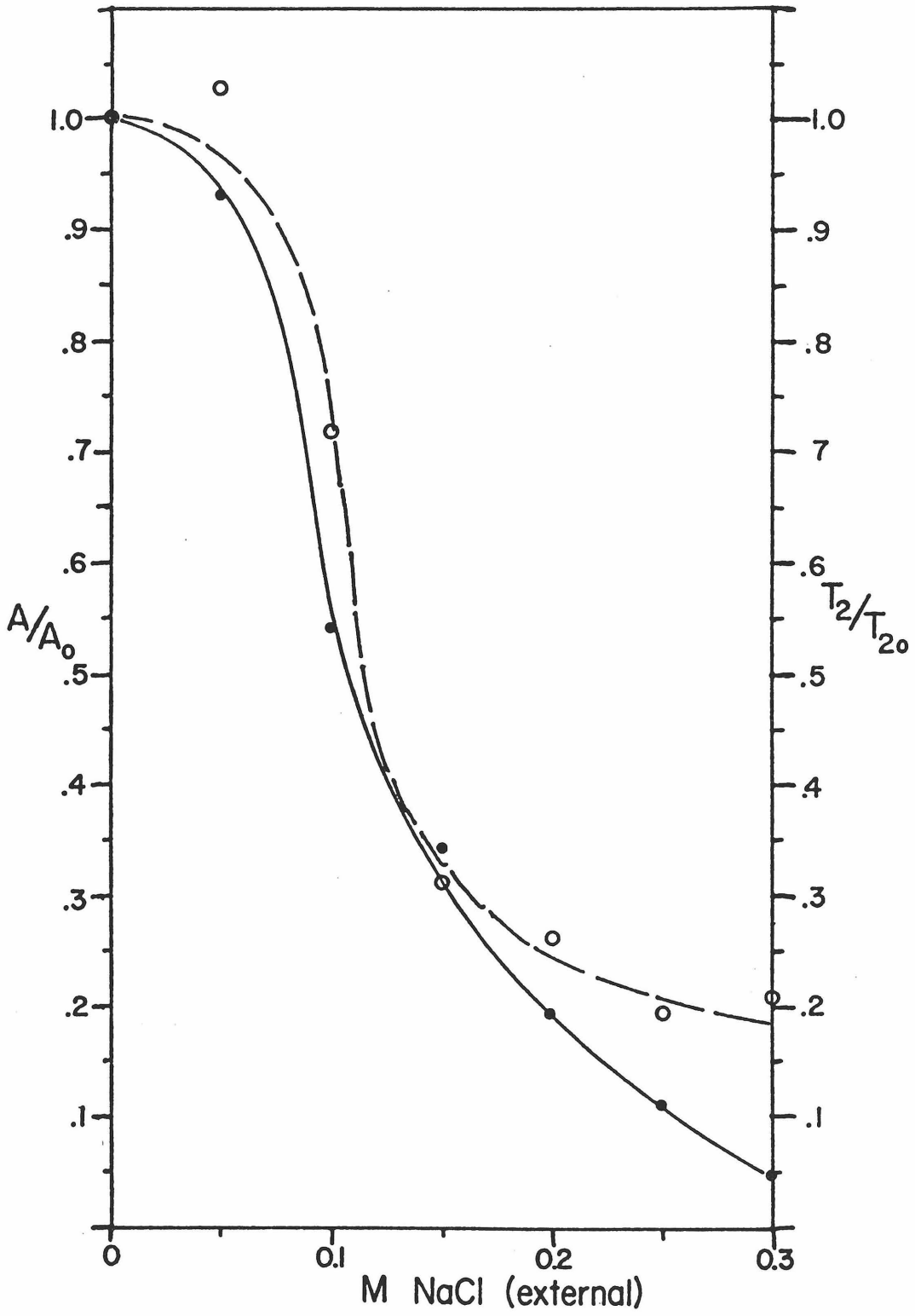
Using an average radius of 150 Å for the small vesicles and 450 Å for the larger vesicles, we compute that the permeability coefficients are 0.16 μ/sec. and 0.39 μ/sec. respectively at 26°C.

3.8. Osmotic Pressure

To test further if curvature is an important factor in fatty acid mobility, we sought to vary the curvature of a vesicle by changing the volume inside. The work of Sheetz and Yau, shown in Fig. 15, demonstrates that the area of the water resonance from inside the vesicle decreases with increasing salt in the surrounding solution. To balance the osmotic gradient the space inside the vesicle has shrunk, most likely through the collapse of the sphere, into a biconcave disc thereby raising the inside salt concentration. The actual volume decrease, however, is greater than predicted to produce isoosmotic solutions. In fact if the original inside volume of water contained 0.06 M $MgCl_2$, we compute that the final volume would contain 1.2 M $MgCl_2$ when the outside solution contained 0.03 M $MnCl_2$, 0.03 M $NiNO_2$ and 0.3 M NaCl or the equivalent of 0.25 M $MgCl_2$. The area of the water resonance is not the only indicator of the change in the inside volume since the pseudo-first order rate constant of water exchange (K_{IO}) as measured by the linewidth of the water resonance is proportional to the volume to surface area ratio. This relationship comes from

FIGURE 15

The external NaCl concentration is plotted versus the ratio of the area (●) or the T_2 (O) of the internal water resonance from vesicles in the presence of added external NaCl to the area or the T_2 respectively of the internal water resonance from vesicles in an isotonic solution (0.06 M $MgCl_2$ inside and 0.03 $MnCl_2$ + 0.03 M $Ni(NO_3)_2$ outside).


1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
A/A₀
T₂/T₂₀
0
0.1
0.2
0.3
M NaCl (external)

the equation for K_{IO}

$$T = K_{IO} = P(S/V)$$

when we assume that P and S do not change with V.

There were two very dramatic changes in the pmr spectrum of the vesicles which accompanied the volume decrease inside the vesicle. First the intensity of the sharp fatty acid resonances decreased, and second, there was an increase in the chemical shift difference between the inside and outside choline resonances. In Fig. 16 we see the decrease in the intensity of the sharp fatty acid proton resonances of small vesicles with increasing salt at 42°C where the effect is most pronounced. At temperatures well above the phase transition there is also a decrease in the intensity but not so great as in this case. For example, in Fig. 17 egg lecithin vesicles show a decrease in the methylene resonance intensity while being some 30° above the Chapman transition temperature.

There is no parallel decrease in the area of the choline methyl resonance with increasing salt even well below the phase transition in the small vesicles. Although the choline resonance does not decrease in intensity, there is an increase in the chemical shift difference between the inside and outside choline resonance as seen in Fig. 18. This chemical shift difference is inversely dependent on the temperature as mentioned earlier, on the vesicle size and on the unsaturation of the fatty acids as shown in Fig. 18. It is interesting to note that the chemical shift separation increases linearly with external salt concentration unlike the decrease of the internal volume.

FIGURE 16

The effect of added external NaCl on the 220 MHz pmr spectra of dipalmitoyl lecithin vesicles containing 0.06 M $MgCl_2$ at 42°C.

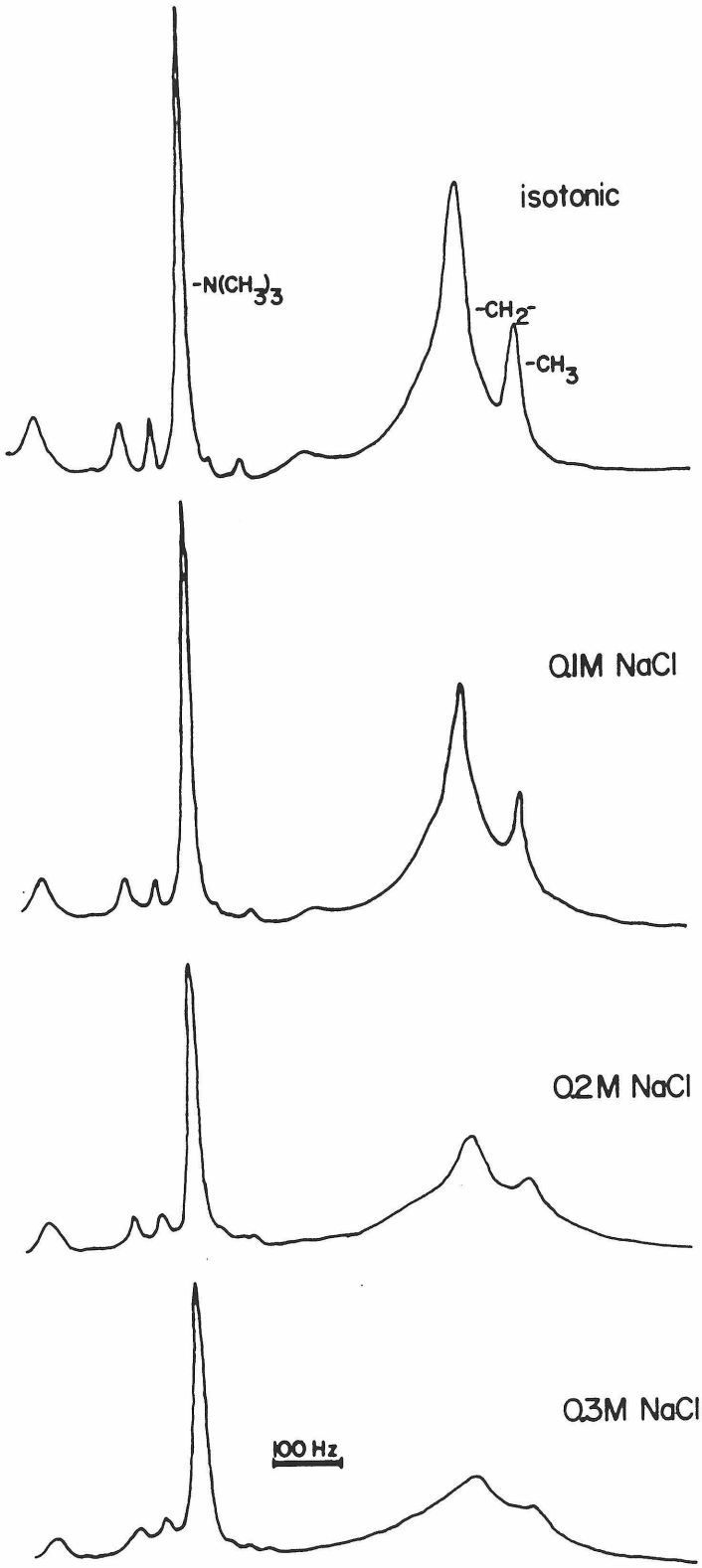

isotonic
-N(CH3)3
-CH2-
-CH3
0.1M NaCl
0.2M NaCl
0.3M NaCl
100 Hz

FIGURE 17

The effect of added external NaCl on the 220 MHz pmr spectra of egg lecithin vesicles containing 0.06 M $MgCl_2$ at 28°C.

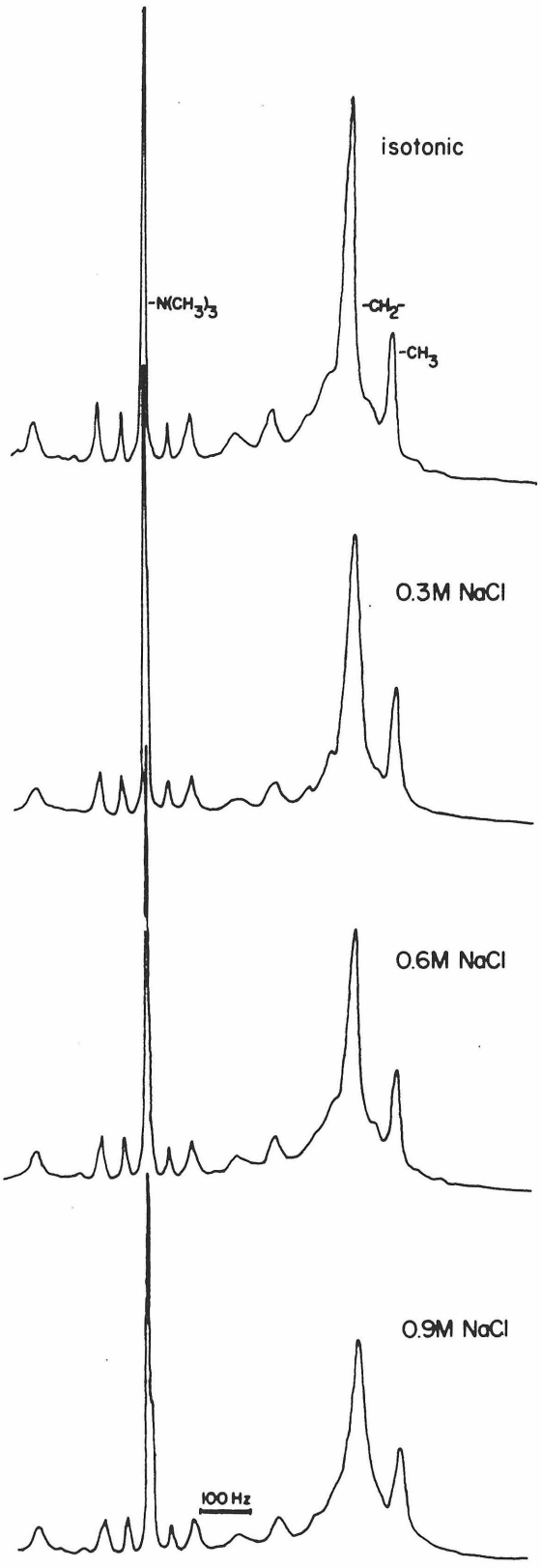
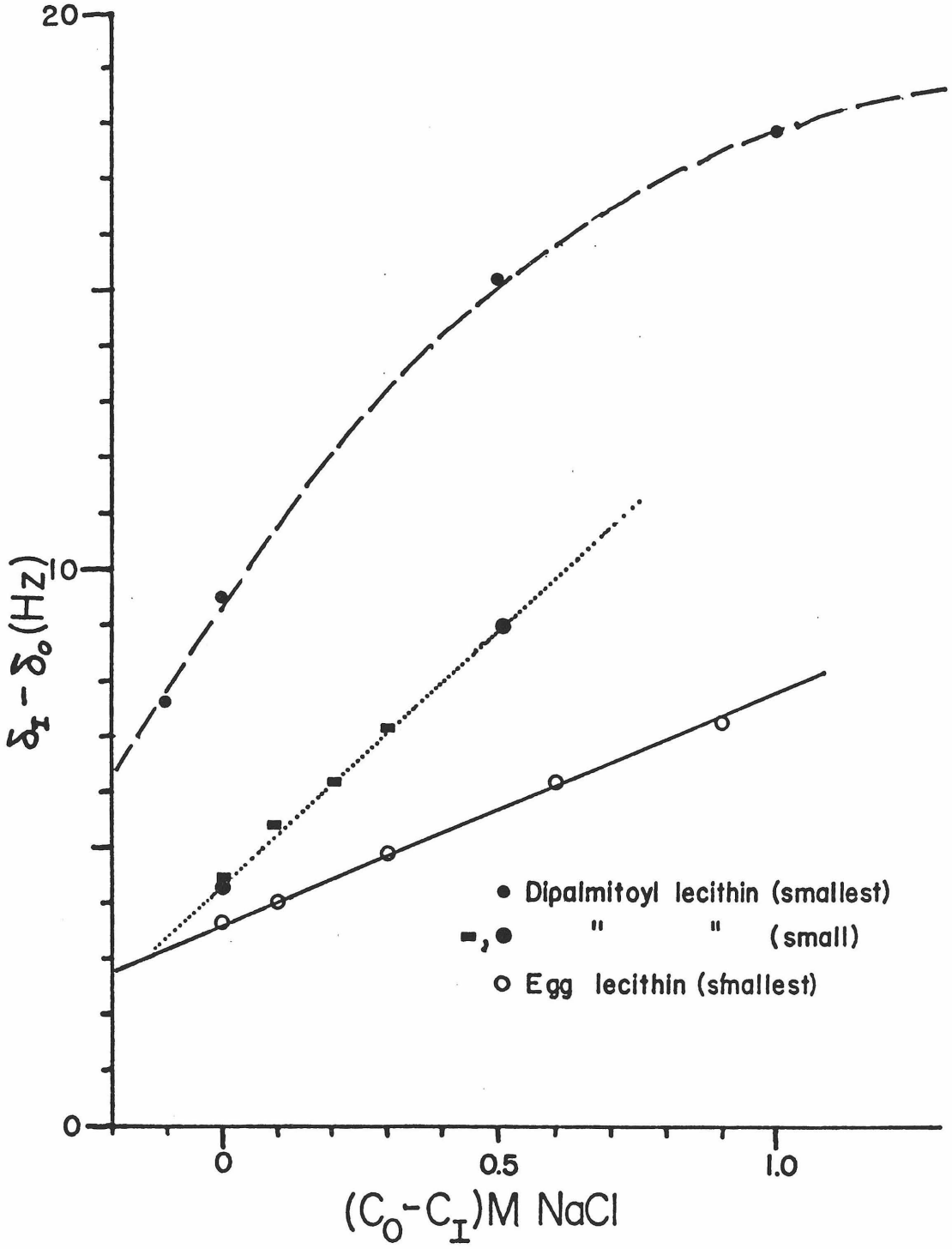

isotonic
-N(CH3)3
-CH2-
-CH3
0.3M NaCl
0.6M NaCl
0.9M NaCl
100 Hz

FIGURE 18

The chemical shift difference at 55 kgauss of the inside and outside choline resonances versus the salt concentration added to or subtracted from the sonicating medium for freshly sonicated dipalmitoyl lecithin vesicles at 5 mg/ml lecithin at 42°C (●), for the same vesicles one week later at 42°C (⊙), for the sonicated dipalmitoyl lecithin vesicles used in Fig. 16 at 42°C (■) and for the egg lecithin vesicles used in Fig. 17 at 28°C (O).


20
10
0
$\delta_I - \delta_I^o$ (Hz)
0
0.5
1.0
$(C_o - C_I)$M NaCl
● Dipalmitoyl lecithin (smallest)
■, ● " " (small)
○ Egg lecithin (smallest)

We have not sized these dipalmitoyl lecithin vesicles by electron microscopy but samples prepared in a similar manner to these small vesicles had an average diameter of 400-600 Å.

4. DISCUSSION

The pmr spectrum of lecithin in unsonicated bilayers is characterized by the following distinct features[80, 94, 95]. Below the Chapman transition no high resolution features are apparent in the spectrum. Above the Chapman transition only the resonances of the choline methyl groups and the chain terminal methyl groups are narrow enough to appear in a delayed Fourier transform experiment. The observed intensities of these resonances are always less than 100% of their expected intensities. These resonances have linewidths of about 150-200 Hz. The spin lattice relaxation times of these protons have also been measured and have been found to be about 0.5 sec. and to increase with increasing temperature. By contrast, the signal due to the bulk of the methylene protons is broad. These protons are characterized by a transverse relaxation time of 10^{-4} sec. and they appear to undergo spin lattice relaxation with a single T_1 of the order of 0.5 sec.

It is known that sonication has a profound effect on the pmr spectrum of lecithin bilayers[75, 82]. The spectrum is now characterized by sharp resonances (linewidths ~10-50 Hz), with intensities approaching 100% of those expected. More recently it has also been shown that different protons along the length of the fatty acid chains exhibit different spin lattice relaxation times T_1's[89]. This

distribution of T_1's indicates greater mobility of the methylene groups as one approaches the methyl end of the fatty acid chain.

There now seems to be little question that the principal source of the line broadening observed for the pmr spectrum of unsonicated bilayers is the nuclear dipole-dipole interactions among spins in the sample. The effective transverse relaxation time of the methylene protons has been shown to be independent of magnetic field, at least up to field strengths of 20 kgauss[80, 81]. In addition Finer et al.[82] have observed that the CW (continuous wave) spectrum of oriented multilayers is narrowed considerably when the chain axis is oriented with respect to the field near the magic angle (54°44'). Both of these observations strongly suggest that the breadth of the CW spectrum and the short transverse relaxation time in the free induction decay of lecithin bilayers is dipolar in origin. Recent work in this laboratory has indicated that it is possible to understand the pmr spectral behavior observed for the unsonicated lecithin bilayers above the phase transition in terms of a model in which the motion of the fatty acid chains is hindered and anisotropic[94].

Since the overall tumbling motion of the bilayer units is extremely slow (seconds or longer in the case of unsonicated lecithin) and there is a uniform distribution of orientation of the chain axis with respect to the magnetic field, the pmr spectrum of the lecithin bilayer should approach that of a powder in the absence of local motion. This is indeed the case below the Chapman transition. It is a reasonable approximation even above the Chapman transition, although it is necessary to invoke some local mobility of the fatty acid chains and the

choline head groups to account for the results of the delayed Fourier transform experiments and the T_1 data. The observation of a powder ^{31}P nmr spectrum both above and below the Chapman transition for the multilayers corroborates this interpretation. These ^{31}P nmr data indicate that whatever local motion does exist is not sufficiently isotropic and rapid to average out the small chemical shift anisotropy of the phosphorus nucleus in the glycerophosphocholine moiety let alone the larger dipolar interactions in the multilayers.

From the nature of this phosphorus chemical shift anisotropy and our knowledge of the motions of the lecithin molecule, we can hope to understand the orientation of the phosphate moiety of lecithin. Rotational motion of the whole lecithin molecule about an axis perpendicular to the bilayer plane should be sufficient to average out this anisotropy in the smectic mesophase, since the rotational correlation time of lecithin should be shorter than 10^{-4} sec. This comes from the fact that esr studies of steroid spin labels indicate a rotational correlation time of 10^{-8} sec. for those molecules in the bilayer. It follows, if motion in the bilayer is fast enough to average out the anisotropy, the parallel shielding tensor is oriented perpendicular to the bilayer. Since the parallel shielding tensor indicates less shielding of the phosphorus nucleus along that axis than perpendicular to it, we can predict that the electron density along that axis is less than along the perpendicular axes, which should be equivalent because of the fast rotation of the molecule. In looking at the phosphate of lecithin this means that the ester oxygens should be oriented perpendicular to the bilayer and the charged oxygens should be in the bilayer

plane with respect to the phosphorus nucleus. This requirement restricts the orientations of the choline to from 30° to 90° with the bilayer plane. We cannot make similar arguments for lecithin in the α-gel state because the rotational motion should be much slower in that case. It should be noted that the anisotropy is somewhat greater in the α-gel than the melted state indicating some motional averaging of the anisotropy in the latter case.

Several important parameters are affected when small vesicles are formed by sonication. First the small vesicles can undergo faster rotational Brownian Motion. For a 250-350 Å diameter vesicle this vesicle tumbling time becomes of the order of 10^{-6} sec.[82] compared to seconds or longer for the multilayers. Secondly, the effects of lateral diffusion can become important. Lateral diffusion of a phospholipid molecule on the surface of a sphere, like vesicle tumbling, tends to average out chemical shift anisotropies and dipolar interactions. Whereas in the case of a large multilayer it takes many seconds to undergo an angular displacement of $\pi/2$, this process could take place in 10^{-4} sec. for a 250-350 Å vesicle, if we assume a lateral diffusion constant of 10^{-8} cm^2/sec. Both of these changes, however, are not expected to lead to the dramatic changes observed in the pmr spectrum of sonicated bilayers. A vesicle tumbling rate of 10^6 $sec.^{-1}$ predicts a dipolar linewidth of 3000 Hz instead of the 10 Hz observed[94]. The fact that we observe no viscosity effect also indicates that the linewidths are not controlled by vesicle tumbling, although this isotropic vesicle tumbling is fast enough to average out the ^{31}P chemical shift anisotropy in the ^{31}P spectrum of these vesicles. Unless the lateral diffusion

constant of phospholipid molecules in the bilayer phase of lecithin is grossly underestimated, the lateral diffusion process is obviously too slow to have any profound effect on the pmr spectrum. Two independent investigators have estimated the translational diffusion constant of lecithin in a bilayer to be about 10^{-8} cm^2/sec.[69, 70]. Moreover, if this lateral diffusion is important, it is difficult to understand why certain protons in a molecule should appear to be more mobile than others in the pmr spectrum. Both vesicle tumbling and lateral diffusion involve motion of the entire phospholipid molecule, and therefore any differential broadening of the resonances could only arise from differences in local or segmental motion.

Sonication can lead to subtle changes in the bilayer structure as a consequence of the increase in the bilayer curvature. This structural change can come about as the result of the pressure which must be maintained across a highly curved surface or from molecular packing considerations. For a 300 Å vesicle a pressure of about 2 atmospheres should exist across the bilayer surface as the consequence of surface tension considerations[93]. This pressure is probably not large enough to affect the bilayer structure, as is evident when we compare the pmr spectra of vesicles with the high salt concentration inside and those which are isoosmotic with the 2 mM PO_4 outside the vesicles. There is no evidence for gross differences between the pmr spectra of these vesicles although an osmotic pressure of 4 atmospheres exists across the bilayer for the vesicles containing high salt.

More important, perhaps, is the change in molecular packing which must presumably accompany the fitting of phospholipid molecules

to form a small vesicle. In a flat bilayer the lateral area available per lecithin molecule is uniform from the surface to the center of the bilayer. For a small vesicle the area available for the polar head group and the fatty acid chains must necessarily be different. A larger or smaller area will be available to one end of the molecule rather than the other end, depending on which half of the bilayer the phospholipid molecule is located. Fig. 19 depicts the ratio of the area available for the charge head to that for the fatty acid chain of the lecithin molecule in a vesicular lamella versus the outside radius of the vesicle for both halves of the bilayer. This simple geometric calculation illustrates that for a molecule on the inside of a 300 Å vesicle, the methyl ends of the fatty acids can occupy an area 1.5 times that covered by the charge group, whereas the opposite is true for a molecule on the outside of the bilayer. The area measurements with Mn^{++} in the outside solution from this study and others indicate that each lecithin molecule covers about the same surface area in the inside and outside halves of the bilayer, which leads to the unlikely geometry presented in Fig. 20. This geometry is unlikely because the volume occupied by the lecithin molecules in the inside of the bilayer is so much greater than that of the molecules in the outside half of the bilayer. To remedy this the inner half of the bilayer must be thinner and/or the outer lecithin molecules must cover more surface area. This further illustrates the changes in packing which must occur to form small vesicles.

The most obvious conclusion which one can derive from the above considerations is that the packing arrangement of the phospholipid

FIGURE 19

The ratios of the area available for the charge head to that for the fatty acid end of the lecithin molecule in a vesicular lamella versus the outside radius of the vesicle for both halves of the bilayer assuming a bilayer thickness of 46 Å.

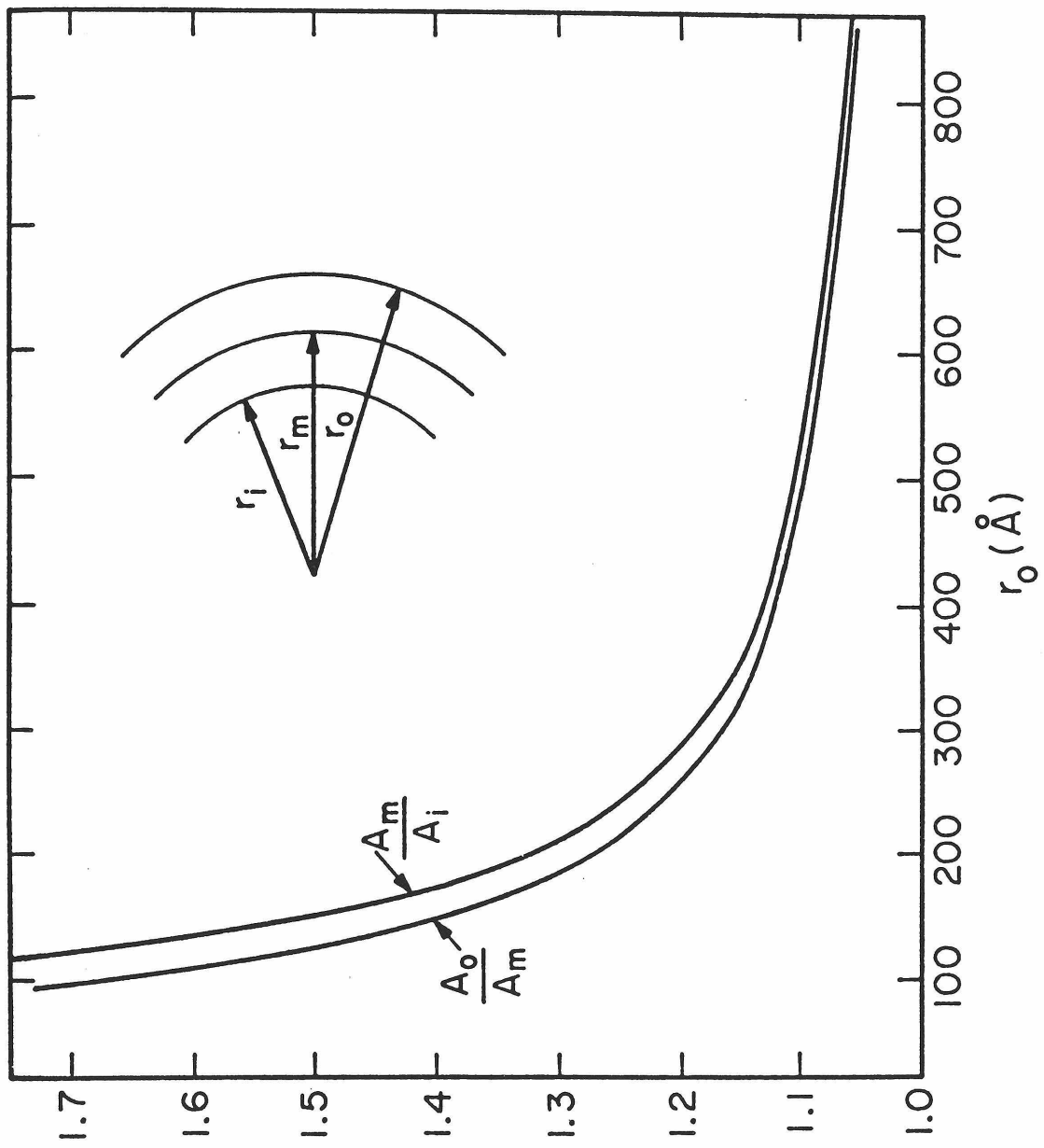
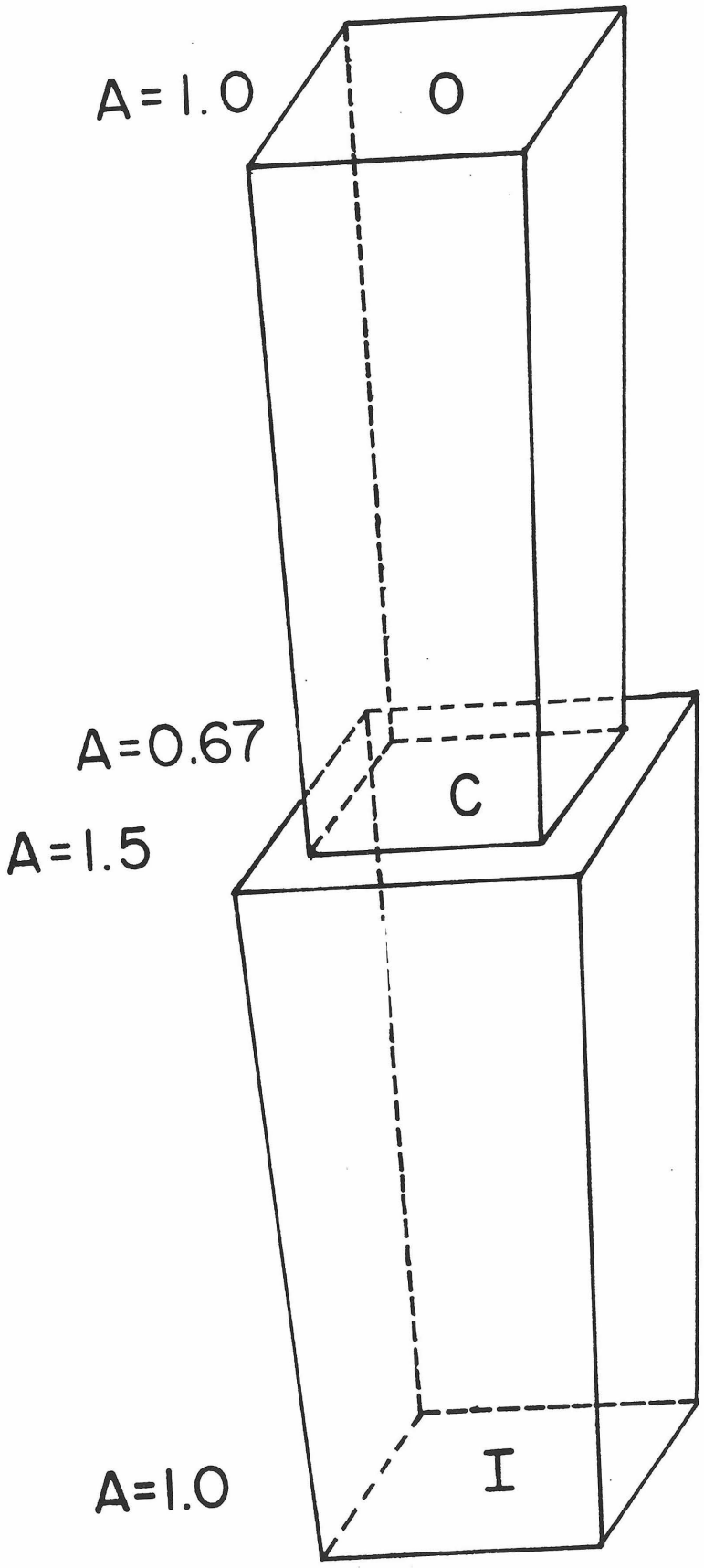

1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
100
200
300
400
500
600
700
800
r_o (Å)
$\frac{A_m}{A_i}$
$\frac{A_o}{A_m}$
r_i
r_m
r_o

FIGURE 20

A diagram of the volume occupied by lecithin molecules on the inside (I) and the outside (O) halves of the bilayer assuming that the surface area covered per lecithin molecule is the same at both surfaces and that the thickness of each half of the bilayer is equal.


A=1.0
O
A=0.67
C
A=1.5
A=1.0
I

molecules in the small vesicles is different or less regular than in the multilayers. This possibility is clearly indicated by the presence of two choline peaks from the small vesicles and by our dilatometry measurements, which showed the apparent molal volume of lecithin to be larger in the small vesicles than in the multilayers, at least below the Chapman transition. Thus, the small vesicles are probably somewhat disordered. The onset of the Chapman transition at lower temperatures and the greater breadth of this transition in the small vesicles, as monitored both by pmr and dilatometry, provide further evidence for this disorder. This disorder, or non-uniform molecular packing, undoubtedly facilitates the formation of kinks via gauche-trans isomerization along the polymethylene chain[31]. When kinks are formed, thinning of the bilayer (46 to 41 Å, Chapman et al.[32]) is accompanied by lateral expansion (51 $Å^2$ to 60 $Å^2$, present data). Above the phase transition the number of kinks formed per chain may or may not be greater in the small vesicles, but it is very likely that these kinks extend further up the chain than is possible in the case of unsonicated lecithin, where the packing is more uniform. Although the apparent molal volume of the lecithin is the same within experimental error between the larger vesicles and the small vesicles above the Chapman transition, one should not assume that consequently there are no structural differences between the two types of bilayers above the phase transition. This is because a large difference in the molal volume is not expected if the less regular structure in the smaller vesicles merely permits the kinks to extend further up the chain. However, this important difference most certainly would lead, on the average,

to a greater segmental motion of the polymethylene chain.

The most striking difference between the small and larger vesicles below the transition temperature is the apparent mobility of the choline group in the small vesicles. If indeed the local mobility of the choline group is greater as we propose, then the surface properties of the small vesicles should be different below the phase transition temperature. From the earlier work on water diffusion across bilayers we know that the flux of water across the membrane is related to the fatty acid composition but the activation energy for the diffusion process appears to be related to the nature of the bilayer surface. The large difference, therefore, that we observed between the activation energy in large (20 kcal/mole) and small (13 kcal/mole) vesicles demonstrates the large difference between the surfaces of the vesicles. In the black lipid membrane studies an activation energy of about 13 kcal/mole was obtained for lecithin in the melted state regardless of the fatty acid composition or the presence of cholesterol[60]. In our case, however, the vesicles were in the α-gel ("solid") phase which studies have shown to affect not only the fatty acids but also the bilayer surface. This is indeed the case for the larger vesicles which exhibit an activation energy 7 kcal/mole higher than normal for water diffusion, but the small vesicles give a value indicative of a melted state in accordance with the pmr spectrum. We feel this independent evidence strongly supports our analysis of the pmr spectra of small vesicles as well as demonstrates the radical change in the bilayer surface which occurs with freezing of the choline groups.

If we change the shape of a spherical vesicle by reducing the

internal volume, we must necessarily produce regions of lower curvature as well as some regions of higher curvature. From our previous studies we would expect that the lower curvature would reduce the local mobility; and although regions of higher curvature would remain mobile, the net effect would be a decrease in the intensity of the sharp fatty acid resonances. In Fig. 17 we do observe this phenomenon for egg lecithin when salt is added to the external medium to reduce the internal volume. Although the work of Sheetz and Yau does show that the internal vesicular volume decreases in response to the osmotic gradient, there is also evidence that a significant osmotic pressure does exist across the bilayer. Because the bilayer is quite permeable to water, this osmotic pressure must be absorbed by the bilayer itself. Necessarily, the osmotic pressure will be equalized by the vertical component of a lateral pressure in the surface of the bilayer. This lateral pressure, like the lower bilayer curvature, can reduce local mobility and, indeed, we note that curvature alone cannot account for the complete stabilization of dipalmitoyl lecithin vesicles at 42°C. The cause then of the decrease in the methylene resonance area with increasing salt can be either or both of the factors mentioned above. At present there are insufficient data to identify one factor as the major cause of the line broadening.

There are also several alternative explanations for the increase in chemical shift difference between the choline methyl protons on the inside and outside vesicle surfaces. The first explanation is that the shift is the result of a conformational change in the bilayer. The conformational change should cause some of the outside choline resonances

to be shifted toward and others on the edge of the disc with higher curvature to be shifted away from the inside choline resonances (Fig. 18) which do not appear to shift. In the spectra, however, the outside choline just shifts downfield away from the inside resonance. It is possible that the lecithins in the regions of high and low curvature are exchanging in a time faster than 0.01 to 0.1 seconds which would produce an average choline resonance. This exchange could be through changes of the regions of curvature or lateral diffusion through 90° on the surface which from Devaux and McConnell's work would occur in about 10^{-4} seconds. Since in a small vesicle a large fraction of the outside lecithins would lie in the edge of the disc, the net effect should be a downfield chemical shift of the external choline resonance. The other explanations that the shift is the result of lateral pressure or the presence of added salt are unlikely. This is because, first, the lateral pressure should bring the outside lecithins closer together and the downfield shift of the outside choline resonance appears to be the result of separation of the outside cholines. Second, the decrease of the volume inside the vesicle with added salt will raise the salt concentration there also and there is no chemical shift change of the inside choline resonance. This phenomenon of the outside choline chemical shift change, therefore, can be attributed to the conformational changes of the vesicle when placed in a hypertonic medium.

The explanations we presented earlier for the observed spectral differences in the small lecithin vesicles appear to be applicable for the changes which occur when the internal volume of the vesicle is decreased. These explanations were based on the premise that the spectral

differences arose from changes in the bilayer structure and not from overall rotation of the vesicle. We should also note that the increased curvature in egg lecithin bilayers upon sonication resulted in similar spectral changes, indicating that with unsaturated fats the molecular packing changes produce greater local mobility in the molecule and cause a chemical shift difference between the inside and outside cholines. Thus we feel that other studies of sonicated vesicles should be interpreted as measurements on an altered lecithin bilayer. Whether or not the measurements will be affected by the different properties of sonicated bilayers is obviously dependent upon what bilayer property is being studied. Bilayer mobility and perhaps passive transport rate studies should show the greatest changes in going from unsonicated bilayers to sonicated bilayers of high curvature.

The work on decreasing the internal volume of the vesicle with added salt can tell us about several other problems concerning the vesicle bilayer. From the osmotic pressure needed to collapse the vesicle we can find the structural rigidity of the bilayer possibly above and below the Chapman transition temperature. Further, from the abnormally low inside concentration of salt we can obtain information concerning the process of vesicle fusion. In the experiment of Sheetz and Yau (Fig. 15) they observed a critical concentration of salt above which the volume inside the vesicle decreased rapidly with increasing salt. The model we have developed to explain that phenomenon considers the vesicle to be similar to a hollow rubber ball which will collapse under a large uniform external pressure to form an ellipsoid or biconcave disc[96]. In the collapsed state the internal pressure

and the bending modulus of the bilayer equalize the external pressure. From the evidence there appears to be quite different behavior of the vesicles above and below the Chapman transition temperature. Below the transition temperature the bilayer appears rigid, so that when a critical pressure is reached it snaps into a biconcave disc. Above the transition temperature the chemical shift change of the outside choline and the intensity decrease of the methylene resonance show no critical pressure indicating plasticity of the membrane.

Provided we knew the magnitude of the pressure on the bilayer, which should arise from the different water activities on either side of the bilayer, we could calculate the bending modulus of the bilayer from the shape which minimum bending energy calculations would give[95]. A discrepancy became apparent, however, when we attempted to calculate the salt activity difference across the membrane. We assumed in making the calculations that the salt concentration originally inside the vesicle was 0.06 M $MgCl_2$, as in the sonicating medium, and that the inside volume decrease was truly measured by the water resonance area. There is no apparent reason for the volume measurement to be low by a factor of 5 and, thus, we feel that the salt concentration inside the vesicle is not as high as originally believed. This is not consistent with measurements of the internal volume of egg lecithin vesicles using radioactive solutes to measure the trapped volume[63], but small egg lecithin vesicles do not fuse into larger vesicles as do dipalmitoyl lecithin vesicles. Provided this fusion process occurs in dipalmitoyl lecithin vesicles without opening the inside of the vesicle to the external medium, as in the case of cell fusion, there would be a significant dilution of the

internal salts upon fusion. For example if we assume that the vesicles we studied, which were from 400-600 Å in diameter, were formed by the fusion of vesicles originally 200 Å in diameter, then the salts in the 200 Å vesicles would have been diluted about seven-fold. Experiments are currently under way to test this hypothesis because of its importance in understanding the vesicle fusion process.

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PART II

PMR STUDY OF THE HUMAN ERYTHROCYTE MEMBRANE UPON PERTURBATION

1. INTRODUCTION

In the first section we have seen how pmr along with other physical studies can be applied to the problem of mechanical stress in the dipalmitoyl lecithin bilayer. In that case pmr was a most sensitive detector of change in the bilayer structure. The application of pmr to the study of structural changes in a biological membrane then seemed a logical next step. There again we sought to supplement the pmr data with other physical and biochemical studies which led us to a deeper understanding of the structural changes we observed.

1.1 Biological Membrane Structure

The functions of biological membranes should be highly dependent upon the structure of the membrane and structural relationships between the various components. For this reason many recent studies of biological membranes have been directed toward the elucidation of membrane structure, with particular emphasis on the state of the lipid phase and the various interactions between the lipids and membrane proteins.

The state of the lipids in artificial and natural membranes has received considerable attention. Most biological membranes contain at most five or six major lipids, and there has been ample evidence to indicate that the bulk of these lipids exist in the form of bilayers. There has also been some experimental evidence[1] to suggest, however, that some of the lipids are linked to the membrane proteins in a specific way, as in the subunit model of Benson[2], or more likely

as in the mosaic model of Glaser et al.[1, 3]. Much of this evidence has come from the effects of phospholipase C, an enzyme which removes the ionic heads of phospholipids leaving diglycerides, on the circular dichroism spectra, pmr spectra and electron microscopy pictures of natural membranes[4, 5]. Moreover, it is known that certain isolated enzymes do require specific lipids for activity, implying specific interactions between these proteins and lipids. Where such specific protein-lipid interaction has been inferred, the exact nature of the interaction is still unknown.

The protein portion of biological membranes is expected to be more heterogeneous than the lipid portion in that proteins must provide the basis for functional specificity. In the case of highly specialized membranes such as sarcoplasmic reticulum and rod outer segment membranes, however, it is not unlikely that only a few proteins are present. Studies of the protein chemistry of sarcoplasmic reticulum membranes, for example, reveal that over 50% of the membrane proteins are accounted for by one protein[6]. In more complicated membranes, such as the erythrocyte membranes, it has been estimated that there are 14-20 proteins[7, 8]. In fact, the molecular weight, solubility properties, amino acid composition[7], enzyme function and spatial positions of certain proteins of the red blood cell membrane have now been characterized[9, 10]. Knowledge concerning the organization of these membrane proteins including their structural relationship with the lipid phase, however, is still fragmentary. Circular dichroism measurements of natural membranes have revealed that as much as 50% of the amino acid residues are in the α-helical

configuration, a result which is not predicted by the Davson-Danielle model but is totally consistent with the mosaic model of membrane structure[3].

The forces which order and organize the membrane components constitute one of the most urgent problems in membrane biophysics. There seems to be little question that the lipid bilayer itself is stabilized by hydrophobic as well as hydrophilic forces. Hydrophobic forces are presumably also important in ordering the membrane proteins among themselves and with respect to the membrane lipids. However, where the protein contains a high percentage of charged residues or where they are in contact with the aqueous medium, one clearly must also consider possible electrostatic interactions between these residues and the charged groups on the lipid bilayer surface. The relative importance of these forces might be reflected in the solubility characteristics of the various membrane proteins. For example, about one-half of the proteins of the red blood cell can be solubilized in water, and aside from 7% which are solubilized in organic solvents along with the lipids, the remaining proteins are only solubilized with surfactants[7].

1.2. Choice of System and Method

We have chosen the human erythrocyte membrane system for the following study because of the vast amount of background information available and the ease with which large amounts of pure membranes could be obtained. The complex protein composition of the membrane was accepted since it was felt that a membrane with general properties

rather than a highly specialized function would be best for a general study of biological membrane structure.

Because of the added complexity of biological membranes as opposed to model bilayer systems, relatively little detailed molecular information has been obtained by physical methods. Many of the techniques discussed in the previous section have been applied to various membrane systems with limited success largely because of the difficulties of interpretation. We, therefore, looked for a method for which the theory was well understood and for a method that was versatile. Proton magnetic resonance spectroscopy was chosen because not only was it possible to interpret the changes in the linewidth of the resonances or their chemical shifts, but also resonances from all the different membrane components were observable on the same spectrum.

Early work on red cell membranes was done at low fields with also low sensitivity and consequently the investigators resorted to adding detergents or sonicating the membranes to obtain observable spectra[11]. Although sonication produces sharp resonances from natural membranes, we know from the earlier work that sonication alters the mobility and configuration of lipid molecules as well as the nature of the bilayer surface, not to mention the possible changes in the protein. It is necessary then to go to higher fields and more advanced pmr instrumentation to gain the resolving power and sensitivity necessary to obtain relevant data from whole biological membranes[12-14]. Some investigators still persist in using sonicated samples in their studies of membranes. As a result of their reluctance to acknowledge the differences between sonicated and unsonicated samples, some

possibly wrong and totally unfounded conclusions have been reached. For example, Metcalfe et al.[15] conclude that the lipid fatty acids in sarcoplasmic reticulum membranes are restricted in the whole membrane compared to the sonicated lipids alone while the choline charge groups have freer motion in the whole membrane. This is not a valid comparison because the mobility of the lipid is altered by curvature in the sonicated bilayers, and they might have observed the same difference by comparing the relaxation parameters of sonicated with unsonicated lipids. We have been careful to avoid these pitfalls by recognizing that changes in structure occur with sonication.

Since it has now been shown that the broad lipid resonances are the result of dipole-dipole broadening and not magnetic field gradients, we can attribute sharp resonances to mobile groups on the membrane. Although the whole membranes undergo isotropic motion on the time scale of seconds, certain portions of the membrane or functional groups may have sufficient local motion to give sharp pmr resonances. Such groups as the choline from lecithin, the ϵ -CH_2 of lysine, etc. are potentially mobile even though other portions of the lipid or protein are not. Unlike earlier workers, we have carefully assigned and quantitated the sharp resonances in the spectra of whole membranes in an effort to obtain specific molecular information about changes in membrane structure. Since some distinct proton groups are found in every important part of the membrane, a single membrane spectrum tells us about the degree of order throughout the membrane. Thus, by following certain spectral regions under a variety of conditions, we can understand how treatments have

changed the structure on a molecular level. This involves careful biochemical controls to know what changes have occurred to correlate with the observed spectral changes. Specifically, we have sought to understand the effects of heating, protein removal, sonication and treatment with phospholipase C on the structure of the erythrocyte membrane. These treatments have been studied by various other techniques, and we hope to show the usefulness of pmr in understanding their effects and the basic structure of the erythrocyte membrane.

1.3. Effects on Human Erythrocyte Membrane Structure.

The effect of temperature on human erythrocyte membranes has been studied extensively from the aspect of functional stability since it is important in blood storage for transfusion purposes. From such work it is known that enzyme activities in the red cell membrane are destroyed at temperatures above about 48°C[16]. Thus at temperatures above this we no longer have a viable or biologically active cell membrane. This is presumably because of the simple denaturation of membrane proteins and not the wholesale disintegration of the membrane since light microscopic studies still observe a whole membrane. There are significant changes in the light scattering from the membranes above 40°C[17] which could also be accounted for by protein denaturation.

The solubilization of proteins from erythrocyte membranes can be extensive under the proper salt and pH conditions. In the specific case of iodosalicylate washes it appears that only one protein is being solubilized[18] but generally a variety of proteins are removed

by salt washes. Some of the solubilized proteins have been localized on the membrane surface[19] but others have been shown to extend through the bilayer[18], precluding any generalizations that just surface proteins are solubilized. From some of the proteins which have been solubilized we have learned that there is a great difference between the inside and outside surfaces of the membrane[19]. This was dramatically illustrated later by experiments on inside-out membranes[20]. The general roles that the solubilizable proteins play in the structure and function of the human erythrocyte membrane are not well understood although certain blood group antigens and enzyme activities have been found in them. Our present study does, however, indicate some basic molecular roles such proteins might play in membrane structure.

Various investigators have studied the specific binding of divalent ions to the erythrocyte membrane[21, 22]. Normally these ions are an integral part of the membrane since it is bathed in about 10^{-3} M of Mg^{++} and Ca^{++} from the plasma. These sites are blocked by local anesthetics[21] and appear to be quite specific for the divalent ions, particularly Ca^{++}. We have observed the effect that divalent ions have on the mobility of various membrane components.

The effect of sonication on red blood cell ghosts has been studied by Rosenberg and McIntosh[23] using electron microscopy and centrifugation. They found after extensive sonication that electron micrographs showed membrane plates as well as small vesicles. Such heterogeneity makes it impossible to analyze the resultant spectra in detail, as earlier pmr studies have shown[11]. We were interested, however, in the general nature of the destabilization

brought about by this treatment.

Phospholipase C is one of the family of lipases from A to D. It catalyzes the cleavage of the glycerol-phosphate ester linkage leaving the diglyceride in the membrane while the phosphorylated amine goes into solution. There are two common sources for the phospholipase C type enzymes, one from the bacterium Cl. perfringens and the other from B. cereus. Although the two enzymes catalyze the same reaction, they have different lipid specifities as indicated by their action on red blood cell ghosts. From two independent studies we find that the action of the Cl. perfringens enzyme leaves nearly all the negatively charged lipids and some phosphatidyl ethanolamine[24] while the B. cereus leaves all of the sphingomyelin and only a small portion of the charged lipids[25]. Since the two enzymes leave quite different lipids, particularly in net charge, it might be expected that the morphology of the cells treated by the different enzymes would be different. Rigorous comparisons of differently treated ghosts were not made nor were radical differences noted. In both cases the remaining diglycerides formed into pools of 3000-10000 Å in diameter; however, Ottolenghi and Bowman did note that mitochondria treated by the Cl. perfringens enzyme contained fewer diglyceride blebs than membranes treated by the B. cereus enzyme[4]. The comparison of these two treatments is important since it provides valuable information about the varied roles, if they are varied, of negatively charged versus zwitterionic lipids in the structuring of the erythrocyte membrane. This work was done in collaboration with Dr. S. J. Singer and Michael Glaser of University of California at San Diego who performed simultaneous circular

dichroism experiments on the same samples that were studied by pmr. This collaboration was necessary to understand the changes in both the lipids and proteins simultaneously.

This work on the human erythrocyte membrane was undertaken as an exploratory venture. We hoped not only to understand the specific features of the human erythrocyte membrane pmr spectrum in terms of molecular structure, but also to develop general techniques applicable in future studies of membrane systems by pmr. For example, by studying the effects of the above treatments systematically and over a wide range of experimental conditions, such as solvent structure, divalent ion concentration, etc., we were able to pinpoint some of those variables which control the resistance of the membranes towards various types of structural changes. It is anticipated that these studies will also provide background information which may be useful for future pmr studies of biological membranes.

2. EXPERIMENTAL

2.1. Preparation of Red Blood Cell Membranes

Red blood cell membranes were prepared from fresh human blood, type O and Rh^+, in A. C. D. by the method of Dodge, Mitchell and Hanahan[26]. The ghosts were lysed in 6 mM phosphate buffer (20:1 lysing buffer to packed cells) at pH 7. 8. They were then washed five times in the same buffer until white and portions were taken for protein assay by the Lowry method[27]. These white membranes were layered onto D_2O (6 mM phosphate at pD 7. 8) and were pelleted

at 40,000 rpm in an SW-50 rotor for 20 minutes. After centrifugation, portions of the supernatant were saved for protein assay. These pellets were resuspended in the D_2O buffer and the procedure was repeated for four additional washes. Care was taken to minimize the exposure of the D_2O suspensions to air since contamination by H_2O occurred readily. The pellets of ghosts were always in two distinct layers. The upper layer was labelled soft-packed ghosts and was pulled off separately from the bottom layer, which we have called hard-packed ghosts. Aliquots of all pmr samples were diluted and assayed for protein.

2.2. Removal of Spectrin

About 30 ml of freshly prepared ghosts were dialyzed against 1000 ml of a 0.001 M EDTA and 0.05 M β-mercaptoethanol solution for 48 hours at 4°C[28]. The ghost suspension was then spun at 14,000 rpm in an SS-35 rotor, and the supernatant withdrawn. A portion of the ghosts was layered onto the D_2O buffered with 1 mM trisma base and 3 mM KCl at pD 7.8 and exchanged into D_2O as before.

2.3. Isolation of Membrane Protein

About 40 ml of freshly prepared ghosts were lyophilized, extracted with 300 ml of ethanol:ether mixture (3:1) at -15°C for four hours, and centrifuged for 30 minutes at 13,000 X g. The extraction was repeated twice with the same solvent mixture. The final pellet was placed in 30 ml of ether at -15°C for three days with occasional shaking and then centrifuged for 30 minutes at 1,300 X g. This

procedure was repeated twice and the final pellet was dried under vacuum and stored at -15 C until used. A weighed sample was suspended in D_2O buffer for pmr spectra.

2.4. Addition of Divalent Cations

Solutions of $MnSO_4$ and $MgSO_4$ (0.1 M) were prepared in D_2O and appropriate μl amounts of these solutions were added directly to membrane samples in pmr tubes with the aid of long capillary tubes.

2.5. Sonication

Several samples of ghosts were sonicated for 15 seconds by a Bronson sonifier with a micro tip at lower level No. 3. These samples were then spun at low speed to pellet whole cells, the particles in the supernatant were drawn off, exchanged into D_2O buffer, and the pmr spectrum recorded.

2.6. High-temperature Centrifugation Experiments

A series of high temperature centrifugation runs were made on a Model E centrifuge. In these experiments the temperature of the buckets was measured before and after each run and the supernatants of the membrane samples were removed while still at the same temperature. The samples were spun at 39,000 rpm for one-half hour in an SW-39 rotor. Other samples were heated to 75°C in a water bath for ten minutes, cooled to 4°C, and centrifuged at 4°C for one-half hour at 39,000 rpm. The supernatant as well as a portion of the original ghost suspension were analyzed for protein.

D_2O and H_2O suspensions of ghosts were carried through the same number of centrifugations. The pH of the supernatant from the final spin was measured. Pelleted membranes were diluted to about 1.0 mg/ml protein before heating and salts were added to these suspensions.

Samples of the supernatant protein and membranes were analyzed by acrylamide gel electrophoresis in sodium dodecyl sulphate according to the procedure of Laico et al.[29]. The amino acid content of the acid digested supernatant protein was analyzed on a Beckman analyzer.

2.7. Preparation of Lipid Suspensions

Lipids obtained from Applied Science Labs came as solutions in organic solvents and measured volumes of these solutions were placed directly into nmr tubes and dried under vacuum. D_2O phosphate buffer (7 mM at pD 8.0) was added to the tubes and they were shaken vigorously to obtain a homogeneous suspension.

2.8. Preparation of Outer Mitochondrial Membranes and Mycoplasma Laidlawii Membranes

Samples of Outer Mitochondrial membranes and Mycoplasma laidlawii membranes were prepared by Dr. Henry Simpkins and Dr. S. J. Singer at the University of California at San Diego. The Outer Mitochondrial membranes were isolated from a sucrose step gradient after mild sonication to separate the inner and outer membranes. Mycoplasma laidlawii membranes were prepared by the osmotic lysis

procedure. All samples were washed five times into low salt D_2O (20 imOsm) buffered at pD 8.0.

2.9. Phospholipase C Treatment

Human red blood cell membranes were prepared from fresh blood by the procedure of Dodge, Mitchell and Hanahan[26]. The phospholipase C experiments shown in this paper were performed with a purified enzyme from B. cereus prepared and generously donated by Dr. A.C. Ottolenghi[30]. Several preliminary experiments were performed with a crude phospholipase C prepared from Cl. perfringens (Obtained from Worthington Co., Freehold, N.J.) which had been heated for ten minutes at 60°C and freed of precipitated protein. The red blood cell membranes were transferred by centrifugation and resuspension (three times) into phosphate-free buffers for the phospholipase C digestions: 5 mM Tris, 5 mM KCl, pH 7.3, in the case of the purified enzyme, and 5 mM Tris, 2 mM $CaCl_2$, and 2 mM KCl, pH 7.3, for the crude enzyme. Approximately one enzyme unit was added per 2.5 mg of membrane protein, and the mixtures were incubated at 37°C for 20 minutes. Ten volumes of cold buffer were then added and the membranes were collected by centrifugation and washed with buffer. After removal of a portion of the sample for CD and analytical measurements, the remainder was transferred by five cycles of centrifugation at 50,000 rpm and resuspension into 7 mM Na phosphate in D_2O, apparent pD 8.1. The final pellet was used for pmr, CD and analytical measurements. Control samples were used through all operations in parallel except that no

phospholipase C was added.

CD spectra were obtained with the J-10 modification of the Durrum-Jasco ORD/UV/CD-5 instrument in 0.5 mm cells. The effect of temperature was observed in 1.0 mm water-jacketed cells; samples were kept at each temperature for 20 minutes before observation. The absorbance did not exceed 2.0 for any measurements. A mean residue weight of 114 was assumed for calculations of the ellipticity.

Analyses were performed as follows: for protein content, the method of Lowry et al.[27] was used, calibrated by Kjeldahl N determinations; for total phosphorus, the method of Bartlett[31] was used; for fatty acid esters, the method of Snyder and Stephens[32] was used with an incubation time of 30 minutes at 75°C; for cholesterol, the method of Abell et al.[33] was used.

2.10. PMR Spectra

All pmr spectra were taken on a Varian HR-220 spectrometer with the aid of a Varian C-1024 time-averaging computer. Samples were equilibrated for 30 minutes at each temperature before spectral observation and the temperature was measured using an ethylene glycol standard and the calibration curve provided by Varian. An external standard of acetone in D_2O doped with $MnSO_4$ served as an intensity standard for intensity measurements. This solution was contained in a capillary and the area of the acetone peak was calibrated against a 0.003 M tetrabutylammonium chloride solution. Care was taken to insure that the same filling factor was maintained throughout all the intensity measurements. Area measurements were made with a

planimeter. Chemical shifts are given in ppm from DSS (3-(Trimethylsilyl)-propane-sulfonic acid sodium salt) and were measured from the external standard, whose chemical shift was in turn standardized against a solution of DSS.

2.11. CD Spectra

Circular dichroism spectra were recorded on a Cary 61 recording spectropolarimeter at Cary Instrument Laboratories, Monrovia, California, using 1 mm quartz cells. The spectra at high temperatures were obtained after the thermostatable cell assembly was allowed to equilibrate for 20 minutes. The protein concentration of the samples was between 0.18 and 0.20 mg/ml in these measurements. A mean residue weight of 114 was used in the calculation of the molar ellipticity.

3. RESULTS

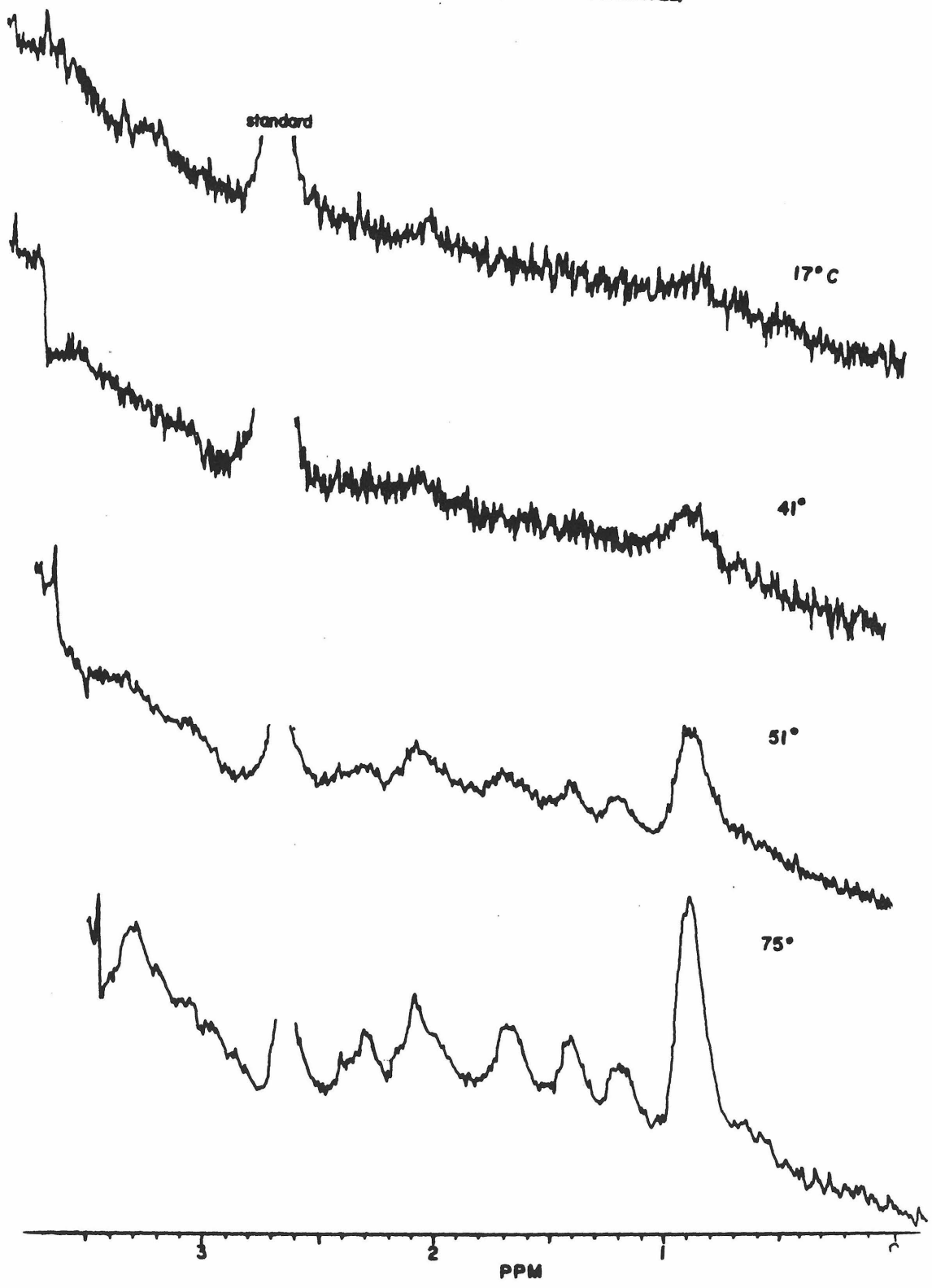
3.1. Analysis of Spectra

The 220 MHz pmr spectrum of soft-packed human erythrocyte membranes exhibits a marked temperature dependence, as shown in Fig. 1, where we have reproduced the spectral region from -0.0 to -3.5 ppm at 17°, 41°, 51° and 75°C. At 17°C, only several broad resonances were evident upon scanning the spectral region from 2 to -9 ppm, aside from the external standard resonance introduced for the intensity measurements and the strong residual HOD resonance at ~-4.5 ppm arising from H_2O contamination of the D_2O. We note

FIGURE 1

220 MHz pmr spectra of whole human erythrocyte membranes (5.0 mg membrane protein/ml) in the region from 0 to -3.5 ppm from DSS.

220 MEGACYCLE P.M.R. SPECTRA OF
HUMAN ERYTHROCYTE MEMBRANES (UNSONICATED)

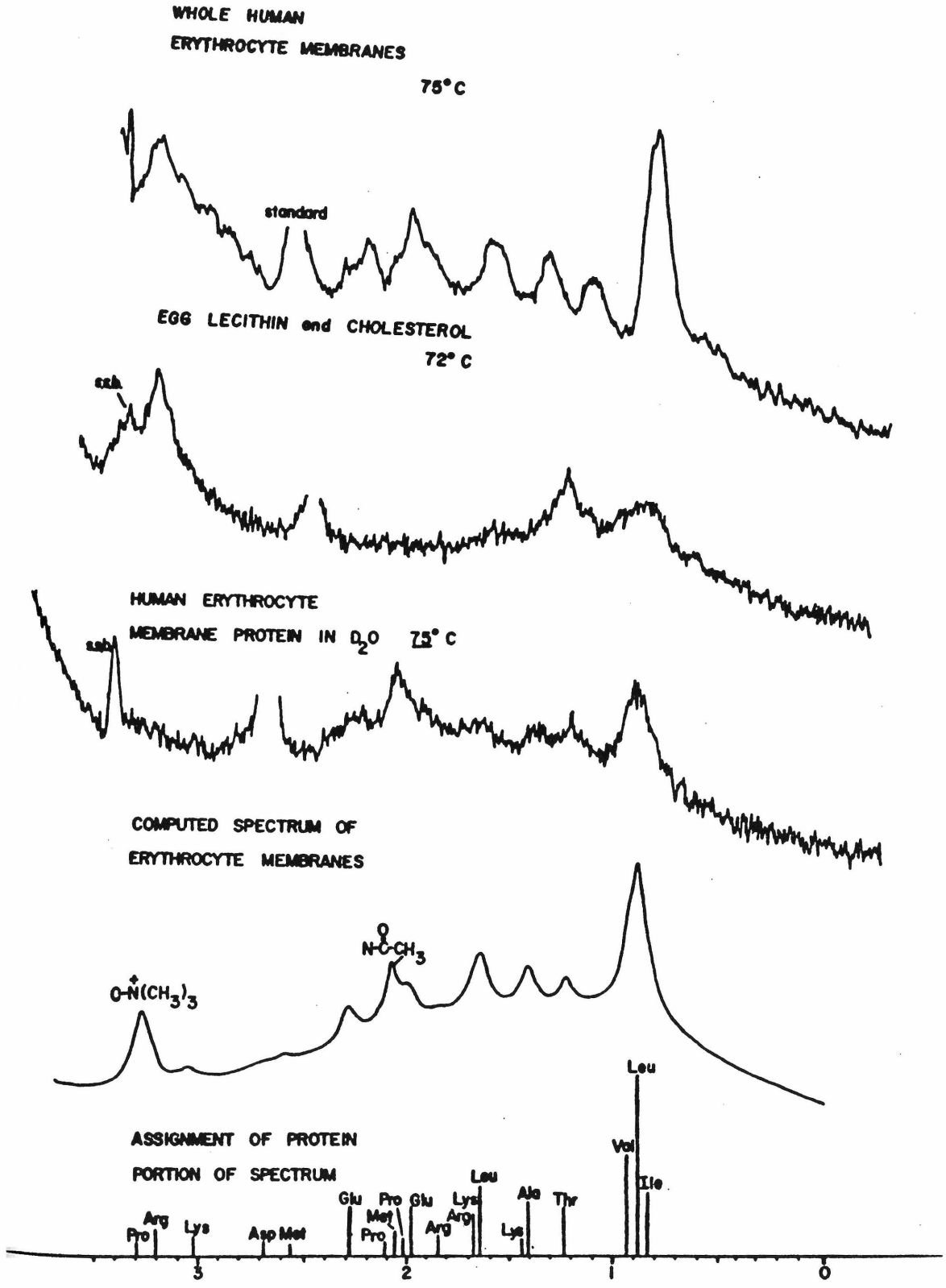


that the apparent spectral base line in the region 0 to -3.5 ppm is actually the composite sum of the high field tail of the strong HOD peak and parts of the very broad resonances. Three resonances, at -0.89, -2.06 and -3.25 ppm, however, became clearly discernible at 41°C. Above this temperature, there was a marked increase in the intensity of the resonance at -0.89 ppm, and five additional peaks also became apparent at -1.22, -1.41, -1.64, -2.26 and -3.02 ppm, as is evident upon a comparison of the spectra recorded at 41°, 51° and 75°C. With increasing temperature, there is a slight concomitant decrease in the linewidths of the various resonance peaks, but this reduction in the linewidths in itself does not account for the observed increase in the height of the resonances. The observed increase in the areas of these peaks clearly indicates that the additional protons are contributing to these resonances. The above observations are largely reproducible from membrane sample to membrane sample irrespective of whether the samples were fresh or had been heated above 75°C.

We have compared the spectra of the "whole" membranes with those of an egg lecithin and cholesterol mixture and the isolated membrane proteins in Fig. 2. This comparison has led us to assign the spectrum of the whole membranes at 75°C to the membrane proteins and the choline methyl groups of the phospholipids. In particular the peak at -0.89 ppm can be assigned to the methyl protons in the protein. The area of this peak, however, indicated only that 21 ± 3% of the methyl protons of valine, leucine and isoleucine are contributing to this resonance. Although these amino acid residues are most likely located in a specific membrane protein fraction, differences in the

FIGURE 2

220 MHz pmr spectra of (a) whole human erythrocyte membranes at 75°C; (b) egg lecithin and cholesterol, 1:1 (20 mg lecithin/ml); (c) delipidated membrane protein from human erythrocyte membranes in D_2O at 75°C (5 mg/ml); (d) computed spectrum for human red cell membrane; (e) assignment of the amino acid resonances in computed spectrum.


WHOLE HUMAN
ERYTHROCYTE MEMBRANES
75° C
standard
EGG LECITHIN and CHOLESTEROL
72° C
ssh
HUMAN ERYTHROCYTE
MEMBRANE PROTEIN IN D_2O 75° C
ssh
COMPUTED SPECTRUM OF
ERYTHROCYTE MEMBRANES
$O\text{-}\overset{+}{N}(CH_3)_3$
$N\text{-}\overset{O}{\overset{\|}{C}}\text{-}CH_3$
ASSIGNMENT OF PROTEIN
PORTION OF SPECTRUM
Pro
Arg
Lys
Asp
Met
Glu
Pro
Met
Pro
Glu
Arg
Lys
Arg
Leu
Lys
Ala
Thr
Val
Leu
Ile
3
2
1
0

amino acid composition among the various protein fractions are not sufficiently large to enable us to draw this conclusion[7]. In the calculation of the membrane protein spectrum we have, therefore, merely used the average amino acid composition for the whole membrane[34, 35]. A computer simulated spectrum of the whole membranes assuming that 20% of the proteins exist in a random coil configuration is given in Fig. 2. For the protons of the amino acid residues in the random coil configuration we have used the chemical shifts and linewidths reported by McDonald and Phillips[36]. The proton resonances of the remaining 80% of the membrane proteins were assumed to have the same spectral position in this calculation as the random coil values but were assigned a linewidth ten times greater. All the lipid proton resonances were assigned a width of 600 cps except for 20% of the choline methyl protons, and their chemical shifts were taken from spectra of lecithin, oleic acid and cholesterol in chloroform as well as the data previously reported by Purcell et al.[37] and Gorkom and Hall[38]. The lipid composition was taken from Dodge and Phillips[39]. In addition, a peak attributable to the acetamide groups of the sugars was added to the computed spectrum at -2.08 ppm and its area was estimated from the amount of neuraminic acid present in the membrane[34]. A complete breakdown of the proton resonances from the human erythrocyte membrane by their chemical shift is given in Table I along with their relative intensities. The calculated spectrum can be seen to be in excellent agreement with the experimental.

The percentage of protein methyls which have been sufficiently mobilized to yield a high resolution pmr spectrum is shown in Fig. 3

TABLE I

Component	Type	Chemical Shift (cps)	Area
C	-C-CH_3	150	20.0
P	Ile, -CH_3	183	31.7
L	-C-CH_3	193	62.4
C	-C-CH_3	194	60.0
P	Lea, -CH_3	195	68.0
P	Val, -CH_3	205	42.6
C	-CH_3	224	20.0
C	9 protons	243	60.0
P	Ile, -CH_2	250	5.3
P	Thr, -CH_3	270	17.6
L	-C-CH_2-C-	277	403.7
C	4 protons	294	26.7
P	Ala, -CH_3	310	24.5
P	Ile, -CH_2	310	5.3
P	Lys, -γCH_2	315	10.4
C	7 protons	325	46.7
L	-CH_2-CH_2C=O	334	35.9
P	Leu, β-CH_2 + γCH_2	360	34.0
P	Arg, γ-CH_2	365	9.1
P	Lys, δ-CH_2 + β-CH_2	370	20.8
C	4 protons	391	26.7
P	Arg, β-CH_2	405	9.1
P	Ile, β-CH	425	5.3
P	Glu, β-CH_2	435	24.3
C	2 protons	441	13.3
P	Pro, γ-CH_2	445	8.5
L	CH_2-C=	446	49.7
S	N-CO-CH_3	454	5.3
P	Met, -CH_3	454	6.1
P	Met, β-CH_2	454	4.0

Component	Type	Chemical Shift (cps)	Area
P	Pro, β-CH_2	465	8.5
C	2 protons	477	13.3
P	Val, β-CH	495	7.1
P	Glu, γ-CH_2	500	24.3
L	-CH_2C=O	503	35.9
P	Met, γ-CH_2	565	4.0
P	Asp, β-CH_2	590	16.9
L	=C-CH_2-C=	616	40.8
P	Phe, β-CH_2	650	4.2
P	Tyr, β-CH_2	655	4.8
P	Cys, β-CH_2	665	2.2
P	Lys, ϵ-CH_2	665	10.4
P	His, β-CH_2	700	4.9
P	Phe, β-CH_2	700	4.2
P	Arg, δ-CH_2	704	9.1
L	-$N^{\oplus}(CH_3)_3$	715	54.5
P	Pro, δ-CH_2	725	8.5
P	Trp, β-CH_2	745	5.0
C	H-C-OH	746	6.7
S	HCOH	815	36.2
L	C-O-CH_2-C	834	13.6
L	P-O-C-CH_2-N	863	15.3
L	C-O-C-CH-C-O	902	9.0
L	P-O-CH_2-C-N	942	18.0
L	C-O-C-C-CH_2-O-P	966	18.0
L	H-C=	1165	68.8
C	H-C=.	1179	6.7
P	Tyr, aromatic O-to Oh	1500	4.8
P	Trp, indole C-5	1549	2.5
P	His, imidazole C-4	1555	2.4
P	Tyr, aromatic M- to OH	1560	4.8
P	Trp, indole C-6	1566	2.5
P	Trp, indole C-2	1584	2.5

Component	Type	Chemical Shift (cps)	Area
P	Phe, aromatic	1598	21.0
P	Trp, indole C-4	1638	2.5
P	Trp, indole C-7	1658	2.5
P	His, imidazole C-2	1740	2.4

P[†] = Protein
L[*] = Phopholipid
C[‡] = Cholesterol
S = Carbohydrate

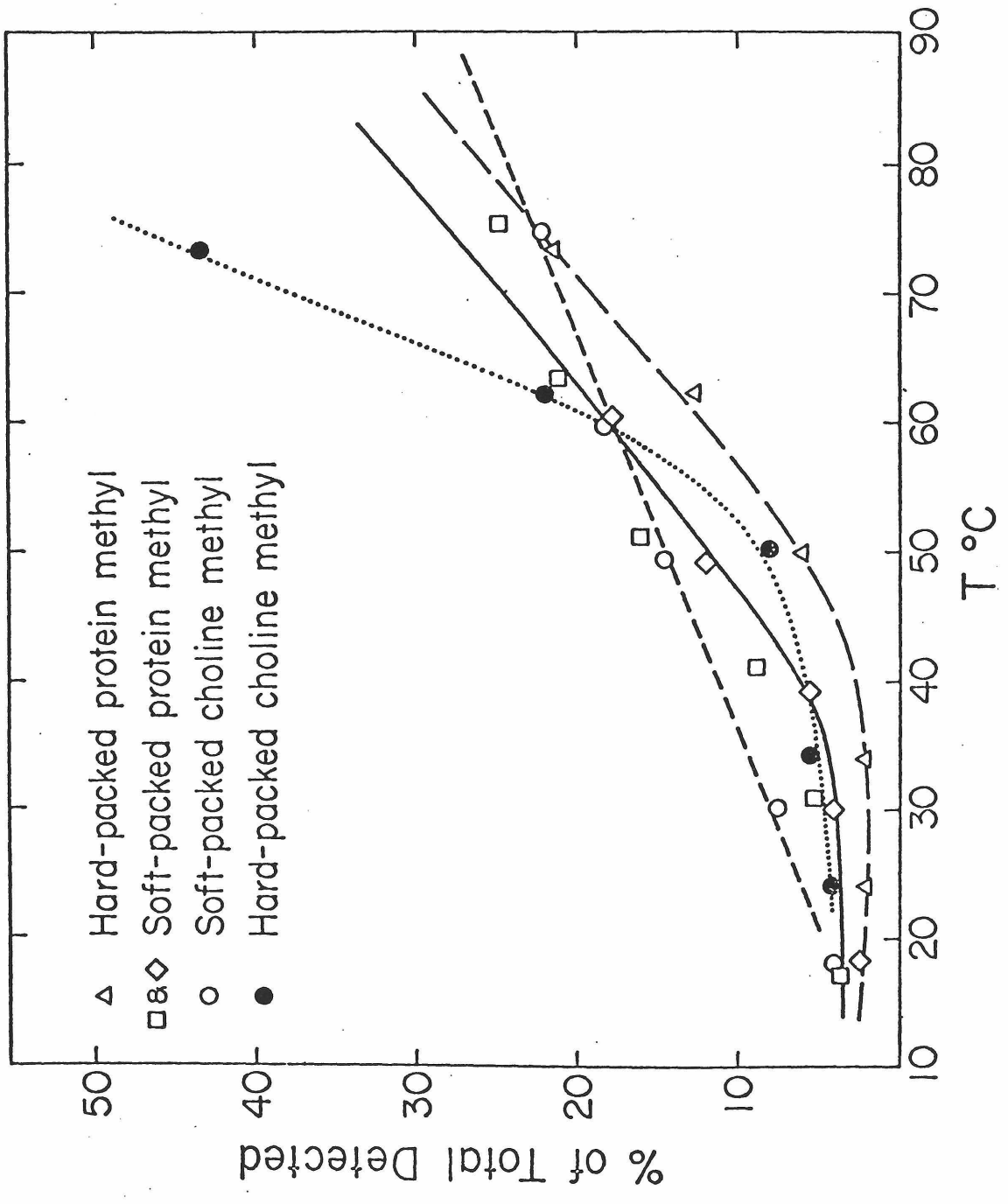
† McDonald, C. C. and W. D. Phillips, J.A.C.S., 91, 1513 (1969).

* Assignments from spectra of phospholipids in $DCCl_3$.

‡ Assignment from spectra of cholesterol in $DCCl_3$.

FIGURE 3

Percentage seen of total versus temperature for: Δ, the aliphatic methyl peak of hard-packed membranes, □ and ◇, the aliphatic methyl peak of soft-packed membranes, ○, the choline methyl peak of soft-packed membranes, and ●, the choline methyl peak of hard-packed membranes.


% of Total Detected
T °C
Hard-packed protein methyl
Soft-packed protein methyl
Soft-packed choline methyl
Hard-packed choline methyl
10
20
30
40
50
60
70
80
90

as a function of temperature for two samples of soft-packed ghosts and one of hard-packed ghosts. The variations in the percentage of protein methyls seen among different samples is ± 3% seen, which is well within the limit of accuracy of the method considering the error in the pmr area measurements (± 10%) and the error in the Lowry protein determination (± 3-5%). A similar plot of the percentage of choline methyls of lecithin and sphingomyelin detected in the pmr spectrum is presented in Fig. 3. The percentage of choline methyls detected is seen to be highly dependent on the state of the membrane. For example, a significantly higher percentage of the cholines was observed in the hard-packed ghosts. We note, however, that the choline resonance is particularly difficult to measure, both because of its intrinsic width (~ 30 cps) and interfering spinning side bands from the HOD peak.

3.2. Protein Solubilization and Denaturation

Because some membrane protein might have been solubilized by heating the membranes to 75 °C, suspensions of human ghosts in D_2O as well as H_2O (buffered with 6 mM phosphate at pD 7.8 and pH 7.8) were heated to 75 °C, centrifuged and the supernatants assayed for protein. It was found that the same percentage of protein was released to the supernatant irrespective of whether the samples were spun at 75 °C or at 4 °C after heating to 75 °C. The latter procedure was therefore used in the majority of these experiments since this procedure was more convenient. We have also noted that equilibrium was reached in less than a minute in these experiments.

The extent of protein solubilization upon heating the ghosts to

75 °C was found to depend on whether the solvent was H_2O or D_2O as well as the pH or pD of the solution. The percentage of protein released, however, was found to be independent of ghost concentration for concentrations of less than 5 mg/ml protein. At a given pH or pD more protein was solubilized in H_2O than in D_2O and the amount of protein solubilized increased with pH near the physiological pH in both solvent systems. Although the amount of protein solubilized does not account for the intensity of the methyl peak observed in the pmr spectrum of the protein in the whole membrane, there appears to be a correlation between the amount of protein released and the percentage of protein detected by pmr. At pD 8.75, for instance, analysis of the supernatant indicates that 13% of the protein was solubilized whereas 30% of the protein was monitored by the intensity of the methyl peak; at pD 7.05 about 15% was detected by pmr and 3% was solubilized. A similar pH dependence of the solubilization was observed in H_2O solutions except that its pH dependence was more pronounced, as shown in Fig. 4.

Protein solubilization was found to be less pronounced in Tris buffer than in phosphate buffer. In Tris buffer it was further suppressed upon the addition of Mg^{++} ions even at concentration levels of less than 10^{-6} moles of Mg^{++} per mg of membrane protein. Although the acetamide peak in the pmr spectrum was not significantly affected there was little evidence of the characteristic membrane protein spectrum in the presence of Mg^{++}, as evidenced by the decrease in the protein methyl peak area with added Mg^{++} shown in Fig. 5. Further clues regarding the stability of the membranes

FIGURE 4

Percentage of the total membrane protein solubilized from human erythrocyte membranes at 75 °C versus the pH (○ and ●) and the pH meter reading of the D_2O suspensions (pD = pH + 0.4) (X).

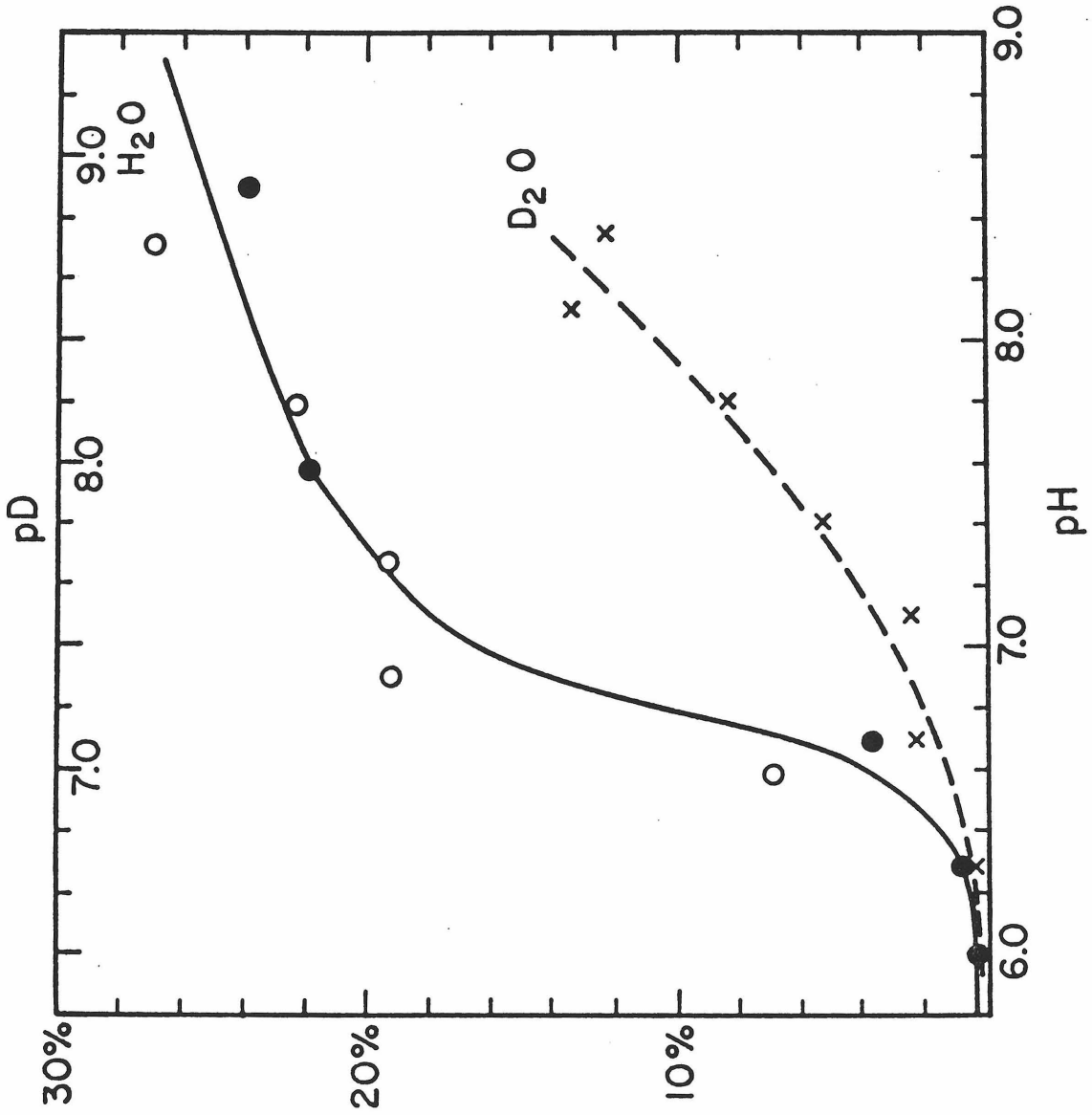
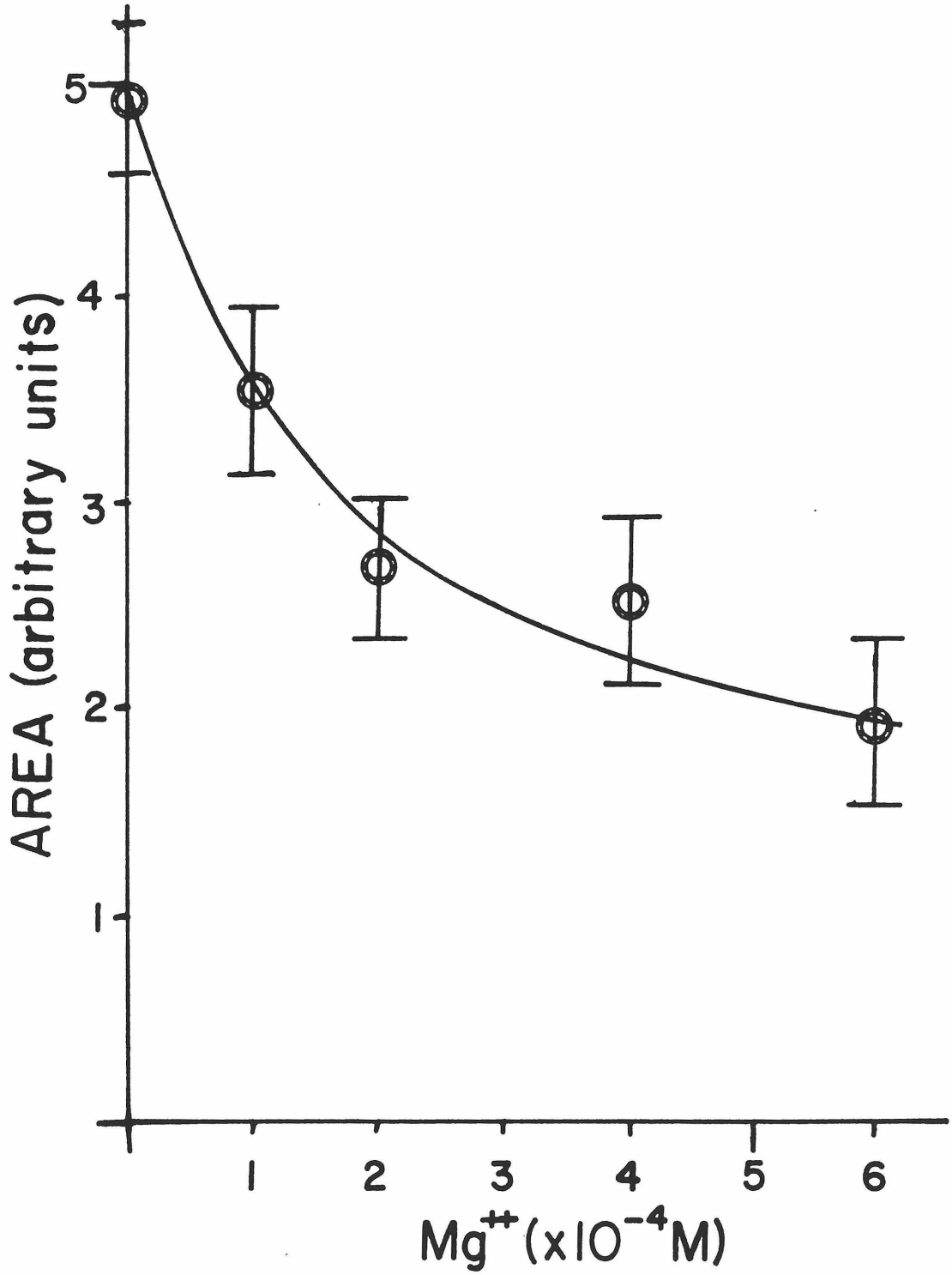

pD
pH
9.0
8.0
7.0
6.0
30%
20%
10%
H_2O
D_2O

FIGURE 5

The area of the aliphatic methyl resonance from soft-packed human erythrocyte ghosts at 75°C versus the added concentration of $MgSO_4$.


AREA (arbitrary units)
5
4
3
2
1
1
2
3
4
5
6
Mg++ (x10-4M)

were provided by the circular dichroism studies. Whereas the CD spectrum of the whole membranes in 7 mM Tris was hardly changed by the addition of divalent cations at 27°C, the addition of 0.001 M Ca^{++} or Mg^{++} had a marked effect on the CD spectrum at 75°C (Fig. 6). Upon the addition of these divalent ions at 75°C the molar ellipticity was reduced in the region from 190 to 215 nm, and the point of cross-over was moved to longer wavelengths (203-205 nm). The CD band at 223 nm was also changed slightly. By contrast Mg^{++} ion had little effect on the pmr of the membranes in the presence of 6 mM phosphate.

An attempt was made to characterize the protein solubilized at high temperatures in phosphate buffer. Sds electrophoresis gels of the protein solubilized from the heated ghost suspensions were compared with gels of the remaining pellet as well as that of the total membrane proteins (Fig. 7). A comparison of the gels showed that the solubilized proteins as well as the protein in the remaining pellet after heat treatment both lacked bands which were present in the gel of the whole membrane. An amino acid analysis of an acid hydrolysis of supernatant protein showed that about 27% of the amino acids were glutamic acid, glutamine, aspartic acid and asparagine, while 11% were arginine and lysine.

In order to further elucidate the location of the membrane proteins which have been mobilized by the above thermal treatment, we have examined the effects which paramagnetic ions may have on the protein spectrum. It is well known that Mn^{++} ion can cause spectral broadening of the nuclear resonances of magnetic nuclei in its immediate vicinity because of magnetic dipole-dipole interactions between

FIGURE 6

Circular dichroism spectra of hyman erythrocyte membranes (0. 21 mg membrane protein/ml) at 75 °C in 7 mM Tris, pH 7. 8 (——), with 1 mM Mg^{++} added (· · · ·), and with 1 mM Ca^{++} added (----).

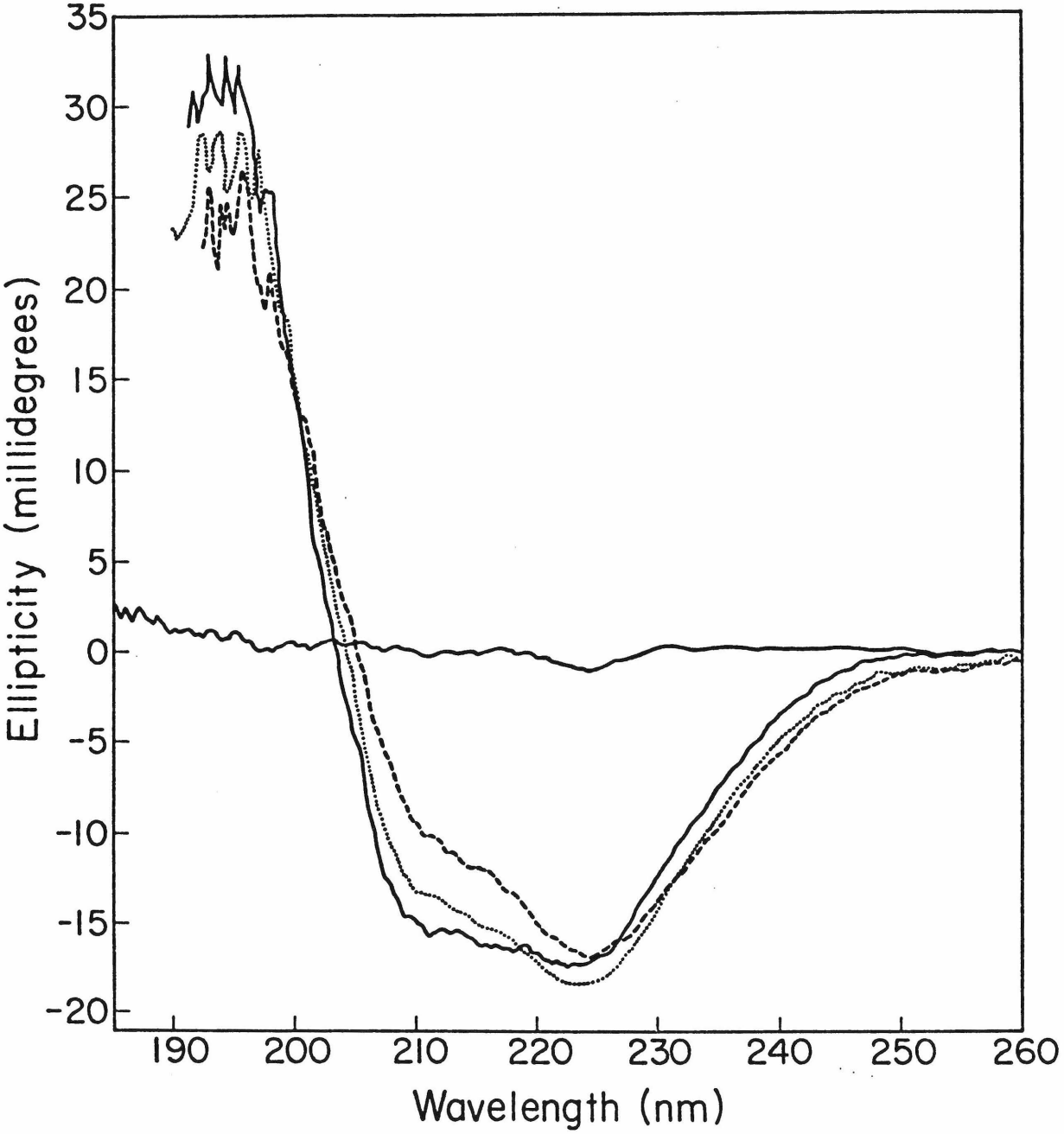
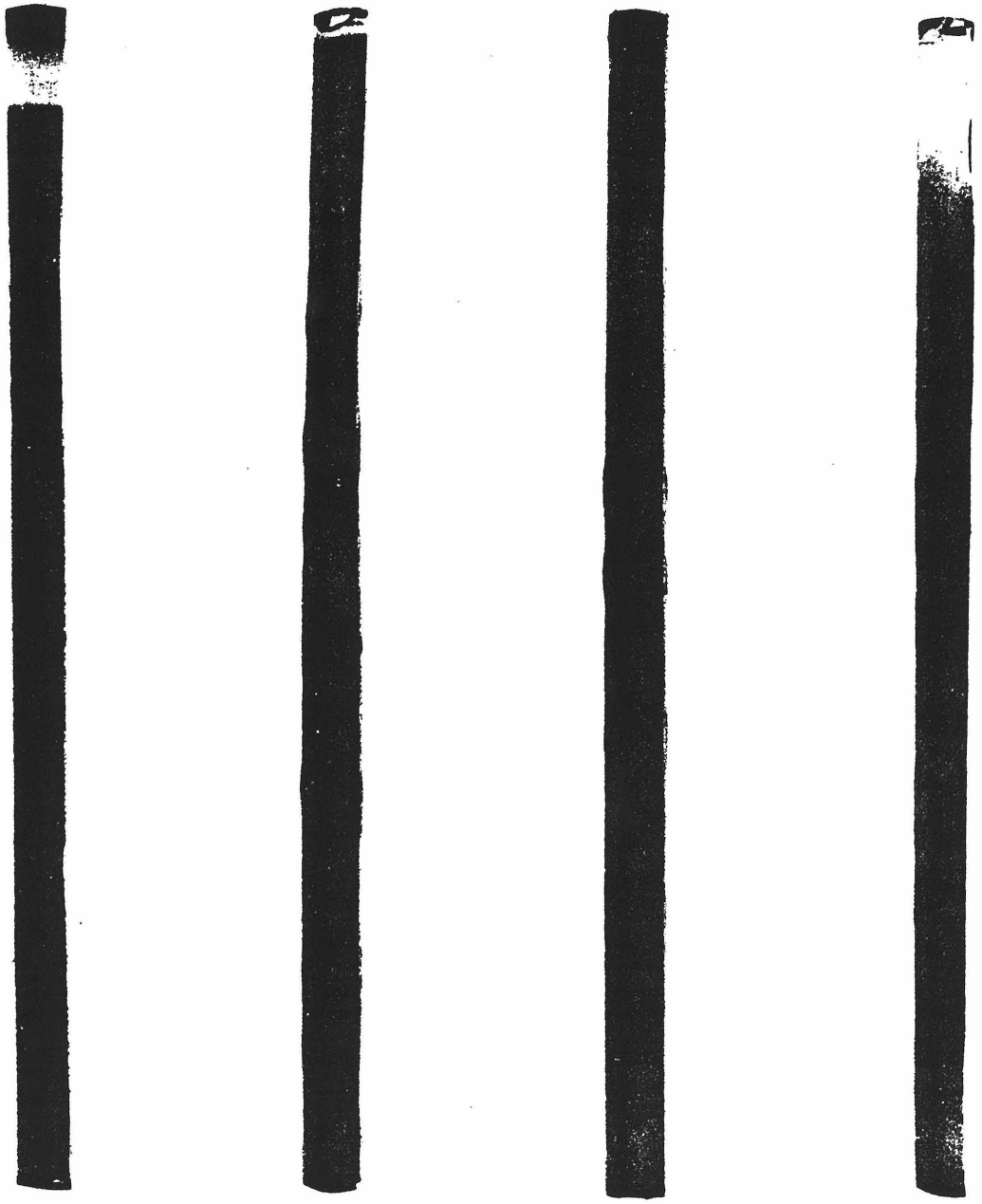

35
30
25
20
15
10
5
0
-5
-10
-15
-20
Ellipticity (millidegrees)
190
200
210
220
230
240
250
260
Wavelength (nm)

FIGURE 7

SDS electrophoresis gels stained with coumassie blue: (A) whole human erythrocyte membranes, (B) protein solubilized by heating membranes to 75 °C (pH 7. 8 in H_2O), (C) membranes after heating to 75 °C with solubilized protein removed, (D) standard sample in (a) insulin (mw. 5700), (b) hemoglobin (16, 000), and γ-globulin ((c) light chain 23, 000 and (d) heavy chain 50, 000).


A
B
C
D

the Mn^{++} ions and the nuclear spins. Since there are few Mn^{++} transport sites in these membranes and Mn^{++} can only bind to the phosphate groups in the lipid portion of the membrane, any spectral broadening of the pmr lines from the protein would indicate that these proteins are located on the surface or in solution. In Fig. 8 we have compared the spectra of human hard-packed ghosts in D_2O with 7 mM phosphate buffer in the presence of 2 mM Mg^{++} or Mn^{++}. The addition of the 2 mM Mn^{++} is seen to lead to a general broadening of all the resonances including the HOD peak. The disappearance of the choline methyls can be attributed to specific Mn^{++} binding to the charge heads of the phospholipid, as demonstrated by similar control experiments with bovine lecithin. When the spectrum of bovine lecithin (20 mg/ml) suspended in D_2O with 1 mM Mn^{++} is compared with that in the presence of the same concentration of Mg^{++} ion, one observes preferential broadening of the choline methyl signal in the solution containing the paramagnetic ion, whereas the methylene and methyl signals of the fatty acids are only slightly affected. By contrast, in the human hard-packed ghost a large part of the protein methyl signal is broadened beyond detection in addition to the choline methyl signal.

3.3. Effects of Nonenzymatic Treatments

We have compared the effects of several types of treatments on red cell ghosts over a wide range of temperatures and have found the most striking differences at elevated temperatures. The spectra of ghosts modified by centrifugation, protein removal and sonication are compared with that of the "whole" membrane at 75°C in Fig. 9.

FIGURE 8

220 MHz pmr spectra of (a) bovine lecithin (20 mg/ml) with 1 mM Mg^{++}, (b) bovine lecithin with 1 mM Mn^{++}, (c) hard-packed human ghosts in 7 mM phosphate buffer with 2 mM Mg^{++} at 65°C, (d) hard-packed human ghosts with 2 mM Mn^{++}.

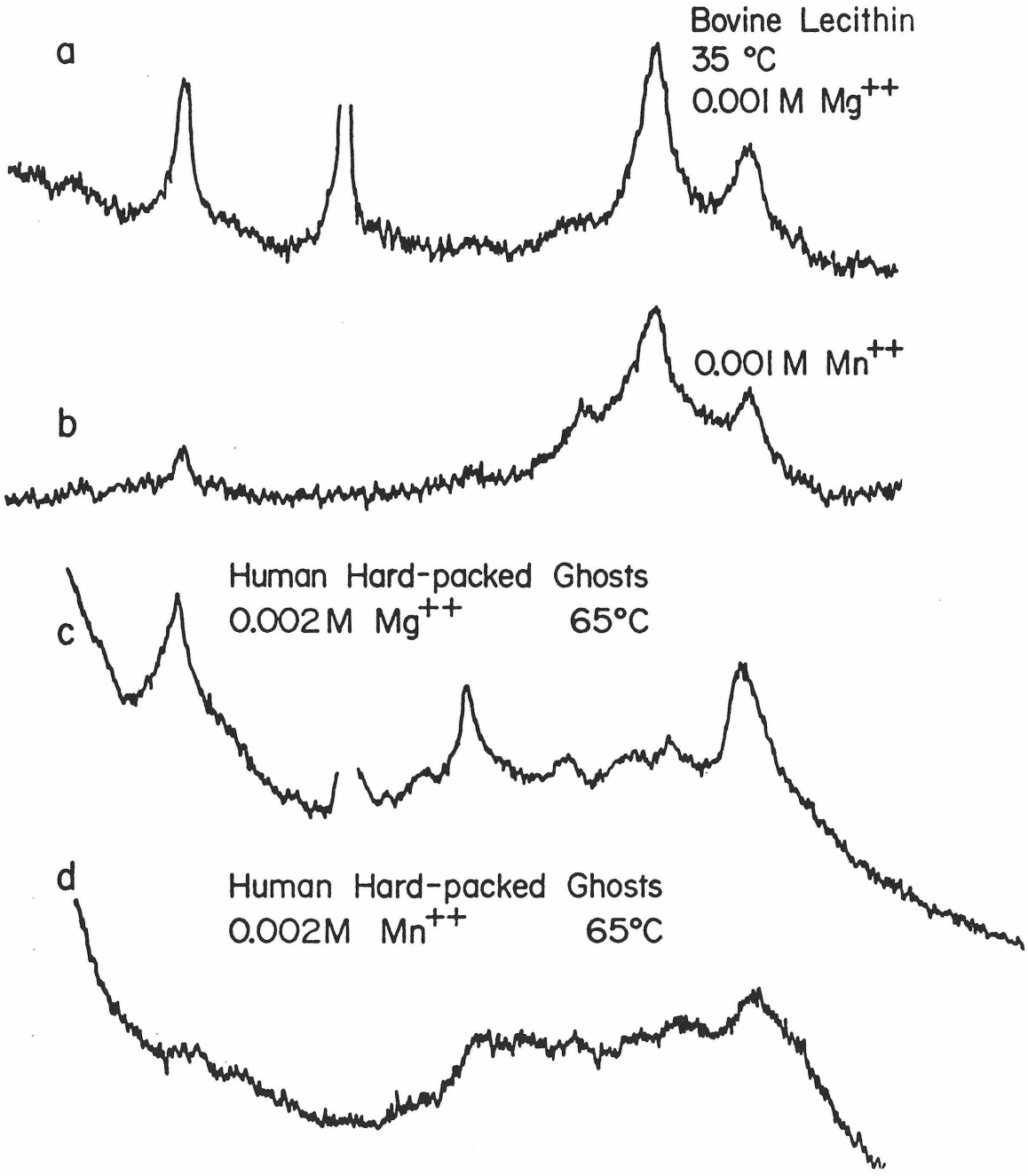
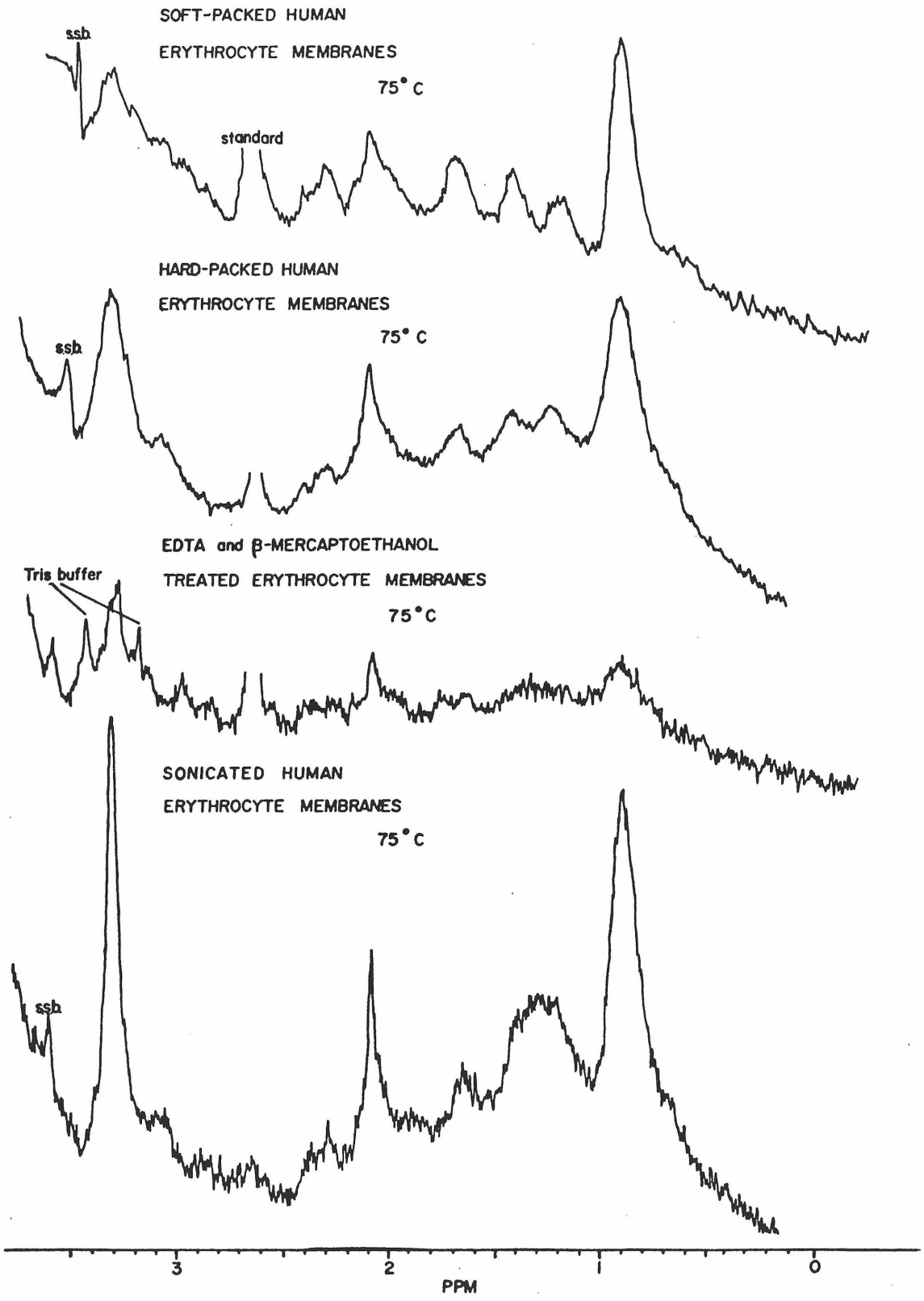

a
Bovine Lecithin
35 °C
0.001 M Mg++
b
0.001 M Mn++
c
Human Hard-packed Ghosts
0.002 M Mg++ 65°C
d
Human Hard-packed Ghosts
0.002M Mn++ 65°C

FIGURE 9

220 MHz pmr spectra of (a) soft-packed human erythrocyte membranes (5 mg protein/ml) at 75°C, (b) hard-packed human erythrocyte membranes (20 mg protein/ml) at 75°C, (c) EDTA and β-mercapto-ethanol washed membranes (6 mg protein/ml) at 75°C, and (d) sonicated human erythrocyte membranes (20 mg protein/ml) at 75°C.


SOFT-PACKED HUMAN
ERYTHROCYTE MEMBRANES
75° C
ssb
standard
HARD-PACKED HUMAN
ERYTHROCYTE MEMBRANES
75° C
ssb
EDTA and β-MERCAPTOETHANOL
TREATED ERYTHROCYTE MEMBRANES
75°C
Tris buffer
SONICATED HUMAN
ERYTHROCYTE MEMBRANES
75°C
ssb
3
2
1
0
PPM

Of these treatments centrifugation is probably the mildest. The application of a centrifugal field, however, can lead to separation of ghosts with differences in the structural strengths such as that which may result from protein loss or fragmentation in the preparation. It might well be that this is the origin of the so-called "hard-packed" and "soft-packed" fractions. In this connection, we note that the hard-packed fraction is characterized by a higher membrane concentration than the soft-packed fraction and it becomes the predominant fraction with aging of the samples.

The pmr spectrum of the hard-packed fraction at 75°C exhibits a choline methyl peak which is twice the area of that of the soft-packed membranes. The trough between the resonances at -1.22 and -1.41 ppm is also diminished significantly in the hard-packed membrane spectrum, presumably reflecting the increased contribution of the methylene protons. In contrast to the differences in the choline methyl signals between the two samples, there is no difference in the protein methyl signals between the samples.

After spectrin removal by EDTA and β-mercaptoethanol which results in the solubilization of ~12% of the membrane proteins, a marked increase in the intensity of the choline methyl was observed. Whereas the percentage of membrane protein monitored in the pmr spectrum at 75°C was only about 15%, the signal at -3.25 ppm now accounted for 50% of the choline methyls present. In addition, there was evidence for a broad resonance centered in the region at -1.27 ppm which we have attributed to the methylene protons of the fatty acids. Thus spectrin removal was seen to produce more dramatic

changes in the lipid portions than the protein portions of the spectrum.

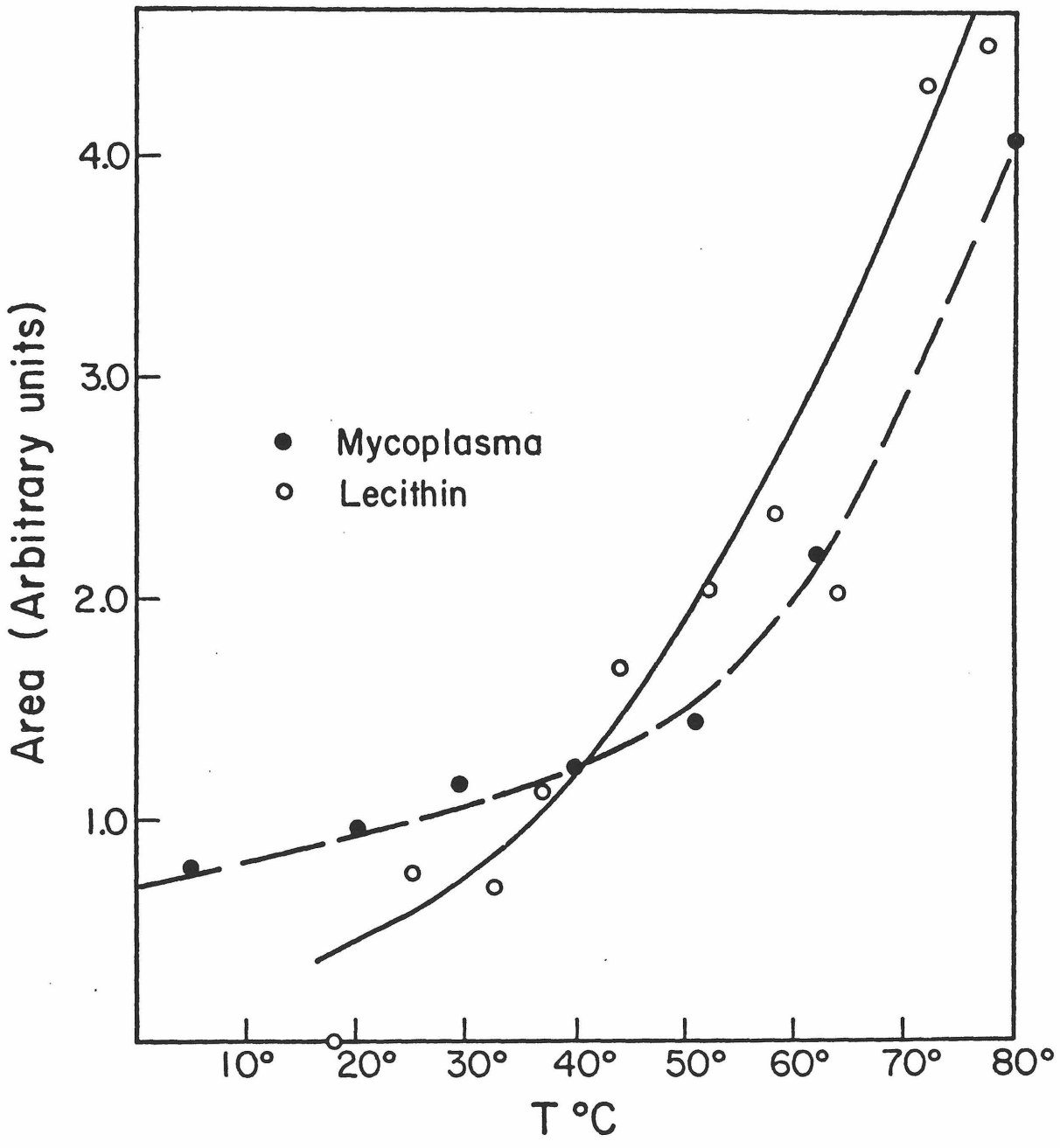
Of the various treatments, sonication caused the greatest damage to the structure of the membrane as is apparent from the prominance of the lipid portion of the spectrum of the membranes. Above 50°C there is now clear evidence for a methylene peak at -1.27 ppm, although the intensity of this peak corresponds to $< 5\%$ of the total methylene hydrogens of the fatty acid side chains. The intensity of this methylene peak increases abruptly with temperature. This spectral behavior is similar to that observed for unsonicated Outer Mitochondrial membranes, Mycoplasma l. membranes, and lecithin suspended in D_2O, except that the "transition" temperature is lower in these cholesterol free membrane systems. For the sake of comparison we have plotted the intensity of the methylene peak versus temperature for Mycoplasma l. membranes and egg lecithin in Fig. 10. In addition to mobilizing some of the fatty acid side chains, sonication leads to a significant sharpening of the choline methyl resonance. The intensity of this peak has also increased. At 75°C the choline methyl peak is about equal in area to the protein methyl resonance at -0.89 ppm from which we deduced that the percentage of choline detected is about twice that of the protein seen.

3.4. Phospholipase C Treatment

The treatment of erythrocyte ghosts by phospholipase C produced dramatic changes in the HR-220 pmr spectrum of the membranes at 18°C as illustrated in Fig. 11a and b. In the control ghost spectrum there are no sharp resonances from the membranes at that temperature.

FIGURE 10

Area of the aliphatic methylene proton peak (-1.25 ppm) versus temperature for (●) Mycoplasma laidlawii membranes and (○) lecithin bilayers.


Area (Arbitrary units)
4.0
3.0
2.0
1.0
Mycoplasma
Lecithin
10°
20°
30°
40°
50°
60°
70°
80°
T °C

The treated spectrum, however, exhibits intense resonances at -0.89, -1.26, -1.52, -2.03, -2.28 and -2.80 with relative areas indicative of a fatty acid proton spectrum. This suggested that the treated membrane spectrum might be assignable to the membrane lipids, as is borne out by the good correlation of the spectrum of the red cell lipids in chloroform (Fig. 11c) with the treated membrane spectrum. Because of the phospholipase C treatment, the choline resonance at -3.25 ppm is not observed and for an unknown reason the cholesterol angular C-18 and C-19 methyl protons are not detected at -0.72 and -0.98 ppm[40]. If we compute the spectrum of the fatty acids alone as in Fig. 11d, we find a good qualitative agreement of the computed and treated membrane spectra. There is, however, quantitative disagreement between the area of the aliphatic methyl resonance of the two spectra. For that reason we computed the spectrum of the fatty acids and cholesterol in Fig. 11e assuming 40 Hz linewidths for the angular methyl protons of cholesterol and 10 Hz for the three methyl groups not bonded directly to the steroid rings. The quantitative and qualitative agreement of this computed spectrum with the treated membrane spectrum is excellent.

The spectrum in Fig. 11b is from ghosts treated with B. cereus phospholipase (to remove 62% of the phosphate groups). If the Cl. perfringens enzyme is used instead to hydrolyze the same percentage of phospholipids, a spectrum of lower intensity and broader linewidths is observed at 18°C, as shown in Fig. 12. At 75°C, however, the spectrum of the sample treated by the Cl. perfringens enzyme in Fig. 12 has roughly the same intensity and linewidth as the sample

FIGURE 11

220 MHz pmr spectra of (a) human erythrocyte membranes (7 mg protein/ml) at 18°C, (b) B. cereus phospholipase C treated human erythrocyte membranes (7 mg protein/ml) at 18°C, (c) $CHCl_3$ extracted lipids from human erythrocyte membranes in $CDCl_3$, (d) computed spectrum of the fatty acids in human erythrocyte membranes, and (e) computed spectrum of the fatty acids and cholesterol in human erythrocyte membranes.

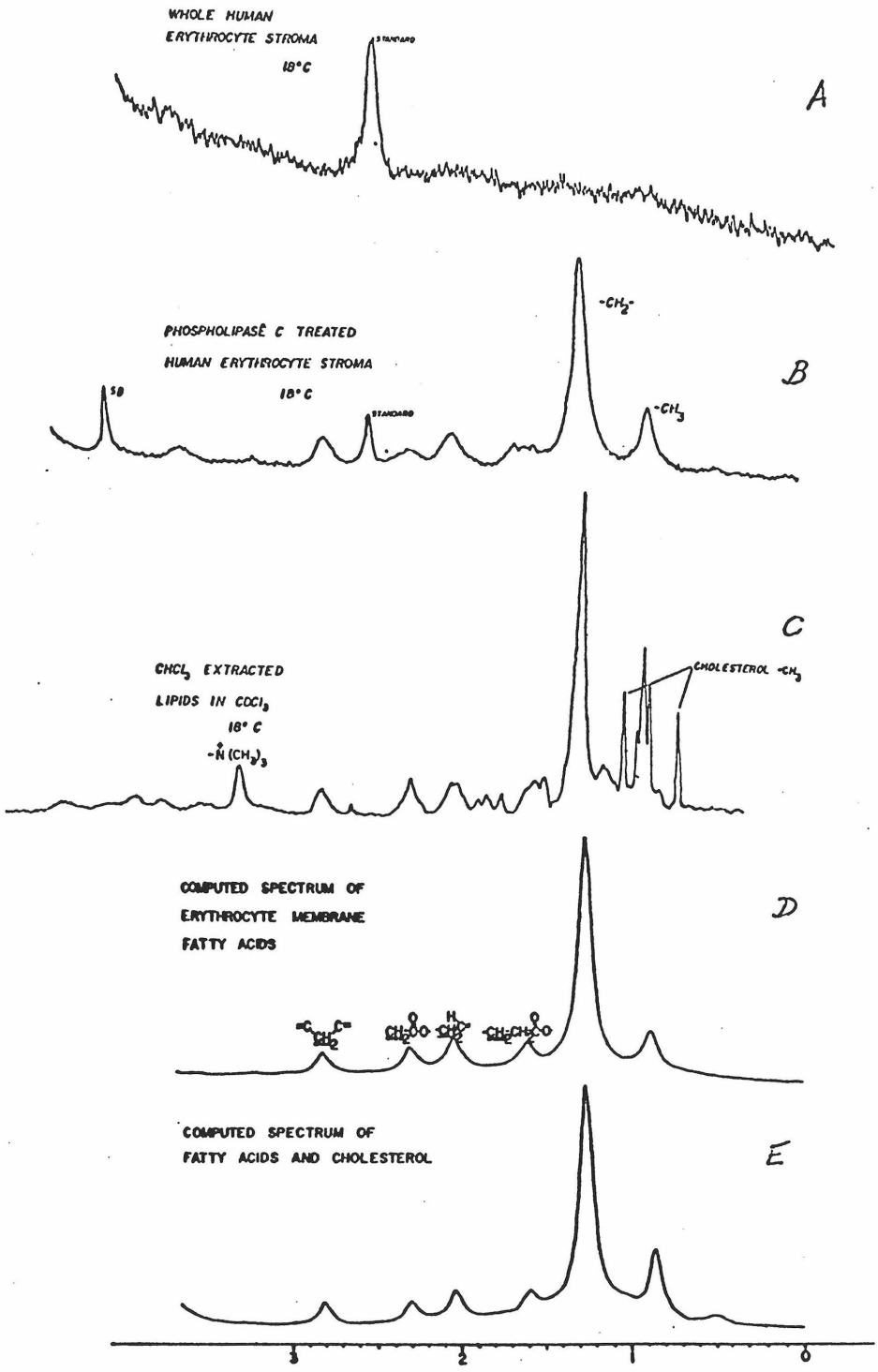
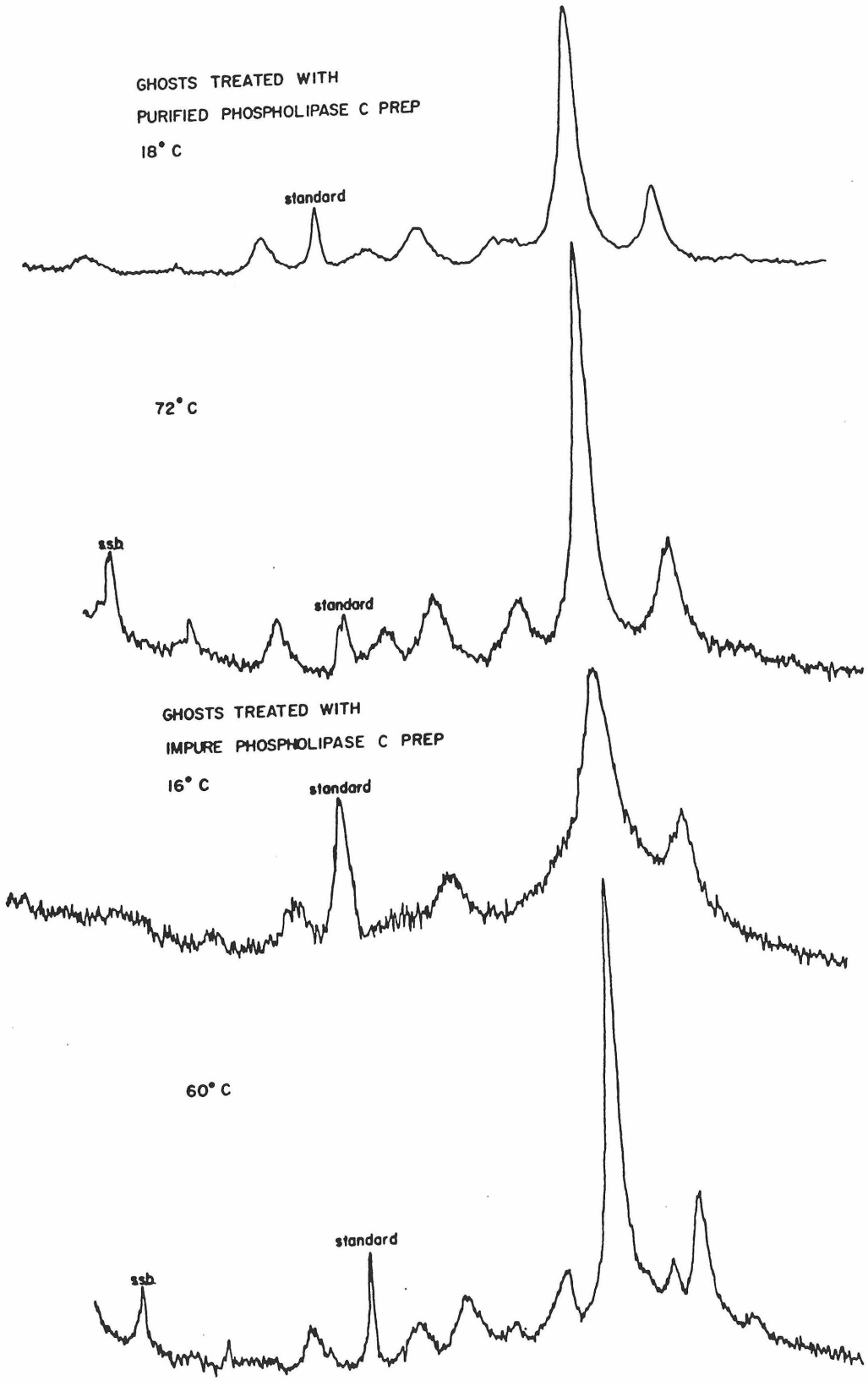

WHOLE HUMAN
ERYTHROCYTE STROMA
18°C
A
PHOSPHOLIPASE C TREATED
HUMAN ERYTHROCYTE STROMA
18°C
SB
STANDARD
-CH2-
-CH3
B
CHCl3 EXTRACTED
LIPIDS IN CCl3
18° C
-N̂(CH3)3
CHOLESTEROL -CH3
C
COMPUTED SPECTRUM OF
ERYTHROCYTE MEMBRANE
FATTY ACIDS
D
COMPUTED SPECTRUM OF
FATTY ACIDS AND CHOLESTEROL
E
3
2
1
0

FIGURE 12

220 MHz pmr spectra of human erythrocyte membranes treated with B. cereus phospholipase C recorded at 18° and 72°C and with Cl. perfringens phospholipase C recorded at 16° and 60°C.


GHOSTS TREATED WITH
PURIFIED PHOSPHOLIPASE C PREP
18° C
standard
72° C
ssb
standard
GHOSTS TREATED WITH
IMPURE PHOSPHOLIPASE C PREP
16° C
standard
60° C
standard
ssb

treated with the B. cereus enzyme at 18°C and 75°C except that the cholesterol angular C-18 and C-19 methyl resonances are narrower from the Cl. perfringens treated membranes. If we plot the change in intensity of the fatty acid methylene signal versus temperature for several different samples, as in Fig. 13, we find that the Cl. perfringens treated samples have significantly different spectral characteristics from the B. cereus treated samples, for in all cases the methylene melting is reversible.

Because the area of the aliphatic methylene resonance did not change with temperature in the B. cereus treated membranes, we assumed that the lipid contribution to the aliphatic methyl peak at -0.90 ppm was constant. The change of the methyl peak area was then plotted versus temperature for the control and B. cereus treated samples in Fig. 14, and a close correspondence was noted between the curves if we subtracted a constant from the methyl peak area of the treated sample.

4. DISCUSSION

It is known that the whole human erythrocyte membrane does not exhibit a high resolution pmr spectrum at physiological temperatures[11, 12]. Although there has been some controversy about the origin of the breadth of the spectral lines, it has become increasingly evident that the lack of a high resolution spectrum reflects the incomplete averaging of the tensor dipole-dipole interaction between nuclear spins as a consequence of slow anisotropic molecular motion of the

FIGURE 13

The observed methylene resonance area versus temperature for Cl. perfringens phospholipase C treated membranes with 75% (X) and 60% (O) of the phosphate removed and for B. cereus phospholipase C treated membranes with 70% (●) of the phosphate removed.

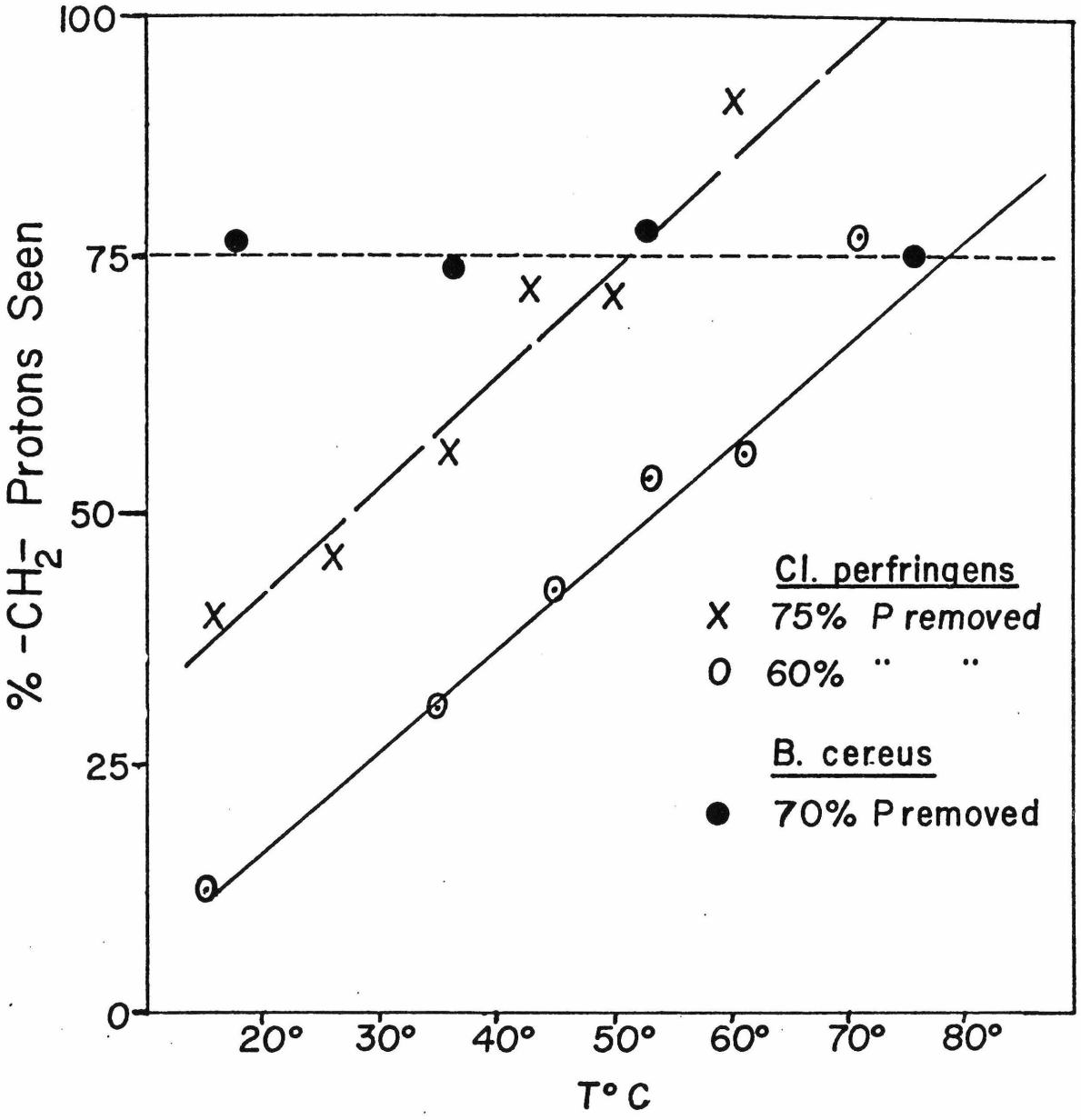
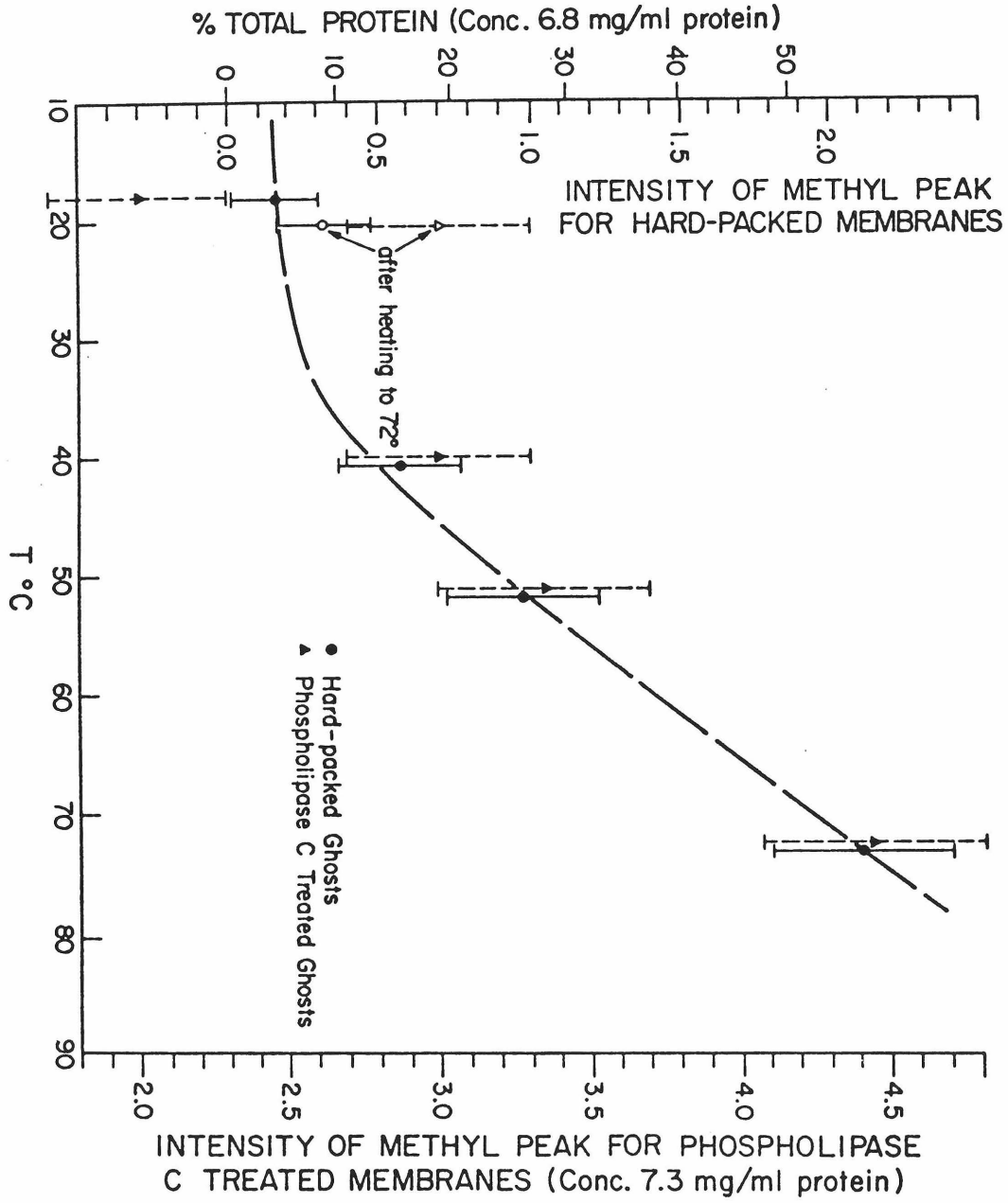

100
75
50
25
0
% -CH2- Protons Seen
20°
30°
40°
50°
60°
70°
80°
T° C
Cl. perfringens
X 75% P removed
O 60% " "
B. cereus
● 70% P removed

FIGURE 14

Area of the aliphatic methyl peak (0. 89 ppm) versus temperature for (●) hard-packed ghosts (6. 8 mg protein/ml) and (▲) for the same membranes treated with phospholipase C (B. cereus enzyme) (7. 3 mg protein/ml).


% TOTAL PROTEIN (Conc. 6.8 mg/ml protein)
0
10
20
30
40
50
0.0
0.5
1.0
1.5
2.0
INTENSITY OF METHYL PEAK FOR HARD-PACKED MEMBRANES
after heating to 72°
T °C
10
20
30
40
50
60
70
80
90
● Hard-packed Ghosts
▲ Phospholipase C Treated Ghosts
2.0
2.5
3.0
3.5
4.0
4.5
INTENSITY OF METHYL PEAK FOR PHOSPHOLIPASE C TREATED MEMBRANES (Conc. 7.3 mg/ml protein)

various components of the membrane[41]. This result is not surprising for an organized and integrated system such as a biological membrane, considering the nature of the forces which must be present to stabilize its molecular structure and to give it its specific functions. While certain groups or molecules can still be relatively mobile, these mobilities need not approach that of the components in the liquid state. The fatty acid side chains of phospholipids, for example, have been shown to be relatively mobile in certain situations; however, this motion is extremely anisotropic and clearly does not approach that of a fluid alkane or that of a fatty acid in a micelle. Thus the lack of a high resolution pmr spectrum for red blood cell ghosts may be taken as evidence for a state of slow molecular motion of the membrane components.

In this work we have observed relatively sharp resonances after the membranes have been subjected to different treatments of varying severity and irreversibility. Heating the membrane to above 40°C gave resonances which we have assigned largely to membrane proteins. Sonication was shown to lead to increased mobility of the fatty acid side chains. While both of these treatments appear to affect different parts of the membrane, it is evident that both change the physical state of the membrane. That the pmr spectra enable us to identify one or the other type of molecule is merely a reflection of the sensitivity of the method to the changes in the local mobility of the molecules in question.

Before commenting on the implications of the pmr results, it would seem appropriate to discuss the basis of our assignment of the pmr spectrum in greater detail. In the thermal treatment of the

membranes, the most prominent resonances occur at -3.25 and -0.89 ppm. Both of these resonances can be assigned to methyl groups, the former to N-methyl protons on the basis of known chemical shifts for the protons in the tetramethyl ammonium ion and the latter to aliphatic methyl protons. Among the membrane components, only lecithin and sphingomyelin have N-methyl groups, and thus we can assign the resonance at -3.25 ppm to these protons unambiguously. Phospholipids, cholesterol and proteins all have aliphatic methyl protons so the assignment of this peak to the appropriate membrane component is less straightforward. Phospholipids and cholesterol, however, both contain a significantly larger fraction of protons as aliphatic methylene protons than aliphatic methyl protons; hence if the mobility of these molecules is sufficiently high to yield a high resolution pmr spectrum, we should expect a sizeable methylene peak at -1.27 ppm as well. The pmr spectrum of sonicated phospholipids suspended in aqueous solution as well as phospholipids and cholesterol dissolved in organic solvents both reveal a more intense methylene peak than an aliphatic methyl peak. This is also the case of phospholipase C treated red blood cell membranes where the fatty acid side chains of the phospholipids have apparently been rendered mobile by enzymatic cleavage of the polar-head groups. The situation is quite different with respect to the membrane proteins. Although there are on the average more methylene protons than methyl protons among the amino acids, a methylene group is more likely to be found in a non-aliphatic environment and the heterogeneity of these chemical environments results in a wide dispersion of chemical shifts for the methylene resonances of the

amino acids. A pronounced central methylene peak is therefore not expected from the membrane proteins at -1.27 ppm. On the other hand, we expect the amino acids valine, leucine and isoleucine to contribute a sizeable aliphatic methyl signal at -0.89 ppm. For the membrane proteins, we therefore expect a well-defined methyl peak at -0.89 ppm and a number of weaker methylene resonances towards lower fields, particularly below -1.27 ppm. These general spectral characteristics are clearly manifested in the pmr spectra of red blood ghosts which have been heated above ~40°C. For this reason, we have assigned the pmr spectra observed in the spectral region -3.0 to -0.8 ppm primarily to the amino acids of the membrane proteins which have somehow been rendered mobile as a result of the heat treatment. This tentative assignment was confirmed by computer simulation of the spectrum from the known average amino acid composition of the red blood cell membrane proteins[34]. The only discrepancy appeared in the region of ~-2.0 ppm, but the resonance appearing at -2.07 ppm can be readily assigned to the acetamide protons of the sugars present in the membrane. That the intense methyl peak at -0.89 ppm is to be assigned to the aliphatic methyls of valine, leucine and isoleucine of the membrane protein component rather than the terminal methyl groups of the hydrocarbon chains of the phospholipids or the methyl groups of cholesterol is clearly evident when we compare the temperature variation of the intensity of this methyl signal between whole ghosts and phospholipase C treated ghosts where 70% of the hydrocarbon chains have been rendered sufficiently mobile to yield high resolution pmr signals. This comparison (Fig. 14) reveals that

there is a component of the aliphatic methyl signal in the phospholipase C treated ghosts which varies in intensity with temperature in exactly the same manner as the untreated whole ghosts. We therefore see that in the case of the phospholipase C treated ghosts, the aliphatic methyl signal is the sum of two components, one due to the mobilized terminal methyl groups of the hydrocarbon chains and possibly the methyl protons of cholesterol, and a second component from the membrane proteins. We had earlier assigned the lipid contribution to the phospholipids alone, as there did not appear to be evidence for peaks at -0.68 and -1.02 ppm which we could otherwise have assigned to the angular C-18 and C-19 methyl resonances of cholesterol. However, we have since compared the intensity of the aliphatic methyl signal observed at -0.90 ppm for the phospholipase C treated ghosts at 18°C with theoretical spectra computed with and without the mobilization of cholesterol, and have found that the observed intensity can only be accounted for by including a contribution from the C-21, C-26 and C-27 methyls of cholesterol which are expected to resonate at this position. Apparently, the C-18 and C-19 methyl resonances of cholesterol are so much broadened that these resonances are not readily discernible. Judging from hindsight, this is perhaps not surprising since we expect rapid reorientation of the C-21, C-26 and C-27 methyl tops, whereas the internal rotation of the C-18 and C-19 angular methyl groups should be highly restricted. If this is the case, we expect the C-18 and C-19 methyl resonances to be three or four times broader than those due to the C-21, C-26 and C-27 methyl tops[42]. A comparison of the observed spectrum of the phospholipase C treated membranes at 18°C with theoretical spectra

of the erythrocyte lipids computed with and without cholesterol mobilization is given in Fig. 11. In these calculations, we have assumed a linewidth of 20 Hz for the terminal methyl protons of the fatty acid side chains as well as for the cholesterol C-21, C-26 and C-27 methyls, while a linewidth of 60 Hz was assumed for the cholesterol C-18 and C-19 angular methyl groups. The calculated spectrum which included contributions from both the fatty acids of the phospholipids and the cholesterol can be seen to be in excellent agreement with experiment. These observations indicate that both the phospholipids and the cholesterol become mobile after this enzymatic treatment.

In this study, we showed that certain membrane proteins were solubilized by heat treatment. Part of the protein spectrum observed is no doubt due to these solubilized membrane proteins. It is likely that the remaining amino acid residues observed by pmr are associated with membrane proteins which are located near the surface of the membrane, as the addition of paramagnetic Mn^{2+} ions was found to produce a general broadening of the entire protein spectrum. Although we do not have direct evidence, it is probable that there has been partial uncoiling of these membrane surface proteins accompanying the protein loss. Should this be the case, we might expect to observe an apparent correlation between the amount of protein detected by pmr and the extent of protein solubilization, and, indeed, we do. As we have shown, the extent of protein loss from the membranes upon heating the ghosts to 75°C was found to be dependent upon whether the solvent was H_2O or D_2O, the pH or pD of the solution, and the presence or absence of small amounts of divalent cations, such as Mg^{2+} and Ca^{++}.

It is known that the membrane surface of red blood cell ghosts is negatively charged. Although this negative charge has been assigned largely to the sialic acid residues because neuraminidase treatment was found to greatly decrease the electrophoretic mobility of the ghosts[43], compositional analyses of the membrane proteins and lipids indicate that these components should bear an average negative charge also. From the amino acid composition of Dodge et al. ghosts, based on two independent amino acid analyses[34, 35], we calculated that the membrane proteins would carry a net negative charge of $\sim 0.6 \times 10^{-4}$ or 1.7×10^{-4} moles/gram of dry membrane depending on whether the histidine residues were protonated or unprotonated; since the normal pK of histidine in proteins is 6.5-7.0, about 20% of the histidine residues would be protonated at pH 7.4, where our experiments were nominally carried out. Similar considerations for the phospholipids and the sialic acid residues yielded negative charge concentrations of 0.7×10^{-4} and 0.5×10^{-4} moles/gram of dry membrane for the phospholipid component and the scialic acids respectively. The scialic acid groups are covalently linked to the membrane proteins and it has been shown that the water soluble proteins are highly negatively charged; thus the electrostatic stabilization of the membrane provided by protein solubilization is great. This provides a possible driving force for the protein solubilization. Analysis of solubilized proteins by sds gel electrophoresis and amino acid analysis has shown that this protein fraction contains a nonrandom distribution of membrane proteins. The fact that the fraction is rich in acidic amino acids correlates well with the work of Rosenberg and Guidotti[7], who have

characterized the water soluble proteins from erythrocyte membranes and have found them to be rich in acidic amino acids.

The marked pH dependence of the extent of protein solubilization in D_2O depicted in Fig. 4 resembles a titration curve and might well be associated with titration of the imidazole base of some of the histidine residues in the membrane surface proteins. Should this be the case, then the enhanced protein loss with increasing pH most likely reflects the decreased stability of the red blood cell ghosts as the negative charge on the membrane surface is increased by deprotonation of these histidine residues. The predominance of this electrostatic contribution to the stability of these erythrocyte membranes is further suggested by the observation that protein release from these ghosts tends to be suppressed at higher ionic strengths. There may, however, be an alternate or an additional explanation for these observations, such as effects which an electrolyte may have on the structure of the solvent. That the solvent structure might also play a role is implicated by the dramatic differences in the extent of protein loss from the erythrocyte membranes at elevated temperature between H_2O and D_2O in the pH range under consideration. The pronounced solvent effect is not readily accounted for by the known discrepancy between the pH and pD of the solution as measured by the glass electrode, but appears to correlate with the known differences in the aggregation properties of proteins between these two solvent systems, i. e., the greater aggregation of phycocyanin in D_2O compared with H_2O[44], as well as the lower critical micelle concentrations for fatty acids in D_2O[45].

The observed suppression of protein solubilization as well as

the non-appearance of a protein pmr spectrum at elevated temperatures in the presence of low concentrations of Mg^{2+} in Tris buffer indicates that the membranes become more resistant towards heat under these conditions. That divalent cations would stabilize the membranes should, perhaps, come as no surprise, if the membrane surface is highly negatively charged. Certain divalent cations such as Mg^{2+} and Ca^{2+} are known to bind to red blood cell ghosts[21], and in light of our earlier discussion, it is likely that this ion-binding involves some of the negatively charged groups on the membrane surface. Although the CD spectrum of the membrane proteins remains unchanged upon the additional physiological concentrations of Mg^{2+} and Ca^{2+} at 27°C, the spectrum at 75°C is sufficiently different to suggest a conformational change in the membrane proteins. Unfortunately, it is not possible to ascertain the nature of the structural changes without a detailed analysis of the CD spectrum. Preliminary experiments in our laboratory indicate stronger binding of Ca^{2+} to the membranes than Mg^{2+}, which might explain the greater CD spectral changes in the presence of Ca^{2+}.

After hard-packing, sonication and spectrin removal, the membranes were found to be less resistant to structural changes upon heat treatment. In the case of hard-packed ghosts and those washed with EDTA and β-mercaptoethanol solution, the structural differences appear to be confined to the region of the lipid polar head groups since the choline methyl peak underwent significant intensity increases after these treatments as compared with normal soft-packed ghosts. In the hard-packed ghosts the membranes are probably weakened

through protein loss as a somewhat larger percentage of membrane proteins appears to be solubilized upon heating compared to soft-packed ghosts (8-10% versus 5-7%). This difference in the protein loss is probably the reason for the almost factor of 2 increase in the choline methyl signal (40% seen versus 20% in normal membranes) between hard and soft-packed ghosts at 75°C. An even greater increase in the intensity of the choline resonance is observed in membranes where 12% of the membrane proteins have been eliminated by spectrin removal. These observations suggest that a cooperative event occurs with protein loss or mild fragmentation. This event might simply be a change in the state of the lipids or might involve alterations in the nature or extent of lipid-protein interactions. The fact that the changes appear to be confined only to the polar head groups of the phospholipids without mobilization of the fatty acid side chains would seem to rule out the possibility that these treatments have caused a phase change in the membrane lipids of a type that might also explain the different reactivities of red cells prior to hemolysis and red cell ghosts toward phospholipase C. Two independent investigators[46, 47] have found that phospholipase C attacks erythrocyte membrane phospholipids at a much slower rate before hemolysis than after as in the ghost. Presumably red cell ghosts differ from whole red cell membranes in the amount of protein lost during the preparation of the ghosts. We therefore are more inclined to attribute the enhanced mobilization of the choline methyl resonance to changes in the protein-lipid interactions on the membrane surface.

Compared with the other nonenzymatic treatments, sonication

was found to produce more severe damage to the structure of the membranes as is evident from the prominence of the lipid resonances in the spectrum of the sonicated ghosts. Both the polar head groups as well as the hydrocarbon side chains of the phospholipids are mobilized upon sonication as has been previously shown by Penkett and coworkers[48], and in view of the gross structural changes which occur, the pmr spectra of sonicated membranes or lipids probably have little bearing on the membrane structure of intact cells.

In the phospholipase C experiments we observed a huge change in the lipid structure with no change, or at most a small change, in the protein structure as determined by circular dichroism experiments done in conjunction with the pmr experiments. This probably indicates that the protein structure is not perturbed greatly by the loss of 75% of the lipids. Although CD could simply be insensitive to the conformational changes which would occur, studies of the effect of small amounts of human growth hormone on the CD spectrum of red cell ghosts indicate the method is sensitive to membrane protein conformational change[49]. The structural independence of a large portion of the membrane lipids and proteins is consistent with freeze etch electron microscopy studies and the mosaic model of biological membranes. In that model, protein units float in a lipid sea and are free to diffuse laterally, as the findings of Frye and Edidin[50] support. Treatment with phospholipase C then removes a large portion of the lipid sea and brings proteins closer together without altering their structure. This theory is entirely consistent with the pmr and CD results of the treatment by the B. cereus enzyme which leaves primarily sphingomyelin in the membrane. The

diglycerides in this case form into pools which are extremely fluid and since very few diglycerides are in the remaining lipid bilayer there is no further increase in the methylene resonance area with increasing temperature. The proteins are forced closer together than normal and thus when they are denatured by heating there is a possibility of interaction and stabilization as seen in the CD spectral behavior.

The case of the Cl. perfringens treated samples is different, but so also is the composition of the remaining lipids different in a very significant way. Nearly all the lipids remaining after Cl. perfringens treatment are negatively charged. If, as in the B. cereus case, all the diglycerides left the bilayer to form into diglyceride pools then the remaining bilayer would have an extremely high charge density which is known to destabilize it. In fact, phosphatidyl serine alone does not form into bilayers primarily because of the unfavorable charge density[51]. It is conceivable then that because of this factor some of the diglycerides would remain in the bilayer to lower the charge density and that at low temperatures they would be sufficiently immobilized to not be observed as part of the high resolution fatty acid signal. At elevated temperatures these fatty acids would be mobilized to give high resolution spectra. The large concentration of phosphatidyl serine could also be the cause of the changes in the CD spectra through the greater charge density or the replacement of neutral lipids by charged lipids in important protein sites. This explanation relating spectral differences to the charge seems to hold, but we cannot rule out a number of alternative explanations such as an extra enzyme activity in the impure Cl. perfringens enzyme preparation or an

alternative role of phosphatidyl serine.

In no case do we have a ready explanation of the role of cholesterol in the two samples. We do not know the solubility of cholesterol in diglycerides and it might have formed into crystals, which would explain its low mobility in the B. cereus case. It is curious, however, that the aliphatic methyls are observed while the ring methyls are not at every temperature. The difference here between the temperature behavior of cholesterol in the two samples is also not understood.

We do not feel the conclusions reached from the pmr studies were particularly unique but we do feel that the method is uniquely sensitive to small changes in membrane structure. An important example is the effect of phospholipase C treatment by two different enzymes. Other methods such as circular dichroism, electron microscopy and light microscopy only recorded minor differences between the two enzyme treatments, whereas by pmr the difference was striking. Specifically, study of this difference helped shed light on the different roles that phosphatidyl serine and sphingomyelin play in membrane structure. Although full understanding of such phenomena can come only by combining pmr and biochemical studies, it is important to note the sensitivity of pmr to changes in the membrane structure and the opportunity of obtaining specific molecular information by pmr.

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PROPOSITION I

Analysis of the Tritium Decay Products of Cytidine-5-^{3}H

Funk and Person[1] recently reported that cytidine-5-^{3}H caused a specific mutation to a thymine-coding base with about 80% efficiency when incorporated into viral DNA, while cytidine-6-^{3}H caused mutations at only 1/200th of this rate. Molecular biologists have found such a specific and efficient mutagen very helpful in genetic studies of viruses[2]. No one has, however, found what base is formed by tritium decay of cytidine-5-^{3}H. It is proposed to begin a study to identify the decay product and to understand how the mutation is effected.

Very recent work by Wood[3] has shown that tritium in naphthalene-1-^{3}H or 2-^{3}H produces a free radical upon decay. The possibility that the β^- particle caused the free radical was ruled out by giving a control sample a similar dose of high-energy radiation. This suggests that decay of tritium in cytidine-5-^{3}H and 6-^{3}H will leave free radicals in those respective positions. Upon formation the two free radicals will react rapidly with neighboring molecules such as water. The cytidine-5-^{3}H product will either hydrogen bond like thymine or be transformable by enzymes to a thymine-like base while the cytidine-6-^{3}H product will not have these characteristics in double-stranded DNA.

Clues as to the identity of the cytidine-5-^{3}H decay product come from photoreactions of cytidine which probably proceed by free radical mechanisms. Grossman[4] has found that uv irradiation of polyribocytidylic acid produces hydration of the 5, 6 double bond of

cytidine and this product codes for U or T in transcription experiments. He postulated that the hydration product favored the imino form of cytidine over the normal amino form, and the imino form hydrogen bonds like U. In hydrated cytidine the amino group can be replaced by an oxygen to form the uracil analog which does not transcribe properly. Hydrated cytidine is possibly the base produced by the decay of cytidine-5-^{3}H, and a different product would be obtained from cytidine-6-^{3}H if the reaction proceeded by a simple hydration mechanism. Experimentation is necessary, however, to identify the actual tritium decay products.

There are several points to be considered in designing the experiments: 1. the molecule of cytosine should be in an environment which resembles the DNA form, 2. there should be a minimum of irradiation of the bases by other β^- decays, 3. the cytosine should be in a form that is easy to analyze and handle efficiently. A compromise procedure is proposed which will not satisfy all the considerations completely and will cater to points 2 and 3. About 10^{-6} moles of cytidine-5-^{3}H and cytidine-6-^{3}H will be prepared or purchased. These samples will be dissolved separately in sterile water (pH 7) and will be placed in dark, sterile vials for about six months. A control sample of 10^{-6} moles of normal cytidine will be prepared with 10^{-6} moles of tritium in the water. After the six months about 3% of the tritium will have decayed to give about 3×10^{-8} moles of products which is sufficient to analyze by ur and ir spectroscopy as well as chromatographic techniques.

The environment of the cytidine in this experiment is unlike

the environment of cytidine in double-stranded DNA; however, water is the most likely reactant in both cases. If the experiment does not explain the biological observations about cytidine-5-^{3}H and 6-^{3}H, it would be necessary to use a phage system to produce a double-stranded DNA with labelled cytidine incorporated. The analysis would be complicated immensely and there would be inefficiency in incorporation of the labelled compound, but most possible sources of artifacts would be eliminated. The possibility of using poly dC and dG in double-stranded form is avoided because of the greater probability of dimerization[5] which would complicate the analysis. Over the six month waiting period the product of tritium decay in these molecules might undergo further reaction as in the common case of deamination of reduced cytidines to form reduced uracils. If the product detected in the original experiment was actually a secondary product, it would be expected to appear in the next experiment with viral DNA unless the environment of cytidine in the viral DNA did not allow the second reaction to occur. Considerable work has been done on the chemistry of pyrimidines which can be used to understand the thymine-like decay product of cytidine-5-^{3}H and the differences of it from the cytidine-6-^{3}H decay product. A final test would be to prepare a single-stranded DNA of the two different decay products and test with an in vitro replicating system unless the results would be obvious.

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PROPOSITION 2

A Double, Ferritin Antibody Labelling Technique

The surface structure of the cell membrane appears to be an important factor in the determination of a cell's properties particularly in reference to the transformations which occur upon oncogeny[1]. Of the many models of the cell membrane structure put forward, the mosaic model elaborated by Singer[2, 3] appears to account for most of the recent experimental findings concerning membrane structure. In that model they propose that amphipathic proteins and lipids are in thermodynamic equilibrium in the nearly two dimensional membrane system. This is in the context that there exists no long-range ordering over major portions of the membrane surface, which is consistent with many recent experiments on membrane structure[2, 4-6]. If this hypothesis is true, generally proteins should aggregate into functional groups on the membrane surface in a reversible binding interaction such that allosteric changes of one or more members of the complex could result in its rearrangement or dissociation.

On the basis of this hypothesis, Singer and Nicolson formulated an alternative explanation for differential agglutinability of malignant cells by the saccharide-binding protein, wheat germ agglutinin (WGA). Burger[7] had proposed that "cryptic agglutinin sites" existed on normal cells and these were exposed after proteolysis or malignant transformation. Singer and Nicolson[3] suggested that the transformation might involve a redistribution of agglutinin sites on the membrane surface, and more recently Nicolson[1] did indeed show that the spatial

distribution of sites was different in the malignant cells.

The technique used in that study involved the use of ferritin conconavalin A to visualize the two dimensional distribution of conconavalin A bound to the membrane surface. The visualization of a major portion of the membrane surface was made possible by spreading the membranes out flat at the water surface upon lysis in a hypotonic solution[8]. The membranes were then mounted on coated electron microscope grids and after conditioning they were stained with ferritin labelled conconavalin A. This method has also been applied to the study of two-dimensional antigenic sites by the use of ferritin-labelled antibodies with marked success. It is limited, however, in usefulness because of the fact that there is no long range ordering in the membrane surface. The two-dimensional distribution of any single antigenic site should be essentially random except for cases where similar sites would self-associate[1]. Therefore, it is proposed that a method for labelling two antigenic sites simultaneously on the same membrane surface should be developed along with a method of analyzing these distributions quickly and impartially.

Multiple staining of antigenic sites was first proposed by Hämmerling et al.[9] using ferritin (~ 100 Å) and a virus (600 Å) to visualize the labelled antigens. This method obviously would give poor resolution, and serious problems of interference could be imagined if the two different sites were closely associated. Ferritin occurs naturally with a wide range of iron hydroxide content from apoferritin to ferritin fully loaded with iron hydroxide[10], and it would be possible to distinguish between fully loaded and half loaded ferritin in the

electron microscope. The different ferritin could be linked to different antibodies by the method of Singer and Shick[11], and a two-dimensional array of the membrane antigenic sites could be obtained by a minor modification of the method described earlier[8]. It is likely that this technique would be first applied to the human red blood cell membrane as a model membrane which is easy to work with and has many well characterized antigens.

The other problem is the analysis of the electron micrographs of the stained cells. This would involve visual discrimination of ferritins with different electron densities and analysis of their relative two-dimensional distributions. Although this could be done manually, the precision and accuracy could be greatly increased if a scanning densitometer were used to read the negatives and a computer program were used to analyze the mean separation distance of like and unlike neighbors. The computer analysis of the mean separation would be the first priority since that analysis would be a paramount task manually. For the future the scanning densitometer could be a valuable tool in distinguishing reproducibly between differently loaded ferritins which could not be distinguished visually with reliability opening the way for multiantibody labelling.

It is believed that the techniques developed as a result of this project would find application by investigators working on the problems of cancer and disease. From all indications the two-dimensional map of the membrane surface which this technique could produce, would greatly aid in our understanding of problems in cellular control and function.

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PROPOSITION 3

The Mode of Binding of Divalent Ions

Considerable interest is growing in the roles of divalent cations in membrane structure and function. Divalent cations are known to play an important part in transmission of nerve impulses[1], in preventing disintegration of Mycoplasma l. membranes[2], in the function of membrane enzymes[3], and in binding of proteins to membranes[4]. There is not, however, a deep understanding of how divalent cations are important in these phenomena. For example, the binding of a protein to a membrane could be the result of Mg^{++} binding to the phospholipids, to the protein itself, or to another protein. In the former case, the Mg^{++} binding to the phosphates, as proposed by Shah and Schulman[5], would result in a positively charged layer to which the protein's negatively charged amino acids would bind. In the second case, Mg^{++} binding would cause a conformation change in the protein which would promote binding to the phospholipid charge groups. In the third case, the Mg^{++} ion would act as an ionic bridge between the negatively charged amino acids of two different proteins. This proposition will be devoted to distinguishing which of these mechanisms is valid for proteins bound to human erythrocyte membranes. In doing this, data will be obtained on the thermodynamic stability given to the membranes by Mg^{++} binding. The major equipment needed will be an LKB 10700 flow microcalorimeter and an atomic-absorption spectrometer. Recent instrumental advances in calorimeters have made measurements of 1 μcal possible. With such instrumental capabilities the application of this technique to

biochemical systems is increasing rapidly, as evidenced by several recent papers[6-9]. It would be employed in these experiments to monitor the heat of interaction of Mg^{++} with the membrane system. The atomic-absorption spectrometer would be used to determine the Mg^{++} content of membranes before and after mixing with Mg^{++} in the calorimeter. The instrument is extremely sensitive for Mg^{++} and can detect 2.5×10^{-6} M.

A typical experiment would be performed as follows. The membrane samples would be divided into two portions for the calorimetry measurements: the first will flow through the reference cell and mix with buffer, and the second will flow through the reaction cell and mix with buffer containing a known concentration of $MgCl_2$. A control to find the heat of dilution of $MgCl_2$ solution will be run independently. After the measurement, the reference sample will be analyzed for Mg^{++}, protein, and phosphorus. The reaction sample will be centrifuged to pellet the membranes and the supernatant will be analyzed for Mg^{++} and possible protein contamination. In this manner the ΔH of interaction and the number of moles of Mg^{++} will be obtained.

The first step in this study would be to find the ΔH of interaction as a function of $MgCl_2$ concentration for whole human red cell membranes prepared by the method of Dodge, Mitchell and Hanahan[10] in a Mg^{++} and Ca^{++}-free Tris buffer (10 mM and pH 7.8). This would also give the number of sites under saturating conditions. The next step would be to treat the membranes with EDTA and to wash away any solubilized protein by centrifugation and collect it. These membranes would then be mixed with different concentrations of Mg^{++}

to give a curve of ΔH as a function of Mg^{++} concentration and a saturation value for the amount bound. In the presence of excess Mg^{++} the membrane would be combined with the protein removed by EDTA treatment when it had been washed free of all the EDTA by dialysis. The results of this experiment would give the amount of Mg^{++} bound by the protein and the enthalpy of binding per mole. If the enthalpy per mole were very large, the first mechanism would be correct since the lipid should be saturated with Mg^{++} and binding of protein would occur without large amounts of Mg^{++} binding. If the enthalpy per mole released were about that in the EDTA treated membranes, either the second or third mechanisms would be correct. In the case that no binding of protein to the membrane did occur under various conditions tried, the problem would be unsolvable by these methods. In this eventuality the thermodynamic data still give us an idea of the importance of Mg^{++} binding in membrane stability.

Assuming the protein did bind and the first mechanism were thereby disproven, the next step would be to prepare ghosts which had 75% of the protein removed by digestion with trypsin. The membranes are reportedly not fragmented by this procedure and the protein left behind has been shown to be largely inside the membrane[11]. Mg^{++} binding to these membranes would be followed, and in the presence of excess Mg^{++} these membranes would be mixed with protein solubilized by EDTA treatment. If the protein did bind, the third mechanism would be eliminated and the second would be proven. In the other case the third mechanism would be proven. There is, however, always the possibility that two or three mechanisms will be important or that the

EDTA solubilized protein will not bind.

Other calorimetry studies have found enthalpy changes on the order of 11 to 15 kcal with α-chymotrypsin binding of inhibitors[6, 7] which they concluded induced conformational changes in the enzyme. A study of glutamine synthetase from E. coli showed ΔH of -7 kcal and -2 kcal for binding L-tryptophan and adenosine 5′ monophosphate[8]. The binding of adenosine 5′ monophosphate, however, was accompanied by a positive entropy change. In the case of Mg^{++} binding to membranes a negative entropy change is expected; therefore, ΔH will be relatively large for binding Mg^{++}. Supposing we can use a concentration of membranes of 5 mg/ml protein, there are 5×10^{-3} M phospholipids present. If one tenth of that concentration of Mg^{++} binding sites are available, a commerical flow microcalorimeter (sensitivity 0.1 μcal/sec) will detect a ΔH of 40 cal/mole. This should give an accuracy of better than ± 5% in measurements of ΔH of -4 kcal or greater as is expected. The atomic absorption studies will not be hindered by the presence of sodium, potassium or calcium since they all have quite different wavelengths of absorption. In the range of 5×10^{-4} of Mg^{++} this apparatus should be able to measure concentrations with an accuracy of more than ± 5%. The assays of protein and phosphate can only measure those concentrations with a slightly better accuracy of ± 3%. The overall accuracy of the ΔH/mole of Mg^{++} will be about ± 10%. Treatment of the amount of Mg^{++} bound in relation to the concentration of sites on the membrane and the concentration of Mg^{++} in solution could give a Keq, but it is difficult to know if all Mg^{++} is still in equilibrium with the water phase.

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PROPOSITION 4

The Stoichiometry of the Alamethicin Pore

Alamethicin is a cyclic polypeptide of molecular weight about 1700 with the structure shown in Fig. 1[1]. It has been studied with particular interest because it produces an action potential-type voltage response to a constant current pulse in a black lipid film[2]. The other antibiotic, EIM, which induces similar phenomena[3] is not as well characterized. In the early work on alamethicin it was found that the conductance increased sharply with increasing alamethicin concentration (roughly according to the sixth power)[4], electrolyte concentration, and applied potential. From this sixth power relationship to the concentration, Mueller and Rudin[4] speculated that alamethicin formed pores in the membrane with aggregates of about six molecules. Subsequent investigators have looked for further evidence to support the presence of some stoichiometric complex of alamethicin as the active species but have not come much closer. In fact, it has only recently been proven that unit conductance states exist for alamethicin pores[5, 6]. The aggregation of alamethicin in water and other solvents has been studied, but without much clarification of the state of alamethicin within the membrane since, like many other surface active compounds, it forms micelles in aqueous solvents at low concentrations, whose sizes are affected by ionic strength, pH, etc.[7]. It is necessary, therefore, to study the stoichiometry in the presence of lipids under conditions which will produce an alamethicin pore.

Some recent black lipid film work sheds some interesting light

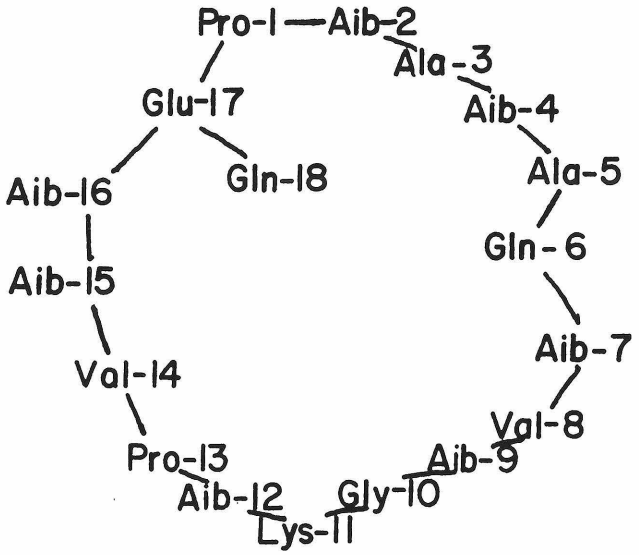

FIGURE 1

The primary structure of alamethicin.

on the possible stoichiometry of the alamethicin[5]. The distribution of conductance states with time has been analyzed and it yields a probability versus conductance curve which normally has definite peaks at near integral values of a unit conductance. From careful analysis of such curves it was discerned that certain half-unit conductances were observed. These were later explained by postulating that the active state of alamethicin in the membrane could have four conductance levels other than zero. The half integral values arose from the fact that the four conductance levels were not whole integral values and two or more active states (patches) would add to give nearly half-integral values. The number 4 is not believed to be the stoichiometry of the patch since the discoverers believe that a single conductance level requires 2-3 alamethicins and a patch would therefore be a complex of 8 or 12 alamethicins. It is difficult to pursue the stoichiometry of alamethicin further in black lipid film work where the concentration of alamethicin in the bilayer is not known.

Another model lipid system, the sonicated phospholipid vesicle, appears to have the most promise for stoichiometric work despite the altered nature of the bilayer in the sonicated vesicle[8]. Hauser et al.[9] have studied the proton magnetic resonance (pmr) spectrum of phosphatidyl choline and serine upon the addition of alamethicin and have found a particularly interesting effect in the phosphatidyl serine vesicles. At a mole ratio of 1 alamethicin to 3000 phosphatidyl serines there is a maximal destabilization of the vesicles analogous to the effect of sodium dodecyl sulphate. Since there are roughly 3000 phosphatidyl serine molecules per vesicle, this corresponds to one

alamethicin molecule per vesicle. As the concentration of alamethicin is increased there is a marked stabilization of the lipid in the vesicle plateauing at 1 alamethicin per 500 phosphatidyl serines or about 6 alamethicins per vesicle of which 4 should be on the outside and 2 on the inside[8]. A similar effect is not noted in the phosphatidyl choline vesicles; and, therefore, although this effect is interesting, it is not solid evidence for a given stoichiometry of alamethicins in a bilayer.

This proposal is to use small dihydrostericuloyl phosphatidyl ethanolamine vesicles and radioactive ^{40}K in a study of the leakage of the ^{40}K from the vesicles as a function of alamethicin concentration. Varying ratios of alamethicin to phosphatidyl ethanolamine will be mixed in methanol where both are soluble and dried under vacuum from the frozen solution to prevent aggregation as the solvent is removed. The mixture at 2% (w:v) will then be sonicated in a solution of 0.001 M KCl (10^6 counts/min/cc of ^{40}K) and 0.001 M Tris at pH 9.0 for ten minutes. The ^{40}K would be dialyzed away by dialysis against 0.001 M NaCl. Just prior to the experiment the external medium would be made 0.001 M in acetic acid making the pH ~4. The sample would then be placed in a hollow fiber dialysis apparatus and dialyzed against the same buffer with 10^{-4} M dinitrophenol in it. The dinitrophenol should create a Nernst potential of about 250 mv. across the membrane, which is normally enough to open alamethicin channels. The opening of the channels would stimulate the efflux of $^{40}K^+$ from inside the membranes and it would dialyze away rapidly because of the large surface to volume ratio in the hollow fiber apparatus. The fast dialysis is necessary to avoid background problems from passive leakage which will

probably be high[10]. As controls, the sample of the vesicles would be counted along with the dialysate and another sample would be treated in the same manner except that no dinitrophenol would be added. From this study a curve for the number of alamethicins per inside of the vesicle versus ^{40}K efflux rate would be obtained, which would help to clarify the stoichiometry of an alamethicin pore.

The lipid in this study was chosen because of the inability of alamethicin to cross black lipid films of it[11], which will mean that dialyzing away the external alamethicins will have no effect on the number of alamethicins inside the vesicle. From studies of the conductance of black lipid membranes there is no reason to believe that the presence or absence of alamethicin on one side of the membrane will affect alamethicin on the other side except in a secondary manner. The presence or absence, then, of alamethicin in the outside half of the bilayer should be of little or no consequence.

A serious problem may be the presence of channels which are open at zero voltage since this occurs at high concentrations of alamethicin. These channels will allow the internal ^{40}K to dialyze out which would merely necessitate plotting the remaining ^{40}K versus the number of alamethicins per inside of a vesicle. If the stoichiometry of an alamethicin patch is fixed and a patch is needed for a conductance level to open regardless of the applied voltage, the above mentioned phenomenon will occur. If this is not true, many curves can be obtained giving the stoichiometry of alamethicin necessary to produce conductance at various applied voltages by simply varying the pH of the external medium.

Depending upon the exact size of the vesicle there are roughly 500-1000 phosphatidyl ethanolamine molecules on the inside of sonicated vesicles. A relatively exact measurement of the vesicle could be made by electron microscopy using a simple negative stain. From the mean outside radius of a vesicle the number of phosphatidyl ethanolamine molecules on the inside surface would be calculated by assuming the thickness of the bilayer to be 54 Å [12] and the lateral area to be about 50 $Å^2$ per molecule.

For the structure of the alamethicin pore to be known, the stoichiometry must be known since many different complexes can be postulated to give a pore. These experiments will, hopefully, clarify that problem and give a greater understanding of the mechanism of action of this very interesting molecule.

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PROPOSITION 5

The Making of a Pure Phospholipid Bilayer for Electrical Studies

The study of black lipid membranes has given us much information about the electrical properties of the phospholipid bilayer. Perhaps it would be more correct to say that the studies have yielded information about the phospholipid and decane bilayer. Several of the investigators on black lipid membranes have gone to great lengths comparing the electrical properties of their membranes with those of biological membranes and always come to the conclusion that the differences are small[1-3]. Still there is the nagging thought in many minds that the effects which are being studied in black lipid films might be quite different in phospholipid bilayers of the same lipids but without the decane or octane used in the black lipid membrane experiment. There are, in fact, several areas where the decane may give special problems. In the study of saturated lecithin the melting point of the lipids in black lipid film is lowered by about 10°C as a result of the presence of decane in the bilayer[4]. In other work, investigators have noted a thinning of the bilayer with increased potential across the bilayer which may indeed be affected greatly by the presence of decane in the bilayer[5, 6]. Another example of the problems which the presence of decane causes is the uncertainty in the lateral area per phospholipid molecule in a black lipid membrane. The calculations of McLaughlin et al.[7, 8] do indicate that assuming a normal lateral area per phospholipid, you can predict the observed charge densities, but that should be qualified by the

relatively large error limits they place on those area figures. From all these problems we can understand that it would be of great value to design a method for preparing phospholipid bilayers in the same configuration as the black lipid membranes without foreign molecules.

This is to be accomplished by a modification of the Langmuir-Blodgett multilayer deposition method[9]. Stated simply, this method involves the deposition of successive monolayers of a water surface film on a glass plate or similar surface by repeatedly dipping the plate into and withdrawing the plate from water covered by the monolayer under pressure. It is proposed, then, to deposit two layers of a phospholipid monolayer such that they separate two isolated water compartments suitable for electrical measurements.

Several important requirements must be met to accomplish this end. First, the phospholipid monolayer must be under a constant surface pressure. Second, the surface must accept the monolayers. Third, the surface must separate two electrically isolated water compartments.

The first requirement can be met in several ways provided the proper apparatus is available. Most monolayer work has been done on a Langmuir film balance[10] which has the capability of measuring the surface pressure of a monolayer. In a normal experiment the surface would first be cleaned and a small amount of a soap in a volatile solvent would be applied between the float and the movable barrier. The float is normally connected to a torsion balance so that the lateral force of the film, which is the difference in surface tension between the clean water surface and the monolayer covered surface, can be measured. When the surface area of the film is condensed, the film pressure

increases gradually at first, then very rapidly until the collapse pressure is reached, as demonstrated in Fig. 1. At the collapse pressure a condensed monolayer is obtained. Since we need a constant pressure as the condensed monolayer is being deposited on the solid surface, the movable barrier must be advanced continuously, preferably by a screw-drive mechanism without vibrational disturbance of the surface. This procedure would require sophisticated apparatus and a lot of time to calibrate the equipment. But there is a simple way of maintaining a constant surface pressure which involves no film balance or elaborate equipment.

The simpler method takes advantage of the fact that certain soap monolayers exert a constant surface pressure when in contact with excess soap. A movable barrier such as a silk thread could be used to separate the phospholipid monolayer and the driving monolayer. The thread would move to maintain the constant surface pressure on the phospholipid monolayer as it was being deposited on the surface. The normal collapse pressure of a phospholipid film is about 40 dynes/cm and a suitable piston oil would be oleic acid[11] which exerts a pressure of 30 dynes/cm, or long chain alcohols which were used by Levine and Wilkins[12] to deposit lecithin films on a glass slide.

For the solid to accept the monolayers it must be quite smooth and have the proper surface properties. Although it has been shown that a continuous soap monolayer can be deposited on a grooved surface or a fine mesh screen[13], abrupt irregularities or sharp points will disrupt the film. It is also important that the film should not have to cover a large surface area unsupported because in this case there is

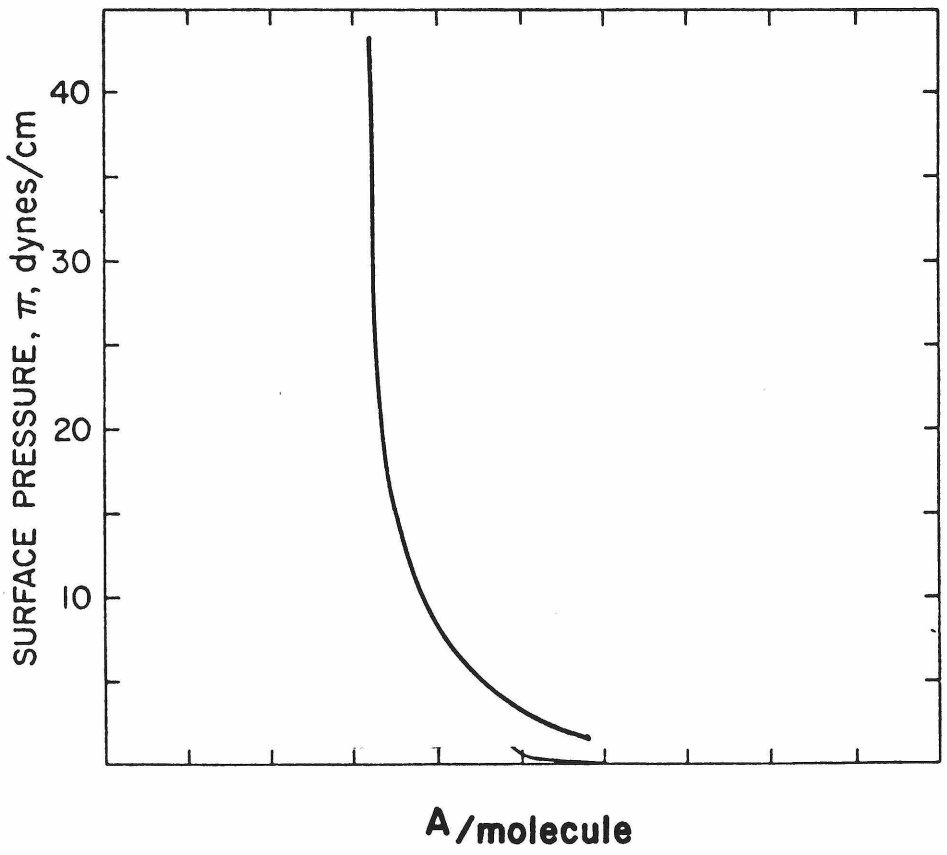


FIGURE 1

Sample pressure versus area curve obtained from a Langmuir film balance experiment.

no torus which can absorb pressure shocks to the membrane. A suitable support may, therefore, be a fine gold electron microscope grid or a cellulose nitrate filtration membrane such as ones recently marketed by Dupont. To form these cellulose nitrate membranes a smooth film was punctured by small beads moving at a high velocity and the resultant holes were uniform with no ragged edges in the scanning electron microscopy. These are not the only possible surfaces available but appear to have the highest probability of success.

The gold surface, in particular, would have to be preconditioned because multilayers are most easily deposited on hydrophobic surfaces[9]. The normal procedure is to condition the surface with a thin film of ferric stearate. The coating is begun by dipping the solid through the monolayer so that the hydrophobic tails of the fats contact the hydrophobic surface as in Fig. 2. When the solid is inserted for the first time, the film covering holes must be broken by a pressure wave to prevent the formation of a trilayer. The support would then be withdrawn and reinserted to produce a normal bilayer as depicted in Fig. 2.

For the bilayer produced in this fashion to be useful, it must be formed so that the bilayer itself is the weakest electrical barrier between two water compartments, or in other words, the conductivity between the two compartments is determined by the bilayer. The electron microscope grid or dialysis membrane must, therefore, be incorporated into a larger structure in a continuous fashion. An example of how this might be done is to use paraffin to affix the microscopy grid in an indentation surrounding a circular hole in a

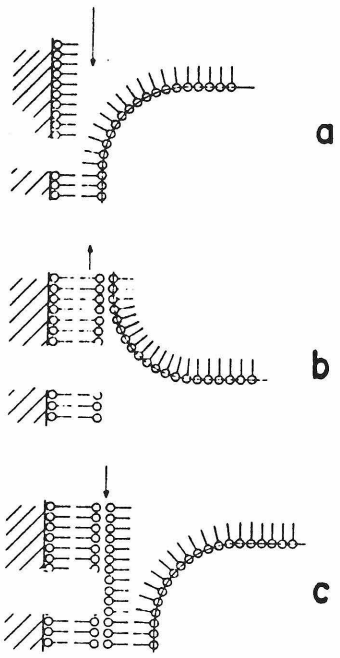
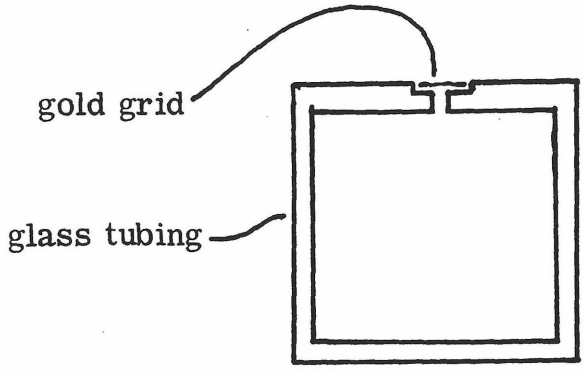


FIGURE 2

Shows the manner in which the bilayer will be formed over holes in the supporting surface.

piece of square glass tubing as illustrated below. By melting the paraffin it would form a smooth, continuous, hydrophobic surface between the ferric stearate coated glass and gold.



In the actual experiment the glass tubing would be inserted vertically through the monolayer twice and on the final insertion would contact a layer of carbon tetrachloride which would insulate the two water chambers. Such a bilayer could be studied without worry of decane contamination and would open the way to many experiments without problems of relevance. Wilkins et al.[12] have demonstrated that phospholipid multilayers can be made, and earlier work shows that monolayers can be deposited on fine mesh screens[13]. There is every reason to believe, therefore, that this project can be made to work, although the method proposed may not be the best to accomplish that end.

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