Proton Magnetic Resonance Studies of Whole Human Erythrocyte Membranes†

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ABSTRACT: An analysis of the proton magnetic resonance (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.

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 (Hubbell and McConnell, 1969a,b; Reinert and Steim, 1970; Wilkins *et al.*, 1971; Glaser *et al.*, 1970; Nicolson *et al.*, 1971; Frye and Edidin, 1970).

Much of the research efforts of our laboratory in recent years has been directed toward gaining a better understanding of the interactions between proteins and lipids. Some of our work has been concerned with structural studies of phospholipid phases and the effects of oligopeptide antibiotics such as valinomycin and gramicidin S on the structure of the lipid phase. Another phase of our work has concerned the study of real membranes. Here we have attempted to perturb biomembranes in certain well-defined ways and then to determine the effects of the perturbation on the lipid and protein components. Our recent work on the effect of phospholipase C on human erythrocyte membranes illustrates this approach (Glaser *et al.*, 1970). In all of our studies we have employed the proton magnetic resonance (pmr) method because this spectroscopic technique has the intrinsic capability of distinguishing between various membrane components as well as allowing an estimation of the concentration and mobility of these components.

The objective of this paper is to further elaborate on our recent pmr studies of erythrocyte membranes. In our earlier work we subjected red cell ghosts to heat as well as enzymatic treatment and the effects which these perturbations had on the pmr spectrum were used to deduce certain conclusions regarding the interactions between the lipid and protein components. In view of the important implications of these findings, we felt compelled to characterize the observed spectral changes more fully. For this reason we have investigated the effects of different types of perturbations of varying severity and irreversibility such as heat treatment, protein removal, sonication, etc., on red blood cell ghosts, and have attempted to characterize the resultant structural changes in the membrane by the pmr method. By studying the effects of these treatments systematically and over a wide range of experimental conditions, such as solvent structure, divalent ion concentration, etc., it is hoped to be able to pinpoint those variables which control the resistance of the membranes toward various types of structural changes. It is anticipated that these studies will provide background information which may be useful for future pmr studies of biological membranes.

Experimental Section

Preparation of Red Blood Cell Membranes. Red blood cell membranes were prepared from fresh human blood, type O and Rh^+, in ACD[1] by the method of Dodge *et al.* (1963). 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 (Lowry *et al.*, 1951). These white membranes were layered onto D_2O (6 mM phosphate at pD 7.8)[2] and were pelleted at 40,000 rpm in a Spinco SW-50 rotor for 20 min. 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

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[1] Abbreviations used are: ACD, acid–citrate–dextrose anticoagulant buffer; imOsm, ideal milliosmolar; DSS, 3-(trimethylsilyl)propanesulfonic acid sodium salt.

[2] In this work, all solution pD's were measured with a Leeds and Northrup 7401 pH meter equipped with miniature electrodes and were calculated from the observed pH meter reading plus the standard correction of 0.4 (Lumry *et al.*, 1951).

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.

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 hr at 4° (Marchesi and Steers, 1968). The ghost suspension was then spun at 14,000 rpm in a Sorvall SS-35 rotor, and the supernatant withdrawn. A portion of the ghosts was exchanged as above into a D_2O solution containing 1 mM Trizma Base and 3 mM KCl at pD 7.8.

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° for 4 hr, and centrifuged for 30 min at 13,00*g*. The extraction was repeated twice with the same solvent mixture. The final pellet was placed in 30 ml of ether at −15° for 3 days with occasional shaking and then centrifuged for 30 min at 1300*g*. This procedure was repeated twice and the final pellet was dried under vacuum and stored at −15° until used. A weighed sample was suspended in D_2O buffer for pmr spectra.

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

Sonication. Several samples of ghosts were sonicated for 15 sec by a Bronson sonifier with a microtip at power level 3. These samples were then spun at low speed to pellet whole cells, the particles in the supernatant were drawn off and exchanged into D_2O buffer, and the pmr spectrum was recorded.

High-Temperature Centrifugation Experiments. A series of high-temperature centrifugation runs of ghost suspensions were made on a Spinco Model E centrifuge for 30 min at 39,000 rpm with an SW-39 rotor. In each case the buckets were equilibrated at 75° before centrifugation and the supernatant was removed while still at that temperature. Other samples were heated to 75° in a water bath for 10 min, cooled to 4°, and centrifuged at 4° for 0.5 hr at 39,000 rpm. After centrifugation, the supernatants as well as the original ghost suspensions were analyzed for protein content, and their solution pH values compared.

The above experiments were carried out for ghosts suspended in both H_2O and D_2O. The original ghost suspensions in the two solvent systems were prepared in an otherwise identical manner, and were diluted to a concentration of about 1.0 mg/ml of protein before heating. The effect of heating was examined as a function of solution pH or pD, and the addition of certain divalent ions.

Samples of the supernatant protein and pelleted membranes after the high temperature centrifugation were analyzed by acrylamide gel electrophoresis in sodium dodecyl sulfate according to the procedure of Laico *et al.* (1970). The amino acid content of the acid-digested supernatant protein was analyzed on a Beckman analyzer.

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.

Preparation of Other Mitochondrial Membranes and Mycoplasma laidlawii Membranes. Samples of outer mitochondrial membranes and *M. laidlawii* membranes were prepared by Dr. Henry Simpkins and Dr. S. J. Singer at the University of California, San Diego, La Jolla, Calif. The outer mitochondrial membranes were isolated from a sucrose step gradient after mild sonication to separate the inner and outer membranes. *M. 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.

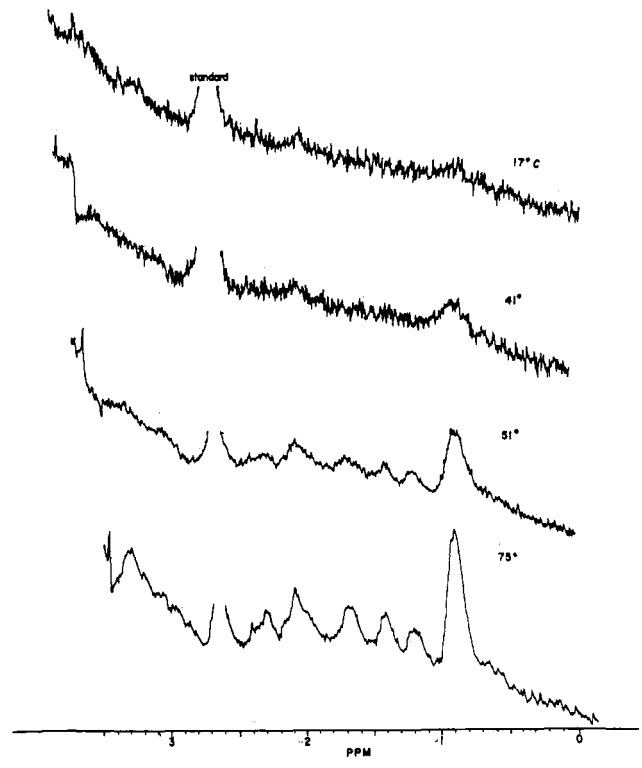

FIGURE 1: Pmr spectra (220 MHz) of whole human erythrocyte membranes (5.0 mg of membrane protein/ml) in the region from 0 to −3.5 ppm from DSS.

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 min 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 and were measured from the external standard, whose chemical shift was in turn standardized against a solution of DSS.

Results

The 220-MHz pmr spectrum of soft-packed human erythrocyte membranes exhibits a marked temperature dependence as shown in Figure 1, where we have reproduced the spectral region from −0.0 to −3.5 ppm at 17, 41, 51, and 75°. At 17°, 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

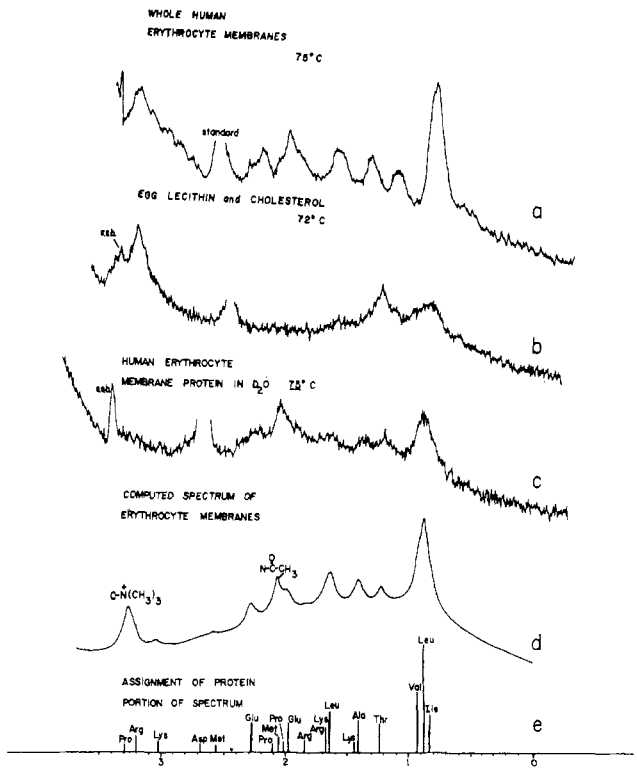

FIGURE 2: Pmr spectra (220 MHz) of (a) whole human erythrocyte membranes at 75°; (b) egg lecithin and cholesterol, 1:1 (20 mg of lecithin/ml); (c) delipidated membrane protein from human erythrocyte membranes in D_2O at 75° (5 mg/ml); (d) computed spectrum for human red cell membrane; (e) assignment of the amino acid resonances in computed spectrum.

and the strong residual HOD resonance at ~ -4.5 ppm arising from H_2O contamination of the D_2O. We note 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°. 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°. With increasing temperature, there is a slight concomitant decrease in the line widths of the various resonance peaks, but this reduction in the line widths 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 reproducible from membrane sample to membrane sample irrespective of whether the samples were fresh or had been heated above 75°, except that upon cooling the samples back to 18° after the heat treatment, the spectrum observed was somewhat more intense than that of a freshly prepared sample.

We have compared the spectra of the "whole" membranes with those of an egg lecithin and cholesterol mixture and the isolated membrane proteins in Figure 2. This comparison has led us to assign the spectrum of the whole membranes at 75° 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

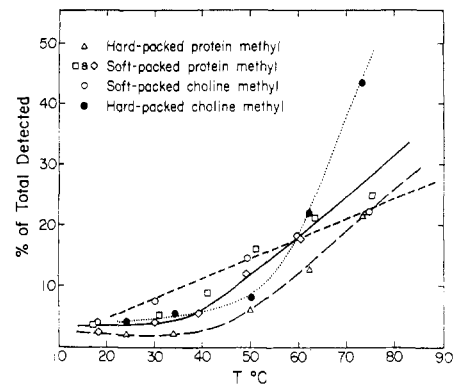

FIGURE 3: Percentage seen of total *vs.* temperature at pH 7.8 for: △, the aliphatic methyl peak of hard-packed membranes, □ and ◇, the aliphatic methyl peak of soft-packed membranes, ○, the choline methyl peak of soft-packed membranes, and ●, the choline methyl peak of hard-packed membranes.

of this peak, however, indicated that only 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 amino acid composition among the various protein fractions are not sufficiently large to enable us to draw this conclusion (Rosenberg and Guidotti, 1969). In the calculation of the membrane protein spectrum we have, therefore, merely used the average amino acid composition for the whole membrane (Rosenberg and Guidotti, 1968; Zwaal and Van Deenen, 1968). A computer-simulated spectrum of the whole membranes assuming that 20% of the amino acid residues of the proteins contribute to the observed spectrum is given in Figure 2. For the protons of the amino acid residues in the random-coil configuration we have used the chemical shifts and line widths reported by McDonald and Phillips (1969). 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 line width 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.* (1966) and Gorkom and Hall (1966). The lipid composition was taken from Dodge and Phillips (1967). 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 (Rosenberg and Guidotti, 1968). 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 Figure 3 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 Figure 3. The percentage of choline methyls detected is seen to be highly dependent on the state of the membrane. For example, a

TABLE I: Percentage of Proteins Released by Heat Treatment and Percentage Detected by Pmr at Various pD Values.

pD	Percentage of Proteins Solubilized after Heat Treatment	Percentage of Proteins Detected by Pmr
7.05	3	15
7.8	7	21
8.75	13	30

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 sidebands from the HOD peak.

Because some membrane protein might have been solubilized by heating the membranes to 75°, 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°, 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° or at 4° after heating to 75°. The latter procedure was therefore used in the majority of these experiments since this procedure was more convenient. We have also noted that the high temperature disintegration was complete in less than a minute in these experiments.

The extent of protein solubilization upon heating the ghosts to 75° 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 of 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 (Table I). 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 Figure 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^{2+} ions even at concentration levels of less than 10^{-6} mole of Mg^{2+} 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^{2+}. By contrast Mg^{2+} 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. Sodium dodecyl sulfate 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 (Figure 5). 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, except different ones, 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

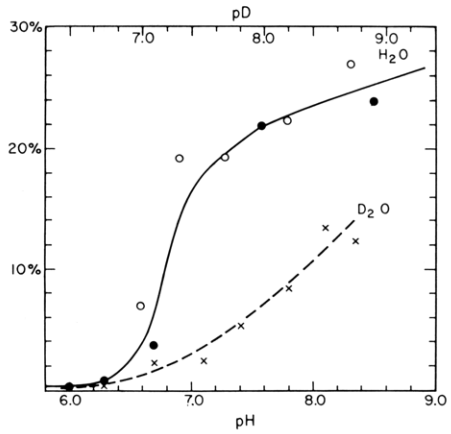

FIGURE 4: Percentage of the total membrane protein solubilized from soft-packed human erythrocyte membranes at 75° *vs.* the pH (○ and ●) and the pD of the D_2O suspensions (×).

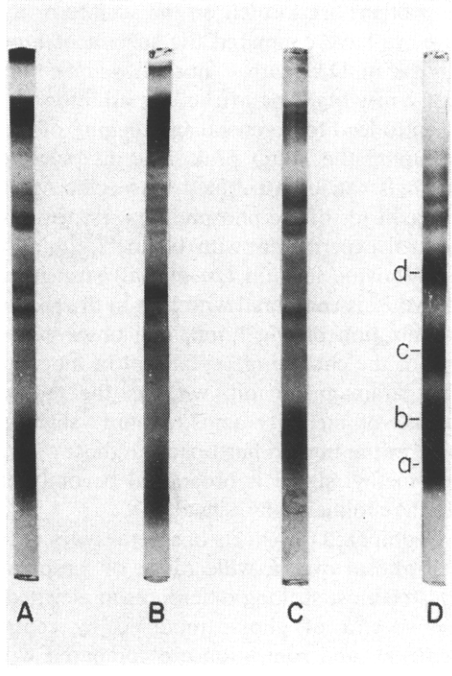

FIGURE 5: Sodium dodecyl sulfate electrophoresis gels stained with coumassie blue: (A) whole human erythrocyte membranes, (B) protein solubilized by heating membranes to 75° (pH 7.8 in H_2O), (C) membranes after heating to 75° with solubilized protein removed, (D) standard sample: (a) insulin (mol wt 5700), (b) haemoglobin (16,000), and (c and d) γ-globulin [(c) light chain 23,000 and (d) heavy chain 50,000].

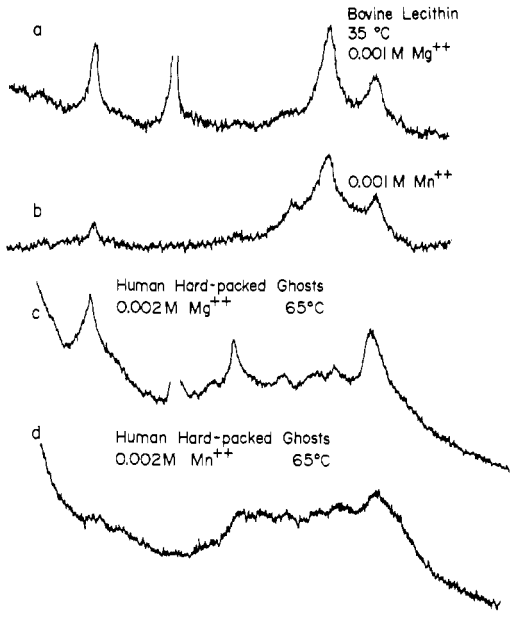

FIGURE 6: Pmr spectra (220 MHz) of (a) bovine lecithin (20 mg/ml) with 1 mM Mg^{2+}, (b) bovine lecithin with 1 mM Mn^{2+}, (c) hard-packed human ghosts in 7 mM phosphate buffer with 2 mM Mg^{2+} at 65°, (d) hard-packed human ghosts with 2 mM Mn^{2+}.

ions may have on the protein spectrum. It is well known that Mn^{2+} ion can cause spectral broadening of the nuclear resonances of magnetic nuclei in its immediate vicinity because of magnetic dipole–dipole interactions between the Mn^{2+} ions and the nuclear spins. Since there are few Mn^{2+} transport sites in these membranes (Weed and Rothstein, 1961) and Mn^{2+} can only bind to the phosphate groups or carboxyl 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 Figure 6 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^{2+} or Mn^{2+}. The addition of the 2 mM Mn^{2+} 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^{2+} 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^{2+} is compared with that in the presence of the same concentration of Mg^{2+} 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.

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° in Figure 7. 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

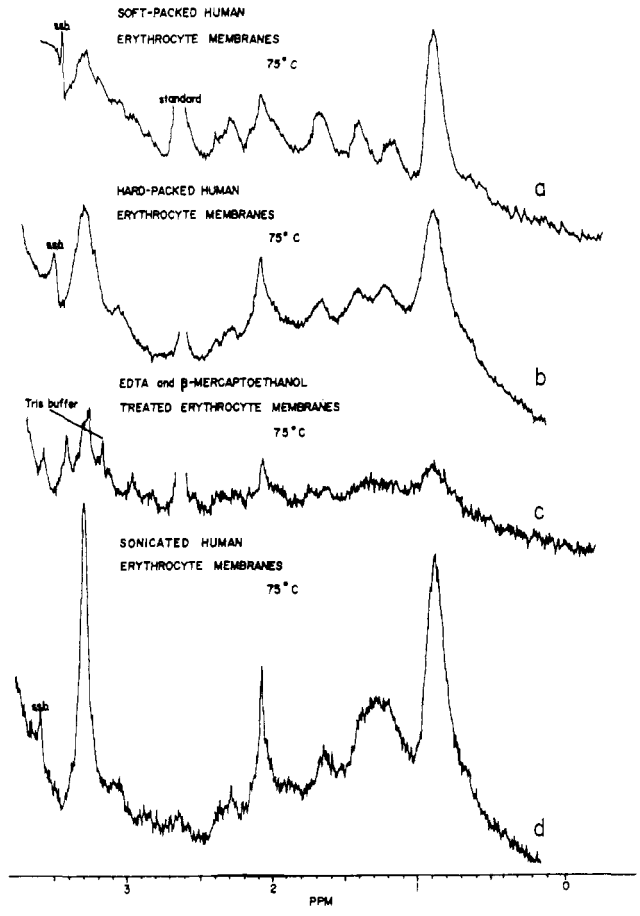

FIGURE 7: Pmr spectra (220 MHz) of (a) soft-packed human erythrocyte membranes (5 mg of protein/ml) at 75°, (b) hard-packed human erythrocyte membranes (20 mg of protein/ml) at 75°, (c) EDTA- and β-mercaptoethanol-washed membranes (6 mg of protein/ml) at 75°, and (d) sonicated human erythrocyte membranes (20 mg of protein/ml) at 75°.

"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° 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° 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 membranes, as is apparent from the prominence of the lipid portion of the spectrum of the membranes. Above 50° 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, *M. laidlawii* 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 *vs.* temperature for *M. laidlawii* membranes and egg lecithin in Figure 8. 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° 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.

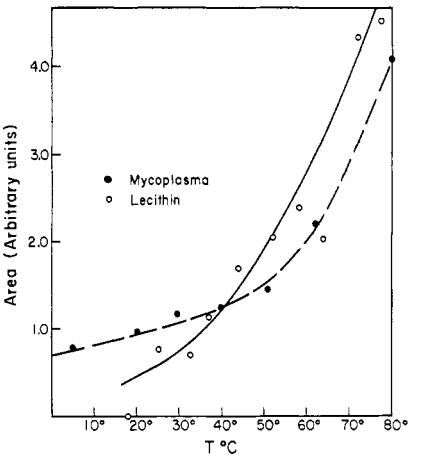

FIGURE 8: Area of the aliphatic methylene proton peak (−1.25 ppm) *vs.* temperature for (●) *M. laidlawii* membranes and (○) lecithin bilayers.

Discussion

It is known that the whole human erythrocyte membrane does not exhibit a high-resolution pmr spectrum at physiological temperatures (Chapman *et al.*, 1968; Kamat and Chapman, 1968). 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 various components of the membrane (Chan *et al.*, 1971). 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° 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.

Assignment of the Pmr Spectra. 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 tetramethylammonium 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 for phospholipase C treated red blood cell membranes where the fatty acid side chains of the phospholipids have been rendered mobile by enzymatic cleavage of the polar-head groups (Glaser *et al.*, 1970). 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 nonaliphatic 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 toward lower fields, particularly below −1.27 ppm. These general spectral characteristics are clearly manifested in the pmr spectra of red cell ghosts which have been heated above ~40°. 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 pro-

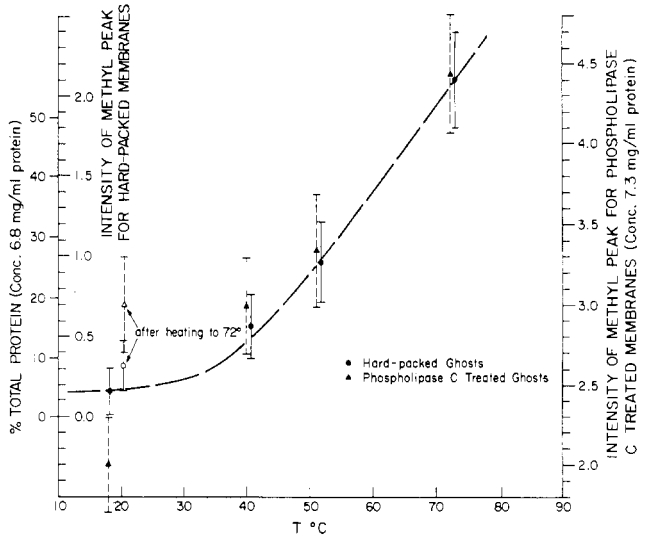

FIGURE 9: Area of the aliphatic methyl peak (−0.89 ppm) *vs.* temperature for (●) hard-packed ghosts (6.8 mg of protein/ml) and (▲) for the same membranes treated with phospholipase C (*B. cereus* enzyme) (7.3 mg of protein/ml).

teins (Rosenberg and Guidotti, 1968). The only discrepancy appeared in the region of ~−2.0 ppm, but the resonance appearing at −2.08 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 (Figure 9) 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.

Protein Solubilization. In the present work, 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 (see data summarized in Table I). As we have shown, the extent of protein loss from the membranes upon heating the ghosts to 75° 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^{2+}.

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 scialic acid residues because neuraminadase treatment was found to greatly decrease the electrophoretic mobility of the ghosts (Seaman and Cook, 1965), 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 red cell ghosts based on two independent amino acid analyses (Rosenberg and Guidotti, 1968; Zwaal and Van Deenen, 1968), we calculated that the membrane proteins would carry a net negative charge of $\sim 0.6 \times 10^{-4}$ or 1.7×10^{-4} mole per g 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 7% of the histidine residues would be protonated at pH 7.8, where our experiments were nominally carried out. Similar considerations yielded negative charge concentrations of 0.7×10^{-4} and 0.5×10^{-4} mole per g 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 sodium dodecyl sulfate gel electrophoresis and amino acid analysis has shown that this protein fraction contains a nonrandom distribution of membrane proteins. The fact that the solubilized fraction is rich in acidic amino acids correlates well with the work of Rosenberg and Guidotti (1969), 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 Figure 4 resembles a titration curve and might well be associated with titration 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 stronger aggregation of hydrophobic groups in D_2O than in H_2O, *e.g.*, the greater aggregation of phycocyanin in D_2O compared with H_2O (Berns *et al.*, 1968), as well as the lower critical micelle concentrations for fatty acids in D_2O (Kresheck *et al.*, 1965).

The observed suppression of protein solubilization as well as the nonappearance 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 re-

sistant 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 (Kwant and Seeman, 1969), 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. The difference in the effect of added Mg^{2+} between Tris and phosphate buffer may be due to a lower magnesium ion activity in the phosphate buffer.

Membrane Fragmentation. 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 (8–10% *vs.* 5–7% for soft-packed ghosts). This difference in the protein loss is probably the reason for the almost factor of 2 increase in the choline methyl signal (40% seen *vs.* 20% in normal membranes) between hard and soft-packed ghosts at 75°. 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 the type that might also explain the different reactivities of red cells prior to hemolysis and red cell ghosts toward phospholipase C. Two independent investigators (Bowman *et al.*, 1971 and Zwaal *et al.*, 1971) 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 that proteins are 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 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 (1968), and in view of the gross structural changes which might occur, the pmr spectra of sonicated membranes probably have little bearing on the membrane structure of intact cells.

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References

Berns, D. S., Lee, J. J., and Scott, E. (1968), *Advan. Chem. Ser. 84*, 21.

Bowman, M. H., Ottolenghi, A. C., and Mengel, C. E. (1971), *J. Memb. Biol. 4*, 156.

Chan, S. I., Feigenson, G. W., and Seiter, C. H. A. (1971), *Nature (London) 231*, 110.

Chapman, D., Kamat, V. B., DeGier, J., and Penkett, S. A. (1968), *Mol. Biol. 31*, 101.

Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963), *Arch. Biochem. Biophys. 100*, 119.

Dodge, J. T., and Phillips, G. B. (1967), *J. Lipid Res. 8*, 667.

Frye, L. D., and Edinin, J. (1970), *J. Cell Sci. 7*, 319.

Glaser, M., Simpkins, H., Singer, S. J., Sheetz, M., and Chan, S. I. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 721.

Gorkom, M. V., and Hall, G. E. (1966), *Spectrochim. Acta 22*, 990.

Hubbell, W. L., and McConnell, H. M. (1969a), *Proc. Nat. Acad. Sci. U. S. 63*, 16.

Hubbell, W. L., and McConnell, H. M. (1969b), *Proc. Nat. Acad. Sci. U. S. 64*, 20.

Kamat, V. B., and Chapman, D. (1968), *Biochim. Biophys. Acta 163*, 411.

Kresheck, G. C., Schneider, H., and Scheraga, H. A. (1965), *J. Phys. Chem. 69*, 3132.

Kwant, W. O., and Seeman, P. (1969), *Biochim. Biophys. Acta 193*, 338.

Laico, M. T., Ruoslahti, E. I., Papermaster, D. S., and Dreyer, W. J. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 120.

Lowry, O. H., Rosenberg, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Lumry, R., Smith, E. L., and Glantz, R. R. (1951), *J. Amer. Chem. Soc. 73*, 4335.

Marchesi, V. T., and Steers, E., Jr. (1968), *Science 159*, 203.

McDonald, C. C., and Phillips, W. D. (1969), *J. Amer. Chem. Soc. 91*, 1513.

Nicolson, G. L., Masouredis, S. P., and Singer, S. J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1416.

Penkett, S. A., Flook, A. G., and Chapman, D. (1968), *Chem. Phys. Lipids 2*, 273.

Purcell, J. M., Morris, S. G., and Susi, H. (1966), *Anal. Chem. 35*, 588.

Reinert, J. C., and Steim, J. M. (1970), *Science 168*, 1580.

Rosenberg, S. A., and Guidotti, G. (1968), *J. Biol. Chem. 243*, 1985.

Rosenberg, S. A., and Guidotti, G. (1969), *J. Biol. Chem. 244*, 5118.

Seaman, G. V. F., and Cook, G. M. W. (1965), *in* Cell Electrophoresis, Ambrose, E. J., Ed., London, J. A. Churchill, Ltd., p 52.

Weed, R. I., and Rothstein, A. (1961), *J. Gen. Physiol. 44*, 301.

Wilkins, M. H. F., Blaurock, A. E., and Engelman, D. M. (1971), *Nature (London) 230*, 72.

Zwaal, R. F. A., Roelofsen, B., Comfurius, P., and Van Deenen, L. L. M. (1971), *Biochim. Biophys. Acta 233*, 474.

Zwaal, R. F. A., and Van Deenen, L. L. M. (1968), *Biochim. Biophys. Acta 150*, 323.