THE GLYCOPHORIN-PHOSPHOLIPID INTERFACE IN RECOMBINED SYSTEMS

A ^{31}P-NUCLEAR MAGNETIC RESONANCE STUDY

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ABSTRACT Glycophorin, the MN glycoprotein from the erythrocyte membrane, was recombined with egg phosphatidylcholine and with the total lipid extract from human erythrocyte membranes in a membranous form. ^{31}P-nuclear magnetic resonance (NMR) spectra of the recombinants resembled spectra obtained from unsonicated phospholipid dispersions and biological membranes. The glycophorin/phospholipid ratio in these recombinants was varied from ~50:1 (lipid/protein) to 200:1, and ^{31}P-NMR spectral intensities were obtained. Comparison of these intensities to that expected based on a pure phospholipid standard revealed that there were two phospholipid environments in the recombinants: one immobilized by the protein, and one slightly disordered and nonimmobilized. A relatively constant number of phospholipids were immobilized per glycophorin at all lipid/protein ratios studied.

Phospholipids and proteins constitute the major components of biological membranes. The structure of these species determines in part the structure of the membrane. Thus the phospholipid bilayer, formed by pure phospholipids, occurs as a major element of membrane structure.

Integral proteins of cell membranes bury a substantial portion of their mass in the hydrophobic interior of the membrane. Therefore, one of the current questions arising about membrane structure concerns the interaction between the integral membrane protein and the phospholipid bilayer in which it is imbedded. The lipid-protein interface potentially provides a unique environment because instead of phospholipid encountering other phospholipids as neighbors, it experiences protein as a neighbor.

Electron spin resonance (ESR) spin labels have been used to examine this interface with several integral membrane proteins, including cytochrome oxidase (1), cytochrome b_5 (2), and Ca^{2+}-ATPase (3). These studies conclude that lipid next to the protein was ordered and immobilized relative to bulk lipid. However, recent ^{2}D-nuclear magnetic resonance (NMR) studies did not detect an analogous phospholipid environment on the NMR time scale (4, 5). The study described here explores the lipid-protein interface with ^{31}P-NMR to obtain a new, independent view of the interactions of lipids and proteins in membranes for one particular membrane protein.

Glycophorin, the integral membrane investigated here, is the major sialoglycoprotein of the human erythrocyte membrane and spans that membrane (5). The NH_2-terminal region of the glycoprotein contains all the carbohydrate and appears on the extracellular surface of the erythrocyte (6). The COOH-terminal region, enriched with charged residues, resides on the

cytoplasmic surface of the membrane. A 23-hydrophobic amino acid residue transmembrane portion connects these two regions and is contained within a tryptic peptide, *T*(is) (7).

The purpose of this study is to determine the nature of the interactions between glycophorin and the phospholipid component of the membrane. Preliminary efforts (8) demonstrated the usefulness of a new approach to such studies, using ^{31}P-NMR. Data from this approach in unsonicated systems, described in detail here, suggest that glycophorin immobilizes more than one layer of phospholipid around it in the bilayer and may disorganize the phospholipid outside of that immobilized layer. The results are compared with another recent study (9) of glycophorin in small, sonicated phosphatidic acid vesicles.

MATERIALS AND METHODS

TPCK trypsin and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp., Freehold, N.J. Freshly out-of-date human red cells were obtained from the local Red Cross Blood Bank. White or slightly pink erythrocyte ghosts were obtained as previously described.[10] Lipids were extracted from the ghosts by chloroform-methanol extraction and phospholipid content verified by thin layer chromatography on silica gel in chloroform/methanol/water (65:25:4). Egg phosphatidylcholine was obtained from Avanti Biochemicals (Birmingham, Ala.), and it exhibited a single spot on a thin layer plate in the above solvent system. Phospholipid concentrations were determined using the method of Bartlett (11) in triplicate on each sample. Protein concentrations were determined using the procedure of Lowry et al. (12) in 3% sodium dodecyl sulfate (SDS) in triplicate. An 18% correction was applied to this value (13) and a molecular weight for the protein part of glycophorin of 14,000 was used to calculate the mole ratio of phospholipid/protein.

Glycophorin was isolated from human red cell ghosts by the lithium diiodosalicylate (LIS) extraction procedure of Marchesi and Andrews (14).

Glycophorin was recombined with pure egg phosphatidylcholine and with the total lipid extract of the erythrocyte membrane. Anhydrous protein and phospholipid were solubilized in 2-chloroethanol and dried under vacuum. The mixture was hydrated in 1 mM histidine, 100 mM NaCl, pH 8, overnight. This material was applied to a 0–40% continuous sucrose gradient and centrifuged at 130,000 *g* overnight in an SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 5°C. Three bands were usually observed: one of unassociated lipid at the top, one of the lipid-protein recombinant at an intermediate position, and one at the bottom of unassociated protein. The lipid-protein complex was harvested, washed three times for NMR measurements by centrifugation for 30 min at 190,000 *g* in the above buffer, and analyzed for lipid and protein as described above. These recombinants were then used for NMR measurements and for trypsin treatment. Salt concentrations are constant throughout these measurements, at 100 mM NaCl.

^{31}P-NMR Method

^{31}P-NMR spectra were obtained at 81 MHz on a Bruker WP 200 (Bruker Instruments, Inc., Billerca, Mass.) at 30°C in 10 mm flat-bottom tubes. Intensities were obtained from spectra measured with gated broadband decoupling 1 W to remove the ^{31}P[^{1}H]nuclear Overhauser effect (15) (40% in unsonicated egg phosphatidylcholine [16]), and to reduce dipolar contributions to the linewidth, so that the effective chemical shift anisotropy is evident. 90°-pulses separated by >5 times T_1 were used to ensure full relaxation. Data were acquired with sweep widths of 10 and 50 KHz with no differences in the results. In the latter case a delay of 5 μs separated the end of the pulse and the acquisition of data. The position and volume of the sample in the coil were kept constant to ensure accurate relative intensities. The same sample tube was used for all intensity measurements on a given day. The standards used were unsonicated dispersions in 100 mM NaCl, 10 mM Tris, pH 7.6, of egg phosphatidylcholine, which has a similar linewidth to the recombinant samples. This precaution is important because a broad resonance is instrumentally attenuated relative to a narrower resonance.

By using a standard with an effective linewidth nearly the same as the sample, the instrumental

attenuation in both resonances is equal and the intensities of the resonance can be accurately compared. Furthermore, all sample spectra were phased with the same parameters as the standard; parameters that were determined on the day of a particular set of experiments. These procedures are essential to obtain accurate intensity measurements of ^{31}P-NMR resonances. 2 K data points were obtained in the time domain. Linebroadening of 200 Hz was used for enhancement of the resonance observed. Intensities of the spectra were evaluated by cutting and weighing photocopies of the spectra. Repeats on spectra produced intensity values within 5% of each other. Multiple standard determinations have demonstrated that the resonance intensity observed is proportional to the amount of phospholipid present.

This approach to lipid-protein interactions detects immobilized phospholipid because it produces a resonance sufficiently broad not to contribute to the resonance intensity observed. An analogy for this result can be found in the temperature-dependence of the ^{31}P resonance of dipalmitoylphosphatidylcholine. As the temperature is lowered below the phase transition temperature, the strength of the dipolar interactions dramatically increases. The weak decoupling power of a high resolution instrument such as the one used here is ineffective at removing the dipolar broadening when the phospholipid is in the gel state. Thus the signal broadens, eventually beyond detection with a normal high resolution instrument. As will be seen in the Results, immobilization by the protein causes a similar effect. Therefore, the phospholipid which is detected is that which has a linewidth similar to the standard without protein, or the nonimmobilized portion of the phospholipid of the membrane.

RESULTS

The glycophorin obtained was analyzed on SDS polyacrylamide gel electrophoresis on 12% gels. A representative gel appears in Fig. 1. The predominant component is glycophorin. The

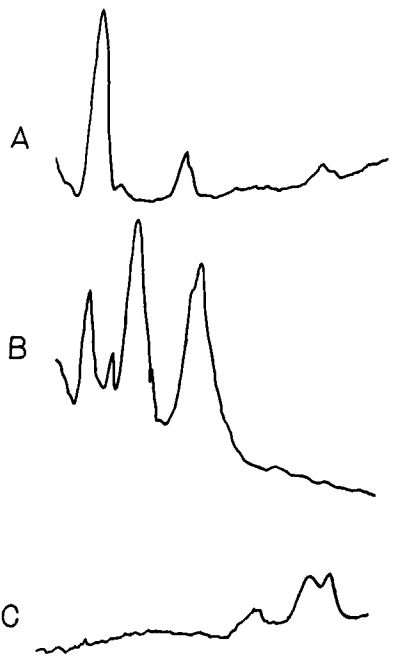

FIGURE 1 SDS polyacrylamide gel electrophoresis on 12% gels of glycophorin, stained by the PAS procedure for glycoproteins. (*A*) glycophorin, as used in these experiments; (*B*) recombinants, as described in the text, treated with trypsin for 2 h at pH 7.5, and spun at 37,000 rpm in a 40 rotor for 45 min to isolate the membranes before application of pellet to gel; (*C*) Coomassie blue stained gels of glycophorin added to preformed membranes and trypsin-treated for 30 min as described in the text.

gel presented is stained by the periodic acid Schiff stain (PAS) procedure for glycoproteins but the same gel pattern obtains with Coomassie blue stain. An identical gel pattern results from glycophorin recombined with phospholipids as described in Materials and Methods.

To assess the structure of the recombinant, the membranes were subjected to trypsin treatment. If the glycophorin is associated with the phospholipids in a transmembrane manner, part of the protein should be protected and remain with the membrane after trypsin treatment. If the protein is bound only to the surface of the membrane, it should be digested into several segments, as described elsewhere (17) and only a small, hydrophobic peptide, not containing any carbohydrate, would remain associated with the membrane.

Recombinants of glycophorin and egg phosphatidylcholine, prepared as described in Materials and Methods, were subjected to trypsin treatment in 10 mM Tris at pH 7.5, 100 mM NaCl for 2 h at 24°C. Trypsin was used in a 1:10 mol ratio with the glycophorin. The membranes were centrifuged and an aliquot of the pellet applied to the 12% polyacrylamide gel. The result is shown in Fig. 1 B. Most of the original glycophorin has been attacked by trypsin and two new glycopeptides appear. Since this comes from the pellet, these glycopeptides are still associated with the membrane. Gels of the sample before centrifugation to obtain the membranes exhibit a considerably more complex banding with PAS stain, indicating water-soluble glycopeptides are released from the membrane by trypsin.

A time course of trypsin hydrolysis was obtained on the same type of recombinant. At various time intervals, aliquots were removed and trypsin hydrolysis stopped with trypsin inhibitor. SDS-polyacrylamide gel electrophoresis of the various aliquots revealed the extent of proteolysis at that time. The results showed the same cleavage pattern at 15, 60, 120, and 300 min.

As a control, glycophorin was mixed with preformed egg phosphatidylcholine membranes and subjected to trypsin hydrolysis as described above. At zero time, the protein did pellet with the membranes but at 30 min the gel shown in Fig. 1 C was obtained. Coomassie blue staining showed two bands, the lower of which did not stain with PAS. The upper band which did stain with PAS decreased in intensity from 30 to 60 min. Thus the major peptide remaining with the membrane after trypsin treatment is small and contains no carbohydrates.

Since a substantial portion of the protein remaining associated with the membrane is protected from trypsin by the recombination procedure, glycophorin must be incorporated in the membranes of these recombinants and not just bound to the surface. With the present data, it is not possible to determine to what extent the incorporation of glycophorin in the phospholipid bilayer is asymmetric. However, since glycophorin is incorporated in the membrane, these recombinants allow a study of the glycophorin-lipid interface, since the lipid to protein mole ratio can be varied.

A further piece of evidence that the protein is incorporated into the membrane is the presence of intramembraneous particles in freeze-fracture electron micrographs of this recombinant (8). Those particles are randomly dispersed, with no indication of high aggregation. Since the predominant PAS band for glycophorin on the electrophoretic gels is a dimer, however, the protein probably does not exist as a monomer in the membrane.

The approach to be used to study the glycophorin-phospholipid interface employs ^{31}P-NMR. ^{31}P-NMR has proven to be a valuable probe of phospholipid headgroup conforma-

tion (for reviews see references 18 and 19) because it is a nonperturbing probe of the phosphate behavior in the phospholipid headgroup. In probing phospholipid-protein interactions, ^{31}P-NMR has proven useful in a study of human low density lipoprotein (20). Careful intensity measurements indicated two environments were possible for the phospholipids: one with high resolution ^{31}P resonances typical of a phospholipid vesicle, and one with resonances so broad that they did not contribute significantly to the resonance intensity observed. The broad resonance probably arises from a large increase in the proton dipolar contributions to the linewidth due to immobilization of a portion of the phospholipid by the B protein of the low density lipoprotein. Thus immobilzation of phospholipids by protein shows up in the ^{31}P resonance as "missing" intensity.

An analogous experimental approach has been applied here to the glycophorin recombinants. In this case the spectra are broad and anisotropic, showing spectral shapes characteristic of bilayer. Fig. 2 *A* shows the ^{31}P-NMR spectrum of unsonicated egg phosphatidylcholine. These multilamellar liposomes are large enough that they do not rotate rapidly on the NMR time scale, and thus can express the effective chemical shift anisotropy reflecting motion of the phospholipid in the bilayer.

Fig. 2 *B* shows the ^{31}P-NMR spectrum of a recombinant of glycophorin and egg phosphatidylcholine, prepared, isolated, and characterized as described in Materials and Methods. The spectrum is similar in shape to Fig. 2 *A*, and to ^{31}P-NMR spectra obtained from the erythrocyte membrane from which glycophorin is derived. It is also similar to spectra obtained from vesicular stomatitus viral membrane (21), chromaffin granule membranes (22), and *Acholeplasma laidlawii* membranes (23). These spectra are all characteristic of phospholipids in a bilayer configuration. A small (<5%), but variable amount of isotropic resonance is frequently seen, as in Fig. 2 *B*. However, some recombinants have shown no isotropic resonance with no significant difference in the number of phospholipids immobilized.

Intensities of the ^{31}P-NMR spectra of the glycophorin recombinants were obtained as described in Materials and Methods, and compared to standard samples of egg phosphatidylcholine. Fig. 3 shows that the intensities of the standards are directly proportional to the phospholipid content. Comparisons in intensity were made on the basis of phosphate analysis of the standard and the glycophorin recombinants. In each of the glycophorin samples, the

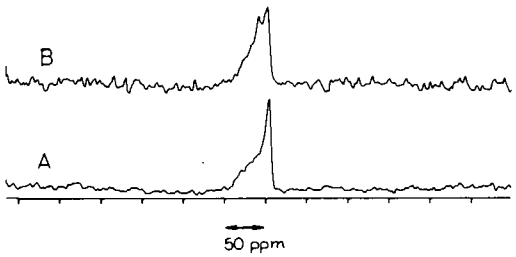

FIGURE 2 81-MHz, ^{31}P-NMR spectra of phospholipids and glycophorin/phospholipid recombinants, obtained at 30°C, with 2 K data points, 7 s repetition rate with 90° pulses, gated broadband proton decoupling (to eliminate the ^{31}P[^{1}H]nuclear Overhauser effect) in 10-mm tubes with 200-Hz linebroadening. 400 scans were obtained for each spectrum. (*A*) Unsonicated egg phosphatidylcholine, 100 mM NaCl; (*B*) glycophorin/egg phosphatidylcholine recombinant, 1 mM histidine, pH 7.2, 100 mM NaCl, phospholipid/protein mole ratio equals 150:1.

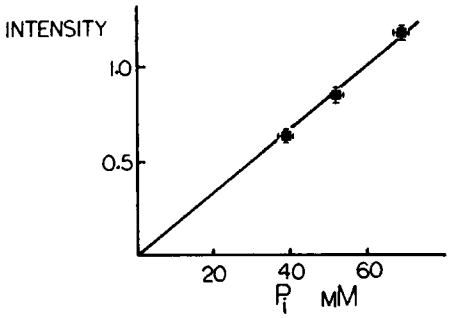

FIGURE 3

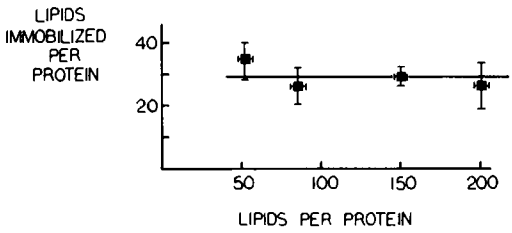

FIGURE 4

FIGURE 3 Plot of ^{31}P-NMR resonance intensity of egg phosphatidylcholine standards as a function of phospholipid concentration in the standards determined by phosphate analysis.

FIGURE 4 Plot of number of phospholipids immobilized per glycophorin as a function of phospholipid/glycophorin mole ratio in the recombinants. The horizontal line is drawn at the arithmetic mean of 29.

intensity measured was less than the intensity expected based on the phosphate content. The intensity missing was then expressed in terms of the number of phospholipids not contributing to the ^{31}P resonance, per mole of glycophorin in the recombinant membrane. The average for eight independent experiments is 29 ± 3, where the uncertainty presented is the standard deviation of the mean. This represents material from several different preparations of glycophorin, and from more than a dozen different blood donors. Because some LIS remains bound to glycophorin after purification, the effect of LIS on the ^{31}P-NMR resonance was measured. At a mole ratio of one LIS (incorporated during hydration in the pH 7.6 10 mM Tris, 100 mM NaCl buffer) per ten phosphatidylcholine, no effect on the resonance intensity was observed, although the resonance shape did change somewhat. Therefore binding of LIS to the recombinant membranes is not the source of the observations reported here.

In Fig. 4, the number of phospholipids immobilized per glycophorin is presented as a function of the phospholipid/glycophorin ratio of the recombined membrane. Over a wide range of ratios, the number of phospholipids immobilized per glycophorin is relatively invariant. Since the percent of the total lipids immobilized by the protein thus, of necessity, does vary with lipid/protein mole ratio, these data are not likely the result of a systematic artifact.

DISCUSSION

These data demonstrate that in the presence of glycophorin, phospholipids in the membrane reside in two different environments. One environment is similar to that of bulk lipids in a bilayer as measured by the ^{31}P-NMR spectral shape, though with some differences as described later. The other environment has a ^{31}P-NMR resonance so broad that it does not contribute to the resonance intensity measured. The number of phospholipids in this second environment is proportional to the glycophorin content of the membrane. Therefore, in this environment, the phospholipid headgroups must be immobilized relative to bulk lipid due to interactions with the protein which lead to strong resonance broadening. Since an average of

29 phospholipids are immobilized, it appears that the protein immobilizes at least one and maybe more than one layer of phospholipids around it.

The phospholipid not immediately adjacent to the protein in the glycophorin recombinants appears to be somewhat disordered relative to phospholipid in pure dispersions because the recombinant ^{31}P-NMR spectra appear narrower. A narrower spectrum implies a smaller order parameter (22) which corresponds to a greater degree of motional freedom for the headgroup. Another possible contribution to this change in spectral shape, however, is the size of the lipid-protein recombinants. If they are small enough to rotate at a rate near the time scale of this experiment (10^{-3} s), some narrowing of the spectra may result. Such a contribution cannot be ruled out at this time.

Previous work (8) has shown that ESR also detects the presence of an immobilized component of phospholipid in recombinants containing a portion of the glycophorin molecule, analogous to the results with cytochrome oxidase (1) and cytochrome b_5 (2), and in qualitative agreement with the ^{31}P-NMR data. In this case only the transmembrane segment of glycophorin (T[is]) is present and 4–5 mol of phospholipid were found immobilized per mole of protein over a wide range of lipid to protein ratios. This value however is less than that found with ^{31}P-NMR here using the whole glycoprotein, possibly indicating that the remainder of the glycoprotein is also involved in lipid-protein interactions. It is important to note that ^{31}P-NMR senses, most directly, the behavior of the phospholipid headgroups, while the ESR experiment reports predominantly the behavior of the hydrocarbon chains. It is not necessary to assume that the protein will affect the two regions of the phospholipid equally. Another factor which must be included is the possible aggregation of T(is). No bulky headgroup of the protein is present to prevent aggregation. Therefore much of the hydrophobic surface of the protein could be involved in protein-protein interactions and thus not available for protein-phospholipid interactions.

^{31}P-NMR results presented here agree qualitatively but disagree quantitatively with those presented earlier (8). In those experiments the same amount of lipid was recombined with protein as was used in the standard. This did not, however, take into account the 10–15 mol of phospholipid isolated with the protein during the LIS extraction procedure for the glycophorin (9). Addition of that number to the 5–7 phospholipids identified previously produces a number which approaches the value reported here. In the present study, total phosphate of recombinants isolated from a single band on a density gradient were obtained, obviating the previous difficulties.

Recently (9), a paper appeared using both calorimetry and ^{31}P-NMR to evaluate the interactions between the phospholipids and glycophorin. In a recombinant with dimyristoyl phosphatidylcholine, calorimetry detected an exclusion of 80–100 phospholipids/glycophorin from the phase transition. Qualitatively those data suggest an effect of the protein on the phospholipids similar to that observed here. The higher number probably arises from the different nature of the measurement, and the different phospholipids. Similar calorimetric data have been obtained using the recombination method described here for glycophorin and dimyristoyl phosphatidylcholine (A. Y. Romans, unpublished data observations).

^{31}P-NMR spectra of sonicated phosphatidic acid vesicles containing glycophorin were also analyzed and only nine phospholipids were found to be immobilized (9). Several factors may

lead to this discrepancy. Phosphatidic acid is quite different from the other phospholipids and may be interacting differently with the protein. The small radius of curvature of these vesicles could lead to packing problems and thus to less advantageous interactions with the protein. Though differences in the systems exist, qualitatively similar interpretations are suggested in the above work and in the present report.

It is of interest to know whether there is any specificity in the interactions between the phospholipids and glycophorin. Diphosphoinositide has been found bound to the protein when glycophorin is isolated by LIS extraction and not delipidated (24), so there appears to be a preferential interaction with some of the phospholipid binding sites on the protein. However, assuming a total of 29, more than half the binding sites are emptied when the glycoprotein is initially purified, perhaps by filling those sites with the detergent, LIS. These sites are able to bind egg phosphatidylcholine showing that the majority of the phospholipid binding sites do not exclusively bind negatively charged phospholipids.

While there is the possibility of exchange of phospholipids between binding sites on the protein and bulk phospholipids not interacting with the protein, such exchange appears to be slow ($<10^3\ s^{-1}$) for glycophorin. The immobilized lipid has a linewidth much broader than that observed for the nonimmobilized phospholipid. If fast exchange was taking place, some of the broad component would mix in with the observed component, and the resulting resonance would be broader than that observed for a bilayer not containing any glycoprotein. Since the observed recombinant resonance is actually narrower than that observed for a pure phospholipid bilayer, the phospholipids are probably in slow exchange.

Slow exchange between two lipid environments in the presence of glycophorin was seen previously with ^{13}C-labeled dipalmitoyl phosphatidylcholine bilayers (25). The ^{13}C label was in the *N*-methyl group. Near, but below, the phase transition temperature, two components are seen in the presence of the protein, indicating slow exchange of phospholipids between two environments. Direct comparison with the data presented here is difficult, however, because of the absence of a gel state in the recombinants described in this report, and because the data just referred to derives only from the observable phospholipid.

Another ^{13}C-NMR experiment with glycophorin has recently been reported (26) where the ^{13}C label was in position 14 of a linoleic acid on a phosphatidylcholine. Two components were observed in the ^{13}C-NMR spectrum of recombinants of glycophorin and the ^{13}C-labeled phospholipid. One component was severely broadened and the intensity of that broad component was proportional to the glycophorin content of the recombinant. The authors suggest that these data implicate about 30 phospholipids in a relatively immobilized environment provided by glycophorin, a suggestion which is in remarkable agreement with the data reported here. The effects on the hydrocarbon chains of the phospholipids by glycophorin appear from the ^{13}C-NMR data to be less strong than the effects on the phosphate region of the phospholipid as reflected by ^{31}P-NMR. This may reflect a differential interaction of glycophorin with various parts of the phospholipid molecule.

The membrane model which these data support is one of a phospholipid-protein complex coexisting with a lipid phase. Since the number of phospholipids immobilized per glycophorin does not vary with phospholipid/protein ratio, a constant boundary of phospholipid must surround the protein. These data are not readily explained by a model in which the immobilization of lipid arises from entrapment in regions of protein-protein contact occurring

through lateral diffusion, because in that model the number of lipids immobilized per protein would not be constant as a function of phospholipid/protein ratio. No attempt is made here to generalize this result to other membrane proteins, however, because different proteins may behave differently.

We thank Dr. Aldefer for the use of his Bruker 200, and N. Littell and J. Bensen for technical assistance.

This research was supported by National Institutes of Health grants RR05400-17 and HL 23853 to P. L. Yeagle.

Received for publication 13 March 1980 and in revised form 4 November 1980.

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