^{31}P NUCLEAR MAGNETIC RESONANCE STUDIES OF THE PHOSPHOLIPID-PROTEIN INTERFACE IN CELL MEMBRANES

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ABSTRACT Both native and recombined membrane systems from the human erythrocyte membrane and the rabbit sarcoplasmic reticulum have been studied with ^{31}P Nuclear Magnetic Resonance (NMR). We compare intensities of the anisotropic ^{31}P resonance exhibited by these membranes with the intensity expected from the known phospholipid content of the membraneous sample. In a recombinant with human erythrocyte glycophorin, a component of the phospholipid is "missing" from the ^{31}P NMR resonance, apparently due to a severe broadening of the resonance of that component. Approximately 29 phospholipid molecules were found immobilized per glycophorin molecule in the membrane, regardless of the phospholipid:protein ratio. Cholesterol may inhibit the immobilization of phospholipids by glycophorin. Recombinants with band three from the human erythrocyte membrane contain an immobilized phospholipid component, analogous to the results with glycophorin. ^{31}P NMR data from the native sarcoplasmic reticulum membrane also revealed an immobilized phospholipid component whose magnitude is independent of temperature between 30°C and 45°C. Extensive papain proteolysis of the membrane completely digests the $Ca^{++}Mg^{++}$ ATPase and removes the immobilization of phospholipids noted in the intact membrane. Limited tryspin cleavage, however, does not completely remove the immobilized component; salt reduces the immobilized component.

INTRODUCTION

The phospholipid-protein interface in cell membranes may provide an important element of membrane structure, modifying phospholipid and protein behavior. Because of its proximity to the relatively large membrane protein, interfacial phospholipid could experience dramatically different motional properties. Furthermore, specific phospholipid-protein interactions at the interface could create an inhomogeneous lateral distribution of phospholipids in the membrane. The interface may play a role in maintaining the asymmetry of the transbilayer distribution of phospholipid classes through specific phospholipid-protein interactions. The phospholipid composition of the interfacial region may modulate membrane-bound enzyme activity. The interface is also likely to be important in membrane biosynthesis and assembly. Electron spin resonance (ESR) of fatty acid and phospholipid spin labels provided early evidence for the presence of such a special environment in membranes containing cytochrome *c* oxidase (1, 2), cytochrome b_5 (3), $Ca^{++}Mg^{++}$ ATPase (4, 5), and rhodopsin (6).

^{31}P NMR is a valuable, nonperturbing probe of phospholipid-protein interactions. It reports primarily on phospholipid headgroup behavior because of the location of the phosphorus atom in the polar headgroup of the phospholipid (7, 8). It is nonperturbing because the NMR-sensitive nucleus of the phosphorus is 100% naturally abundant, so that no probes need be added nor chemical modifications made. ^{31}P NMR has proven sensitive to phospholipid-protein interactions in human low density lipoproteins (9), vesicular stomatitus virus (10), and recombinants of phospholipid and human erythrocyte glycophorin (11, 12, 13). In the first and last systems, the protein has been shown to immobilize a fraction of the phospholipid that exchanges slowly with the bulk, largely unperturbed, phospholipid. In the case of glycophorin, the presence of a special phospholipid environment induced by the protein has recently been further demonstrated by ^{13}C NMR (14), ESR spin label data (11), calorimetry (12), and Raman studies (15).

In the present study, ^{31}P NMR is applied to several membrane systems. The effect of glycophorin and band 3, the major integral membrane proteins of the human erythrocyte, on the phospholipids of their membranes are determined separately in recombined systems and compared to the intact erythrocyte membrane. Two phospholipid environments are found: one mobile, which resembles pure phospholipid dispersions, and one immobilized by the protein. Immobilization can apparently be reduced by incorporation of cholesterol in the membrane of the glycophorin recombinant. Sarcoplasmic reticulum is also shown to contain an immobilized component, removable by papain digestion but not by trypsin digestion. These

data support the concept of a special phospholipid environment at the phospholipid headgroup-protein interface. Effects on the nonimmobilized phospholipid component are also chronicled.

MATERIALS AND METHODS

Egg phosphatidylcholine and bovine brain phosphatidylserine were obtained from Avanti Biochemicals (Birmingham, AL) and showed a single spot on two-dimensional thin layer chromatography (1:$CHCl_3$-CH_3OH-NH_3 [65:25:5]; 2:$CHCl_3$-$[CH_3]_2$ CO-CH_3OH-CH_3 COOH-H_20 [6:8:2:2:1]). Triton X-100 was purchased from Calbiochem, San Diego, CA. Dodecyltrimethylammonium bromide (DTAB) and cytochrome *c* were obtained from Sigma Chemical Co., St. Louis, MO. Sodium dodecylsulfate (SDS) was obtained from Bio-Rad Laboratories, Richmond, CA. Con A-Sepharose 4B affinity media was purchased from Pharmacia, Inc., Piscataway, NJ.

Freshly out-of-date human red cells were obtained from the local Red Cross Blood Bank. White or slightly pink erythrocyte ghosts were prepared according to the procedure of Dodge et al. (16), and stored at 4°C under nitrogen for short periods. Oxygen levels in all buffers used for ghost preparations and band 3 preparations were lowered by extensive diffusing with N_2 before use. Spectrin-depleted ghosts were prepared by the procedure of Wolosin et al. (17), which results mainly in depletion of spectrin from the ghost.

Sonicated egg phosphatidylcholine vesicles were prepared by hydration of lyophilized phospholipid in 100 mM NaCl, 10 mM histidine, pH 7 buffer, 1 mM in EDTA. Sonication was performed in a Branson 350 Sonifier (Branson Sonic Power Co., Danbury, CT) with three 5-min irradiations in an ice bath under N_2. The vesicles were then centrifuged at 45,000 rpm in a Beckman 50 rotor (Beckman Instruments Inc., Fullerton CA) for 45 min at 5°C. Vesicles were harvested from the upper 85% of the clear supernatant.

Glycophorin was isolated from human erythrocyte ghosts by the LIS extraction procedure of Marchesi and Andrews (18). Glycophorin was recombined with phospholipids as described previously (13) and isolated as a single band from a sucrose gradient.

Purified band 3 was prepared from the erythrocyte ghosts by Con A affinity chromatography in 100 mM DTAB (19). Band 3 in DTAB was then recombined with egg phosphatidylcholine initially by cosolubilization in 100 mM DTAB. Extensive dialysis (three days, three changes of 10 mM histidine, pH 7, 10 mM NaCl, 100-fold vol excess) results in formation of recombinant membranes, as obtained previously (19). These recombinants were then incubated for one day at room temperature with sonicated egg phosphatidylcholine vesicles to further remove DTAB. After centrifugation at 45,000 rpm for 15 min to remove most of the vesicles, these recombinants were applied to a 0–40% sucrose gradient, centrifuged at 25,000 rpm overnight in a Beckman SW 27 rotor, and a single band at ~ 30% sucrose was noted and harvested. It was washed free of sucrose by centrifugation and analyzed for protein, phospholipid, and cholesterol content and on 6% SDS gels. Only a single major band is noted in the latter, in the position of band 3, when stained with Coomassie. Recombinants made by this technique have previously been shown to be active in anion transport (19).

Phospholipid concentrations were determined using the phosphate analysis of Bartlett (20). Protein concentrations were measured using the procedure of Lowry et al. (21). For glycophorin-containing solutions, the Lowry protein value was increased by an 18% correction (22). Cholesterol was determined using the cholesterol oxidase assay in Triton X-100 (23).

Sarcoplasmic reticulum was isolated from the white hind leg muscle of New Zealand white rabbits according to the procedure of Eletr and Inesi (24). ATPase activity was assayed in the presence and absence of calcium at 37°C (25). Some samples were further purified on a sucrose gradient as described (26). Adenosine triphosphate (ATP), phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase and NADH were obtained from Sigma.

Total lipid extracts were obtained from the erythrocyte ghosts and sarcoplasmic reticulum by $CHCl_3$-CH_3OH (2:1) extraction of the membranes. Trypsin, soybean trypsin inhibitor, and papain were obtained from Sigma.

^{31}P NMR spectra were obtained at 81 MHz on a Bruker WP 200 Fourier transform nuclear magnetic resonance spectrometer (Bruker Instruments, Inc., Billerica, MA), at 30°C in 10 mm flat-bottom tubes using quadrature detection. 50 kHz spectra were accumulated with a 5 μs delay, using 2048 points and with the resonance centered near the pulse. Intensities of the ^{31}P NMR phospholipid resonance were determined using a number of precautions. The spectra were obtained using 90% pulses (11 μs) separated by five times the phospholipid spin lattice time, T_1, to ensure full relaxation between pulses. The broad band ^1H decoupler (1 Watt) was gated to remove the ^{31}P [^1H] nuclear Overhauser effect (NOE) (27). A 40% enhancement of the signal intensity is observed from the NOE for phospholipids (28). The position of the sample in the receiver coil and the sample volumes were kept constant in all experiments. The same sample tube was used for all intensity measurements, both samples and standards, on a given day. To provide a standard curve for evaluating intensities, samples of pure unsonicated egg phosphatidylcholine or total lipid extracts from the human erythrocyte ghosts or sarcoplasmic reticulum at three different concentrations were measured each time experiments were performed. These standards have approximately the same linewidth and spectral shape as the membrane samples. This precaution is important because a broad resonance is instrumentally attenuated relative to a narrower resonance. Furthermore, a small amount of isotropic resonance sometimes appears in the membrane preparations which would overlap the resonance position of inorganic phosphate. Thus internal inorganic phosphate is not a satisfactory standard. Once the ^{31}P NMR spectra were obtained, intensities were evaluated by cutting and weighing photocopies of the spectra. Line broadening of 200 Hz was used for sensitivity enhancement, and 2K data points were collected. T_1 values were determined using the $180° - \tau - 90°$ pulse sequence.

RESULTS

Characterization of the Spectrin-depleted Ghosts

Normal human erythrocyte ghosts used in these experiments exhibit the profile seen in Fig. 1 on sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis stained with Coomassie blue. Material prepared according to the method described above for spectrin depletion was subjected to sucrose density gradient centrifugation at 100,000 × *g* overnight in a SW 27 rotor. On a 0–40% sucrose gradient only one band appeared, indicating the preparation was fairly homogeneous. Examination by sodium dodecylsulfate polyacrylamide gel electrophoresis on 6% gels shows bands corresponding to spectrin to be diminished; little else is affected, as seen in Fig. 1. When gels of the band 3 preparation, run simultaneously with the above gels, were stained by the PAS procedure for carbohydrates, two new bands appeared. These are the PAS bands associated with glycophorin from the human erythrocyte, indicating that glycophorin also is not removed.

Glycophorin Recombinant Preparation

Glycophorin was recombined with egg phosphatidylcholine and cholesterol, using 2-chloroethanol, as has been

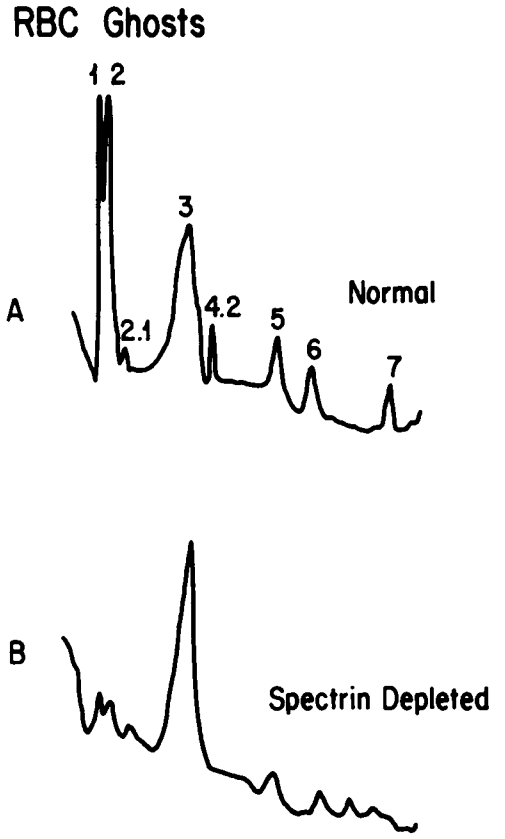

FIGURE 1 SDS polyacrylamide gel electrophoresis on 6% gels stained with Coomassie blue of: *A*, human erythrocyte ghosts; *B*, spectrin-depleted ghosts.

described previously (11, 13). The recombinants were isolated as a single diffuse band on a 0–40% sucrose density gradient and characterized with respect to phospholipid:protein ratio. The details of the measurements on these recombinants using ^{31}P NMR are given in reference 13.

Band 3 Recombinant

Band 3 was isolated and recombined with egg phosphatidylcholine as described above. When isolated from the sucrose gradient, a very thin band was observed near 30% sucrose, indicative of a homogeneous preparation. Cholesterol analysis demonstrated that < 1 cholesterol mol/protein mol remained in the recombinant. A single phospholipid:protein ratio was examined. When 6% SDS polyacrylamide gels of this recombinant were stained with the PAS procedure for carbohydrate, bands corresponding to glycophorin appeared. It was thus necessary to obtain a quantitative analysis for the glycophorin content by subjecting standard solutions of pure glycophorin of known concentration to SDS polyacrylamide gel electrophoresis on the same batch of 6% gels as the band 3 recombinants. For this experiment all gels were run at the same time and stained by the PAS procedure simultaneously. The gels were then scanned using a Gilford gel scanner (Gilford Instrument Laboratories, Inc., Oberlin, OH) and the intensity of the glycophorin bands determined by cutting and weighing photocopies of the scans. A plot of band intensity vs. glycophorin concentration produced a straight line. Using this plot as a standard curve, the glycophorin content of the band 3 recombinant could be determined. Greater than 95% (by weight) of the protein in these recombinants is found in band 3, i.e., the phospholipid-protein interactions in these recombinants involve primarily band 3 protein.

Sarcoplasmic Reticulum

A 10% SDS gel of the sarcoplasmic reticulum appears in Fig. 2. The major protein observed is the $Ca^{++}Mg^{++}$ ATPase. Activity measurements yielded activities on these samples of 1.5–2.5 μmol P/min/mg protein. Samples were kept refrigerated under N_2 until just before NMR

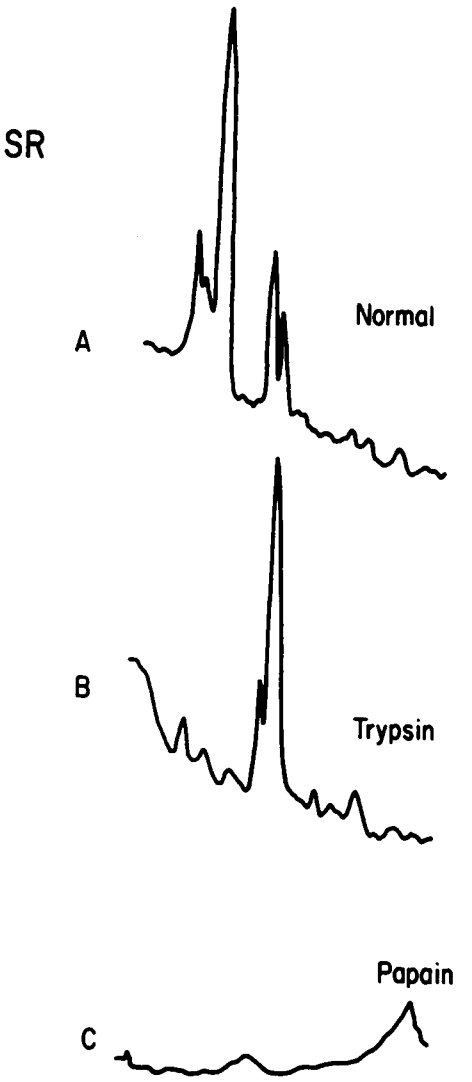

FIGURE 2 SDS polyacrylamide gel electrophoresis on 10% gels stained with Coomassie blue of: *A*, sarcoplasmic reticulum; *B*, trypsin-treated sarcoplasmic reticulum; *C*, papain-treated sarcoplasmic reticulum.

measurements to ensure that the data were obtained from enzymatically active membranes. Usually measurements were made within two or three days of preparation and retained most of their original activity.

Trypsin and papain were used to degrade the sarcoplasmic reticulum for further measurements. By procedures described by others (29), trypsin led to partial degradation, in agreement with previous studies (29, 30). A weight ratio (trypsin:membrane protein) of 1:400 was incubated 7 min at 23°C before the reaction was stopped with soybean trypsin inhibitor (2:1 weight ratio with trypsin). An SDS gel in Fig. 2 shows a large new band overlapping a band from calsequestrin near 55,000 mol wt which, according to previous studies, represents a breakdown product of the $Ca^{++}Mg^{++}$ ATPase. In agreement with these studies, no evidence is seen for proteolysis of calsequestrin or the high affinity binding protein.

Papain treatment for 1 h at a 1:20 weight ratio produces extensive degradation of the sarcoplasmic reticulum. As seen in Fig. 2, the $Ca^{++}Mg^{++}$ ATPase is completely degraded, with no large molecular weight fragments remaining. A more complete description of the papain degradation is given elsewhere.[1]

^{31}P NMR Measurements of Phospholipid Immobilization

The 81-MHz ^{31}P NMR spectra of human erythrocyte ghosts and sarcoplasmic reticulum appear in Fig. 3. These anisotropic resonances exhibit the residual ^{31}P chemical shift anisotropy characteristic of phospholipids in a bilayer (7). The spectrum represents a partially motionally averaged chemical shift tensor, averaged predominantly by the rapid axial rotation of the phospholipid about an axis perpendicular to the bilayer surface, with some wobble of that axis included (31).

Similar spectra have been obtained previously for erythrocyte ghosts (32), as well as for chromaffin granule membranes (32), *Acholeplasma laidlawii* membranes (33), vesicular stomatitis viral membranes (10), and rod outer segment disk membranes (34), which are consistent with the phospholipid bilayer thought to be a major component of these systems. Egg phosphatidylcholine dispersions show the same spectral shape (13). ^{31}P NMR spectra of proteolysed sarcoplasmic reticulum, spectrin-depleted human erythrocyte ghosts, and glycophorin, and band 3 recombinants with egg phosphatidylcholine are also similar (data not shown).

To be able to calibrate the intensity of the ^{31}P resonances, unsonicated egg phosphatidylcholine dispersions of known concentration were measured and the intensity of the ^{31}P NMR phospholipid resonances (obtained as described in Methods) were plotted as a function of the

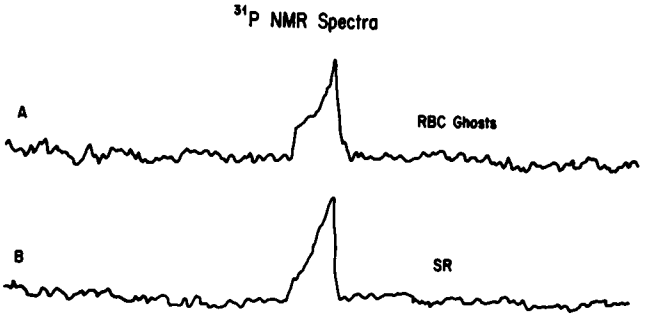

FIGURE 3 80 MHz ^{31}P NMR spectra at 30°C of: *A*, human erythrocyte ghosts; *B*, sarcoplasmic reticulum. Full spectral width displayed is 50 kHz.

phospholipid concentration determined from phosphate analysis. Dispersions of total lipid extracts from the sarcoplasmic reticulum and erythrocyte membrane were measured in the same manner. An example of such a plot appears in Fig. 4. The linear relationship is clear. The absence of any cholesterol effects on the intensities of the standards is also apparent since the sarcoplasmic reticulum extracts contain little cholesterol, while the erythrocyte membrane extracts have a high cholesterol content. Though not shown, pure egg phosphatidylcholine dispersions fall on the same line. Standard plots were obtained every time intensity measurements were performed on membrane samples.

The ^{31}P NMR resonance intensity measured for the membrane preparations could be compared to the intensity expected from the measured phospholipid content of the membrane sample. The expected intensity could then be determined, assuming all the phospholipid in the membrane contributed to the ^{31}P NMR resonances. For most of the unperturbed membrane systems studied here the observed ^{31}P NMR resonance intensity was less than

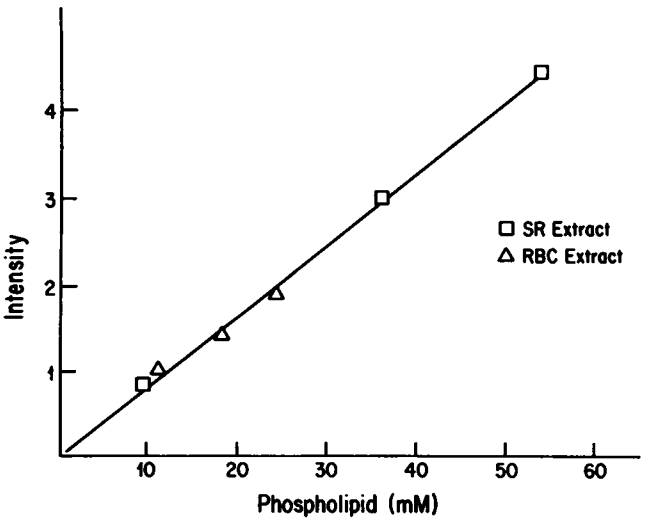

FIGURE 4 ^{31}P NMR resonance intensity as a function of phospholipid concentration in dispersion of total lipid extracts from the sarcoplasmic reticulum and human erythrocyte ghosts.

[1]Albert, A. D., M. Lund, and P. L. Yeagle. Evidence for the influence of the protein-phospholipid interface on sarcoplasmic reticulum Ca^{++} Mg^{++} ATPase activity. Manuscript submitted.

that expected. The "missing" intensity is postulated to be due to phospholipids that are immobilized by proteins in the membrane, thereby broadening the ^{31}P NMR resonance of those phospholipids such that they do not contribute significant resonance intensity in the region measured. In the absence of protein no such broadening of a component of the phospholipid is observed. This procedure is valid because the only major phosphate containing species in these preparations are phospholipids. Fig. 5 provides a hypothetical example of how the immobilized component fails to contribute to the observed intensity. Though visually deceiving, the immobilized component in Fig. 5 corresponds to 25% of the total intensity.

The results of these measurements on a number of different membrane systems are presented in Table I. Data from several independent preparations of these membrane systems are summarized as the mean and the standard deviation of the mean, and are listed as the percent of the total phospholipids in the membrane which appear to be immobilized. For glycophorin recombinants eight independent preparations were used. For glycophorin/cholesterol recombinants, three or four independent preparations were used for each cholesterol concentration. Two band 3 recombinants were studied with a 200:1 phospholipid:protein ratio. Material from 20 animals was used to obtain the sarcoplasmic reticulum data. For each entry in Table I for which ≥ 3 preparations were studied, a standard deviation of the mean is provided. Where only two preparations were measured, the range of values is provided. Each preparation is normally measured two to five times in the NMR and two determinations in triplicate for the phosphate.

In the case of the glycophorin data and the band 3 data, with a single type of protein in the membrane, the percent of phospholipids immobilized can be normalized by the protein content of the membrane. The number of phospholipids immobilized per protein can thus be calculated. The value shown in Table I is constant over a wide range of phospholipid:protein ratios (50:1–200:1) for the glycophorin recombinants (13), which makes it unlikely that the results from these experiments are due to an artifact. A mol wt of 93,000 was used for the calculations with band 3 (35, 36).

It is worth noting that perturbing the membrane can perturb the immobilized component, further reducing the chance that these observations are artifact. For example, in the case of papain-treated sarcoplasmic reticulum,

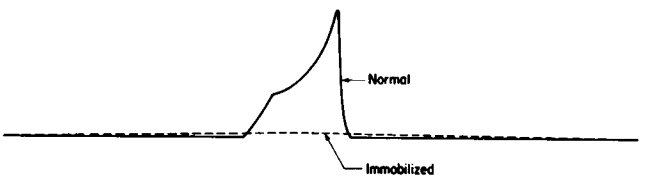

FIGURE 5 Diagram of hypothetical immobilized ^{31}P NMR spectrum underlying a normal ^{31}P NMR spectrum.

TABLE I
^{31}P NMR MEASUREMENTS OF PHOSPHOLIPID IMMOBILIZATION

Membrane	% Phospholipids Immobilized	Phospholipids Immobilized	No. Preparations
glycophorin/egg phosphatidylcholine recombinant (13)	variable	29 ± 3	8
glycophorin/egg phosphatidylcholine cholesterol recombinants*			
20% mole cholesterol	—	14 ± 5	3
40% mole cholesterol	—	8.6 ± 0.8	3
60% mole cholesterol	—	6.2 ± 0.7	4
Band 3/egg phosphatidylcholine recombinants	20%	40%	2
sarcoplasmic reticulum (no salt)[1]	32 ± 1%	—	5
sarcoplasmic reticulum (100 mM NaCl)[1]	10 ± 2%		6
papain-treated sarcoplasmic[1] reticulum	1 ± 3%	—	7
Trypsin-treated sarcoplasmic[1] reticulum	21 ± 3%	—	2
sarcoplasmic reticulum (100 mM KCl, 300 mM sucrose)	0 ± 2%	—	2

*Yeagle, P. L. Evidence for cholesterol-protein interactions involving a human erythrocyte membrane protein. Manuscript submitted.

digestion of the major membrane proteins removes the immobilization observed in the intact membrane which ties the immobilization to the protein component. Addition of cholesterol to the glycophorin recombinants also reduces the immobilized component.

An important additional effect is noted in the sarcoplasmic reticulum data. In the presence of 10 mM Tris, but no NaCl, a substantially larger immobilized component is seen than in the presence of an additional 100 mM NaCl. Such a result suggests that electrostatic forces are involved in the measured phospholipid immobilization. The possible involvement of a peripheral membrane protein in phospholipid immobilization was explored by additional experiments. Trypsin, which cleaves only the $Ca^{++}Mg^{++}$ ATPase protein under the conditions of these experiments, does not remove most of the phospholipid headgroup immobilization. Papain, which attacks all the proteins of the sarcoplasmic reticulum under the conditions of these experiments, does remove all of the immobilization.

Data for the intact human erythrocyte ghost membrane are not presented in Table I because of a variability in the results obtained so far that has not been observed in any other membrane system studied by this technique. In a minority of samples no immobilized component could be detected, in agreement with other published results (37). However, in a majority of the samples studied, less ^{31}P NMR intensity was observed than expected. The source for this variability is not known though it may be because each sample studied comes from a different individual.

Variable cholesterol contents may also play a role. Although variable results were observed for the human erythrocyte, highly reproducible results were obtained for all the other systems studied.

Motional Characteristics of Nonimmobilized Phospholipids

Two measurements of phospholipid headgroup motional behavior were used to study the behavior of the nonimmobilized or visible phospholipids in the membranes studied. First is T_1, the spin lattice relaxation time, which, most directly, is a measure of the rate of rotation of the phospholipid headgroup (27). Values for T_1 for several membrane preparations are presented in Table II. These values represent the average of three to five determinations on independent preparations in each system. Since the ^{31}P NMR resonances are anisotropic, one might expect the T_1 also to be anisotropic. However, careful examination of the spectra reveal no anisotropy in T_1 in any of the systems studied.

The residual chemical shift anisotropy is a measure of motional freedom or order of the phospholipid headgroup. Thus with this measurement plus the T_1 both motional rate and motional order can be examined. The chemical shift anisotropy was measured as described elsewhere (32) in random dispersions. The values for several systems are listed in Table III.

DISCUSSION

These data indicate that two distinctly different phospholipid environments exist in several different membrane systems. In one environment the phospholipids resemble sufficiently the properties of pure phospholipid dispersions that an anisotropic ^{31}P NMR resonance is observed which is similar in shape to pure phosphatidylcholine dispersions of the total lipid extract of the membrane under study. Possible perturbations of that environment will be discussed later. The other environment for phospholipids in the membranes produces a ^{31}P NMR resonance so broad that under the conditions of the experiments reported here these phospholipids do not contribute significantly to the observed resonance intensity. Since this phenomenon is reserved to membranes containing protein, the broadening has been assigned to lipid-protein interactions.

TABLE II
SPIN-LATTICE RELAXATION TIMES OF PHOSPHOLIPIDS IN MEMBRANES

Membranes	$T_1(s)$
Unsonicated egg phosphatidylcholine	1.4 ± 0.1
Human erythrocyte ghost	1.5 ± 0.2
Spectrin-depleted ghost	1.6 ± 0.2
Sarcoplasmic reticulum[1]	1.3 ± 0.2
Erythrocyte ghost extract	0.7 ± 0.1
Sarcoplasmic reticulum extract[1]	0.7 ± 0.1
Vesicular stomatitus virus (10)	0.14 ± 0.05
Vesicular stomatitus virus extract (10)	0.8 ± 0.1

TABLE III
EFFECTIVE ^{31}P CHEMICAL SHIFT ANISOTROPY IN MEMBRANES

Membranes	No. Measurements	CSA (*ppm*)
Unsonicated egg phosphatidylcholine	10	45 ± 1
Human erythrocyte ghost	7	46 ± 2
Spectrin-depleted erythrocyte ghost	3	37 ± 2
Cholesterol-depleted erythrocyte ghost	4	49 ± 3
Erythrocyte ghost extract	3	39 ± 5
Band 3 recombinant	3	43 ± 5
Glycophorin recombinant	7	39 ± 3
Glycophorin-cholesterol recombinant	4	38 ± 2
Sarcoplasmic reticulum	8	38 ± 5
Papain-treated sarcoplasmic reticulum	3	44 ± 3
Trypsin-treated sarcoplasmic reticulum	2	40 ± 2
Sarcoplasmic reticulum extract	3	41 ± 2
Vesicular stomatitus virus (10)	2	35 ± 5
Vesicular stomatitus virus extract (10)	2	33 ± 5

The resonance broadening by the protein may arise from at least two mechanisms. One is dipolar broadening caused by an increase in motional correlation time due to interactions between the phospholipid and the protein. Because the protein is much larger than the phospholipid, association with the protein could lead to adoption by the phospholipid of the motional characteristics of the larger protein. In the case of band 3, the rotational rate of the protein (38) is five or six orders of magnitude slower than the rotational rate of the nonimmobilized phospholipids (27, 39). Thus a powerful effect on ^1H-induced dipolar broadening could be expected. A second possible effect is an increase in the residual chemical shift anisotropy which would broaden the ^{31}P NMR resonance. The full ^{31}P chemical shift anisotropy for the phospholipids possible in an anhydrous powder is ~ 200 ppm (7). A residual chemical shift anisotropy of ~ 45 ppm is normally seen in egg phosphatidylcholine dispersions due to partial motional averaging of the chemical shift tensor by phospholipid motion. If less freedom of motion is available when the phospholipid is interacting with the protein, the chemical shift anisotropy expressed may increase, thereby increasing the linewidth of the resonance. Experimental evidence is not available at this time to distinguish between these possibilities; both may contribute.

Making effective use of the implications of this NMR data in understanding membrane structure and function requires data from independent probing techniques. In the case of glycophorin recombinants, quantitative agreement with the ^{31}P NMR results was obtained with ^{13}C-labeled phosphatidylcholine in which the ^{13}C label was located in the hydrocarbon chain (14). Thirty phospholipid mol/protein mol were found in an immobilized environment,

independent of lipid:protein ratio. Qualitative agreement with these NMR studies is offered by the results of ESR studies (11), differential scanning calorimetry studies (12), and Raman studies (15). Each of these studies also detects two different environments for phospholipids in glycophorin-phosphatidylcholine recombinants.

Sarcoplasmic reticulum and its Ca^{++} Mg^{++} ATPase also have been studied by several other techniques. ^{13}C NMR revealed that ~ 1/4 of the phospholipids of intact sarcoplasmic reticulum were immobilized as detected with *N*-methyl resonance from the phosphatidylcholine in the membrane, which agrees with the ^{31}P NMR data (40). Calorimetry data show that ~ 40 phospholipids are removed from the phase transition by the Ca^{++} Mg^{++} ATPase in recombinants (42). ESR spin labels likewise show two environments for phospholipids in the presence of the Ca^{++} Mg^{++} ATPase (43, 44).

This background permits better interpretation of Table I. By assigning the broadened component to phospholipids at the lipid-protein interface, questions can be asked concerning the effects and structure of that interface. A striking observation is the reduction in the population of the glycophorin-immobilized component due to glycophorin induced by cholesterol. One interpretation (though not the only one) is that cholesterol binds directly to the protein, thereby displacing phospholipid. This could prove to be an important element of plasma membrane structure. A similar proposal has been advanced for cholesterol-band 3 interactions from monolayer data (45). Thus, while both glycophorin and band 3, the two major integral membrane proteins of the human erythrocyte, are capable of immobilizing phospholipid, cholesterol may inhibit such immobilization in the native erythrocyte membrane. The variability in ^{31}P NMR results for the erythrocyte noted earlier may thus be due to differences in cholesterol content from one individual to another as well as to other factors. Further study is required on this subject.

The data from the sarcoplasmic reticulum suggest a somewhat different picture than those from the glycophorin and band 3 containing phosphatidylcholine recombinants. While it seems simplest to assign the observed immobilized component to the lipid-protein interface of the Ca^{++} Mg^{++} ATPase, the predominant integral membrane protein, this assignment is likely incorrect. Salt can readily disrupt the immobilization observed, suggesting the possibility of surface interactions with peripheral membrane proteins. Preferential digestion of the Ca^{++} Mg^{++} ATPase without concurrent degradation of the peripheral membrane proteins of the sarcoplasmic reticulum does not remove the phospholipid immobilization. These data implicate the peripheral membrane proteins in the immobilization more strongly than the major integral membrane protein, the Ca^{++} Mg^{++} ATPase. This conclusion is supported by recent ^{31}P NMR data on recombinants of purified Ca^{++} Mg^{++} ATPase with phospholipids in which no immobilization of phospholipids is detected (52). The difference between this interpretation of the ^{31}P NMR data and ESR and calorimetry data on this system mentioned above is not difficult to rationalize. ^{31}P NMR most directly measures the behavior of the phospholipid headgroup, and thus may be insensitive to the behavior of the hydrocarbon chains of the phospholipids. Therefore, the lipid-protein interactions involving the Ca^{++} Mg^{++} ATPase may be predominantly hydrophobic and may not involve the phospholipid headgroups. Quite a different situation obtains for glycophorin and band 3 described here, and for rhodopsin.[2]

It is now appropriate to turn to the behavior of the nonimmobilized phospholipid component in these membrane systems. ^{31}P NMR provides several measures of the behavior of the phospholipid headgroup. Data from two of those measures appear in Tables II and III. The ^{31}P T_1 can be interpreted in terms of the rate of rotation of the phospholipid headgroup (27). In most of the systems studied by this approach, no remarkable differences are observed in headgroup rotational rate between intact membranes (in which only the nonimmobilized component is measured) and the total lipid extracts of those membranes. Only in the viral system is there a strong effect which was attributed to the presence of a viral protein (G) in the encapsulating membrane (10). In the other membranes, the rate of motion of the nonimmobilized phospholipid headgroups seems little affected by the presence of the protein. The reproducible differences between the ^{31}P T_1 of the intact membranes and the total lipid extracts of the same membranes appear to be real. This observation would be consistent with a ~ two-fold increase in headgroup rotation rate in the membranes containing protein, according to a previously described model for phospholipid headgroup rotation (27). A concurrent disordering of the phospholipid headgroup, which would be detected in the chemical shift anisotropy, does not seem to occur. Why such a difference in rotational rate is observed must be the subject of further study. Since pure egg phosphatidylcholine exhibits a ^{31}P T_1 like the membranes, study should probably be focused on the reason for the shorter T_1 values in the total lipid extracts.

The ^{31}P chemical shift anisotropy provides a measure of motional order of the phospholipid headgroup (7, 32). Table III shows, in most cases, no striking effects of membrane proteins on the motional order of the nonimmobilized phospholipid component. One possible exception is the difference noted between the normal erythrocyte ghost and the spectrin-depleted erythrocyte ghost. Cholesterol depletion does not have the same effect as spectrin depletion. The apparent greater order in the spectrin-containing membrane may imply a spectrin-phospholipid interaction, which would be consistent with data from model systems

[2]Albert, A. D., B. J. Litman, and P. L. Yeagle. Rhodopsin-phospholipid interactions in the rod outer segment disk membrane. Manuscript submitted.

(46) in which spectrin is shown to bind to the surface of negatively charged phospholipid vesicles. The relevance of this observation to the intact human erythrocyte is enhanced by the known location of phosphatidylserine on the cytoplasmic side of that membrane, where spectrin is also located (47).

Recent data from rhodopsin of the rod outer segment disk membrane also show such an effect on the chemical shift anisotropy.[3] In recombinants of low lipid:protein ration, an increase in the effective ^{31}P chemical shift anisotropy with an increase in protein content is noted. Apparently rhodopsin can cause some ordering of the phospholipid headgroups of nonimmobilized phospholipids. However, the mechanism must be different for the integral membrane protein rhodopsin than for the peripheral membrane protein spectrin.

Glycophorin aparently has an opposite effect on the nonimmobilized phospholipids in the recombinant studied here. The observable phospholipid exhibits less effective chemical shift anisotropy in the recombinant than in the pure phospholipid. This may reflect some long-range disordering of the phospholipid headgroups by the protein, though the apparent differences noted are marginal and may not be reliable.

The lack of strong effects in most cases studied here on the properties of the nonimmobilized phospholipids indicates that the phospholipids are in "slow" ($\tau > 10^{-3}$ s) exchange with phospholipids of the lipid-protein interface. Only in the viral systems does "fast" exchange seem to be occurring when an averaged T_1 is observed that reflects immobilization. ^{13}C NMR data show the same slow exchange seen in the ^{31}P NMR data for glycophorin recombinants and sarcoplasmic reticulum (14, 40).

Recent ^{2}D NMR data from studies devoted to exploring the properties of the lipid-protein interface have been interpreted as reflecting a disordering of the lipid hydrocarbon chains at the interface and a rapid exchange ($\tau < 10^{-4}$ s) between the interface and bulk membrane lipid (48, 49). Another reasonable interpretation is that the protein increases the distribution of order parameters of the lipid, but does not change the average order parameter. These interpretations do not reflect the same picture of membrane structure presented above. The question then arises as to whether there is a single model which would be consistent with all the data.

If the interpretation that these ^{31}P NMR data reflect headgroup immobilization by the protein is correct, then part of the model should include a relatively long-lived complex between the phospholipid headgroup and the protein. Because ^{31}P NMR directly reflects the polar headgroup behavior, this interpretation seems reasonable. However, an immobilization of the headgroup does not require an immobilization of the hydrocarbon chains of the phospholipid on the same time scale. The observation of two components in the ESR spectra and a single component in the ^{2}D NMR data can be reconciled by the considerable difference in time scales of the two experiments; effects which appear to be averaged over all the lipid in the ^{2}D NMR experiment are "frozen" in the ESR experiment. The ESR data therefore detect an immobilized environment at the lipid-protein interface. The increase in distribution of order paramenters detected by ^{2}D NMR may reflect a rough protein surface upon which the lipids are immobilized on the ESR time scale, but not on the ^{2}D NMR time scale.

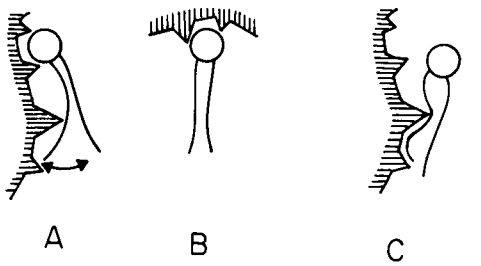

FIGURE 6 Model described in the text for the interaction between phospholipids and proteins in membranes. The circle represents the polar head and the lines represent the hydrocarbon tails of the phospholipid. *A*, long-lived headgroup interactions and short-lived tail interactions; *B*, headgroup interactions alone; *C*, tail interactions alone.

Therefore one model which would be consistent with these data is that the phosphate of the phospholipid may bind to the protein on a relatively long time scale ($\tau > 10^{-3}$ s), while the hydrocarbon chains of the same phospholipid are moving on and off the protein or from one orientation to another on the protein, on that same time scale. Such movement on a time scale between 10^{-5} s and 10^{-7} s would still lead to an immobilized spectrum detectable by ESR. This model is represented schematically in Fig. 6.

Finally, it should be noted that membrane proteins, as in the case of water-soluble proteins, can be expected to exhibit a wide variety of structures and behaviors. A single model of the structure of the lipid-protein interface is not applicable to all membrane proteins.

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DISCUSSION

Session Chairman: V. Adrian Parsegian *Scribe*: Nejat Düzgünes

FEIGENSON: Can you distinguish angular restriction from slower motion in the lipid component which you cannot see in the spectrum? The protein could be acting as a rigid body merely restricting lipid motion without attractive force for the lipid.

YEAGLE: No, we cannot make this distinction at this point. Experiments with glycophorin-phospholipid recombinants suggest that the motional rates slow down. Here, 28–30 phospholipids/glycophorin appear to be in a motionally-restricted environment. If you reduce the ratio of phospholipids:protein in the recombinant to this number, then all the phospholipids should be in the restricted environment. In such an experiment all the phospholipid is in an "unseen" state because it has a very long T_1. In our intensity measurements we select the phospholipids that behave like pure phospholipid dispersions, i.e., those with $T_1 \sim 1$ s, obliterating or saturating any signals with $T_1 > 1$ s.

FEIGENSON: In that case you don't have any component which has a short T_1. So, with very long pulse delays you should be able to see all the lipid around the glycophorin.

YEAGLE: That's correct. We are attempting to get those data.

FEIGENSON: The effect of cholesterol on the glycophorin-phospholipid interaction might be related to the effect of cholesterol in inhibiting immobilization of the gel phase, and it might inhibit the immobilized fraction, if there is one, around the glycophorin without competing with the phospholipid for the sites on the glycophorin.

YEAGLE: On the basis of NMR data alone it would be difficult to distinguish between the two possibilities. We have other spectroscopic evidence for cholesterol-protein interactions. We can also make pure cholesterol-glycophorin recombinants which band uniformly on a sucrose gradient.

FEIGENSON: I would like to suggest an interpretation for your observation that salt reduces the immobilized lipid component in sarcoplasmic reticulum. It might be related to the well-known effect of multivalent cations in creating an immobile component in the headgroup region; 100 mM salt would be able to compete with multivalent cations and reduce the immobilization.

YEAGLE: That is a viable interpretation; but there are alternative possibilities such as an ionic interaction between the phospholipids and proteins. The protein could be the intrinsic membrane protein, that is, Ca^{++}-Mg^{++}ATPase or possibly a peripheral membrane protein. Another possibility which we cannot rule out at the moment is protein aggregation that may lead to trapped lipid, and that is not observed in the presence of high salt.

BLOOM: Controversial conclusions drawn from "missing intensity" components should always be treated with skepticism, as a considerable effort is often required to eliminate all possible alternative reasons for the apparent absence of some fraction of the anticipated total spectral intensity. My main criticism of the paper is that there are many possible explanations for your results. What is the maximum possible width of the missing intensity components? This may be estimated from the rigid-lattice second moment of the ^{31}P NMR line which depends, in this case, only on the geometry of the polar head group. Can the width be >50 kHz? If not, the "immobilized component" should have been easily observed as a large, fast-decaying initial part of the free induction signals whose Fourier transforms generated the spectra of Fig. 3. Were these signals examined for such a contribution? In asking this question, I assume that the effective band-width of the detection system, including effects associated with the recovery of the amplifier following the rf pulse, is at least as large as 50 kHz.

YEAGLE: I would appreciate your help in calculating the line width of the induction decay. I agree with you that if we had a receiver with minimal dead-time and if we waited long enough between pulses, we would obtain the situation you are describing, in which case there should be no missing intensity. Since there appears to be a very long T_1 in this system, long periods of data acquisition would be required. In our high-resolution instrument the component with high T_1 is saturated relative to the short T_1. We not only have to obtain many acquisitions for adequate signal:noise ratio, but we also have to wait between acquisitions for the system to relax. This time could be in the range of seconds to minutes. Moreover, we have a finite dead-time in the receiver. Thus, the component with a short T_2 is instrumentally attenuated and may not appear in our spectra.

BLOOM: Would you say that the true band-width of the system you used was <50 kHz?

YEAGLE: The band-width of the nonimmobilized component is <50 kHz. At 80 MHz this component would be ~3.6 kHz. The chemical shift anisotropy, which is the maximum line width you can get in the absence of dipolar interactions and in the absence of any motion, is of the order of 200 ppm, which is within the 50 kHz range.

BLOOM: Could the "missing intensities" have been due to changes in

the electrical properties of the rf-tuned circuit caused by the dielectric properties of the samples?

YEAGLE: Variations of salt levels do not in themselves produce intensity changes. The simple addition of a noninteracting protein to a phospholipid dispersion also does not produce an effect within the uncertainty of our measurements.

BLOOM: One possible source of broad ^{31}P NMR lines in the presence of proteins, having nothing to do with immobilized head groups, involves increased homogeneous broadening, characterized by a decreased T_2, of the ^{1}H NMR spectrum by the proteins by analogy with previously-observed effects on ^{2}H NMR spectra (Kang et al. 1979. *Biochemistry.* 18:3257–3267; Paddy et al. 1981. *Biochemistry.* 20:3152–3162). A substantial decrease in the proton T_2 would make it more difficult to decouple the protons from the ^{31}P spins. I am not aware of any published theory for this effect but it is easy to see that the decoupling efficiency would be adversely affected when T_2 became so short that $\gamma_P H_{1P} T_2 < 1$, where γ_P is the proton gyromagnetic ratio and H_{1P} the amplitude of the proton decoupling field. No numbers on H_{1P} are presented in your paper, so I can't estimate the effectiveness of the proton decoupling. Another related effect associated with the decreased effectiveness of the proton decoupling for intermediate values of the correlation time associated with the ^{1}H-^{31}P dipolar interactions has been discussed recently by W. P. Rothwell and J. S. Waugh (1981. *J. Chem. Phys.* 74:2721–2732) and should also be taken into account.

YEAGLE: In experiments with phospholipid-glycophorin recombinants containing only the immobilized lipid, in which we should not expect to see a resonance intensity, we do not see it. Moreover, if the spectrum is obtained with 6–7 gauss of decoupling power we do not observe a significant resonance intensity under fast pulsing conditions. Thus, I think we are dealing with a T_1 effect.

M. BROWN: In a given spectrum what is the estimated error in the integrated intensity? How were the quoted errors derived?

YEAGLE: They were arrived at by standard-deviation analysis of multiple determination of multiple samples, i.e., individual samples measured several times and such measurements on independent samples.

M. BROWN: But in a given spectrum what would you estimate the error in the integrated intensity to be?

YEAGLE: We can produce spectra that are within 5% of each other.

M. BROWN: In the gel state of DPPC, which can be taken as representative of the type of "immobilization" that might be expected in a lipid bilayer, an increase in the ^{31}P chemical shielding anisotropy is observed from ~ -41 ppm in the liquid crystalline state to ~ -0 ppm, depending on the temperature (Michael Brown, unpublished results; M. F. Brown and J. Seelig. 1978. *Biochemistry.* 17:381). Some additional dipolar broadening is also observed *vis-à-vis* the liquid-crystalline state. Thus, under conditions of "immobilization" anticipated to occur in a lipid bilayer, the ^{31}P chemical-shielding anisotropy is experimentally observed to increase by about a factor of two relative to the liquid-crystalline state. As a consequence, $\leq$20% of gel-type lipid should be clearly detectable with the relatively large spectral windows and flat base lines reported in this study, particularly in those recombinants with low lipid:protein ratios. If such "immobilized" or gel-type lipid exists, why aren't the corresponding spectra components *directly* observed)

YEAGLE: With respect to the phospholipid headgroups, the gel-state lipid is not an adequate model of immobilization because the headgroups are not immobilized to a great extent in the gel state relative to the liquid-crystalline state. This is different from the behavior of the hydrocarbon chains. If a phospholipid headgroup is binding to a protein in a way similar to other phosphate compounds, then it will bind to a nonrotational site. When a small organic phosphate molecule binds to a water-soluble protein, it takes on the correlation time of the protein and does not rotate itself. It is immobilized, but moves only because the protein confers movement on it.

M. BROWN: Alan Deese, Edward Dratz, and I have recently published a ^{31}P NMR study of retinal-rod outer-segment membranes, which contain the visual pigment rhodopsin (1981. *FEBS (Fed. Eur. Biochem. Soc. Lett.* 124:93–99). In this paper we state that we do not observe any immobilized or gel-state lipid. Since then, Alan Deese has attempted to reproduce your results for rhodopsin-containing membranes, reported as a footnote in your paper. Based on the results of a single experiment he sees 100% of the ^{31}P NMR signal intensity. That is, for the case of ROS disk membranes we haven't been able to reproduce your results. Could you please comment on this?

YEAGLE: I think it is a bit unfair, with all due respect to Alan Deese, for you to take a single experiment and say that the multiple experiments that we have performed are incorrect. I talked to Alan Deese and he said he was going to do more experiments and get back to me on how his results come out. I would like to emphasize that our reported data are reproducible. There is only one variable system, human erythrocyte ghost, that has not produced reproducible data. All we report on that system is to mention its irreproducibility.

With respect to rhodopsin, we have a discussion poster (Albert et al., this volume) reporting temperature effects that differ between a simple papain-treated membrane and a normal membrane in intact disks. I must emphasize that we are working with intact disks and SR. This has disadvantages and advantages. There are very interesting, reproducible, and dramatic differences between those two preparations that cannot be ascribed to artifact because both experiments were done under identical conditions, over periods of months. If you take the SR membrane under low-salt conditions that produce an artificial environment and artificial effect in the membrane, and thoroughly papain-treat it and remove protein, the immobilization effects are gone. That is not an artifact. The salt effects that we see are reproducible and are dependent upon the salt concentration. They are also not an artifact. The results of the glycophorin-phospholipid-cholesterol system, in which the amount of immobilized lipid/normalized protein content is decreased as cholesterol is added, also cannot be attributed to a systematic artifact.

DEESE: By applying a 200-Hz line broadening, could you be losing some of your intensity at the edges of your spectra with the signal:noise level you show in the spectra?

YEAGLE: We use 200-Hz line broadening when we are at 80 MHz. At 40 MHz we only use 100 Hz. We have done NMR experiments with both 40 and 80 MHz, and we get the same result. We use only 100 Hz because we are concerned about the line broadening being too large a percentage of the chemical shift anisotropy.

McLAUGHLIN: In our recent work (A. C.McLaughlin, et al. 1981. *Biochem. Biophys. Acta.* 643:1–16) we see all of the intensity in the phosphorus signal in the SR. Those experiments were done with no proton decoupling. Also, we measured the T_1 and it relaxes with a single short relaxation time (~1 s). We have done these experiments in the native SR and reconstituted SR at lipid:protein ratios of 100:1, 60:1, and 42:1, and see exactly the same results in all four preparations.

Another comment: It has been shown before in the red cell ghost (P. R. Cullis, and C. Grathworhl. 1977. *Biochim. Biophys. Acta.* 471:213–226) (and I think those results were fairly reproducible) that you do see all of the phosphorus signal, i.e., $\geq$ 97%. I don't know what that means in terms of your results with reconstituted glycophorin. It may imply that glycophorin in the reconstituted system is not typical of most proteins in the red cell ghost membranes.

YEAGLE: With respect to the SR, when we do the experiments under your conditions we get the same answer. We get no immobilized phospholipids, no missing intensity, zero plus or minus a couple of percent. In that case the data were based on just two different preparations. Most of our data, however, were based on many different preparations. We have a paper on SR coming out shortly (1981, *Biophys. J.* 36:393–407) that involves material from 25 different animals. That number of experiments, I think, leads to something on the order of the certainty you have in your data. With the SR, again we get the same result you do if we do the experiment under the same conditions. If I may make one further point, we have one observation on recombination experiments and physical measurements that disturbs me. We took an intact SR preparation, subjected it to a deoxycholate-exchange type of reaction with excess (but not highly excess) phospholipids, and isolated what remained on the sucrose gradient, and it was active. When we measured the intensity, under conditions in which we had seen an immobilized component (NaCl, but no sucrose, no KCl), we do not see the same result we see in the absence of the detergent treatment. I am not certain at this point whether the residual detergent might be speeding up the exchange rate.

FLEISCHER: When well-defined functional normal or reconstituted sarcoplasmic reticulum is studied by ^{1}H, ^{2}H or ^{31}P NMR measurements, a homogeneous lipid environment is observed. Therefore, if more than one lipid environment exists, the exchange rate is rapid on the NMR time scale. (McLaughlin et al., this volume; Fleischer et al., this volume; Deese et al., this volume; Seelig et al. 1981. *Biochemistry.* 20:3922–3932; McLaughlin et al. 1981. *Biochim. Biophys. Acta.* 643:1–16).

In ^{31}P NMR studies of immobilized lipid you find 32% immobilized in no salt and 10% immobilized in 100 mM NaCl. When one studies SR in 0.3 M sucrose/100 mM salt, conditions we developed to preserve the function during prolonged storage of the sample event at room temperature, one also finds no immobilized phospholipid. Too often the function of a membrane preparation is not adequately considered in an otherwise elegant biophysical study. The point to be made is that the preparation is of utmost importance.

How does function, that is, Ca^{++}-ATPase and more importantly, Ca^{++}-pumping, correlate with the immobilized lipid? How do you relate the immobilization you find to function, that is, what is the significance of the immobilization?

YEAGLE: I hope that is where this conference will head. There may be no physiological significance of the immobilization. Under conditions where we get this larger amount of immobilized lipid in the sarcoplasmic reticulum we get high levels of aggregation as revealed by SDS gels. We do not lose any significant ATPase activity. In the presence of Na^{+} or K^{+} we can get a stimulation of ATPase activity. We have not yet measured Ca^{++}-pumping activity. We have attempted to use a more purified preparation of SR by the procedure described by Hidalgo (1980. *Biochim. Biophys. Acta.* 599:522) and have gotten considerable enrichment in specific Ca^{++}-ATPase activity. The results with this system were similar to the other preparations. With respect to the SR, the immobilized component in low salt in particular could very well be an artifact of protein aggregation, which we have been able to detect by looking at our systems more carefully.

FLEISCHER: In our experience the hypotonic condition under which you get 30% immobilization leads to loss of function in both ATPase and Ca^{++}-pumping activity.

GRIFFITH: I have a comment on making absolute measurements in biochemistry. Absolute intensity measurements are difficult with any spectroscopic technique. They are a problem in optical spectroscopy, they are harder in spin-labeling; they are even more difficult in some of the NMR experiments. Phil Yeagle's paper has attracted a lot of comment because he ascribes significance to a component he doesn't see. There are also a large number of publications that report a single environment without documenting just exactly how much lipid they could see. We have to analyze those papers carefully.

There is a system that could serve as a useful control. Leonard Banaszak and Joachim Seelig have recently studied an unusual phospholipid-containing system, lipovitellin-phosphitin, in which there are phosphates coupled to the protein that can be used as an internal standard. Pat Jost and Bruce Burrell have also finished a study with Len Banaszak and Joachim Seelig on this system. The spin-labeling data reveal two components, and ^{31}P NMR sees on average one component. The chemical analysis confirms that ^{31}P NMR is seeing essentially all of the phospholipids. So, this is one case that is well-documented.

This comment is neither a criticism nor support of your work, but if there is a general problem with different groups reporting different results on the same system, they might want to use lipovitellin:phosphitin as a control.

YEAGLE: With respect to the intensity measurements, we are not making absolute intensity measurements, but rather, intensity measurements relative to standards. We have arranged with Len Banaszak to do that experiment.

PADDY: I have made bacteriorhodopsin reconstitutions which contain deuterium-labeled lipids and which also contain two-dimensional crystals. The spectra show no highly-ordered component and, in fact, the orientational order of the system is less than for dimyristoyl phosphatidylcholine by itself. I observe what seem to be two components in the spectra. One interpretation of my result is that there is one component inside and the other outside the crystal, with the lipid inside the crystal being less ordered than the lipid outside.

ONG: Our data do not support the idea of immobilized lipid. One can interpret the ^{13}C NMR data of Stöffel in a different way: the morphological state difference will make the line broaden. James Prestegard and I have observed no significant line broadening or intensity loss in our reconstituted system, which is obtained by a different procedure (*Biochemistry* 20:4283). Our phosphorus NMR data suggest that there is no loss in intensity.

I would like to suggest an experiment. Could you add a paramagnetic ion to shorten the T_1 to facilitate the measurements if you have a problem with long T_1? Or, could you consider doing the temperature-dependence of the intensity in the case of a slow exchange in comparison to controls in which you have 100% intensity?

YEAGLE: You are dealing with a small vesicle system; we are dealing with a large vesicle system. I don't know if there is an inherent difference there. The data we obtain with the large vesicles are not quantitatively but qualitatively similar to the data of the Utrecht group, who used small vesicles (1978. *Biochim. Biophys. Acta.* 514:9). I suggest that you try to repeat their experiments and if we can identify some discrepancy there either in the preparation or the way the measurements were made we might understand better these sets of data. Temperature over the physiological range is not sufficient to speed up the exchange between the two environments that we observe in the case of glycophorin or the rod outer segment disk membranes. The presence of detergent might speed up the exchange, but temperature is not sufficient.

WATTS: We've been looking at headgroup interactions with proteins, using lecithin and phosphatidylglycerol deuterated at the headgroup and reconstituted with glycophorin. We have not made accurate intensity measurements because of all the problems previously alluded to by Hayes Griffith, but with the same instrumental conditions we can account for the vast majority of the deuterium intensity from the lipids. Both the T_1 and quadrupole splittings decrease in the presence of glycophorin compared with the pure lipid but we don't see any appreciable intensity in the baseline, which is completely flat throughout the 50 KHz band-width

of our machine. This is at lipid:protein ratios of 15:1 and 30:1, where you would expect at least to see a considerable decrease in the deuterium intensity if the headgroups were being "immobilized." So we believe we see all the intensity. From Phil Yeagle's contribution, it appears that lipid headgroup interaction with proteins is a differential effect, and when we talk about headgroups we have to be specific whether we are talking about phosphate atoms or the carbons or nitrogen atoms that may be comparable with the motional changes occurring at the acyl chain end of the molecule.

The phosphorus spectra we get are very noisy and we cannot say anything about the intensity in these samples because of instrumental problems. If you take PC and mix it with another lipid, for example PG in a 1:1 mixture, then you yet again see a decrease in the quadrupole splitting. In other words, another lipid can perturb the interaction of the lecithin headgroups, although they have very similar physical properties. It is therefore clear that one has to be rather specific about which part of the lipid molecule you are talking about, and headgroups are simply not specific enough.

I would like to follow this up with a comment. The order and rate of motion of lipids are two properties which are physically quite different, but they are often confused. Maybe we should be a little more specific about these terms to avoid confusion, in particular with respect to the thermal properties of membranes.

YEAGLE: One thing we haven't discussed here is the measurement of T_1, the spin-lattice relaxation rate of the phosphorus nucleus in various circumstances. I would like to bring to your attention that in one system we do see something that can be explained by a rapid exchange between an environment which has motional restriction and one that does not. That case is the VSV system (Table II) where the T_1 is markedly decreased in the intact virus. But if you remove the coat G protein from that virus, then the effects on the T_1 disappear. So this must be a protein-mediated effect, and because we see a single T_1, it must be an averaged effect. Now, in these other cases, for example in the ROS disk membrane, we see a single T_1, which is very much like pure phospholipid T_1. Tony Watts' point is well taken, that what we are most directly measuring is the phosphate; that with phosphorus you do not see rapid mixing of an immobilized component or of a perturbed component that might be next to a protein because the T_1 is not affected. If there were a rapid mixing, the T_1 should be like that in the case of the VSV, whereas in fact it is shortened and probably does reflect an averaging of at least two different environments.