which are on the exposed surface of the receptor protein. The structural difference between the agonists and the antagonists must therefore reside in the catechol moiety. The antagonists are characterized by replacement of the catechol with a strongly hydrophobic group. It is noteworthy that the catecholamines are inactivated physiologically by catechol *O*-methyltransferase which converts the catechol hydroxyl groups to methoxy groups. This, of course, changes the nature of the catechol from hydrophilic to hydrophobic. It would seem that the hydrophobicity–hydrophilicity of this terminus is crucial to the biological activity exhibited by the compound.

The results of the fluorescence studies reported here make it possible to understand the above relationships. It is apparent that the hydrophobic center of the antagonist enters a pocket in the receptor which cannot be occupied by the hydrophilic catechol. In some manner as yet undetermined, the physical occupancy of this hydrophobic pocket of the receptor prevents the activation normally brought about by the surface interactions of the catecholamine. The reason that such a hydrophobic site would exist on the β receptor is certainly not understood. Such a site might be simply a consequence of protein folding and be devoid of natural function. At present, no natural substrate for this hydrophobic "propranolol receptor" has been recognized. This of course does not rule out the possibility that a natural substrate might exist.

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Small Unilamellar Vesicles Containing Glycophorin A. Chemical Characterization and Proton Nuclear Magnetic Resonance Studies†

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ABSTRACT: Glycophorin A, a major glycoprotein of the red blood cell, is reconstituted in small lipid vesicles (250–300 Å in diameter) by using cholate detergent solubilization followed by rapid removal of cholate on a molecular sieve column. The extent of glycophorin incorporation is found to be critically dependent on the amount of cholate used, with higher amounts yielding vesicles with higher percentages of glycophorin. Vesicles with as much as 1 molecule of protein per 20 molecules of lipid can be prepared. Data on the vesicles obtained by using hydrolytic enzymes such as neuraminidase and trypsin, combined with amino acid analysis, suggest that glycophorin is incorporated in a transbilayer fashion with a high fraction of the molecules oriented with the carbohydrate-containing amino terminus to the vesicle exterior. Interaction of the protein with the hydrophobic portion of the bilayer is apparent in proton nuclear magnetic resonance spectra, and lipid line-width increases have been used to characterize the strength and stoichiometry of interaction. Glycophorin is found to affect directly as many as 40 lipid molecules per molecule of protein; however, the magnitude of the effects is not large.

The interaction of protein and lipid constituents of membranes is an important aspect of many membrane-centered phenomena. A variety of techniques have been employed, and a variety of lipid–protein systems studied, in an effort to elucidate the molecular details of those interactions [for a review, see Chapman et al. (1979)]. It is now apparent that a number of integral membrane proteins may have hydrophobic segments of appropriate length to span a lipid bilayer when in an α-helical configuration. A protein which is well characterized, is known to be transbilayer, and has a single such hydrophobic segment would seem an ideal choice for the study of the basic properties of postulated lipid–hydrophobic helix interactions.

Glycophorin A (GPA),[1] the major glycoprotein in human erythrocyte membranes, would seem an ideal protein. This

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[1] Abbreviations used: GPA, glycophorin A; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DSS, 5,5-dimethylsilapentane-2-sulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; R_1, spin–lattice relaxation rate (T_1^{-1}); R_2, spin–spin relaxation rate (T_2^{-1}).

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protein, with a molecular weight of 31 000, has been purified and its primary structure elucidated (Tomita et al., 1978). The series of some 23 nonpolar amino acid residues from 73 to 95 (Furthmayr et al., 1978) are believed to exist as a single transmembrane α helix in the red blood cell (Cotmore et al., 1977).

A number of authors have recognized the potential utility of GPA and have pursued studies in reconstituted GPA membranes. The reconstitution approaches vary considerably, ranging from simple addition of protein (Tosteson et al., 1973), to sonication (Van Zoelen et al., 1978a,b), to detergent solubilization followed by dialysis (Grant & McConnell, 1974), to ethanol injection (Redwood et al., 1975; Juliano & Stamp, 1976), to rehydration of lipid–protein films (MacDonald & MacDonald, 1975; MacDonald, 1980). Glycophorin does have some significant disadvantages with respect to its study in reconstituted systems. One is that, aside from carrying MN blood-group determinants and receptor sites for some lectins and viruses, it has no known function. Successful reconstitution is therefore difficult to assay. In addition, glycophorin is somewhat unique among integral membrane proteins in that it is highly water soluble due to the presence of 60% by weight carbohydrate. It readily forms 10–20 monomer aggregates in aqueous solution (Springer et al., 1966) and may therefore not reconstitute easily. Although efforts to assay reconstitution have been made in several studies, difficulties in characterization have certainly contributed to considerable variability in interpretation of lipid–protein interactions. For example, the stoichiometry of lipid–protein interactions deduced ranges from 4 to 135 boundary lipid molecules (Van Zoelen et al., 1978c; Romans et al., 1979; Utsumi et al., 1980; Taraschi & Mendelsohn, 1980). In view of reconstitution and assay difficulties, it is important that physical studies be accompanied by adequate characterization of the reconstituted system to be used.

^{13}C, ^{1}H, ^{19}F, and ^{31}P nuclear magnetic resonance (NMR) relaxation studies have all been employed in the investigation of physical and dynamic properties of phospholipid membranes. In principle, NMR relaxation studies allow determination of a very detailed quantitative picture of phospholipid and protein motions on time scales from 10^{-5} to 10^{-13} s. They can therefore contribute significantly to the characterization of lipid–protein interactions. Indeed, some NMR characterizations of glycophorin systems have been presented (Romans et al., 1979; Brulet & McConnell, 1976; Utsumi et al., 1980). In many cases, NMR relaxation parameters are influenced not only by internal motions and lipid–protein interactions but also by morphological or dynamic properties of the reconstituted particle. This is particularly the case with vesicles of less than 1000 Å in diameter (Sheetz & Chan, 1972). Thus, characterization of reconstituted morphology is also important for NMR interpretation.

We present here a successful reconstitution of glycophorin in phospholipid vesicles using a modification of the cholate solubilization procedure of Brunner et al. (1976, 1978). A similar technique has also recently been used to reconstitute the hydrophobic segment of glycophorin (Allen et al., 1980). With whole glycophorin, the preparation results in homogeneously sized glycophorin-containing vesicles of 250–300 Å in diameter. The similarity in size to that of non-protein-containing vesicles allows a series of NMR investigations with minimal sensitivity to the long-disputed size effect on NMR line-width measurements (Bloom et al., 1978; Petersen & Chan, 1977; Gent & Prestegard, 1977). The vesicles are characterized with respect to mole percent of vesicle-bound protein and asymmetry of protein incorporation using gel permeation chromatography and selective enzyme hydrolysis of exposed glycophorin residues.

Proton NMR results will be presented as an indication of lipid–protein interaction strength and stoichiometry. Proton NMR offers advantages over NMR studies using other nuclei. The sensitivity of proton NMR is high, and the more exposed position of protons on the surface of molecules allows greater response to intermolecular interactions. Our analysis here will be quite empirical. A more quantitative analysis of proton relaxation will be presented along with ^{13}C and ^{19}F NMR data in a later publication.

Materials and Methods

Materials. Egg yolk phosphatidylcholine (PC) was isolated and purified on a silicic acid column according to Singleton's procedure (Singleton et al., 1965). Phosphatidylethanolamine (PE) was obtained from *Escherichia coli* extracts. Phosphatidylserine (PS) from bovine brain extracts purchased from Sigma (St. Louis, MO) was further purified on a silicic acid column as the sodium salt (Marinetti et al., 1958). All the lipids employed were shown to be pure by thin-layer chromatography using silica gel G prepared by Analtech (Newark, DE).

Neuraminidase from *Clostridium perfringens* (type VI) and carboxypeptidase A-PMSF from bovine pancreas (type II) were purchased from Sigma. Bovine pancreas trypsin treated with TPCK was obtained from Worthington (Freehold, NJ). For column chromatography, Sepharose 2B and Sephadex G-100 were the products of Pharmacia (Uppsala, Sweden). Silicic acid and Bio-Gel A-1.5m were purchased from Bio-Rad (Richmond, CA). Sialic acid and cholic acid (sodium salt) were obtained from Calbiochem (San Diego, CA). Ammonyx-LO was purchased from Onyx (Jersey City, NJ). All the buffers used such as phosphate, Tris-HCl, and citrate were made from chemicals of reagent grade.

Glycophorin A was isolated from human erythrocytes following the general procedure of Tomita et al. (1978). Spun human blood cells were obtained from the blood bank at Yale–New Haven Hospital and used within a week. Following the procedure of Dodge et al. (1965), the red cells were subjected to hemolysis and washed at 4 °C. Glycoproteins were then isolated by lithium diiodosalicylate–phenol extraction (Marchesi & Andrews, 1971). Delipidation followed with chloroform–methanol, chloroform–methanol–HCl, and ethanol extractions. Remaining glycoproteins were further purified by using a Bio-Gel A-1.5m column equilibrated with 0.1% of the nonionic detergent, *N*,*N*-dimethyllaurylamine *N*-oxide (Ammonyx-LO) (Furthmayr et al., 1975). The elution profile as monitored at 230 nm shows a major peak ($\gtrsim$70%) arising from glycophorin A (GPA) but also a number of minor peaks. The major peak was collected and dialyzed to remove detergent. The yield for GPA was estimated at 20 mg/unit of blood. In a few experiments, product contaminated with the minor components was employed. No differences in reconstitution behavior or NMR results were noted.

Sample Preparations. In general, ^{1}H NMR sample buffer was made up of 100 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.02% w/v NaN_3 at pH 7.4. pH values in $^{2}H_2O$ (99.8%, Bio-Rad) buffer were read directly from a pH meter without corrections. Lipid vesicles were manufactured by two major procedures, nondetergent sonication and detergent solubilization. For sonicated samples, weighed lipids were first dissolved in a small amount of chloroform to ensure complete mixing. The organic solvent

was then removed under vacuum, and enough Tris buffer was added to disperse the lipid film as a 1–2 mg/mL dispersion. Sonication was carried out in a bath-type Branson sonifier operating at 40 kHz and thermostated at 25 ± 5 °C. Samples were repeatedly flushed with nitrogen and degassed. A period of about 2 h was usually sufficient to achieve clear solutions from ~5% w/v vortexed lipid dispersions. For the preparation of GPA vesicles, an extra hour of sonication was performed after the protein had been added to the preformed lipid vesicles.

The detergent solubilization method was a modification of Brunner's liposome preparation (Brunner et al., 1978). Normally, 1% w/v lipid dispersion (or sonicated vesicles) was solubilized with 2% w/v recrystallized cholate. In the case of GPA vesicles, slightly more cholate was usually needed. After overnight incubation at 4 °C, the resulting micellar solution with a volume of ~0.5 mL was loaded onto a Sephadex G-100 column (1.5 × 30 cm) at 4 °C preequilibrated with Tris buffer. As the cholate micelle and cholate solubilized materials were retarded by the column, only fractions at the void volume containing vesicles were collected. Less than 1% residual cholate of the total applied contaminated the lipid or lipid–protein fractions. Exhaustive dialysis against the same buffer, sometimes containing outdated lipid, facilitated the removal of residual cholate. The last dialysis was carried out in ^2H_2O buffer for NMR measurements. Unlike the sonicated samples which tended to aggregate, cholate–Sephadex-treated samples remained stable at 4 °C for weeks.

Sample Characterization. Vesicle size distributions and protein distributions were characterized by using gel permeation chromatography. A Sepharose 2B column (2.6 × 40 cm) was continuously monitored with a differential refractometer (Waters Associates). However, for the improvement of sensitivity and quantitation, total phosphorus in collected fractions was analyzed according to Bartlett's procedure (Bartlett, 1958), and the protein content was examined with the Lowry assay (Lowry et al., 1951). Coincidence of protein and lipid profiles was used to evaluate protein incorporation. For NMR samples prepared by cholate solubilization, the presence of any residual cholate after dialysis was checked by chemical analysis (Singer & Fitschen, 1961).

For determination of the possible preferential orientation of GPA in vesicles, accessibility of sialic acid, which resides exclusively on the amino terminus, was assayed with neuraminidase. Liberated sialic acid was measured following procedures described by Warren (1959). H_2SO_4 (0.1 N) hydrolyzed sample aliquots were used as controls in these measurements (Spiro, 1966). A sample of 0.02 unit of neuraminidase (1 unit/mL) was found sufficient to hydrolyze samples of 0.15 μmol/0.1 mL sialic acid in an hour at 25 °C. Incubations proceeded at least 2 times as long to allow adequate opportunity for hydrolysis. The enzyme activity was halted by acidification, and the bulk sample was analyzed for free sialic acid.

An attempt to assay the accessibility of the carboxy terminus was first made with carboxypeptidase A (Ambler, 1972). It was then pursued further with the well-established trypsin digestion pattern of glycophorin (Tomita & Marchesi, 1975). Aliquots of 20 μL of reconstituted samples containing ~1 mg/mL glycophorin were treated with 1 μg of trypsin (1 mg/mL) at 37 °C for 8 h. The samples were applied to a short (approximately 0.5 × 7 cm) Sephadex G-100 column and eluted with distilled water. Void volume samples containing vesicles with protected peptide fragments were collected and subjected to amino acid analysis. Analysis proceeded with hydrolysis in 6 N HCl at 110 °C for at least 24 h, followed by application to a Beckman 121M amino acid analyzer.

NMR Measurements. All the ^1H NMR studies were carried out on a Bruker HX-270 superconducting spectrometer operating at 270 MHz in the Fourier-transform mode and interfaced to a Nicolet BNC-12 computer. The probe temperature was controlled within ±1 °C. Typical spectra were recorded with quadrature detection and HO^2H gated presaturation, using the internal ^2H signal of ^2H_2O for the field lock. The free induction decay was time-averaged over about 100 scans and then Fourier transformed. Chemical shifts are reported in parts per million downfield relative to 5,5-dimethylsilapentane-2-sulfonic acid (DDS).

For spin–lattice relaxation rate (R_1) measurements, a $(180°–\tau–90°–AT–D5)_n$ pulse sequence, provided by the Nicolet T1 IR program, was used. HO^2H saturation power was turned on all the time except during the acquisition time (AT). The sum of AT and pulse delay, D5, was 3 s, which is at least 5 times the R_1^{-1} of interest. The observed peak heights were assumed to be representative of the resonance intensities and were later plotted against the delay time, τ, between 180° and 90° pulses, i.e., ln $(I_\infty - I_\tau)$ vs. τ, where τ ranged from 0.0 to 0.5 s and $I_{2.5}$ was used for I_∞. The resulting line was straight in most cases, and its slope gave $-R_1$. Exceptions occurred at high GPA content and high temperature where the difference in R_1 for overlapping GPA and lipid resonances became more pronounced, and thus biphasic log plots were apparent. Approximately ±10% experimental errors were estimated for R_1's by using signal to noise ratios in individual spectra to place error bars on points in the semilog plot. However, somewhat larger errors are expected for the biphasic R_1's.

Spin–spin relaxation rates (R_2's) were simply determined by measuring the line widths at the half-height ($\Delta\nu_{1/2}$) of the resonances in individual spectra and using $R_2 = \pi\Delta\nu_{1/2}$. NMR lines in pure lipid vesicle spectra are sufficiently well resolved to make this method adequate in most cases, and field-dependence studies of line widths suggest heterogeneity contributions to width to be small. Lines are also sufficiently narrow that the receiver dead time of 100 μs will not reduce significantly the observed intensities of lipid resonances. For more severely overlapping resonances, the line widths were approximated by measuring twice the distance between the hypothetical center line and the edge toward the less perturbed direction. For protein-containing vesicles, some lipid and protein resonances overlapped in the region of methylene and methyl. Though protein resonances accounted for a very small portion of the overlapping resonances in most samples, substracting the contribution of GPA was necessary in the most concentrated sample in order to extract representative line widths of lipid resonances. As a reasonable approach, it was assumed that the methyl–methylene portion of the GPA spectrum in solution did not undergo significant changes upon incorporation into lipid vesicles, and a GPA solution spectrum was used in the subtraction. The R_2 data presented were estimated to be accurate to ±20%.

Results

Sample Characterization. Size distribution and protein content data for glycophorin-containing vesicles reconstituted with cholate concentrations ranging from 2 to 7% are presented in Figure 1. Sepharose 2B gel elution profiles have been analyzed for lipid by phosphate analysis and for protein by Lowry analysis. Over 90% of the total amounts of lipid and protein in the aliquot applied to the Sepharose column were accounted for in the eluent, and no siginificant variations with time were observed after sample preparation.

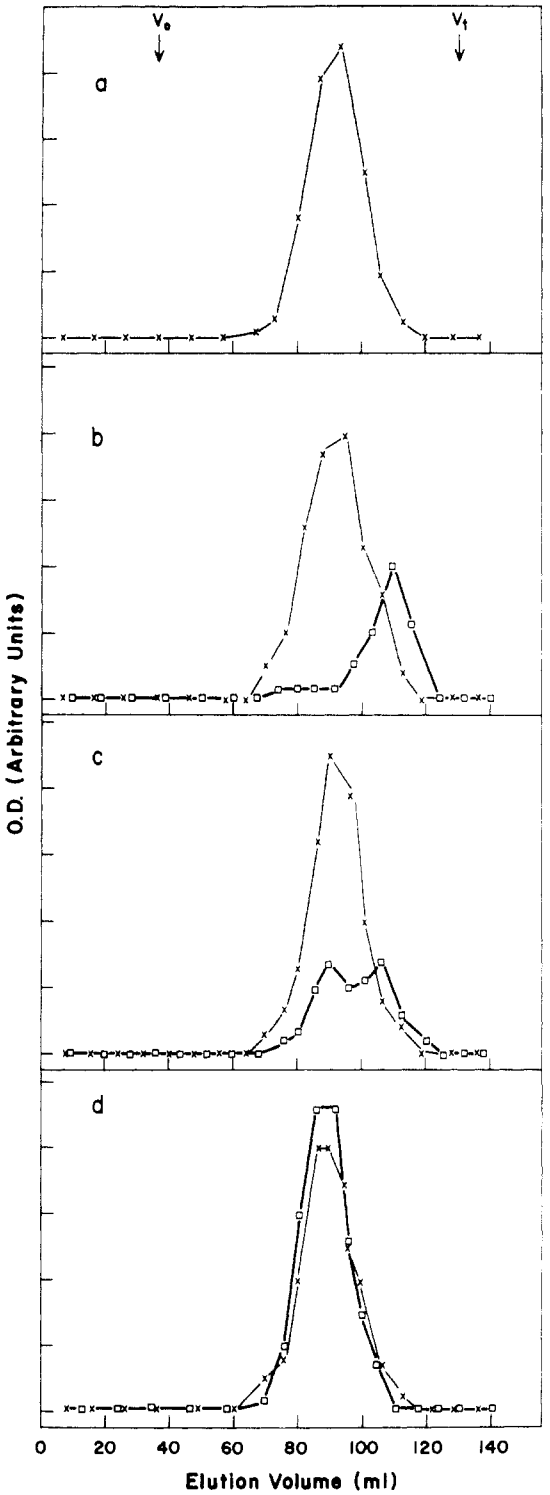

FIGURE 1: Elution profiles from the Sepharose 2B column analyzed for phospholipid (×) and protein (□). (a) Vesicles without GPA were used as the control for ^{1}H NMR measurements. Samples with an initial lipid/GPA molar ratio of 40:1 were treated with (b) 2%, (c) 5%, and (d) 7% cholate and collected after Sephadex G-100 column chromatography. All the profiles are scaled with respect to the total amount of lipid present in each elution. Arrows indicate the void volume and the total volume of the Sepharose 2B column.

Vesicle size distributions can be deduced from the position of phosphate maxima in the elution profiles. With Ackers' equation (Ackers, 1967) and sonicated egg yolk phosphatidylcholine vesicles as a standard, the vesicles containing protein were found to have size distributions centered around 250–300 Å in diameter. Vesicles prepared without glycophorin also fall in this range, making comparison of data on lipids with and without protein relatively independent of morphological differences. Sizes are similar to those of sonicated preparations, but distributions are somewhat narrower.

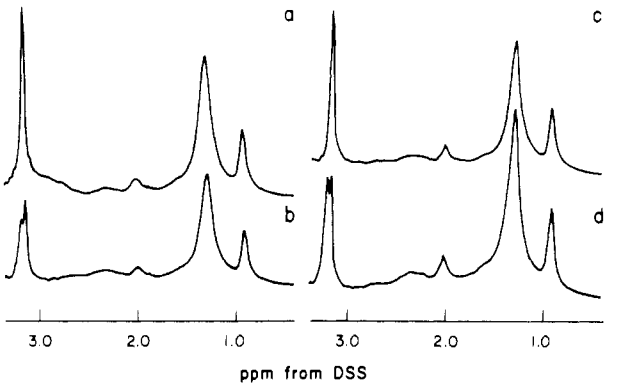

FIGURE 2: ^{1}H NMR spectra of cholate-prepared vesicles recorded at 24 °C in the presence of external Pr^{3+}. (a) PC vesicles without GPA in the absence of Pr^{3+}. (b) PC vesicles without GPA in the presence of Pr^{3+}. (c) PC vesicles with a lipid/GPA molar ratio of 240:1 in the absence of Pr^{3+}. (d) PC vesicles with a lipid/GPA molar ratio of 240:1 in the presence of Pr^{3+}.

Incorporation of protein can be assessed by comparison of protein and lipid elution profiles. Protein aggregates and lipid vesicles both elute at the void volume of the Sephadex preparative column, but on the Sepharose analytical column, protein aggregates elute at significantly larger volumes than do vesicles. Overlap of protein profiles with lipid vesicle profiles, therefore, gives a good indication of protein incorporation. For low glycophorin content samples with initial lipid to protein ratios higher than 80:1, the normal procedure using 2% cholate resulted in nearly perfect coincidence. For higher glycophorin content samples (40:1) prepared with 2% cholate, a major proportion of protein was not incorporated, and it eluted at a volume consistent with the expected size of a 10–20-molecule aggregate (Figure 1b). Raising cholate concentrations to 5% resulted in a reduction in the percentage of aqueous aggregates (Figure 1c). Using 7% cholate resulted in complete incorporation of glycophorin (Figure 1d). Analysis of lipid to protein ratios based on elution profiles suggests lipid to GPA molar ratios of 100:1, 60:1, and 20:1 for the vesicles. In the latter case, it is apparent that a significant amount of lipid was removed with cholate on the Sephadex preparative column. Samples in results which follow were prepared with cholate concentrations adequate to ensure complete incorporation, and reported protein to lipid ratios are based on analysis of peak fractions from a Sepharose column elution.

Analysis of sonicated preparations shows successful incorporation of glycophorin but a somewhat broader distribution of protein concentrations among vesicles. Similar results have been discussed in reports of Van Zoelen et al. (1978a,b) and De Kruijff et al. (1978).

Integrity of vesicles is an important consideration, especially when asymmetric incorporation of protein is to be studied. Also, the presence of residual cholate, although below the level of direct detection in the present samples (<10 μg/mL), has been suggested to contribute to vesicle instability. ^{1}H NMR in the presence of impermeable shift reagents can be used to assess vesicle integrity. Normally, the ^{1}H NMR spectra for PC-containing vesicles of small size show a sharp choline methyl peak at 3.25 ppm which is composed of inside and outside choline methyls in the bilayer structure. Pr^{3+} addition to the exterior of vesicles normally results in splitting of inside and outside choline methyl resonances (Huang et al., 1974). If the vesicles are not well-defined bilayers, or are unstable due to contamination with an ionophoric detergent, the choline peak will, instead of splitting, be shifted and broadened as a

single resonance. Figure 2 shows spectra of pure lipid and low GPA containing vesicles prepared by the cholate method and tested for ion impermeability by addition of Pr^{3+}. Splitting of the choline peak is seen in both cases. Results suggest that the bilayer structure stays intact and is impermeable to ions over the time course of NMR measurements. It is, however, noted that the retention time of vesicle ion impermeability decreases as the GPA content in the sample increases. In a sample prepared with a lipid to protein ratio of 40:1, substantial leakage of Pr^{3+} could be detected after 15 min. Thus, the GPA vesicles can be regarded as closed structures, but either GPA promotes ion transport or the stability of the bilayer must be questioned.

The effect of cholate was examined independently in a study in which up to 2 mol % cholate added to a sonicated egg yolk PC vesicle without GPA did not alter the choline peak splitting in the presence of external 1 mM Pr^{3+}. Since all the NMR samples prepared by cholate solubilization were shown to contain less than 0.1 mol % cholate relative to lipid by chemical analysis, the effect of residual cholate on bilayer structure is expected to be minimal.

The sharp peak at 2.05 ppm downfield from DSS seen in Figure 2c has been assigned to the *N*-acetyl methyl on the carbohydrate moiety of glycophorin. Since all the carbohydrate is on the N-terminal side of the hydrophobic segment and since sialic acid which bears many of the *N*-acetyl methyl groups is a potential cation binding site (Sillerud et al., 1978), it would seem probable that the degree of splitting of this peak on addition of Pr^{3+} could be used to assess asymmetry of glycophorin incorporation. The addition of large quantities (30:1 mole ratio) of Pr^{3+} to GPA in solution resulted in a few hertz downfield shift of the entire *N*-acetyl methyl peak in addition to line broadening of the resonance. Similar results observed in cholate-prepared samples with a low content of GPA suggest that all of the N-terminal ends are on the outside of the vesicle. The small spectral perturbations and slight permeability to ions, however, preclude any definitive statements for samples with a high content of GPA.

There are other means of assaying the exposure of sialic acid to the external vesicle surface. Van Zoelen et al. (1978c) have used neuraminidase to remove exposed sialic acid in vesicles reconstituted by the method of MacDonald & MacDonald (1975). Because of its larger size, an enzyme should be less prone to permeate the bilayer and complicate analysis of results. Neuraminidase at a level of 0.02 unit was added to a 5 mol % GPA vesicle sample of 0.1 mg of GPA/0.1 mL. Release of free sialic acid was completed after an hour incubation at 25 °C, as shown by the tendency to plateau in Figure 3. As a control, an aliquot of the initial sample was completely hydrolyzed in 0.1 N H_2SO_4. The result that the plateau is near the control value indicates that 100% sialic acid was hydrolyzed in the vesicle sample and would suggest that the N terminus containing the carbohydrate moiety is located exclusively outside the vesicle. This is consistent with the observation of Eylar et al. (1962) that nearly 60% of the total sialic acid in human red cells can be removed. Similar curves were obtained for GPA vesicles of lower protein content (2.5 mol %) and sonicated GPA vesicles.

In the proton NMR studies to follow, it is particularly important to establish that glycophorin is incorporated in a transbilayer fashion and not simply surface bound. The most direct evidence would come from protection of the carboxy terminus. The recent use of carboxypeptidase A to remove amino acids from and identify the location of the carboxy terminus of bacteriorhodopsin in a reconstituted system

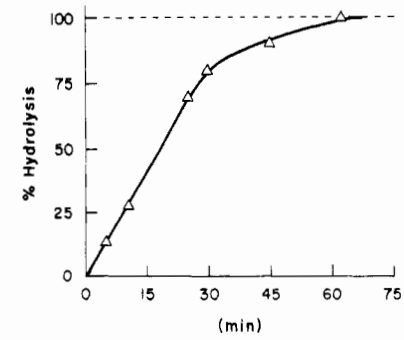

FIGURE 3: Extent of sialic acid hydrolysis (Δ) on 5.0 mol % GPA-containing vesicles prepared by cholate solubilization as a function of incubation time at 25 °C. A 0.02-unit sample of neuraminidase (1 unit/mL) was added to 150–200 μg/0.1 mL GPA-containing samples. Amounts of free sialic acid were measured by the thiobarbituric acid procedure of Warren (1959). Hydrolysis of 100% was determined from the aliquots treated with 0.1 N H_2SO_4.

(Gerber et al., 1977) suggests that the same approach might be used for glycophorin. No activity toward glycophorin-reconstituted vesicles was observed, suggesting protection of the carboxy terminus. However, glycophorin in solution (Tomita et al., 1978) and glycophorin in 0.2% sodium dodecyl sulfate ($NaDodSO_4$), even after heating at 100 °C, also failed to show significant activity of carboxypeptidase A, suggesting that factors in our samples other than transmembrane encapsulation may protect the carboxy terminus.

Trypsin is active toward glycophorin both in solution and in reconstituted vesicles (Tomita & Marchesi, 1975; MacDonald & MacDonald, 1975; Van Zoelen et al. 1978c). In solution, cleavage sites on the carboxy terminus beyond the hydrophobic segment do exist. Although these sites appear to be protected in resealed inside-out ghosts (Steck, 1972), the reason for protection is unclear, and hydrolysis at these sites may still provide an indication of an exposed carboxy terminus in vesicle preparations. On trypsin treatment, fragments released from glycophorin-containing vesicles are easily separated from vesicles by Sephadex chromatography, and portions remaining with the vesicle can be analyzed for amino acid composition as described under Materials and Methods. A control run with intact GPA and a small amount of PC, without trypsin treatment, shows little interference by PC and a good agreement in amino acid composition with literature values (Tomita et al., 1978). Given that the amino terminus is exposed, several hydrolysis patterns on trypsin treatment remain possibilities; among these are the following: (a) hydrolysis from the amino end as would occur in the native orientation (release of fragments T_1 and T_2),[2] labeled native; (b) hydrolysis from both ends as would occur with only the hydrophobic segment anchored and both ends exposed to the outer surface (release of fragments T_1, T_2, and T_4 and even T_3), labeled surface bound; (c) a more complete hydrolysis of surface-bound GPA, leaving only the hydrophobic fragment T_6; and (d) complete protection, leaving intact GPA. Table I gives vesicle-retained amino acid compositions expected for each of these cases, along with experimental observations for cholate-prepared samples. The experimental observations are scaled to produce agreement with Phe, which is conserved in each of the models. Similar results of amino acid analysis were obtained for sonicated samples. For comparative purposes,

[2] Since the peptide T_3, residues 40–61, has never been found in trypsin digestion of intact cells (Furthmayr, 1978), it is reasonable to assume that the solution tryptic cleavage site at residue 61 may be protected in GPA vesicles.

Table I: Amino Acid Analysis Results from Cholate Reconstitution and Comparison with Amino Acid Compositions for Expected Models after Trypsin Treatment

	model				
amino acid	intact GPA	native, residues 40–131	surface bound, residues 40–101	T_6, residues 62–96	exptl[b] results (mol/mol of GPA)
Asx[a]	8.0	5.0	0.0	0.0	4.7
Thr	15.0	6.0	4.0	2.0	8.7
Ser	18.0, 19.0	9.0	4.0	2.0	9.5
Glx[a]	14.0, 15.0	13.0	9.0	3.0	8.8
Pro	10.0	9.0	3.0	1.0	4.7
Gly	5.0, 6.0	5.0	5.0	4.0	4.6
Ala	6.0	3.0	3.0	2.0	4.4
Val	11.0	9.0	6.0	3.0	6.5
Met	2.0	1.0	1.0	1.0	1.0
Ile	11.0	10.0	9.0	7.0	7.9
Leu	7.0, 8.0	7.0	5.0	4.0	6.7
Tyr	4.0	2.0	2.0	1.0	2.3
Phe	2.0	2.0	2.0	2.0	2.0
His	5.0	3.0	3.0	2.0	3.2
Lys	5.0	3.0	2.0	0.0	3.0
Arg	6.0	5.0	4.0	1.0	3.7
total	131.0	92.0	62.0	35.0	81.7
σ	3.7	1.8	2.3	4.1	

[a] Asx includes Asp and Asn; Glx includes Glu and Gln. [b] Samples with a PC/GPA molar ratio of 50:1 were used in the reconstitution. Results of amino acid composition are obtained from several experiments and presented here without corrections for any losses during acid hydrolysis.

a standard deviation of experiment from each model was calculated. The best overall fit is that for "native" hydrolysis from only the amino terminus. The fit can be improved by allowing for partial hydrolysis of GPA.[3] Raising the unhydrolyzed GPA content as high as 50%, however, makes both native and "surface-bound" configurations fit equally well. It is, therefore, apparent that vesicles of well-defined size can be prepared with varying proportions of glycophorin using cholate solubilization. Although a definitive statement with respect to asymmetry cannot be made, enzyme hydrolysis data and NMR data strongly support the fact that glycophorin is incorporated in a transbilayer fashion with the carbohydrate-containing amino terminus on the vesicle exterior.

^{1}H NMR Results. Figure 4 shows typical ^{1}H NMR spectra of 1.7 mol % GPA vesicles prepared by cholate solubilization along with those of pure lipid vesicles recorded in the temperature range from 10 to 50 °C. Lipid methyl and methylene resonances dominate the spectrum at 0.85 and 1.25 ppm from DSS. Line broadening of these resonances in the presence of protein is apparent, particularly at low temperatures, indicating that protein does interact with, and perturb, lipid hydrocarbon chains. A quantitative measurement of line-width variations as a function of protein content and as a function of temperature can provide an indication of the strength and stoichiometry of interaction. However, such measurements are complicated in ^{1}H NMR spectra by the potential overlap of lipid and protein resonances.

Protein resonances in fact make a minor contribution to spectra such as those in Figure 4, but their contribution could be removed completely by using difference spectroscopy techniques and a spectrum of the lipid-solubilized GPA molecule. Lipid-solubilized spectra are not easily obtained;

[3] A total of 50–60% of sialic acid is retained after trypsin treatment and separation of oligopeptide fragments. Some retention is expected for cleavage after residue 39, but the rather high value suggests incomplete hydrolysis.

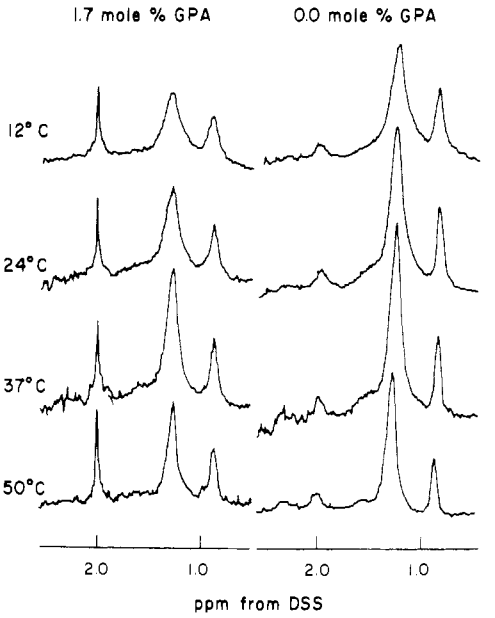

FIGURE 4: ^{1}H NMR spectra recorded at 12, 24, 37, and 50 °C of 1.7 mol % GPA vesicles (left) and pure lipid vesicles (right). Both samples were prepared by cholate solubilization.

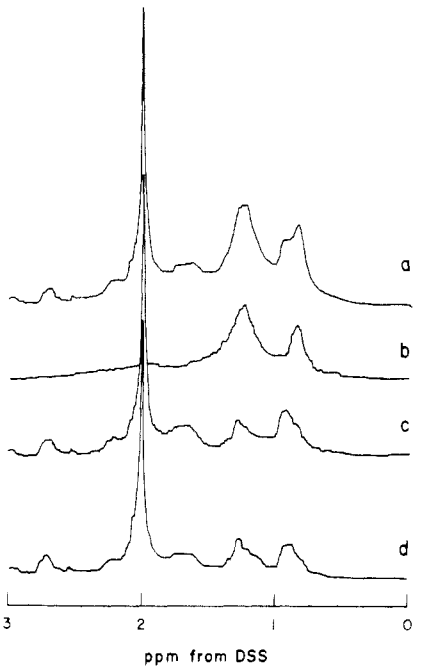

FIGURE 5: Illustrations of residual GPA ^{1}H NMR resonances in a 5.0 mol % GPA vesicle sample prepared by cholate solubilization. (a) ^{1}H NMR spectrum of 5.0 mol % GPA vesicle sample at 24 °C. (b) ^{1}H NMR spectrum recorded at 3 °C of lipid only vesicles. (c) Difference spectrum (a) – (b). (d) GPA solution spectrum recorded at 24 °C for comparison with (c).

^{1}H NMR spectra of GPA in aqueous solution are more readily obtained (Cramer et al., 1980; Egmond et al., 1979) as shown in Figure 5d. Such spectra, however, are marked by the absence of resonance intensity in positions expected for many of the residues in the hydrophobic region of GPA (Egmond et al., 1979). There is ample opportunity for additional resonances to appear or disappear on lipid solubilization. Enhanced resolution of hydrophobic residues has, in fact, been noted by other authors on solubilization of membrane proteins in perdeuterated $NaDodSO_4$ (Brown, 1979).

If we assume that broadening of lipid resonances occurs in a manner similar to that which occurs on lowering the temperature, an approximation of a lipid-solubilized spectrum can

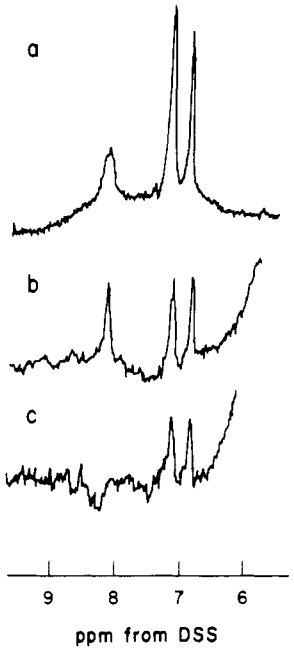

FIGURE 6: Aromatic proton region of GPA spectra. (a) ^1H NMR spectrum of GPA in aqueous solution at pH 7.4. Peak assignments from the left end are His C-2, His C-4 overlapping with Tyr C-2,6, and Tyr C-3,5. (b) ^1H NMR spectrum of 5.0 mol % GPA vesicles prepared by cholate solubilization. (c) ^1H NMR spectrum of 4.0 mol % GPA vesicles prepared by sonication. For spectra of (b) and (c), approximately 6000 scans were accumulated at 24 °C.

be obtained. Figure 5c shows a computer-processed difference spectrum resulting from subtracting a control spectrum from a GPA vesicle spectrum (Figure 5a). The lipid only control spectrum, Figure 5b, was recorded at 3 °C, at which point lipid methylenes show a line broadening equivalent to that observed for 5.0 mol % GPA vesicles at 24 °C. Comparison of the difference spectrum with the GPA solution spectrum in Figure 5d indicates an overall similarity. There are small differences. There is, for example, a slight intensity enhancement of the resonances around 0.9 ppm or diminishment of resonances around 1.3 ppm downfield from DSS. Many hydrophobic residues would contribute resonance intensity to these regions. Another difference is observed on comparison of the low-field aromatic region. Figure 6 shows a difference in line shape for the His C-2 protons of GPA, which are centered at 8.1 ppm downfield from DSS. In aqueous solution, the resonance is unusually broad (Figure 6a) and difficult to detect, while in lipid vesicles (Figure 6b), the His C-2 resonance is sharp and well resolved. The broadening in aqueous solution has been attributed to either a heterogeneity of His pK_a values or an intermediate exchange rate with solvent (Cramer et al., 1980). Solubilization in lipid might be expected to reduce heterogeneities found in aqueous aggregates of 10–20 monomers. It might equally as well change protonation–deprotonation rates. It is interesting that solubilization in Amonyx-LO, a nonionic detergent, produces a similar effect. Preparation of GPA vesicles by sonication resulted in spectra without an observable His C-2 resonance (Figure 6c). This could be the result of additional broadening, or sonication, which is carried out at somewhat elevated temperatures, could have promoted exchange of the C-2 proton for deuterium (Dwek, 1973).

Despite these differences, one is struck by the similarlity of aqueous and lipid-solubilized GPA spectra. Either might be used to remove protein contributions from vesicle spectra such as those in Figure 4. Because of the improved signal to noise ratio in Figure 5d and the approximations necessary to obtain Figure 5c, we have chosen to use the aqueous GPA spectrum for difference processing.

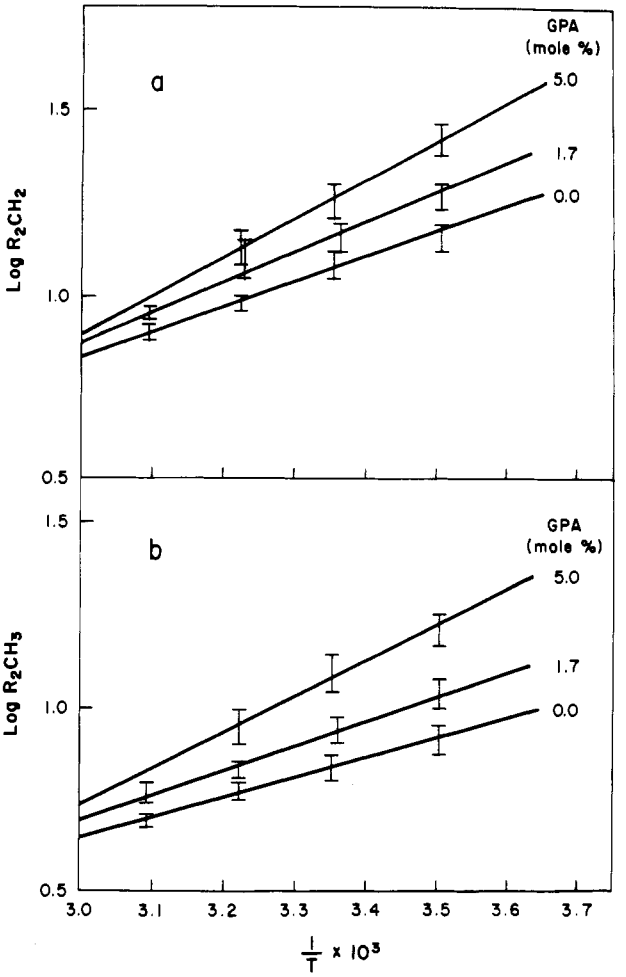

FIGURE 7: Semilogarithmic plot of spin–spin relaxation rate, R_2, as a function of reciprocal temperature, $1/T$, for cholate-prepared samples with different GPA contents. (a) Lipid methylene R_2; (b) lipid methyl R_2. In (a) and (b), top, 5.0 mol % GPA vesicles; middle, 1.7 mol % GPA vesicles; and bottom, pure lipid vesicles.

Line-width measurements of lipid methylene and methyl resonances in the presence and absence of GPA are plotted vs. temperature (log R_2 vs. $1/T$) in Figure 7. The line widths for resonances in the presence of GPA are obtained as described under Materials and Methods from spectra in which protein contributions have been subtracted by scaling the GPA spectrum in Figure 5d so that the well-resolved *N*-acetyl methyl resonance at 2.05 ppm in Figure 5a is completely eliminated.

As the temperature increases, the line widths of methylene and methyl resonances are seen to decrease in all cases. However, there is a small increase in slope for GPA vesicle samples. Extracting apparent activation energies (±0.5 kcal/mol) from the plots, one notes a change from 3.1 to 5.1 ppm for the methylene resonance, and a change from 2.3 to 4.0 ppm is noted for the methyl resonance on adding 5.0 mol % GPA to the lipid vesicle. These changes suggest GPA–lipid interactions are slightly stronger than the lipid–lipid interactions normally present.

Line-width broadening at 24 °C for lipid methyl and methylene resonances of phosphatidylcholine is plotted vs. GPA content in Figure 8. A linear increase of line width with protein content is noted below a protein to lipid mole percentage of 2.5 mol %, reaching a level of 60% broadening at 2.5 mol % GPA. Above that point, a plateau is reached, on which further addition of GPA results in little change in lipid

Table II: Spin-Lattice Relaxation Times of Lipid Methylene (CH_2) and Methyl (CH_3) Resonances Measured at Different Temperatures for Different GPA Contents in the Cholate Samples

	T_1 (s)									
	lipid vesicle		1.7 mol % GPA		2.5 mol % GPA		4.0 mol % GPA		5.0 mol % GPA	
temp (°C)	CH_2	CH_3	CH_2	CH_3	CH_2	CH_3	CH_2	CH_3	CH_2[a]	CH_3[a]
12	0.20	0.32	0.22	0.27			0.27	0.30	0.23	0.20
24	0.30	0.42	0.30	0.38	0.33	0.40	0.30	0.23	65% 0.26, 35% 0.05	70% 0.23, 30% 0.064
37	0.38	0.57	0.40	0.54	0.43	0.54	0.43	0.50	65% 0.25, 35% 0.10	70% 0.30, 30% 0.087

[a] Including superimposed resonances under lipid resonances.

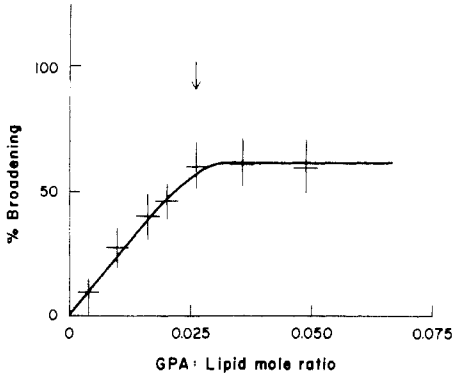

FIGURE 8: Extent of lipid methylene resonance broadening as a function of GPA content in cholate-prepared vesicle samples at 24 °C. The crossbars represent the experimental errors of both the sample composition and the line broadening in each measurement. The arrow indicates approximately the stoichiometry for the GPA–lipid interaction.

line width. The results suggest that, beyond 2.5 mol %, protein binds to vesicles in a manner that results in little further mobility restriction for the observable hydrocarbon chains. Vesicles with different lipid compositions, for example, 1:2 phosphatidylethanolamine/phosphatidylcholine or 1:4 phosphatidylserine/phosphatidylcholine, showed similar line-broadening effects.

With cholate-prepared vesicles, little broadening of the choline methyl resonance was observed. Insignificant changes in ^{31}P NMR line widths (in preparation) also support a minimal perturbation of the polar head-group region. Simple addition of GPA to a vesicle preparation produced no significant changes in line width except for the choline methyl. Sonication in the presence of GPA did produce some line broadening of methyl and methylene resonances but not to the same extent as the cholate-solubilized preparation. The choline methyl is also broadened significantly in sonicated samples. These results suggest that there may be more than one possible mode of GPA incorporation.

In addition to line-width measurements, spin–lattice relaxation time measurements (T_1 measurements) were made for cholate-prepared samples with varying levels of GPA. These results are presented in Table II. Because of the large number of spectra involved in a T_1 determination by the inversion recovery method, no attempt was made to subtract protein contributions from the spectra. In practice, no significant changes (±10%) in lipid methyl and methylene or choline methyl T_1's could be detected in cholate-prepared GPA vesicles with less than 2.5 mol % GPA, and a single exponential decay fit the inversion recovery data. For the 5.0 mol % GPA sample, the methyl and methylene resonances do begin to show a biexponential recovery. The most likely explanation for the short- and long-component recoveries is contributions from protein and lipid, respectively.

Choline methyls show minimal T_1 changes for cholate-prepared vesicles, but parallel with line-width data, the choline methyl for a sonicated preparation shows a significant decrease in T_1 for a GPA vesicle compared to a protein-free vesicle (0.22 s compared to 0.30 s).

T_1's for the *N*-acetyl methyl of incorporated GPA are very similar to those for GPA in solution for both types of preparation. This is consistent with the ESR observation of Lee & Grant (1979) which suggests that the N terminus of GPA is relatively free from lipid–protein interactions.

Discussion

Data presented above demonstrate that cholate solubilization followed by elution on a Sephadex G-100 column is a reasonable way to reconstitute glycophorin into lipid vesicles. Vesicles of constant size containing different amounts of GPA can be prepared. It is however, important to use sufficient cholate to ensure complete incorporation and to assay lipid to protein ratios after preparation. Enzymatic hydrolysis of the incorporated glycophorin is consistent with a transbilayer incorporation in which the carbohydrate-bearing amino terminus is on the vesicle exterior. The possible protection of certain tryptic cleavage sites via proximity to membrane surfaces remains a concern, but it is significant that the hydrolysis results are in many respects similar to observations on whole cells or ghosts in which the incorporation is known to be transbilayer with the amino terminus out. For example, tryptic cleavage after residue 39 rather than 39 and 61, as occurs in solution, produces a good fit to the data. Similar cleavage in natural membranes has been found in that only fragments T_1 and T_2 of glycophorin were released (Furthmayr, 1978). Also, inclusion of unhydrolyzed GPA in our data analysis is supported by the observations of Steck (1972) and Tillack et al. (1972), which suggests the partial retention of phytohemagglutinin receptor site on glycophorin. Several authors have also observed that only 50% of glycophorin-bound sialic acid can be released by trypsin in natural membranes [Jackson et al. (1973) and references cited therein; Winzler et al. (1967)]. This would be consistent with the improvement of fit to trypsin hydrolysis noted here when the presence of some intact GPA is included in our models. The transbilayer configuration suggested is also supported by the fact that the most extensive NMR perturbations occur for resonances from the hydrophobic lipid acyl chains.

There are now several examples in the literature of glycophorin reconstitution in lipid bilayer systems. Samples prepared by the hydrated film method of MacDonald & MacDonald (1975) have been studied most extensively (Van Zoelen et al., 1978c). Although there is no complete agreement on protein orientation, there is evidence that incorporation in that system is only slightly asymmetric with the carbohydrate moiety of the molecule exposed to the outer surface. There is also evidence that GPA vesicles prepared by sonication (Van Zoelen et al., 1978c) are appreciably asymmetric, based on neuraminidase treatment.

Our own experiments on sonicated preparations gave enzyme hydrolysis results similar to those obtained by the cho-

late–Sephadex method, supporting a high level of asymmetry. However, NMR results showing slightly lower perturbation to lipid methylene and higher perturbation to head-group resonances would suggest the possibility of some surface-bound protein. The greater breadth of size and protein distributions also suggests that the cholate–Sephadex preparation would be preferred for physical studies.

The most important characteristics of the cholate-prepared vesicles from the point of view of future physical studies are therefore the following: (1) high levels of protein incorporation can be obtained (as high as 5 mol %); (2) the incorporated protein is transbilayer and possibly asymmetric across the bilayer; (3) the vesicles are homogeneous and of a size similar to non-protein-containing vesicles prepared by the same method.

Given that the cholate-prepared vesicles can be characterized with respect to mode and level of incorporation, the proton NMR results presented here invite some preliminary comments about the nature of lipid–protein interactions. It is widely accepted that spin relaxation as reflected in inversion recovery experiments or in NMR line widths provides a sensitive probe of molecular motion. The relationship between motions and various spin relaxation pathways is, in fact, a complex one. Motions of lipids are anisotropic, and, for proton NMR, intermolecular as well as intramolecular motions are important. Several correlation times must therefore be used to describe motions, and these correlations times are not reflected equally in different types of spin relaxation experiments. On a qualitative level, spin–lattice relaxation times of lipid methylenes are more sensitive to rapid internal chain rotations or isomerizations while spin–spin relaxations or line widths are more sensitive to slower chain-wagging motions and intermolecular associations (Gent & Prestegard, 1977). We will defer a quantitative assessment of spin relaxation data to a paper including ^{13}C NMR results as well. But the relaxation data presented here can be used in an empirical way to assess stoichiometry of interactions and compare glycophorin–lipid interactions to observations in other systems.

In very general terms, line widths increase as the mobility of molecular segments to which the protons are attached decreases. Increases in lipid methylene line widths on incorporation of glycophorin could, therefore, be interpreted as restriction of lipid chain-wagging motions by the protein. Proton NMR line widths in small unilamellar vesicles are also strong functions of vesicle size (Sheetz & Chan, 1972). In our preparations, however, the similarity of sizes of vesicles with different amounts of glycophorin minimizes the possibility of indirect contributions to changes in line width due to glycophorin-induced changes in vesicle size, and line-width increases can be interpreted directly in terms of lipid–protein interactions.

The fact that the entire lipid methylene line appears to broaden with no apparent indication of multiple components in either line shape of T_1 relaxation would indicate that all lipid molecules interact with protein equally on the time scale of the spin–spin relaxation times, 0.01 s. In other words, perturbed and unperturbed lipids are in rapid exchange. The simplest model would allow just two states, perturbed or "boundary lipid" and free lipid. The observed line width would be a weighted average of line widths for lipid in the two states. Addition of increasing amounts of glycophorin would result in increased fractions of boundary lipid and broader lines until no free lipid remained. Beyond this point, no further broadening would occur. This is the behavior observed in Figure 7. The line width at the plateau could be taken to represent a property of boundary lipid and the mole percentage at the beginning of the plateau an indication of protein–lipid interaction stoichiometry (1:40).

It is possible to give a molecular interpretation to the observed plateau stoichiometry by assuming the transmembrane segment of glycophorin to be a single α helix for the sake of simplicity. A typical radius of an α helix is 7–8 Å (Chapman et al., 1979). Given that the radius of a hydrocarbon chain is 2.4 Å and the axes of cylinders representing the α helix and hydrocarbon chain are likely to be parallel, there is room for 10–15 hydrocarbon chains adjacent to the protein in each half of the bilayer. With two chains per molecule, this represents a relatively small number of lipid molecules. If there is preferential association of the α chain of a lipid as suggested by Zumbulyadis & O'Brien (1979), however, we could approach 30 lipids, in reasonable agreement with the onset of the plateau region. It is, of course, not necessary to give such a literal interpretation to our two-state model. It is quite possible that the observed stoichiometry is simply indicative of a composition beyond which two phases exist, one nearly pure protein and one of the observed protein–lipid composition.

If we accept a physical boundary layer of 30 lipid molecules, it is useful to compare the perturbation they experience (23 Hz additional line broadening) to perturbations observed in other membrane systems. The cholesterol–phosphatidylcholine system is now among the better studied membrane systems. It also provides a simple model for interaction of an acyl chain with a rigid hydrophobic moiety. A transbilayer pair of cholesterol molecules could be treated as a rigid cylinder with a cross-sectional area of 35 $Å^2$ (Levine & Wilkins, 1971) and a length corresponding to the thickness of the hydrophobic portion of the bilayer, 36 Å. On the basis of this geometry, and no preferential chain association, there would be approximately seven lipid molecules around the cholesterol cylinder (3.5 from each monolayer), as compared to the 30 found in the GPA vesicle. At a cholesterol to phosphatidylcholine molar ratio of 1:3, approximately 30% increase in line width is observed (Gent & Prestegard, 1974). Although some of this increase might be attributed to increased vesicle size in the case of cholesterol, it provides an estimate for potential perturbations. If the system obeyed a two-state model and were saturable as in the case of GPA vesicles, this 30% increase would be near the plateau. Similarity to the 60% line broadening in a 2.5 mol % GPA vesicle (a factor of 2) suggests that the perturbations produced by glycophorin are consistent with those expected for a relatively rigid hydrophobic segment.

It is also significant that below 2.5 mol % GPA, little perturbation of lipid hydrocarbon T_1's was noted. This is also the case for addition of cholesterol to vesicles, where it has been interpreted as a minimal perturbation of chain rotation or coupled isomerizations. Thus, although protein–hydrocarbon chain interactions exist, these need not be especially strong or specific. They are qualitatively similar in their effects to the predominantly steric interactions between lipid hydrocarbon chains and the cholesterol ring structure.

It is also interesting to compare results from the glycophorin system to results on other transmembrane proteins. ^1H NMR studies of rhodopsin-containing membranes have been published (Brown et al., 1977a,b; O'Brien et al., 1977). Approximately 40% line broadening can be estimated from published spectra at 1 mol % rhodopsin. Estimates of 24 boundary lipids/protein have been obtained by ESR methods for this system (Watts et al., 1979). The results would therefore suggest a line broadening of boundary lipid not more than 3 times that observed here.

Acknowledgments

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