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# PROTON NMR STUDIES OF VESICLES INCORPORATING GLYCOPHORIN

Arthur L.Y. LAU * and David COWBURN

*The Rockefeller University, New York, NY 10021, U.S.A.*

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The effects of incorporation of glycophorin, the major sialoglycoprotein of the human erythrocyte membrane, on the lipid of small vesicles have been studied using proton NMR and electron microscopy. In contrast to the incorporation of other peptides, the major effect is apparently the clustering of vesicles without fusion. The relative mobility of lipids of the vesicle, monitored by changes in proton spin-lattice time, is only moderately effected by the presence of protein. The methylene protons of the lipid chains are subject to a somewhat greater restriction of motion following the incorporation of glycophorin than are the protons of the head group.

## 1. Introduction

Glycophorin is the major sialoglycoprotein found in human erythrocyte membranes. Its amino acid sequence has been determined [1] and its molecular weight found to be about 50000, of which $\approx 60\%$ is carbohydrate and the rest protein [2,3]. Several methods for isolation of this protein from erythrocyte ghosts have been reported [4]. It has been claimed that the polypeptide chain of glycophorin consists of three distinct segments, each with its unique chemical properties. In the intact erythrocyte membrane it is thought to span the entire thickness of the cell membrane, with the C-terminus exposed towards the inside of the cell membrane and the N-terminus facing the outside [6]. This protein is of great interest because of its orientation in the natural membrane and it offers a model system for the study of protein interactions with membranes.

Extensive characterization of glycophorin has been performed by a variety of chemical techniques [2,7–9]. Using freeze-etch electron microscopy, the interaction of glycophorin with lipid bilayers was studied by Segrest et al. [10] and also by Grant and McConnell [11]. Their data suggest that the protein is being incorporated into the hydrocarbon phase of the phosphatidylcholine bilayers. A detailed study of the assembly of vesicles bearing the protein has been presented [8].

This report concerns an investigation on the interaction of externally introduced glycophorin with phosphatidylcholine bilayer vesicles, using proton NMR spectroscopy coupled with other techniques such as electron microscopy and gel chromatography. Proton NMR spectroscopy, with its sensitivity towards structural details and environmental changes, has been employed extensively in the investigations of the structural and hydrodynamic properties of bilayer membranes (see, e.g., refs. 12–20). More recently, NMR has also been successfully used in the studies of the interactions of various oligopeptides with phospholipid bilayers [21–27].

Because of the difficulties associated with the manipulation of large relatively planar cell membranes most investigations with bilayers involving the use of NMR spectroscopy have been concerned with the model membrane systems, such as the unsonicated multiamellar structure and the sonicated bilayer vesicles. These two systems offer convenient and interrelated models for membrane studies. The difference in their physical properties is due chiefly to the much larger surface curvature of the small vesicles which results in a looser and

* Present address: Miles Laboratories Inc., Elkhart, IN, U.S.A.

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less stable structure [27]. Recently, Brulet and McConnell [28] reported an investigation with $^{13}$C-NMR spectroscopy on the effect of glycophorin on the choline head groups of unsonicated lipid multilayers. Studies on the effect of this sialoglycoprotein on small bilayer vesicles are reported here, providing further insight into the nature of interactions of membrane protein with lipid bilayers.

## 2. Experimental section

### 2.1. Materials

L-α-Dipalmitoylphosphatidylcholine was purchased from Calbiochem. The product was examined by thin-layer chromatography and was used without further purification. $D_2O$ (99.8% $^2$H) was from Stohler Isotope Chemicals. Europium nitrate was from Thompson Packard, Inc. Methanol and chloroform, both used in the extraction of glycophorin from erythrocyte ghosts, were either J.T. Baker analyzed reagent grade or Matheson Coleman and Bell spectrograde products. All chemicals were used as received.

### 2.2. NMR instrumentation

The Fourier transform (FT) spectra of sonicated lecithin bilayer vesicles were obtained with a Varian HR-220/Nicolet Technology TT-220 NMR spectrometer operating at 220 MHz for protons. Intensities of NMR signals were measured against a standard chloroform capillary treated with the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Chemical shifts were calibrated against an external tetramethylsilane (TMS) capillary without correction for variations in the bulk magnetic susceptibility. Sample temperature was controlled by a Varian 4540 temperature control unit, and was determined from the spectrum of a standard ethylene glycol sample. $T_1$ values were calculated from a least-squares fit, using both slope and intercepts as adjustable parameters. ('NTCFT', Nicolet Technology Corporation, 1976.)

### 2.3. Isolation of glycophorin

Human blood, less than 2 weeks old, was purchased from the New York Blood Center. Erythrocyte ghosts were prepared by hypotonic lysing and washing of these cells with 10 mM potassium phosphate buffer at pH 7.8 following the procedure of Dodge et al. [29]. Erythrocyte ghosts obtained were either white or with a faint trace of pink due to residual hemoglobin. The water-soluble glycophorin was isolated from the ghosts using a procedure adapted from that of Hamaguchi and Cleve [5]. To 1 volume of the ghost suspension (protein concentration $\approx 2$ mg/ml), 9 volumes of a 2:1 mixture of chloroform/methanol was added. The mixture was stirred rigorously at room temperature for about an hour with a magnetic stirring bar, and was then centrifuged at 1000 $g$ in a Sorvall Superspeed RC-2 centrifuge. The upper aqueous phase which contained the glycophorin, was transferred to a round-bottom flask. Care was taken not to include any interfacial material in the transfer. The aqueous phase was then concentrated in a rotary evaporator at 37°C to about one-tenth of its original volume. The product after evaporation was clear but slightly viscous.

Ethanol precipitation of the aqueous phase components was brought about by the addition of 9 volumes of absolute ethanol to 1 volume of the concentrated aqueous phase. The mixture immediately turned cloudy. Actual precipitation occurred rather slowly, however, and was essentially complete by standing the mixture in the cold room at 4°C for about 2 days. The precipitate was then centrifuged at 12000 $g$ for 30 min and the bulk ethanol removed by aspiration. The precipitate, which was white in color, was vacuum dried overnight. The purity of the product was checked by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (see below) before use. This preparation was repeated once with freshly drawn human blood, and the result found was identical. Protein concentration was determined by the method of Lowry [30] using bovine serum albumin as standard.

### 2.4. SDS-polyacrylamide gel electrophoresis.

The gel used for electrophoresis was a SDS-acrylamide gel with an acryamide concentration gradient from 20% at the bottom (positive end) to 6% at the top [31]. After the material to be electrophoresed was layered on the top, a constant current of 10 mA was applied for about 12 h. Pyronin Y was used as a front tracking dye to determine the actual length of time required. The gel was washed in a mixture of 50% methanol/7% acetic acid aqueous solution overnight. It was then washed in a 7% acetic acid aqueous solution for about 30 min, and then in a mixture of 1% periodic acid and 7% acetic acid solution for 1 h at 35°C. After several additional washings with 7% acetic acid solution for a total of about 4 h to remove excess periodic acid, 300 ml of Schiff's reagent (0.46 basic fuchsin in 0.8% sodium metabisulfite and 0.1 M HCl, decolorized by charcoal filtration before use) were quickly added, the trough containing the gel was securely wrapped up in an aluminum foil, and the mixture was gently shaken in the dark for about 2 h at 35°C. The liquid was then carefully aspirated off, and the gel washed repeatedly with several 300-ml portions of 0.5% sodium metabisulfite in 7% acetic acid solution, until the washing solution gave a negative color reaction to formaldehyde. Additional washings with 7% acetic acid solution for a total of 3 h removed the excess metabisulfite.

Subsequent staining of the gel with Coomassie brilliant blue was performed by immersing the gel in a solution of 0.25% Coomassie blue, 50% methanol and 7% acetic acid for 5 h or more, followed by destaining of the background with 7% acetic acid/50% methanol mixture. The entire procedure was carried out at room temperature unless otherwise specified, and with continuous gentle shaking.

### 2.5. Amino acid analysis of glycophorin

Approximately 2 mg of the extracted glycophorin was weighed into a test tube, to which 2 ml of 6 M HCl were added. After the protein was completely dissolved and the solution degassed, the tube was sealed and left in an incubator at 110°C for about 48 h. When the hydrolysis was completed, the seal was broken, and the hydrolysate lyophilysed overnight to remove the solvent. The dried material was then dissolved in 2 ml of citrate buffer (pH 2.2) and its amino acid content analysed with a Beckman/Spinco amino acid analyzer.

A single column analysis was carried out with a 30 cm column of Durrum DC-6A resin with a protocol using a three-step increase in ionic strength. Conventional ninhydrin detection was employed [32]. An additional sample containing a mixture of known quantities of the common amino acids was run under identical conditions to provide the calibration standard.

### 2.6. Preparation of samples for NMR spectroscopy

Sonicated bilayer vesicles were prepared using a Branson Sonifier. A measured quantity of dipalmitoylphosphatidylcholine was weighed into a centrifuge tube, to which a known volume of $D_2O$ was added. The $D_2O$ contained 2 mM of potassium phosphate and was at pD 7.4 [33]. Sonication of the mixture for about 15 min produced a clear, almost colorless, transparent solution. This suspension of bilayer vesicles was centrifuged at 17400 g for about 30 min to remove metallic particles and residual multilamellar structures from the small vesicles. The latter was used in all subsequent NMR and electron microscopy experiments. A known quantity of glycophorin was weighed into a 2 ml volumetric flask, to which $D_2O$ containing 2 mM phosphate at pH 7.4 was added to form a homogeneous solution. The mixture was allowed to stand overnight at 4°C to ensure complete solubilization of the protein.

NMR samples containing both bilayer vesicles and glycophorin were prepared by pipetting measured quantities of each solution into the NMR tube, and the mixture was homogenized with a vortex mixer, and incubated at 60°C for about 30 min before NMR spectra were taken.

### 2.7. Preparation of samples for electron microscopy

A typical procedure is as follows: a drop of the vesicle solution (with or without glycophorin), di-

luted to about 0.5 mg dipalmitoylphosphatidylcholine/ml, was applied onto a 400 mesh copper grid coated with a film of colloidion and sprayed with a thin layer of carbon, for about 30 s. The grid was washed with three drops of staining solution (% phosphotungstic acid, buffered at pH 7.4 by phosphate). An additional drop of phosphotungstic acid was then applied to the grid for about 30 s, and the grid was blotted dry by a piece of fine filter paper. The grid was allowed to dry overnight prior to observation under a Hitachi-Perkin Elmer HU-11C-1 electron microscope operating at 75 kV.

## 3. Results and discussion

### *3.1. SDS-polyacrylamide gel electrophoresis patterns of glycophorin*

Glycophorin can be identified and its purity determined using SDS-polyacrylamide gel electrophoresis coupled with periodic acid-Schiff's reagent (PAS) and Coomassie brilliant blue double-staining technique.

When stained for carbohydrate by the PAS procedure, the electrophorogram of both the isolated glycophorin as well as the glycophorin-containing erythrocyte ghosts gave the characteristic three-band pattern in pink as reported earlier by Fairbanks et al. [34]. Heating of the glycophorin sample prior to electrophoresis did not appear to have a noticeable effect on the relative intersities of these three bands. It was found that the weaker bands II and III could be removed with Sephadex-100 column chromatography. Thus, it appears that bands I, II and III are due to different species not easily interconvertible, rather than aggregates of different sizes of the same species. This conclusion agree with some previous work [35], but not others [36].

Subsequent staining of the gel with Coomassie brilliant blue revealed no additional bands with glycophorin. The pattern of the ghosts contained many additional bands similar to those reported earlier [37] in addition to the glycophorin bands which remained pink (fig. 1).

The PAS I band of glycophorin was also observed by staining the gel with Coomassie brilliant blue alone. In this case the PAS I band is very faint, observable only with heavy staining and could be masked if other stronger bands were present nearby. For example, this band was usually not observed with the ghost because of the stronger bands III and IV located just above and below it. The position of this band is identical to that revealed by PAS staining, and is definitely not band III as has been claimed [8], at least as determined in this gel system.

The result of amino acid analysis is shown in table 1. The experimental error of this method of determination is $\approx \pm 10\%$. It can be seen that, within experimental error, the amino acid content of our glycophorin sample is compatible with that reported earlier [1]. The molar percentages of alanine and leucine were somewhat higher than the published results, whereas those of serine and proline were lower. We do not consider these discrepancies serious and believe that the protein we worked with was the same glycophorin used by others.

### *3.2. Interaction of externally added glycophorin with lipid bilayer vesicles*

Upon the addition of glycophorin to dipalmitoylphosphatidylcholine vesicles, and a subsequent incubation period of 30 min, changes in the turbidity of the vesicle solution were noticed. This observation is similar to that when other polypeptides such as alamethicin or poly(L-glutamic acid) [22,23] were added to lipid vesicle solution. However, in contrast to the situation with alamethicin which causes severe cloudiness and large alteration in homogeneity of the magnetic field of the vesicle sample, the changes in the glycophorin-containing vesicle solution was relatively mild. A measurement of the intensities of both the choline methyl and fatty acid chain methylene protons as a function of glycophorin concentration revealed that both signal intensities decrease with increases in glycophorin in the sample (fig. 2). This suggests that some change in the physical state of the vesicles has occurred, and we have confirmed this expectation with electron microscopy observation.

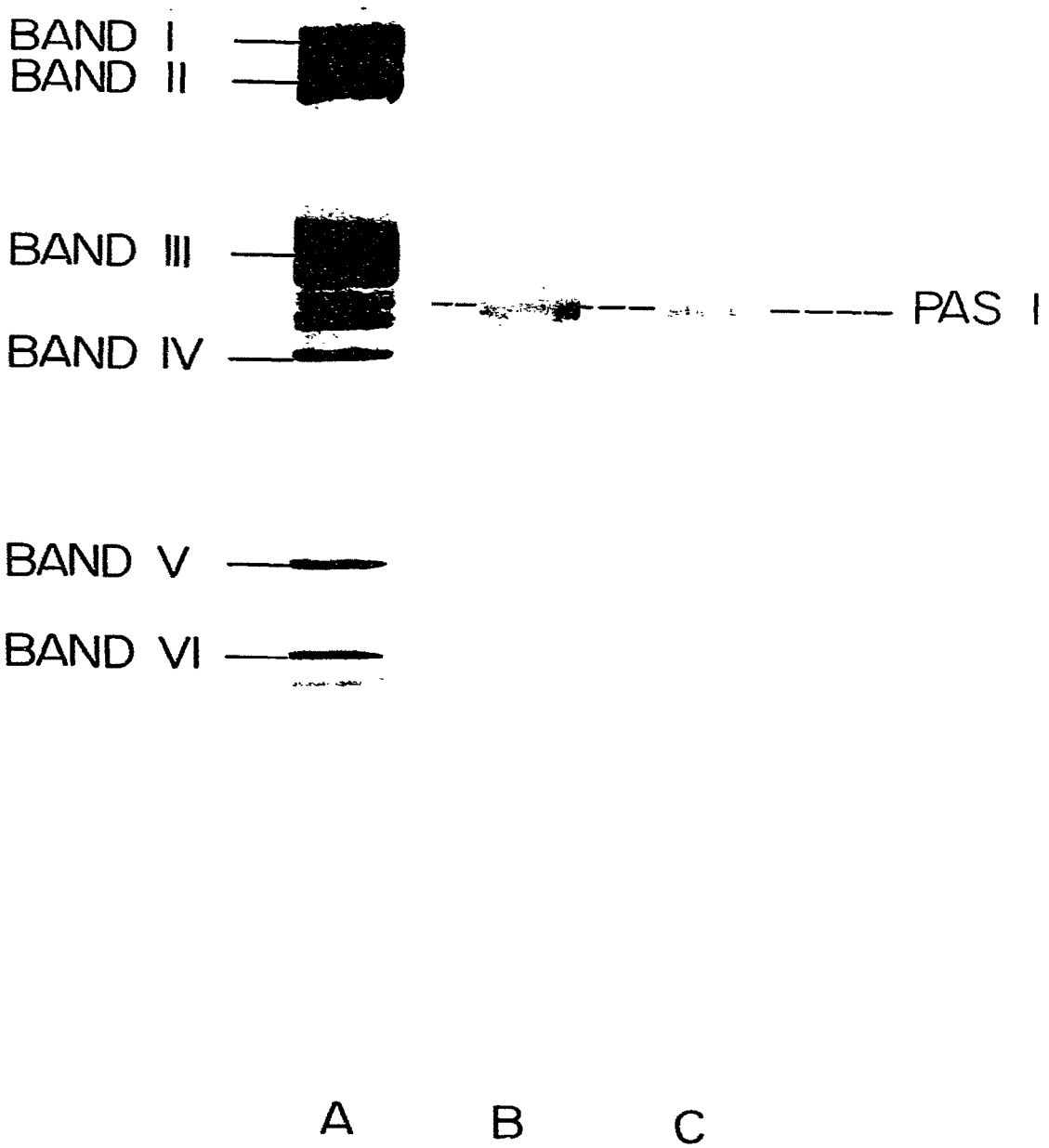


Fig. 1. SDS-polyacrylamide gel electrophoresis of erythrocyte ghosts (A) and purified glycophorin (B, C), using two different staining procedures. A and B, Coomassie brilliant blue stain; C, periodic acid-Schiff's reagent stain.

Table 1

Spin-lattice relaxation times of choline and chain methylene protons

| The protons of | | $T_1$ values (s) in the solvents | | |
|---|---|---|---|---|
| | | $D_2O$ | 10% sucrose/$D_2O$ | 20% sucrose/$D_2O$ |
| Choline head group | without glycophorin [a] | 0.66 (0.02) [b] | – | – |
| | with glycophorin | 0.65 (0.01) | – | – |
| Methylene in alkyl side chain of lipid | without glycophorin | 0.64 (0.02) | 0.65 (0.01) | 0.63 (0.03) |
| | with glycophorin | 0.58 (0.03) | 0.60 (0.03) | 0.58 (0.02) |

[a] Solutions and concentrations of glycophorin are described in the Experimental section.
[b] Number in brackets is the standard deviation of the $T_1$ value, calculated from a least-squares fit of the data.

It was revealed by negative-stain electron microscopy that the glycophorin caused coagulation of vesicles. Fig. 3 shows that, in specimens containing only phosphatidylcholine bilayer vesicles, most of the vesicles exist as isolated individuals, whereas in specimens containing glycophorin as well, vesicles form clusters with one another. In some cases the clustering resulted in the compressing of vesicles to form stacked rice-shaped structures. Of the vesicles that still retain a more or less spherical shape, there was little change in their mean diameter, however, in contrast to the situation with alamethicin which caused a drastic increase in the average vesicle size due to extensive vesicle fusion. Thus, externally added glycophorin did not cause extensive fusion of vesicles but only coagulation of them.

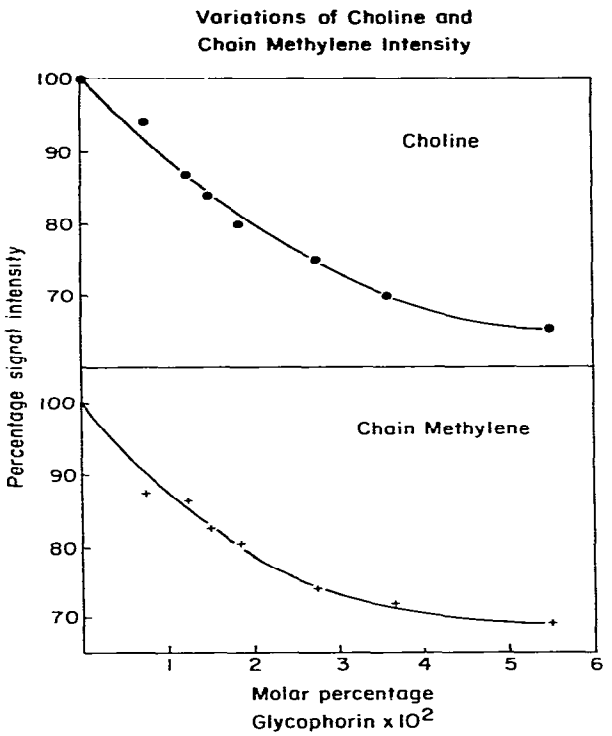


Fig. 2. 220-MHz $^1$H-NMR intensities of choline methyl and fatty acid methylene protons in vesicles with and without glycophorin. See text.

In a previous study [24], it was shown that fusion of vesicles induced by alamethicin resulted in the mixing of the internal contents of the vesicle with the external medium. This mixing was detected using the europium(III) ion as a paramagnetic shift reagent. If $Eu^{3+}$ was added to a solution containing the vesicles, two proton NMR signals from choline methyl protons could be detected, corresponding to the methyls in the inner and outer monolayers. Fusion results in a merging of the two choline signals.

We added europium(III) nitrate to the extravesicular solution prior to the addition of glycophorin. Two detectable signals were observed from the methyl groups. No detectable upfield shift of the inner choline proton signal was observed, subsequent to addition of glycophorin. It is concluded that the incorporation does not lead to extensive fusion of vesicles in which the external and internal media are mixed.

We also attempted to quantify the amount of glycophorin strongly associated with lipid bilayer vesicles. After an incubation period of about 30 min at 60°C, the bilayer vesicle/glycophorin mix-

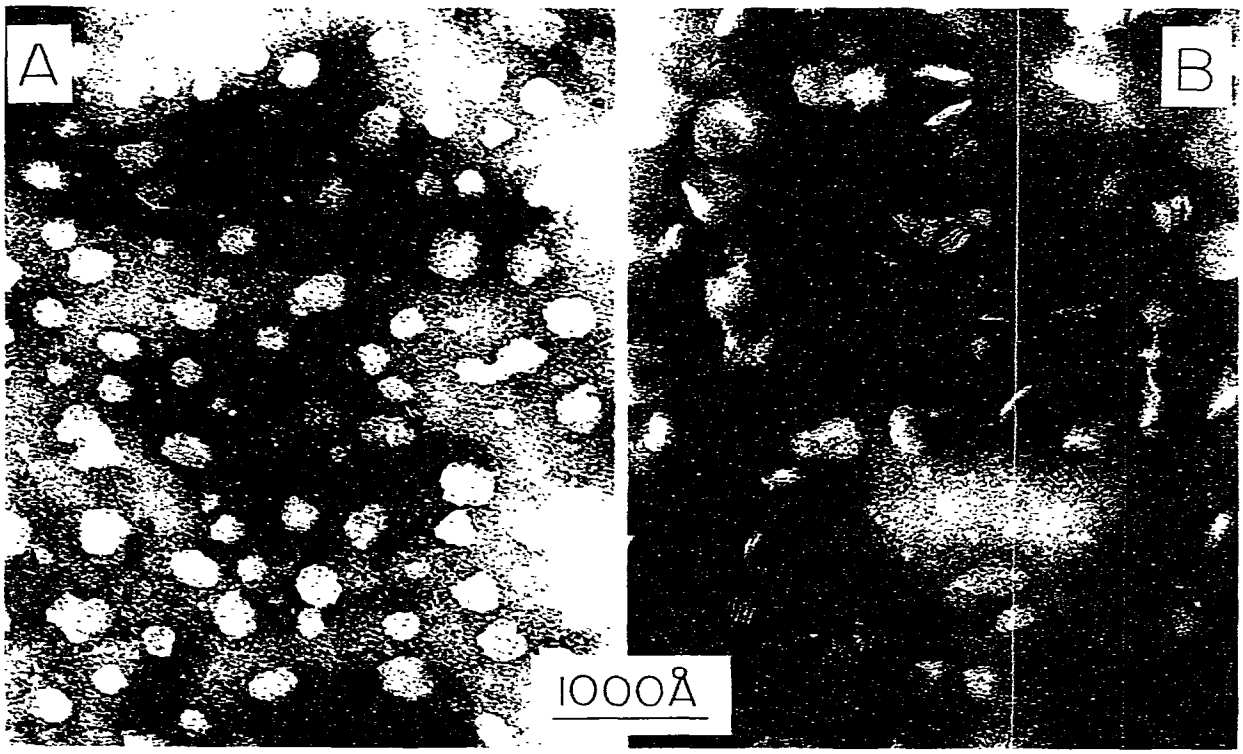


Fig. 3. Electron micrographs of phosphatidylcholine vesicles before and after addition of glycophorin. See text.

ture was applied to a 30 cm Sepharose 4B column and eluted with a solution of 2 mM sodium phosphate buffered at pH 7. The elution profile of the lipid vesicles was determined by observing the optical density at 300 $\mu$m and that of glycophorin by determining the amount of sialic acid present in each eluent fraction using the method of Warren [37]. Results of such determinations are depicted in fig. 4. It could be seen that some of the glycophorin was eluted together with the major absorption peak of bilayer vesicles, and there was a long tail probably representing free glycophorin molecules in solution. Assuming that each glycophorin molecule contains 28 sialic acids, and that the molar extinction coefficient of the sialic acid-thiobarbituric acid complex is 57000 [37] in their experiment, $\approx$ 10% of the added glycophorin became associated with the vesicles equivalent to 2 to 3 protein molecules per vesicle of average size 350 Å.

### *3.3. Effect of glycophorin on the NMR spectral properties of phosphatidylcholine vesicles*

We have measured the spin-lattice relaxation time ($T_1$) of the dipalmitoylphosphatidylcholine bilayer vesicles at 220 MHz with and without the presence of glycophorin. The relaxation rates of both the choline methyl as well as the chain methylene protons are depicted in fig. 5 as a function of temperature. It can be seen that the $T_1$ values of the choline methyl protons are not affected greatly by the presence of the glycoprotein; the small effect observed being somewhat more pronounced at lower temperatures. On the other hand, the $T_1$ of the chain methylene protons was decreased with

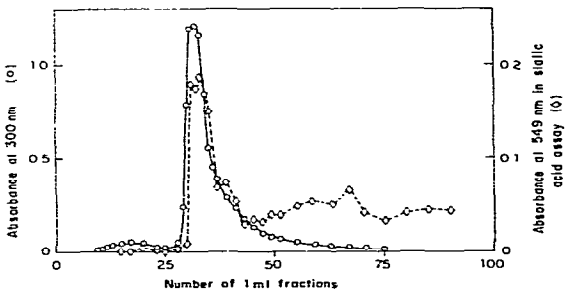


Fig. 4. Elution profile of vesicles of phosphatidylcholine and glycophorin. Phosphorus determinations (O) and sialic acid assay (+) are shown. See text.

the presence of 0.5% glycophorin in the vesicle sample, although the temperature dependence remained approximately the same.

The observed values of $T_1$ for the phospholipids contain contributions from several different relaxation mechanisms and can be expressed as

$$T_{1,\mathrm{obs}}^{-1} = T_{1,\mathrm{D}}^{-1} + T_{1,\mathrm{SA}}^{-1} + T_{1,\mathrm{SC}}^{-1} + T_{1,\mathrm{SR}}^{-1} + T_{1,\mathrm{Q}}^{-1}$$

where the subscripts refer to the various mechanisms; D, dipole-dipole coupling; SA, chemical shift anisotropy; SC, scalar coupling; SR, spin rotation; and Q, quadropolar interactions. The last term can be readily excluded. A substantial body of experimental evidence [12–27] suggests that the other terms, particularly $T_{1,\mathrm{D}}^{-1}$ and $T_{1,\mathrm{SA}}^{-1}$ are significant, and are dependent on inter- and intramolecular motion, incorporating correlation times for overall motion of the vesicle ($\tau_v$), and motions parallel ($\tau_{\parallel}$) and perpendicular ($\tau_{\perp}$) to the plane of the bilayer.

It has been established that the spin-lattice relaxation rates, at 220 MHz, in phosphatidylcholine vesicles are dominated by intramolecular effects [38]. Because the number of glycophorin molecules incorporated per residue is relatively low, we consider it justified to assume that intermolecular effects are not responsible for the changes in $T_1$ observed, which then must result from alteration of the anisotropic motions of the lipids produced by the incorporation of the protein.

With regard to changes in the vesicle tumbling rate, changes in viscosity or coagulation of vesicles obviously would cause a decrease in the tumbling rate of the aggregate. This point was investigated by addition of sucrose to the vesicle suspension. With the presence of 10 or 20% sucrose in the sample, the $T_1$ of the chain methylene protons was not noticeably affected. The vesicles are probably still tumbling with a sufficiently fast rate even in the aggregated state with glycophorin so that any small change in this rate does not contribute significantly to the spin-relaxation time.

It has been proposed [21,27] that the effects of $\tau_{\parallel}$ and $\tau_{\perp}$ can be phenomenologically expressed in

$$T_{1,\mathrm{obs}}^{-1} = A\tau_{\parallel} + B/(\omega_0^2\tau_{\perp})$$

where $A$ and $B$ are constants and $\omega_0$ is the frequency of observation. The observed decrease in $T_{1,\mathrm{obs}}$ (methylene) might then be the result of an increase in $\tau_{\parallel}$, or a decrease in $\tau_{\perp}$, or some combination of changes in both values. It appears unlikely that the incorporation of a molecule of the

Table 2

Result of amino acid analysis of glycophorin

| | Percentage determined [a] | Percentage reported by Tomita and Marchesi [b] |
|---|---|---|
| Ala | 7.0 | 4.7 |
| Arg | 4.5 | 4.6 |
| Asx [c] | 7.0 | 6.1 |
| Glx [d] | 11.4 | 10.7 |
| Gly | 5.2 | 4.6 |
| His | 3.2 | 3.8 |
| Ile | 7.9 | 7.6 |
| Leu | 8.5 | 6.1 |
| Lys | 4.4 | 3.8 |
| Met | 1.8 | 1.5 |
| Phe | 2.2 | 1.5 |
| Pro | 5.2 | 6.9 |
| Ser | 10.0 | 14.5 |
| Thr | 11.0 | 11.5 |
| Tyr | 3.0 | 3.1 |
| Val | 8.0 | 8.4 |

[a] Expressed as percentage of total number of amino acids present.

[b] V.T. Marchesi and M. Tomita, Proc. Natl. Acad. Sci. USA 72 (1975) 2964.

[c] Asx = Asn + Asp.

[d] Glx = Gln + Glu.

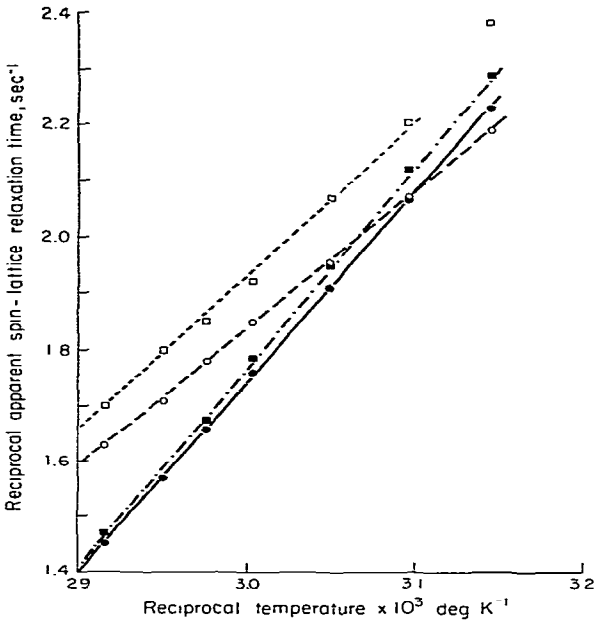


Fig. 5. Temperature dependence of the apparent spin-lattice relaxation terms of protons of choline head group (————, — - - —) and methylene side chains (— —, ----), with (■, □) and without (○, ●) glycophorin. See text.

size of glycophorin, and subsequent aggregation, could lead to a more fluid situation in which $\tau_{\perp}$ might decrease. If, indeed, $\tau_{\parallel}$ is truly the variable responsible for the decrease in $T_{1,obs}$ (methylene), then its increase is readily understandable as a reduction in rotational isomerization of the lipid side chains. Such a reduction would be expected for lateral tightening of the bilayer structure, a phenomenon proposed earlier for the phosphatidylcholine/polyglutamic acid system [21].

The changes in observed signal intensities of choline and methylene protons closely parallel each other. It seems likely that this reduction of apparent intensity is the result of transferring the signal strength to the undetectable broad signal underlying the observed signals [13]. This could be the result of either changes in the overall rotation of the vesicle aggregates, or from the creation of a fraction of the lipids which are more significantly immobilized by the added glycophorin than the remainder. Since aggregation state and coagulation are experimentally controlled by the techniques described here, it is most reasonable to conclude that the incorporation of glycophorin into small unilamellar vesicles under the conditions used here gives rise to a greater restriction of the motions of the methylenes of the lipid chain compared to the head group. This conclusion is confirmatory of that reported recently on the basis of $^{13}$C-NMR measurements in a similarly reconstituted system [39]. In that case direct observation of the partially immobilized chains was possible. It is widely accepted that glycophorin is anchored in its natural milieu by the interaction of a sequence in residues 73–91 of relatively hydrophobic residues, with the lipid matrix [1]. Our and other [39] observations on the NMR properties of lipids in vesicles incorporating glycophorin are consistent with the reconstitution leading to a similar structure in this artificial case.

## Acknowledgements

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