# Lectin–Receptor Interactions in Liposomes: Evidence That Binding of Wheat Germ Agglutinin to Glycoprotein–Phosphatidylethanolamine Vesicles Induces Nonbilayer Structures†

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ABSTRACT: Glycophorin, the major integral membrane glycoprotein of the erythrocyte membrane, known to bear receptors which serve as the MN blood group determinant and for the binding of wheat germ agglutinin (WGA) has been incorporated into unilamellar vesicles of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). The effect of WGA binding on the phospholipid organization in the glycophorin–lipid vesicles was studied by using $^{31}P$ NMR (at 81.0 MHz) and freeze–fracture electron microscopy. Addition of WGA to the DOPC–glycophorin vesicles (200/1 mol/mol) induces aggregation of the vesicles but not fusion. This aggregation could be reversed by *N*-acetyl-D-glucosamine (GlcNac) which is a known sugar hapten inhibitor of WGA agglutination. DOPE in the presence of excess water undergoes a bilayer → hexagonal ($H_{II}$) phase transition above 0 °C. We have previously shown that cosonication of DOPE with glycophorin in a 25/1 molar ratio stabilizes the bilayer organization whereas sonicaton of DOPE alone results in unstable lipid aggregates which precipitate from solution [Taraschi, T. F., de Kruijff, B., Verkleij, A. J., & van Echteld, C. J. A. (1982) *Biochim. Biophys. Acta 685*, 153–161]. Incorporation experiments employing sonication revealed that a DOPE/glycophorin molar ratio of <475/1 was required to obtain a vesicle populaton comprised of only unilamellar, bilayer vesicles. Addition of WGA to bilayer vesicles of DOPE/glycophorin (200/1) triggered a bilayer → hexagonal ($H_{II}$) phase transition in these vesicles. This process was found to be irreversible upon addition of GlcNac. The WGA-induced bilayer → hexagonal ($H_{II}$) transition was proven to result from a specific binding event with the carbohydrate portion of the protein as GlcNac addition to the vesicle solution prior to lectin titration was found to totally inhibit the bilayer → $H_{II}$ transition. The relationship between protein aggregation, fusion, and the hexagonal ($H_{II}$) phase is discussed, as is the possible role of nonbilayer lipid structures in cell functional events.

During the past 10 years considerable attention has been focused on the complex contact events which occur at the eucaryotic cell surface. These cellular phenomena are thought to be specifically mediated in part by complex carbohydrates that are covalently linked to membrane lipids and proteins and coat the surface of cells (Hughes, 1976). This specificity results from the recognition of particular carbohydrate sequences on glycoproteins and/or glycolipids by complementary sites on proteins. Other factors such as topological distribution and mobility of the receptor in the membrane and the distance of the saccharide determinant from the membrane are also likely to play key roles in effector binding to the cell. Saccharide determinants are thought to be involved in the mediation of basic cell functions such as adhesion (fusion) (Gartner & Podleski, 1975; Huang, 1978), the immune response (Hughes & Sharon, 1978), control and differentiation of cell growth (Edelman, 1976), and the agglutination of normal and transformed cells (Sharon & Lis, 1972). Furthermore, evidence has been presented that these membrane-bound carbohydrates function as receptors for the selective binding of enzymes (Hughes & Sharon, 1978), antibodies (Edelman, 1976), toxins (Sandvig et al., 1976), lectins (Goldstein & Hayes, 1978), hormones (Fishman & Brady, 1976), and viruses (Haywood, 1974) to the plasma membrane. It has been proposed that following the initial recognition of the effector a chain of events occurs that eventually evokes a metabolic response by the cell (Nicolson, 1976). Despite these recent advances, however, the mechanistic details concerning the events that transpire following the recognition of the ligand by the receptor and the resulting response remain largely a mystery.

Several related theories have appeared that propose that receptor-mediated cellular responses are coordinated by an assembly of interacting macromolecules consisting of cell surface receptors which are attached to membrane proteins and lipids and which are capable of laterally diffusing in the membrane and a submembranous fibrillar structure (Edelman, 1976; Nicolson, 1976). Accordingly, cell surface receptors may undergo changes in mobility and distribution following binding and lead to modification of the cytoskeletal structure with which they are associated.

Studies involving effector–receptor interactions have evolved in many directions during the past few years. These include the quantitation of high-affinity binding sites, their surface distribution and alterations in their number and distribution to the binding event (Hakomori, 1975; Nicolson, 1976; Singer, 1974, and references contained therein), the isolation and characterization of extracellular carbohydrates bound to glycolipids and glycoproteins (Goldstein & Hayes, 1978), and the head-group dynamics of membrane glycoprotein oligosaccharide moieties in response to specific recognition events (Lee & Grant, 1980). None of these or other studies, however, have directly addressed the possibility that the binding event and subsequent receptor rearrangement may lead to an alteration in the molecular organizaton of part of the lipids which comprise the membrane in which glycolipids and glycoproteins bearing carbohydrate receptors are embedded. This hypothesis is particularly appealing in that certain prominent membrane lipids [unsaturated phosphatidylethanolamine (Cullis & de

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Kruijff, 1978), monoglucosyldiglyceride (Wieslander et al., 1978), cardiolipin (Cullis et al., 1978), and phosphatidic acid (Verkleij et al., 1982)] form nonbilayer structures under relevant physiological conditions which have been implicated as key components in fundamental membrane functional events such as transbilayer transport, fusion, and endo- and exocytosis (Cullis & de Kruijff, 1979; Verkleij et al., 1979; Cullis et al., 1980).

Glycophorin–phospholipid-reconstituted systems have been shown to be viable systems for the study of effector–receptor interactions that occur at cell surfaces (Redwood et al., 1975; Lee & Grant, 1980; Ketis & Grant, 1982). Glycophorin, the major integral sialoglycoprotein of the human erythrocyte membrane, is an extremely well-suited glycoprotein for ligand–receptor studies. It is known to contain receptors for several sugar-specific plant lectins (e.g., wheat germ agglutinin) due to its high content of GlcNac and sialic acid (Verpoorte, 1975) to which WGA[1] is known to specifically bind (Nagata & Burger, 1974; Allen et al., 1973; Privat et al., 1974; Burger & Goldberg, 1967), MN blood group substances (Marchesi et al., 1972), and influenza virus (Marchesi et al., 1972). In addition, glycophorin is biochemically well characterized, has been the subject of many physicochemical studies in reconstituted phospolipid systems (Taraschi & Mendelsohn, 1980; Taraschi et al., 1982; van Zoelen et al., 1978a,b, and references contained therein), and has resultantly been found to cause extensive perturbation of phospholipid hydrocarbon chain conformation and packing (Mendelsohn et al., 1981; Taraschi & Mendelsohn, 1980).

The utility of $^{31}P$ NMR for investigating phospholipid phase organization in model and biological membranes has been firmly established (Cullis & de Kruijff, 1979; Seelig, 1978). In this investigation we present $^{31}P$ NMR and freeze–fracture electron microscopic data concerning the effect of wheat germ agglutinin binding on the phospholipid organization in two glycophorin-containing membrane systems.

## Experimental Procedures

### *Materials*

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine ($18:1_c/18:1_c$, phosphatidylethanolamine) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine ($18:1_c/18:1_c$, phosphatidylcholine) were synthesized according to estabilished methods (Cullis & de Kruijff, 1976; van Deenen & de Haas, 1964) and purified by high-performance liquid chromatography (HPLC).

Wheat germ agglutinin was purchased from Boehringer Mannheim Biochemicals (West Germany). Neuraminidase from *Vibrio cholerae* (protease free) was obtained from Koch-Light Ltd. (U.K.). Trypsin (type XI, diphenylcarbamyl chloride treated to remove chymotrypsin activity), trypsin inhibitor, and *N*-acetyl-D-glucosamine (GlcNac) were purchased from Sigma (St. Louis, MO). Percoll and calibrated density marker beads were obtained from Pharmacia (Sweden). All other chemicals were of analytical grade.

### *Methods*

Glycophorin was isolated from human erythrocyte ghosts and purified as described extensively before (Taraschi et al., 1982). Reconstitution of glycophorin into small, unilamellar lipid vesicles at 4 °C was achieved by adding 1.2 mL of a solution (100 mM NaCl, 10 mM Tris-HCl, and 0.2 mM EDTA, pH 7.4) containing the appropriate amount of glycophorin to a glass vial containing the desired amount of lipid which had previously been dried from a chloroform solution by evaporation with nitrogen and placed under oil pump vacuum for 2 h to remove final solvent traces. The solution was sonicated in the glass vial 2 × 30 s by using a Branson tip sonicator (power setting 5), transferred to a cellulose centrifugation tube, and further sonicated 8 × 30 s. During sonication a stream of nitrogen was directed into the sample vial or tube containing the lipid–protein recombinants which was immersed in an ice bath. Following sonication, the resulting opaque solution was centrifuged for 20 min at 10000 rpm, 4 °C, in the SS-34 rotor of a Sorvall centrifuge to remove metal particles from the probe and large lipid aggregates which contained little or no protein. Protein incorporation was measured by determination of the sialic acid to phosphorus ratio in the centrifuged sample after being subjected to further centrifugation (15 min, 15000 rpm) on self-generating gradients of Percoll (25% v/v stock Percoll in 150 mM NaCl, 10 mM Tris-HCl, and 0.2 mM EDTA, pH 7.4; density range 1.0–1.3 g/mL) in which unincorporated protein remains in the buffer on top of the Percoll material. Phosphorus content was determined by using a modification of the Fiske–Subbarow procedure (Böttcher et al., 1961) and glycophorin by the method of Warren (1959) by using a value of 2.1 μmol of sialic acid/mg of protein. Details of the neuraminidase, trypsin treatment, and WGA titration experiments are given in the respective figure captions.

*NMR.* Proton noise decoupled $^{31}P$ NMR spectra were obtained on a Bruker WP 200 FT NMR spectrometer operating at 81 MHz. Approximately 1.0 mL of 100 mM NaCl, 10 mM Tris-HCl, and 0.2 mM EDTA, pH 7.4, containing the phospholipid–glycophorin recombinants and 0.2 mL of the $^2H_2O$ analogue of this buffer were transferred to a 10-mm NMR tube and examined at 30 °C. Spectra were accumulated from up to 3000 transients by employing a 90° pulse (18 μs), a 25-kHz sweep width, and a 1-s interpulse delay using gated proton noise decoupling (4-W input power during the 0.0819-s acquisition time). Line widths of the isotropic spectra were estimated by measuring the full spectral width at half-height. Reported line widths in the text have been corrected for the 50-Hz line broadening which was applied to all spectra. The percent hexagonal ($H_{II}$) phase present in each of the NMR spectra was measured by cutting and weighing of the spectral components.

*Freeze–Fracture Electron Microscopy.* Freeze–fracture electron microscopy was performed as described previously (Ververgaert et al., 1973); 25% (v/v) glycerol was added to the samples to prevent freeze damage. Samples were quenched from 30 °C.

*Viscosity Measurements.* The viscosity of 1.5 M *N*-acetyl-D-glucosamine (33.19 g/100 mL) was determined at 30 °C according to standard procedures (Shoemaker & Garland, 1962).

## Results

One of the basic requirements for model systems involving ligand–receptor interactions is that the system under consideration resembles the in vivo situation. Therefore, model systems should be comprised of relevant lipids and proteins which are arranged in an organization similar to that present in a plasma cell membrane including an asymmetric orientation of the receptor toward the extracellular medium. Furthermore, these systems should be biochemically well-de-

[1] Abbreviations: $\nu_{1/2}$, full spectral line width at half-maximal intensity; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; WGA, wheat germ agglutinin; GlcNac, *N*-acetyl-D-glucosamine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

Table I: Biochemical Characterization of DOPE–Glycophorin Recombinants at Varying Lipid/Protein Ratios

| initial lipid/protein[a] ratio (mol/mol) | final lipid/protein[b] ratio (mol/mol) | morphological state[c] | density on 25% Percoll gradients[d] band 1 | band 2 |
|---|---|---|---|---|
| 2000/1 | 1600/1 | 90 | >1.00, <1.017 | >1.017, <1.029 |
| 1500/1 | 1300/1 | 80 | >1.00, <1.017 | >1.017, <1.029 |
| 1000/1 | 800/1 | 75 | >1.00, <1.017 | >1.017, <1.029 |
| 500/1 | 450/1 | 60 | >1.00, <1.017 | >1.017, <1.029 |
| 200/1 | 200/1 | 0 | >1.00, <1.017 | |
| 25/1 | 25/1 | 0 | >1.00, <1.017 | |

[a] Ratio present prior to sonication. [b] Ratio present following centrifugation on Percoll. [c] Determined from the $^{31}P$ NMR spectra; % hexagonal phase present in the DOPE–glycophorin recombinants. [d] Centrifuged 15 min, 15 000 rpm, 4 °C.

fined as well as be of well-controlled complexity. Unsaturated phosphatidylcholines (e.g., DOPC) and phosphatidylethanolamines (e.g., DOPE), two of the most prominent lipid classes present in biological membranes, were chosen for this investigation in light of their differing polymorphic phase properties. DOPC always forms lipid bilayers upon dispersion in aqueous buffer, whereas DOPE is organized in a hexagonal ($H_{II}$) phase above 0 °C. In addition, a good deal of information regarding their incorporation into and behavior in reconstituted systems with glycophorin (van Zoelen et al., 1978a,b; Taraschi et al., 1982) is available.

We have previously reported (Taraschi et al., 1982) that cosonication of an aqueous dispersion (pH 7.4) of DOPE and glycophorin in a 25/1 molar ratio results in the formation of stable, unilamellar bilayer vesicles (300–1000-Å diameter) with protein particles visible by freeze–fracture electron microscopy. This experiment clearly demonstrated the remarkably strong bilayer stabilizing capacity of the protein as attempts to produce sonicated vesicles of pure phosphatidylethanolamine have been unsuccessful (Gammack et al., 1964; Litman, 1973) except at low ionic strength and high pH (9.4) (Stollery & Vail, 1977). Thus, the DOPE–glycophorin system and the polymorphic phase properties of DOPE add a new dimension to effector–receptor interactions.

In our initial study of the DOPE–glycophorin cosonicated recombinant, the specific details regarding the origin and extent (i.e., the number of glycophorin molecules necessary to stabilize the bilayer) of the bilayer stabilization were not addressed in detail. Therefore, before the potential profitability of the DOPE–glycophorin system for the WGA investigations could be realized, biochemical characterization of a series of DOPE–glycophorin sonicated recombinants was carried out by using density gradient centrifugation, $^{31}P$ NMR, and freeze–fracture electron microscopy.

*Incorporation Experiments.* The biochemical characteristics of the cosonicates prepared at DOPE/glycophorin ratios ranging between 2000/1 and 25/1 (moles of lipid to moles of protein) are summarized in Table I. A comparison of the final lipid/protein ratios, which were determined by the procedure outlined under Methods, reveals that only in recombinants with ratios of 200/1 or less all the lipid and protein was associated. At higher ratios (>200/1) it was observed that following centrifugation a pellet consisting of unincorporated lipid (the pellet was found to be devoid of sialic acid) was obtained along with the nonpelletable glycophorin–lipid sonicates.

For determination of the homogeneity of the preparations, the recombinants were subjected to centrifugation on self-generating gradients of Percoll (density range 1.0–1.3 g/mL).

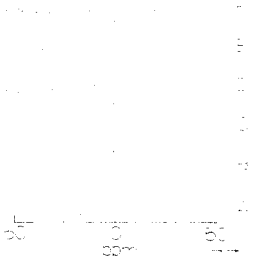

FIGURE 1: Proton-decoupled $^{31}P$ NMR spectra (81.0 MHz) of aqueous cosonicated recombinants of DOPE (~30 μmol) and glycophorin as a function of decreasing glycophorin concentration at 30 °C. (A) 25/1 (moles of lipid per mole of protein); (B) 200/1; (C) 450/1; (D) 800/1; (E) 1300/1; (F) 1600/1.

A blank tube containing Percoll and calibrated density marker beads was simultaneously centrifuged with the tubes containing the lipid–protein sonicates to determine the density of the resulting band(s). The results of this exercise are summarized in Table I. It was observed that following centrifugation only one sharp band was observed in the 200/1 sample. A somewhat broader band was observed for the 450/1 sample, and a broader diffuse band which covered a wider density range resulted from the 800/1 sonicate. Two bands were clearly visible in the 1300/1 sample, with some aggregated clumps of material apparent in the lower denser band. This appearance was even more pronounced in the 1600/1 sample, with the majority of material present as clumps in the lower band. Comparison of these bands with the tubes containing the density marker beads revealed the density of the upper band in the three different DOPE–glycophorin preparations to be >1.0 and <1.017 whereas the lower denser band in the 1300/1 and 1600/1 samples was estimated to lie between 1.017 and 1.029.

*$^{31}P$ NMR.* The use of $^{31}P$ NMR to detect phospholipid molecular organization is well documented [for review, see Cullis & Kruijff (1979) and references contained therein]. Briefly, three major $^{31}P$ NMR lines shapes may be observed when individual lipid species from biological membranes are hydrated. Handshaken liposomes (radius ≥ 2000 Å) exhibit bilayer spectra characterized by a high-field peak and a low-field shoulder separated by ~40 ppm. Hexagonal ($H_{II}$) phase forming lipids exhibit line shapes that are narrower and have a reversed asymmetry compared to the bilayer situation. Last, lipids in inverted micelles, rhombic or cubic configurations, and sonicated vesicles give rise to narrow, symmetric "high-resolution" $^{31}P$ NMR spectra as effectively isotropic motion occurs due to fast lateral diffusion and/or particle tumbling.

The $^{31}P$ NMR spectra obtained from the various DOPE–glycophorin recombinants are presented in Figure 1. The 25/1 sample (Figure 1A) consists of a narrow, symmetrical resonance ($\nu_{1/2}$ = 100 Hz) arising from phospholipids present in small, bilayer vesicles. Freeze–fracture electron microscopy confirmed that the 25/1 preparation consisted entirely of small, unilamellar vesicles (250–400-Å diameter) containing incorporated protein (Figure 2A). A similar, slightly broader ($\nu_{1/2}$ = 140 Hz) spectrum is observed for the 200/1 recombinant which was comprised of larger protein-containing bilayer vesicles (300–1000-Å diameter) (Figure 2B). In the 450/1 sonicate, however, an asymmetry in the isotropic peak is evident with the appearance of a spectral component having a low-field peak and a high-field shoulder which is typical of lipid organized in the hexagonal ($H_{II}$) phase. Freeze–fracturing revealed that vesicles (up to 4000-Å diameter) con-

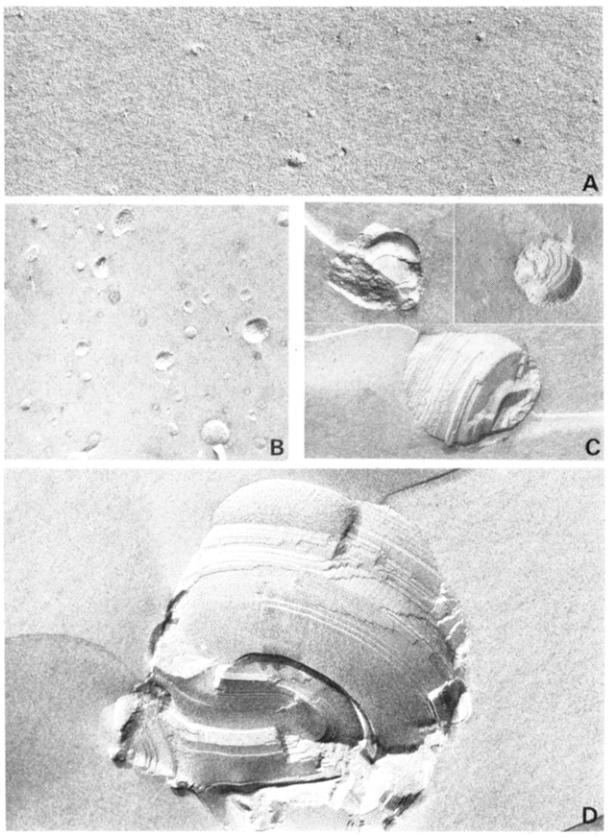


FIGURE 2: Freeze–fracture electron micrographs of cosonicated DOPE–glycophorin (moles of lipid per mole of protein) recombinants. (A) 25/1; (B) 200/1; (C) 450/1; (D) 1600/1. Magnification 40000×. Samples quenched from 30 °C. Glycerol was added prior to quenching to prevent freeze damage.

taining areas of hexagonal ($H_{II}$) phase tubes, lipidic particles, and protein-containing bilayer lipid were present in addition to small, bilayer vesicles (Figure 2C). A further increase in the hexagonal spectral component is observed in the 800/1 and 1300/1 samples (Figure 1D,E) until a situation is reached whereby 90 ± 10% of the lipid is present in a hexagonal ($H_{II}$) organization in the 1600/1 sample (Figure 1F). When viewed by freeze–fracture electron microsocpy, the 1600/1 preparation consisted almost exclusively of extended hexagonal ($H_{II}$) cylinders, with some stabilized regions of bilayer phase containing protein still evident (Figure 2D). The percent of hexagonal ($H_{II}$) phase present in the $^{31}P$ NMR spectrum of each of the sonicates is summarized in Table I. These investigations demonstrate that glycophorin can stabilize only a finite amount of DOPE into a bilayer phase (e.g., up to 200/1), and beyond this ratio the sonicated preparations contain increasing amounts of lipid which is not directly influenced by the protein and is present in a hexagonal ($H_{II}$) phase. On the basis of these results, the 200/1 DOPE/glycophorin sonicate was chosen to be the subject of investigation for the WGA titration experiment.

*WGA Titration.* (*A*) *DOPC–Glycophorin.* The DOPC–glycophorin sonicated recombinant was selected as a control for the WGA titration studies since these vesicles have been biochemically well characterized (van Zoelen et al., 1978a,b) and are similar in composition to a glycophorin–phosphatidylcholine–WGA system previously investigated by Redwood et al. (1975). The effect of wheat germ agglutinin addition on the phospholipid molecular organization of the DOPC–glycophorin vesicles (200/1) was studied by $^{31}P$ NMR. In the absence of WGA, a narrow, symmetric line shape ($\nu_{1/2}$ = 70 Hz) typical of phospholipids present in small, bilayer vesicles

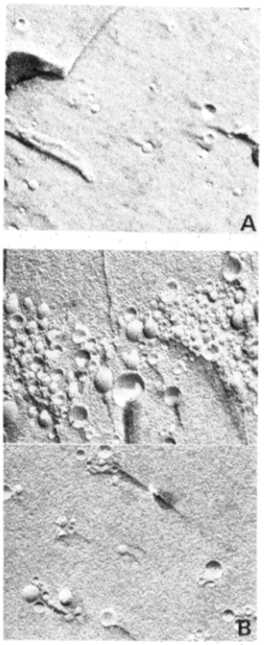


FIGURE 3: Freeze–fracture electron micrographs of cosonicated DOPC–glycophorin vesicles (200/1) in the absence (A) and presence (B) of 250 nmol of WGA (WGA/glycophorin = 1.25). Samples quenched from 30 °C. Glycerol was added prior to quenching to prevent freeze damage.

was observed. The vesicles were estimated to be 250–300 Å in diameter from freeze–fracture electron micrographs (Figure 3). Negligible change in both the visual appearance and $^{31}P$ NMR spectrum of the vesicles was observed after the addition of 50 nmol of WGA (WGA/glycophorin $\simeq$ 0.2 mol/mol). Upon further lectin addition, the sample became cloudy in appearance, and a 40% broadening ($\nu_{1/2}$ = 100 Hz) of the isotropic $^{31}P$ NMR spectrum was observed (WGA/glycophorin = 1.0). The aggregation of the vesicles which results from WGA binding to glycophorin is clearly visualized in Figure 3B. The aggregation process was found to be reversible as addition of the inhibitory sugar hapten *N*-acetyl-D-glucosamine (GlcNac) to the aggregated vesicles resulted in a reversion back to a clear solution having a $^{31}P$ NMR spectrum identical with that originally observed in the absence of WGA. This behavior is consistent with the previous observations of Redwood et al. (1975) that glycophorin liposomes interacted with WGA to form unfused aggregates that could be dissipated by GlcNac addition. The combined NMR and freeze–fracture electron microsopic results clearly demonstrate that the binding and subsequent vesicle aggregation caused by WGA proceed without structural reorganization of the membrane phospholipid bilayer.

(*B*) *DOPE plus Glycophorin.* The $^{31}P$ NMR spectra of DOPE–glycophorin vesicles (200/1) in the absence and presence of varying amounts of WGA are shown in Figure 4. A narrow, symmetric ($\nu_{1/2}$ ~130 Hz) isotropic $^{31}P$ NMR line shape typical of small bilayer vesicles is detected in the absence of WGA (Figure 4A). Addition of a small amount of lectin (WGA/glycophorin = 0.25 mol/mol) induced a cloudiness in the sample accompanied by a 20% increase in the line width ($\nu_{1/2}$ = 160 Hz) of the isotropic NMR spectrum (Figure 4B). This preparation was found to consist of vesicles (1000–2000 Å) comprised of $H_{II}$ phase cylinders, most likely stabilized by a monolayer of lipid containing the protein, lipidic particles and also some bilayer vesicles (Figure 5). Following the next lectin addition (100 nmol of WGA) the visual appearance of the sample drastically changed, and large clumps of material

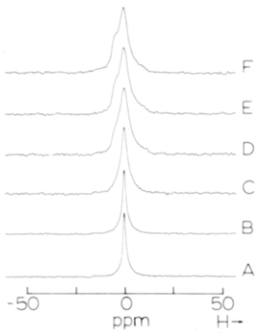


FIGURE 4: Proton-decoupled $^{31}P$ NMR spectra (81.0 MHz) of DOPE–glycophorin vesicles (200/1, 37.5 μmol of DOPE, 7.5 mg of glycophorin) in the absence and presence of WGA at 30 °C. (A) 0 nmol; (B) 50 nmol (WGA/glycophorin = 0.2); (C) 100 nmol (WGA/glycophorin = 0.5); (D) 150 nmol (WGA/glycophorin = 0.75); (E) 200 nmol (WGA/glycophorin = 1.00); (F) 250 nmol (WGA/glycophorin = 1.25). WGA was added to the NMR tube in aliquots from a stock solution (1.1 mM WGA in 100 mM NaCl, 10 mM Tris-HCl, and 0.2 mM EDTA, pH 7.4), vortexed, and allowed to incubate at 30 °C for 15 min prior to data acquisition.

were observed floating in and adhered to the sides of the NMR tube. The NMR spectrum became considerably broader, and an asymmetry could be detected in the isotropic line shape (Figure 4C). As the lectin concentration was further increased (WGA/glycophorin ~1.0), the sample appeared to be highly aggregated, and an NMR line shape typical of lipids organized in an extended hexagonal ($H_{II}$) phase was observed (Figure 4D–F). Our suspicion that WGA triggered a bilayer → hexagonal ($H_{II}$) phase transition in the DOPE–glycophorin vesicles was further confirmed by freeze–fracture electron microscopy which revealed large extended areas of hexagonal ($H_{II}$) phase cylinders whose fracture face was composed of long, parallel lines which had a "ribbed appearance" (Figure 5). Organized rows of lipidic particles that are associated with fusion were also observed in a transitional region between bilayer and hexagonal ($H_{II}$) phase lipid (Figure 5C). Attempts to revert the hexagonal ($H_{II}$) phase material back to the original bilayer situation by adding GlcNac were unsuccessful. Interestingly, spectra 4F containing the highest amount of WGA (WGA/glycophorin ~1.25) also contained a small spectral component (note the low-field shoulder) which typically arises from phospholipids present in large bilayer structures (radius ≥ 2000 Å).

The combination of the $^{31}P$ NMR and freeze–fracture electron microscopy data unequivocally demonstrate that WGA binding to the surface of glycophorin containing model membranes results in a perturbation and complete structural reorganization of the phospholipid matrix.

(*C*) *DOPE–Glycophorin–GlcNac–WGA*. Several studies involving lectin binding in model membrane systems have demonstrated that WGA displays negligible binding to lipid bilayers (Ketis et al., 1980; Ketis & Grant, 1982; Boldt et al., 1977). Further evidence that the bilayer → hexagonal ($H_{II}$) phase transition observed in the DOPE–glycophorin system results from a specific binding of WGA to the carbohydrate portion of glycophorin was provided by $^{31}P$ NMR. Prior to lectin titration, GlcNac, which is known to inhibit agglutination by WGA, was added to the sample (1.5 M, GlcNac/WGA with respect to the highest amount of lectin added was 6500/1 mol/mol). The sample and experimental conditions used were identical with those used in the previous lectin titration experiment. The resultant $^{31}P$ NMR spectrum was broadened by ~40% ($\nu_{1/2}$ 100→140 Hz) compared to the DOPE–glycophorin vesicles in the absence of GlcNac. Addition of WGA in increments to an amount sufficient to induce the

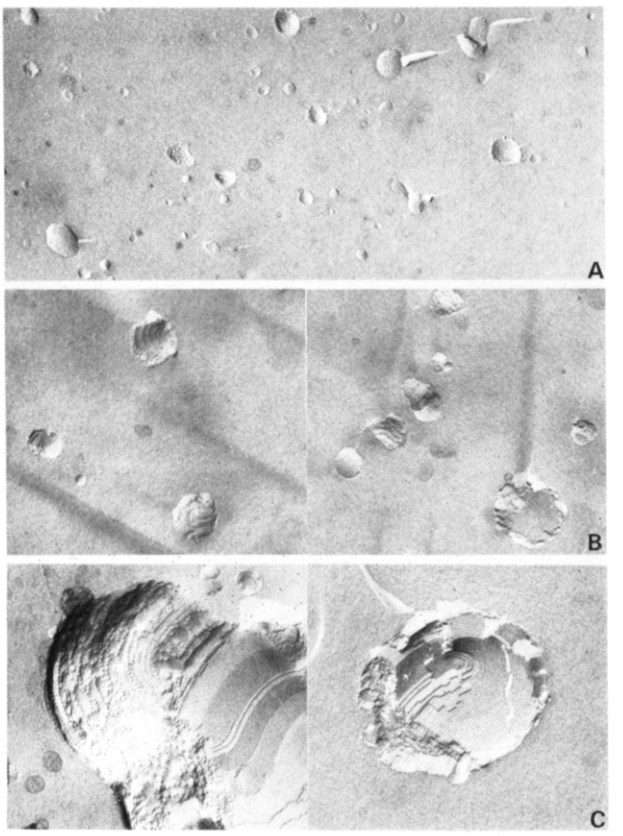


FIGURE 5: Freeze–fracture electron micrographs of cosonicated DOPE–glycophorin vesicles (200/1) in the absence (A) and presence (B) of 50 nmol of WGA (WGA/glycophorin = 0.25) and in the presence of (C) 250 nmol of WGA (WGA/glycophorin = 1.25). Samples quenched from 30 °C. Glycerol was added prior to quenching. Magnification 40000×.

bilayer → hexagonal ($H_{II}$) transition in DOPE–glycophorin vesicles devoid of GlcNac had negligible effect on the vesicles as evidenced from both $^{31}P$ NMR and freeze–fracture electron microscopic examination. This control provides unambiguous evidence that the binding of lectin to the vesicles is mediated solely by the carbohydrate residues of glycophorin.

(*D*) *Enzyme Treatment*. In order to fully characterize the specific origin and nature of the strong and complete bilayer stabilization which was observed in the 25/1 and 200/1 DOPE/glycophorin samples and thus gain insight into the factors responsible for the dramatic changes observed in the DOPE–glycophorin WGA system, the vesicles were initially treated with neuraminidase (50 units/mg of protein) to remove the negatively charged sialic acid residues on glycophorin, and subsequently trypsin was added (5% w/w protein) to remove the bulky, hydrophilic carbohydrate head group residues of glycophorin at the outside of the vesicle. Previous investigations have shown in both DOPC–glycophorin (van Zoelen et al., 1978b) and DOPE–glycophorin (Taraschi et al., 1982) cosonicated vesicles 95 ± 5% of the sugar residues were oriented toward the outside of the vesicles.

The $^{31}P$ NMR spectra obtained for the enzymatically treated vesicles are shown in Figure 6. In both instances (200/1 and 25/1) removal of the sialic acid residues had no effect on the structural integrity of the vesicles (compare parts A and B with parts D and E of Figure 6). This result was further verified by freeze–fracture electron microscopy (data not shown). Marked differences were observed, however, following incubation with trypsin (2 h, 37 °C). In the case of the 200/1 sample, a nearly complete bilayer → hexagonal ($H_{II}$) phase transition was triggered with 95% of the $^{31}P$ NMR

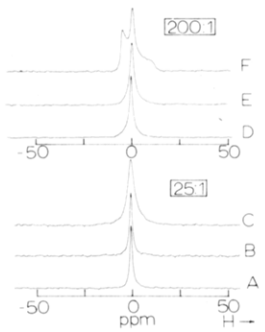


FIGURE 6: Proton-decoupled $^{31}P$ NMR spectra (81.0 MHz) of cosonicated vesicles of DOPE and glycophorin in the absence [(A) 25/1; (D) 200/1] and presence (B and E) of neuraminidase (50 units/mg of protein; 2 h, 37 °C) and in trypsin (C and F) (5% w/w; 2 h, 37 %C) at 30 °C.

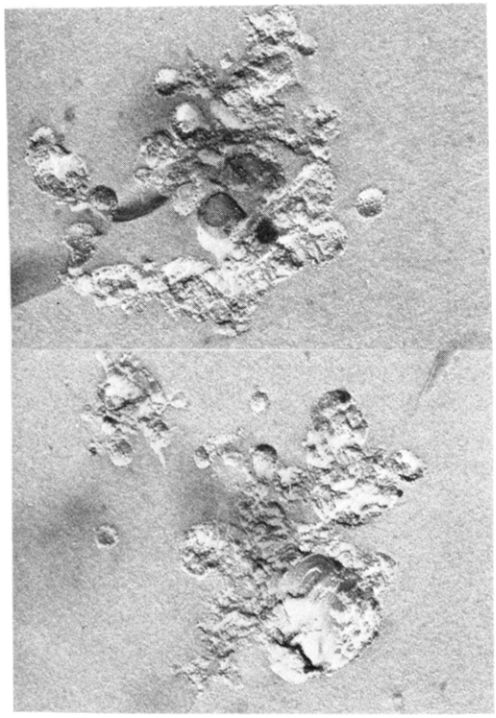

FIGURE 7: Freeze–fracture micrographs of trypsin-treated DOPE–glycophorin vesicles (25/1). Magnification 40000×. Glycerol was added to prevent freeze damage. Quenched from 30 °C.

signal arising from lipid in the hexagonal ($H_{II}$) phase whereas the 25/1 sample showed only an increase in line width of the isotropic NMR line shape ($\nu_{1/2}$ 100→310 Hz). This sample was found to contain bilayer vesicles with larger protein particles than were observed in the nontrypsin-treated vesicles, fused and consequently larger bilayer structures containing large protein particles, and also some small regions of hexagonal ($H_{II}$)phase (Figure 7).

## Discussion

In the current investigation it is demonstrated that binding of wheat germ agglutinin to phospholipid vesicles containing glycophorin results in a drastic reorganization of the phospholipid from a bilayer to nonbilayer [hexagonal ($H_{II}$)] phase. Before the implications of such a remarkable event are considered, a discussion of the factors which stabilize the bilayer organization in DOPE–glycophorin vesicles is warranted.

Unsaturated phosphatidylethanolamines, unlike bilayer-forming lipids like phosphatidylcholines which have a cylindrical shape, possess an overall cone shape due to the relatively small area occupied by the polar head group in comparison to the larger hydrocarbon chain area (Hauser et al., 1981). Consequently, these lipids are more favorably accommodated in a hexagonal ($H_{II}$) arrangement (Cullis & de Kruijff, 1979).

Attempts to produce sonicated vesicles of phosphatidylethanolamine have been unsuccessful except at low ionic strength and high pH (9.4; Stollery & Vail, 1977). In a previous investigation (Taraschi et al., 1982) we reported that cosonication of DOPE and glycophorin (25/1 mol/mol) in aqueous solution (pH 7.4) resulted in the formation of small, stable, unilamellar vesicles. In the current study these results were extended by an investigation into the extent and origin of the bilayer stabilization. $^{31}P$ NMR and freeze–fracture electron microscopic results revealed that a DOPE/glycophorin cosonicated recombinant with an upper molar ratio limit of approximately 200/1 is required to produce a glycophorin-containing vesicle population comprised solely of bilayer vesicles. Assuming a diameter of 1 nm for the $\alpha$-helical membrane-spanning region of glycophorin, it can be roughly estimated that the bilayer stabilizing effect of the protein extends to about five surrounding lipid layers. Beyond this range, the preparations become contaminated with small vesicles containing short, highly curved hexagonal ($H_{II}$) phase tubes (Figure 2). The presence of these tubes, which are enclosed in vesicles stabilized by a monolayer of lipid containing the protein, is manifested as an asymmetry (e.g., the appearance of a low-field peak and high-field shoulder) in the isotropic $^{31}P$ NMR spectrum (Figure 1C). The fact that these structures give rise to hexagonal and not isotropic line shapes is worth noting in that a controversy has arisen in the literature concerning discrepancies which may exist between results obtained by using NMR techniques and other techniques such as freeze–fracture electron microscopy or X-ray diffraction (Hui et al., 1981).

Two bilayer vesicle populations were prepared (200/1 and 25/1) which differed only in protein content in order to gain insight into the origin of the bilayer stabilization observed for the DOPE–glycophorin system. In both cases treatment with neuraminidase, which removes the negatively charged sialic acid residues present on the carbohydrate moieties of glycophorin, had a negligible effect on the vesicles (Figure 6). Drastically different results were obtained, however, following trypsin treatment of the samples. Incubation of glycophorin-containing vesicles with trypsin removes the hydrophilic sugar-containing portions of glycophorin (Tomita & Marchesi, 1975) which in the case of the DOPE–glycophorin vesicles is known to be almost totally (95 ± 5%) oriented toward the outside of the vesicle.

$^{31}P$ NMR and freeze–fracture electron microscopic examination of the vesicles following incubation with trypsin revealed that the 200/1 vesicles underwent a nearly 100% bilayer → hexagonal ($H_{II}$) transition whereas the 25/1 recombinant had a substantially broader ($\nu_{1/2}$ 100→310 Hz) spectral line width but contained small bilayer vesicles with larger protein particles, larger protein-containing fused bilayer structures, and only a negligible amount of lipid in the hexagonal ($H_{II}$) phase. From these results it is obvious that a combination of factors are responsible for the bilayer stabilization. It can be postulated that two closely apposed bilayers must come in contact and fuse with one another to form the hexagonal ($H_{II}$) phase. Removal of the intervesicle repulsion arising from the large, hydrophilic sugar moieties of glycophorin with trypsin allowed the majority (>90%) of the bilayer vesicles (200/1) to fuse and form the hexagonal ($H_{II}$) phase. In addition, the bilayer stabilization may be partially resultant from an ex-

tensive interaction between the hydrophilic head group of the protein and the lipid head groups, which becomes disrupted following treatment with trypsin. Some lipid remains closely associated with the protein and is prevented from undergoing a bilayer → $H_{II}$ phase transition, giving rise to the isotropic NMR signal. That this [the bilayer → hexagonal ($H_{II}$) transition] did not occur in the 25/1 sample must be the result of a direct bilayer stabilization by the intrinsic, hydrophobic portion of the protein, in good agreement with previous observations (Taraschi et al., 1982). Some morphological alteration did occur in the preparation; however, sufficient protein was available to maintain a bilayer organization. Thus, the bilayer stabilization in the DOPE–glycophorin vesicles arises from two factors: at low glycophorin concentration, bilayer stabilization results from intervesicle steric hindrance by the bulky, hydrophilic carbohydrate moieties of glycophorin which present a barrier to fusion; at high glycophorin concentrations, this factor is accompanied by a strong, hydrophobic interaction between the membrane-spanning region of glycophorin and the lipid hydrocarbon chains, which becomes the dominant bilayer stabilizing force. Quantitation of the extent of the hydrophobic stabilizing effect may be possible as a result of ongoing investigations with the hydrophobic peptide of glycophorin.

Wheat germ agglutinin addition to glycophorin–DOPC vesicles induced agglutination of unfused aggregates which could be dissipated by *N*-acetyl-D-glucosamine. Similar observations have been previously reported for intact cell–lectin interactions (Allen et al., 1973; Rapin & Burger, 1974; Sharon & Lis, 1972). In the current investigation the aggregation process was manifested as a 40% increase in the $^{31}P$ NMR line width and visualized by freeze–fracture electron microscopy. It is well established that in small systems such as sonicated vesicles the narrow, symmetric high-resolution $^{31}P$ NMR spectrum which is observed results from isotropic motional averaging arising from lateral diffusion of phospholipids around the vesicle and Brownian tumbling of the entire vesicle (Cullis, 1976). In the fast correlation time limit (e.g., for sonicated vesicles) the line width ($\nu_{1/2}$) varies as

$$\frac{1}{T_2} = M_Z T_c + C \qquad (1)$$

where $1/T_2 = \nu_{1/2}\pi$, $M_Z$ is the residual second moment obtained after averaging due to the restricted anisotropic motion of the lipid in the plane of the membrane is taken into account (Cullis, 1976), $T_c$ is the correlation time for isotropic motion, and $C$ is a portion of the line width ($\nu_{1/2}$) that is not dependent upon vesicle tumbling or lipid lateral diffusion (McLaughlin et al., 1975). The correlation time, $T_c$, for isotropic rotational motion of a spherical vesicle can be related to vesicle size by

$$\frac{1}{T_c} = \frac{6}{r^2}(D_t + D_{diff}) \qquad (2)$$

where $D_t = KT/(8\pi rn)$ is the tumbling dependent part of Brownian rotational diffusion of the lipid molecule in the bilayer (MacKay et al., 1978; Cullis, 1976). By use of a value of $n_{30°C} = 0.7975 \times 10^{-2}$ P for the viscosity (Burnell et al., 1980) and $r = 300$ Å [determined by freeze–fracture electron microscopy; this study and van Zoelen et al. (1978)], $D_t$ was calculated to be $6.97 \times 10^{-8}$ cm$^2$/s. Assuming $D_{diff} = 2.8 \times 10^{-8}$ cm$^2$/s for the diffusion rate of DOPC in vesicles (Galla et al., 1979), $T_c$ was calculated to be $1.530 \times 10^{-5}$ s. Comparison of values obtained for the contributions due to tumbling ($6.97 \times 10^{-8}$ cm$^2$/s) and diffusion ($2.8 \times 10^{-8}$ cm$^2$/s) reveals that for the DOPC–glycophorin vesicles 75% of the contribution to $T_c$ arises from tumbling. Thus, the 40% line-width increase which is observed upon WGA addition can be easily accounted for by a decreased tumbling rate of the large (up to 5000-Å diameter) aggegated units which result.

The WGA-induced bilayer → hexagonal ($H_{II}$) phase transition in the DOPE–glycophorin vesicles was proven to result from a specific lectin–glycophorin interaction as addition of the sugar hapten inhibitor GlcNac prior to lectin titration prevented the transition. In the presence of 1.5 M GlcNac, the resultant $^{31}P$ NMR spectrum was broadened by ~25% compared to that of the DOPE–glycophorin vesicles in the absence of GlcNac. The viscosity of the buffer solution containing the vesicles ($n_{30°C} = 0.8$ cP) is higher in the presence of 1.5 M sugar ($n_{30°C} = 2.5$ cP), leading to a decrease in the rate of tumbling (see eq 2), an increase in the correlaton time ($T_c = 4.59 \times 10^{-8} \rightarrow 8.24 \times 10^{-8}$), and a concomitant increase in the spectral line width ($\nu_{1/2}$ 100→140 Hz). Thus, the increase in line width does not reflect a change in the morphology of the vesicles (freeze–fracturing reveals bilayer vesicles identical with the control vesicles) but rather a decreased rate of vesicle tumbling in the presence of the sugar.

It is widely believed that following effector (e.g., lectin) binding to the carbohydrate receptor, rearrangement of the receptor molecules takes place (Edelman, 1976; Nicolson, 1976). In an elegant study using fluorescent wheat germ agglutinin, Ketis & Grant (1982) observed a phenomenon similar to "receptor patching" in a fluid system comprised of DMPC, glycophorin, and cholesterol, following addition of WGA. This information, in conjunction with the results obtained for the DOPC–glycophorin–WGA and enzymatically treated DOPE–glycophorin systems, provides the basis from which a plausible interpretation of the morphological changes observed in the DOPE–glycophorin–WGA system can be formulated.

Following WGA binding to the DOPE–glycophorin vesicles, cross-linking of the receptor head groups of glycophorin within the individual vesicles by the multivalent WGA results in aggregation of the protein into "patches", thus removing the extensive bilayer stabilizing effect of the previously randomized protein molecules. As a result of this event, large domains of lipid are freed from the influence (both hydrophobic and steric) of the protein and may spontaneously form intrabilayer hexagonal ($H_{II}$) phase cylinders or fuse with protein-free lipid areas present in adjacent vesicles to form the hexagonal ($H_{II}$) phase. It is also likely that concurrent with these processes, intervesicle receptor cross-linking may act to "pull" the vesicles together and induce fusion and consequent $H_{II}$ phase formation. Interestingly, lipidic particles, which have been suggested to represent inverted micellar intermediates formed within one single bilayer or at the nexus of intersecting bilayers during the fusion event (Verkleij et al., 1979; Cullis & de Kruijff, 1979), were observed by freeze–fracture electron microscopy in these preparations (Figure 5C). A small portion of the lipid will always remain associated with and under the influence of the protein and gives rise to the isotropic and bilayer components which are present in the $^{31}P$ NMR spectra in Figure 4. Thus, varying degrees of cellular-mediated protein aggregation in a membrane may trigger lipid phase transitions to nonbilayer structures which may participate in and facilitate certain cellular functional processes.

Nonbilayer lipids have been implicated as key components that mediate cellular events such as fusion, exo- and endocytosis, and transbilayer transport (Cullis & de Kruijff, 1978). The phosphatidylinositol (PI) effect that accompanies receptor-mediated membrane phenomena suggests a direct in-

volvement of the lipid in the cellular response to stimuli (Michell, 1975). It is tempting to speculate that the receptor-mediated increase in cytosol $Ca^{2+}$ levels may result from an ionophoric effect of phosphatidic acid, a product formed following receptor-mediated breakdown of phosphatidylinositol, which has recently been shown to form nonbilayer structures (inverted micelles) under relevant physiological conditions that are postulated to function as membrane ionophores (Verkleij et al., 1982).

It is also conceivable that nonbilayer lipids could play a significant role in the mechanism by which hormones exert their biological effects. Hormones such as insulin and epidermal growth factor, following binding to specific carbohydrates at the cell surface, move into the plane of the membrane to surface regions called "coated pits" and are later endocytotically drawn into the cell where they exert their effect. An effector-induced fusion event, followed by the formation of nonbilayer (e.g., inverted micellar) structures in part of the membrane, could provide a pathway whereby the hormone could pass the hydrophobic interior of the membrane and be endocytotically taken up into the cell cytoplasm. It should be emphasized that while the above-mentioned hypotheses are highly appealing, they are at this point rather speculative.

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# Properties of Clathrin Coat Structures†

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ABSTRACT: Clathrin polymerizes to form characteristic coat structures (baskets) closely resembling those found on coated vesicles. Two sizes of baskets are formed from clathrin, depending on the purity of the preparation and on other factors. A protein of $M_r$ 110 000 has been separated from clathrin by lysine–Sepharose chromatography which is needed for the formation of 150S baskets. In its absence, polymerization results in the larger size baskets, i.e., 300S. Addition of $Ca^{2+}$ or $Mg^{2+}$ stimulates 300S formation in the presence of the 110 000 protein.

The coated pit regions of plasma membranes have been shown to be the site of localization of receptor-bound ligands (conjugated and nonconjugated proteins, nonprotein hormones, etc.) prior to internalization (Goldstein et al., 1979; Brown & Goldstein, 1979; Willingham & Pastan, 1980; Wall et al., 1980). The coated pits have also been implicated in other cellular processes involving membrane surfaces: in membrane recycling (Heuser & Reese, 1973), in intracellular protein translocation (Ockleford & Whyte, 1977; Pearse & Bretscher, 1981), and in exocytosis of newly synthesized protein (Rothman et al., 1980). The transfer of ligands has been assumed to occur by the intermediary of coated vesicles pinched off from coated pits (Goldstein et al., 1979; Brown & Goldstein, 1979; Pearse, 1980, 1982). The details of this processing have been reexamined recently, and a modified mechanism has been suggested (Willingham & Pastan, 1980).

The characteristic coat structure of coated pits and vesicles has been shown by Pearse to be formed principally by one protein called clathrin (Pearse, 1975, 1976; Crowther et al., 1976). The conformation of clathrin is well adapted to form the pentagons and hexagons of the coat since it has three equal arms radiating from a central locus (Ungewickell & Branton, 1981). Clathrin has a sedimentation constant near 8 S and a molecular weight of 610 000 (Pretorius et al., 1981; Ungewickell & Branton, 1981). It is readily released from coated vesicles and can be polymerized to form a polygonal structure closely resembling that found on coated vesicles (Pearse, 1978; Schook et al., 1979; Keen et al., 1979; Woodward & Roth, 1979; Nandi et al., 1980). The re-formed coat structure, in the absence of membrane, is usually referred to as baskets. A second, much smaller protein may form a part of the coat structure (Lisanti et al., 1981; Kurchhausen & Harrison, 1981). A tryptic fragment of clathrin of $M_r$ 110 000 has been shown to be capable of self-associating to form baskets (Schmid et al., 1982).

We have reported elsewhere that when native clathrin is polymerized, two sizes of baskets, i.e., 150S and 300S, are produced (Pretorius et al., 1981) with average molecular weights of $25 \times 10^6$ and $100 \times 10^6$, respectively (Nandi et al., 1980). We have now tried to determine the factors and conditions that influence the size of the basket formed by polymerization of clathrin in slightly acidic solutions. With the present method of isolating coated vesicles, a preparation of clathrin is obtained which gives only 150S. We have fractionated clathrin on a lysine–Sepharose column and isolated a protein fraction which appears to be needed for the formation of the 150S basket.

## Materials and Methods

*Preparation of Clathrin.* Coated vesicles were prepared by a procedure based on that published by Pearse (1975), which, however, avoided high concentrations of sucrose; it is to be described in another report on the properties of coated vesicles (Nandi et al., 1982). The vesicles gave a single, symmetrical band on sucrose gradients (10–30%) and sedimented with an average $s_{20,w} \simeq 200$ S.

Clathrin was solubilized from this homogeneous preparation of coated vesicles by extracting in a 2 M urea solution, pH 8.0, 10 mM Tris,[1] and 5 mM $NaN_3$ for 2 h. The solution was then dialyzed for 4 h against 10 mM Tris, pH 8.0, and 5 mM $NaN_3$ in order to remove urea and then centrifuged at 150000*g* for 70 min. Velocity centrifugation of the supernatant showed a single moving boundary with a sedimentation coefficient ($s_{20,w}$) typical of native clathrin, i.e., ~8 S. The protein composition of this preparation was analyzed by $NaDodSO_4$ gel electrophoresis in overloaded gels (50–100 μg of protein) (Figure 1, gel on left) by the technique reported previously with 8% gels and 5% stacking gels (Nandi et al., 1980). Smaller amounts of nonclathrin components were present than in earlier clathrin preparations.

*Lysine–Sepharose Column.* Clathrin could be fractionated somewhat further by chromatography on a lysine–Sepharose

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[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; LS-clathrin, clathrin isolated from lysine–Sepharose column in 0.075 M NaCl; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate.