PROPERTIES OF GLYCOPHORIN A AND ITS DEGLYCOSYLATED DERIVATIVES; PHOSPHOLIPID VESICLE FORMATION AND THE INCORPORATION OF INTEGRAL MEMBRANE PROTEINS

by

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Date: March 17, 1981
Approved:

[Signatures]

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in the Graduate School of Duke University

1981
ABSTRACT

(Biochemistry)

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The physical properties of native and deglycosylated glycophorin A and of membrane protein reconstituted vesicles were investigated. The goals of these studies were twofold. First, solutions containing glycophorin A were examined prior to and following removal of covalently attached carbohydrate from the protein to determine the contribution of carbohydrate to the glycoprotein amphiphilic properties. Second, the properties of glycophorin reconstituted vesicles were studied to address the question: Does the incorporation of a transmembrane protein into a membrane vesicle significantly increase bilayer premeability to ions?

Glycophorin A, the major sialoglycoprotein of the human erythrocyte membrane, is approximately 31,000 daltons: 55% by weight is carbohydrate and 45% is protein. Neuraminidase was used to remove 99% of the sialic acid producing asialoglycoprotein and endo-α-N-acetylgalactosaminidase was used to remove 90% of the serine and threonine linked carbohydrate chains producing apoglycophorin A.

Removal of carbohydrate results in increased binding of sodium dodecyl sulfate (SDS) to the polypeptide chain at saturating SDS concentrations. This
increased binding indicates that sialic acid residues exhibit little or no SDS binding and actually inhibit SDS binding to the peptide chain. In detergent free solutions, native, asialo, and apoglycophorin are heterogeneously aggregated. SDS associates with aggregated glycophorin below its critical micelle concentration indicating the presence of noncooperative monomer binding sites; however, as with other membrane proteins, the association of SDS to glycophorin occurs principally as a cooperative transition above the critical micelle concentration. The additional binding of SDS to the deglycosylated forms does not produce significant changes in the peptide conformation as measured by circular dichroism.

Removal of carbohydrate also produces corresponding changes in molecular weight, electrophoretic mobility on SDS gels, and distribution coefficients on gel chromatographic columns. At saturating levels of SDS binding, glycophorin and its deglycosylated derivatives were determined to be predominantly monomeric by sedimentation equilibrium. The relative mobility of the three forms on SDS gels increases with increasing carbohydrate removal. Relative mobility depends upon ionic strength, SDS concentration, and protein concentration. Native glycophorin and apoglycophorin in solution as a 1:1 molar mixture behave as noninteracting species by sedimentation equilibrium and gel filtration.

Glycophorin incorporated vesicles were prepared by removal of detergent from solutions containing mixed micelles of egg phosphatidylcholine, octyl glucoside, and purified glycophorin. Glycophorin was not uniquely oriented in the membrane as shown by trypsin and neuraminidase treatment of vesicles. Vesicles that were formed in the presence or absence of glycophorin were similar in size and morphology (2300 ± 400 Å in diameter) as imaged in the electron microscope. The permeability of these large unilamellar vesicles to
Cl⁻, Na⁺, and Rb⁺ followed first order kinetics with rate constants of $2.1 \times 10^{-5}$, $2.6 \times 10^{-7}$, and $9.0 \times 10^{-7}$, respectively. The presence of glycophorin in the membrane at levels up to 220 copies per vesicle increased the permeability by less than a factor of five. This demonstrated that incorporation of membrane protein into vesicles does not necessarily lead to dramatic increases in membrane ion permeability, and therefore; this methodology may provide a useful control in the reconstitution of ion pumps into vesicles.

To determine whether some biological activities can be maintained or restored under these conditions two integral membrane proteins, dopamine-β-hydroxylase and hepatic asialoglycoprotein receptor, were incorporated using similar procedures. These proteins were found to either retain or regain activity upon incorporation into vesicles. Both proteins are oriented in these vesicles such that all substrate binding sites are exposed on the vesicle exterior.
ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. Jacqueline Reynolds for her continued guidance and support over the past four years. I would also like to thank Dr. Charles Tanford for his instruction and counsel. A special debt of gratitude is due to Dr. Lowry Glasgow, Dr. Guido Zampighi, Dr. Yas Nozaki, Dr. Joseph Albanesi, and Dr. Tom Andersen for their collaborations on various experiments.

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LIST OF ABBREVIATIONS

A
ASOR
BSA
C_{12}E_8
CMC
DβH
DOC
EDTA
egg PC
Fuc
Gal
GalNAc
GlcNAc
HAR
Man
NeuAc
θ
OD
OG
r
SDS
TES
Tris

Absorbance
Asialoorosomucoid
Bovine serum albumin
Dodecyl octaoxyethylene glycol monoether
Critical micelle concentration
Dopamine-β-hydroxylase
Sodium deoxycholate
Ethylendiaminetetraacetic acid
Egg yolk phosphatidylcholine
Fucose
Galactose
N-acetylgalactosamine
N-acetylglucosamine
Hepatic asialoglycoprotein receptor
Mannose
N-acetylneuraminic acid
Molar ellipticity
Optical density
Octyl glucoside
Radial distance
Sodium dodecyl sulfate
N-tris (hydroxymethyl)methyl-2-aminomethane-sulfonic acid
Tris (hydroxymethyl) amino methane
Every living cell is bounded by a plasma membrane. This structure is a phospholipid bilayer interspersed with many proteins and serves as a selective permeability barrier between the cell's internal constituents and the external environment. A pure lipid bilayer provides only a nonspecific hydrophobic barrier to water-soluble molecules; thus, it is the protein interspersed in the bilayer which provides the functional specificity of the membrane and permits passage of specific information between the cell interior and exterior.

Membrane proteins are important mediators of all cell interaction and communication. They act as specific receptors for large and small chemical species (from low density lipoprotein to $K^+$), and as enzymes, channels, or pumps to move molecules across the bilayer. The mechanism by which a plasma membrane function can be understood only through studies of these specific proteins.

Membrane proteins have been classified operationally as either peripheral or integral (Green, 1972). Peripheral (extrinsic) membrane proteins can be removed from the membrane easily by manipulating the ionic strength of the suspending medium. These proteins are presumably associated with the membrane by polar bonds on the bilayer surface. The integral (intrinsic) membrane proteins cannot be extracted unless the entire membrane is

To my family and friends
CHAPTER I
INTRODUCTION

Every living cell is bounded by a plasma membrane. This structure is a phospholipid bilayer interspersed with many proteins and serves as a selective permeability barrier between the cell's internal constituents and the external environment. A pure lipid bilayer provides only a nonspecific hydrophobic barrier to water soluble molecules; thus, it is the protein interposed in the bilayer which provides the functional specificity of the membrane and permits passage of specific information between the cell interior and exterior.

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disrupted by detergent, organic solvent, or chaotropic agents, suggesting that they are deeply embedded in the membrane. Many of these integral membrane proteins are glycoproteins and are oriented in the membrane such that the bound carbohydrate is always located on the external surface of the cell. For many cells this surface sugar is believed to be involved in intercellular contact and recognition; however, in other cells, such as the red cell, the role of bound carbohydrate is not known.

There are many approaches to the study of integral membrane proteins ranging from electron microscopy of intact membranes to chemical composition analysis of isolated proteins. The approach taken in this and many other laboratories is to isolate and characterize proteins in detergent solutions. Subsequent reconstitution of these proteins into lipid vesicles then allows the study of structural and functional features that require compartmentalization and orientation. Using this approach, we chose to investigate two important questions:

1. How do bound carbohydrate moieties contribute to the structural and physical properties of an integral membrane protein?

2. Does the incorporation of a transmembrane protein significantly increase the passive permeability of the bilayer to ions? Therefore, can such vesicles be employed for specific ion transport measurements?

Role of bound carbohydrate

Glycoproteins are ubiquitous in living organisms; most serum proteins and many integral membrane proteins are glycoproteins (Marshall, 1972). The sugar content varies from only a small weight fraction of the protein as in Band 3 from erythrocytes (5-10%) to a significant weight fraction as in glycoporphin (55%). Since oligosaccharide structures are rigid, branched, and very water soluble, their presence might be expected to alter significantly
the protein structure and properties. Unfortunately, the effect of bound sugar residues on the properties of most proteins remains essentially unknown. The role of sugar may be investigated by deglycosylating the protein and then comparing the solution properties of the native and deglycosylated protein. In most cases, deglycosylation has been accomplished by specific glycosidase cleavage in vitro or by the use of tunicamycin, an antibiotic which specifically inhibits de novo oligosaccharide chain biosynthesis in cells. Most glycoproteins possessing enzymatic activity have relatively small amounts of bound carbohydrate (5-20%). All studies to date have shown that carbohydrate removal has no effect on these enzymatic activities (Tarentino et al., 1974; Wang and Hirs, 1977). Small changes in some properties, e.g. tyrosine exposure to water (Wang and Hirs, 1977), susceptibility to proteolysis and denaturation (Olden et al., 1978), and electrophoretic mobility on gels (Leach et al., 1980) have been observed. In one case the glycoprotein, hepatic asialoglycoprotein receptor, loses lectin binding activity upon removal of its terminal sugar residues (Hudgin et al., 1974; Paulson et al., 1977). As discussed in Chapter V, this inhibition of activity occurs because the protein binds its own sugar chains. In glycoproteins containing considerable weight ratios of carbohydrate, one might expect larger changes in protein properties when sugar is removed. This has been shown to be the case for two water soluble glycoproteins. Dramatic decreases in the ability of antifreeze glycoprotein of arctic fish to lower the freezing point of water (Ahmed et al., 1973) and decreases in the viscosity of solutions containing submaxillary mucin (Gottschalk and Thomas, 1961) are observed upon carbohydrate cleavage from the polypeptide. Sugar residues appear to be involved in mucin aggregation, since the native protein is probably a tetramer whereas the deglycosylated protein is monomeric in solution (Hill et al., 1977). Some
glycoproteins have been shown to behave anomalously in certain transport measurements (Leach et al., 1980). These anomalies will be discussed in greater detail in Chapter III.

Most studies on the role of carbohydrates in membrane glycoproteins have been concerned with the biosynthesis and expression on the cell surface of a membrane protein lacking its full complement of oligosaccharide (Olden et al., 1978; Olden et al., 1979; Leavitt et al., 1977; Gibson et al., 1979; Helfetz and Lennarz, 1979). This deficiency in bound carbohydrate is produced by incubating cells with tunicamycin. It has been suggested that the carbohydrate may act as a "ticket" permitting transport of the protein from its site of biosynthesis to the plasma membrane, since some proteins are not transported efficiently to the surface when sugar biosynthesis is inhibited (Leavitt et al., 1977). Leavitt has evidence that protein solubility properties also may be affected. In this respect, it is important to understand the effect of carbohydrate on the amphiphilic properties of a membrane glycoprotein.

Reconstitution of integral membrane proteins

Many functions of membrane proteins, e.g. ion transport or membrane internalization processes, can be studied only in intact cells or in analogous systems where two aqueous compartments are separated by a membrane as in vesicles. While vesicles of defined protein and lipid composition can be produced, the asymmetry of the native membrane may not be maintained. Protein incorporated into vesicles may be inside out or randomly oriented with respect to the native membrane (Goldin, 1977). Functional asymmetry may be imposed on the system by adding impermeable inhibitors or substrates to one side of the membrane. Several ion transport proteins have been reconstituted
into vesicles and examined in some detail; however, few questions concerning mechanism and control of pump action have been resolved by such procedures. Answering these types of questions requires a system in which transport activity, size, structure, ion permeability and polypeptide orientation and content of the vesicles are defined. Kagawa and Racker (1971) and others have shown that many membrane proteins can be incorporated into vesicles and thereby retain or regain some function. Attempts to incorporate ion pumps, e.g. the Ca\(^{+2}\) transporting ATPase from sarcoplasmic reticulum, often lead to incorporation of only a small fraction of the original ATPase activity, uncoupling, or low efficiency of ion transport activity measured as ions transported/ATP hydrolysed (Meissner et al., 1973; Repke et al., 1976; Racker, 1972). Low pumping efficiency may be due to inactivation of protein, removal of factors necessary for pumping, presence of vesicles that are very permeable to ions, etc. In this respect, it is especially important to measure the passive ion permeability of vesicles and to determine whether the incorporation of a membrane protein increases ion permeability nonspecifically.

Scope of this work

Glycophorin, the major sialoglycoprotein of the red cell membrane, was chosen to examine the questions posed above because (1) it may be easily isolated in large quantities; (2) the primary structure of both protein and carbohydrate moieties have been determined, and enzymes are available to cleave the carbohydrate linkages; and (3) it has been shown to have a transmembrane orientation in the native membrane.

In the first part of this thesis, the contribution of bound carbohydrate was evaluated by comparing some solution properties of native and deglycosylated glycophorin, including amphiphile binding, peptide chain conformation, electrophoretic mobility, and protein association state. Through
these studies a better understanding of protein-protein and protein-amphiphile interactions relevant to the type of association occurring in the native membrane may be attained.

In the second part of the thesis, vesicles incorporating glycophorin were prepared from solutions containing egg phosphatidylcholine, octylglucoside, and protein. These vesicles were characterized with respect to size, protein orientation, protein composition, and passive ion permeability. To assure that biological activity could be regained or maintained in this reconstitution procedure, two other integral membrane proteins possessing measurable biological activity were incorporated into vesicles and studied similarly. The information obtained is useful not only in revealing the interaction of these proteins with membranes, but also for future reconstitutions of ion transport proteins.

Before describing this work further, the present knowledge regarding glycophorin will be reviewed with emphasis on the characteristics it shares with other glycoproteins and integral membrane proteins.

Glycophorin

General information

Glycophorin is the primary carrier of cell surface carbohydrate in the human red cell. Although it is present in the membrane at about 750,000 copies per cell, its physiological function is not known.

Glycophorin was the first integral membrane protein for which both the amino acid sequence and topology in the membrane were determined (Marchesi et al., 1976; Furthmayr, 1977a). It has a molecular weight of 31,000 daltons, 55% of which is carbohydrate and 45% protein. The peptide sequence of 131 amino acids (shown in Figure 1) is divided into 3 domains: 2 hydrophilic segments separated by a stretch of 23 nonpolar amino acids. As shown in
Fig. 1. Amino acid sequence of glycophorin A from Tomita et al. (1978). CHO indicates the amino acid residues to which oligosaccharide chains are attached. There are two glycophorin allotypes in the human population termed M and N which have differences at positions 1 and 5. The residues in parentheses on top correspond to the N allotype and those below them correspond to the M allotype.
Figure 2, the amino terminal domain contains all bound carbohydrate and exists on the outside of the cell, the hydrophobic domain penetrates and spans the bilayer, and the carboxy terminal sequence is located on the cell interior (Gottsch et al., 1977; Meister et al., 1976). Glycophorin was the first of many integral membrane proteins shown to contain a hydrophobic domain (Segrest et al., 1973; Segrest, 1977). Analogous domains have been found in all such proteins sequenced to date, including the G domain protein.

[Diagram of protein sequence]

(Leu) - Ser - Thr - Thr - (Glu) - Val - Ala - Met - His - Thr - Thr - Ser - Ser - Val - Ser - Lys - Ser - Tyr - 10

Ser - Thr - Thr - Ser - Ser - Val - Ser - Lys - Ser - Tyr - 20

Ile - Ser - Ser - Glu - Thr - Asn - Asp - Thr - His - Lys - Arg - Asp - Thr - Tyr - Ala - Ala - Thr - Pro - Arg - Ala 30

His - Glu - Val - Ser - Glu - Ile - Ser - Val - Arg - Thr - Val - Tyr - Pro - Pro - Glu - Glu - Glu - Thr - Gly - Glu 40

Arg - Val - Gln - Leu - Ala - His - Phe - Ser - Glu - Ile - Glu - Ile - Thr - Leu - Ile - Ile - Phe - Gly - Val 50

Ile - Val - Gln - Leu - Ala - His - Phe - Ser - Glu - Ile - Glu - Ile - Thr - Leu - Ile - Ser - Tyr - Gly - Ile - Arg - Arg - Leu - Ile - Lys 60

Met - Ala - Gly - Val - Ile - Gly - Thr - Ile - Leu - Ile - Leu - Ile - Ser - Tyr - Gly - Ile - Arg - Arg - Leu - Ile - Lys 70

Lys - Ser - Pro - Ser - Asp - Val - Lys - Pro - Leu - Pro - Ser - Pro - Asp - Thr - Asp - Val - Pro - Leu - Ser - Ser 80

Val - Gly - Ile - Glu - Asp - Pro - Glu - Thr - Ser - Asp - Gly - Lys - 90

Ser - Asp - Val - Lys - Pro - Leu - Pro - Ser - Pro - Asp - Thr - Asp - Val - Pro - Leu - Ser - Ser 100

Val - Glu - Ile - Glu - Asp - Pro - Glu - Thr - Ser - Asp - Gly - Lys - 110

The significance of this linkage is not known. The carboxy terminal domain is composed of 36 amino acids, six of which are charged. On the basis of differential labeling and proteolytic cleavage of carboxy terminal peptides in lysis and intact red cell ghosts, Blatchler
Figure 2, the amino terminal domain contains all bound carbohydrate and exists on the outside of the cell, the hydrophobic domain penetrates and spans the bilayer, and the carboxy terminal sequence is located on the cell interior (Cotmore \textit{et al.}, 1977; Marchesi \textit{et al.}, 1976). Glycophorin was the first of many integral membrane proteins shown to contain a hydrophobic domain (Segrest \textit{et al.}, 1973; Segrest, 1977). Analogous domains have been found in all such proteins sequenced to date, including the fd phage protein (Nakashima and Konigsberg, 1974), bacteriorhodopsin (Khorana, \textit{et al.}, 1979; Orchinikov \textit{et al.}, 1979), HLA antigen, cytochrome b\textsubscript{5} (Ozols and Gerard, 1977; Fleming \textit{et al.}, 1978), membrane Ig\textsubscript{M} (Kehry \textit{et al.}, 1980), and Semliki forest viral coat proteins (Garoff \textit{et al.}, 1980). The existence of these domains may distinguish integral membrane proteins from others. It is likely, however, that some integral membrane protein may lack long hydrophobic sequences but instead contain sequences that fold to produce hydrophobic surfaces which associate with the bilayer.

The amino terminal domain of glycophorin contains a high percentage of serine and threonine residues. Fifteen of these are covalently linked to a tetrasaccharide, the structure of which is shown in Figure 3 (Marchesi \textit{et al.}, 1976 and Thomas and Winzler, 1969). An additional oligosaccharide chain is bound to asparagine 26 and its structure (shown in Figure 3) was determined recently (Yoshima \textit{et al.}, 1980). These carbohydrate structures are typical of many O-linked or N-linked chains. One unique feature of the N-linked chain is the N-acetylglucosamine attached $\beta$1-4 to mannose at the carbohydrate chain branch point. The significance of this linkage is not known.

The carboxy terminal domain is composed of 36 amino acids many of which are charged. On the basis of differential labeling and proteolytic cleavage of carboxy terminal peptides in leaky and intact red cell ghosts, Bretcher
Fig. 2. Topology of glycophorin in the membrane. Glycophorin is a transmembrane protein with the sugar moieties facing the outside of the cell. The nonpolar region from Ile$^{73}$ through Ile$^{95}$ passes through the bilayer, and the carboxyterminus is situated inside the cell. (Taken from Cantor and Schimmel, 1980)
Cell membrane

1
73
95
131
Fig. 3. Carbohydrate structures of glycophorin. Fifteen O-linked chains (top) and one asparagine linked chain (bottom) exist per glycophorin molecule. The amino acid residues to which they are attached are indicated in Fig. 1. O-linked and asparagine linked sequences were determined by Thomas and Winzler (1969) and by Yoshima et al. (1980), respectively.
NeuAc2-6GlcNAc1-3\[4\]Galβ1-3GalNAc1-O-Thr/Ser

NeuAc2-6Galβ1-4GlcNAcβ1-Manβ1-3(6)Manβ1-4GlcNAcβ1-4GlcNAc-Asn

Fucα1-6

CHO

CHO
(1971) and others (Segrest et al., 1973) have concluded that this portion of the glycophorin molecule is located on the inside of the cell. This was confirmed by the observation of Cotmore et al. (1977) that ferritin-conjugated antibodies directed against the carboxy terminal peptides were found exclusively on the cytoplasmic side of leaky ghosts. This was the first definitive proof that an integral membrane protein can be oriented to span the membrane. One might predict from this membrane spanning orientation that glycophorin could act to transmit signals across the bilayer; however, its physiological function remains a mystery. In fact, individuals who completely lack glycophorin, En(a−) blood type, show no physiological signs of any deficiency (Gahmberg et al., 1976).

Structure of the protein in solution and in the membrane

The amino terminal peptide of glycophorin contains virtually no helix or β structure, being predominantly in an unordered form as determined by circular dichroism analysis (Schulte and Marchesi, 1979). "Unordered" is not meant to imply random structure, because it is possible that the presence of large amounts of carbohydrate and the high charge density from sialic acid residues may constrain the peptide chain in a rather specific manner. Similarly, the carboxy terminal peptides have little apparent structure. This is not surprising considering the large number of prolines and negatively charged residues which would serve as helix and β sheet breakers. A tryptic peptide of glycophorin, Tis, contains the hydrophobic region and is water insoluble. It has therefore been studied in organic solvents and in anionic detergent solutions. The CD spectra indicates that this fragment is 75 to 100% α helical (Segrest, 1977; Schulte and Marchesi, 1979), and this amount of helix would account for all of the helical structures estimated for intact
glycophorin (which in water is about 27% helix). This evidence by no means proves that the protein is helical in the bilayer, since the conditions for solubilization also induce helix formation. It is interesting that secondary structure predictions from amino acid sequence of this region using the rules of Chou and Fasman (1974) would indicate a β sheet conformation.

Detailed structural information for an integral membrane protein has been obtained only in the case of bacteriorhodopsin which exists as a two dimensional crystalline array in the membrane. A three dimensional structure of the repeating unit cell of this array was determined using transmission electron microscopy and electron diffraction. Henderson and Unwin (1975) found that bacteriorhodopsin consisted of 7 short helical domains lying side by side in the membrane. Whether other membrane proteins exist in the bilayer as helices has not been determined to date. Two dimensional arrays of glycophorin have not been produced and, therefore, analysis of its structure by this method is not yet possible.

Associations of glycophorin in vivo

Little is known about the specific interactions between phospholipid and proteins and between proteins themselves in the red cell membrane. Armitage et al. (1977) have shown that diphosphoinositide remains tightly bound to glycophorin after extraction of the membrane with lithium diiodosalicylate (LIS) and phenol. The lipid moiety as well as residual LIS are preferentially bound to the Tis fragment (Romans and Segrest, 1978). This evidence coupled with the fact that this peptide can be incorporated into phosphatidylcholine vesicles (Segrest et al., 1974) further demonstrates, as was indicated from the sequence, that the Tis fragment contains the hydrophobic portion of the protein and that this portion is responsible for membrane attachment. Furthermore, glycophorin molecules perturb lipid in model
phospholipid bilayers. This perturbation has been measured by line width broadening of C\textsuperscript{13} NMR spectra of lipid (Utsumi et al., 1980) and by changes in the cooperative gel to liquid crystalline phase transition of the bilayer during differential scanning calorimetry (van Zoelen et al., 1978).

The most convincing evidence that glycophorin interacts with other membrane proteins in the plane of the bilayer comes from rotational diffusion measurements (Nigg et al., 1980). These measurements of labeled Band 3, a major integral membrane of the red cell, were performed in the presence and absence of glycophorin A antibodies. Since intact antibodies but not Fab fragments act to partially immobilize Band 3, Nigg et al. (1980) conclude that preexisting Band 3-glycophorin complexes are crosslinked in the bilayer. Nothing is known about the state of association of glycophorin with itself. Some authors have suggested that intramembranous particles seen by freeze fracture of red cell membranes contain complexes of glycophorin A and Band 3 (Pinto de Silva and Nicolson, 1974). However, the membranes of En(a-) blood group erythrocytes which entirely lack glycophorin A have a normal distribution and number of particles (Gahmberg et al., 1976). Chemical crosslinking studies have yielded equivocal results (Ji, 1974). There is also no direct evidence for interaction between glycophorin and the red cell cytoskeleton, which is found closely apposed to the cytoplasmic surface of the bilayer (Bennett and Stenbuck, 1979).

**Associations of glycophorin in solution**

Like several viral membrane glycoproteins, glycophorin is soluble but highly aggregated in aqueous, detergent free buffer forming species of several hundred thousand daltons. (See Chapter III, also Verpoorte, 1975). The hydrophobic region is believed to be largely responsible for this association
since nonionic detergents bind glycophorin and significantly reduce the size of these aggregates (Furthmayr, 1977b). Membrane proteins like cytochrome b$_5$ and the coat glycoprotein of the Semliki Forest virus form micellar type structures with their hydrophobic portions sequestered together. These regions are excluded from water with the water soluble peptide portions forming a hydrophilic "shell" around them. These structures may appear as rosettes when negatively stained and imaged by electron microscopy. A similar arrangement may exist for glycophorin.

Because of this aggregation, glycophorin is usually purified from other red cell membrane glycoproteins by gel filtration in the presence of Ammonyx 10 or SDS (see Chapter II). Two of these other glycoproteins have been termed glycophorin B and C (Furthmayr, 1978a; Furthmayr, 1978b); however, in this thesis, only purified glycophorin A was studied. Therefore, all references to glycophorin are exclusively glycophorin A unless specified otherwise.

Detergents may provide the membrane protein with a microenvironment that mimics the lipid bilayer in having hydrophobic and hydrophilic regions. Thus the association of amphiphile with protein in solution may be illustrative of interactions of lipid with protein. The binding of SDS to glycophorin is unlike that to water soluble proteins. With the exception of serum albumin (Reynolds et al., 1967) and β-lactalbumin (Jones and Wilkinson, 1976), water soluble proteins in their native state do not have high affinity binding sites for SDS. In the range of $10^{-4}$ to $10^{-5}$ M unbound detergent almost all such proteins cooperatively bind a large number of detergent molecules and undergo a concomitant conformational change. These changes result in saturation binding of SDS to water soluble polypeptides having reduced disulfide bonds of about 1.4 g SDS/g protein; lower weight ratios are seen in
unreduced proteins (Reynolds and Tanford, 1970; Pitt-Rivers and Impiombato, 1968). For intrinsic membrane proteins, e.g. cytochrome b₅ (Robinson and Tanford, 1975), Ca⁺² ATPase of sarcoplasmic reticulum (Nozaki and Tanford, 1979), coat protein of bacteriophage fl (Makino et al., 1975), and glycoporphin (Grefrath and Reynolds, 1974), cooperative binding occurs at the detergent critical micelle concentration (CMC), not below it as with water soluble proteins. The amount of SDS bound at saturation levels is generally consistent with one or more micelles being bound. A shift in the detergent CMC which is brought about by a change in buffer ionic strength results in a corresponding shift in the binding isotherm (Grefrath and Reynolds, 1974). Therefore, this cooperative binding is independent of monomeric concentration and is a consequence of SDS micellization. This "comicellization" process is primarily due to association of SDS with the hydrophobic regions of the protein; however, unlike water soluble proteins the region may not be denatured in the process as indicated by circular dichroism studies. At saturating levels of SDS binding, Grefrath and Reynolds (1974) found that glycoporphin binds 6±1 g SDS/g polypeptide. A considerably lower binding level was determined in this thesis work and by others (Egmond et al., 1978). This binding will be discussed with respect to other glycoproteins and integral membrane proteins in Chapter III.

Another anionic detergent, sodium deoxycholate (DOC), also binds glycoporphin cooperatively at its CMC. It is important to note that significant binding of both DOC and SDS occur below their CMC, indicating the presence of one or more high affinity sites for monomeric detergent (Grefrath, 1974).

Association state of glycoporphin in SDS

A large number of studies have been directed toward understanding the
state of association of glycophorin in SDS. Grefrath and Reynolds (1974), using rigorous methods to determine the molecular weight of glycophorin at saturating levels of SDS binding, found that the protein exists as a monomer.

Much confusion has been generated from studies of the electrophoretic mobility of this protein on SDS polyacrylamide gels. Glycophorin was first identified by its migration on gels and was visualized with periodic acid-Schiff's reagent (PAS) which stains carbohydrate and is particularly sensitive to sialic acid residues (Fairbanks et al., 1971). The results from many labs (Potempa and Garvin, 1977; Furthmayr and Marchesi, 1976) indicate that under various conditions glycophorin may migrate as PAS 1 band, as PAS 2 band or as a "species" of intermediate mobility termed PAS 4. (See Figure 30.) The band or bands produced depends on SDS concentration, temperature, ionic strength and protein concentration of the sample and gel buffers. Furthmayr and Marchesi (1976) have suggested that PAS 1 is a glycophorin dimer and that PAS 2 is the monomer form. Dohnal et al. (1980) claim that PAS 4 is a unique monomer species distinct from PAS 2. In fact, gels containing only PAS 1, only PAS 2, only PAS 4, PAS 1 and PAS 2, or PAS 1, 2, and 4 have been reported. The problem of which association state of glycophorin is represented by a particular band is unresolved at present, but will be discussed in the Appendix.

Order of presentation

The results of this thesis are presented in the following order:

Chapter II describes the purification and preparation of glycophorin A and its deglycosylated derivatives. The amino acid and sugar composition of these species were examined to quantitate sugar removal and to determine that the peptide backbone remained intact during enzymatic digestion.
Chapter III examines some solution properties of glycophorin and its derivatives, e.g., SDS binding, peptide chain conformation, electrophoretic mobility and protein association state. The effect of bound carbohydrate on these glycophorin properties will be evaluated. The association of SDS with glycophorin is compared to that of other glycoproteins and integral membrane proteins.

An Appendix at the end of the thesis provides additional information regarding the association state of glycophorin in SDS containing solutions. Attempts are made to account for the multiple bands seen on SDS gels and to identify factors affecting interconversion of these bands. Possible interaction between native and apoglycophorin are also investigated.

Chapter IV describes a procedure for producing phospholipid vesicles and for incorporating glycophorin into them. These vesicles are characterized with respect to vesicle size and shape, amount of protein incorporated, and protein orientation. A theoretical discussion of vesicle formation and protein incorporation from solutions containing mixed micelles is given.

Chapter V is an extension of this work, in which two other integral membrane proteins, dopamine-β-hydroxylase and hepatic asialoglycoprotein receptor, are incorporated into vesicles to determine whether biological activities can be maintained or restored in this reconstitution system.

Chapter VI addresses the question: Does the incorporation of a transmembrane protein like glycophorin significantly increase the passive permeability of the bilayer to ions? The results have important implications for later reconstitutions involving ion transport proteins.

Chapter VII gives a brief summary of results and suggests directions for future research.
Most serum proteins and many membrane proteins are glycosylated. As discussed in Chapter I, however, the effect of bound carbohydrate on the protein properties and structure is known in only a few cases.

To determine the role of carbohydrate on some properties of a membrane glycoprotein, native and deglycosylated forms of the protein may be compared. Deglycosylated forms are usually produced by specific enzymatic or chemical cleavage of sugar chains from the native molecule.

The membrane glycoprotein glycophorin A was chosen for these studies and is 55% carbohydrate by weight. All of its oligosaccharide chains have been sequenced and may be cleaved by specific and available glycosidases. Since glycophorin has no known functional or enzymatic activity, only solution properties of the molecule could be investigated.

This chapter describes the purification of glycophorin A and the production of two deglycosylated derivatives, asialoglycophorin and apoglycophorin. The properties of these forms are discussed at length in Chapter III and its Appendix.

Indicative of its hydrophobic association with the membrane, glycophorin can be extracted only by reagents that disrupt the bilayer. One commonly used method of glycophorin extraction is that of Marchesi and Andrews (1971), using lithium diiodosalicylate (LIS): however, LIS remains tightly bound to glycophorin even after treatment with detergent or organic solvent,
leaving the protein product with a brownish hue (Grefrath, 1974; Romans and Segrest, 1978). Alternative extraction procedures have been developed using organic solvents: butanol (Azuma et al., 1973), ethanol (Fletcher and Woolfork, 1971), phenol (Springer et al., 1966), pyridine (Zvilichovsky et al., 1971), and chloroform:methanol (Hamaguchi and Cleve, 1972); or using detergents: Triton X-100 (Yu et al., 1973), and SDS (Grefrath, 1974). The chloroform:methanol procedure was chosen for the purification described herein. This procedure leads to solubilization of the membrane lipids in the organic phase and partitioning of glycophorin and some glycolipids into the aqueous phase. Other membrane proteins enter the organic phase or form a precipitate at the interface (Hamaguchi and Cleve, 1972; Grefrath, 1974).

Glycophorin A has been purified from the other components in the aqueous phase by various chromatographic methods: (1) gel filtration in SDS (Grefrath, 1974); (2) gel filtration in Ammonyx LO detergent (Furthmayr et al., 1975); (3) hydrophobic adsorption chromatography in SDS (Simmonds and Yon, 1977); or (4) affinity chromatography in SDS (Kahane et al., 1976).

As described in Chapter I, purified glycophorin A has 15 O-linked tetrasaccharide chains and one asparagine-linked chain. Treatment with neuraminidase and endo-α-N-acetylgalactosaminidase should cleave O-linked chains from the peptide.

**Materials and Methods**

**Materials.** Sodium dodecyl sulfate ("Specially pure" grade, green label) was obtained from BDH Chemical Corp.; gas liquid chromatography of an acid hydrolysate of this product showed no alcohols other than dodecanol to be present. Sodium dodecyl [35S] sulfate was purchased from Amersham/Searle; a sample of the dodecanol used in the radio-chemical synthesis was provided by that firm and was shown to be free of alcohols of other
chain lengths. Na\textsuperscript{125}I was purchased from New England Nuclear. Sepharose 4B and 6B and Sepharose S-200 were Pharmacia products. Norleucine and amino acid standards were obtained from Pierce Chemical and Beckman concentrated buffers were used for amino acid analysis. N-acetylneuraminic acid was obtained from Boehringer Mannheim. Anion exchange resin Dowex AG1-X2 was purchased from Bio-Rad. Other chemicals were reagent grade products.

**Analytical methods.** Sialic acid was determined by the periodate-resorcinal method (Jourdain et al., 1971) using N-acetylneuraminic acid as a standard. Free N-acetylgalactosamine and Gal β1→3 GalNac were assayed by the Morgan-Elson reaction (Reissig et al., 1955).

Amino acid and amino sugar determinations were carried out on a Beckman Model 118C amino acid analyzer using the collagen buffer system (Beckman, 1975) 119C-AN-003 application notes). Amino sugars were determined by 10, 18, and 24 hr hydrolyses and amino acids by 24, 48, and 72 hr hydrolyses. Norleucine was included in all determinations as an internal standard. All hydrolyses were performed in evacuated tubes in 6 N HCl, 0.1% phenol at 108±2°.

Determination of the amino terminal amino acid was conducted by using the dansylation method of Gray (1972). Dansylated amino acids from Pierce were used as standards.

Protein concentrations were routinely measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Calibration of this procedure was accomplished by quantitative amino acid analysis.

**SDS polyacrylamide gel electrophoresis**

SDS was purchased from BDH and other electrophoretic chemicals were obtained from Bio-Rad. The method of Weber and Osborn (1969) was routinely
used with the following specifications and modifications: (1) Ten centimeter gels of 5-10% total acrylamide concentration were used. (2) The sodium phosphate concentration of the gel and reservoir buffers were halved to 0.05 M and 0.1 M, respectively. Variations in ionic strength were used in experiments reported in Chapter III. Calibrating standards were always run to estimate apparent molecular weights. Samples were brought to between 0.4% and 5% SDS and heated at 100°C for 3-5 minutes before application to the gels. Gels were stained with Coomassie brilliant blue or PAS reagent (Fairbanks et al., 1971) and scanned with a Gilford spectrophotometer equipped with a Beckman photoelectric scanning system.

Purification of glycophorin A. Human red blood cell ghosts were prepared from freshly drawn blood by the method of Dodge et al. (1963) using 0.155 M NaHCO₃, pH 8.2 to wash intact red cells. Intact red cells were separated from white blood cells by underlayering cells with a hypaque-ficoll solution of density 1.080 and spinning at 2100 rpm in JA-10 preparative rotor at room temperature for 20 minutes. White cells formed a suspended layer above the red cells and were removed by aspiration. 10 mM NaHCO₃, pH 8.2 was used for lysis of red cells and for subsequent washes of the red cell ghosts. A final wash with 5 mM NaHCO₃ produced white ghosts free from hemoglobin. Spectrin was removed from white ghosts by the method of Reynolds and Trayor (1971) or by the method of Tyler et al. (1980). These ghosts were washed twice with 1 mM NaN₃, 10 mM Tris-HCl pH 7.4 and stored at -20°C for up to several months. All lysis and subsequent washes were performed at 4°C.

Glycophorin A was isolated from ghosts as described by Grefrath (1974) and Grefrath and Reynolds (1974). Frozen ghosts were diluted to about 2 mg/ml "Lowry" protein in 10 mM Tris-HCl, pH 7.4, 1 mM NaN₃, and homogenized by 5 strokes in a homogenizer. "Lowry" protein refers to protein determined by
the Lowry method (1951). Glycoprotein and some glycolipid are extracted with chloroform:methanol (2:1) by the method of Hamaguchi and Cleve (1972). The aqueous phase from this extraction was diluted with deionized water and centrifuged at 2500 rpm to remove emulsified chloroform and any contaminating interface material. This aqueous phase was rotary evaporated long enough to permit freezing. Samples were lyophilized to a concentration of 4 mg/ml "Lowry" protein, and centrifuged again to remove any insoluble material. Protein solutions were brought to 10% SDS and heated at 80°C for 30 minutes before chromatography. Glycophorin solutions were chromatographed on a Sepharose 4B column (2.5 X 100 cm) equilibrated and eluted in 0.1 M sodium phosphate buffer, pH 7.1 (µ = 0.26 M), 10 mM SDS and 1 mM NaN₃ (Figure 4). Fractions from the front half of the sialic acid containing peaks (cross-hatched area in Figure 4, Kd = 0.608) were pooled and exhaustively dialyzed against deionized water 10 mM Tris-HCl, pH 7.5, 1 mM NaN₃ containing ion exchange resin until all SDS had been removed (4 days). Glycophorin A solutions were concentrated by lyophilization and stored at -20°C.

Enzymes

Neuraminidase and endo-α-N-acetylgalactosaminidase were gifts from Dr. Larry Glasgow. Their purification from Streptococcus pneumoniae is described in Glasgow et al. (1977). This neuraminidase is an exoglycosidase which hydrolyzes bonds formed by unsubstituted sialic acid residues at the non-reducing ends of oligosaccharides. The endo-α-N-acetylgalactosaminidase has been shown to hydrolyze glycosidic bonds formed by N-acetylgalactosamine and the hydroxyl group of either serine or threonine when in the sequence Gal β1→3 GalNAc-α-1-OSer/Thr (Endo and Kobata, 1976; Bhavanandan et al., 1976). These enzymes were free of contaminating glycosidic, hemolytic, and proteolytic activities. Enzymes were stored for several months at -20°C with
Fig. 4. Elution profile of the aqueous phase from the chloroform: methanol extract of red cell ghosts on a Sepharose 4B column (2.5 X 100 cm). The column was equilibrated in 0.1 M sodium phosphate, pH 7.1, 10 mM SDS, 1 mM NaN₃ at 24°C. The column was calibrated with DNA, BSA, cytochrome c, and β mercaptoethanol. Vo, B, CC, and Vt indicate the void volume, the elution positions of BSA and cytochrome c, and the total volume respectively. G represents the glycophorin peak which may also be identified by sialic acid assays (not shown). Tubes from the crosshatched region are pooled and concentrated for further studies and have been shown to contain 95% pure glycophorin A.
little loss of activity.

Preparation of glycophorin derivatives

Glycophorin A (2-7 mg/ml) free of SDS was incubated for 72 hours at 37°C in 50 mM sodium cacodylate, pH 6.0, to give glycophorin A (control); with neuraminidase (0.08 units/ml) to give asialoglycophorin A; or with neuraminidase (0.08 units/ml) and endo-α-N-acetylgalactosaminidase (0.2 units/ml) to give apoglycophorin. The glycophorin derivatives were usually isolated from released sugars by gel filtration on a Sepharose S-200 column (0.8 cm X 70 cm) equilibrated in 0.1 M sodium phosphate, pH 7.1, 13.9 mM SDS. (See Figure 8)

Results

The purification procedure for glycophorin A is similar to that of Grefrath (1974); however, spectrin was often removed from ghosts using the method of Tyler et al. (1980). A crucial step in this preparation is the removal of membranous contaminants from the aqueous phase of the chloroform-methanol extraction. This removal is effected by centrifugation of the aqueous phase both prior to and after concentration by lyophilization. Band 3 protein and residual spectrin are the major contaminants if this is not done. In this purification, the aqueous phase contains 80-90% of the red cell sialic acid corresponding to a yield of 1-3% of the total ghost "Lowry" protein. (Since the Lowry method detects only protein and not carbohydrate, this 1 to 3% yield represents the percentage contribution of the polypeptide portion of glycophorin to the total protein detected. Glycophorin is 55% carbohydrate by weight, and therefore, in terms of total glycoprotein weight, the yield is 2-6% of total ghost protein.)

Glycophorin A is separated from other glycophorins and glycolipids in
the aqueous phase by gel chromatography on Sepharose 4B equilibrated in 10 mM SDS, 0.1 M sodium phosphate, pH 7.1. The front half of the sialic acid peak is at least 95% glycophorin A as determined by SDS gel electrophoresis (Figure 5) and quantitative amino acid analysis (Table 1). The trailing half of the peak contains glycophorin A and some glycophorin B which electrophoreses as a PAS 3 band (Furthmayr et al., 1976).

Fluorescence and UV absorbance spectra of glycophorin A show the presence of tyrosine residues with no apparent contribution from tryptophan residues. This absence of tryptophan in the spectrum is consistent with the protein sequence and indicates little contamination from glycophorin B which contains tryptophan.

**Digestion**

Because both neuraminidase and endo-α-N-acetylgalactosaminidase are inactive in 10 mM SDS, SDS was removed from glycophorin solutions prior to incubation with enzymes. Up to 99% of all sialic acid residues were cleaved by neuraminidase producing asialoglycoprotein. This corresponds to removal of 46% of all bound carbohydrate by weight which represents 25% of the total glycoprotein by weight. The time course of enzymatic cleavage of Gal β1→3 GalNAc from asialoglycoprotein is given in Figure 6. Up to 90% of all GalNAc residues are removed in 20 hours corresponding to cleavage of 13 of the 15 O-linked carbohydrate chains from the protein. No further cleavage occurred at longer incubation times. The glucosamine composition was not affected by enzyme digestion (Table 1) indicating that the asparagine linked chain was not cleaved. In apoglycophorin, 77% of total carbohydrate of native glycoprotein by weight is removed representing 42% of total glycoprotein weight.

SDS polyacrylamide gels show the presence of one band for each of the three forms corresponding to apparent molecular weights 74,000, 63,000, and
Fig. 5. SDS gel electrophoresis of the three glycophorin forms (l-r) native, asialo, and apoglycophorin were electrophoresed on 7.5% polyacrylamide gels containing 0.05 M sodium phosphate, pH 7.1, 0.4% SDS. Gels were stained with Coomassie blue. The black mark at the bottom of the gel indicates the location of the tracking dye. About 10 µg of protein was loaded per gel.
Fig. 6. Time course of N-acetylgalactosamine removal from asialoglycophorin. Asialoglycophorin (0.5 mg/ml protein) in 50 mM sodium cacodylate, pH 6.0 was incubated at 37° with endo-α-N-acetylgalactosaminidase (activity = 1 μmole/min). Aliquots of the reaction mixture were removed at various times and assayed for free GalNAc as described in Materials and Methods. After 48 hours incubation apoglycophorin was separated from free sugar by gel chromatography on a small BioRad P-2 column, and GalNAc remaining bound to apoglycophorin was quantitated by amino acid and amino sugar analysis.
All values in terms of moles/mole peptide

From sequence of Toots et al. (1978).

From Furthmayr et al. (1975).

From Graff (1974).

These are averages of two preparations.

Extrapolated to zero time from 48 hr data (Yoshiki).

Extrapolated to zero time from 48 hr and 72 hr data (Yoshiki).

N.D. is not determined.
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<td>Fucose</td>
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All values in terms of moles/mole peptide

*From sequence of Tomita et al. (1978).*
*From Furthmayr et al. (1975).*
*From Grefrath (1974).*
*These are averages of two preparations.*
*Extrapolated to zero time from 24, 48 hour hydrolysis.*
*Value from the 72 hour hydrolysis.*
*Extrapolated to zero time from 10, 18 and 24 hr hydrolysis.*
*N.D. is not determined.*
The amino acid and amino sugar composition of glycophorin, asialoglyco-
phorin, and apoglycophorin after separation of protein from released sugar by
gel filtration chromatography are shown in Table 1. The compositions re-
ported by other investigators are given for comparison. There is generally
good agreement among the compositions; however, isoleucine, even after a 72
hour hydrolysis, gave lower values than those determined by sequence analysis.
Grefrath also reported low isoleucine values, and this may be due to incom-
plete hydrolytic cleavage of Ile-Ile or Leu-Ile in the molecule. It should
be noted that the reported sequence of the hydrophobic region has been modi-
fied over the past few years and some discrepancies in the sequence may exist.
Amino terminal analysis of glycophorin A and its deglycosylated derivatives
from MM and NN blood types gave one spot corresponding to serine or leucine,
respectively. For most glycophorin preparations, ghosts from MN donors or
mixtures of ghosts from MM, NN, and MN donors were used.

Discussion

Glycophorin A was purified to 95% homogeneity from red cell ghosts by a
variation of the method of Grefrath (1974). This procedure avoids the use of
LIS which cannot be easily removed from the glycoprotein. Although glyco-
phorin has been desialylated with neuraminidase treatment by other investi-
gators they did not determine its physical properties. No further deglyco-
sylation has been reported previously. The recent purification and isolation
of endo-α-N-acetylgalactosaminidase has allowed extensive cleavage of up to
90% of the 0-linked chains from glycophorin. Specific glycosidases like the
ones used here have proven to be useful not only for deglycosylation but also
as analytical tools to prove the exact primary structure of carbohydrate
moieties. The fact that almost all of the 0-linked chains are removed by
this enzyme adds further proof that the structural assignments of Thomas and Winzler (1969) are correct. Unlike some chemical methods, the enzymatic de-glycosylation is specific and occurs without the cleavage of the polypeptide backbone.

No attempt to cleave the asparagine-linked carbohydrate chain from the polypeptide was made; however, enzymes are now commercially available which would make this possible.

As a first step in attaining an understanding of the functional relevance of covalently bound oligosaccharide chains, a membrane glycoprotein like glycophoria must be chemically and physically characterized. In the previous chapter, the chemical composition of native glycophoria and two de-glycosylated derivatives were examined. This chapter addresses the question: How does bound carbohydrate contribute to the physical properties of an integral membrane glycoprotein?

In this work, the conformation and aggregation state of these glycophoria forms in aqueous and SDS containing buffer as well as their electrophoretic mobility on SDS polyacrylamide gels were investigated. Interpretation of these studies requires knowledge of the association of SDS with the three glycophoria species. In addition, the ability of native glycophoria to associate with zygophoria was investigated.

Amphiphile binding properties of soluble proteins, glycoprotins, and membrane proteins indicate that each group has distinctive features. The differences between soluble and membrane proteins were described in the introduction. Leach et al. (1986) have found that glycoprotins not only bind less than the normal ratio of SDS (1.4-1.5/g glycoprotein) but also exhibit deviant hydrodynamic properties. These properties will be discussed later in this chapter. Glycophoria, being part membrane protein (containing a hydrophobic sequence) and part glycoprotein (12% carbohydrate by
CHAPTER III

PROPERTIES OF GLYCOPHORIN AND ITS
DEGLYCOSYLATED DERIVATIVES

As a first step in attaining an understanding of the functional relevance of covalently bound oligosaccharide chains, a membrane glycoprotein like glycophorin must be chemically and physically characterized. In the previous chapter, the chemical composition of native glycophorin and two deglycosylated derivatives were examined. This chapter addresses the question: How does bound carbohydrate contribute to the physical properties of an integral membrane glycoprotein?

In this work, the conformation and aggregation state of these glycophorin forms in aqueous and SDS containing buffer as well as their electrophoretic mobility on SDS polyacrylamide gels were investigated. Interpretation of these studies requires knowledge of the association of SDS with the three glycophorin species. In addition, the ability of native glycophorin to associate with apoglycophorin was investigated.

Amphiphile binding properties of soluble proteins, glycoproteins, and membrane proteins indicate that each group has distinctive features. The differences between soluble and membrane proteins were described in the introduction. Leach et al., (1980) have found that glycoproteins not only bind less than the normal ratio of SDS (1.4 ± 0.2 g SDS bound/g glycoprotein) but also exhibit deviant hydrodynamic properties. These properties will be discussed later in this chapter. Glycophorin, being part membrane protein (containing a hydrophobic sequence) and part glycoprotein (55% carbohydrate by
weight) would be expected to display physical properties indicative of both protein types.

The physical properties of native glycophorin have been studied extensively as described in the introduction with respect to peptide chain conformation by CD spectra (Schulte and Marchesi, 1979; Schulte and Marchesi, 1980), state of association in SDS solutions by sedimentation equilibrium (Grefrath, 1974; Grefrath and Reynolds, 1974; Dohnal et al., 1980), state of association in detergent free buffer (Verpoorte, 1975), amphiphile binding properties (Grefrath, 1974; Grefrath and Reynolds, 1974), and electrophoretic mobility and apparent molecular weight on SDS gels (Segrest et al., 1971; Siverberg et al., 1976; Furthmayr and Marchesi, 1976; Marton, 1974; Potempa, 1978; Tuech and Morrison, 1972). The properties of neuraminidase treated glycophorin or other deglycosylated derivatives, however, have not been systematically investigated previous to this work.

Schulte and Marchesi (1980) have found no difference in peptide conformation between native and asialo-glycophorin in detergent free buffer as determined by CD spectra. Asialo-glycophorin is more susceptible to trypsin cleavage (Tomita et al., 1976) and has greater mobility on SDS gels than native protein (Fairbanks et al., 1971). Furthermore, MN antigenic activity of native glycophorin was shown to depend on the integrity of sialic acid residues (Sadler et al., 1979).

**Materials and Methods**

**Materials.** $^{35}$S-SDS was obtained from Amersham and unlabeled SDS was from BDH (green label).

**Analytical methods**

SDS polyacrylamide gel electrophoresis was carried out in phosphate
buffer as described in Chapter II according to the method of Weber and Osborn (1971). 7.5% polyacrylamide gels were polymerized in 13.9 mM SDS, sodium phosphate concentrations of 0.05 M (ionic strength, μ = 0.13) or 0.1 M (μ = 0.26), pH 7.1. The tray buffer was identical in composition to the gel buffer for these runs. The pretreatment conditions of the glycophorin sample usually included heating the sample at 100° for 3 to 5 minutes. Specific details of the sample conditions are given in figure legends and in the text.

Protein concentrations were routinely measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Calibration of this procedure was accomplished by quantitative amino acid analysis.

**SDS binding measurements**

The critical micelle concentration (CMC) of SDS in 0.1 M sodium phosphate buffer (μ = 0.26) has been reported as 0.72 mM (Grefrath and Reynolds, 1974). Above the CMC, the amount of SDS bound was measured using either 13.9 mM or 2.6 mM SDS solutions containing appropriate amounts of $^{35}$S-SDS as eluting buffer on a Sephacryl S-200 or a Sepharose 6B column (0.6 x 60 cm) and determining the concentration of SDS and protein in collected fractions. In all cases columns were eluted and equilibrated in buffers containing SDS and 0.1 M sodium phosphate, pH 7.1 at 25°C. Samples were prepared for chromatography as follows. Glycophorin samples at 1-4 mg/ml in distilled water or in 0.05 M sodium cacodylate, pH 6.0, were made 0.1 M in sodium phosphate, pH 7.1 by addition of 1.0 M sodium phosphate buffer. Amounts of a 10% SDS solution in excess of that needed to saturate binding and to produce an unbound SDS concentration of 13.9 mM were added to the glycophorin sample. The 10% SDS solution contained $^{35}$S-SDS and was also used to make up the column buffer. Therefore, the same specific activity exists in the sample buffer and in the column buffer. Usually the protein-SDS mixture (0.6 to 0.4 ml)
was heated at 100° for 5 minutes prior to loading on the column, but samples which were not heated gave the same binding results. The excess $^{35}$S counts which were applied to the column eluted as an SDS micellar peak that was well separated from the glycophorin elution position.

For measurements below the CMC, protein solutions lacking SDS were dialyzed in Lucite cells with 1 ml chambers versus varying concentrations of radioactive SDS. Aliquots were taken from both sides of the chambers and counted. $^{35}$S-SDS radioactivity was measured in a Beckman LS-100 scintillation counter, using a toluene-Triton X-100 (2:1) based cocktail. An aliquot was also taken from the sample side to determine protein concentrations which were in the range of 0.2 to 0.25 mg/ml. From the known specific activity, the free and bound SDS concentrations could be determined. When the binding remained constant for 24 hours, the samples were deemed to be at equilibrium. These equilibrium dialysis experiments were conducted at 24-25°C in 0.1 M sodium phosphate buffer, pH 7.1.

Circular dichroism

Glycophorin samples were equilibrated in 13.9 mM SDS, 0.1 M sodium phosphate, pH 7.1 by passage down the columns described above or in detergent free 0.1 M sodium phosphate buffer. Circular dichroic spectra of the three forms were recorded on a Jobin-Yvon Dicrographe Mark III calibrated with a 0.1% aqueous solution of d-10 camphorsulfonic acid. A 0.1 cm path cell was used for most measurements, and protein concentrations ranged from 0.1 mg/ml to 0.5 mg/ml. Molar ellipticity values were determined using a mean residue weight of 109 which was calculated from amino acid compositions given in Table 1.
Analytical centrifugation

Sedimentation equilibrium was carried out on a Beckman Spinco Model E analytical ultracentrifuge equipped with a photoelectric scanner. The concentration of protein as a function of radial distance from the center of rotation was determined by scanning the cell at 280 nm. Centrifugation was carried out at rotor speeds between 10,000 and 20,000 rpm for samples in SDS containing buffers and at 8000 rpm for samples in detergent free buffer. 100 µl of samples were used in each case. Equilibrium was deemed to have been achieved when no change in the slope of a plot of ln OD. vs r² was seen in scans taken 8 hours apart. Equilibrium of samples (initial protein concentrations 0.25 to 1.0 mg/ml) above 25°C was usually achieved within 24 hours at a given speed.

In sedimentation equilibrium the following equation applies:

\[ M = \frac{2RT}{\omega^2} \frac{d\ln C}{(dr^2)} \frac{1}{(1-\phi'\rho)} \]  

where R is the universal gas constant \((8.31 \times 10^7 \text{ ergs mole}^{-1} \text{ K}^{-1})\), T is temperature in °K, and \(d\ln C/dr^2\) is the slope of a plot in lnC vs r² which is obtained directly from the experiment at rotor speed \(\omega\) in radians per second. \(\rho\) is the solvent density and \(\phi'\) the effective partial specific volume. In aqueous buffer for a protein having no preferential binding terms, \(\phi'\) is equivalent to \(\bar{v}_{GP}\), the partial specific volume of the glycoprotein. This is not true in SDS buffer where detergent binding to the protein occurs. For this case, it is an excellent approximation to divide \((1-\phi'\rho)\) into independent contributions from the protein and carbohydrate moieties and from bound detergent:
(1-\(\rho'\)) = (1-\(\bar{\rho}p\)) + \(\delta_{\text{CHO}}\) (1-\(\bar{\rho}_{\text{CHO}}\)) + \(\delta_{\text{SDS}}\) (1-\(\bar{\rho}_{\text{SDS}}\)) \quad (2)

Where \(\delta_{\text{CHO}}\) and \(\delta_{\text{SDS}}\) are g carbohydrate and g SDS bound per gram protein, respectively. The partial specific volumes of carbohydrate \(\bar{\rho}_{\text{CHO}}\), were calculated from compositional data (in Table 1) using the data of Gibbons (1966), and of the peptide moiety, \(\bar{\rho}_p\), from amino acid composition by the method of Cohn and Edsall (1943). These values are given in Table 3.

Results

Lowry protein determination. Determination of glycophorin protein concentration was routinely performed by the Lowry method (1951) using BSA as a standard. This procedure gave values 22 \(\pm\) 5% lower than the actual protein concentration as obtained by quantitative amino acid analysis. A similar correction factor had been previously reported by Grefrath and Reynolds (1974). Lowry protein concentrations determined for asialoglycophorin are 18% lower than the actual value. Apoglycophorin gives color yields identical to BSA within experimental error, and thus its protein concentration needs no correction. Apparently, removal of carbohydrate from the polypeptide chain leads to enhanced Lowry color formation.

Association with SDS. No significant differences in SDS binding among the three glycophorin forms are observed below the detergent CMC (See Figure 7). At the highest SDS concentration measured below the CMC, 0.45 mM unbound SDS, in 0.1 M sodium phosphate, pH 7.1 \((\text{CMC} = 0.72 \text{ mM})\), 0.25 g SDS bind per g of protein. (Note that the binding is reported in terms of grams protein not in terms of total glycoprotein weight. These differences are noted in Table 4). Most of the detergent binding occurs as part of a cooperative transition
Fig. 7. Association of SDS to glycophorin and its deglycosylated derivatives. All binding measurements were performed in 0.1 M sodium phosphate, pH 7.1 ($\mu = 0.26, \text{CMC} = 7.2 \times 10^{-4} \text{M SDS}$) at 25°. Open figures were obtained by equilibrium dialysis as described in Methods. Filled figures were determined by column chromatography. Triangles, circles and squares represent native, asialo, and apoglycophorin, respectively. For column runs protein loading concentrations were generally in the range of 3 to 0.5 mg/ml and for dialysis in the range 0.3 to 0.2 mg/ml. The binding values obtained by chromatography at 13.9 mM SDS were the average from at least two experiments. The binding to asialo and apoglycophorin above the CMC was the same within experimental error.
Fig. 8. Determination of the binding of SDS to apoglycophorin by gel chromatography on a Sephacryl S-200 column (0.6 x 60 cm) equilibrated in 0.1 M sodium phosphate, pH 7.1, 13 mM SDS, at 25°C. About 0.4 ml of 2 mg/ml apoglycophorin was loaded on the column. Protein concentration was determined by corrected Lowry method (1951) and SDS concentration was determined using $^{35}$S-SDS. G, Asialo, Apo, and SDS indicate the elution position of glycoporphin, asialoglycophorin, apoglycophorin, and the SDS micelle peak, respectively.
<table>
<thead>
<tr>
<th>Protein</th>
<th>$\frac{v}{\nu}$ cm$^3$/g</th>
<th>Initial Protein Concentration (mg/ml)</th>
<th>Estimated % Recovery</th>
<th>Molecular Weight$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycoporphin</td>
<td>0.67</td>
<td>0.53</td>
<td>75</td>
<td>340,000</td>
</tr>
<tr>
<td>Asialglycoporphin</td>
<td>0.695</td>
<td>0.35</td>
<td>61</td>
<td>400,000</td>
</tr>
<tr>
<td>Apoglycoporphin</td>
<td>0.711</td>
<td>0.42</td>
<td>25</td>
<td>600,000</td>
</tr>
</tbody>
</table>

$^a$ The three glycoporphin forms were equilibrated in 0.1 M sodium phosphate, pH 7.1 by passage down a G-50 column equilibrated in this buffer.

$^b$ Centrifugation was carried out at 21° at 8000 rpm. All plots of $\ln$ absorbance vs $r^2$ were highly curved. These values were calculated from the slope of a line fitted by the least squares method through points obtained from the bottom half of the cell.
TABLE 3
MOLECULAR WEIGHT OF GLYCOPHORIN IN SDS SOLUTION

<table>
<thead>
<tr>
<th></th>
<th>M(1-(\varphi))</th>
<th>SDS Bound</th>
<th>Carbohydrate Bound</th>
<th>(\frac{\varphi}{\text{CHO}})</th>
<th>Protein Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycophorin</td>
<td>1.25 x 10^4</td>
<td>1.06 ± 0.2</td>
<td>1.20</td>
<td>0.612</td>
<td>15,600 ± 1,000</td>
</tr>
<tr>
<td>Asialoglycophorin</td>
<td>1.04 x 10^4</td>
<td>1.77 ± 0.2</td>
<td>0.592</td>
<td>0.640</td>
<td>14,700 ± 1,000</td>
</tr>
<tr>
<td>Apoglycophorin</td>
<td>0.88 x 10^4</td>
<td>1.80 ± 0.4</td>
<td>0.211</td>
<td>0.640</td>
<td>15,100 ± 1,800</td>
</tr>
</tbody>
</table>

\(a\) Obtained from sedimentation equilibrium in 0.1 M sodium phosphate, pH 7.1, 13.9 mM SDS (\(\mu = 0.26\)). Values of M(1-\(\varphi\)) taken at the meniscus.

\(b\) Average of at least two measurements.

\(c\) Determined from N-acetylated sugar, sialic acid, and hexose analysis.

\(d\) Calculated from carbohydrate composition using the method of Gibbons (1966).

\(e\) Determined from M(1-\(\varphi\)) where,

\[
(1-\varphi) = (1-\varphi_p) + \delta_{\text{CHO}}(1-\varphi_{\text{CHO}}) + \delta_{\text{SDS}}(1-\varphi_{\text{SDS}})
\]

\(\rho\) is solution density, \(\varphi_{\text{SDS}} = 0.87, \varphi_p = 0.726, \delta_{\text{SDS}} = \text{g of SDS bound per g protein} \) and \(\delta_{\text{CHO}}\) is \(\text{g carbohydrate bound/g protein}\).
above the detergent CMC. It can be seen that saturating levels of SDS binding to glycophorin are obtained at 13.9 mM SDS in this buffer, and these solution conditions were employed for most of the physical studies described below.

Binding above the detergent CMC was determined by column chromatography on Sephacryl S-200. A typical elution profile for apoglycophorin is shown in Figure 8. Apoglycophorin as well as the other two glycophorin forms, chromatograph as a symmetric peak well separated from the $^{35}$S-SDS micelle peak, which arises from the SDS concentration in the loaded sample in excess of the column SDS concentration. Since the binding ratio was constant (within experimental error) across the eluted protein peak and did not change on re-chromatography or upon dialysis against elution buffer, these values indicate the binding at equilibrium. At saturating levels of detergent binding apo and asialoglycophorin bind $1.8 \pm 0.4$ and $1.77 \pm 0.2$ g SDS per g protein, whereas native glycophorin binds significantly less, about $1.06 \pm 0.2$ g/g. This equates to 52, 87, and 88 moles of SDS bound per mole of native, asialo and apoglycophorin, respectively. Since a micelle at these ionic strengths contains about 114 SDS monomers, these values would correspond to one half and three quarters of a micelle being bound per protein molecule.

Grefrath and Reynolds (1974) reported similar binding isotherms with cooperative transitions occurring above the CMC; however, they calculated a saturation binding level of $6 \pm 1$ g of SDS bound per g protein for native glycophorin. This value is considerably higher than that measured here and is higher than that of other membrane proteins shown in Table 4. This binding corresponds to about 290 moles of SDS bound per mole of glycoprotein, which would be 2.5 SDS micelles bound per molecule. One possible source of error in their measurements results from the use of Schwartz-Mann SDS which
TABLE 4

SDS BINDING BY GLYCOPROTEINS AND INTEGRAL MEMBRANE PROTEINS

<table>
<thead>
<tr>
<th>Molecule</th>
<th>% CHO</th>
<th>g SDS/g Molecule</th>
<th>g SDS/g Polypeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA *</td>
<td>0</td>
<td>1.45±0.15</td>
<td>1.45±0.15</td>
</tr>
<tr>
<td>Glycogen</td>
<td>100</td>
<td>&lt;0.03</td>
<td></td>
</tr>
<tr>
<td>*Glycophorin</td>
<td>55</td>
<td>0.5</td>
<td>1.10</td>
</tr>
<tr>
<td>α1 Acid glycoprotein *</td>
<td>41</td>
<td>0.7</td>
<td>1.28</td>
</tr>
<tr>
<td>*Asialoglycoporin</td>
<td>29</td>
<td>1.24</td>
<td>1.77</td>
</tr>
<tr>
<td>Tamm-Horsfall * urinary glycoprotein</td>
<td>28</td>
<td>0.99</td>
<td>1.37</td>
</tr>
<tr>
<td>Ovomucoid *</td>
<td>23</td>
<td>1.10</td>
<td>1.43</td>
</tr>
<tr>
<td>Fetuin *</td>
<td>23</td>
<td>1.23</td>
<td>1.60</td>
</tr>
<tr>
<td>*Apoglycophorin</td>
<td>13</td>
<td>1.56</td>
<td>1.80</td>
</tr>
<tr>
<td>Cellobiohydrolase C *</td>
<td>13</td>
<td>1.28</td>
<td>1.46</td>
</tr>
<tr>
<td>*Ca2+ ATPase</td>
<td>0</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>*Cytochrome b5 *</td>
<td>0</td>
<td>1.24</td>
<td>1.24</td>
</tr>
<tr>
<td>*fl phage</td>
<td>0</td>
<td>1.65</td>
<td>1.65</td>
</tr>
</tbody>
</table>

* Indicates integral membrane protein.

Compiled by Leach et al. (1980) from the work of many authors. All values presumably represent saturating levels of SDS binding.

Binding determined by Nozaki and Tanford (1979).

Binding determined by Robinson and Tanford (1975).

Binding determined by Makino et al. (1975).
has been shown to have considerable heterogeneity in the alkyl chain length. Dohnal and Garvin (1980) report that 18.8% and 3.7% of the alkyl chains are actually C₁₄ and C₁₆, respectively. Reynolds showed that 30% of the chains were C₁₄, 70% were C₁₂, and trace amounts were C₁₀ (personal communication). All tested brands of SDS except BDH are significantly contaminated by these longer alkyl chain sulfates, and thus BDH was chosen for all work presented in this thesis. It is important to note that Dohnal and Garvin (1980) demonstrated that tetradecyl sulfate has greater affinity for glycophorin and other proteins than dodecyl sulfate, but actual quantitative binding measurements were not performed.

From the Sephacryl S-200 column native, asialo and apoglycophorin have distribution coefficients, Kᵰ, of 0.07, 0.14 and 0.18 respectively at protein loading concentration in the range of (1-4 mg/ml) (Kᵰ = Ve-V₀/Vᵳ-V₀, where V₀ is the void volume, Vᵳ is the internal volume and Ve is the protein peak volume, all expressed as grams eluate.) This indicates that the size of the protein decreases with increased carbohydrate removal. However, interpretation of the chromatographic behavior may not be straightforward for glycoproteins.

Leach et al. (1980) have shown that the presence of large amounts of carbohydrate on a protein dramatically affects the chromatographic behavior of glycoproteins. They illustrate this point by comparing three proteins of about the same molecular weight in SDS: aldolase contains no carbohydrate and has molecular weight 40,000; acid glycoprotein is 36,000 daltons, 41% of which is bound carbohydrate; and fetuin is 44,000 daltons, 23% of which is carbohydrate. On gel chromatographic columns equilibrated in SDS, aldolase, α acid glycoprotein, and fetuin elute with apparent Stokes' radii of 57Å, 48.5Å, and 67Å, respectively. Thus as Leach et al. (1980) point out, the two glycopeptides deviate in opposite directions when compared to the aldolase -
SDS complex. Leach et al. (1980) suggest that the hydrodynamic volume contribution per residue by the oligosaccharide side chain of fetuin are over twice that for α acid glycoprotein. This assumes that the polypeptide backbone of each interacts with SDS in the usual fashion to produce a typical complex.

**SDS polyacrylamide gel electrophoresis**

Figure 9 shows densitometry scans of the three forms on 7.5% polyacrylamide gels. Glycophorin samples (0.1 - 0.4 mg/ml) were heated at 100°C for 3 to 5 minutes prior to electrophoresis, but heating of the sample or the presence or absence of β-mercaptoethanol in the incubation mixture had no effect on the electrophoretic mobility. One peak is seen for each form with mobilities corresponding to apparent molecular weights of 74,000, 63,000 and 39,000 for native, asialo and apoglycophorin, respectively. These values are considerably higher than the actual molecular weights. The apparent molecular weight for native glycophorin is consistent with previous reports (Tuech and Morrison, 1971). The reasons for this anomalous behavior will be addressed in the discussion and appendix. Peak broadening occurs with increased carbohydrate removal and this may be due to heterogeneity in the number and location of carbohydrate residues removed. A slight shoulder on the leading edge of apoglycophorin is often seen. Removal of sialic acid from the protein greatly enhances the staining by the anionic dye Coomassie brilliant blue. (See Figure 9). This enhancement may be due to elimination of charge repulsion between the negatively charged sialic acids and Coomassie blue dye. As expected, removal of carbohydrate significantly decreases the ability to stain protein by the PAS procedure. Apoglycophorin is barely visualized, further demonstrating that a considerable amount of the carbohydrate had been removed.
Fig. 9. Densitometry profile of native (A,D), asialo (B,E) and apoglycophorin (C,F) on 7.5% polyacrylamide gels in 0.1 M sodium phosphate, pH 7.1, 0.4% SDS. Electrophoresis, staining, and spectrophotometric scanning of the gels were carried out as described in Methods. A–C are stained with Coomassie blue and D–F are stained by the PAS procedure of Fairbanks et al. (1971). The peak areas when normalized to amount of protein loaded (10–15 μg) have the ratios of Coomassie staining of 1:2.0:2.0 for native, asialo, and apoglycophorin, respectively. Relative PAS staining ratios of 1.0:.48:<.1 were obtained for native, asialo, and apoglycophorin respectively. Using five protein standards, the apparent molecular weights of these 3 forms are 74,000, 63,000, and 39,000.
Circular dichroic spectra

The far ultraviolet circular dichroic spectra of native, anato, and apoglycophorin in 13.9 mM SDS, 0.1 M sodium phosphate buffer, pH 7.1 are shown in Figure 10. Protein was equilibrated in this buffer by passage down a Sephadex G-200 column. All spectra were taken at 218 nm. The more shallow of the 218 nm peaks in each of the spectra is similar to that found in some fractions of the native protein. The peaks at 100° for B, C, and D are not as pronounced as in other spectra. Apoglycophorin yields a spectrum very similar to that of native glycophorin, with slightly higher values at 100°. The peak at 100° for F is almost flat, indicating a lack of native conformations in a rather open state. The spectra for anato are rather aqueous-like, and the carbohydrate nature of anato glycoporin is seen as a shoulder peak at 218 nm. This phenomenon has been previously described by Akeson et al. [10].

The carbohydrate nature of glycophorin makes an anato analogue in the CE.
Circular dichroic spectra

The far ultraviolet circular dichroic spectra of native, asialo, and apoglycophorin in 13.9 mM SDS, 0.1 M sodium phosphate buffer, pH 7.1 are shown in Figure 10. Protein was equilibrated in this buffer by passage down a Sephacryl S-200 column described in Methods. No large changes in peptide backbone conformation among the three forms are seen from the CD spectra. All forms have a deep trough at 207-208 nm and a shallower one around 218 nm. The more negative ellipticity of the 208 nm trough relative to the 218 nm one is similar to that seen with other proteins in saturating concentrations of SDS (Jigensons, 1976; Visser and Blout, 1971). Heating of glycophorin at 100° for 5 minutes did not affect the spectra.

Using the ellipticity at 208 nm as a measure of α-helical content as recommended by Greenfield and Fasman (1969), native, asialo and apoglycophorin yield values of 40, 39, and 41% α-helix. Apoglycophorin had a slightly decreased molar ellipticity at 218 nm compared to asialo and native glycophorin, but this difference is only about 7-8%. The relatively low values of molar ellipticity at 208 and 220 nm indicate that glycophorin is almost 60% unordered. Use of the word "unordered" should not be misinterpreted. It is possible that the peptide backbone may be constrained in a rather specific manner distinct from α helix or β conformations.

The spectra of the three forms in detergent free sodium phosphate buffer are similar in shape to those in SDS, but the magnitude of ellipticities are reduced by 25-30%. In addition the trough at 208 nm is shallower with respect to the one at 220 nm. This damping of the molar ellipticities in aqueous buffer may result from light scattering produced by aggregated glycophorin. This phenomenon has been previously described by Holzworth (1972).

The carbohydrate moiety of glycophorin makes no contribution to the CD
Fig. 10. CD spectra of native (---, peptide concentration = 0.254 mg/ml) asialo (........, .366 mg/ml) and apoglycophorin (----, 0.45 mg/ml) after passage down a Sephacryl S-200 column to equilibrate the samples in 0.4% SDS, 0.1 M sodium phosphate (μ = 0.26) pH 7.1.
...spectra at these wavelengths as was shown by Schulte and Marchesi (1979) and is consistent with the observations of Nasi (personal communication). These spectra of glycophorin are similar in shape and magnitude to those shown by Schulte and Marchesi (1979).

Sedimentation equilibrium of glycophorin in detergent-free buffer
spectra at these wavelengths as was shown by Schulte and Marchesi (1979) and is consistent with the observations of Nozaki (personal communication). These spectra of glycophorin are similar in shape and magnitude to those shown by Schulte and Marchesi (1979).

**Sedimentation equilibrium of glycophorin in detergent free buffer**

All three forms of glycophorin were soluble but highly aggregated in detergent free buffer (0.1 M sodium phosphate, pH 7.1, \( \mu = 0.26 \)) as determined by sedimentation equilibrium. Plots of \( \ln \text{OD} \) vs \( r^2 \) were highly curved yielding molecular weights at the bottom one-third of the cell which correspond to decamers or higher molecular weight species (Table 2). The recovery of apoglycophorin was significantly lower than that of the other two forms indicating that a considerable portion of the protein sedimented near the cell bottom at 8000 rpm. Protein aggregation is also apparent by gel filtration of the native form on Sepharose CL-4B equilibrated in detergent free buffer (elution position is indicated in Figure 19, Chapter IV). Glycophorin elutes as a broad peak with a Stokes' radius centered around 100Å.

**Molecular weight determination in SDS**

Plots of \( \ln \text{OD} \) vs \( r^2 \) for all three forms in 13.9 mM SDS, 0.1 M sodium phosphate, pH 7.1 usually yielded slightly curved lines at all rotor speeds. Sedimentation data may be plotted as \( M(1-\varphi) \) vs. OD, OD being a function of protein concentration. Figure 11 shows the range of values of \( M(1-\varphi) \) vs. OD for native glycophorin and similar plots were made for asialo and apoglycophorin. At low native glycophorin concentrations \( M(1-\varphi) \) ranged from 1.25 - 1.52 \( \times 10^4 \), which corresponds to a glycoprotein molecular weight of 31,000 to 38,200 daltons. Using a glycoprotein weight of 31,000 and assuming that monomer and dimer glycophorin bind equivalent amounts of SDS on a
Fig. 11. Plot of $M(l-\bar{0}^\prime_p)$ vs. OD for native glycophorin in 13.9 mM SDS, 0.1 M sodium phosphate, pH 7.1. Crosshatched area shows the range of values of $M(l-\bar{0}^\prime_p)$ obtained from sedimentation equilibrium data of three native glycophorin preparations. At least two rotor speeds were used for each preparation. An $M(l-\bar{0}^\prime_p)$ value of $1.25 \times 10^4$ corresponds to a glycoprotein weight of 31,000 $d$. Assuming that dimeric glycophorin binds the same amount of SDS on a weight/weight basis as monomeric glycophorin, then an $M(l-\bar{0}^\prime_p)$ value of $1.61 \times 10^4$ would correspond to the presence of 19% dimer, and 81% monomer.
weight per weight basis, these values indicate the presence of 0 to 13% dimer on a molar basis. At the highest M(1-θ')s detected, calculations based on the above assumptions indicate that the solution contained 16% dimer and 84% monomer.

A native glycophorin dimer binding 3.0 g/g polypeptide would be expected to yield a M(1-θ') of 2.56 x 10⁴ or if no detergent were bound a value of 1.98 x 10⁴. This is much higher than the M(1-θ') obtained experimentally.

100° for 3 to 5 minutes prior to analysis by SDS gel electrophoresis, gel chromatography, or sedimentation equilibrium. This sample was applied to 7.5% polyacrylamide gels poured and electrophoresed in this buffer. There is no evidence for interaction between the two species on these gels - i.e., both species migrate as they would if electrophoresed separately. This is not the
weight per weight basis, these values indicate the presence of 0 to 13% dimer on a molar basis. At the highest $M(1-\varnothing')$ detected, calculations based on the above assumptions indicate that the solution contained 19% dimer and 81% monomer.

A native glycophorin dimer binding 1.0 g/g polypeptide would be expected to yield a $M(1-\varnothing')$ of $2.54 \times 10^4$ or if no detergent were bound a value of $1.98 \times 10^4$. This is much higher than the $M(1-\varnothing')$ obtained experimentally (Figure 11). To attain this measured $M(1-\varnothing')$, a glycophorin dimer would have to exhibit negative values of SDS binding which is clearly impossible. The $M(1-\varnothing')$ and the calculated polypeptide molecular weights for the three forms of glycophorin are shown in Table 3. These represent values taken at the meniscus, and are reasonably close to the actual value of about 14,500 expected for monomeric polypeptide. Curvature is usually seen in the $\ln \text{OD}$ vs. $r^2$ plots of all three forms at the higher protein concentrations, indicating the presence of larger molecular weight species. This curvature could be accounted for by assuming the presence of less than 20% dimer in the sample solution at the highest protein concentrations measured.

**Interaction of native glycophorin and apoglycophorin**

To determine whether apoglycophorin and native glycophorin associate in SDS containing solutions, these two species were mixed in 13.9 mM SDS, 0.1 M sodium phosphate, pH 7.1 $($\(\mu = 0.26\)$). This mixture was routinely heated at 100° for 3 to 5 minutes prior to analysis by SDS gel electrophoresis, gel chromatography, or sedimentation equilibrium. This sample was applied to 7.5% polyacrylamide gels poured and electrophoresed in this buffer. There is no evidence for interaction between the two species on these gels - i.e. both species migrate as they would if electrophoresed separately. This is not the
Fig. 12. Elution profile of a 1:1 molar mixture of native and apoglycophorin on a Sephacryl S-200 column equilibrated and eluted in 0.1 M sodium phosphate (μ = 0.26), pH 7.1, 0.4% SDS at 25°C. A 1:1 molar mixture of glycoporphin and apoglycophorin (protein concentrations about 1 mg/ml for each species) was heated at 100°C for 5 minutes before loading on the column. Native glycoporphin was detected by sialic acid (---) and 125I labeled apoglycophorin was detected by counts in a gamma counter. G and ApoG indicate when native and apoglycophorin elute if applied separately at these protein concentrations.
case when electrophoresis is carried out at lower ionic strengths as discussed in the appendix (Figure 34).

Figure 12 shows the elution pattern of a 1:1 molar mixture of native and apoglycoprotein on Sephacryl S-200 equilibrated and eluted in the buffer described above. Although the two protein peaks are not completely resolved, the elution pattern suggests that the two proteins chromatograph as noninteracting species. Sedimentation equilibrium of a similar 1:1 mixture is shown.

Quantitation of protein whether by Coomassie blue staining, 2D electrophoresing or Lowry (1951) color formation is strongly affected by the amount of carbohydrate on the protein. Thus, all such methods should be calibrated first by quantitative amino acid analysis.

Removal of carbohydrate led to corresponding changes in molecular
case when electrophoresis is carried out at lower ionic strengths as discussed in the appendix (Figure 34).

Figure 12 shows the elution pattern of a 1:1 molar mixture of native and apoglycophorin on Sephacryl S-200 equilibrated and eluted in the buffer described above. Although the two protein peaks are not completely resolved, the elution pattern suggests that the two proteins chromatograph as noninteracting species. Sedimentation equilibrium of a similar 1:1 mixture is shown in Figures 13 and 14. The plots of ln OD vs $r^2$ for apoglycophorin, native glycophorin and a mixture of two are shown in Figure 13. The curve for the mixture is intermediate in slope to that of the two species sedimenting separately. The experimental and theoretical curves (Figure 14) calculated on the assumption that apoglycophorin and native glycophorin mixed in solutions behave as noninteracting species are virtually superimposable. A heterodimer of apoglycophorin and native glycophorin binding 0.5 g/g SDS has a calculated value for $M(1-\Phi'\rho)$ or $1.70 \times 10^4$. An even larger value would be obtained at higher values of SDS binding. The largest $M(1-\Phi'\rho)$ observed at the bottom of the cell only approaches $1.5 \times 10^4$ which is the value obtained experimentally for native glycophorin alone.

Thus in high ionic strength phosphate buffers ($\mu = 0.26$) and at saturating levels of SDS binding, sedimentation equilibrium data indicates that these two proteins behave as noninteracting monomers.

**Discussion**

Quantitation of protein whether by Coomassie blue staining, PAS staining or Lowry (1951) color formation is strongly affected by the amount of carbohydrate on the protein. Thus, all such methods should be calibrated first by quantitative amino acid analysis.

Removal of carbohydrate led to corresponding changes in glycoprotein
Fig. 13. Sedimentation equilibrium plots of native (control) and apoglycophorin and a 1:1 molar mixture of the two in 0.1 M sodium phosphate, 13.9 mM SDS, pH 7.1. Samples were centrifuged at 15,000 rpm, T = 25°C.
Fig. 14. Sedimentation equilibrium plot of a 1:1 molar mixture of native and apoglycophorin. Experimental values (○) and theoretical values (—) of ln absorbance vs. \( r^2 \) are given. The theoretical values were calculated with the assumption that native and apoglycophorin behave as non-interacting species using the following equations:

1. \[ C_T = C_G + C_A \]

2. \[
C_i = \frac{C_o B_i (r_b^2 - r_m^2)}{B_i (r_b^2 - r_m^2) e^{B_i (r_b^2 - r_m^2)}} e^{B_i (r_b^2 - r_m^2) -1} \]

where \( B_i \) = slope of a plot of ln absorbance vs. \( r^2 \) for the component \( i \)

\[ = \frac{M_i (1-\phi'\rho) \omega^2}{2RT} \]

\( C_i \) is the concentration of species \( i \) at some distance \( r \) from the center or rotation. \( C_o \) is the initial concentration of species \( i \). \( r_b \) and \( r_m \) are the distances to the bottom of the cell and to the meniscus, respectively.
molecular weight, electrophoretic mobility on SDS gels, and distribution coefficients, \( K_d \), on gel chromatographic columns. At saturating SDS levels, all forms of glycoporphin were determined to be predominantly monomeric, but in aqueous, detergent-free buffer all species were aggregated. Apoglycoporphin appeared to be the most highly aggregated, and therefore it may be generally true that removal of carbohydrate from a membrane glycoprotein may lead to increased aggregation. The molecular weight must be identical to that calculated for the soluble protein. These conditions are not met by some glycoproteins. Lenoir et al. (1980) have compared the hydrodynamic properties of the SDS-holding, 2.5M SDS, charge to size ratio of several glycoproteins (Figure 4 and 5). Values of SDS binding for soluble and membrane proteins, unbound glycoporphin, and the three forms of glycoporphin are shown in Table 1. Lenoir et al. (1980)
molecular weight, electrophoretic mobility on SDS gels, and distribution co-efficients, $K_D$, on gel chromatographic columns. At saturating SDS levels, all forms of glycophorin were determined to be predominantly monomeric, but in aqueous, detergent free buffer all species were aggregated. Apoglycophorin appeared to be the most highly aggregated, and therefore it may be generally true that removal of carbohydrate from a membrane glycoprotein may lead to increased aggregation of the protein. It is interesting in this respect that removal of carbohydrate from the water soluble protein, mucin, led to a decrease in protein aggregation state (Hill et al., 1971). All data presented here and by others is consistent with the notion that protein-protein interaction is mediated primarily by the hydrophobic region. At high ionic strength and saturating levels of SDS binding native and apoglycophorin at a molar ratio of 1:1 in solution do not appear to interact with each other to any significant extent.

The apparent molecular weights as determined by gel electrophoresis for all three forms are more than twice the actual value. Most investigators have interpreted the native glycophorin bands on gels in terms of glycophorin dimer. However, any molecular weight determination by SDS gel electrophoresis is suspect since this technique demands (1) the charge to mass ratio of the glycoprotein SDS complex be the same as water soluble proteins used as standards and (2) the relationship between hydrodynamic size of the glycoprotein and its molecular weight must be identical to that relationship for water soluble protein. These conditions are not met by most glycoproteins. Leach et al. (1980) have compared the hydrodynamic properties, the SDS binding, and the charge to mass ratio of several glycoproteins (Tables 4 and 5). Values of SDS binding for soluble and membrane proteins, selected glycoproteins, and the three forms of glycophorin are shown in Table 4. Leach et al. (1980)
noted that the amount of SDS bound to glycoproteins on a g/g basis is reduced by about the same percentage as the weight percentage of the bound carbohydrate. These data suggest that the polypeptide moiety binds the normal weight ratio of SDS while the carbohydrate exhibits little or no SDS binding. It is interesting that glycophorin and its derivatives display characteristics consistent with this behavior. Although all three forms of glycophorin have binding isotherms indicative of membrane proteins (suggesting that cooperative SDS binding occurs to the hydrophobic region), the magnitude of SDS binding to the native form on a g/g glycoprotein basis is much lower than the normal ratio. This was predicted previously by Banker and Cotman (1972) on the basis of Ferguson plots of glycophorin electrophoretic mobility. The fact that binding to glycophorin on a g SDS bound per g polypeptide basis is considerably increased when sialic acid is cleaved from the chain indicates that covalently bound sialic acid exhibits little or no SDS binding; and, in fact, may mask hydrophobic sites thereby preventing SDS binding to the peptide chain. This masking of the peptide chain could be due to steric hindrance, to charge repulsion effects, or to a combination of these factors. Whether increased SDS binding shown by deglycosylated forms represents additional binding to the hydrophobic peptide or to the amino terminal region is not known. This additional binding brings about no apparent change in the CD spectra, suggesting that much of the binding is to the hydrophobic region. Studies of SDS binding to glycophorin peptides would elucidate this point. The charge to mass ratio of native glycophorin and several other glycoproteins complexed to SDS is considerably lower than the ratio of most proteins used as standards (Table 5). This may account in part for the anomalous behavior of glycoproteins on gels. From Table 5, one can see that the increased binding of SDS of asialoglycophorin more than compensates for the
### TABLE 5

**CHARGE TO MASS RATIO OF VARIOUS PROTEINS COMPLEXED WITH SDS**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\delta_D$</th>
<th>$Z_{pp}$</th>
<th>$Z_{CHO}$</th>
<th>$Z_{TOT}$</th>
<th>$\overline{Q}$</th>
<th>$\overline{Q} / M \times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average of 12 common hydrophilic proteins</td>
<td>1.4±0.1</td>
<td></td>
<td></td>
<td></td>
<td>-4.7±0.4</td>
<td></td>
</tr>
<tr>
<td>Histone H1</td>
<td>1.4</td>
<td>+56</td>
<td>-102</td>
<td>-2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferridoxin</td>
<td>1.4</td>
<td>-11</td>
<td>-30</td>
<td>-6.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha_1$-Acid glycoprotein</td>
<td>0.7</td>
<td>-26</td>
<td>-95</td>
<td>-3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetuin</td>
<td>1.23</td>
<td>-21</td>
<td>-183</td>
<td>-4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tamm-Horsfall urinary glycoprotein</td>
<td>0.99</td>
<td>-12</td>
<td>-261</td>
<td>-3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta$-K-casein</td>
<td>2.0</td>
<td>+4</td>
<td>-81</td>
<td>-6.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>1.10</td>
<td>-4</td>
<td>-105</td>
<td>-4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Integral membrane proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycophorin A</td>
<td>0.5</td>
<td>-11.5</td>
<td>-28</td>
<td>-39.5</td>
<td>-93.3</td>
<td>-3.01</td>
</tr>
<tr>
<td>Asialoglycophorin</td>
<td>1.24</td>
<td>-11.5</td>
<td>-2</td>
<td>-13.5</td>
<td>-100.2</td>
<td>-4.35</td>
</tr>
<tr>
<td>Apoglycophorin</td>
<td>1.56</td>
<td>-11.5</td>
<td>-1</td>
<td>-12.5</td>
<td>-100.6</td>
<td>-5.6</td>
</tr>
<tr>
<td>Cytochrome b$_5^b$</td>
<td>1.24g/g</td>
<td></td>
<td></td>
<td>-70</td>
<td>-4.2</td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$ ATPase $^b$</td>
<td>2.6 g/g</td>
<td></td>
<td></td>
<td>-990</td>
<td>-9.0</td>
<td></td>
</tr>
<tr>
<td>fd coat protein $^b$</td>
<td>1.65g/g</td>
<td>0</td>
<td>-30</td>
<td>-5.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

*a Much of this table is taken from Leach *et al.* (1980). The average net charge $\overline{Q}$ on a (glyco) polypeptide-SDS complex may be expressed as:

$$
\overline{Q} = \frac{\delta_D M Z_D}{M_D} + \overline{Z}_{TOT}
$$

where $\delta_D$ is the binding ratio in g SDS per g glycoprotein $Z_D$ is the charge per SDS molecule ($=-1$), $M$ is the molecular weight of the (glyco) polypeptide, $M_D$ is the molecular weight of the SDS ($=288d$), and $Z_{TOT}$ is the
average net intrinsic charge of the (glyco) polypeptide. \( Z_{\text{TOT}} = Z_{\text{CHO}} + Z_{\text{pp}} \) where \( Z_{\text{CHO}} \) and \( Z_{\text{pp}} \) are the average net intrinsic charge on the carbohydrate and polypeptide moieties respectively.

b Intrinsic charge on the protein is small compared to charge provided by bound SDS; therefore, intrinsic protein charge is ignored in computing the charge to mass ratio.
loss of negative charge by removal of sialic acid from glycophorin. Asialo-glycophorin as well as apoglycophorin complexed with SDS have larger charge to mass ratios than the native form, and this along with changes in size probably accounts for the differences noted in electrophoretic mobility among the forms. In this regard, Leach et al. (1980) also found that removal of some of the sialic acid residues from α acid glycoprotein or fetuin elicited a small increase in their electrophoretic mobilities.

Membrane proteins, like Ca\textsuperscript{2+} ATPase and fd coat protein have higher charge to mass ratios than standards and therefore might also be expected to yield anomalous molecular weights by this method. Taken as a whole, the work reported in this chapter indicates that removal of carbohydrate from glycophorin leads to changes consistent with the known behavior of other membrane proteins and glycoproteins.

Many functions of membrane proteins, e.g. ion transport or membrane internalization processes, can be studied only in intact cells or in analogous systems such as phasmid virus. These studies elucidate some principles involved in the incorporation of integral membrane proteins into vesicles and provide a model for later reconstitutions involving ion pumps and transporters. This chapter describes the formation and characterization of vesicles formed in the presence and absence of the transmembrane protein glycophorin. In Chapter V, the reconstitution of membrane bound dopamine-β-hydroxylase and hepatic asialoglycoprotein receptor are discussed. In Chapter VI, data concerning ion influx and efflux from pure phosphatidyl choline vesicles and glycophorin containing vesicles are examined.
CHAPTER IV

VESICLE FORMATION AND GLYCOPHORIN INCORPORATION

Many functions of membrane proteins, e.g. ion transport or membrane internalization processes, can be studied only in intact cells or in analogous systems where two aqueous compartments are separated by membrane, such as phospholipid vesicles. Unfortunately, few vesicle preparations have been well defined with respect to size distribution, protein orientation in the membrane, and passive permeability; this knowledge is essential for the interpretation of ion transport data.

These properties were investigated for vesicles produced in a simple ternary system: egg yolk phosphatidylcholine/octyl glucoside/purified integral membrane protein. These studies elucidate some principles involved in the incorporation of integral membrane proteins into vesicles and provide a model for later reconstitutions involving ion pumps and transporters. This chapter describes the formation and characterization of vesicles formed in the presence and absence of the transmembrane protein glycophorin. In Chapter V, the reconstitution of membrane bound dopamine-β-hydroxylase and hepatic asialoglycoprotein receptor are discussed. In Chapter VI, data concerning ion influx and efflux from pure phosphatidyl choline vesicles and glycophorin containing vesicles are examined.

Procedure for vesicle formation and protein incorporation

Previous methods of glycophorin incorporation into vesicles involved
the drying of this protein together with lipid in organic solvent (MacDonald and MacDonald, 1975; van Zoelen et al., 1978a). These procedures and others which use organic solvents produce a very heterogeneous vesicle preparation and would not be generally applicable for the incorporation of functional membrane proteins which are inactivated in these solvents. Because most membrane proteins are generally purified and characterized in detergent solubilized form, a reconstitution method that allows formation of protein containing vesicles from protein-detergent mixtures was chosen for these studies.

In these methods, vesicle formation and incorporation of protein depend on the removal of detergents from detergent/phospholipid/protein mixed micelles. This removal is facilitated if the detergent has a high CMC (and therefore a relatively high monomer concentration). The bile detergents sodium deoxycholate and sodium cholate have been used extensively for reconstitution studies (Kagawa and Racker, 1971; Razin, 1972; Korenbrot, 1977). These detergents can be easily removed from a lipid mixture by gel filtration (Brunner et al., 1976), centrifugation (Warren et al., 1974) or dialysis (Kagawa and Racker, 1971) to produce small unilamellar vesicles. In these cases the rate of removal and the initial detergent to lipid ratio appear to be important determinants of the vesicle size. The treatment of egg phosphatidylcholine (PC) with sodium cholate at a 1:2 molar ratio followed by gel filtration to remove detergent results in the formation of a homogeneous population of 30 nm vesicles (Brunner et al., 1976). However, 100 nm vesicles were prepared by Enoch and Strittmatter (1979) using an egg PC:DOC ratio of 2:1. (According to Szoka and Papahadjopoulos (1980) cholate may be substituted for DOC with similar results.) In this technique, detergent removal was effected by passage of the PC/DOC mixture through two consecutive Sepharose G-25 columns. Employing an initial egg PC:DOC ratio of 1:1.6 and
removing detergent by fast controlled dialysis, Milsmann et al., (1978) produced a relatively homogeneous population of 60 nm diameter vesicles. Use of these procedures for protein incorporation has not been fully developed.

Cholate and deoxycholate have the disadvantage that they inactivate many membrane proteins and that their solubility is limited under certain buffer and temperature conditions. To circumvent these problems, researchers from this lab and others have turned to nonionic detergents for formation of protein containing vesicles.

Vesicle formation from Triton X-100 has been described and is based on the ability of Biobeads SM-2 to absorb Triton X-100 selectively and rapidly over phospholipid (Gerritson, et al., 1978). These vesicles have not been well characterized.

For the work presented in this thesis, the nonionic detergent octyl glucoside was chosen because it is commercially available in pure form, offers no restriction on buffer choice or temperature in the ranges of concern here, and has a high CMC around 20 mM (de Grip and Bovee-Geurts, 1979).

Octyl glucoside has been used in other reconstitution studies of viral membrane glycoproteins (Helenius et al., 1977; Petri and Wagner, 1974), HLA antigens (Engelhard et al., 1978; Curman et al., 1980), and the proton pumps of chloroplasts and mitochondria (Racker et al., 1979). The general procedure in this thesis has been to deposit egg phosphatidyl choline as a thin film on the walls of a glass tube, to dissolve the film in a solution containing protein-detergent mixed micelles, and then to remove octyl glucoside from the lipid/detergent/protein micelles by dialysis or gel filtration.

This procedure is similar to that of Helenius et al. (1977); however, a much higher lipid/protein ratio was used because the functional studies on ion transport to which the technique will eventually be applied may best be
performed with a relatively small number of protein molecules per vesicle. In addition, a high detergent concentration was employed so that from statistical considerations alone the protein is expected to be distributed among micelles in monomeric form. It is important to note, however, that some membrane proteins will probably require detergent with longer alkyl chains (and therefore lower CMC's) than octyl glucoside for maintenance of structural integrity.

**Materials and Methods**

**Materials.** Hen egg lecithin was purchased from Lipid Products, South Nutfield, U.K. It was tested by high pressure liquid chromatography and found to contain no component other than diacyl phosphatidylcholine (detection limit 0.1% by weight). Octyl glucoside (octyl-β-D-glucopyranoside) was purchased from Calbiochem, and \(^{14}\text{C}\)octyl glucoside was obtained from New England Nuclear Corp. \(\text{Na}^{125}\)I was purchased from Amersham Corp. TPCK-trypsin and papain were Worthington products, and neuraminidase was a gift from Dr. L. Glasgow. Sodium dodecylsulfate (chain length purity 99.8%) was purchased from BDH Chemicals, Ltd., Poole, U.K. Resins used for gel chromatography (Sephadex G-25, G-50, Sepharose 4B, 4B-CL) were obtained from Pharmacia, Inc. Water was deionized, and filtered to remove particulate matter. All other chemicals were reagent grade.

**Glycophorin A solutions for reconstitution.** Glycophorin was radioiodinated by the chloromine T method of Cuatrecasas (1971). A small amount of \(^{125}\)I-glycophorin (in 1 to 2 ml) was added to 20 to 300 ml of unlabeled glycophorin at a concentration of 1 mg/ml. 0.2 to 0.5 ml of 20 mg/ml (68.5 mM) octyl glucoside solution was added to the protein solution. Since octyl glucoside has a CMC near 20 mM (de Grip and Bovee-Geurts, 1979) and a micelle
aggregation number near 100 (D. McCaslin, unpublished data), these conditions correspond to a range of about 1 to 130 detergent micelles per protein molecule. For reconstitution studies, this solution was added to dried lipid or dried lipid-octyl glucoside mixtures as described below.

Formation of vesicles

Procedure I

5 to 10 mg of egg lecithin was dissolved in 2:1 (v/v) chloroform: methanol in a 30 ml Corex tube, and the mixture dried to a thin film under a stream of argon or nitrogen, and then put under vacuum for several hours to remove any residual traces of organic solvents. This lipid film was redis­ solved in 0.4 to 0.6 ml of an appropriate octyl glucoside (OG) or octyl glu­coside/protein solution (usually around 68.5 mM in OG). Enough solid octyl glucoside was added to make the molar ratio of octyl glucoside to phospholipid 5-6:1; however, in some cases higher detergent ratios of up to 15:1 were used. Solubilization was carried out with hand shaking and bath sonica­tion (2-10 min) until the solution was clear and no discernable lipid remained on the glass surface of the Corex tube. This mixture was chromatographed on a Sephadex G-25 column (total column volume = 44.0 ml, sample size 0.4 - 0.6 ml, column presaturated with lipid) equilibrated and eluted in 0.01 M Tris- HCl, pH 7.5 or 8.5 and 0.25 M in whichever salt (generally NaCl or KCl) it was desired to trap in the vesicles. The vesicles which were formed during gel filtration elute at the void volume as a turbid solution. These vesicles were then passed down a Sepharose CL-4B column (total volume 45.2 ml, equili­brated with the same salt and buffer) to minimize the amount of residual de­tergent and to separate free protein from vesicle incorporated proteins. The column steps were performed at 4°C.
Procedure II

2-10 mg of egg lecithin was dried in a Corex tube as in Procedure I. In some preparations octyl glucoside (at a weight ratio of 4:1, OG:egg PC) was codeposited with lipid, but no advantage in doing so was observed. This lipid or lipid-detergent film was dissolved in 0.4 to 0.6 ml of a detergent or detergent-protein solution such that the final molar ratio of detergent to lipid was 15:1. This solution was dialyzed against two 1 liter changes of buffer for 12 hours each in 0.01 M Tris, pH 7.5 or 8.5 and in 0.25 NaCl or 0.25 KCl for glycophorin reconstitution experiments. Most of the detergent was removed at this point and the solution became turbid. These vesicles were then chromatographed on a Sepharose CL-4B column as in Procedure I. The two procedures differed in (1) the method of detergent removal and (2) the starting detergent to lipid ratios.

Sucrose density gradient centrifugation

Sedimentation velocity and flotation analysis were carried out in 12 ml linear sucrose gradients. Centrifugation was performed in a Beckman Ti41 swinging bucket rotor at 40,000 rpm. Samples subjected to flotation analysis were placed at the bottom of the tube, and contained 5% greater concentration of sucrose than the highest concentration in the gradient. In sedimentation velocity measurements samples were layered on top of the gradient. After centrifugation, the gradients were eluted from the bottom of the tube.

Enzymatic digestion of vesicles

Papain digestion of 125I-glycophorin containing vesicles was carried out at 37°C for 1 hour. Vesicles containing less than 1 µg of protein were mixed with 74 mg of papain in 0.5 ml of 0.01 M TES, pH 7.0, 0.1 M NaCl. The lipid vesicles were analyzed directly by flotation in linear sucrose
gradients without separation of enzyme and digestion products prior to analysis.

Trypsin or neuraminidase digestion of $^{125}$I glycophorin A containing vesicles was carried out at 37° for 1 hr. These vesicles in equilibration buffer or solubilized in detergent were mixed with 10 μg trypsin or 1 unit activity of neuraminidase. These glycophorin containing vesicles were analyzed by SDS gel electrophoresis and by counting of gel slices in a gamma counter.

Ferritin labeling of vesicles

Ferritin was conjugated to wheat germ agglutinin by the method of Kishida et al. (1975) and incubated with the vesicle preparation at 24°C for 30 min. Vesicles were separated from unbound ferritin by Sepharose CL-4B column chromatography and then subjected to negative staining as described below.

Electron microscopy. Dr. Guido Zampighi performed all electron microscopy on these vesicle preparations. For negative staining, vesicle suspensions were applied to carbon-coated grids, washed with stain solution (2% uranyl acetate in water), and blotted with filter paper. Freeze fracture experiments employed the technique of Costello and Corless (1977). Vesicle suspensions were sandwiched between two copper strips and plunged into liquid propane (-190°C). Specimens were fractured in a Balzers 360 M freeze-fracture apparatus at -160°C, and were shadowed with platinum and carbon, without etching.

A Philips EM-301 electron microscope was used, with an objective aperture of semi-angle 0.005 radian. Grids were scanned at low magnification to select regions having a good distribution of vesicles and an even deposit of
stain. These areas were imaged randomly.

**Internal volume.** Total trapped volume in a vesicle suspension was obtained by passing vesicles prepared in 0.25 M NaCl or KCl down a Sephadex G-50 column to exchange the Cl\(^-\) in the external medium for NO\(_3^-\). External Cl\(^-\) concentrations after this exchange (C\(_o\)) were measured using chloride electrodes (see Chapter VI) and were generally between 10\(^{-5}\) and 10\(^{-4}\) M. The concentrations were measured again after lysis of the vesicles by addition of excess C\(_{12}E_8\) (C\(_\infty\)). The ratio of internal to total volume is obtained as (C\(_\infty\) - C\(_o\))/(0.25). The phospholipid concentration was measured in terms of inorganic phosphate by the method of Bartlett (1959), to allow calculation of the internal volume per mol phospholipid.

**Results**

**Formation of vesicles**

In these studies, relatively high lipid to protein ratios were used, and thus the properties of the vesicle preparation are determined primarily by the detergent to lipid ratio in the initial solution rather than by the detergent to protein ratio. To determine the detergent to lipid ratio necessary for a good lipid dispersion and for formation of reasonably uniform vesicles, protein free octyl glucoside-egg PC systems were examined. Detergent solubilization of the lipid films was conducted in two ways. Either a detergent solution was added to the lipid directly, or a portion of the desired final octyl glucoside concentration was deposited with the lipid and the remainder added in solution, as described by Helenius et al. (1977). The two procedures gave identical results. Variations among preparation were largely dependent on the final detergent to lipid ratio in solution before detergent removal. No difference between the two procedures was observed in later...
experiments in which protein was incorporated into vesicles.

Generally the amount of lipid deposited on the vessel walls and volumes of added solution were such that a final concentration of 20 mM egg PC was obtained. To obtain a clear solution the presence of at least a 4:1 molar ratio of OG to lipid was required. (Since the CMC of OG is about 20 mM the molar ratio of detergent to lipid on the mixed micelle would be 3:1). After detergent removal from mixed micelle solutions, the resulting cloudy solution was examined by electron microscopy, which showed the presence of unilamellar vesicles (See Figure 15). When the amount of detergent was close to the minimum required for obtaining a clear solution (5:1, OG:egg PC), only about 50% of the lipid was found in vesicular form. The remainder consisted of other kinds of aggregates, as shown in Figure 15A. Much better vesicle preparations were obtained with OG:egg PC molar ratios of 10:1 or greater. The electron micrographs of Figure 15B–F were taken of samples prepared as a 15:1 OG to egg PC ratio, corresponding to a 14:1 ratio in mixed micelles. Approximately 90% of the lipid appeared to be in a vesicular form in these preparations by electron microscopy. This estimate was confirmed by comparing measured internal volume with internal volume calculated on the basis of vesicle dimensions. The results of this calculation are shown in Table 6 and plotted in Figure 16. Volumes were determined for spherical unilamellar vesicles assuming a bilayer thickness of 4.0 nm and an average surface area per phospholipid molecule of 0.7 nm. Similar calculations were performed by Hauser et al. (1973) and by Enoch and Strittmatter (1979) using a bilayer thickness of 4.6 nm but their results are not significantly different from those shown in Table 6. A membrane thickness of 3.7 nm was measured on the basis of trapping volumes for egg PC vesicles which are much smaller than the vesicles formed here. Egg PC packing constraints between the two leaflets of the
Fig. 15. Electron microscopy of egg phosphatidylcholine vesicles. A. Vesicles prepared using a molar detergent/lipid ratio of 5:1, examined by negative staining. The electron micrograph shows one of the worst portions of the total field. The origin of the small round particles in the background is not known (x 50,000). B. Vesicles prepared using a molar detergent/lipid ratio of 15:1, examined by negative staining. The electron micrograph shows an average area of the total field (x 50,000). C. Representative freeze-fracture electron micrograph of vesicles prepared at a 15:1 molar detergent/lipid ratio (x 40,000). D. The same preparation as in B, examined at lower magnification to show the homogeneity of the preparation. Dark round shapes in the background probably represent vesicles that collapsed during the staining procedure (x 15,000). E. Glycophorin-containing vesicles (50 protein molecules per vesicle) prepared at a 15:1 molar detergent/lipid ratio. Freeze-fracture studies were not made (x 30,000). F. Visualization of membrane-bound glycophorin, using ferritin-conjugated wheat germ agglutinin, which was added to the vesicle preparation before the final chromatography step. This preparation contained an average of 20 glycophorin molecules per vesicle. The most prominent ferritin molecules seen on negative staining with uranyl acetate are indicated by brackets (x 90,000).
<table>
<thead>
<tr>
<th>External diameter, nm</th>
<th>Phospholipid molecules per vesicle</th>
<th>Internal volume per vesicle $\mu l \times 10^{13}$</th>
<th>Internal volume per mol lipid $\mu l$</th>
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</thead>
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<tr>
<td>100</td>
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<td>0.400</td>
<td>2360</td>
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<tr>
<td>150</td>
<td>290,000</td>
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### TABLE 6
**CALCULATED PROPERTIES OF VESICLES**

<table>
<thead>
<tr>
<th>External diameter, nm</th>
<th>Phospholipid molecules per vesicle</th>
<th>Internal volume per vesicle mL x 10^15</th>
<th>Internal volume per mol lipid mL</th>
</tr>
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<td>100</td>
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<td>150</td>
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<td>200</td>
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</tr>
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<td>220</td>
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<td>7170</td>
</tr>
<tr>
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<tr>
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<tr>
<td>260</td>
<td>588,000</td>
<td>8.38</td>
<td>8580</td>
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*a Calculated by method of Huang and Mason (1978) based on a bilayer thickness of 4.0 nm, and an average surface area of 0.70 nm^2 per phospholipid molecule.*
Fig. 16. Internal volume and number of phospholipid molecules per vesicle as a function of the diameter of unilamellar egg phosphatidylcholine vesicles. These theoretical curves were calculated assuming a bilayer thickness of 4 nm and an average surface area of 0.7 nm$^2$ per phospholipid molecule (calculated by the method of Huang and Mason (1978)).
smaller vesicles were considerably different due to the high degree of curvature (Johnson, 1973; Huang and Muen, 1978). From Table 6, the internal volume per mole PC is expected to be about 7500 ml for vesicles with an external diameter of 200 nm. Using the chloride contents of the sample described in the latter sections, the internal volume of vesicles prepared at 3000 to 5000 m-atm was considerably larger than that under the latter sections. Larger vesicles were also smaller.

\[ \text{Internal Volume (ml/mole of Phospholipid)} \times 10^{-3} \]

\[ \text{ Vesicle Diameter in nm} \]

\[ \text{Phospholipid Molecules per Vesicle} \times 10^{-5} \]

Electron micrographs of vesicles prepared from a 3:1 water/methanol showed that these vesicles had about the same size as those prepared at higher purification.
smaller vesicles were considerably different due to the high degree of curvature (Johnson, 1973; Huang and Mason, 1978). From Table 6, the internal volume per mole PC is expected to be about 7500 ml for vesicles with an external diameter of 230 nm. (See below). Using the chloride trapping procedure described in Methods, values of 6000 to 7400 ml were measured for vesicles prepared at a molar detergent/lipid ratio of greater than 10:1; values of 3600 to 5000 ml were measured at 5:1 OG to egg PC ratio, indicating that a considerably larger fraction of the lipid was incapable of trapping solute under the latter conditions.

Characteristics of pure lipid vesicles

Lipid vesicles eluted at the void volume when chromatographed on Sepharose CL-4B (as shown in Figure 18), indicating that they are considerably larger than egg PC vesicles prepared by sonication (Huang, 1979) or by detergent removal from cholate-lipid mixed micelles (Brunner et al., 1976). Many preparations as examined by electron microscopy contained vesicles with mean diameters of greater than 200 nm. Typical electron microscopy of vesicles prepared at a 15:1 OG to egg PC ratio are shown in Figure 15B. The vesicles were smooth and round. The smooth fracture faces shown in Figure 14E are representative of the entire field, and the absence of multistep surfaces unambiguously demonstrated the unilamellar nature of the preparation. The vesicles were also fairly homogeneous in size. Figure 17 shows a size distribution histogram of one such preparation, which yielded a mean diameter of 240±60 nm. Fewer than 20% of the vesicles had diameters outside the range 175-325 nm.

Electron micrographs of vesicles prepared from a 5:1 OG:PC mixture showed that these vesicles had about the same size as those prepared at higher
Fig. 17. Size distribution histograms of vesicles prepared at a 15:1 molar detergent/lipid ratio. A represents pure lipid vesicles and B represents vesicles containing about 50 glycophorin molecules per vesicle. Diameters of 75 randomly chosen vesicles were measured in each case from representative fields of the electron micrographs. Average values were $240 \pm 60$ nm in A and $225 \pm 85$ nm in B. Particles with a diameter of $< 100$ nm (mostly non-vesicular) were not included in this analysis. (Histogram prepared by Guido Zampighi.)
Fig. 18. Elution profile of cytochrome c (measured at 550 nm) and phospholipid vesicles (measured by optical density at 330 nm arising from light scattering) chromatographed on Sepharose CL-4B column. Cytochrome c was added to the lipid-detergent mixture before vesicles were formed, and the portion co-eluting with lipid (20%) represents the protein trapped in the internal vesicular volume (see text). Chromatography was performed in 0.01 M Tris-HCl, pH 7.5, 0.1 M NaCl at 4°C. The elution profile of the lipid in this experiment is typical of all vesicle preparations used in these investigations.
detoxificant/lipid ratio; however, a considerable amount of the lipid was seen to be present in nonvesicular form (Figure 15A). In addition to the trapping of the vesicular nature of preparation could be demonstrated by cytochrome c trapping (Figure 1B). Vesicles were prepared from a cytochrome c-cone, also dissolved in a solution as in Procedure II with a lipid concentration of 30 mM. Vesicles (with internal volume 7.5 liters/mole PC) formed under these conditions are expected to trap 22% of cytochrome c, and this corresponded within experimental error to the fraction of cytochrome c eluting in the void volume.

Characterization of chlorophyll-containing vesicles

Vesicles prepared from mixed solutions containing chlorophyll and photosynthetically indistinguishable from pyridine-free vesicles (Figure 2B). However, they have a broader size distribution as shown in a typical size distribution.
detergent/lipid ratios; however, a considerable amount of the lipid was seen to be present in nonvesicular form (Figure 15A). In addition to ion trapping, the vesicular nature of preparation could be demonstrated by cytochrome c trapping (Figure 18). Vesicles were prepared from a cytochrome c, octyl glucoside, PC solution as in Procedure II with a lipid concentration of 30 mM. Vesicles (with internal volume 7.5 liters/mole PC) formed under these conditions are expected to trap 22% of cytochrome c, and this corresponded within experimental error to the fraction of cytochrome c eluting in the void volume. The trapped fraction of cytochrome c could not be reduced with sodium ascorbate (to which lipid vesicles are impermeant), but it was reducible after the vesicles were lysed with the detergent C₁₂E₈. Cytochrome c added to pre-formed vesicles did not coelute with vesicles in the void volume.

To determine the amount of detergent remaining in the vesicle preparation, ¹⁴C labeled octyl glucoside was present during vesicle formation. From the number of counts coeluting with lipid on a chromatographic column, the amount of residual detergent was calculated. After the first chromatographic step (Sephadex G-25) the residual detergent was less than 0.01% of what was added originally, corresponding to about 1 octyl glucoside molecule per 200 lipid molecules. After chromatography on Sepharose CL-4B the radioactivity coeluting with lipid was barely detectable, about 1+1 molecule of detergent per 400 lipid molecules. This small amount of residual octyl glucoside may represent detergent molecules trapped in the internal aqueous space.

Characterization of glycophorin containing vesicles

Vesicles prepared from mixed micelles containing glycophorin are morphologically indistinguishable from protein-free vesicles (Figure 15), but they have a broader size distribution as shown in a typical size distribution.
histogram (Figure 17). The average diameter is $225 \pm 85$ nm compared to $240 \pm 60$ nm obtained in the absence of protein. These statistical size measurements were made on only one preparation; however, all preparations superficially appeared identical as imaged by electron microscopy. Residual detergent was determined for one preparation and was similar to that obtained for protein-free vesicles.

Figure 19 shows a typical elution profile of glycophorin-containing vesicles on Sepharose CL-4B. In this experiment the added amount of glycophorin corresponded to about 100 molecules per vesicle, but only about half that amount eluted with lipid at the void volume. Most of the glycophorin not incorporated into vesicles eluted at a position where aggregated glycophorin elutes in aqueous solution in the absence of lipid and detergent, but some radioactive counts were also found in a peak of smaller particle size. Rechromatography or sucrose gradient centrifugation showed that all of the glycophorin counts in the void volume coeluted or comigrated with lipid. Figure 20 shows flotation analyses of a preparation in which about 30% of glycophorin was incorporated. Since the separation in this case was on the basis of density rather than particle size, protein coeluting with lipid in Figure 19 must be incorporated with lipid in the same particle. Similar results were obtained with 4 different preparations at molar ratios of protein to lipid ranging from 1:500 to 1:4400. This corresponds to 100 to 900 glycophorin molecules added per vesicle formed. The fraction of protein molecules incorporated, on the basis of coelution with lipid, ranged from 25 to 50%. Glycophorin incubated with preformed vesicles did not elute with lipid in the void volume of Sepharose 4B indicating that glycophorin does not bind preformed vesicles.

The initial phospholipid concentration used to prepare vesicles for these experiments was lower than that employed for the cytochrome c trapping
Fig. 19. Elution profile of glycophorin-containing vesicles on Sepha­rose CL-4B. The protein was 125I-labeled, and was monitored by radioactivity; lipid was monitored on the basis of optical density at 280 nm arising from light scattering. Half of the glycophorin was observed to co-elute with lipid at the void volume ($V_0$). Much of the remainder elutes at the same position ($G$) as glycophorin aggregates that normally form in aqueous solution when the protein is dissolved in the absence of detergent or lipid. Initial protein solution contained 3.5 μM glycophorin, 68.5 mM octyl glucoside, corresponding to about 130 detergent micelles per protein molecule. Final concentrations prior to detergent removal were 3.5 μM glycophorin, 16 mM phospholipid, 240 mM octyl glucoside, corresponding to 7 lipid molecules per average micelle and 600 micelles per protein molecule.
experiment (Figure 15), and therefore the calculated internal volume was smaller. In Figure 19, the lipid concentration was 15.6 nM, allowing the trapping of 11% of volume. The amount of glycoporin eluting with lipid was 50X and in all cases was considerably higher than the percentage that could have been trapped in the vesicular internal volume. Therefore, most of the protein re-eluting with lipid was membrane bound.

This conclusion was confirmed by showing that the glycoporin was ex-

The best characterized unilamellar phospholipid vesicles used in the present study were obtained by sonication of vesicles consisting of phospholipid. The vesicles obtained by this procedure are generally small (membrane diameter less than 30 nm) and because of phospholipid limiting constituent to highly curved membranes, asymmetries in composition occur between bilayer.
experiment (Figure 18), and therefore the calculated internal volume was smaller. In Figure 19, the lipid concentration was 15.6 mM, allowing the trapping of 11% of volume. The amount of glycophorin eluting with lipid was 50% and in all cases was considerably higher than the percentage that could have been trapped in the vesicular internal volume. Therefore, most of the protein co-eluting with lipid was membrane bound.

This conclusion was confirmed by showing that the glycophorin was susceptible to the action of neuraminidase, trypsin and papain. (Figures 20, 21) (Cytochrome c was found to be inaccessible to chemical reagents.) Approximately 75% of glycophorin molecules were converted to asialoglycophorin by neuraminidase treatment, whereas 100% were converted when the vesicles were first lysed with $C_{12}E_8$. Although glycophorin is relatively resistant to trypsin treatment, a considerable amount of the glycophorin was converted to a smaller species as detected by SDS gel electrophoresis, and addition of $C_{12}E_8$ enhanced this proteolysis. Papain cleavage of vesicle showed that 60% of $^{125}I$ glycophorin counts could be cleaved from vesicles (Figure 20). These data are consistent with the view that membrane bound glycophorin does not have a unique orientation in the membrane.

Membrane incorporation of glycophorin was also demonstrated by use of ferritin conjugated wheat germ agglutinin as shown in the electron micrograph (Figure 15F).

Discussion

The best characterized unilamellar phospholipid vesicles prior to the present study were obtained by sonication of aqueous suspensions of phospholipid. The vesicles obtained by this procedure are generally small (external diameter less than 30 nm) and because of phospholipid packing constraints in highly curved membranes, asymmetries in composition occur between bilayer
Fig. 20. Flotation centrifugation of papain digested $^{125}$I labeled glycophorin incorporated into vesicles. Samples were loaded onto 12 ml 15% to 60% linear sucrose gradients and were centrifuged at $4^\circ$ in a Beckman Ti41 rotor at 40,000 rpm for 20 hrs. Fractions were collected from the bottom and counted for $^{125}$I directly. An aliquot of $^{125}$I labeled glycophorin-vesicle suspension after the removal of detergent by dialysis was centrifuged is shown in A. $^{125}$I glycophorin, papain digested $^{125}$I glycophorin, and an aliquot of the internal volume peak of 4B-CL column from Fig. 18 migrate identically and are shown in B. Aliquots from the void volume of the Sepharose CL-4B column shown in Fig. 18 containing $^{125}$I glycophorin incorporated vesicles was incubated at $37^\circ$ for 1 hr in the absence (C) and presence (D) of papain prior to centrifugation.
Fig. 21. SDS gel electrophoresis of trypsin (A) and neuraminidase (B) digested vesicles containing 125I glycophorin. Vesicles from the void volume of the Sepharose CL-4B column (shown in Fig. 18) were digested with trypsin or neuraminidase as described in Methods. Control 125I glycophorin vesicles with no enzyme present during incubation (---), 125I glycophorin vesicles with enzyme present (---), and 125I glycophorin vesicles solubilized with C12E8 with enzyme present (....). TD indicates the tracking dye.
Lee et al. (1973; Litwin, 1974). The unilamellar vesicles described in this thesis work were obtained by removal of detergent from mixed micelles of phospholipid and octyl glucoside and were much larger in size (average diameter greater than 200 nm). Although concentration studies using octyl glucoside have been carried out in other laboratories, the vesicles obtained (as imaged in electron micrographs) were more heterogeneous and generally smaller than those obtained here.

---

(3) Orientation of proteins in vesicles:

**Initial state.** Octyl glucoside, whether solubilized and reconstituted after lipid layer in aqueous solution, produced negative orientation of proteins which upon dialysis form fairly ordered membrane-like structures.

![Graphs showing relative mobility](image)
leaflets (Michaelson et al., 1973; Litman, 1974). The unilamellar vesicles described in this thesis work were obtained by removal of detergent from mixed micelles of phospholipid and octyl glucoside and were much larger in size (average diameter greater than 200 nm). Although reconstitution studies using octyl glucoside have been carried out in other laboratories, the vesicles obtained (as imaged in electron micrographs) were more heterogeneous and generally smaller than those obtained here.

**Vesicle formation**

The mechanism of vesicle formation and the reconstitution of membrane proteins warrants further investigation, but the present study offers some insight into the process.

Liposome or vesicle formation is a spontaneous process which occurs upon hydration of diacyl phospholipid and is a consequence of head group repulsion, packing constraints of diacyl lipid chains, and the hydrophobic effect. These have been described in detail by Tanford (1980). The concern here is to understand vesicle formation and protein incorporation from ternary mixtures of detergent, protein, and phospholipid in aqueous solution.

Three aspects of vesicle formation and protein incorporation will be discussed:

1. Initial state of 3 components before detergent removal,
2. Changes that occur during detergent removal,
3. Orientation of protein in vesicles.

**Initial state**

Octyl glucoside, whether codeposited and dried with lipid or added to lipid later in aqueous solution, produced clear solutions of mixed micelles which upon dialysis form fairly uniform vesicles. Although the method of
addition of detergent to egg PC did not appear crucial, the ratio of detergent to lipid was shown to be critical for good vesicle preparation. At OG:PC molar ratios less than 4:1, solutions were cloudy presumably due to light scattering of larger egg PC particles. Above this ratio, solutions were clear. However, solutions containing this minimal ratio produced considerable amounts of nonvesicular lipid aggregates. These aggregates were probably present before detergent was removed. The ratio of detergent to lipid in the micelles is 4:1 when the total ratio in solution is 5:1, at the concentrations of OG and PC used here, approximately 100 mM and 20 mM respectively. This 4:1 ratio is an average value, and statistical considerations would predict that the actual ratio in individual particles will be distributed over a broad range; about one-fourth of the mixed micelles at a 4:1 average ratio are likely to have an actual OG/PC ratio of less than 3:1 (Boas, 1966). No studies of phase separations within mixed micelles have been reported, but patches of aggregated lipid may exist in mixed micelles in which more than one-third of the constituent molecules are phospholipids. (Since each lipid contributes two hydrocarbon chains, the molar ratio of lipid hydrocarbon chains to the much shorter OG chain becomes 1:1 when the molar ratio of OG/PC is 2:1.) Therefore, increased disruption and dispersal of PC aggregates in mixed micelles at higher detergent concentrations would be expected to produce greater vesicle yields. This was borne out as shown earlier at 10:1 and 15:1 ratios in which 90% of PC after detergent removal was found in vesicular form.

In like manner the dispersal of protein is also important if incorporation into vesicles is to take place. Detergent removal from membrane proteins always results in extensive protein aggregates which may or may not be soluble in aqueous solution. Inactivation of the protein often occurs during
this process. The protein aggregates may be hard to disrupt even with the addition of detergents (Nozaki et al., 1978). (Glycophorin for example forms decamers or higher oligomeric species in detergent free solution and even at saturating levels of nonionic detergent binding may remain partially dimeric.) Although the octyl glucoside concentration in solution is such that from statistical considerations alone the protein can be distributed among micelles in monomeric form, the protein may remain highly aggregated. These aggregates like those of lipid may not participate in vesicle formation. Therefore, a starting protein/lipid/detergent mixture in which lipid and protein are disaggregated and well dispersed in mixed micelles is preferable. However, from considerations presented below, if one starts with a mixture of lipid and protein in separate micellar solutions, detergent will equilibrate rapidly between particles but the other 2 components may not. This may lead to a kinetically stable mixture in which a relatively small number of the protein detergent micelles contain any phospholipid. Removal of detergent from a system such as this could lead to the formation of pure phospholipid vesicles and aggregated protein.

General considerations about detergent removal

The removal of detergent from these mixed micelles may be visualized as the reversal of the detergent solubilization process (Helenius and Simons, 1975). Removal of detergent decreases the solubility of lipid and protein moieties leading to an aggregation of detergent-depleted mixed micelles. However, upon further removal of detergent, these larger particles may not necessarily take the form of protein containing vesicles. Two events can occur in the aggregation process: (1) changes in local composition within a particle due to removal of detergent and exchange of lipid and protein
between particles; (2) molecular rearrangement in response to this compositional change.

This first process requires movement of species through the aqueous solution. The rate at which a given constituent can participate in a compositional change therefore depends on its solubility in an unassociated state in the aqueous medium. This solubility is very small for phospholipid and for most intrinsic membrane proteins. The attainable concentration for unassociated detergent depends on its CMC but is always much higher than that of lipid and protein. Thus detergent removal and aggregation of the remaining lipid and protein are expected to occur without much change in the local lipid to protein ratio. (Fixing this ratio by starting with mixed micelles containing protein and lipid in the same particle at the desired value before detergent removal appears advantageous.) From these considerations, it seems reasonable to expect that the yield of incorporated protein depends on protein aqueous solubility. Glycophorin is not typical of most membrane proteins being 55% by weight carbohydrate and having considerable but limited solubility in aqueous solution (however, it is highly aggregated even at low concentrations). Thus the percent of glycophorin reconstituted may represent the thermodynamic distribution between soluble and membrane bound states at the time vesicles are formed. In this case better yields of incorporated protein can be expected for more typical intrinsic membrane proteins of lower aqueous solubility.

Little is known about the hydrodynamics of local molecular rearrangements that occur as local composition changes. Multilayered structures may represent a more stable form than single bilayers. Unilamellar vesicles form only if lipid is sufficiently well dispersed originally to prevent more extensive aggregation at a comparable rate. Therefore the probability of formation of
protein-containing vesicles is enhanced if the particles within which rearrangement is to take place already contain protein and lipid uniformly distributed in nonaggregated form.

A unilamellar vesicle is probably a metastable species, a "kinetic trap," rather than an equilibrium state. Kinetic events during removal of octyl glucoside from mixed micelles in some way dictates the formation of a bilayer with less curvature than when cholate is used or when vesicles are formed by sonication. The effect of the rate of detergent removal on vesicle size requires further study.

Orientation of protein

If formation of vesicles from mixed micelles were a random assembly process, then one would not expect the native vectorial orientation of the protein to be regained upon detergent removal. Indeed, in previous reconstitution studies of membrane proteins into large vesicles, the proteins appear to be oriented randomly in the membrane. In octyl glucoside vesicles, glycophorin is not uniquely oriented in the membrane. Whether glycophorin molecules actually span the bilayer in these vesicles is not known. The asymmetric orientation of lipid and protein that occurs in small vesicles with high membrane curvature is probably the result of molecular packing constraints and steric hindrance (van Zoelen et al., 1978c). The bulkiest part of the protein molecules and phospholipids with large head groups are positioned on the vesicle exterior. However, unique orientations have been reported for some integral membrane proteins incorporated into somewhat larger vesicles, where steric effects would not be expected to be quite as pronounced (Helenius et al., 1975; Curman et al., 1980; Huang et al., 1980). It is possible that asymmetries may be attributed to packing constraints in a smaller vesicle precursor or micellar aggregate, and these asymmetries somehow are
maintained throughout the vesicle formation process. The orientation and mode of incorporation of membrane proteins will be discussed in more detail in the following chapter.
CHAPTER V

INCORPORATION OF DOPAMINE-ß-HYDROXYLASE AND HEPATIC ASIALOGLYCOPROTEIN RECEPTOR INTO VESICLES

Introduction

In the previous chapter, the preparation and properties of glycophorin incorporated vesicles are described. These vesicles were formed by removal of detergent from an octyl glucoside/egg phosphatidylcholine/protein mixture. To show that some biological activities can be maintained or restored under these conditions, two integral membrane glycoproteins, dopamine-ß-hydroxylase (DSH) and hepatic asialoglycoprotein receptor (HAR), were incorporated using similar procedures. Measurements of biological activity permit determination of the fraction of protein incorporated and determination of the protein orientation in the bilayer.

The results of these studies coupled with knowledge of the protein's ability to bind preformed vesicles may suggest mechanisms by which protein is incorporated during vesicle formation.

Dopamine-ß-hydroxylase

Two forms of DßH exist in the chromaffin granule of the bovine adrenal medulla. One form soluble DßH (sDßH) is released by osmotic shock from the chromaffin granule and is soluble in aqueous solution. The other form membrane bound DßH (mDßH) must be extracted from the membranes and solubilized with detergents. Although the two forms have different locations, they have no obvious enzymatic, structural or conformational differences as determined by various physico-chemical methods (Albanesi, 1988). However, small differences in amino acid sequence or in the length of peptide chain would be difficult to detect by the methods employed. Bjerre et al. (1979) demonstrated that sDßH binds adenosine deaminase whereas the soluble DßH does not. Some involvement of a short hydrophobic amino acid sequence (Albanesi, 1988). No evidence has been previously reported in his thesis.

By examining the relative abilities of the two forms to incorporate into vesicles, one may gain some understanding of factors that are important in determining the ability of the protein to bind to membranes, thereby inhibiting its binding to other vesicles.
determined by various physico-chemical methods (Albanesi, 1980). However, small differences in amino acid sequence or in the length of peptide chain would be difficult to detect by the methods employed. Bjerrum et al. (1979) demonstrated that mDSH binds nonionic detergents whereas the soluble DSH does not. Some investigators have suggested that mDSH associates with membranes by means of a short "tail" or "stalk" composed of hydrophobic amino acids (Albanesi, 1980). No evidence exists to suggest that it is transmembrane. By examining the relative abilities of the two forms to incorporate into vesicles, one may gain some understanding of the factor or factors responsible for this differential location. Reconstitution involving DSH were done in collaboration with Joseph Albanesi and the results of this work have been previously reported in his thesis.

Hepatic asialoglycoprotein receptor

Hepatic asialoglycoprotein receptor (HAR, also called hepatic lectin) binds desialylated serum glycoproteins and mediates their internalization into liver cells. HAR is an integral membrane sialoglycoprotein which when desialylated binds its own sugar chains, thereby inhibiting its binding to other asialoglycoproteins. Although it has been purified to homogeneity and is obtainable in milligram amounts, HAR topography and state of association in the membrane (like most other membrane proteins) is not known.

While the work described here was in progress, two preliminary reports of HAR incorporation into phospholipid vesicles appeared in the literature (Klausner et al., 1980; Baumann et al., 1980).

Klausner found that HAR associates with preformed small unilamellar vesicles which were prepared by sonication. Interaction between protein and lipid was indicated in three ways: perturbation of lipid phase transition by protein, specific HAR activation by lipid, and change in vesicular density in
the presence of protein; however, several important controls were not re-
ported.

Baumann et al. (1980) produced protein incorporated vesicles by dialyz-
ing detergent from a ternary mixture: phospholipid/deoxycholate/HAR. Their
attempts to determine protein orientation suggest that about 70% of the re-
ceptor is oriented with its binding sites accessible to the vesicle exterior.
Their most important finding was that mouse L cells after fusion with these
vesicles acquire the ability to bind and internalize asialoglycoproteins.
The role of HAR and other proteins in the internalization process is not known.

In the studies presented in this chapter, emphasis was placed on deter-
mining the yields of protein incorporated into vesicles by the octyl gluco-
side procedure, the orientation of protein within the vesicle, and the abil-
ity of protein to bind preformed vesicles. This work was performed in col-
aboration with Tom Andersen.

Materials and Methods

Materials

Triton X-100 was a Rohm and Haas product and BRIJ 58 was from Sigma.
Neuraminidase and orosomucoid were gifts from Robert Hill. Na$^{125}$I was pur-
chased from New England Nuclear.

Preparation of soluble and membrane bound dopamine-$\beta$-hydroxylase

The two forms of the enzyme were purified from chromaffin granules by
the method of Albanesi (1980). The soluble form was prepared and stored in
the absence of detergent, whereas the membrane bound enzyme was purified in
0.1-0.2 mg/ml BRIJ 58. Exchange of BRIJ 58 with octyl glucoside was carried
out by adsorbing mD$\beta$H to ConA-Sepharose as described in Albanesi (1980), then
eluting in buffer containing 10% a methyl-D-mannoside and 25 mM octyl
glucoside. In some cases following exchange, octyl glucoside removal was ef-
fected by dialysis and was monitored by loss of \([C^{14}]\) octyl glucoside counts from the dialyzed sample.

**DBH incorporation into vesicles**

Incorporation of protein into phospholipid vesicles was performed by methods described in Chapter IV with slight modifications. The dried lipid film (5 mg) was redissolved in 0.4-0.6 ml of a protein detergent solution (20 mM TES pH 7.0, 0.1 M NaCl containing from 1-20 μg protein and 140-170 mM octyl glucoside). The final molar ratios were 0.5-10 μmoles protein/mole phospholipid/11 moles octyl glucoside. Detergent was removed from this solution by dialysis at 4°C against two 1-liter changes (12 hrs.each) of detergent-free buffer. The resulting cloudy solution which contained vesicles was chromatographed on a Sepharose CL-4B column (1.5 X 30 cm) equilibrated in the same buffer at 4°C to remove any residual detergent. Pure phospholipid vesicles were prepared using a protein free buffer solution.

**DBH assays**

Dopamine-β-hydroxylase was assayed by the method of Nagatsu and Udenfriend (1972) as described in detail in Albanesi (1980). In every case, protein was diluted 10 fold into an assay mixture giving a final volume of 1.0 ml.

**Preparation of \( ^{125}I \) asialoorosomucoid (ASOR) and HAR**

Orosomucoid was treated with neuraminidase to yield asialoorosomucoid (ASOR), which is the binding substrate for HAR (Andersen et al., 1981). ASOR was specifically labeled with \( ^{125}I \) using the Biorad Enzymobead procedure (Andersen et al., 1981) to a specific radioactivity of 0.4 to 0.5 μCi/μg.
Rabbit HAR was isolated, delipidated, and purified in Triton X-100 as described by Hudgin et al. (1974). Protein was obtained in aqueous solution free from detectable detergent by ethanol precipitation of protein by the method of Andersen et al. (1981). Precipitated protein was dissolved in 0.1 M NaCl, 0.01 M TES, pH 7.8 solution containing 40 mg/ml octyl glucoside to produce a clear solution at the desired protein concentration.

**Incorporation of HAR into phospholipid vesicles**

The procedure for incorporation was similar to that described for DBH. 5 mg of egg phosphatidylcholine was dried to a thin film. To this film was added 0.5 ml of 0.1 M NaCl, 0.01 M TES, pH 7.8 (buffer A) containing 40 mg/ml octyl glucoside and 0.608 mg/ml of HAR. To dissolve the lipid film, bath sonication and gentle vortexing were carried out until the solution was clear and there were no visible signs of lipid on the tube walls (5-10 minutes). This clear solution was dialyzed for 36 hours with 3-1 liter changes of buffer A at 4°C. In this particular case the starting molar ratio of constituents are 11 mol OG:1 mol PC:15 μmole protein (wt. ratio 4 mg OG:1 mg PC:0.06 mg protein). Other starting ratios are specified in figure legends.

**Incubation with preformed vesicles**

Pure phosphatidylcholine vesicles were prepared as above in the absence of protein. HAR in 40 mg/ml octyl glucoside containing buffer A was dialyzed for 24 hrs with 2-1 liter changes of detergent free buffer A to remove octyl glucoside. 0.7 ml of this solution containing 0.23 mg of protein was added to 0.6 ml of preformed vesicles containing 4 mg of phosphatidylcholine. This solution was incubated at 4°C for 3 days.
Sucrose density centrifugation

12 ml sucrose density gradients from (7 to 30%) were centrifuged at 4°C for 20 hours at 40,000 rpm in a Beckman Ti41 swinging bucket rotor. Samples were loaded on the top of the gradient and after centrifugation fractions were collected from the bottom.

Binding assay

The binding assay of ASOR to HAR was performed in the presence or absence of 0.1% Triton X-100 by Method A of Hudgin et al. (1974).

Tritium labeling. Sialic acid residues of HAR were labeled by periodate oxidation and $^3$H-borohydride reduction by the method of Peters et al. (1979). Tritiated HAR gave a native absorption spectra and retained full binding activity; however, the labeled sialic acid residues could not be cleaved by treatment with neuraminidase.

Other. Phospholipid was determined as inorganic phosphate after hydrolysis by the method of Bartlett (1959). Guido Zampighi performed all electron microscopy by methods described in Chapter IV.

Results

Soluble and membrane bound DβH

Figure 22 shows the elution profile of mDβH on Sepharose CL-4B column (1.5 X 30 cm) which is equilibrated and eluted in detergent free buffer. The protein solution loaded on the column contained 0.5 mg/ml BRIJ 58. This detergent concentration in the loaded sample was sufficient to permit greater than 80% elution from the column and to maintain mDβH in solution following chromatography. It is important to note that less than 25% of protein eluted from the column when the loaded protein sample contained no detergent. This
Fig. 22. (A) Elution profile of membrane-bound DβH on a Sepharose CL-4B column. Protein (≈ 2 μg corresponding to a total activity of 18.8 μmoles/min) in a volume of 0.5 ml containing 0.5 mg/ml BRIJ 58 was loaded on a column equilibrated and eluted in detergent free buffer, 0.1 M NaCl, 0.01 M TES, pH 7.0, at 4°. Eighty-two percent of activity was recovered from the column. Crosshatching indicates fractions which were pooled for rechromatography.

(B) Elution profile of membrane-bound DβH following incorporation into phosphatidylcholine vesicles, by method described in the text. These vesicles (0.5 ml) were loaded onto the column in (A) and eluted with detergent free buffer. Of the added activity, 76% was recovered by elution, and 73% of the total eluted activity appeared in the void volume.
poor recovery and the trailing of the elution pattern seen in Figure 22A may be the result of protein binding to the column matrix. The peak tubes from this column were pooled, concentrated, brought to 150 mM OG, and then subjected to the incorporation procedure described in Methods. These peak tubes apparently did not retain enough BRIJ 58 to interfere with vesicle formation. The protein vesicle solution was rechromatographed on the same Sepharose CL-4B column (Figure 22B) and about 75% of mDβH activity coelutes in the void volume with phospholipid suggesting that incorporation into vesicles had occurred. Performing the enzyme assays in detergent to disrupt vesicles revealed no latent activity, thus, all of the enzyme active sites are exposed to the external solution. When a similar incorporation procedure was performed with sDβH (Figure 23A and B) only 5% of activity coeluted with phospholipid in the void volume. This low activity was only detectable following disruption of the vesicles with BRIJ 58, indicating that sDβH is trapped in the internal volume of vesicles (just as is cytochrome c, see the preceding chapter) and that intact vesicles are not permeable to substrate.

To determine whether mDβH can also interact with preformed vesicles, the concentration of detergent in the protein solution was lowered to prevent disruption of the egg phosphatidylcholine vesicles. BRIJ 58 in the protein solution was exchanged with octyl glucoside on a ConA-Sepharose column as described in Methods. Matrix bound mDβH was subsequently eluted in a solution containing octyl glucoside and α-methyl-D-mannoside. After removal of 99.8% of the octyl glucoside by dialysis, mDβH remains in solution and retains 90% of its original activity. This solution containing 20 μg of mDβH was incubated with vesicles for 12 hours at 4°C and then passed down a Sepharose CL-4B column (Figure 24). Some incorporation into preformed vesicles occurs, but is significantly lower than the 75% incorporation obtained when protein
Fig. 23. (A) Elution profile of soluble DβH on Sepharose CL-4B equilibrated in 0.1 M NaCl, 0.01 M TES, pH 7.0, at 4°. 3-4 μg of protein (activity = 16.4 μmole/min) in a volume of approx. 0.5 ml was added to the column (dimensions 1.5 X 30 cm). Vo and Vt indicate the void volume and total volume respectively. The sample contained no detergent. Ninety-one percent of the loaded activity was recovered from the column.

(B) Elution profile of soluble DβH after incorporation attempt. The method of incorporation is described in the text. Activity was measured as described in Methods with 0.2 mg/ml BRIJ 58 in the final assay mixture. Ninety-three percent of activity added to the column was recovered; 6% of this recovered activity eluted in the void volume with phospholipid.
Fig. 24. Elution profiles of membrane-bound DβH on Sepharose CL-4B. The chromatographic behavior of membrane-bound DβH following incorporation into phosphatidylcholine vesicles (closed circles), incubation with preformed vesicles (open circles) and removal of detergent (squares) are compared.

Octyl glucoside was first exchanged for BRIJ 58 associated with protein, as described in Methods. This detergent exchange resulted in no loss of activity and greater than 90% of initial activity was retained following incorporation, addition to preformed vesicles, or removal of detergent. Similar aliquots of sample (activity = 21-23 μmoles/min) were loaded on the column in each case.

mDβH, upon removal of octyl glucoside by dialysis, retained 90% of the activity it displayed in the presence of detergent. However, only 24% of the activity loaded on the column was recovered by elution.

A sample of mDβH from which octyl glycoside had been removed by dialysis was incubated overnight with preformed vesicles. Fifty-eight percent of the activity added to the column was recovered and of this recovered activity, 45% eluted with the phospholipid peak.

Protein was incorporated into vesicles as described in Methods. Eighty-one percent of the activity added to the column was recovered by elution and 75% of this activity eluted with vesicles in the void volume.
is present during vesicle formation. Less than 50% of the eluted activity coelutes with preformed vesicles and moreover, only 30% of activity loaded on the column was recovered after elution. When added in detergent-free and lipid-free solution was chromatographed on a Sephacryl CL-4B column (Figure 26), less than 25% of the added activity was recovered from the column and only about 5% of this eluted in the void volume suggesting that some aggregation had occurred.

Because vesicles also elute in the void volume, unincorporated protein could not be separated from protein incorporated during or after assembly. Figure 26 shows that the alternate procedure, sodium dodecyl sulfate treatment, did allow separation of free from bound protein. In Figure 26 (c)–(f), 2-AB equilibrated with aqueous buffer was added to preformed vesicles, and was incorporated into the vesicles. The incorporation of 2-AB was measured in the fractions of the column.
is present during vesicle formation. Less than 50% of the eluted activity coelutes with preformed vesicles and moreover, only 58% of activity loaded on the column was recovered after elution. When mDSH in detergent free and lipid free solution was chromatographed on a Sepharose CL-4B column (Figure 24) less than 25% of the added activity was recovered from the column and only about 5% of this eluted in the void volume suggesting that some aggregation had occurred.

sDSH was also incubated with preformed vesicles, however, no activity eluted in the void volume of the Sepharose CL-4B column indicating no incorporation. This is similar to the result obtained with cytochrome c and glycoporphin.

**Hepatic asialoglycoprotein receptor**

To separate HAR containing vesicles from unincorporated protein, two methods were used: column chromatography on Sepharose CL-4B and sucrose density centrifugation. Figure 25 shows the elution profile of HAR on the Sepharose CL-4B column equilibrated in detergent free buffer. The protein solution loaded on the column was also free of detergent. Seventy percent of the protein elutes in the void volume and the remainder as a broad included peak, indicating that the protein is soluble, but highly aggregated under these solution conditions. The protein has no binding activity in aqueous solution, but protein in all column fractions may be reactivated by addition of 0.1% Triton X-100.

Because vesicles also elute in the void volume, unincorporated protein could not be separated from protein incorporated vesicles by this method. Figure 26 shows that the alternate procedure, sucrose density centrifugation, did allow separation of free from bound protein. In Figure 26 (A-C), HAR in aqueous buffer, HAR added to preformed vesicles, and HAR incorporated into
Fig. 25. Elution profile of hepatic asialoglycoprotein receptor on a Sepharose CL-4B column equilibrated and eluted in 0.01 M TES pH 7.8, 0.1 M NaCl at 4°. 0.6 ml of HAR (0.19 mg) in detergent free buffer was loaded on the column. The binding assay was performed in 0.1% Triton X-100 as described in Methods. Values from this assay are given for the void volume and included volume tubes. (Fraction 15 represents the void volume.)
Fig. 26. Sucrose density gradient centrifugation of HAR in detergent free buffer (A,D), HAR added to preformed vesicles (B,E), and HAR incorporated into vesicles (C,F). HAR activity was assayed by 2 different methods. In A–C, HAR containing samples were made 20 mM in CaCl₂ and incubated with 1²5I-ASOR prior to centrifugation. In D–F, 1²5I-ASOR was not added to samples before centrifugation. After centrifugation, gradient fractions in D–F were assayed for 1²5I ASOR binding in the presence of 0.1% Triton X-100 (Hudgin et al., 1974).

0.3 to 0.5 ml of samples prepared as described in Methods were loaded on a 12 ml, 7-30% sucrose gradient. Centrifugation was carried out at 4°C in a Beckman Ti41 rotor at 40,000 rpm for 20 hrs. Fractions were collected from the bottom. Light scattering due to vesicles was measured as absorbance at 280 nm (open circles) and 1²5I ASOR was counted in a gamma counter. Solution density was determined by refractometry. Vesicles in C and F were produced in solution containing an initial lipid to protein ratio of 16:1 (see Methods).
vehicles were incubated with $^{125}$I-adenine for binding equilibrium through 1 h. 
CaCl$_2$ solution was added to make each solution 50 mM CaCl$_2$ to maintain 
conjugation. Sucrose gradients were also 50 mM CaCl$_2$. High CaCl$_2$ is necessary to
maintain binding activity during centrifugation. However, high CaCl$_2$ 
concentrations could not be tolerated during the separation 
of 30S sucrose gradient 
shocks, which produce complete dissociation of 70S ribosomes 
into subunits. About 95% of the $^{125}$I-adenine was 
recovered in the presence of 50 mM CaCl$_2$. However,
vesicles were incubated with $^{125}\text{I}-\text{ASOR}$ under binding conditions (enough 1 M CaCl$_2$ solution was added to make each solution 20 mM CaCl$_2$) prior to centrifugation. Sucrose gradients were also 20 mM CaCl$_2$, Ca$^{2+}$ being necessary to maintain binding activity during centrifugation. Vesicles floated on the 7-30% sucrose gradient whereas protein entered the gradient and was well separated from lipid. Almost all of the $^{125}\text{I}-\text{ASOR}$ comigrates with vesicles produced in the presence of HAR; however, virtually no counts migrate with preformed vesicles incubated with HAR. This indicates that $^{125}\text{I}-\text{ASOR}$ exhibits little nonspecific binding to vesicles and that HAR is either not incorporated into preformed vesicles or that it is incorporated in an inactive form.

In Figure 26 (D-F), $^{125}\text{I}-\text{ASOR}$ was not added to samples prior to centrifugation but was used to assay activity in gradient fractions collected after centrifugation. Assays were performed in 0.1% Triton X-100 as described in Figure 26 and Methods. This amount of detergent is sufficient to disrupt vesicles and to activate protein. (Note that activation here refers to an increase in measured activity in the binding assay.) Greater than 50-60% of the binding activity of HAR comigrates with the vesicle fractions when vesicles are formed in the presence of HAR (Figure 26E). Similar results were obtained when $^3\text{H}-\text{HAR}$ was used in the reconstitution procedure and when vesicles were formed at a 1:1 weight ratio of PC:protein. Only about 15% of the activity comigrated with lipid when the protein was incubated with preformed vesicles (Figure 26E). A comparison of the migration of $^{125}\text{I}-\text{ASOR}$ (Figure 26A) and HAR (D) indicates, that HAR has no substrate binding activity in detergent-free, lipid-free solution; however, interactions with lipid (Figure 26C) or detergent (D) restore the binding activity. Incubation of protein with preformed vesicles does not lead to substantial incorporation or to subsequent activation of binding activity (Figure 26B, E).
HAR orientation in the vesicles

Two tests for protein orientation indicate that all HAR molecules are oriented such that the ASOR binding sites are on the vesicle exterior.

HAR incorporated vesicles were assayed for binding in the presence and absence of 0.1% Triton X-100, this concentration being sufficient to disrupt vesicles and maximally activate binding activity. Detergent disrupted and intact vesicles showed identical binding activity. This was true for vesicles prepared at 1:1 and at 1:16 protein to detergent weight ratios. Thus all sites are oriented outward and protein is activated to the same extent whether incorporated into a bilayer or solubilized in detergent solution as measured in this assay system.

The second series of experiments to test sidedness were based on the fact that neuraminidase treatment leads to inhibition of HAR binding activity. Purified HAR protein initially dissolved in BRIJ 58, acetate buffer pH 5.5 (the pH at which neuraminidase shows maximal activity) shows a 70% loss in activity after incubation with neuraminidase. HAR incorporated vesicles (prepared at both 1:1 and 1:16 ratios; separated from unincorporated HAR) that had been disrupted in BRIJ 58 showed an 86-89% loss in activity after treatment with enzyme. In fact vesicles (both 1:1 and 1:16) treated with neuraminidase prior to disruption by detergent in the binding assay displayed the same loss of activity (86-90%), indicating the absence of latent binding activity. Vesicles have been shown previously to be impermeable to chemical reagents and soluble proteins like neuraminidase (Chapter IV). It is important to note that no attempt was made to separate neuraminidase from vesicles, because control experiments had demonstrated that the presence of neuraminidase during the 15 minute binding incubation at pH 7.8 does not alter lectin activity significantly.
The identical loss of lectin activity in both intact and disrupted vesicles demonstrates that almost all binding sites are oriented on the outside of the vesicles.

**Negative staining**

Visual inspection of electron micrographs of negatively stained vesicles showed that those prepared at HAR to egg lecithin weight ratios of 1:16 and 1:1 are apparently different in size and shape. 1:1 vesicles appear more asymmetric in shape and on the average are 2 to 4 times smaller in diameter than 1:16 vesicles. Vesicles prepared at 1:16 ratio are round and superficially similar to pure phospholipid vesicles shown in Chapter IV; however, there appears to be a greater size heterogeneity among vesicles and a greater proportion of smaller vesicles of 100 nm in diameter. Almost all vesicles prepared at 1:1 ratio have sizes between 100 and 50 nm with only few larger vesicles. No knoblike protein structure extending from the membrane may be visualized.

**Summary and Discussion**

Three integral membrane proteins were incorporated into vesicles formed by removal of detergent from egg PC/OG/protein mixed micelles. 25-50% of glycophorin molecules in the initial mixture were incorporated into vesicles, 50-60% of HAR, and about 75% of mD\(\beta\)H. In all cases, most or all of the protein is oriented with its active site and/or carbohydrate moieties exposed on the vesicle exterior. This incorporation was also much greater than could be accounted for by protein trapping within the vesicles. Trapping was demonstrated for several species including ions and the soluble proteins, cytochrome c and sD\(\beta\)H. The amount trapped depends on lipid and protein concentration during vesicle formation and upon the size of vesicles formed. This
ability to trap components is exploited in the ion permeation investigations presented in Chapter VI and has found important application in vesicular drug delivery studies (Papahadjopolous and Szoka, 1980). The factors affecting the yield of incorporation of membrane proteins into bilayers have not been systematically studied, but higher yields are probably possible under slightly different solution conditions. In this regard, one may note that the extent of HAR reconstitution into vesicles did not depend significantly on initial protein to lipid weight ratios at least in the range from 1:1 to 1:16. For this lectin, Baumann et al. (1980) obtained yields similar to those reported here.

**Binding to preformed vesicles.** The soluble proteins cytochrome c and sDβH as well as the transmembrane protein, glycophorin, do not bind preformed vesicles. About 15% of HAR and about 29% of mDβH activity were bound to preformed vesicles. In all cases, the incorporation of membrane proteins into preformed vesicles was dramatically less than the incorporation when vesicles were formed in the presence of protein, i.e. from detergent/lipid/protein mixed micelles. These results are different from those of Klausner et al. (1980) who found that 95% of the HAR dissolved in detergent free solution could bind 25 nm sonicated vesicles.

Spontaneous binding of integral membrane proteins to preformed vesicles or to other membranes has been demonstrated in a number of cases: cytochrome oxidase (Eytan and Broza, 1978), D-B hydroxybutyrate dehydrogenase (McIntyre et al., 1979), and cytochrome b₅ and its reductase (Roseman et al., 1979; Robinson and Tanford, 1974; Enoch et al., 1979; Leto and Holloway, 1979). The best characterized of these proteins, cytochrome b₅, is composed of 2 domains: one comprising the bulk of the protein is hydrophilic and contains biological activity and the other is a 15 residue hydrophobic segment at the
The integral membrane proteins mentioned above may be structurally similar to cytochrome b$_5$ in containing a small hydrophobic "tail" which may insert into the bilayer. The existence of such a sequence on mDSH may distinguish it from the soluble DSH form. Peptide mapping and sequence analysis of the two forms will resolve this point.

The modes of association of mDSH and HAR with the membrane in vivo are not known. Like cytochrome b$_5$ they are soluble in water after detergent removal; however, in detergent free, lipid free solution HAR loses all measurable activity. mDSH remains active under these conditions. Clearly, an active HAR conformation depends on the binding of amphiphilic ligands, but whether HAR has a significantly larger hydrophobic domain than mDSH or cytochrome b$_5$ awaits further research.

Leto and Holliday (1979) have addressed the question as to the mode of association of cytochrome b$_5$ with phospholipid vesicles. They found that monomeric cytochrome b$_5$ in aqueous solution exists in equilibrium with a protein aggregate, probably an octomer. Their data indicate that the monomer form, but not the octomer, interacts with vesicles, suggesting that the hydrophobic regions are "masked" in the aggregated protein so as to preclude their direct interaction with vesicles. Therefore the extent to which cytochrome b$_5$ interacts with vesicles depends on the relative partitioning of monomer into protein aggregate or into vesicles. Many integral membrane proteins, specifically some transmembrane proteins like bacteriorhodopsin, are very different from cytochrome b$_5$, in having little or no aqueous solubility and in being situated in the native membrane such that the bulk of the protein is buried within the bilayer. For these proteins the number of monomeric species existing in aqueous solution at any time would be expected to be
quite low, leading to little interaction with preformed vesicles. That only a minor portion of mDBH and HAR bind preformed vesicles suggests that their hydrophobic regions are shielded in protein aggregates produced after detergent removal and that the dissociation rate of aggregated species to monomer is slow. If this is the case, then it is possible that the method of detergent removal from protein in detergent solution (which has been shown to affect protein solubility) is an important factor in determining final incorporation yields (Helenius and Simons, 1975).

An alternative mechanism might involve the transfer of protein from a protein aggregate to a vesicle during a collisional process, but there is no evidence for this. In related work some investigators have reported the transfer of intrinsic membrane proteins between artificial phospholipid vesicles and intact cells without detectable vesicle fusion (Cook et al., 1980; Buoma et al., 1977); however, the mechanism by which this occurs is not known.

**Orientation of the protein**

Whereas glycophorin does not appear to be uniquely oriented in the vesicle, DgH and HAR are incorporated into vesicles with all active sites oriented outward. If formation of vesicles from protein/lipid/detergent mixed micelles is a random assembly process, one would not expect a unique orientation of the protein within the bilayer. For vesicles having a small internal volume, asymmetric incorporation of protein may occur for purely steric reasons. The asymmetric orientation observed in larger vesicles are not well understood (Helenius et al., 1975; Curman et al., 1975; Huang et al., 1980). In these cases and in those reported in this chapter, asymmetries may result from packing constraints in smaller vesicle precursors or micellar aggregates and these asymmetries may somehow be maintained throughout the vesicle
formation process, e.g. small vesicles may form first and may fuse to form larger vesicles and in so doing retain orientation. Curman et al. (1975) has suggested that vesicle formation precedes protein incorporation into vesicles thus accounting for protein asymmetry. Proteins like cytochrome b5 could be incorporated in this manner; however, it is not clear how a transmembrane protein which has hydrophilic groups on both sides of the membrane in the native state could integrate in a transmembrane orientation by simple addition of protein to a vesicular system. Moreover, the production of vesicles from lipid/detergent/protein mixed micelles always leads to much greater incorporation than incubation of protein with preformed vesicles suggesting that incorporation occurs during vesicle formation as described in Chapter IV.

The fact that vesicles produced at high protein to lipid ratios are smaller than those produced at lower ratios may be significant. Further investigations into this observation are required.
The passive ion permeability of pure egg phosphatidylcholine vesicles and glycophorin incorporated vesicles was investigated.

This property is particularly important because the ability to use protein-incorporated vesicles for studying the function of ion pumps and ion exchange proteins depends on maintenance of the membrane permeability barrier. If incorporation of a transmembrane protein significantly increases nonspecific permeability of the vesicle, then the measurement of specific ion fluxes over background ion leakage may be rendered difficult or impossible. Glycophorin was used in this work to determine whether the incorporation of a transmembrane protein with no known transport function will significantly alter the passive permeability of the membrane. In this regard, it is reasonable to hope that glycophorin will be typical of transmembrane proteins in general.

The ion permeability properties of phosphatidylcholine vesicles have been systematically studied only in small vesicles having an external diameter of about 25 nm. These vesicles formed by sonication (Huang, 1969) or from cholate-lipid mixed micelles (Brunner et al., 1976) have an internal volume of $2 \times 10^{-18}$ ml per vesicle, which corresponds to inclusion of only one molecule of solute per vesicle when the internal solute concentration is 1 mM. The large vesicles described in this thesis have a 1000 fold larger internal volume and therefore provide a considerable advantage for transport studies.
Materials and Methods

The materials and methods used for preparation of pure egg phosphatidylcholine vesicles and glycophorin incorporated vesicles are given in Chapter IV. The methods for vesicle preparation and internal volume determination are also described there.

Materials

$^{22}\text{NaCl}$, $^{36}\text{Cl}$, and $^{86}\text{RbCl}$ were purchased from Amersham Corporation.

Cl efflux. To measure Cl efflux, the external solution of vesicles prepared in 0.25 M NaCl or KCl was exchanged with 0.01 M Tris-$\text{HNO}_3$, 0.25 M NaN0$_3$, pH 7.5 or 8.5 on a Sephadex G-50 column. The column dimensions were 2 X 8 cm and the exchange was completed in 10 to 16 minutes. External Cl was monitored continuously for 2 to 3 hours using a solid state ($\text{Ag}_2\text{S}$-$\text{AgCl}$) electrode obtained from Orion Research, Inc., together with a home-made reference calomel electrode (Nozaki and Tanford, 1967), which was modified to provide a second bridge solution (1 M NaN0$_3$) between the KCl salt bridge and the sample solution. The electrode assembly was housed in an aluminum chamber for shielding and attached to a Radiometer model 4 pH meter for emf measurements. The instrument was calibrated daily with KCl solutions in 0.25 M NaN0$_3$ and readings were found to be stable for several hours and unaffected by the presence of vesicles or detergent. All measurements were made at room temperature.

Influx measurements. For influx measurements using $^{22}\text{Na}$ and $^{86}\text{Rb}$, the internal and external buffer and salt compositions were the same. For $^{22}\text{Na}$ permeation studies internal and external solution contained 0.25 M NaCl, and for $^{86}\text{Rb}$ studies contained 0.25 M KCl.

10-20 μl of a solution containing the radioactive ion of interest were
added to 2-3 ml of isotope free vesicle suspension at zero time. Permeation of ions into the vesicles was measured by methods similar to those of Toyoshima and Thompson (1975). At appropriate times, 0.1-0.2 ml aliquots were removed and passed down a Sephadex G-50 column (0.6 X 7 cm) equilibrated and eluted in nonradioactive aqueous buffer at 24°C. Chromatography was completed within 10 minutes. The column eluent was collected in 5-7 drop fractions directly into scintillation counting vials or into test tubes. The elution profile showed two distinct peaks of radioactivity, one in the void volume representing radioactive ions eluting with vesicles and the other in the total volume due to radioactivity in the external aqueous space. The ratio of internal vesicle volume to total volume is required to calculate $C_\infty - C_0$, and was determined by measuring internal volume with Cl electrodes as described in Chapter IV on one aliquot of each preparation. During these influx experiments, preparations were kept under argon at room temperature, 24°C.

Other methods. Radioactivity of $^{36}$Cl and $^{86}$Rb were measured in a Beckman LS-100 scintillation counter, using a toluene Triton X-100 (2:1) based cocktail. $^{22}$Na was measured directly in a Beckman Model 3000 gamma counter.

Results

Ion permeation measurements obeyed first order kinetics within experimental error. First order kinetics of permeation are described by the equation,

$$-\rho \frac{dCex}{dt} = \frac{dCin}{dt} = -k (Cex - Cin)$$ (1)

where Cin and Cex are internal and external concentrations of the ion respectively. $k$ is the first order rate constant, and $\rho$ is the ratio of internal
volume to external volume. The change in Cex is proportional to the change in Cin by the factor, \(-\rho\). Conservation of mass requires that

\[ \rho \, \text{Cin} + \text{Cex} = (1 + \rho) \, \text{C}_\infty \]  

(2)

where \(\text{C}_\infty\) is the concentration inside and outside the vesicles at equilibrium, i.e. at time = \(\infty\). (Derivation in Note 1) By substituting equation (2) into equation (1) and integrating, one obtains the equation:

\[ \ln (\text{C}_\infty - \text{C}_t) = \ln (\text{C}_\infty - \text{C}_0) - (1 + \rho) \, \text{kt} \]  

(3)

where \(\text{C}_t\) is the measured concentration at time \(t\) and \(\text{C}_0\) the initial value. (Derivation for efflux and influx in Note 2) This equation is the same for efflux measurements in which \(\text{Cex}\) is determined as a function of time and for influx in which Cin is determined. Experimental plots of \(\ln (\text{C}_\infty - \text{C}_t)\) vs. time show that this equation is obeyed within experimental error for Cl\(^-\) efflux and Cl\(^-\), Na\(^+\) and Rb\(^+\) influx (Figures 27-29). Vesicles become diluted during the chromatographic steps used in the measurements of these rates, and therefore the ratio of internal to total volume is considerably smaller than when the vesicles are first formed. Since in these cases \(\rho\) is much less than 1, the slopes of the plots of Figures 27-29 give the rate constant \(k\).

These rates along with the permeability coefficient, \(P\), are compiled in Table 7. \(P\) is calculated from the rate constant by the relation:

\[ P = k \, \frac{\text{Vi}}{\text{Ai}} \]  

(4)

where \(\text{Vi}\) and \(\text{Ai}\) are the internal volume and surface area per vesicle, respectively. (\(\text{Vi}/\text{Ai}\) for these calculations was \(3.62 \times 10^{-6} \text{ cm.}\)) This equation follows from the definition of \(P\) as the ion flux per unit membrane area at unit concentration gradient, and therefore, the use of \(P\) allows comparison of
Fig. 27. Chloride efflux from vesicles at 24°C plotted according to the first order rate equation. (Equation 3 in the text) Chloride efflux was monitored continuously with a Cl\textsuperscript{-} sensitive electrode. Protein containing vesicles contained 50 glycophorin molecules per vesicle.
Fig. 28. Rubidium influx into vesicles at 24°C plotted according to equation 3. Influx was measured using $^{86}$RbCl as described in the text. Protein containing vesicles contained 70 glycophorin molecules per vesicle. Note that the concentration scales are different between influx and efflux experiments because the external solution volume is much larger than the internal vesicular volume, so that $C_\infty$ is close to 0.25 M in an influx experiment, but less than 1 mM in an efflux experiment.
Pure Lipid

With Glycophorin

\( \ln (C_{\text{g}} - C) \) MOLAR UNITS

TIME IN HOURS

0 20 40 60 80 100
Fig. 29. Sodium influx into vesicles at 24°C plotted according to equation 3. Influx was measured using $^{22}$NaCl as described in the text. Protein containing vesicles contained 70 glycophorin molecules per vesicle.
\[ \frac{\text{Ln} (C_8 - C_1)}{\text{MOLAR UNITS}} \]

**TIME IN HOURS**

- **Pure Lipid**
- **With Glycophorin**
TABLE 7

ION FLUX MEASUREMENTS$^a$

<table>
<thead>
<tr>
<th>Method</th>
<th>Glycophorin molecules/vesicles</th>
<th>k, sec$^{-1}$</th>
<th>P, cm/sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl$^-$</td>
<td>b none</td>
<td>2.1 ($\pm$ 0.6) x 10$^{-5}$</td>
<td>7.6 x 10$^{-11}$</td>
</tr>
<tr>
<td></td>
<td>c 50</td>
<td>4.7 x 10$^{-5}$</td>
<td>1.7 x 10$^{-10}$</td>
</tr>
<tr>
<td></td>
<td>c 70</td>
<td>8.3 x 10$^{-5}$</td>
<td>3.0 x 10$^{-10}$</td>
</tr>
<tr>
<td></td>
<td>c 220</td>
<td>9.3 x 10$^{-5}$</td>
<td>3.4 x 10$^{-10}$</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>d none</td>
<td>2.6 x 10$^{-7}$</td>
<td>9.5 x 10$^{-13}$</td>
</tr>
<tr>
<td></td>
<td>d 70</td>
<td>4.4 x 10$^{-7}$</td>
<td>1.6 x 10$^{-12}$</td>
</tr>
<tr>
<td>Rb$^+$</td>
<td>e none</td>
<td>9.0 x 10$^{-7}$</td>
<td>3.3 x 10$^{-12}$</td>
</tr>
<tr>
<td></td>
<td>e 70</td>
<td>1.3 x 10$^{-6}$</td>
<td>4.7 x 10$^{-12}$</td>
</tr>
</tbody>
</table>

$^a$ At 24°C, pH 7.3-8.5, 0.25 M salt concentration. Glycophorin molecules per vesicle were obtained from measured protein/lipid ratios by assuming a vesicle diameter of 230 nm, which corresponds to Table 6 by 460,000 lipid molecules per vesicle.

$^b$ Average of six measurements: four efflux measurements using chloride electrodes, with NaCl inside and NaNO$_3$ outside; one similar measurement with KCl inside and NaNO$_3$ outside; one influx measurement using 36Cl$^-$ with KCl inside and outside. Three of these measurements were performed by G. Zampighi and Y. Nozaki.

$^c$ Efflux measurements using chloride electrodes, with NaCl inside and NaNO$_3$ outside.

$^d$ $^{22}$Na influx, with 0.25 M NaCl on both sides of the membrane.

$^e$ $^{86}$Rb influx, with 0.25 M KCl on both sides of the membrane.
flux rates between vesicles of different sizes. The permeabilities of vesicles produced using octyl glucoside was found to be extremely low (Table 7). These results are comparable to permeability data for protein free vesicles produced by sonication (Hauser et al., 1973; Toyoshima and Thompson, 1975), in spite of the fact that the sonicated vesicles are about ten times smaller in diameter, i.e. 20-30 nm external diameter, and were prepared in the absence of detergent. The permeability coefficients obtained by these researchers is given in Table 8. One may note that the measurements of Hauser et al. were made at 4°C where permeability is about 7 fold lower than room temperature and that the Cl⁻ versus NO₃⁻ measurements presented in this thesis were made in buffered solutions where one would not expect to see the large difference between Cl⁻/NO₃⁻ and Cl⁻/Cl⁻ exchange that has been observed in unbuffered solutions (Toyoshima and Thompson, 1975). Less extensive studies of vesicles formed by removal of cholate from cholate lipid mixed micelles show that these vesicles have similar dimensions to those made by sonication and that they are equally resistant to ion permeation (Brunner et al., 1976). The data in Table 7 show that incorporation of glycophorin into vesicles only slightly increases the permeability to ions, and this is perhaps the most significant aspect of these results. At the highest levels of glycophorin incorporation (220 copies per vesicle) the Cl⁻ efflux rate from vesicles was increased only 4.4 fold. Permeability values of vesicles containing fewer copies of glycophorin were correspondingly lower, but were still higher than those of pure lipid vesicles. This data differs from pervious reports which show strong enhancement of lipid bilayer permeability when glycophorin is incorporated (Tosteson et al., 1973; Tosteson, 1978; van Zoelen et al., 1978a,b).

Some of the Cl⁻ efflux data of Table 7 (including the experiment in
**TABLE 8**

COMPARATIVE ION FLUX MEASUREMENTS

<table>
<thead>
<tr>
<th>Ion measured</th>
<th>Method and temperature</th>
<th>( k, \text{sec}^{-1} )</th>
<th>( P \text{ cm/sec} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Na}^+ ) efflux</td>
<td>a 4°</td>
<td>( 0.3-1.3 \times 10^{-7} ) avg. ( 7.0 \times 10^{-8} )</td>
<td>( 0.53-2.2 \times 10^{-14} ) avg. ( 1.2 \times 10^{-14} )</td>
</tr>
<tr>
<td></td>
<td>b 20°</td>
<td></td>
<td>( 3.4 \times 10^{-14} )</td>
</tr>
<tr>
<td>( \text{Cl}^- ) efflux</td>
<td>c 4°</td>
<td>( 3.3 \times 10^{-5} )</td>
<td>( 5.6 \times 10^{-12} )</td>
</tr>
<tr>
<td></td>
<td>d 4°</td>
<td>( 8.0 \times 10^{-5} )</td>
<td>( 1.3 \times 10^{-11} )</td>
</tr>
<tr>
<td></td>
<td>e 4°</td>
<td>( 3.4 \times 10^{-6} )</td>
<td>( 5.7 \times 10^{-13} )</td>
</tr>
<tr>
<td>( \text{Cl}^- ) influx</td>
<td>f 3.2°</td>
<td>( 1.79 \times 10^{-4} )</td>
<td>( 1.5 \times 10^{-11} )</td>
</tr>
<tr>
<td></td>
<td>g 3.2°</td>
<td>( 1.19 \times 10^{-5} )</td>
<td>( 1.0 \times 10^{-12} )</td>
</tr>
</tbody>
</table>

a Sonicated egg lecithin vesicles of average diameter 250Å ± 8. Efflux measured with 0.145 M NaCl on both sides of the membrane, pH 5.5, no buffer specified. (Hauser et al., 1973)

b Egg lecithin vesicles prepared using sodium cholate. Average vesicle diameter is 300Å. Same solution conditions as a. (Brunner et al., 1976)

c Sonicated egg lecithin vesicles as in a efflux measured at pH 6.4.

d Vesicles as in a. Internal cavity contained 0.145 M NaNO₃, pH 6.4.

e Vesicles as in c, pH 10.0.

f Sonicated egg lecithin vesicles in unbuffered 2.0 M KCl (pH 5.5-6.0) on both sides of the membrane (Toyoshima and Thompson, 1975).

g Vesicles as in f in unbuffered 1.0 M KCl in external medium, 1.0 M KNO₃ in internal cavity.
which 220 copies of protein are incorporated per vesicle) were obtained with vesicles prepared from solutions at the minimal detergent to lipid ratio. The permeability properties of these vesicles did not differ from those of vesicles prepared from solutions at higher detergent to lipid ratios.

Discussion

The vesicles prepared by the use of octyl glucoside described here have a ten-fold larger diameter and therefore a 1000-fold larger internal volume than the small vesicles that are formed by sonication or from cholate-lipid mixed micelles. Because of this larger size more ions may be trapped per vesicle thereby facilitating measurement of transport processes. It is important to note that this larger size does not significantly affect passive permeability of the vesicle wall. The small effect of glycophorin incorporation on passive permeability (if it proves to be representative of inserted membrane proteins in general) is especially encouraging, because it suggests that transport studies can be carried out in reconstituted vesicles that contain only a very small number of copies of a purified transport protein. For example, the highest permeability for Cl⁻ given in Table 7 (for 220 glycophorin molecules per vesicle) corresponds to a leak rate of fewer than 50 Cl⁻ ions per second per vesicle at a transmembrane concentration gradient of 0.15 M. This may be contrasted with the turnover number of the erythrocyte anion exchanges, which corresponds to the transport of about $5 \times 10^4$ Cl⁻ ions per second per binding site (Knauf, 1979). Thus even a single exchange molecule per vesicle would lead to a transfer rate 1000 times higher than the passive permeability measured in these studies.

Since ion transporters like the ATP driven Na⁺, K⁺ pump have turnover rates of 160 Na⁺ ions per second per binding site which is about 300 times slower than Cl⁻ exchange (Joiner and Lauf, 1978), passive leak rates would
contribute proportionally more to the measured ion flux in this reconstituted pump system. The presence of glycophorin may cause slight discontinuities in the lipid bilayer, since increasing the number of glycophorin molecules per vesicle results in slightly enhanced membrane permeability (up to 4.4 times that of pure lipid vesicles under conditions previously described).

These methods for the incorporation of membrane proteins into vesicles and for the determination of ion influx and efflux should be applicable to the study of the functional properties of specific ion transport proteins.

\[
\begin{align*}
(4) \quad M_t &= \text{CinVt} + \text{Cax Vex} \\
(5) \quad M_t &= \text{Vex} (\text{pCin} + \text{Cax}) \\
(6) \quad M_t &= \text{Vex} \\
(7) \quad \text{VtC}_{\text{p}} &= \text{Vex} (\text{pCin} + \text{Cax}) \\
(8) \quad \text{Gy} - \text{Vex} (1 + p) &= \text{Vex} (\text{pCin} + \text{Cax}) \\
(9) \quad G_p (1 + p) &= \text{pCin} + \text{Cax} \\
(10) \quad \text{Cin} &= G_p (1 + p) - \text{Cax}
\end{align*}
\]

and \( \text{Cax} = G_p (1 + p) - \text{pCin} \)
NOTE 1

Resolving Cin in Terms of Cex and C∞

(1) \( V_t = V_{in} + V_{ex} \)

Vt, Vex, Vin are total, external and internal volumes, respectively by definition.

(2) \( \rho = \frac{V_{in}}{V_{ex}} \) and \( \rho V_{ex} = V_{in} \)

(3) \( V_t = V_{ex} (1 + \rho) \)

(4) \( M_t = C_{in} V_{in} + C_{ex} V_{ex} \)

Mt is total mass in solution.

(5) \( M_t = V_{ex} (\rho C_{in} + C_{ex}) \)

Substituting (2) into (4) and factoring.

(6) \( M_t = V_t C_{\infty} \)

(7) \( V_t C_{\infty} = V_{ex} (\rho C_{in} + C_{ex}) \)

(8) \( C_{\infty} V_{ex} (1 + \rho) = V_{ex} (\rho C_{in} + C_{ex}) \)

(9) \( C_{\infty} (1 + \rho) = \rho C_{in} + C_{ex} \)

(10) \( C_{in} = C_{\infty} (1 + \rho) - C_{ex} \)

and \( C_{ex} = C_{\infty} (1 + \rho) - \rho C_{in} \)

In the experimental procedure, Cin is measured as a function of time. To solve this equation, one integrates between the above boundary conditions and substitute eq. (10) from Note 1 for Cin yielding:

\[
\int_{0}^{t} \frac{dC}{C - C_{ex}} = \int_{0}^{t} \frac{1}{C_{\infty} - C_{in}} \frac{dC}{(1 + \rho) M_t}
\]

When \( \rho \) is small, \( (1 + \rho) \) goes to 1 and the rate equation for both
NOTE 2

Derivation of First Order Rate Equation for Influx and Efflux

From the first order rate:

\[-\rho \frac{dC_{ex}}{dt} = \frac{dC_{in}}{dt} = -k(C_{ex} - C_{in})\]

1. Influx

\[\int_{0}^{t} \frac{dC_{in}}{(C_{ex} - C_{in})} = \int_{0}^{t} -k \, dt\]

In the experimental procedure, \(C_{in}\) is measured as a function of time. To solve the rate equation above, one integrates between the above boundary condition and substitute eq. (10) from Note 1 for \(C_{ex}\) yielding:

\[\ln(C_{\infty} - C_{t}) = \ln(C_{\infty} - C_{0}) - (1 + \rho) \, kt\]

2. Efflux

\[\int_{0}^{t} \frac{-\rho dC_{ex}}{(C_{ex} - C_{in})} = \int_{0}^{t} -kt\]

In the experimental procedure, \(C_{ex}\) is measured as a function of time. To solve this equation, one integrates between the above boundary conditions and substitute eq. (10) from Note 1 for \(C_{in}\) yielding:

\[\ln(C_{\infty} - C_{t}) = \ln(C_{\infty} - C_{0}) - (1 + \rho) \, kt\]

When \(\rho\) is small, \((1 + \rho)\) goes to 1 and the rate equation for both
efflux and influx reduces to:

\[ \ln(C_\infty - Ct) = \ln(C_\infty - Co) - kt \]
CHAPTER VII

DISCUSSION

Chapters II and III and the appendix to Chapter III present one of the first efforts to determine the effects of carbohydrate removal on specific properties of a purified integral membrane protein, glycophorin A. In addition, chapters IV through VI provide some insight into problems concerning the reconstitution of purified integral membrane proteins into phospholipid vesicles.

A battery of specific glycosidases are now available and may be used to selectively remove significant amounts of carbohydrate from glycoproteins. Using two such enzymes, neuraminidase and endo-\(\alpha\)-N-acetylgalactosaminidase, most of the \(\text{O}-\)linked sugars were cleaved from glycophorin A. Removal of carbohydrate led to corresponding changes in molecular weight, electrophoretic mobility on SDS gels and, distribution coefficients, \(K_D\), on gel chromatographic columns. At saturating SDS levels all forms of glycophorin were determined to be monomeric, but in aqueous, detergent-free buffer all species were aggregated. Apoglycophorin appeared to be the most highly aggregated. All data presented here and by others is consistent with the notion that protein-detergent as well as protein-protein interactions are mediated primarily by the hydrophobic region. Carbohydrate has no or only a minor role in such interactions and instead may act to inhibit binding. SDS binding isotherms to the three forms were typical of all membrane proteins in having a cooperative binding transition at the detergent CMC. It is not known whether the increased SDS binding shown by deglycosylated forms represents additional
binding to the hydrophobic peptide or to the amino terminal region. This additional binding brings about very little change in CD spectra, again suggesting that much of the binding is to the hydrophobic region. All data presented herein indicates that removal of carbohydrate has no apparent effect on any protein-protein interactions.

An interesting finding is that quantitation of protein whether by Coomassie blue staining, PAS staining, or Lowry (1951) color formation is dramatically affected by the amount of carbohydrate on the protein. Thus, all methods should be calibrated first by quantitative amino acid analysis.

The role of bound carbohydrate on the red cell membrane is not known, although removal of sialic acid residues decreases the red cell's lifetime in circulation. Using a variety of glycosidases, one might remove the bulk of bound carbohydrates from the red cell and assess its lifetime in circulation or study its rheological properties. It seems likely however, that carbohydrate plays a more subtle role in membrane protein biosynthesis or as a receptor during hematopoiesis, i.e. red cell differentiation and maturation (Jokinen et al., 1979). In this regard, the recent discovery of a hematopoietic cell line that expresses glycophorin on its surface, will allow the investigation of mammalian membrane glycoprotein (and particularly glycophorin) biosynthesis. Certain partially glycosylated glycophorin precursors are presently being identified in these cell lines. The identification and characterization of precursors may be aided by knowledge of properties of deglycosylated forms like those described here. While bound carbohydrate may increase aqueous solubility, it appears that most amphiphilic properties of a membrane protein are determined primarily by the hydrophobic region.

In the second part of this thesis, protein incorporated vesicles were prepared by removal of detergent from solutions containing mixed micelles of
egg phosphatidylcholine, octyl glucoside and integral membrane proteins. These vesicles are considerably larger than vesicle preparations described by previous investigators who used sonication or cholate dialysis. This larger size facilitates measurement of passive ion permeability of the vesicle and allows considerable trapping not only of ions but of larger molecules like cytochrome c. These larger vesicles were shown to possess permeability properties comparable to small vesicles. The most significant conclusion from this work is that the incorporation of a transmembrane protein, glycophorin, into the bilayer does not significantly increase the passive permeability of the vesicles to ions. Furthermore, two other integral membrane proteins, mD8H and HAR, regain or retain activity upon incorporation into vesicles. Thus, the procedure by which these proteins were incorporated appears applicable to the study of ion transporters, e.g. Ca\(^{+2}\) ATPase. Questions concerning counter ion transport, mechanism of pump action, and identity of the functional pumping unit may be approached in these two compartment systems.

A theoretical discussion of vesicle formation is given, but details as to how protein is incorporated, and particularly, how unique orientation may arise during formation remain unanswered.

Properties of integral membrane proteins may be explored using vesicle incorporated protein. As Albanesi (1980) has suggested, the incorporation of mD8H into vesicles allows one to selectively label the putative hydrophobic domain with a radioactive acyl azide. These chemicals are known to partition preferentially into bilayers and to react after flash photolysis with peptide regions inserted into the bilayer (Klip and Gitler, 1974; Cerlitti and Schatz, 1979). Peptide mapping might then allow identification of the lipid associated segment.

In addition, some receptor functions, like those of HAR, may be
investigated in vesicles containing incorporated receptor. Structural changes in protein and surrounding lipid attendant to ligand binding may be monitored by a variety of techniques. Furthermore, fusion of such vesicles with intact cells may allow definition of factors required for ligand internalization. Some progress in this area has already been reported (Baumann et al., 1980).

Moreover, these vesicles have higher trapping efficiencies for large molecules than some liposomes, thus this entrapment method could be applied as a vesicular drug delivery system (Papahadjopolous and Szoka, 1980).
APPENDIX

TRANSPORT STUDIES OF GLYCOPHORIN

Glycophorin has been shown by a number of investigators to behave anomalously by SDS polyacrylamide gel electrophoresis (Lenard, 1970; Segrest et al., 1971; Banker and Cotman, 1972; Furthmayr and Marchesi, 1976). Glycophorin migrates on some gel systems as multiple bands. All of which have an apparent molecular weight higher than the actual value. It must be stressed that SDS gel electrophoresis is not a rigorous method for molecular weight determination and is based on two assumptions given in the discussion of Chapter III which are not met by glycophorin and many other proteins. An understanding of multiple band formation by a purified protein requires examination of the equation describing transport.

Electrophoretic mobility of a species on polyacrylamide gels is described by the following equation:

\[ v = \frac{\varepsilon q}{f \Phi(r)} \]

Where \( v \) is the steady state velocity of the species transported, \( \varepsilon \) is the field strength, \( \Phi(r) \) is the retardation effect on gel pores, and \( q \) and \( f \) are the total charge and the frictional coefficient of the species transported, respectively. Since \( \varepsilon \) and \( \Phi(r) \) depend on the gel system employed, changes in the mobility of a species and formation of multiple bands result from differences in a species charge, frictional coefficient, or a combination of the two. The total charge of a species in an SDS gel electrophoretic system
depends on the amount of SDS bound to the transported species and upon the intrinsic charge of the species. This binding may depend on any number of factors such as ionic strength, temperature, SDS concentration, and pH. The frictional coefficient depends on the size and shape of the migrating species which is affected by changes in the state of association or the conformation of the species. Generally, the state of association is dependent on protein concentration, whereas conformation is not. Thus, when an apparently homogeneous species migrates as multiple bands, one cannot assume that oligomerization has occurred. In this regard, it is important to recognize that gel electrophoresis is a transport measurement and does not give results necessarily representing thermodynamic equilibrium. If one is dealing with an interacting system, e.g., monomer $\leftrightarrow$ dimer, the interpretation of one band or multiple bands is not straightforward. The formation of multiple bands is dependent upon rates of association and dissociation relative to the rate of transport and upon protein concentration (Cann, 1970).

Transport phenomena

On Fairbanks gels (1971) which are prepared in Tris acetate buffer $\mu = 0.1$, pH 7.4, 1% SDS, glycophorin A forms two PAS stainable bands, PAS 1 and PAS 2 (Figure 30). Several investigators have shown that these two bands are reversibly interconvertable (Marton and Garvin, 1973; Tuech and Morrison, 1974; Furthmayr and Marchesi, 1976). The relative distribution of material between the two bands depends on (1) the concentration of SDS in the applied protein sample and in the gel, (2) the duration and temperature of incubation of the applied protein sample prior to electrophoresis, (3) protein concentration in the sample, (4) the ionic strength of the applied protein sample and of the gel, and (5) the use of the Tris buffer system.

All of the work presented in this thesis, including gel electrophoresis,
Fig. 30. Mobilities of glycophorin A on SDS gels prepared according to Fairbanks et al. (1971). Protein sample buffer was 6% SDS, 4 M urea, 20 mM Tris-HCl (pH 6.8), 2 mM EDTA. Sample was electrophoresed after incubation for 30 min at 37°C (A) or an additional 2 minutes at 100°C (B). PAS 1, PAS 4, and PAS 2 positions are indicated. Gels were scanned photometrically at the wavelength shown. TD is tracking dye. (Taken from Furthmayr and Marchesi, 1976)
was conducted in phosphate buffered solutions. Furtherly and Hipplewell (1976) claim that interconversion between gliospherin bands are observed only in the 
Trij-buffered gels and not in phosphate buffer, but data presented here show that electron microscopy. 

Saturates

The presence of PAS 1 and PAS 2 were ruled out. 

The presence of PAS 2 was ruled out. Either explanation is not possible. 

Furtherly and Mariscal examined whether PAS 2 species with 

was conducted in phosphate buffered solutions. Furtherly and Hipplewell (1976) claim that interconversion between gliospherin bands are observed only in the 
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Trij-buffered gels and not in phosphate buffer, but data presented here show that electron microscopy.
was conducted in phosphate buffered solutions. Furthmayr and Marchesi (1976) claim that interconversion between glycophorin bands are observed only in the Tris buffered gels and not in phosphate buffer, but data presented here show that electrophoretic mobility changes can be observed in the latter system. Saturating levels of SDS binding are used in all of these studies.

Pretreatment of the sample

The distribution of staining between two bands depends on the treatment of the glycophorin sample before electrophoresis. Heating of glycophorin preparations in 20 mM Tris HCl, 6% SDS, 0.7 mM SDS, 8 M urea prior to electrophoresis on the Fairbanks gel system produces a shift in material from PAS 1 to PAS 2 (Furthmayr and Marchesi, 1976). Calculations indicate that the amount of SDS in the incubation mixture should be sufficient to allow saturation of SDS binding. Glycophorin, however, is a highly charged molecule (37 negative charges/molecule) and at this ionic strength, it is not known whether intrinsic charge might interfere with SDS binding. Furthmayr and Marchesi have interpreted this shift as an interconversion of glycophorin dimer, PAS 1, to monomer, PAS 2, upon heating. This is not the only possible explanation since changes in SDS binding or in protein conformation were not ruled out.

The PAS 2 form may be reconverted to PAS 1. When a PAS 2 band is eluted from the gel, reheated, and electrophoresed, a shift in material from PAS 2 to PAS 1 is observed (Figure 30, Furthmayr and Marchesi, 1976). If a heated glycophorin sample in 2% SDS, \( \mu = 0.008 \) (apparently at saturating levels of SDS binding) is frozen, thawed, and electrophoresed, a corresponding conversion of PAS 2 to PAS 1 occurs (Marton, 1975).

Furthmayr and Marchesi showed a similar shift between two species with different elution volumes expressed in terms of \( K_D \), the distribution
coefficient on a gel filtration column eluted in 1% SDS, 10 mM Tris HCl, 1 mM EDTA, pH 8.0, \( \mu = 0.017 \) at 45°C. Assuming no interaction between transported molecules and the support gel, \( K_D \) is a function of the hydrodynamic radium of the migrating species. However, changes in this radius can come not only from changes in the state of association of the protein, but also changes in SDS binding or in protein conformation.

The addition of salt during preincubation also resulted in increased conversion of PAS 1 material to PAS 2 (Potempa, 1978) thus affecting at least one of the changes mentioned above. Higher concentration of protein in the applied sample resulted in greater amounts of material migrating as PAS 1 relative to PAS 2. If PAS 2 represents monomer and PAS 1 is dimer, then an association constant of about \( 1.5 \times 10^5 \) liters/mole may be calculated from the initial protein concentrations and the relative peak areas of bands given by Furthmayr and Marchesi (1976). The magnitude of the concentrating effects of electrophoresis at the gel-sample solution interface is not known.

**Ionic strength.** Potempa and Garvin (1978) discovered that as the ionic strength in the Fairbanks (1971) gel is raised from 0.1 to 0.3, material in the PAS 1 and PAS 2 regions shifts to form another band PAS 4 of intermediate mobility to these two. (See Figure 30 for location of PAS 4). These samples were generally 2% SDS, \( \mu = 0.01 \) and at the protein concentration used, had saturating levels of SDS. Since at intermediate ionic strengths significant amounts of PAS 1, 2, and 4 were observed on the same gel, Potempa and Garvin (1976) have suggested that PAS 4 was a unique glycophorin species distinct from PAS 1 and 2. An interconverting two species mixture (e.g. monomer \( \rightarrow \) dimer) may give three bands or boundaries in a transport measurement when reactions are so slow that chemical equilibrium cannot keep pace with transport, but fast enough that appreciable changes take place during the course of
electrophoresis. The existence of one band at higher ionic strengths may then be the result of faster interconversion of monomer and dimer relative to the rate of transport. These results suggest that "kinetic trapping" of glycophorin between these two states may be occurring under these conditions. If there were a high energy of activation for the interconversion between monomer and dimer then attainment of equilibrium after a perturbation such as heating may be a slow process. Marton (1974) provides some evidence for this slow equilibration in Tris buffers.

Two lines of evidence suggest that the interconversion of glycophorin between PAS 1 and PAS 2 forms is mediated through the hydrophobic region. Furthmayr and Marchesi (1976) showed that incubation of glycophorin with the Tis fragment (the 23 amino acid peptide containing the hydrophobic region) leads to increased shift in material from PAS 1 to PAS 2. The labeled Tis fragment migrates with PAS 2 but not PAS 1 indicating some interaction with PAS 2. Furthermore, carboxymethylation of Methionine 81, a residue in the hydrophobic region, also leads to a shift in material from PAS 1 to PAS 2 (Silverberg et al., 1976).

**Studies in phosphate buffers**

Figure 31 showed the migration pattern of native, asialo and apoglycophorin A on polyacrylamide gels in 0.05 M sodium phosphate, pH 7.1, 0.4% SDS, $\mu = 0.14$. Two different initial protein loading concentrations were studied. At the higher protein concentration, one band is observed as was described in Chapter III, however, at a much lower protein concentration, a single band of considerably greater mobility is seen for all three forms. These bands may be analogous to the PAS 2 bands seen on Fairbanks gels. Therefore, mobility of glycophorin on phosphate buffered gels, also depends on protein loading concentration. This dependence was further characterized as shown in Figure
Fig. 31. Profiles of native glycophorin (A), asialoglycophorin (B),
and apoglycophorin (C) on 7.5% polyacrylamide gels in 0.05 M sodium phosphate,
pH 7.1, 13.9 mM SDS. 125I labeled glycophorin (< 0.05 μg) [----] or 125I
labeled glycophorin added to 4 μg of unlabeled glycophorin [-----] in gel buf­
fer were heated at 100° for 3 minutes, then applied to the gel. After elec­
trophoresis, the gels were stained with Coomassie blue and scanned photomet­
rically or sliced into 2 mm sections and counted. Staining profiles exactly
coincide with 125I counts [-----] at protein concentrations detectable by
staining. At low protein concentrations native, asialo, and apoglycophorin
migrate with apparent molecular weights 43,000, 35,000 and 23,000 respectively
and at the higher protein concentrations at 74,000, 63,000 and 39,000,
respectively
32. On page 136 of the original manuscript, one band is seen at all protein concentrations, but the relative mobility of the band increases with decreasing protein concentration.

If association of substrate to DNA occurs in monolayer, at x < 0.5 the equilibrium equation can be written:

\[ \frac{[S]}{[S]_0} = k_a x \]

where:
- \([S]_0\) is the initial substrate concentration
- \([S]\) is the substrate concentration at equilibrium
- \(k_a\) is the association constant
- \(x\) is the fraction of substrate associated

This chromatographic behavior is also due to monomer-dimer interconversion, then the apparent association constant is \(9.3 \times 10^6\) liters/mole (based on initial loading concentration). This is considerably higher than that estimated from equilibrium data (40 times lower than the \(K_a\) determined from gel electrophoresis). These differences are
32. On 0.1 M sodium phosphate containing SDS gels, one band is seen at all protein concentrations, but the relative mobility of the band increases with decreasing protein concentration.

If association of monomer to form dimer is occurring, at \( \mu = 0.26 \) the equilibrium equation may be written:

\[
2A \rightleftharpoons A_2
\]

\[
K_a = \frac{[A_2]}{[A]^2}
\]

Since \([A_T] = 2[A_2] + [A]\), \(K_a\) is equal to \(1/[A_T]\) at the point where \([A] = 1/2[A_T]\). An apparent association constant of \(1.68 \times 10^6\) liters/mole based on initial loading concentration) may be estimated from this gel data. This is considerably higher than the association constant calculated from sedimentation equilibrium data, \(1.6 \times 10^3\) liters/mole \(\pm 0.8 \times 10^3\), and 10 fold higher than the association constant calculated from the gel data of Furthmayr and Marchesi (1976), \(1.5 \times 10^5\) liters/mole.

The distribution coefficient, \(K_D\), of glycophorin on gel filtration columns also depends on protein loading concentrations (Figure 33). Chromatography was performed in a jacketed column containing Sepharose 6B or Sephacryl S-200 equilibrated in 0.1 M sodium phosphate, 0.4% SDS, pH 7.1 at either 24 or 50°C. Glycophorin eluted as a single, symmetric peak, the elution volume of this peak increased with decreasing protein loading concentration. If this chromatographic behavior is also due to monomer-dimer interconversion, then the apparent association constant is \(9.5 \times 10^4\) liters/mole (based on initial loading concentration). This is considerably higher than that estimated from sedimentation equilibrium data but 40 times lower than the \(K_a\) determined from gel electrophoresis. These differences are
Fig. 32. Effect of protein concentration on relative electrophoretic mobility of glycophorin on 7.5% polyacrylamide gels in 0.1 M sodium phosphate, pH 7.1, 13.0 mM SDS. Five microliters of 125I-glycophorin was added to unlabeled glycophorin samples of varying concentrations in gel buffer. This mixture was heated at 100° for 5 minutes prior to loading on the gel. After electrophoresis gels were stained with Coomassie blue or sliced into sections and counted. Relative mobility was calculated using the tracking dye, bromophenol blue.
Fig. 33. Effect of protein concentration on gel chromatographic $K_D$. Unlabeled glycophorin samples of varying protein concentration were "spiked" with $^{125}$I labeled glycophorin and heated at $100^\circ$ for 5 minutes prior to loading on the column. A jacketed column (0.8 X 65 cm) containing Sepharose 6B (triangles) or Sephacryl S-200 (circles) was equilibrated and eluted with 0.1M sodium phosphate, pH 7.1, 13.9 mM SDS buffer at $24 \pm 1^\circ$ (open symbols) or at $50^\circ \pm 2^\circ$ (closed symbols). $K_D$, the distribution coefficient, was determined from the equation $K_D = \frac{V_t - V_e}{V_t - V_o}$, when $V_t$ is the total volume, $V_o$ is the void volume, and $V_e$ is the elution volume of the glycophorin sample. $V_o$ and $V_t$ were determined using DNA and $\beta$ mercaptoethanol, respectively.
reflected in previous literature (Skadrisk and Bolmeke, 1974; Partlow and Marchesi, 1971) and the reasons for these differences have not been resolved yet.

It is important to note that the binding measured on Sepharose 2B columns was performed at high initial loading concentrations of glyophosphate such that the glyophosphate eluted with a C of around 0.2 M. For binding across the protein peak, the conditions and the column packed with protein were the same.
reflected in previous literature (Grefrath and Reynolds, 1974; Furthmayr and Marchesi, 1971) and the reason for these differences have not been resolved yet.

It is important to note that SDS binding measured on Sephacryl S-200 columns was performed at high initial loading concentrations of glycophorin such that the glycophorin eluted with a K_D of about 0.08. SDS binding across the protein peak was constant and thus apparently independent of protein concentration. As mentioned previously, upon rechromatography of this eluted peak or upon dialysis against column buffer no change in binding occurred within experimental error.

Native glycophorin-apoglycophorin interactions

Although there is little evidence for interaction between native and apoglycophorin in 0.1 M sodium phosphate, 13.9 mM SDS, \( \mu = 0.26 \), some association between these species is indicated by electrophoresis on 0.05 M sodium phosphate, 13.9 mM SDS, \( \mu = 0.13 \), containing polyacrylamide gels. Figure 34 shows a shift in material from both control and apoglycophorin bands to a new band of intermediate mobility. Ratios of relative staining intensity suggest that the intermediate species may be a native glycophorin-apoglycophorin heterodimer. Figure 35 shows that heating the samples at 100°C prior to electrophoresis increases the conversion of material to the intermediate species. This data is consistent with the interpretation of glycophorin interconversion discussed previously in this appendix.
Fig. 34. Densitometry scan of 7.5% polyacrylamide gels of glycophorin and apoglycophorin. 15 μg of native glycophorin (A,D), 15 μg of apoglycophorin (B,E), or a 1:1 mixture of the two (C,F) were heated at 100°C for 3 minutes before application to the gels. The gel buffer conditions were identical to those in Fig. 31. After electrophoresis, parallel gels were stained with Coomassie blue (A-C) or by the PAS procedure (D-F) and scanned at the appropriate wavelength. The apparent molecular weight of the intermediate band is about 61,000.
Fig. 35. Effect of heating on the interaction of glycophorin and apoglycophorin on 7.5% polyacrylamide gels in 0.05 M sodium phosphate, pH 7.1, 13.9 mM SDS. A mixture of glycophorin (≈ 10 μg) and apoglycophorin (≈ 15 μg) in 0.1 M sodium phosphate, pH 7.1, 13.9 mM SDS were incubated at room temperature for 10 minutes (A) or incubated for 7 minutes at room temperature then heated at 100°C for 3 minutes (B) prior to application to the gels. Gels were scanned photometrically as described in Methods. TD represents the tracking dye, bromophenol blue.
ABSORBANCE AT 585 nm

RELATIVE MOBILITY

A

B

TD

TD
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