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HUMAN ERYTHROCYTE MEMBRANE PROTEINS

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HUMAN ERYTHROCYTE MEMBRANE PROTEINS

CHAPTER I

INTRODUCTION

The human erythrocyte (RBC) has been the subject of numerous investigations which have been performed over a period of decades. These studies have been carried out at many different levels of evaluation. For instance, the RBC's function in oxygen transport has been examined extensively in the intact RBC, both in vivo and in vitro. Another example of RBC studies has been the very productive area of hemoglobin structure and composition, which led to major advances in the understanding of protein structure. In addition, the human RBC has served as a model for the exploration of membrane composition and function, the use which is most pertinent to this effort.

The human RBC has been used for many investigations of membrane structure, function, and composition. The constituents of the red blood cell membrane have been examined in many different ways and at several different stages of preparation. These include the RBC in whole blood, the washed intact RBC, the lysed RBC (ghost), and the lipid extracted ghost. The RBC ghost as a source for probing into the protein content of membranes is a critical feature of this work.

Studies on the RBC ghost in the past have centered on its lipid

content, while investigations on the protein content have been limited. The major reason for studying the lipids of the red blood cell ghost has been that lipid methodology is well-established and relatively simple. The proteins are more difficult to study, because of problems relating to solubility, aggregation, and the many different proteins present in the ghost. The initial phase of this study was designed to deal with those problems, thereby leading to the isolation and characterization of all ghost proteins. The efforts were partially successful and led to a second phase in which aqueous fractionation was evaluated as a technique for the separation of ghost proteins. When the results of the two phases were combined and correlated, a more definitive understanding of erythrocyte ghost proteins evolved and led to the proposal of a classification system for the ghost proteins. The sequence of events leading to the proposal will now be described.

CHAPTER II

LITERATURE REVIEW

The study of membrane composition has become an area of increasing activity. Three major membrane systems have been used for the study of animal membranes and their components. Myelin, a membraneous covering over both central and peripheral nervous tissue, has served as a model system of membranes having the capacity to insulate the transmission of nervous impulses from the surrounding tissues. Myelin is characterized by a very high lipid and a low protein content; most proteins appear to be of relatively low molecular weight. At the opposite end of the spectrum, the inner mitochondrial membrane has been extensively investigated as an example of membranes with major metabolic functions. The inner mitochondrial membrane and other membranes of the same type are characterized by a high protein content and a small amount of lipid. In addition, the lipid moiety tends to be more uniform in composition with fewer types of lipids present. In between these two major types of membranes lies the erythrocyte membrane, which has been studied extensively as an example of cellular plasma membranes. It consists of almost equal proportions of qualitatively heterogenous lipids and proteins.

The mature human erythrocyte has multiple advantages as a source of material for the study of plasma membranes. The most obvious advan-

tage is that there are no intracellular organelles which require complex separation techniques for their removal. A second advantage is that the major protein of the mature red blood cell (RBC) is hemoglobin, which can be readily removed from the mature erythrocyte. The extraction of hemoglobin from the mature RBC is possible because the erythrocyte membrane has a capacity to reform intact membranes spontaneously, a property common to many plasma membranes. Another advantage of the erythrocyte membrane as a model is that previous extensive studies have already been performed regarding the cells' function, composition, enzymes, and antigens. This makes it possible for many components, especially enzymes and antigens, to be identified on the basis of their previously determined characteristics. Finally, it has been demonstrated that in some human hematologic disorders, changes in the RBC membrane have been noted which may be related to the etiology of the disease. Thus, if an adequate understanding of the composition of the membrane can be developed, a number of clinical disorders are available for study.

Unfortunately, the erythrocyte membrane does have some disadvantages as a model for membrane studies. The first is that the mature RBC, although circulating in the blood, is a nonmotile cell and is not representative of all cellular plasma membranes. A second disadvantage is that the mature RBC is uniquely a non-nucleated cell in its functional state. A third problem with the erythrocyte membrane is that, although hemoglobin is readily removed, it is impossible to prevent a simultaneous removal of other non-hemoglobin proteins from the membrane. Whether or not important structural proteins of the RBC membrane are removed at the same time as hemoglobin will have to be determined. A final disadvantage

is that the effects from hemolytic techniques on the membrane itself are not clearly understood. For example, major alterations in membrane structure and composition may be caused by osmotic lysis, the method commonly used to remove hemoglobin. Although what is lost from the membrane during this process has been partially identified, all changes produced in the membrane during this process have not been determined. Nevertheless, because of its many advantages, the human erythrocyte membrane was chosen for investigation in this study, as it has been for many previous studies on cellular plasma membranes.

Structure of the Erythrocyte Membrane

The reports on membrane structure are so numerous that a review of all major papers on the subject is not possible. Consequently, only those papers which are of importance for erythrocyte membrane structure will be considered. The studies have been done on materials as diverse as mackerel egg oil and muscle cell endoplasmic reticulum. However, all these reports have contributed to the understanding of the structure of the erythrocyte membrane.

The first paper of major significance on the composition of erythrocyte membranes was published in 1925 by Gorter and Grendel (1). These workers attempted to determine the composition of the surface of erythrocytes. Using careful procedures to separate the plasma from the red cells, they extracted the erythrocytes repeatedly with large amounts of pure acetone. The lipids were applied to a Langmuir trough and the surface area covered by a single layer of lipid molecules was measured. Then, by an indirect measurement technique, the total surface area of the extracted erythrocytes was calculated. Combining these two findings, the

authors were able to show that there was in the erythrocytes an amount of lipid adequate to cover the cell surface in a layer 2 molecules thick. This was approximately true for all six species studied. Although subsequent attempts to repeat the experiment showed that the method of lipid extraction was not satisfactory for complete removal of lipids and that the technique for measuring cell surface was inadequate, the basic assumption that the surface of the erythrocyte is covered by a lipid bilayer has continued to hold true.

The next major advance in understanding the structure of the erythrocyte membrane was made by Danielli and coworkers. In the first of these papers, Danielli and Harvey (2) measured the surface tension of the oil drop of the mackerel egg. The low value (0.6 dyne/cm) at the interface between the oil drop and the protoplasm contrasted markedly with the much higher value of approximately 9.0 dynes at interfaces between mackerel egg oil and different buffer systems. The authors postulated that the explanation for this difference must be that an additional substance was present which had lowered the tension. After a series of experiments involving tension measurements between brombenzene and an aqueous interface, the authors concluded that the material which was responsible for the reduction of interfacial tension was protein in nature. They suggested that the low tension observed at the surfaces of living cells must be due to the adsorption of proteins on a lipid layer.

The second paper by Danielli and Davson (3) was on the subject of the permeability of thin lipid films and considered how it was possible for different molecules to move across a lipid film. They were particularly concerned with how a membrane could distinguish between

molecules of different sizes and solubility characteristics, as well as ions of different charge. They concluded that a very thin lipid layer with a protein adsorbed upon the surface would be the most likely structure to possess the required characteristics. Within the text itself, it was suggested that only one surface, the inner surface, of the membrane would have to be covered by a protein film. Nevertheless, the authors proposed a specific model consisting of a bimolecular layer of lipids with the hydrophobic tails placed internally and the hydrophilic heads placed externally in the aqueous media. The proteins were adsorbed onto the hydrophilic heads of the lipids to form the membrane structure. This concept was to provide the basic model for membrane structure for many years and still has not been refuted completely.

In third and final paper, Davson and Danielli (4) further refined their model to include an adsorbed layer of protein on the polar heads of the phospholipids, forming the bimolecular leaflet. They specified that the protein was bound to phospholipid through electrostatic interactions. Their conclusion that the proteins bound ionically to the polar heads of the phospholipids has caused a major controversy about membrane structure. Davson and Danielli also discussed the binding of proteins, especially globular proteins, to lipids at an oil-water interface. They suggested that the hydrocarbon residues of the amino acids are incorporated into the lipid layer to form a stable film with the lipid. As a result of this combination of lipid and protein, it was possible for stable films to be formed and to impart to the remainder of the membrane a structural strength which lipids alone could not achieve. The specific interaction of lipid and protein did not require polar interac-

tions, but did require that the hydrocarbon portion of the amino acids reside within the oil layer, while the polar groups remained in the aqueous phase.

The next major advance in the understanding of the structure of membranes came about as a result of the development of the electron microscope. Although many workers reported their findings with electron microscopy, the work of Robertson was clearly the most important one. Basing most of his studies on myelin, Robertson (5) proposed in 1959 a model for Schwann cell membranes which consisted of a single biomolecular leaflet of lipid, with the polar surfaces of the lipids covered by monolayers of non-lipid material. This formed a trilayered membrane structure approximately 75 Å wide. The 75 Å wide structure was made up of two 20 Å dense lines, separated by a 35 Å light central zone. This model was presumed to be common to all membranes, including the red blood cell membrane. In 1964, Robertson (6) proposed the concept that the trilayered appearance observed by electron microscopy was produced by a so-called membrane, which was common to all membranous structures. Although recognizing the similarity, Robertson pointed out that his model differed from the original Danielli-Davson concept in three major ways. The first major difference was that the Danielli-Davson model did not indicate how many layers of lipid were present in the membrane core. His studies clearly indicated that the unit membrane concept restricted the number of lipid monolayers to two. The second major difference was that Danielli and Davson could not show whether the non-lipid components on the margins of the membrane were present as a spread film or as a sequence of globular molecules. Robertson's theory specified that the non-lipid portion

of the membrane was spread over the surface as monolayers rather than as a globular protein monolayer. This finding was based primarily on X-ray studies of myelin and Robertson questioned whether this concept was applicable to all cell membranes. The third and final difference was that the unit membrane concept proposed an asymmetrical structure for the membrane. The surface on the outside of the cell was shown by electron microscopy to be different from that of the inside of the cell. Robertson's concept of the unit membrane further strengthened the view that the lipid bilayer is the major feature of membrane structure.

The next major advance in the study of membrane structure also involved the use of electron microscopy, but was based on a technique different from the typical thin sectioning method. The procedure of freeze cleaving or freeze etching of intact cells was introduced by Moor and Muhlethaler (7) in their study of the internal structure of yeast cells. However, it was not until 1966 that Branton (8) first described the use of this procedure for studying biological membranes of yeast cells, algae, and human RBC. Branton noted that previous observations had not revealed membrane faces whose morphological features were consistent with what was previously known about membrane surfaces. He concluded that the process of cleaving resulted in separation of the cell membrane along internal faces and not at the surface of the membrane. He based his conclusion on three different pieces of evidence. The first was that, where the membrane could be clearly defined as leading from a tangential view to the cross-sectional view, an overall cross-sectional thickness of 75 Å for the intact membrane was not compatible with a thickness of 40 Å, which was the cross-sectional thickness found after freeze

cleaving. The second line of evidence was that the fractured membrane surfaces could not be etched, as would be expected if the surface was either the outer or the inner membrane surface. The final piece of evidence was that the cleaved membrane faces did not show the structural features associated with true membrane surfaces. These findings were consistent with the concept that the biological membrane is organized, at least in part, as an extended lipid bilayer.

A somewhat different concept using the same technique of freeze cleaving was proposed by Weinstein and Bullivant (9) in 1967. Using mature erythrocyte membranes from both the mouse and humans, the authors described a technique for studying the structure of RBC membranes. They noted that the cell surface was smooth and partially covered with small particles which might represent antigens, enzymes, or structural proteins. More particles were observed on the external than on the internal surface of the membrane. They considered the possibility, proposed by Branton, that the fracture plane might be through the membrane itself, but rejected that concept for lack of evidence. Their observation about the surface being covered by a large number of small spherical particles, which are relatively uniform in size, was substantiated in 1968 by the work of Koehler (10), who investigated the same problem in the plasma membranes of frog erythrocytes.

In further studies on the erythrocyte membrane surface by the freeze cleaving technique, Weinstein (11) again described the numerous particles on the external surface as opposed to the smaller number of particles on the internal surface. This was true both for intact red blood cells and for red blood cell ghosts. However, he did note at this

time that there was an external coating over the cell surface, the composition of which could not be determined. In addition, he observed that the membrane-associated particles would occasionally penetrate through the membrane surface, representing molecules exposed to both the internal and external surface. The concept that the erythrocyte membrane is covered by a thin exterior coat, the removal of which exposes an underlying granular surface, has been further supported by the work of Pinto da Silva and Branton (12), as well as Tillack and Marchesi (13), both of whom used labelling techniques to coat the erythrocyte surface. The difference is that according to Weinstein (11), the lipid layer of the membrane was actually fractured, whereas the latter two groups maintained that the fracture line separated the lipid layers of the membrane. The only definite conclusion that can be reached from these studies is that an outer coat is removed from the erythrocyte membrane surfaces during the process of freeze cleaving. Whether or not this actually exposes the hydrophobic regions of the erythrocyte membrane is yet to be determined.

The most recent advance in the understanding of biological membrane structure has come about as the result of the use of spectroscopic techniques. These have included infrared spectroscopy, fluorescent spectroscopy, optical rotatory dispersion, circular dichroism, and proton magnetic resonance. In 1966, Wallach and Zahler (14) used optical dispersion techniques to evaluate the physical status of proteins within the plasma membranes of Ehrlich ascites carcinoma cells. They found that the proportion of membrane proteins in the beta conformation was limited and that the proteins resided in a medium of high refractive index, where

they interacted with lipids through hydrophobic interactions. They further noticed that there was a specific group of membrane proteins possessing both structural and functional activities. In addition, the proteins had unusual amino acid sequences, which may be a necessary structural requirement for their lipid binding properties.

In 1966, Lenard and Singer (15) used optical rotatory dispersion and circular dichroism for evaluation of protein structure within the membranes of human red blood cells and Bacillus subtilis. They found that one-quarter to one-third of the membrane protein is in an α-helical conformation, with the remainder probably in random coil form. Since this appeared to be a common feature of proteins from many different membranes, the authors suggested that membranes are formed and stabilized through the interaction of lipids with the hydrophobic portions of membrane proteins.

Additional physical studies using improved techniques were reported by Glazer et al. (16), who studied the effects of temperature and purified phospholipase C on human red blood cell membranes. Phospholipase C removed 60-70% of the phosphorylated amines from the membrane without removing any cholesterol, fatty acid, or protein. After phospholipase treatment, the average conformation of the proteins determined by circular dichroism was not detectably changed. However, proton magnetic resonance spectra showed that the physical state of the fatty acid chains and phospholipids was markedly altered. About three-fourths of the fatty acid chains were more mobile in the treated membrane than they had been in the untreated membrane. Conversely, the effect of temperature was substantially different on the protein and lipid components of

the membrane. With untreated membranes at elevated temperatures, as much as two-fifths of the protein was noted to have changed in physical state, whereas there was no evidence of a change in the lipid fatty acid chains. The authors concluded that a large proportion of both the phospholipids and the proteins could change independently of one another within the RBC membrane. These findings were most compatible with a mosaic membrane structure, in which most of the lipid and part of the protein were not interacting. Thus, lipid-protein interaction with the RBC membrane did not involve all membrane proteins and lipids.

In 1971, Singer (17) extensively reviewed the major data relating to molecular organization of biological membranes and more fully described the features of the lipid-globular protein mosaic (LGPM) model. This membrane model evolved primarily from the need to account for hydrophobic interactions between the lipids and proteins. The membrane proteins were divided into peripheral and integral proteins. The peripheral proteins were defined as ionically-bound proteins, which could easily be removed from the membrane. In addition, they were not associated with lipid after removal from the membrane and were soluble in aqueous buffers. The integral proteins were difficult to remove from the membrane and required stronger methods for solubilization (e.g., the use of detergents). In addition, these proteins were usually associated with lipids, the removal of which led to the formation of highly insoluble protein aggregates. Within the membrane, the lipids and the globular integral proteins were arranged in an alternating mosaic pattern. The hydrophobic segments of the integral proteins and the phospholipids were isolated within the hydrophobic interior of the membrane and away from

the aqueous exterior. At the same time, the ionic groups of the lipids and the charged residues of the peripheral proteins were both in contact with water on the exterior surface of the membrane. The exterior surface was also the site where the carbohydrate portions of both glycoproteins and glycolipids were found. This arrangement allowed for maximum hydrophobic and hydrophilic interactions between the different components of the membrane.

The mosaic model proposed a considerably different role for both the lipid and proteins than the Danielli-Davson-Robertson (D-D-R) model. The proteins in the mosaic model were an integral portion of the membrane and were inserted into the membrane interior, primarily in a globular conformation. This differed markedly from the D-D-R model according to which the surface of the membrane was covered with proteins possessing the beta-configuration. The lipids also differed in major ways from the earlier model and, although still assumed to be a bilayer, were not present as an uninterrupted continuum. The ionic groups of the lipids were not buried and constrained by a layer of lipids, but were in contact with the aqueous phase. Thus, the LGPM model proposed by Singer provided a modification of the lipid bilayer model, which was more consistent with the accumulated knowledge of membrane composition.

Singer then reviewed critically the evidence for the D-D-R model, the Benson model (a model similar to the lipid globular model in which the lipid and protein are joined together into lipoprotein subunits), and the LGPM model. Two pieces of evidence, i.e., the railroad track appearance found by thin section electron microscopy and the X-ray diffraction data on myelin, were compatible with all three systems. Five

major pieces of evidence were thought to be incompatible with the D-D-R model, but compatible with the Benson and the LGPM model. They were: 1) the unaltered staining pattern of membranes after delipidation, 2) the particulate structures found by freeze-etching techniques, 3) the circular dichroism measurements showing a 30-40% α-helix protein content, 4) the cleavage of phosphorylated amines of phospholipids by phospholipase C, and 5) the binding of a fluorescent dye to phospholipids and proteins in a similar manner. One piece of evidence, the occurrence of a phase change in lipids demonstrable by differential calorimetry and X-ray measurements, was incompatible with the Benson model. None of the data selected were inconsistent with the lipid-globular protein mosaic model.

In summary, the current data would suggest that the lipid-globular protein mosaic model is the most acceptable model for all cellular membranes. Most of the information which was used to develop the model was obtained from studies on erythrocyte membranes, the material selected for study in this investigation. The data to be presented later should provide further arguments for the acceptance of the LGPM model as the best current model for erythrocyte membrane structure.

Erythrocyte Membrane Composition

The human erythrocyte membrane is a complex mixture of proteins, lipids, and carbohydrates. Of these, none has been studied more extensively than the lipid moiety. The studies on lipids developed because lipids were assumed to play a critical role in the structure of cellular membranes, and an advanced methodology was available for their qualitative and quantitative determination. Carbohydrate is the smallest com-

ponent of the red cell membrane on a percentage basis and is covalently bound to either a lipid or a protein molecule. On a weight basis protein is the largest component, although it is actually a very heterogeneous mixture. The relative proportion of the major classes of compounds in the red cell membrane is given in Table 1 from the data of Rosenberg and Guidotti (18).

Lipid Composition

The content of the individual lipids, as reported by Sweeley and Dawson (19), is given in Table 2. On a molar basis, cholesterol accounted for 43%, the phospholipids for 54%, and the glycosphingolipids for 2.7% of the total lipids. As far as is presently known, lipid classes, such as cholesterol esters, triglycerides, and free fatty acids, which are normally present in the plasma, have not been found in the RBC membrane.

Data obtained by major investigators on the distribution of individual phospholipids are reviewed in Table 3. Although there was some variation among the different results and considerable variation in the methods used for determining the phospholipid content, certain features were consistently reported. The total cholesterol and lipid phosphorus content of individual RBC was quite consistent. Only a slight predominance of lipid phosphorus over cholesterol was found on a molar basis. The method of lipid extraction, as demonstrated in the work of Ways and Hanahan (23), influenced the total content of phospholipid and cholesterol. The data of Crowley *et al.* (24), and Neerhout (27) both demonstrated an increase in the total phospholipid and cholesterol in erythrocytes obtained from umbilical cord blood, when compared to red blood

TABLE 1

MAJOR COMPONENTS OF THE HUMAN RBC MEMBRANE[a]

Component		
Protein	49.2%	
Lipid (Total)	43.6%	
Phospholipid		32.5%
Cholesterol		11.1%
Carbohydrate (Total)	7.2%	
Neutral sugars		4.0%
Hexosamines		2.0%
Sialic Acids		1.2%

[a]Rosenberg and Guidotti (18).

TABLE 2

LIPIDS OF THE HUMAN RBC MEMBRANE[a]

	$\mu M \times 10^{-10}$/Cell	Percent Total Lipid	
Cholesterol	3.20	43.2	
Phosphatidyl Choline	1.20	16.2	
Phosphatidyl Serine	0.60	8.1	
Phosphatidyl Ethanolamine	1.10	14.8	54.0
Phosphatidyl Inositol	0.03	0.4	
Sphingomyelin	1.0	13.5	
Lysophosphatidyl Choline	0.04	0.5	
Phosphatidyl Acid	0.04	0.5	
Glycosphingolipids	0.20	2.7	
Total	7.41	99.9%	

[a]Sweeley and Dawson (19).

TABLE 3

PHOSPHOLIPID DISTRIBUTION IN THE HUMAN RBC MEMBRANE

Reference	Lipid P mgsX10-11/ Cell (μMX10-13)	Cholesterol mgsX10-10 Cell (μMX10-13)	Phosph. Ethanolamine	Phosph. Serine
Reed et al. (20)	1.15 (3.73)	1.13 (2.92)	24.6	14.9
Farquhar (21)			29	10
Bradlow et al. (22)			27.5	13.3
Ways & Hanahan (23)	1.27 (4.12)	1.26 (3.26)	25.7	15.0
Crowley et al. (24)	1.27 (4.12)	1.26 (3.26)	24.7	14.8
(Cord Blood)	1.41 (4.58)	1.50 (3.88)	22.2	15.2
Williams et al. (25)			27.9	3.9
Dodge & Phillips (26)	0.995 (3.23)	1.547 (4.00)	27.5	14.8
Neerhout (27)	1.22 (3.96)	1.33 (3.44)	31.2	13.1 (With PI)
(Cord Blood)	1.54 (5.00)	1.79 (4.63)	29.1	15.2 (With PI)
Jaffe & Gottfried (28)	1.121 (3.64) (gMX10-10)	1.330 (3.44) (μMX10-10)	29.5	13.8 (With PI)
Cohen & Derksen (29)			28.3	14.5
Phillips et al. (30)	H.L. { Young		26.4	15.0
	H.L. { Old		26.4	14.2
	D.S. { Young		29.7	15.5
	D.S. { Old		29.0	12.2
Turner & Rouser (31)	1.05 (3.41)	1.18 (3.05)	26.0	13.4
Dodge et al. (32)	1.28 (4.16)	1.54 (3.98)	29 29	

TABLE 3--Continued

Moles Per Cent of Phosphorus					
Phosph. Inositol	Phosph. Choline	Lysophosph. Choline	Sphingo-myelin	Phosphatidyl Acid	Minor or Unknown
4.0	30	2.5	22.0	-	2
-	36	-	21	-	3
-	30.5	2.9	24.6	N.D.	1.2
2.2	29.5	0.5	23.8	-	3.3
(Without Sphing)	29.9	-	25.3	-	5.0
(Without Sphing)	27.6	-	28.9	-	6.1
2.5	35.6	1.7	28.4	-	-
0.6	29.2	1.0	25.4	-	1.5
	29.5	1.2	24.1	1.0	-
	27.7	1.0	26.0	0.9	
	28.2	1.4	26.0	1.0	-
< 1.0	33.6	-	23.4	-	-
0.5	29.7	1.6	26.3	-	0.6
0.4	30.4	1.4	26.2	-	1.0
0.8	28.8	1.5	23.4	-	0.4
0.6	31.3	1.2	35.4	-	0.4
1.1	28.3	1.1	24.6	2.1	3.5
(Without Sphing)	33	-	PS + PI	2.0	-
	49 (Phos.Chol + Sphing)			3.0	7.3

cells from normal human adults.

Three specific phospholipids (phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin) accounted on the average for over 80% of the total phospholipids in the membrane. Only phosphatidyl serine, which accounted for approximately 13% of the lipid phosphorus, could be considered another significant phospholipid component of the membrane. There was a slight difference in the percentage of the three major phospholipids in erythrocytes from adult blood and from umbilical cord blood. Both phosphatidyl ethanolamine and phosphatidyl choline were reduced with an almost equal increase in the amount of sphingomyelin. The difference between the two types of blood could not be explained on the basis of two cell populations, one old and one young, since the data from Phillips *et al.* (30) demonstrated that there was no consistent difference in the distribution of phospholipids between young and old erythrocytes. Special consideration should be given to the data of Turner and Rouser (31) since they were obtained with the best available methodology for the separation of phospholipids. Although surprisingly little information was available, the phospholipid distribution did not appear to be much different in RBC ghosts than in intact cells, as suggested by the work of Dodge *et al.* (32).

The distributional pattern of individual phospholipids in the red cell membrane did not fully express their complexity. One reason is that not all of the phospholipids are present as di-acyl phosphoglycerides, but rather contain a mixture of the di-acyl form and the plasmalogen form. The plasmalogen content of the major phospholipids is presented in Table 4. Approximately 50% of the ethanolamine phosphoglycer-

TABLE 4

PLASMALOGEN CONTENT OF PHOSPHOGLYCERIDES

Reference	Percent as Plasmalogen			
	Ethanolamine Phospho-glycerides	Serine Phospho-glycerides	Choline Phospho-glycerides	Sphingo-myelin
Farquhar (21)	67	8	10	0
Ways and Hanahan (23)	35	1.5	2.0	0
Williams et al. (25)	52.0	0.0	3.9	0
Cohen and Derksen (29)	46.3	0.0	3.6	0

ides were found to be in the plasmalogen form. The more recent data suggested that the serine phosphoglycerides and sphingomyelin did not contain any plasmalogens. The biological significance of the variation in plasmalogen concentrations has not been determined.

In addition to the phospholipid distribution within the red cell membrane, the fatty acid composition of human red cells has also been extensively studied and the results are reviewed in Table 5. The major fatty acids present in the RBC membrane included palmitic acid, stearic acid, oleic acid, linoleic acid, and arachidonic acid. In addition, the 22-carbon and 24-carbon fatty acids accounted for approximately 15% of the total fatty acid composition. There was also a difference between the fatty acid composition of erythrocytes from normal adults and from the umbilical cord. The cord blood erythrocytes contained more palmitic and oleic acid and less linoleic acid. Although slight differences did occur, there appeared to be little difference in the fatty acid composition of old and young red cells, as demonstrated by Phillips et al. (30).

Major differences in the fatty acid composition of the different phospholipids in the RBC membrane were quite impressive, as shown in Table 6. Although palmitic acid was a minor component of phosphatidyl serine and phosphatidyl ethanolamine, it was the major fatty acid of both phosphatidyl choline and sphingomyelin. Stearic acid was the major fatty acid component of phosphatidyl serine, but a relatively minor component of phosphatidyl ethanolamine, phosphatidyl choline and sphingomyelin. The mono-unsaturated fatty acid, oleic acid, was a major component of phosphatidyl ethanolamine and phosphatidyl choline, but a minor component of phosphatidyl serine and sphingomyelin. Linoleic acid, the

TABLE 5

FATTY ACID COMPOSITION OF HUMAN RBC MEMBRANES
(MOLES %)

Reference		% 14:0	% 16:0	% 16:1	% 18:0	% 18:1 ω9	% 18:2 ω6	% 20:3 ω6	% 20:3 ω9	% 20:4 ω6	% 22 (All)	% 24 (All)
Farquhar (21)		0.5	29.9	0.7	15.0	19.9	10.8	1.3		10.0	8.7	-
Ways and Hanahan (23)		0.7	24.5	0.2	19.0	16.4	11.2	1.5		15.1	5.1	5.7
Crowley et al. (24) (Cord Blood)		0.6	25.2	2.0	17.1	13.4	3.4	2.5		16.7	8.5	9.3
Dodge and Phillips (26) (Fatty Acid		-	21.4	0.9	14.0	12.6	9.0	1.2	0.3	11.9	10.6	8.0
Neerhout (27) (Cord Blood)	Adult	0.2	17.0	0.7	15.3	14.6	10.9	1.4		17.4	7.3	7.5
	Cord Blood	0.2	21.3	1.0	16.3	11.9	3.4	2.7		19.6	7.4	8.9
Phillips et al. (30)	Young	-	19.3	-	15.9	12.8	8.3	1.3	0.2	16.2	N.E.	N.E.
	Old	-	20.7	-	15.5	14.3	10.1	1.1	0.1	14.2	N.E.	N.E.

TABLE 6

FATTY ACID COMPOSITION OF INDIVIDUAL PHOSPHOLIPIDS

Reference	Phosphatidyl Ethanolamine					Phosphatidyl Serine				
	16:0	18:0	18:1	18:2	20:4	16:0	18:0	18:1	18:2	20:4
Farquhar (21)	18.9	8.0	25.2	7.0	21.9	7.1	41.6	13.0	2.8	19.7
Ways and Hanahan (23)	15.5	14.1	17.2	5.6	21.8	4.4	39.7	9.8	2.6	23.5
24C fatty acids					0					7.8
Crowley et al. (24) [Cord Blood]	24.4	12.0	18.3	2.5	20.0	4.9	46.4	5.4	1.4	21.7
24C					6.3					4.3
Williams et al. (25)	13.8	5.8	19.0	6.4	27.1	12.3	10.2	19.1	6.2	22.0
24C					0.6					0.6
Dodge and Phillips (26)	12.9	11.5	18.1	7.1	23.7	2.7	37.5	8.1	3.1	24.2
24C					0.3					0.7
Cohen and Derksen (29)										
RBC	16.6	11.2	15.6	4.7	24.8					
Platelets	8.5	17.2	6.7	1.9	37.2					

TABLE 6--Continued

Phosphatidyl Choline					Sphingomyelin				
16:0	18:0	18:1	18:2	20:4	16:0	18:0	18:1	18:2	20:4
33.0	11.7	20.6	18.2	5.0	Not Determined				
34.7	13.8	21.1	21.9	6.7	41.3	9.1	5.2	3.7	0.7
				0					30.5
41.5	10.3	18.2	6.8	12.7	41.2	13.2	2.4	0.3	0
				0.7					31.3
36.1	10.1	18.6	23.2	5.2	45.7	7.3	1.1	0.3	0
				0.2					32.5
31.2	11.8	18.9	22.8	6.7	23.6	5.7	0.8	0.2	1.4
				0.2					50.6
33.6	12.7	19.7	22.8	6.2					
23.8	16.0	20.6	11.5	14.7					

essential fatty acid, was a major component of phosphatidyl choline, but only a relatively small percentage was present in the other major phospholipids. Arachidonic acid, a chemical relative of linoleic acid, was a major component of phosphatidyl ethanolamine and phosphatidyl serine, while almost nonexistent in sphingomyelin and only a minor component of phosphatidyl choline. The most remarkable difference in fatty acid composition was noted when the 24-carbon fatty acids are compared between the different phospholipid classes. They represented a minor component of all of the phospholipids, except for sphingomyelin, where it accounted for 30 to 50% of the total fatty acids in the phospholipid.

The same differences were not found when the fatty acid composition of platelets, another circulating component of the blood, was studied. As shown at the bottom of Table 6, there was a substantial difference between the fatty acid composition of both phosphatidyl ethanolamine and phosphatidyl choline for the different membranes. Only the oleic acid content of phosphatidyl choline appeared to be similar on a percentage basis. The biological significance of the differences in fatty acid composition between both the individual phospholipids of the red cell membrane and between the phospholipids of RBC's and platelets has yet to be determined. It would not be surprising to find that this distribution may ultimately have a major role in determining the relationship between the phospholipids and proteins of the RBC membranes.

Carbohydrate Composition

The carbohydrate composition of the erythrocyte membrane has been studied in several different manners. In Table 7 are listed the results obtained by several investigators. Bakerman and Wasemiller re-

TABLE 7

CARBOHYDRATE COMPOSITION OF HUMAN ERYTHROCYTE MEMBRANES

Reference		Weight Percentage of Whole Membrane							
		Total	Neutral Sugars			Hexosamines		Sialic Acid	Fucose
Bakerman and Wasemiller (33) (neutral sugars, hexosamines, sialic acids)		10.0							
Rosenberg and Guidotti (18)									
Ghosts		7.2		4.0		2.0		1.2	
Lipid-extracted Ghosts		7.5		2.5		2.6		2.4	
Poulik (34)									
Butanol supernate:	pH 2.0	7.3		2.8		2.3		2.0	0.2
	pH 6.0	11.7		4.3		2.7		4.5	0.2
Formic acid extracted stroma:	pH 2.0	7.1		3.5		2.1		1.5	0.1
	pH 6.0	7.3		3.7		1.7		1.6	0.1
			Galactose	Glucose	Mannose	Acetyl Galacto-samine	Acetyl Gluco-samine		
Winzler (35)		8.0	1.8	0.8	0.4	1.8	0.8	2.1	0.3

ported (33) that the total amount of neutral sugars, hexosamines, and sialic acids accounted for 10% of the whole membrane. A somewhat lower figure was obtained by Rosenberg and Guidotti (18), who studied both intact and lipid-extracted RBC ghosts. A figure of approximately 7% was obtained for each, although the distribution of the different types of sugars was quite different. Poulik (34), using a butanol extraction technique after pre-incubating the ghosts with formic acid at pH 2 or pH 6 to remove proteins, also obtained values of approximately 7% with a relatively stable distribution of the four major sugar groups for both pH values studied. A more complete study of the membrane carbohydrates was reported by Winzler (35) in 1969. Galactose, acetylgalactosamine, and sialic acid were the major sugar components, accounting for 70% of the total sugars.

More precise data on the carbohydrate content of the RBC membrane are difficult to obtain because they are covalently linked either to a lipid moiety or to a protein moiety. The only data which gave the distribution of carbohydrate between the lipid and protein moieties were those of Rosenberg and Guidotti (18), who compared carbohydrate composition for ghosts and lipid-extracted ghosts. In the intact ghosts, carbohydrates accounted for 7.2% of the membrane mass, including 4% as neutral sugars, 2% as hexosamines, and 1.2% as sialic acid. In the lipid-extracted ghosts they accounted for 7.5%, with 2.5% as neutral sugars, 2.6% as hexosamines, and 2.4% as sialic acid. Correcting the figures for an 8.7% loss of protein during the process of lipid extraction and assuming that the proteins lost do not have a carbohydrate composition different from that of the whole membrane, an estimate of the carbohydrate re-

maining bound to protein is possible. On this basis, 51.4% of the original ghost carbohydrate is still present in the lipid-extracted ghost. On an individual sugar basis, this represents 3.8% of the neutral sugars, 64% of the hexosamines, and 98.3% of the sialic acid from the intact ghosts. Consequently, an estimate can be made that approximately one-half of the carbohydrate is lost from the membrane during the process of lipid extraction. The carbohydrate-containing material which is removed (presumably glycolipids) contains approximately two-thirds of the neutral sugars and one-third of the hexosamines originally present in the membrane. This suggests a substantial difference in the distribution of individual sugars between the glycolipid and the glycoprotein.

The carbohydrate present in the glycolipid fraction has considerable biological significance because of its role in determining cellular antigenicity. All glycolipids of the RBC membrane are glycosphingolipids. In Table 8 the different types of glycosphingolipids are divided into neutral glycosphingolipids, the acidic glycosphingolipids, and the glycosphingolipids with blood group activity. As noted, neutral glycosphingolipids account for 70%, and the acidic and those containing the blood group activities for 30% of membrane glycolipids. It is not possible to quantitate these two latter groups. The gangliosides are made up of the ceramide hexosides which contain N-acetyl neuraminic acid. The glycosphingolipids with blood group activity are characterized only by a terminal sugar sequence, which gives them specific antigenic properties. In contrast to the glycolipids of nervous tissue, human erythrocyte membranes do not contain galactosyl ceramides, sulphatides, or more complex neutral glycosphingolipids.

TABLE 8

GLYCOLIPIDS OF THE HUMAN RBC MEMBRANE

	Structure	Concentration (μmoles/100 ml blood)	Designation	% of Total
Neutral Glycosphingolipids (Globosides)				
Glucosyl Ceramide	Glu-Ceramide	0.50	GL-1	70%
Lactosyl Ceramide	Gal(β1→4)Glu-Ceramide	1.43	GL-2	
Trihexosyl Ceramide	Gal(β1→4)Gal(β1→4)Glu-Ceramide	1.27	GL-3	
Tetrahexosyl Ceramide	NAcGal(β1→3)Gal(β1→4)Gal(β1→4)Glu-Ceramide	7.05	GL-4	
Acid Glycosphingolipids (Gangliosides) Contain sialic acid	-Sugar-Sugar-Ceramide | NAcNA			30%
Glycosphingolipids with Blood Group Activity Contain terminal sugar sequences which give specific antigenic properties	Sugar-Sugar-Sugar-Ceramide | Sugar			

Human erythrocyte membranes normally do not contain galactosyl ceramides, sulfatides, or more complex neutral glycosphingolipids.

An additional interesting feature about the composition of the erythrocyte glycolipids was that substantial variation occurred in the fatty acid composition of the various glycolipids. The distribution of fatty acids in glycolipids as well as the distribution of fatty acids in a single neutral glycosphingolipid from different human tissues are presented in Tables 9 and 10, respectively. The neutral glycolipids were found to have a distribution different from that of glycolipids which are associated with blood group activities, especially as to the content of C_{24} fatty acids. Since the variation between the different blood groups results from small changes at the nonreducing end of the carbohydrate chain, the glycolipids with A, B, and H activities had a similar fatty acid composition. A more interesting observation was the variation in the fatty acid composition of lactosyl ceramide from human red blood cells, leukocytes, and splenic tissue. The erythrocytes again had a very high percentage of C_{24}-fatty acids, whereas leukocytes and splenic tissue contained less C_{24}-fatty acid and more palmitic acid. The specific biological significance of these changes in fatty acid composition is not known, but may have some bearing on the process of cellular differentiation.

The other major source of carbohydrate in the RBC membrane is the carbohydrate which is covalently linked to proteins. Data of Rosenberg and Guidotti (18) indicated that slightly over 50% of the carbohydrate in the ghost membrane was attached to protein. Essentially all of the sialic acid, a large portion of the hexosamines, and a small portion of the hexoses were associated with the protein. The distribution and composition of glycoproteins are not well understood, but will

TABLE 9

FATTY ACID COMPOSITION OF HUMAN ERYTHROCYTE GLYCOLIPIDS[a]

	Human Globoside	Blood Groups		H and Le
		A	B	
16:0	6.0	3.8	5.1	4.6
16:1	0	4.4	3.9	4.2
18:0	2.0	1.2	1.2	1.5
18:1	0	2.2	1.4	2.0
20:0	1.0	11.4	11.6	9.8
21:0	0	4.2	2.2	2.0
22:0	12.0	9.2	12.8	10.5
22:1	0	16.2	16.7	14.5
23:0	3.0	0	0	0
24:0	35.0	19.3	19.5	20.5
24:1	40.0	17.9	18.1	16.1

[a]Sweeley and Dawson (19).

TABLE 10

FATTY ACID COMPOSITION OF LACTOSYL CERAMIDE FROM DIFFERENT HUMAN SOURCES[a]

	Human RBC's	Human Leukocytes	Human Spleen
16:0	6.0	27.4	46.0
18:0	4.0	3.9	3.9
18:1		5.6	0.1
20:0	2.0	0.6	4.4
22:0	14.0	4.3	13.0
22:1		1.1	
23:0			5.0
24:0	48.0	12.9	24.2
24:1	24.0	38.3	2.7
Other	2.0	5.9	0

[a]Sweeley and Dawson (19).

be considered at considerable length in the next section on protein composition.

Protein Composition

The proteins of the erythrocyte membrane are probably the most poorly understood of all of the components of the membrane. The protein content has been reported to vary from as low as 45% (36) to as high as 55% (33) of the total dry weight of the ghost membrane. In addition, the proteins of the erythrocyte membrane represent a very diverse group both with respect to composition as well as to function of individual members. The best known of the proteins probably are those which carry antigenic activity, such as blood group ABH or blood group MN activity, and are the type of proteins to which carbohydrates are attached. There are proteins which bind to the lipid of the membrane to form the lipid-protein structure which is common to all membranes. Still other proteins function to transport substances across the membrane and are probably enzymes which have a specific role as transport proteins. Other enzymes must also be present to supply the energy needed to maintain the integrity of the membrane. These would include glycolytic enzymes as well as enzymes which specifically metabolize agents capable of directly destroying the membranes. Finally, a group of proteins may exist whose sole purpose is to determine the structure of membranes. These structural proteins have quite controversial background, and a major question still exists as to their existence. This section will first consider the efforts which have been made to separate all of the proteins into different groups or types. A discussion of attempts to obtain RBC membrane proteins with specific chemical or biological properties will conclude

the review.

Preparation of Membranes for Study

The preparation of RBC membranes usually involves the simultaneous performance of two different operations. The first one is the separation of the mature red blood cells from circulating leukocytes and platelets, and the second one is the disruption of the RBC membranes to remove hemoglobin. This approach is possible because whole blood contains only a small number of other cell types (relative to the mass of red blood cells), and their removal can be combined with methods used to extract hemoglobin from the red cells. Many methods are available for the isolation of RBC membranes, but most represent modifications of a single basic process.

The classical method for the preparation of erythrocyte membranes for many years was the addition of distilled water to a suspension of red blood cells. This readily removed most of the hemoglobin from within the cells, and repeated washings produced an almost complete removal of hemoglobin. Careful chemical studies have not been carried out to demonstrate the major features of the end product of the process. However, the process has been used in the relatively recent studies of Post *et al.* (37) on erythrocyte adenosine triphosphatase activity and Green (38) on studies of the Rhesus antigens of the RBC.

In 1953, the general principles of the method most commonly used for the lysis of erythrocytes were described. Hillyer and Hoffman (39) proposed that a gradual hemolytic technique would produce the best possible end product. The process required repeated cell washes with solutions of progressively lower tonicity, starting at a concentration of

0.03 M sodium chloride, the level at which 100 per cent hemolysis occurred in normal human red cells. Repeated washings were then performed with solutions of reduced tonicity until the cellular membranes were devoid of hemoglobin. The authors carried out careful electron microscopic studies to define the physical state of the membrane and to describe the specific changes which resulted from the hemolytic procedure.

In 1956, Danon et al. (40) modified the procedure of Hillyer and Hoffman to produce a more gradual exposure to hypotonic solutions. The original method required that the hypotonic sodium chloride solutions be added directly to the blood cells, whereas the Danon method involved the addition of hypotonic solutions by dialysis. The gradual osmotic lysis produced a membrane with no major ruptures or alterations which might have resulted from the cells' subjection to sudden osmotic shock.

Dodge et al. (32) described a major modification in the technique of gradual osmotic lysis, including a very thorough chemical evaluation of the effects of pH and osmolarity of the hypotonic solution on removal of hemoglobin and non-hemoglobin protein from the erythrocytes. They found that buffer concentrations between 10 and 20 ideal milliosmolar (imOsm) and pH values of 5.8 to 8 resulted in maximum hemoglobin removal from the RBC's. They also demonstrated a maximum binding of hemoglobin to the membrane at a pH of 5.8. Conditions for optimal hemolysis in a single-stage procedure involved the use of phosphate buffers at an osmolarity of 20 imOsm and a pH of 7.4. Under these conditions there was an almost complete removal of hemoglobin with very little concomitant loss of lipid. The amount of non-hemoglobin nitrogen-containing material lost during the process was sizable, but no evaluation of

the material removed was made. This study resulted in a procedure which has become a standard method for the preparation of erythrocyte membranes by osmotic lysis.

Other major procedures for the preparation of erythrocyte membranes have received limited use in studies of erythrocyte membrane protein. The technique of Mazia and Ruby (41), which involved the use of Triton X-100, a non-ionic detergent, has been followed by a few workers. However, the method suffers severely from the fact that membranes can be completely solubilized by this detergent and the loss of lipid from the membrane has not been determined during the process of hemoglobin removal. The procedure of freezing and thawing red blood cells, which has been commonly used for the study of intracellular enzymes, has not been widely utilized in studies on erythrocyte membrane proteins because of the major physical changes which are produced by the process. A few methods have been described which are based on the addition of the chelating agent, ethylene-diamine-tetracetic acid (EDTA), to the buffer systems. However, all of the techniques involve the process of osmotic lysis, and no evaluation of the changes produced has been performed. Thus, at the present time, only the techniques involving the gradual osmotic lysis of the erythrocytes with simple hypotonic buffers have been widely studied and definitely determined to be satisfactory for the study of the RBC membranes.

Solubilization of Membrane Proteins

Once satisfactory RBC membranes have been prepared, the next step is to solubilize the proteins for further study. The major problem of membrane protein chemistry has been the lack of solubility of some,

if not most, of the membrane proteins. The difficulty arises because of the hydrophobic nature of proteins attached to lipids. In fact, there is a question even as to what constitutes solubility in an aqueous medium for a membrane protein which is normally associated with membrane lipid. In general, soluble membrane proteins have been defined as those proteins present in the clear supernate after centrifugation of membranes to which a solubilizing agent had been added. Many chemical agents have been used to solubilize membrane proteins with varying degrees of success. The types of agents and the difficulties involved in their use will now be discussed.

Organic solvents were probably the first agents to be widely used for the purpose of solubilizing membrane proteins. Maddy (42) used a mixture of water and butanol which resulted in separation of soluble proteins into the aqueous phase and lipids into the butanol phase. Excellent solubilization was achieved with ox erythrocytes, although the same system has not been as effective in the study of human erythrocytes. Blumenfeld (36) used 33% pyridine in water to solubilize 35 to 40% of the membrane protein essentially free of phospholipid. Winzler (35) has used a mixture of phenol and water (1:1) to extract a mixture of glycoproteins and proteins, from which further purification of the glycoprotein was possible. Zahler and Wallach (43) have had extensive experience with the use of a mixture of 2-chloroethanol and water for solubilizing membranes. They have achieved essentially complete solubilization of all membrane protein, although further purification of solubilized proteins has been difficult.

The so-called dissociating agents have been widely used in the

study of soluble proteins to separate the proteins into their individual peptides. Both guanidinium hydrochloride (at concentrations up to 6 molar) and urea (at concentrations up to 8 molar) have also been extensively studied for the purpose of solubilizing RBC membrane proteins. Results obtained by different workers have been quite variable with the use of these agents, even when used at the same concentration. No explanation for the variation in results is available at the present time.

The most widely used agents at the present time are the detergents. Anionic, cationic, nonionic, and bile salt detergents have all been used for the purpose of solubilizing membrane proteins. The most effective detergent based on its effectiveness in the hands of many different investigators has been the anionic detergent, sodium dodecyl sulphate (SDS). It has been used for the complete solubilization of RBC membrane protein and as an agent in the analysis of the peptides. The nonionic detergent, Triton X-100, has also been widely used, but the data have been somewhat confused by its use in systems for the preparation of ghosts. The bile salt, sodium deoxycholate, has been frequently evaluated as a solubilizing agent, but has also produced quite variable results from one investigator to another. However, it has not been as widely studied as the other detergents, and further studies are necessary to establish its real value. Cationic detergents have not been effective.

Many aqueous systems, including double distilled water, have been used to obtain soluble proteins. Marchesi and Steers (44) utilized such a system to dialyze ghost suspensions against a dilute solution of adenosine triphosphate and β-mercaptoethanol. The method was further

modified (45) by switching to dilute solutions of sodium EDTA, which have also been used in a large number of studies to obtain at least part of the soluble membrane proteins. Acids, especially acetic acid and formic acid, have been employed for the same purpose, although varying degrees of solubility have been obtained. Hypertonic salt solutions have been used by some investigators (46), both alone and as part of a series of steps in the solubilization procedure.

A limited number of studies have been carried out for the purpose of comparing the effectiveness of various agents in solubilizing the RBC membrane proteins. Bakerman and Wasemiller (33), using RBC ghost suspensions as substrate, evaluated several agents. Approximately 90% or more of the protein could be solubilized with sodium dodecyl sulphate, Kryo EO (a non-ionic detergent), and 6 M urea (pH 11.0). They found that 75 to 85% of the protein was soluble in 66% acetic acid or in sodium hydroxide solution (pH 13.0). Sodium deoxycholate (1.0 M), sodium sulfide (1.0 M), and 1.5 M guanidinium hydrochloride could dissolve 45 to 75% of the protein.

In a slightly different study, using lyophilized lipid-extracted membrane protein, Rosenberg and Guidotti (18) attempted to solubilize the protein using varying agents. Little effect could be obtained with guanidinium hydrochloride, urea, glacial acetic acid or Triton X-100. About 97% solubility was obtained in 88% formic acid; approximately 98% solubility was obtained in 1% SDS; and 100% solubilization was obtained by succinylation of the membrane protein.

In summary, the problem of solubilizing the RBC membrane protein is a complex one. Many agents have been both proposed and used on the

basis of careful evaluation. The most complete solubilization would appear to be obtained with detergents, especially sodium dodecyl sulphate. The dissociating agents and organic solvents may also be very useful for this purpose, although the final physical state of the protein after such treatment has not been determined. A problem with the use of the dissociating agents and organic solvents is that the extraction procedure may be selective for specific groups or families of the proteins. The aqueous systems appear to be able to solubilize only part of the protein present in the membrane, depending upon the system chosen. The data on solubilization indicate that methods are available for a complete solubilization of all of the proteins, although the mechanism is yet to be determined. Partial solubilizations, even on a selective basis, are also available for the study of membrane proteins. A better understanding of the protein composition of the RBC membranes requires a better understanding of both these processes.

Investigations Pertinent to All RBC Membrane Proteins

The characterization of red cell membrane proteins is a relatively new undertaking, primarily because of the difficulties encountered in solubilization of the proteins. The first major step toward characterization was made in 1966 by Maddy (42) when he described a method for the solubilization of membrane proteins of the ox erythrocyte. By using a butanol-water fractionation procedure, he isolated from the aqueous phase a protein with an estimated molecular weight of 300,000. He demonstrated that the preparation consisted of particles of different sizes, probably as aggregates of a smaller unit. Ultracentrifugation separated

the protein into two fractions with sedimentation coefficients of 5S and 10S. By a variety of techniques, 90% of the protein was found to be a sialoprotein. Only a trace of lipid was present, and carbohydrates accounted for about 8% of the protein fraction.

In 1967, Bakerman and Wasemiller (33) made a major contribution toward the understanding of membrane proteins. Using a hypotonic phosphate buffer system for membrane preparation, they analyzed the ghosts and demonstrated that they were a lipoglycoprotein containing 55% protein, 35% lipid, and 10% carbohydrate. Solubilization of the membrane was almost complete in anionic detergents, nonionic detergents, and urea. Using column chromatography on polyacrylamide gels, membranes dissolved in SDS were studied using a sequence of gel particles from P-30 through P-300. With the P-300 gel, 98% of the SDS solubilized membrane was retarded on the column. The SDS solubilized membranes were separated by column chromatography into two principal molecular weight classes which had different amino acid distributions and different equilibrium characteristics. When the membranes were dissolved in other agents, the gels were less effective in separating the membrane proteins. The authors concluded on the basis of sedimentation equilibrium experiments that the smallest molecular weight class isolated on gel filtration columns had a molecular weight of 40,400 with the protein portion having a molecular weight of 22,200. They suggested that the two molecular weight classes represented the repeating units of erythrocyte structural membrane.

Rega et al. (47) in 1967 extended the observations of Maddy to human erythrocyte ghosts and were able to solubilize 83% of the membrane proteins in the butanol-saturated water phase. Following a single ex-

traction, only 5% of the solubilized material was lipid and approximately 9% was carbohydrate. The protein was determined to be glycoprotein containing hexose, hexosamine, glucose, and sialic acid. Almost 100% of the protein had a single electrophoretic mobility on agarose gel electrophoresis. Only a single peak was found with Sephadex G-100 column chromatography, although ultracentrifugation studies demonstrated that the protein was heterogeneous. Little effect of solubilization was noted upon the content of sulfhydryl groups. Although demonstrable in the intact ghost, both cation-dependent nucleoside triphosphatase and acid phosphohydrolase activities were destroyed in the process of solubilization. The results indicated that the technique was effective for solubilizing most of the membrane proteins, but was not adequate for further study of the peptides.

The utilization of another organic solvent was reported in 1968 by Zahler (48). Using a 2-chloromethanol water mixture at a pH between 2 and 3, he completely dissolved the membrane proteins of the erythrocytes. The membrane solutions were then partitioned by molecular sieving on Sephadex LH-20, completely separating the membrane proteins from the lipids. The solubilized protein was noted to be heterogeneous by polyacrylamide gel electrophoresis, although an effort was made to explain the heterogeneity on the basis of aggregation. The blood group A substance was still present in the protein, but the Rh activity had been destroyed. The acetylcholinasterase, as well as the acid and alkaline phosphatase activities were destroyed in the process. The author suggested that his technique might be helpful in the study of the heterogeneity of isolated membrane proteins, but would not be satisfactory for

studying biological membrane function.

In 1968, Poulik (34) summarized his results with modifications of the butanol solubilization techniques. He compared the effects of three different conditions: 1) in the absence of urea, 2) in the presence of urea, and 3) after pre-treatment with formic acid. Solubilization under all three conditions was quite variable, ranging from 20% to 55%, with an overall average of about 25%. The proteins in the water phase were nearly free of lipid, contained a large amount of carbohydrate, and retained A, M, and N serological activities. As previously noted, the Rh antigen was destroyed during the procedure. The water phase (pH 2) was further separated by gel filtration on Sephadex G-100 into an excluded fraction (approximate molecular weight 200,000) and a retarded fraction (approximate molecular weight 50,000). The two fractions differed in both carbohydrate content and serological activity. Urea starch gel electrophoresis demonstrated that the water phase and the two subfractions were both quite heterogeneous. The data suggest that the proteins extracted by butanol, even under varying conditions, are a mixture of proteins, which did not include all of the major membrane proteins. The authors were not able to reproduce the 83% solubilization previously reported by Rega et al. (47).

Also in 1968, Rosenberg and Guidotti (18) published the first of two major studies on the solubilization, fractionation, and partial characterization of RBC membrane proteins. The authors used exhaustive lipid extraction with ethanol-ether to remove essentially all of the lipid before studying the lipid-extracted membrane proteins. The data on the basis of end-group analysis, electrophoresis, ultracentrifugation,

and gel chromatography in various solvent systems demonstrated heterogeneity of membrane proteins. Most of the molecular weights were in the range of 50,000, although further fractionation was not performed. They suggested that the preparation technique and the solvent systems developed would be valuable for further studies on the individual protein molecules in the red cell membrane.

In the second paper, Rosenberg and Guidotti (49) in 1969 reported their efforts to fractionate and partially characterize all of the membrane proteins of red cells. Their approach was to progressively extract the membrane with a series of solvents before a final solubilization procedure. The RBC ghosts were sequentially extracted with: 1) 1.0 mM sodium EDTA and 50 mM β-mercaptoethanol, 2) 0.8 M sodium chloride, and 3) a mixture of ethanol-ether (3:1, v/v), followed by repeated washings with ether. This removed 11%, 41% and 7%, respectively, of the total erythrocyte membrane protein, for a total extraction of 59% of the protein. The remaining protein residue was solubilized in 3% SDS and chromatographed on Sephadex G-200, using unbuffered 1% SDS for elution. The protein residue was separated into 5 major fractions, representing the other 41% of the total protein. The composition of the eight fractions (Fractions I-III from the extractions and Fractions IV-VIII from Sephadex G-200 chromatography), was then further studied. Four of the protein fractions (I, III, VI, VII) were found to contain large amounts of sialic acid. Several fractions (IV, V, VI) contained more of the non-polar amino acids than the other membrane proteins. Studies with polyacrylamide gel electrophoresis and N-terminal amino acid analysis showed that there were at least 12 different membrane proteins present

in significant amounts, with molecular weights ranging from about 10,000 to 150,000. The authors were able to account for all of the membrane proteins. Results of this study demonstrated clearly the heterogeneity of the membrane proteins on the basis of molecular size and composition of the several fractions.

In 1970, Lenard published two papers on the protein components of the erythrocyte membrane. The first (50) was on human erythrocyte membranes employing a technique by which the proteins were completely solubilized in 1% SDS solution and separated by polyacrylamide gel electrophoresis in the presence of SDS. The entire membrane was used for electrophoresis without any prior extraction as performed by Rosenberg and Guidotti (49). Lenard identified 14 different molecular weight classes which could not be further fractionated by treatment with detergents, alkali, or urea. This suggested that many of the different proteins in the membrane were not aggregates formed from smaller units by non-covalent binding. Four intensely stained bands were specified as the major protein components of the RBC membrane and accounted for 60-65% of the total protein membrane. The molecular weights of the four proteins as determined by polyacrylamide gel electrophoresis were about 255,000, 240,000, 108,000 and 86,000. Staining the gels for carbohydrate revealed that only the 108,000 molecular weight component contained significant amounts of carbohydrate, although additional bands were found to contain small amounts of carbohydrates. In the second paper in 1970, Lenard (51) studied the RBC membrane proteins from pig, sheep, rats and dogs in addition to those of man. Eight major bands with a molecular weights ranging from 22,000 to 255,000 were found to be present in all

of the species examined. The 108,000 molecular weight protein, which had been shown to be glycoprotein in human membranes, was found to be present in all of the species studied. Identification of sulfhydryl groups with radioactively-labelled N-ethylmaleimide showed that the two very large protein bands contained about half of the radioactivity. These two and other sulphydryl-containing proteins were quantitatively and qualitatively similar in all species. The author concluded that two-thirds of the membrane proteins from the different species studied were very similar to one another.

In 1971, Fairbanks et al. (52) presented their findings on the electrophoretic analysis of RBC membrane proteins. Using polyacrylamide gel electrophoresis in 1% SDS, six major protein bands (I-VI) were found to account for two-thirds of the membrane protein. Component III (molecular weight 89,000) was the dominant protein and constituted 30% of the total protein. Components I and II (molecular weights around 250,000) made up 25% of the total protein. The molar amounts of I plus II, IV (molecular weight 77,500), V (molecular weight 41,300), and VI (molecular weight 36,200) were similar, with a range of 3.4 to 4.6 x 10^5 chains per ghost. Identification of the carbohydrate-containing bands was performed by the use of periodic acid-Schiff (PAS) reagent. The most rapidly migrating PAS-positive zone corresponded to the membrane lipids, but the other three bands of lower mobility were sialoglycoproteins. The major glycoprotein had an approximate molecular weight of 83,500 and contained at least 57% of the sialic acid. The PAS-positive bands were not stained by protein stains and had altered mobilities after treatment with sialidase. Attempts to further reduce the large proteins with a series of

denaturing agents was unproductive. No polypeptides with a molecular weight less than 15,000 could be demonstrated in the gels. A major alteration in the bands distribution could be produced by heating ghosts in solutions containing low levels of SDS and high levels of salts. The pattern of the bands became more diffuse with a lower average molecular weight. The change was assumed to be due to the activity of proteinases. Selective solubilization of some of the major components was possible by use of different agents. Components I, II, and V were extracted by incubating the ghosts at low ionic strength. Component VI could be removed by washing with buffered saline in concentrations above 0.1 M. The removal was rapid, complete, and selective. In addition, the patterns produced in the electropherograms were complementary when the released and retained materials were compared. The material removed contained a minimal amount of sialic acid and no PAS-positive lipids. The authors concluded that there were two classes of membrane proteins present in the erythrocyte membrane. Components I, II, V and VI composed one major class. This class made up 30 to 35% of the membrane protein and was very weakly bound to the membrane, probably by ionic bonds. The second class, which included components III, IV, and glycoproteins, along with various minor components, constituted 65 to 70% of the protein. The second class of proteins was tightly bound to the membrane, reflecting their involvement in hydrophobic protein-protein and protein-lipid interactions.

Steck et al. (53) studied the orientation of membrane proteins by treating both normal and inside-out vesicles (i.e., membranes treated to invert the inner membrane surface to the outside) with proteolytic

enzymes which are not capable of penetrating into the vesicle's interior. Inside-out vesicles were found to be less susceptible to proteolysis than ghosts and normal vesicles. All major bands stained with Coomassie Blue were digested, except for one protein exposed at the outer membrane surface. One protein was attacked only by digestion of inside-out vesicles. The three glycoproteins which were detected only by carbohydrate staining were digested by proteolytic attack at either surface. The authors concluded that the membrane was highly asymmetric as to protein orientation, with some of the protein spanning the thickness of the membrane.

Confirmation of many of the previous studies was presented in 1972 by Kobylka et al. (54). They studied the proteins and glycoproteins of the erythrocyte membrane by polyacrylamide gel electrophoresis after using three different buffer systems for hypotonic hemolysis. Although major variations in membrane morphology were produced by each of these system, no major variation was found in patterns of the major membrane proteins. Several species were studied and all showed a similar pattern consisting of nine common bands. The only significant differences which could be demonstrated in the protein patterns were attributed to proteolytic digestion of the membranes during preparation. White blood cells in the membrane preparations were thought to account for the problem of proteolytic digestion. The glycoproteins were analyzed both by polyacrylamide gel electrophoresis and by column chromatography. Each species was found to have a different major glycoprotein or group of glycoproteins. A problem was noted with the use of polyacrylamide gel electrophoresis for molecular weight determination of glycoproteins. Sub-

stantial variations in molecular weight values were produced by changes in gel concentration. Values obtained by polyacrylamide gel electrophoresis did not correspond to those obtained by gel filtration.

Investigations Pertinent to Selected RBC Membrane Proteins

Spectrin

In 1968, Marchesi and Steers (44) described a protein extraction method which involved the dialysis of guinea pig erythrocyte ghosts against a solution of adenosine triphosphate and 2-mercaptoethanol. The technique removed approximately 20% of the membrane-bound protein. This protein produced a single major band on polyacrylamide gel and a single boundary in free boundary electrophoresis. It could be polymerized in the presence of divalent cations to form coiled filaments visible by electron microscopy. Antibodies to the protein reacted specifically with red blood cells or their ghosts, but did not react with serum, erythrocyte cytoplasm, or other blood cells. The authors thought that the functional role of the protein was not known, but appeared to be involved in maintaining the structure of red cell membrane. They suggested that the protein be called "spectrin", since it was obtained from membrane ghosts.

In 1970 a further extraction and purification with partial characterization was reported by Marchesi *et al.* (45). They found that the protein could be solubilized from the membranes by low ionic strength aqueous solutions containing sodium EDTA. Further purification was achieved by gel filtration and confirmed by polyacrylamide gel electrophoresis. Depending upon the medium in which the protein was dissolved,

either one or two major species could be found. Using equilibrium ultracentrifugation and polyacrylamide gel electrophoresis, the molecular weight of the monomeric unit was estimated to be 140,000. Cyanogen bromide cleavage was consistent with monomeric units of 140,000 to 150,000.

In another report from the same group, Tillack et al. (55) demonstrated that the protein could be solubilized not only from human ghosts but also from guinea pig, horse, sheep, and rabbit erythrocyte ghosts. It constituted approximately 20% of the total membrane protein in all species and was free of both carbohydrate and lipid. Preparations from each species were quite similar when studied by gel filtration, polyacrylamide gel electrophoresis, and immunoprecipitin reactions. Amino acid compositions were nearly identical, and antisera to both guinea pig and human spectrin cross reacted with all the other species. However, cyanogen bromide cleavage of human, horse, and sheep spectrin showed that the peptides produced were not the same.

In 1971, Juliano et al. (56) reported that a protein, which they had previously designated as P-II, was essentially the same protein as spectrin. The protein had a molecular weight around 150,000 and was bound to the membrane through ionic interactions, which could be disturbed by alterations of pH, ionic strength, or divalent cation concentrations. On SDS polyacrylamide gels a slow running doublet accounted for 80-90% of the protein fraction. He noted that the release of P-II resulted in fragmentation of the membrane. As performed in the study of Marchesi et al. (45), the membrane proteins were extracted by a single exposure to a chelating agent solution of low ionic strength.

These results, in conjunction with data of workers attempting

to characterize all of the membrane proteins, strongly support the concept that there are two major proteins of high molecular weight which can be extracted by chelating agents of low ionic strength. These proteins appear to have a structural role in the RBC membrane, although definitive proof does not exist at the present time.

Glycoproteins

Several investigators have been interested in the extraction and purification of glycoproteins from the RBC membrane. Blumenfeld (36) in 1968 described a method by which membrane ghosts were extracted with an aqueous pyridine solution. She concluded that the solubilization procedure resulted in total extraction of the sialoprotein present in the membrane. It contained 35-40% of the total protein, essentially no lipid, and all of the sialic acid. Only a portion of the hexoses and hexosamines were removed. The author postulated that there were only two types of proteins in the erythrocyte membrane. One type was water soluble and contained all of the sialic acid, but essentially no lipid. The other was insoluble in water, free of sialic acid, and associated with the lipids of the membrane.

In 1970, Blumenfeld et al. (57) further expanded their work on pyridine extraction of RBC membranes. The two previously mentioned protein types were found to show multiple bands on polyacrylamide gel electrophoresis and had similar band patterns for both of the protein fractions and for intact ghost membranes. Only the sialoglycoprotein, containing the virus receptor activity, was present uniquely in the water soluble fraction. In addition, they noted that the water soluble proteins had a very strong tendency for aggregation, especially in the pres-

ence of certain salts. The aggregates could be reduced by SDS gel electrophoresis into about 20 bands. These data demonstrated clearly that the product of the extraction was a heterogeneous group of proteins and that the extraction was not selective.

Winzler (35) in 1969 summarized his extensive studies on the use of a mixture of phenol-water (1:1, v/v) for the extraction of glycoproteins from the RBC membrane. The glycoprotein contained most of the sialic acid as well as about half of the hexose and hexosamine content of the erythrocyte. The glycoprotein combined with influenza viruses to cause hemagglutination and carried the M and N specific antigens. Chemical assays showed that the glycoprotein was composed of 37.5% as amino acids, 1.0% as lipid, and 64.5% as carbohydrate. Trypsin treatment of the glycoprotein produced a glycopeptide containing the M, N, and MN activity corresponding to the blood group of the donor erythrocytes. The glycopeptide obtained by the trypsin treatment contained 20.7% as amino acids and 78.6% as carbohydrate, with no lipid present. The insoluble residue after trypsin treatment of the stromal glycoprotein contained 95.0% amino acids, 1% lipid and 3.4% carbohydrate. Major subsequent interest has been on the carbohydrate moiety of the sialoglycopeptide, and its relationship to the antigenic activity of the cell.

The study by Zvilichovsky *et al.* (58) clarified the relationship between pyridine and phenol extraction procedures. By adding an ethanol fractionation step to the aqueous pyridine solubilization procedure, the authors isolated a preparation which contained glycoproteins. The amino acid and carbohydrate composition of this preparation resembled that obtained by extraction with phenol. In addition, the antigens pres-

ent were also similar to those obtained with phenol extraction. It was suggested that the proteins described by the two groups were essentially the same protein, with varying degrees of purification.

Two publications in 1971 produced information about the location of the glycoprotein in the membrane. Phillips and Morrison (59), using an enzyme, lactoperoxidase, to catalyze the iodination of proteins of intact membranes, were able to demonstrate that a glycoprotein with an approximate molecular weight of 60,000 was labelled prior to the procedure and occupied an exposed position on the membrane. It was the major glycoprotein in the erythrocyte membrane and appeared to be the same glycoprotein which had been studied by a number of investigators. Two minor glycoproteins which were also demonstrated did not appear to be exposed on the surface of the cell. Bretscher (60) used a radioactive labelling reagent, which could not penetrate the erythrocyte membrane, to demonstrate that the glycoprotein extended through the membrane and was exposed on both surfaces. The carbohydrate-containing portion was found to be on the external surface while the other portion resided on the inner surface of the membrane. The glycoprotein had a molecular weight of about 31,400 and was about one-third protein.

The most definitive characterization of a glycoprotein from the RBC membrane was reported in 1972 by Marchesi et al. (61). They isolated the major glycoprotein from human erythrocyte membranes by treatment of RBC membranes with lithium diiodosalicylate. The protein was a single polypeptide chain with a molecular weight of about 50,000 and contained approximately 60% carbohydrate and 40% protein. In addition, the glycoprotein carried multiple blood group antigens, the receptors for influ-

enza viruses, and receptors for various plant agglutinins. Tryptic digestion of the glycoprotein demonstrated four unique carbohydrate-containing peptides. Their sequence in the molecule was determined by tryptic digestion of intact erythrocyte membranes and of partially digested glycoprotein fragments. Cleavage with cyanogen bromide produced five fragments, two of which contained most of the carbohydrate in the molecule and were derived from the N-terminal half of the polypeptide chain. The nonpolar amino acids of the glycoproteins were located predominantly in the C-terminal fragment.

Blood Group Antigens

Many workers have studied the blood group antigens of the red cell membrane. An unequivocal location of all major red cell antigens has not yet been determined. Certain blood group antigens, however, have been studied to the point where considerable understanding of the role of protein in blood group activity can be evaluated. Green (38) in 1965 studied the Rh antigen starting with a preparation of lyophilized cell ghosts. He found that a protein or peptide was the crucial part of the antigen, although whether the protein was a glycopeptide, glycoprotein, or lipoprotein could not be determined. One or more disulfide bonds and one or more free sulphydryl groups were necessary for antigen activity. In 1967, he (62) expanded his studies using twice lyophilized human RBC membranes which had been solubilized and disaggregated with SDS. The detergent was then completely removed by ion-exchange column chromatography. Gel filtration of the solubilized protein in the presence of the detergent showed multiple peaks, indicating a heterogeneous group of molecules. When the detergent was removed, only a single peak

was found at the void volume. The material remaining after detergent removal contained ABH and MN antigens appropriate for the starting material. However, the Rh antigenic activity was no longer present.

In 1968, Green (63) again contributed to the knowledge of the Rh antigen by studying the effects of extraction of RBC membranes with 100% n-butanol. The extraction procedure destroyed all of the Rh antigenic activity. However, the addition of either butanol extract of membrane or chloroform-methanol extract of human plasma could regenerate the activity. Using chromatography with silicic acid, DEAE cellulose, and thin layer silica gel as well as snake venom digestion, the active component was found to be phosphatidyl choline. He concluded that the presence of lecithin was necessary for the expression of Rh antigenic activity and that in addition the lecithin must contain unsaturated fatty acids.

No other major worker has demonstrated data contrary to those of Green, and so it must be concluded that the Rh antigen does involve the proteins of the RBC membrane. In addition, a lipid, especially phosphatidyl choline, must be present with the protein in order for the activity to be regenerated. Further understanding of the role of configuration in membrane structure will probably be necessary before the Rh antigen and its associated proteins can be adequately evaluated.

The studies on blood group ABH substances have been equally as complex. As already discussed, some of the ABH activity occurs in glycolipids. However, it has been only recently that glycoproteins have been clearly identified as a source of such antigenic activity. Whittemore *et al.* (64) in 1969 presented the first evidence that A, B, and H blood

group activities were not limited to glycolipids, but could be found in human erythrocyte membrane glycoprotein. After butanol extraction of stroma, over 80% of the protein was soluble in the aqueous phase. A repeated butanol extraction of the aqueous phase removed all detectable lipid. The solubilized glycoprotein was found to possess A, B, and H blood group activity at a level comparable to intact ghosts at the same protein concentration.

Fiori et al. (65) in 1971 solubilized erythrocyte stroma with the nonionic detergent, Triton X-100, and then fractionated the solubilized material on both Sephadex G-200 and G-100. The blood group specific activity appeared in three separate fractions. Although one of the fractions was clearly a glycolipid, the other two fractions were glycoprotein in nature and were similar to glycoproteins from salivary secretions. Their data showed that previous workers were not able to obtain the blood group specific glycoproteins because they were precipitated by ethanol, which had been used to isolate the active substances.

An interesting aspect of the nature of the blood group specific activity in glycoproteins was reported in 1971 by Gardas and Koscielak (66). They found that blood group A, B, and H activities were present in the RBC glycolipid fractions, independent of the salivary secretor status of the blood donor. However, blood group activity could be found only in the RBC glycoprotein fractions obtained from salivary secretors of blood group activity. Glycoprotein extracts of stroma from nonsecretors did not contain A and B specificity. This study, along with the previous studies, clearly demonstrates that ABH blood group activity is associated with the glycoproteins of the erythrocyte membrane. However,

the association of ABH activity with glycoproteins may be dependent upon the secretor status of the individual from whom the blood is obtained.

Enzymes

Many of the proteins from human erythrocyte membranes have been shown to have enzymatic activity. In 1966, Firkin and Wiley (67) summarized the enzymatic activities which had been reported to be associated with the erythrocyte stroma, and their list of different activities is given in Table 11. Many of the enzymes included in the list are probably not an integral part of the membrane, but rather are loosely bound to the membrane surface. In this review only those enzymes which have been clearly demonstrated to be closely associated with the membrane will be discussed.

Adenosine triphosphatase. One of the major functions of the human erythrocyte membrane is to maintain a high potassium, low sodium intracellular concentration as opposed to the high sodium, low potassium concentration of the plasma. Maintenance of the gradient requires the active transport of sodium and potassium across the membrane. Post et al. (37) in 1960 presented very strong evidence that this active transport function was related to the enzyme, adenosine triphosphatase (ATPase). Analyzing human erythrocyte membranes prepared by disruption with distilled water, they showed that the enzyme (ATPase) and active transport shared an unusual group of properties: (a) location in the membrane, (b) utilization of adenosine triphosphate, rather than inosine triphosphate, (c) a requirement for the presence of both sodium and potassium ions, (d) inhibition by ouabain, (e) the substitution of ammonium ions for potassium ions, but not for sodium ions, (f) a requirement for

TABLE 11

ENZYMES ASSOCIATED WITH THE ERYTHROCYTE MEMBRANE[a]

1. Adenosine triphosphatase	11. Monacyl phosphatide acylase
2. Adenylic acid deaminase	12. Myokinase
3. Acetylcholinesterase	13. Nucleoside phosphorylase
4. Adenyl cyclase (pigeon erythrocytes)	14. Phosphoribose isomerase
5. Aldolase	15. Phosphoglycerate kinase
6. Carboxylesterase (ali-esterase)	16. Phosphoketopentose epimerase
7. Diglyceride kinase	17. Phosphatidic acid phosphatase
8. DPNH-cytochrome c reductase	18. Pyrophosphatase
9. DPN- and TPN-ase	19. Transketolase
10. Glyceraldehyde phosphate dehydrogenase	20. Peptidase
	21. Proteinase

[a]Firkin and Wiley (67).

the same concentrations of sodium ions, potassium ions, ouabain, and ammonium ions for half maximal activity.

Hokin and Reasa (68) in 1964 were in general agreement with these findings, but found that sodium or potassium alone could have effects on ATPase activity which were not related to sodium or potassium transport. They suggested that all of the ATPase activity was not involved in the same process.

Marchesi and Palade (69) in 1967 located the sites of activity of ATPase in the red cell ghost membrane. They modifed a lead salt method to demonstrate chemically the sites of ATPase activity by lowering the concentration of lead for incubation with unfixed red cell ghosts. The sites of chemical reactivity were localized exclusively along the inner surface of the ghost membrane for both magnesium ATPase and sodium-potassium ATPase. The findings indicated that the ATPase catalyzed the release of inorganic phosphate on the inside of the ghost membrane. No reaction product could be found deposited on the outer surface of the ghost membrane. There was no difference in distribution between reaction products for magnesium ATPase and those of sodium-potassium ATPase.

Dunham and Hoffman (70) in 1970 attempted to isolate the sodium-potassium ATPase from RBC membranes. They incubated ghosts with tritiated ouabain in the presence of ATP, magnesium, and sodium, which are required for ouabain to bind to the sodium-potassium transport sites with the highest specificity. The labelled membranes were then solubilized with SDS, leaving most of the tritiated ouabain bound to a solubilized component. Solubilized sodium-potassium ATPase could be obtained after removal of SDS by dialysis. The ouabain-membrane complex and

sodium-potassium ATPase appeared to be identical and were purified about 8-fold relative to the starting material. This represented a major step toward the isolation of the cation transport system in red cell membranes.

In 1970, Rosenthal et al. (71) attempted to isolate the ATPase activity from RBC membranes. By extracting the membrane with a dilute EDTA solution, they obtained a group of proteins with a fibrillar appearance. Within that group of proteins, they found a ouabain-insensitive ATPase activity. The activity was calcium-dependent and inhibited in the presence of magnesium ions. GTP failed to serve as substrate for the enzyme. By studying the erythrocyte membrane before solubilization, they observed that a system of fibrils similar to the isolated fibers was present on the inner surface of the membrane. This suggested that the calcium-activated, magnesium-inhibited ATPase and the associated fibrils might be involved in the maintenance of erythrocyte deformability. It was clear from their studies that the ouabain-insensitive ATPase was not an integral part of the membrane.

Heller and Hanahan (72) studied enzyme activities in human, bovine, and porcine erythrocytes. Treatment of the human erythrocyte membrane with high concentrations of sodium iodide in the presence of ATP solubilized part of the ouabain-insensitive ATPase, but would not remove the sodium-potassium activated ATPase. Their data support the concept that sodium-potassium ATPase was not extractable from the membrane in aqueous systems as was the calcium-activated ATPase. Also, in 1972, Hanahan and Ekholm (73) presented more data on changes in ATPase activity in human erythrocytes during osmotic lysis. They found that with each washing step in the lytic procedure there was a decrease in

total ATPase activity and an increase in sodium-potassium ATPase activity. These changes were not modified by "freeze-thaw" treatment of the membrane. They postulated that this represented removal of one type of activity while the membrane is opened up to make more sodium-potassium ATPase activity available. Thus the erythrocyte membrane obtained by osmotic lysis must be considered a first derivative of the intact erythrocyte membrane.

Acetylcholinesterase. The presence of acetylcholinesterase activity in both red blood cells and plasma has been known for many years. In 1949, Michel (74) described a method for the quantitation of the enzyme activity in red blood cells based on its ability to produce a reduction in pH. The pH change resulted from the release of acetic acid during the hydrolysis of acetylcholine. In 1966, Mitchell and Hanahan (46) used hypertonic sodium chloride solutions to partially solubilize the erythrocyte membrane. Ultracentrifugation of the solubilized material in sodium bromide solutions (density = 1.21 g/ml) yielded two protein fractions. One of the fractions floated in this density medium and contained all of the lipid from the red cell membrane, whereas the other fraction sedimented and was free from lipid. This suggested that the enzyme activity was associated with the lipid moiety of the membrane and could not be separated from the lipid without loss of activity. Bellhorn et al. (75) in 1972 studied the enzyme further using tritiated diisopropylfluorophosphate (DIFP) to selectively label the enzyme. The labelled membrane protein was then solubilized in SDS for separation by polyacrylamide gel electrophoresis. The authors demonstrated that the acetylcholinesterase activity could be selectively labelled and visual-

ized as a distinct band in the gel. Their evidence indicated that the enzyme existed as a dimer of about 180,000 molecular weight and could be broken down into two 90,000 molecular weight components in the presence of β-mercaptomethanol.

In 1972, Heller and Hanahan (76) found that activation of the enzyme by sodium and calcium ions was independent of the acetylcholine concentration, but that the optimum concentration of magnesium was dependent upon substrate concentration. Solubilization of 10-45% of the erythrocyte membrane protein by such aqueous solvents as sodium chloride, calcium chloride, sucrose, or EDTA-β-mercaptoethanol resulted in only a 10-20% loss of acetylcholinesterase activity in the ghost preparation. The detergents, Triton X-100 and SDS, were both found to partially solubilize the membrane. The enzyme activity was irreversibly lost with SDS but some enzyme activity still remained when the membrane was solubilized with Triton X-100. The changes produced by these agents may be the result of alterations in conformation of the enzyme protein.

Miscellaneous enzymes. In 1969, Zamudio et al. (77) studied the relationship between membrane structure and the enzyme activity, NADH:(acceptor) oxidoreducatase, of erythrocyte ghosts. They evaluated the effects of hypotonic treatment, sonication, temperature, and divalent cations upon both intact and fragmented ghosts. Procedures affecting the membrane structure altered the rate of the enzyme reaction. The enzyme was completely inactivated by SDS, but was not affected by the phospholipases A and C. Treatment of freeze-dried membranes with anhydrous organic solvents did not affect the enzyme activity. The authors concluded that NADH:(acceptor) oxidoreductase of red blood cell ghosts was

a structural enzyme of the membrane.

Also in 1969, Nilsson and Ronquist (78) used a density gradient centrifugation technique to separate components of the RBC ghost. The main component obtained was found to have glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase activities. The authors concluded that the enzymes were associated with the erythrocyte membrane. Results of a study by Tanner and Gray (79) in 1971, however, do not agree with the preceding authors' conclusion. They isolated a protein from ghosts by extraction with EDTA solution after identifying the band on polyacrylamide gel electrophoresis. The molecular weight of the polypeptide was estimated to be 33,000 and the protein accounted for approximately 5% of the total membrane protein. By comparing the N-terminal sequence of the protein with that of known protein sequences, the data suggested that the protein might be erythrocyte D-glyceraldehyde-3-phosphate dehydrogenase, and direct enzyme assay confirmed this postulate. The authors concluded that, although the enzyme is strongly retained by erythrocyte ghosts during hemolytic procedures, it is probably not an integral part of the structure of the erythrocyte membrane. Their conclusion about this glycolytic enzyme is probably true for all of the major glycolytic enzymes.

A recent report in 1972 by Kim et al. (80) has shown that within the erythrocyte membrane there exists a galactosyl transferase which catalyzes the transfer of galactose from UDP-galactose to acceptors. The significance of the enzyme is that it can produce alterations in ABH antigens and may be responsible for determining the specific ABH antigens. Definitive demonstration that the enzyme was actually bound into

the membrane has not been presented.

Lipoproteins

The association of lipid and protein in the erythrocyte membrane has been described. However, one of the major areas of controversy about membrane structure has been whether or not true lipoprotein units exist within the membrane structure. A very limited number of investigations have been performed which suggest that a lipoprotein does exist as a separate entity within the membrane. In 1966, Morgan and Hanahan (81) attempted to solubilize and characterize the lipoprotein from erythrocyte stroma. They isolated a soluble lipoprotein component which contained 94% lipid and 6% protein. The component was prepared by ultrasonication in a 10% n-butanol solution followed by density gradient ultracentrifugation. The lipoprotein contained 60-80% of the original lipid, but only 9-20% of the original protein. In the analytical ultracentrifuge, a single peak was obtained and electrophoresis by three different techniques demonstrated a homogeneous band with a mobility comparable to that of plasma alpha-2 lipoproteins. N-terminal analysis revealed two amino acids, serine and glutamic acid. Amino acid composition was noted to differ from that of other erythrocyte proteins. Removal of lipids from the lipoprotein fraction did not change the data obtained on characterization. On the basis of equilibrium ultracentrifugation, the lipid-free protein had an average molecular weight of 163,000.

A very strong criticism of the use of ultrasonication to obtain lipoproteins was published in 1968 by Rosenberg and McIntosh (82). On the basis of chromatographic, ultracentrifugal, and electron microscopic studies, sonication of red blood cell membranes was shown to break the

membranes into small vesicles and linear fragments with an intact unit membrane structure. The fragments ranged in size from 100 to 600 Å and did not sediment under conditions commonly used to define solubility. Morgan and Hanahan probably studied such fragments produced by sonication.

In 1971, Juliano and Rothstein (83) took a somewhat different view of erythrocyte membrane lipoprotein and attempted to partially characterize proteins which were closely associated with the lipids. After a water extraction system to remove 50% of the total membrane protein, they obtained membrane vesicles from which an additional 25% of the remaining protein could be removed by incubation in 8 M urea. The remaining lipoprotein fraction contained 50% of the total lipid in the ghost and another 25% of the total protein. It was solubilized with detergents and electrophoresed on polyacrylamide gel. The major protein component, representing over 80% of the total protein in the fraction, had a molecular weight of 95,000, contained most of the protein bound hexose, and was intensely labelled by application of non-penetrating protein reagents to the intact cell. The lipids associated with the protein were not essentially different from those of the original ghost preparation. The authors suggested that a small fraction of the RBC lipid is hydrophobically bound to protein and that the remainder of the lipid is held in the membrane by lipid-lipid interactions.

Water Extractable Proteins

Many references have already been made to the extraction of red cell membrane proteins by aqueous systems. Some specific studies have been performed in an attempt to characterize the water soluble proteins

of the human RBC membrane. In 1970, Hoogeveen et al. (84) used a double extraction of ghost membrane to obtain two different groups of proteins. The first extraction was with water at pH 7.0 and resulted in the release of a protein fraction which they called P-I. The fraction contained four major components with molecular weights ranging from 30,000 to 48,000. A second fraction was obtained by solubilization with 1.0 mM EDTA at pH 9.0. It consisted primarily of a single molecular weight component of about 150,000, which tended to aggregate at higher ionic strengths and in the presence of calcium. The water soluble proteins were present at the inner face of the membrane and were distinctly different from those proteins remaining in the erythrocyte residue.

In 1971, Maddy and Kelly (85) used dilute acetic acid as a solvent to liberate 30 to 40% of the membrane proteins free of phospholipid and sialoprotein. Multiple analytical techniques demonstrated that the proteins extracted were essentially the same as those obtained with the dilute EDTA solutions. Also in 1971, Reynolds and Trayer (86) demonstrated that aqueous solutions of EDTA could extract as much as 89% of the protein at a concentration of 5 millimolar EDTA. Increasing the EDTA concentration reduced the amount of protein which could be solubilized.

Hamaguchi and Cleve (87) also published on the water-dissolved membrane proteins of human erythrocytes using an extraction system which included a 0.1% (w/v) aqueous solution of Triton X-100. They obtained about 40% of the protein from the membranes. A special note of caution should be entered here since Triton X-100 has already been shown to solubilize membrane proteins by removing part of the lipid. These data should therefore be interpreted with a great deal of caution.

CHAPTER III

MATERIALS AND METHODS

Materials

Source of Erythrocytes

The erythrocytes used for this study were obtained from the Blood Bank of the University of Oklahoma Hospitals in the form of whole blood containing either ACD (acid-citrate-dextrose) or CPD (citrate-phosphate-dextrose) anticoagulants. Most of the blood used was recently outdated blood, but in many cases the blood had been drawn less than 21 days before its use. Although all ABO blood types were used, most of the studies were done with blood group A type red blood cells. Except for occasional units of blood, the Rhesus typing was positive. No further typing of the blood was done except when blood group MN typing had to be performed to determine which blood group activity should be assayed by hemagglutination inhibition.

Methods

Preparation of Ghosts

Erythrocyte ghosts were prepared with the hypotonic phosphate buffer system of Dodge *et al.* (32) according to a procedure in Figure 1. Other procedures for the preparation of erythrocyte ghosts, such as the

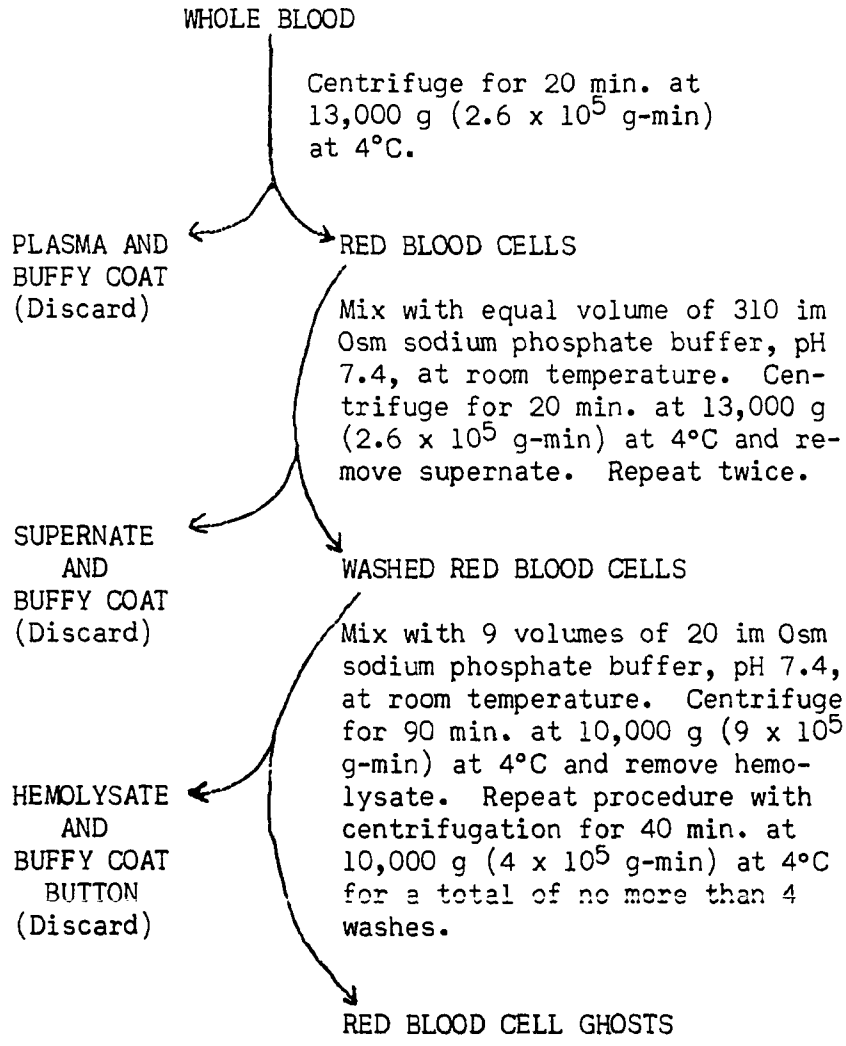

Figure 1. Preparation of erythrocyte ghosts (Dodge et al. (32)).

technique of Mazia and Ruby (41), were also evaluated. However, they were not as effective either because the product was not completely free of hemoglobin or because the method was technically too difficult to perform.

Fractionation of Erythrocyte Ghosts

Further fractionation of the erythrocyte ghosts was carried out with a number of aqueous systems. The procedure used for extraction of proteins with 1.0 millimolar sodium EDTA is given in Figure 2. The same procedure was also used with other aqueous solvents, which included 1) water at pH 7.0, 2) 0.5 M NaCl, 3) 0.1 M tetramethylammonium bromide (TMAB), and 4) 0.1 (w/v) Triton X-100. The basic procedure was modified for a set of experiments which involved the use of a single EDTA extraction (60 minutes) followed by a second extraction with either ammonium hydroxide, pH 10.5, or sodium hydroxide, pH 11.0 (15 minutes).

The extraction procedure resulted in a number of different products for which abbreviations will be used. Abbreviations ending in G, R, and S signify that the products being discussed are ghosts, residues and combined extraction supernates, respectively. A product ending in C is the supernate obtained from a single extraction. The initial letter of the abbreviation identifies the agent used for the extraction procedure. These include: 1) E for 1.0 millimolar sodium EDTA, 2) W for water a pH 7.0, 3) N for 0.5 M NaCl, 4) TM for 0.1 M TMAB, and 5) TX for 0.1% Triton X-100. Numbers in the middle of the abbreviation designate from which extraction in the series the product was derived. For example, a product abbreviated as W3C is the supernate obtained from the third water extraction of RBC ghosts only, whereas W3S is the combined super-

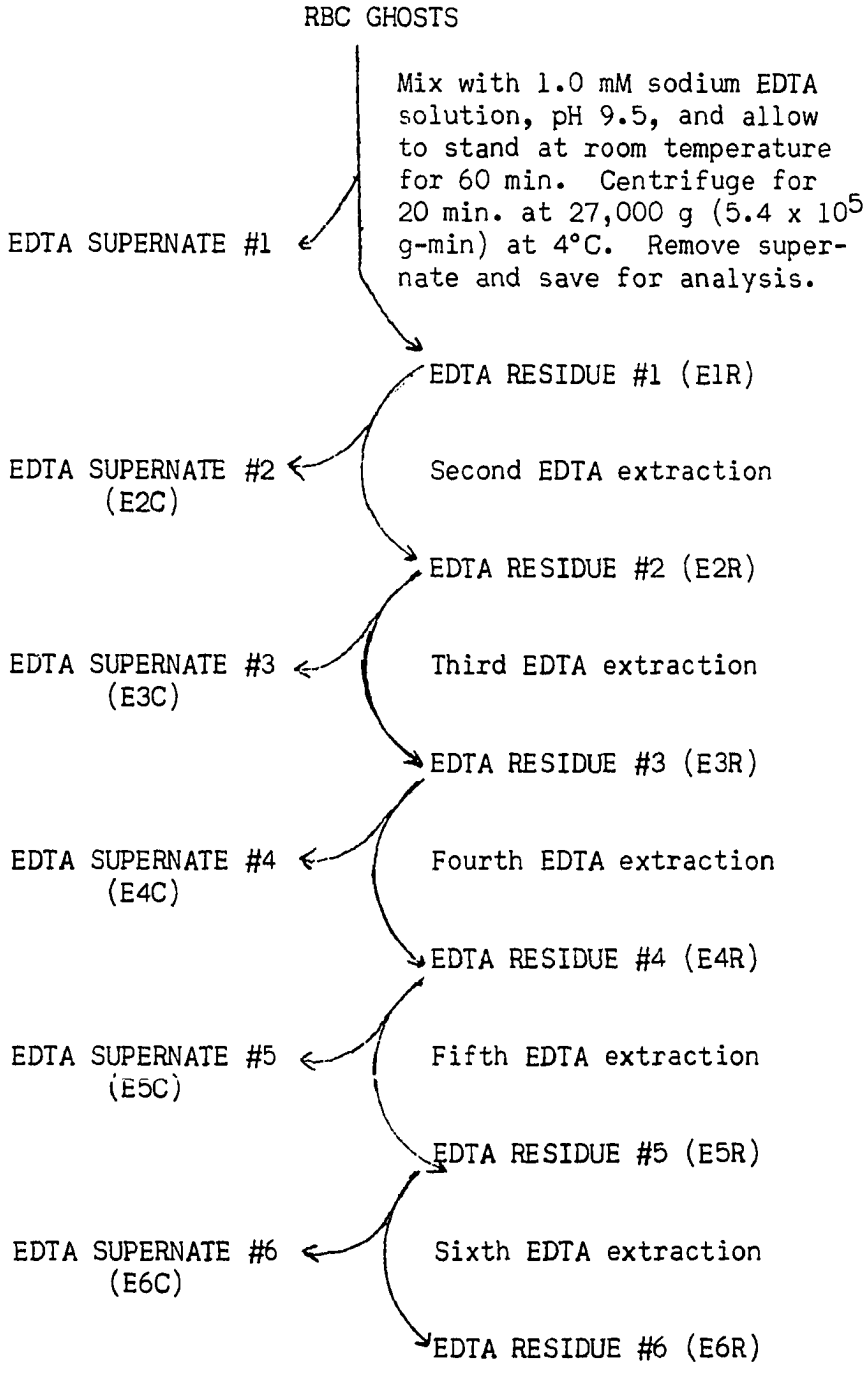

Figure 2. Extraction of RBC ghosts with hypotonic EDTA solution.

nates from the first three water extractions. Other examples are E5R and E6R, which are the residues of RBC ghosts remaining after the fifth and sixth extractions, respectively.

Solubilization

In the initial experiments, the solubility of RBC ghost proteins in various solvent systems was determined quantitatively. Ten milliliters of RBC ghost suspension (approximately 4 mg protein/ml) were mixed with 1.0 ml of a concentrated solution of the test solubilizing agent in order to obtain the desired final concentration. For example, 10.0 ml of ghost suspension was mixed with 1.0 ml of a 11.0% (w/v) SDS solution to obtain a final 1.0% SDS concentration. The only exception to this technique was in the guanidinium HCl experiment in which the agent was weighed and added directly to the ghost suspension. After thorough agitation, the ghost-solvent mixture was incubated at room temperature for 60 minutes before centrifugation at 100,000 x g for 60 minutes (6×10^6 g minutes). When solubilization was incomplete, a button (infranate) of undissolved ghosts was visible in the bottom of the centrifuge tube. The clear supernate above the button was removed by aspiration through a Pasteur pipette. The amounts of protein in the supernate and the infranate were measured in order to determine the per cent of the protein solubilized.

For gel filtration studies in which only SDS was used as a solubilizing agent, a different technique was followed. About 5 ml of ghost suspension was mixed with enough concentrated SDS solution to obtain a final SDS concentration of 2-3%. This mixture was incubated at 37°C for 15 to 60 minutes. If the mixture was clear, then a sample was applied

to the column. However, if it was not clear, additional concentrated SDS solution was added until the solution was clear before application to the column.

Solubilization for polyacrylamide gel electrophoresis was performed in a different manner and will be discussed as part of that technique.

Gel Filtration

Most of the gel filtration studies were performed with Sepharose 4B. The gel was equilibrated with 0.05 M Tris buffer (pH 7.6) containing 0.1% (w/v) sodium dodecyl sulphate (SDS). The same buffer system was used for elution unless otherwise specified. Because of difficulties which arise in maintaining the flow of buffer systems containing detergents, the gels were contained in 2.5 cm x 90 cm glass columns with an outer water jacket in order that a temperature of 37°C could be maintained. Fractions from the column were collected with an ISCO Golden Retriever fraction collector by the drop-counting method. The drop counter was set at 192 drops, which produced a volume of about 4.2 ml for each fraction. The volume did vary slightly from that value, depending upon how much protein was present. The elution pattern was determined by measuring the optical density at 280 nm ($O.D._{280}$), the protein content, the organic phosphorus content, and the carbohydrate content, depending upon the experimental objective. Additional gel filtration was also performed using Sephadex G-200 in 2.5 x 90 cm glass columns with two different buffer systems and at two different temperatures. When the buffer was 0.05 M Tris (pH 7.6), the procedure was carried out at room temperature. However, if the buffer contained SDS, the procedure

was carried out at 37°C in a water-jacketed column. When needed for additional studies, fractions obtained from the column were concentrated by ultrafiltration with the Amicon Diaflo Ultrafiltration System 202. A 62 mm PM 10 filter was used under pressure from a compressed nitrogen source.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed by a technique similar to that described by Lenard (50). The membrane material was solubilized by the addition of an equal volume of an aqueous solution containing 3.0% or more SDS and 1.0% β-mercaptoethanol. The mixture (approximately 2.0 mg protein/ml) was incubated at 37° for two hours rather than boiled for 3 minutes as done by Lenard. Then 25-100 µl of the sample were mixed with an equal volume of a 40% sucrose solution applied to the polyacrylamide gel. A 5% polyacrylamide gel catalyzed by the addition of ammonium persulfate was used for the study with no sample or stacking gel. The buffer for electrophoresis was 0.1 M sodium phosphate buffer, pH 7.4, containing 1.0% SDS and 1.0% β-mercaptoethanol. After pre-running for 60 minutes to remove the ammonium persulfate, the electrophoresis was carried out at room temperature for a period of two and one-half hours. Longer periods were used when a better separation was desired for the larger proteins. The current for the procedure was 5.0 milliamperes per tube. The gels were fixed in a mixture of ethanol, acetic acid, and water (50:5:45, v/v/v) as described by Kaplan and Criddle (98). Protein staining was performed with 0.125% Coomassie Blue in the ethanol-acetic acid mixture. The excess Coomassie Blue stain was removed with a Hoefer Destainer (Hoefer Scientific Instruments, San Francisco, Calif.)

containing 5.0% acetic acid. An alternate staining method for Coomassie Blue stain was utilized which produced better bands for scanning. The charcoal adsorbent was removed from the center of the Hoefer Destainer before the addition of a very dilute aqueous solution (approximately 0.002%) of Coomassie Blue. The solution was continuously agitated by the magnetic stirrer for several hours until readily visible bands were noted. This resulted in a minimal amount of background staining. The carbohydrate bands were visualized by the periodic acid-Schiff (PAS) staining technique described by Zacharius et al. (99). After staining, the gels were scanned by a Gilford Recording Spectrophotometer with a gel scanning attachment. A wavelength of 540 nm was used for scanning both Coomassie Blue and PAS stained gels.

Analytical Methods

Proteins. Protein was determined by a modification of the Lowry method (88).

Lipids. Phospholipid was determined either as the total phosphorus (89) minus the inorganic phosphorus (90) or as the total phosphorus only, depending upon the specific experimental situation. The phosphorus value was multiplied by 25 to obtain a value for phospholipid. Total cholesterol was determined using a cholesterol determination kit from Hycel, Inc. (Houston, Texas). Lipid extraction of ghosts and residue was carried out with chloroform-methanol according to the method of Folch et al. (91).

Carbohydrates. Total hexose content was determined by using the phenol-sulphuric acid method of Dubois et al. (92). To obtain the total carbohydrate content, the hexose content was divided by a factor

(0.41) obtained from the data of Winzler (35) to correct for the sialic acid and amino sugars, which the Dubois et al. method does not measure. Individual hexose contents were determined by gas-liquid chromatography on a Barber-Colman Series 500 Gas Chromatograph employing a glass column packed with 3% ECNSS-M on Gaschrom Q, 80/100 mesh, according to the method by Holme et al. (93). Individual hexosamine contents were determined by the amino acid analyzer. Sialic acid was determined by the method of Warren (94).

Amino acid analysis. Amino acid analyses were carried out on a Beckman Model 120C Amino Acid Analyzer. The amino acid analysis of protein bands separated by polyacrylamide gel electrophoresis was carried out as described by Houston (95). The gels were first stained with either Coomassie Blue or with PAS stain. The bands produced were removed by careful sectioning using a razor blade to cut the gel. Several gel sections from the same protein band were hydrolyzed with 5.7 N hydrochloric acid in evacuated, sealed tubes at 110° for 24 hours before application onto the analyzer.

Enzyme Assays

Acetylcholinesterase was determined by the method of Michel (74). NADH:(acceptor) oxidoreductase was measured by the method of Zamudio et al. (77). Glucose-6-phosphate dehydrogenase, aldolase, and lactate dehydrogenase were determined with kits from CalBiochem, Inc.

Immunological Methods

Hemagglutination inhibition. A, B, H, M, and N blood group activities were determined by the hemagglutination inhibition method with

a modification (96) of the microdiluter system of Takatsy. Commercially available antibodies for the studies were obtained from Ortho Diagnostics, Inc., Raritan, N. J. Known type A and type B red blood cells were also obtained from Ortho Diagnostics, Inc.

Immunodiffusion. Immunodiffusion studies were performed using the Ouchterlony double diffusion technique (97). The studies were carried out on agar gel plates containing 1.0% (w/v) agar in 0.05 M Tris buffer (pH 7.6). This technique was also utilized to detect antigens in unfixed polyacrylamide gels. The gels were divided into 12 equal sections from top to bottom. The gel sections were then embedded in 1% agar and immunodiffusion was performed against previously prepared antibodies.

Antibody production. Antibodies for the immunodiffusion studies were obtained from rabbits in the following manner. Antigens used to stimulate antibody production were prepared from several sources, including RBC ghost suspensions, concentrated column fractions, extraction residue suspensions, and polyacrylamide gel sections. For liquid antigenic material, 1.0 ml of the sample was vigorously mixed with 1.0 ml of Freund's adjuvant (complete or incomplete) to form an emulsion and injected into rabbits both subcutaneously and intraperitoneally. The antigens were administered at weekly intervals for at least 4 weeks and for longer periods in cases where antibodies were slow to appear. The plasma was initially harvested after two weeks and then at weekly intervals until precipitating antibodies against the antigens could be demonstrated by immunodiffusion.

When polyacrylamide gels were used for antibody induction, the carbohydrate bands were stained by the PAS method and removed by cutting

out the bands with a razor blade. The gel sections (usually four) without adjuvant were injected every other day into rabbits for a total of 4 injections. Thereafter, the sections were given on a weekly schedule until precipitating antibodies appeared.

Electron Microscopy

Both the erythrocyte ghosts in hypotonic phosphate buffer and the EDTA-extracted residue in EDTA buffer were examined by electron microscopy. They were fixed with glutaraldehyde-osmic acid and embedded in epoxy resin. The embedded material was sectioned and photomicrographs were taken at magnifications of 10,000X, 30,000X and 300,000X.

CHAPTER IV

RESULTS

Isolation and Characterization of Erythrocyte Ghosts

Composition

The relative composition and concentration (expressed in mg/mg protein) of the major components of RBC ghosts are given in Table 12. The major component in the ghost is protein, accounting for almost one-half of the total weight. An almost equal percentage of the membrane is made up of cholesterol and phospholipid. The approximate values for the phospholipid:protein, the cholesterol:protein and the carbohydrate:protein ratios are 0.6, 0.4, and 0.15, respectively. Assays of individual sugars reveal that sialic acid and galactose are the two major sugars present. The hexosamines, glucosamine and galactosamine are the next most common sugars with minor amounts of glucose, mannose and fucose present.

The enzymatic activities found in erythrocyte ghosts are given in Table 13 under Experiment A. The two glycolytic enzymes (glucose-6-phosphate dehydrogenase and aldolase) are present in small amounts, whereas no lactic dehydrogenase activity could be demonstrated. The two membrane-associated enzymes, acetylcholinesterase (AChEase) and NADH:(ac-

TABLE 12

COMPOSITION OF RBC GHOSTS AND EXTRACTED RESIDUE (E6R)

Major Component	Percent by Weight		mg/mg Protein	
	Ghosts	Residue	Ghosts	Residue
Protein	47.3	36.1	1.0	1.0
Phospholipid	27.8	33.2	0.588	0.918
Cholesterol	17.8	19.3	0.376	0.535
Carbohydrate	7.0	11.4	0.148	0.316
Carbohydrate Comp.	Percent by Weight		µg/mg Protein	
Galactose	21.5	23.0	22.4	39.8
Glucose	6.8	13.9	7.1	23.9
Mannose	6.4	3.0	6.7	5.2
Fucose	5.3	1.8	5.5	3.0
Glucosamine	14.3	15.3	14.9	26.4
Galactosamine	14.0	10.4	14.5	17.8
Sialic Acid	31.7	32.7	33.0	56.3

TABLE 13

ENZYME CONTENT OF VARIOUS COMPONENTS[a]

	LDH-P	G-6-P-D	Aldolase	AChEase	NaOase
EXPERIMENT A:					
RBC Ghosts	0	0.655 mμ O.D./min	11.49 mμ O.D./min	4.23	2.292
EDTA Extr. Residue (E6R)	0	0	0	2.90 pH μ/min	0.698 mM FeCN/min
EXPERIMENT B:					
EDTA Supernate #1	70.56 mμ/min	0	6.03 mμ/min	0.52 pH μ/min	0.090 mM FeCN/min
#2	0	0	0	0.38	0.102
#3	0	0	0	1.68	0.209
#4	0	0	0	0	0.220
#5	0	0	0	0	0.250
#6	0	0	0	0	0.435

[a] Δ O.D. X 10^3/min/mg protein.

ceptor) oxidoreductase (NaOase), are both present in substantial amounts.

Amino acid analysis of RBC ghosts is reported in Table 14. The two major amino acids are glutamic acid and leucine. Although less abundant, aspartic acid, serine, alanine and glycine are also major amino acids. The other amino acids are equally distributed except for minor quantities of tyrosine and histidine. The presence of both galactosamine and glucosamine can be shown by amino acid analysis.

Photomicrographs of RBC ghosts are given in Figures 3, 4, and 5. At the lower magnification of 10,000X and 30,000X, the ghosts have the same basic physical shape and appearance as normal erythrocytes which have not been hemolyzed. The membrane itself seems to be intact with no sites of rupture. At the highest magnification the membrane is again found to be intact, having smooth outer surface and a fuzzy inner surface, but a trilaminar structure cannot be seen.

Solubilization of RBC Ghosts

Five different agents were compared for their effectiveness in solubilizing RBC ghost proteins. The results obtained at three different concentrations of each agent are given in Figure 6. The most effective agent was the anionic detergent, sodium dodecyl sulphate (SDS), which solubilizes 98% of the protein at a concentration of 0.5% (w/v). At the same concentration sodium deoxycholate, a bile salt detergent, solubilizes slightly over 70%, Triton X-100, a nonionic detergent, almost 55%, and Cetavlon, a cationic detergent, less than 5% of the total protein. The dissociating agent, guanidinium hydrochloride, at 6 M concentration solubilizes slightly less than 80% of the protein.

The effectiveness of SDS as a solubilizing agent was demonstrated

TABLE 14

AMINO ACID ANALYSIS OF GHOSTS AND RESIDUE[a]

	Ghosts	Residue
Lysine	61.2	45.5
Histidine	32.4	23.7
Arginine	61.3	46.4
Aspartic acid	87.4	81.0
Threonine	53.8	60.5
Serine	82.7	91.2
Glutamic acid	137.2	121.9
Proline	65.1	76.0
Glycine	71.2	82.8
Alanine	79.9	76.2
Valine	53.0	61.5
Methionine	-	-
Isoleucine	39.5	43.5
Leucine	104.7	110.5
Tyrosine	29.2	22.7
Phenylalanine	41.6	58.1
Galactosamine	10.0	17.4
Glucosamine	10.3	25.8

[a]Values are given as the moles of the individual amino acid per 1000 moles of the total amino acids in the sample.

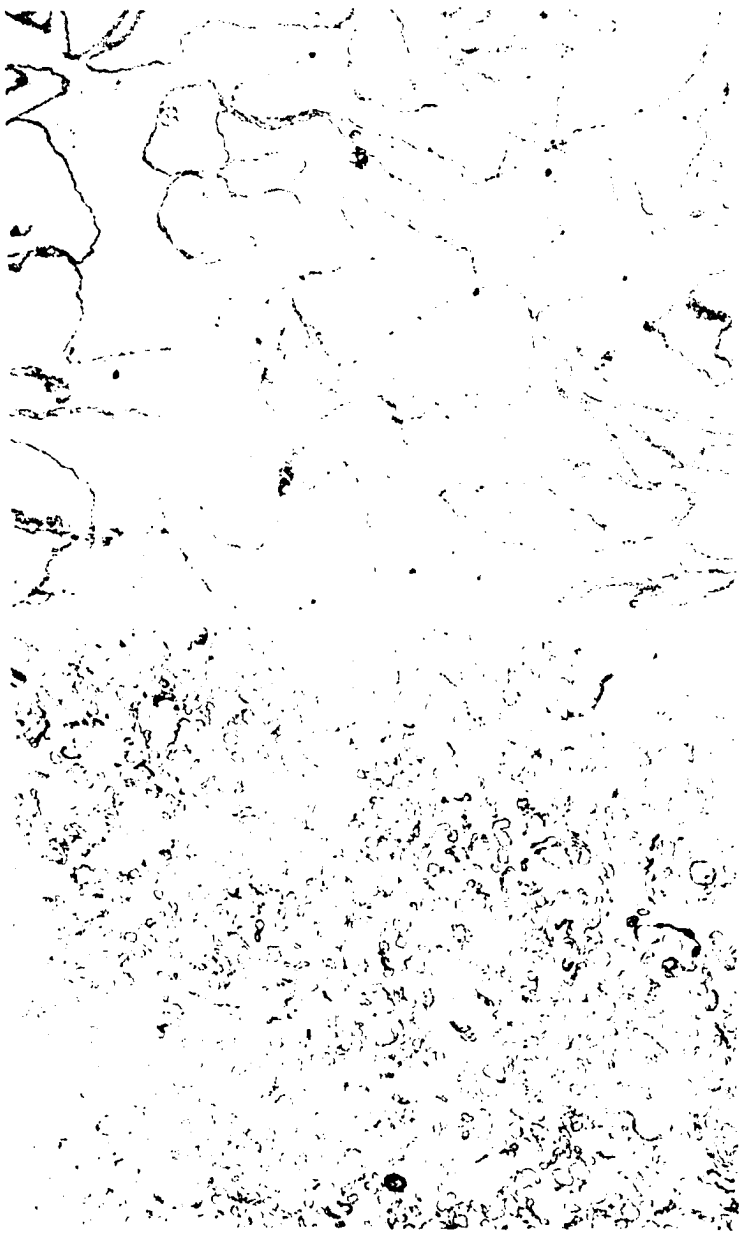

Figure 3. Electron photomicrographs of human erythrocyte ghosts and EDTA-extracted residue.

Suspensions of human erythrocyte ghosts (upper picture) and EDTA-extracted residue (lower picture) were prepared as described under Results and photographed at 10,000X magnification.

Figure 4. Electron photomicrographs of human erythrocyte ghosts and EDTA-extracted residue.

Suspensions of human erythrocyte ghosts (upper picture) and EDTA-extracted residue (lower picture) were prepared as described under Results and photographed at 30,000X magnification.

Figure 5. Electron photomicrographs of human erythrocyte ghosts and EDTA-extracted residue.

Suspensions of human erythrocyte ghosts (upper picture) and EDTA-extracted residue (lower picture) were prepared as described under Results and photographed at 300,000X magnification.

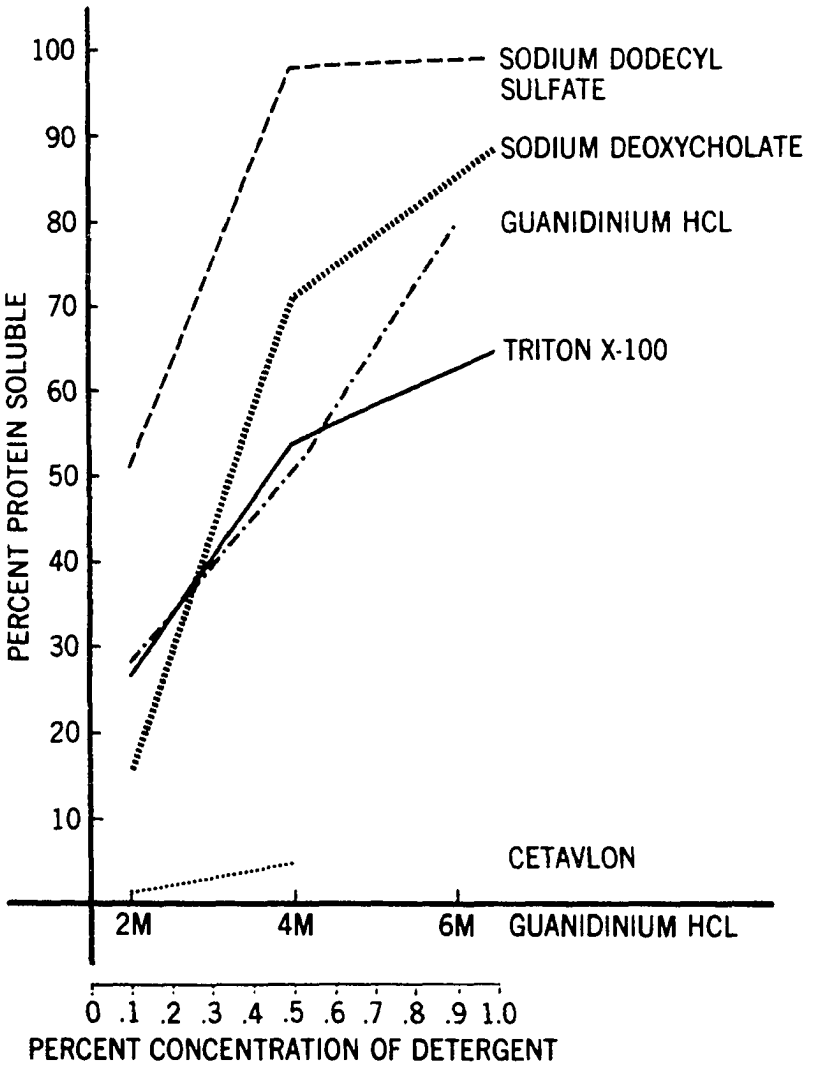

Figure 6. Solubilization of RBC ghosts with various agents.

The upper scale on the abscissa is for the concentration of guanidinium hydrochloride only. The lower scale is for the concentration of all detergents. No value is given for Cetavlon at 1% concentration because quantitation was technically not possible.

in a series of experiments in which RBC ghosts were solubilized at various SDS concentrations (0.075%, 0.1%, 0.5%, 1.0%) and fractionated by Sepharose 4B gel filtration with elution using an SDS buffer system at the same SDS concentration as that used for solubilization. As shown in Figures 7 and 8, very little protein was eluted at either 0.075% or 0.1% SDS concentration, whether measured as $O.D._{280}$ or Lowry protein. However, in Figures 9 and 10, three distinct protein peaks can ben seen for the 0.5% and 1.0% SDS concentrations, respectively, and there appears to be little difference between the two figures. Thus, an SDS concentration of 0.5% is necessary for solubilizing the ghost proteins before gel filtration. This finding was confirmed in a study, the results of which are shown in Figure 11. The pellet remaining after solubilization at 0.1% SDS concentration was resuspended in 1.0% SDS and applied to a Sepharose 4B column with elution by a 1.0% SDS buffer system. Major protein peaks are demonstrated by both $O.D._{280}$ and Lowry protein measurements. These gel filtration data agree with the results presented in Figure 6. Consequently, SDS was used to solubilize RBC membrane proteins before analytical studies.

Gel Filtration of Solubilized Ghosts

Fractionation of the RBC membrane proteins was performed by gel filtration on Sepharose 4B gel as previously described. The proteins are separated into three major fractions as shown in Figure 12 and no protein elutes at the void volume. The peaks are designated as Fraction I (FI), Fraction II (FII) and Fraction III (FIII), starting with the least retarded fraction (FI) and ending with the most retarded fraction (FIII). After combining the tubes from each of the three fractions and concen-

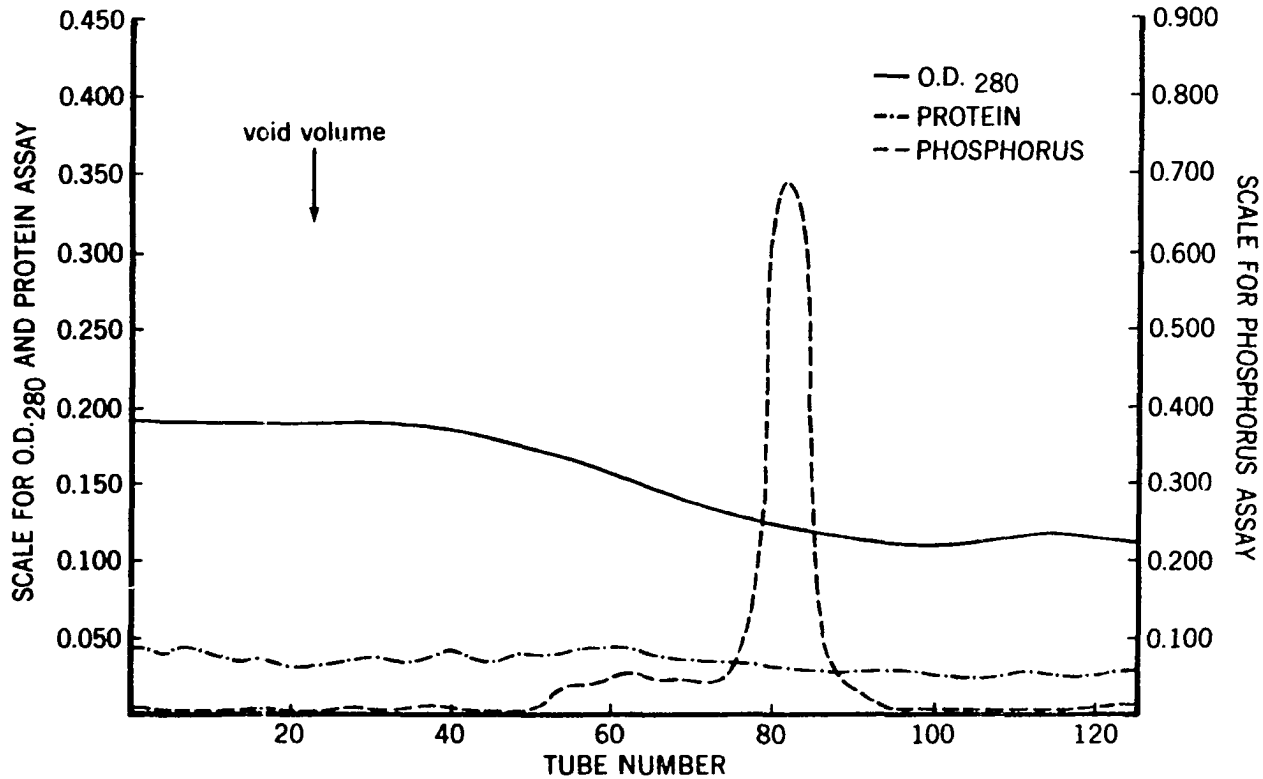

Figure 7. Gel filtration of RBC ghost supernate at 0.075% SDS concentration.

The solubilization technique with SDS is described in Methods. The supernate from the procedure was applied to a Sepharose 4B gel column (2.5x90 cm) which had been equilibrated with 0.075% SDS in 0.05 M Tris HCl (pH 7.6). The material was eluted with the same SDS buffer system at 37°C with a flow rate of 10-20 ml per hour. Individual fractions were collected containing approximately 4 ml per tube. The optical absorption of every tube was measured at 280 nm. The protein (Lowry method) and total phosphorus contents were measured in every third tube. The scale for the $O.D._{280}$ and Lowry protein is on the left ordinate and the scale for the phosphorus is on the right ordinate.

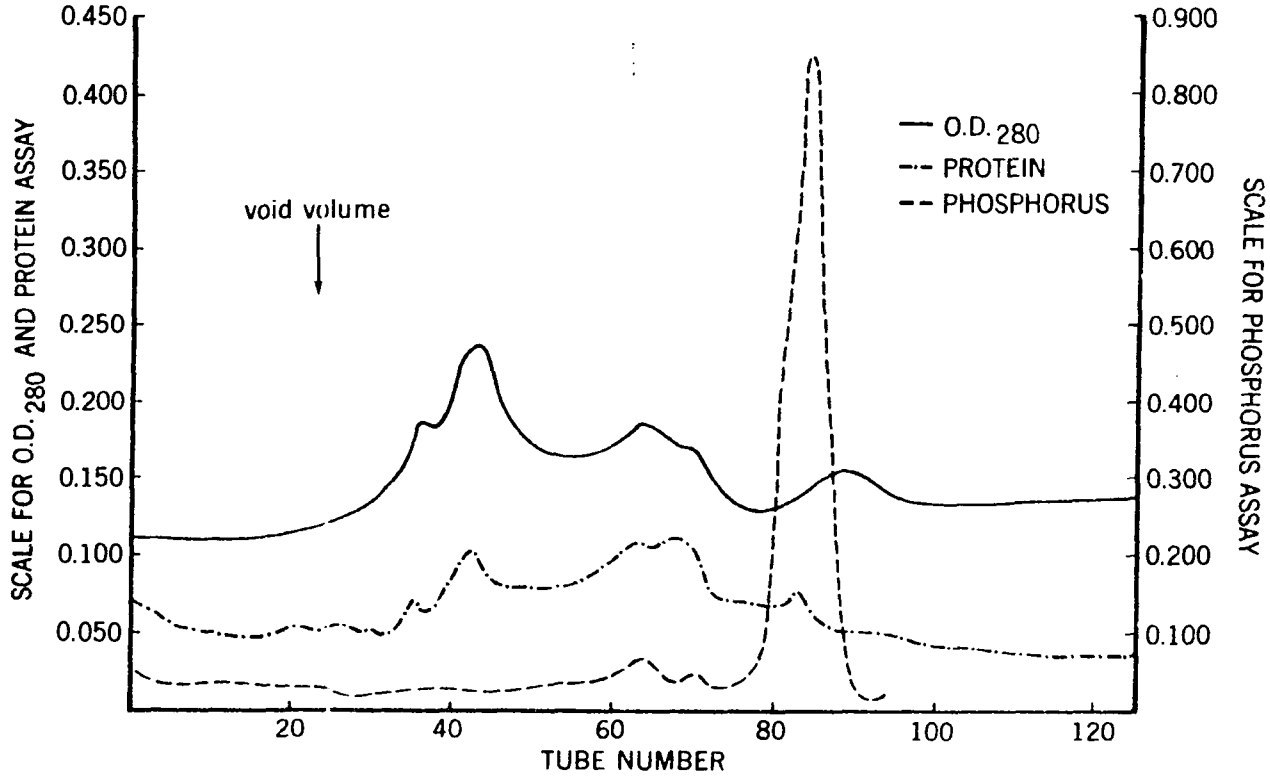

Figure 8. Gel filtration of RBC ghost supernate at 0.1% SDS concentration.

The solubilization procedure is described in Methods. The conditions for fractionation are the same as those described under Figure 7, except that the SDS concentration was 0.1% throughout the experiment.

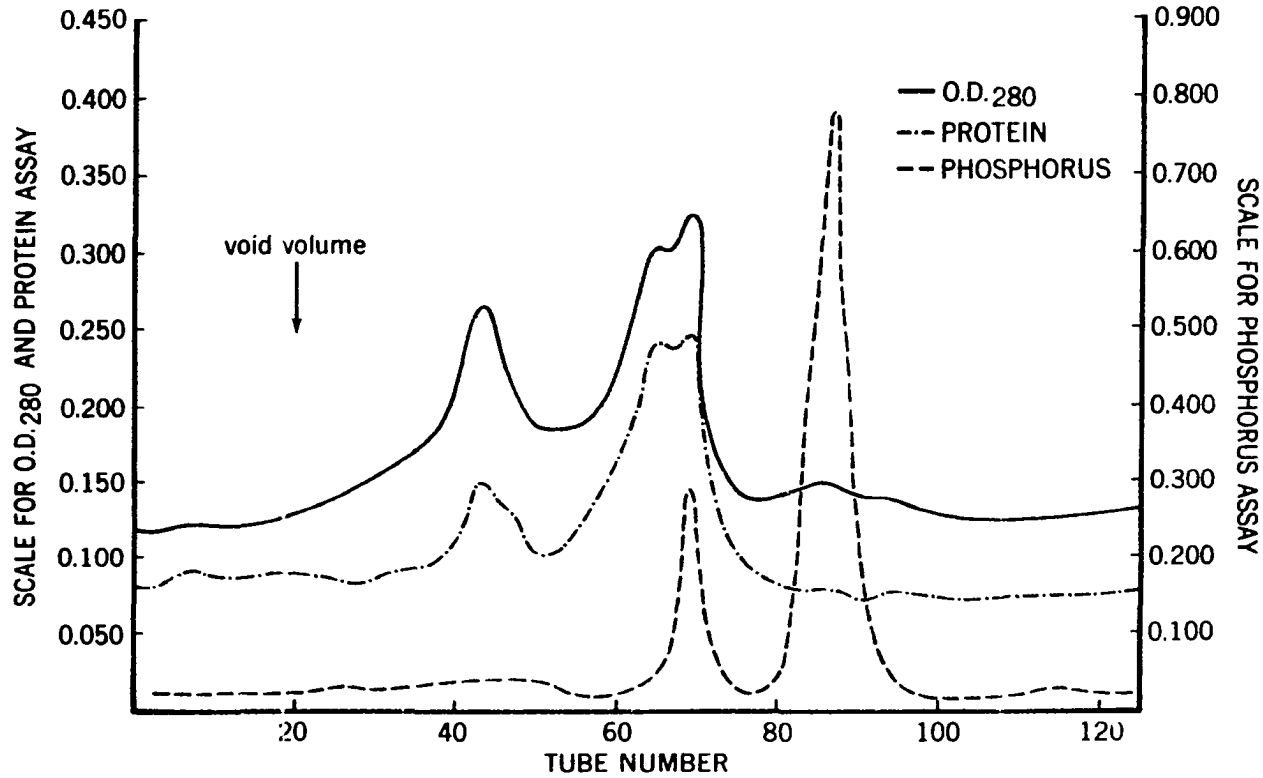

Figure 9. Gel filtration of RBC ghost supernate at 0.5% SDS concentration.

The solubilization procedure is described in Methods. The conditions for fractionation are the same as those described under Figure 7, except that the SDS concentration was 0.5% throughout the experiment.

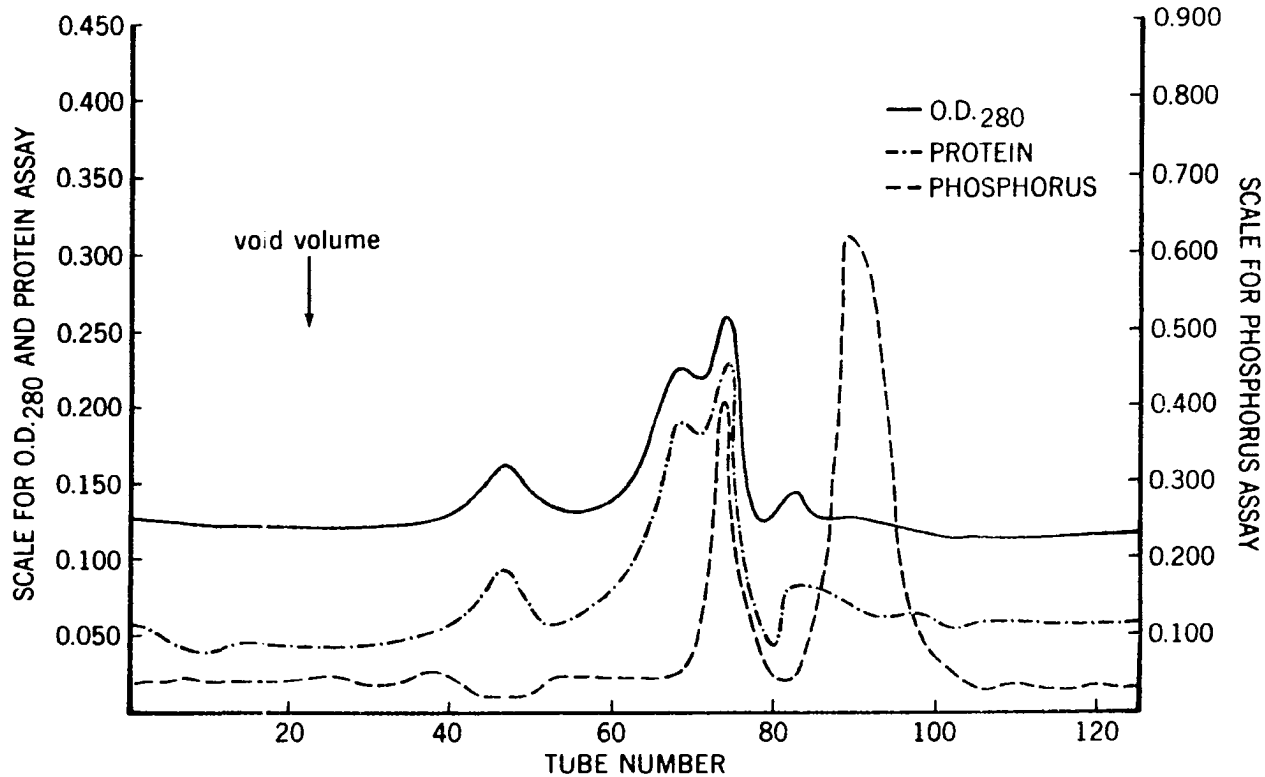

Figure 10. Gel filtration of RBC ghost supernate at 1.0% SDS concentration.

The solubilization procedure is described in Methods. The conditions for fractionation are the same as those described under Figure 7, except that the SDS concentration was 1.0% throughout the experiment.

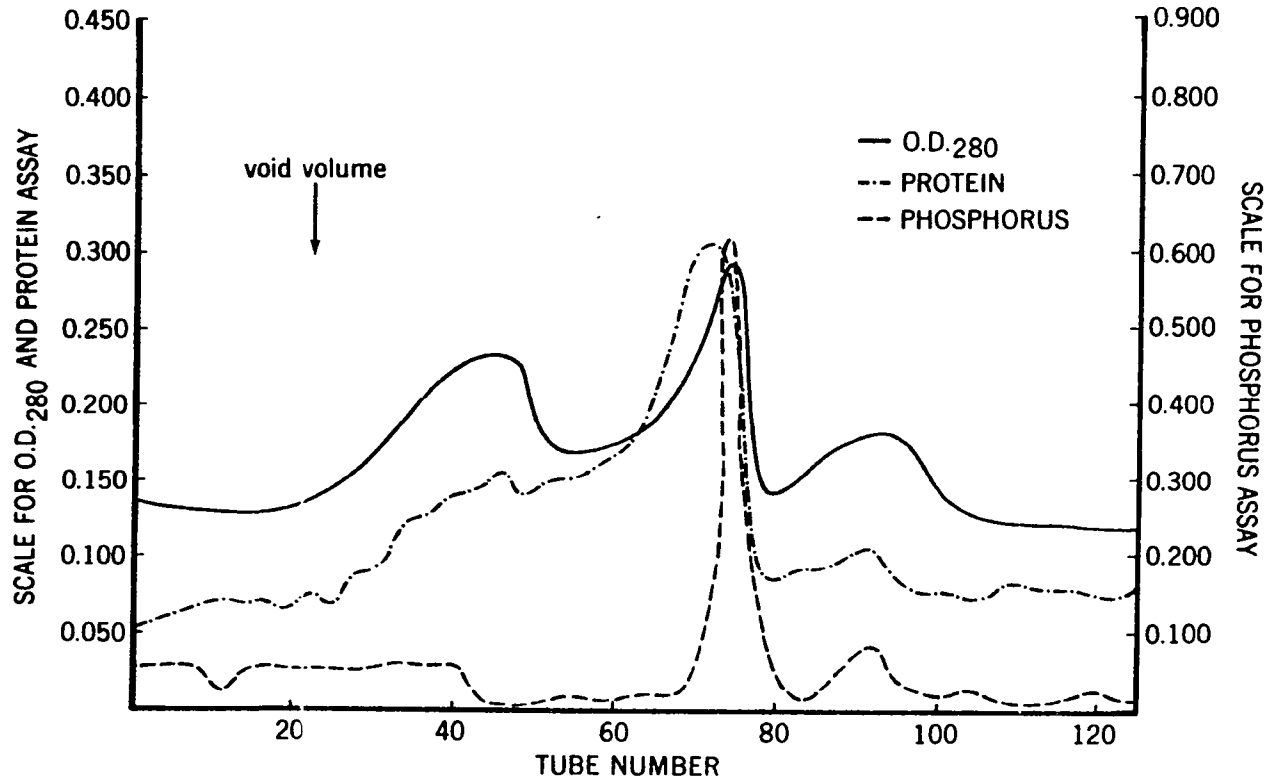

Figure 11. Gel filtration of RBC ghost residue from the 0.1% SDS concentration experiment.

The residue remaining after solubilization at 0.1% SDS concentration was completed solubilized at a final 1.0% SDS concentration and applied to the Sepharose 4B. Otherwise the conditions were the same as described under Figure 7.

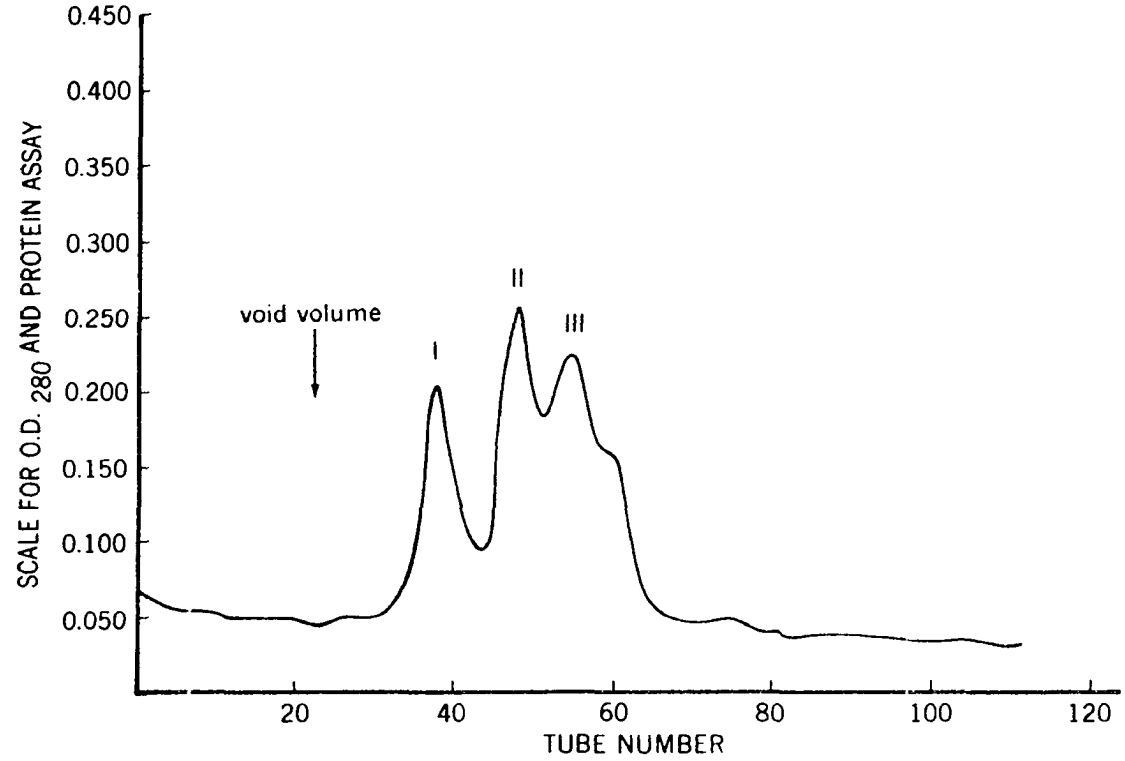

Figure 12. Sepharose 4B gel filtration of SDS-solubilized RBC ghosts.

Intact RBC ghosts were solubilized at a final SDS concentration of about 3%, incubated at 37°C for 30-60 minutes, and applied to a Sepharose 4B gel column. The material was eluted at 37°C with 0.1% SDS in 0.05 M Tris HCl buffer (pH 7.6) and was monitored by measuring the absorbance at 280 nm only.

trating the protein by ultrafiltration, each fraction was rechromatographed on Sepharose 4B as shown in Figures 13, 14, and 15. Each fraction elutes as a single peak at its appropriate elution volume, except for the small amount of protein which is usually found in the void volume.

The Sephadex G-200 was also used to rechromatograph the individual fractions. The column was eluted with 0.05 M Tris buffer only in the absence of SDS. The results for Fractions I, II, and III are shown in Figures 16, 17, and 18, respectively. The use of Sephadex G-200 did not improve the protein separation. FI eluted as a single peak at the void volume as did most of the proteins of Fractions II and III. Apparently, the molecular weight limit for exclusion by Sephadex G-200 was not high enough to allow all RBC membrane proteins to be retarded for separation. The possibility existed that the membrane proteins were not separated by Sephadex G-200 because SDS was absent. However, as shown in Figure 19 elution of the proteins in the presence of SDS did not result in an improved separation of proteins. Consequently, for fractionation of solubilized ghost protein, Sepharose 4B in the presence of SDS is superior to the Sephadex G-200, either in the presence or absence of SDS.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electropherograms of solubilized ghosts are given in Figures 20 and 21. In the former figure, eleven major protein bands are seen in the photoscan. The most rapidly migrating band is not a protein band, but results from the staining of the membrane lipid. In the latter figure, two major carbohydrate-containing bands are seen on the scan plus several minor bands. The major peak closer to the cathode

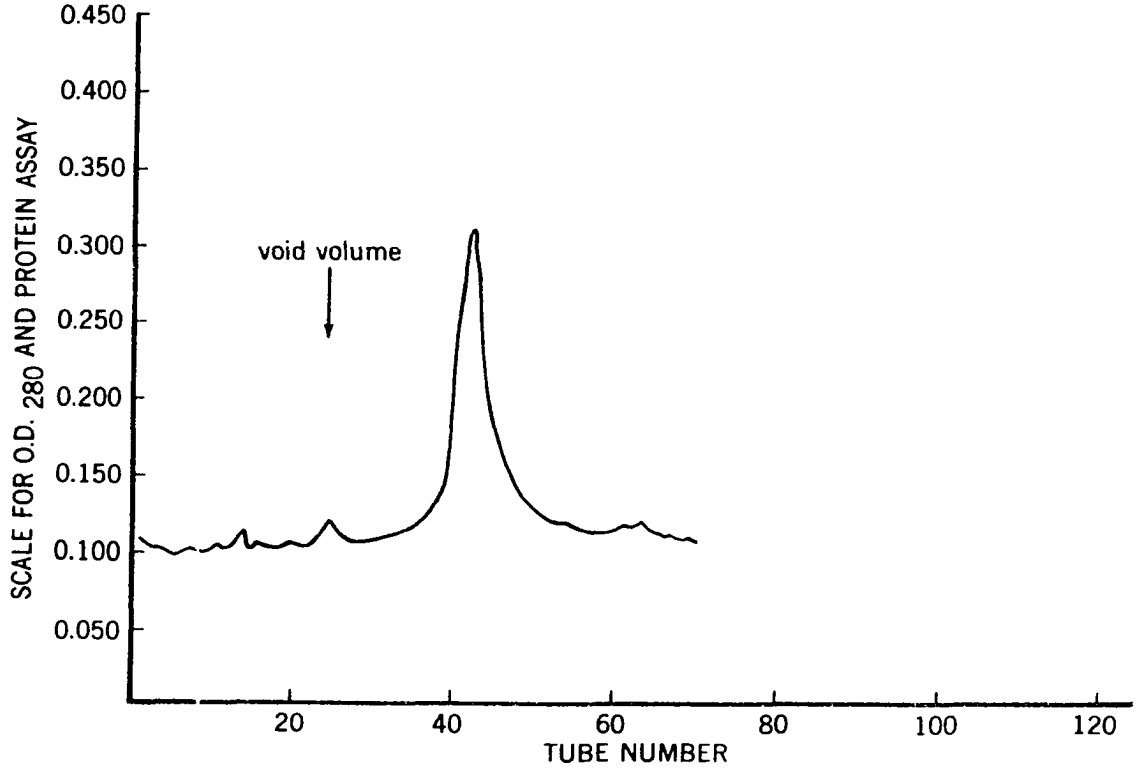

Figure 13. Sepharose 4B gel filtration of fraction I.

The eluant tubes containing Fraction I as shown in Figure 12 were combined before being concentrated by ultrafiltration. The concentrated Fraction I was re-chromatographed under the conditions described under Figure 12.

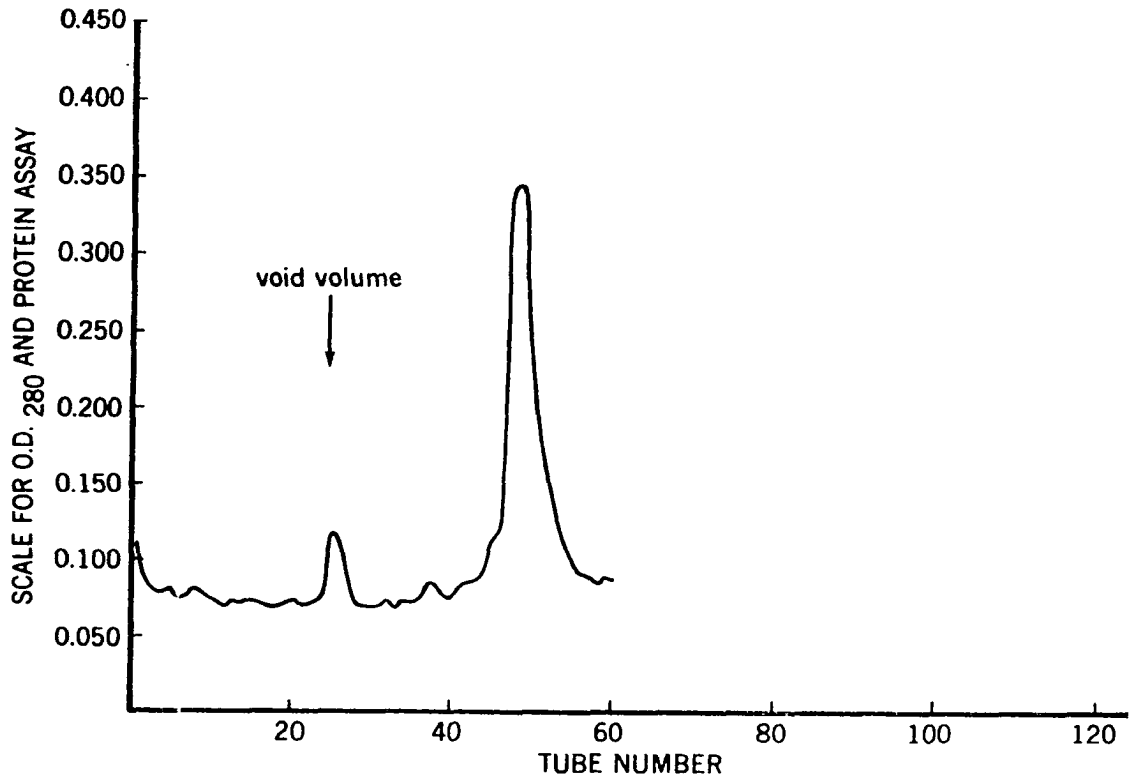

Figure 14. Sepharose 4B gel filtration of fraction II.

Fraction II was re-chromatographed as described in Figure 13.

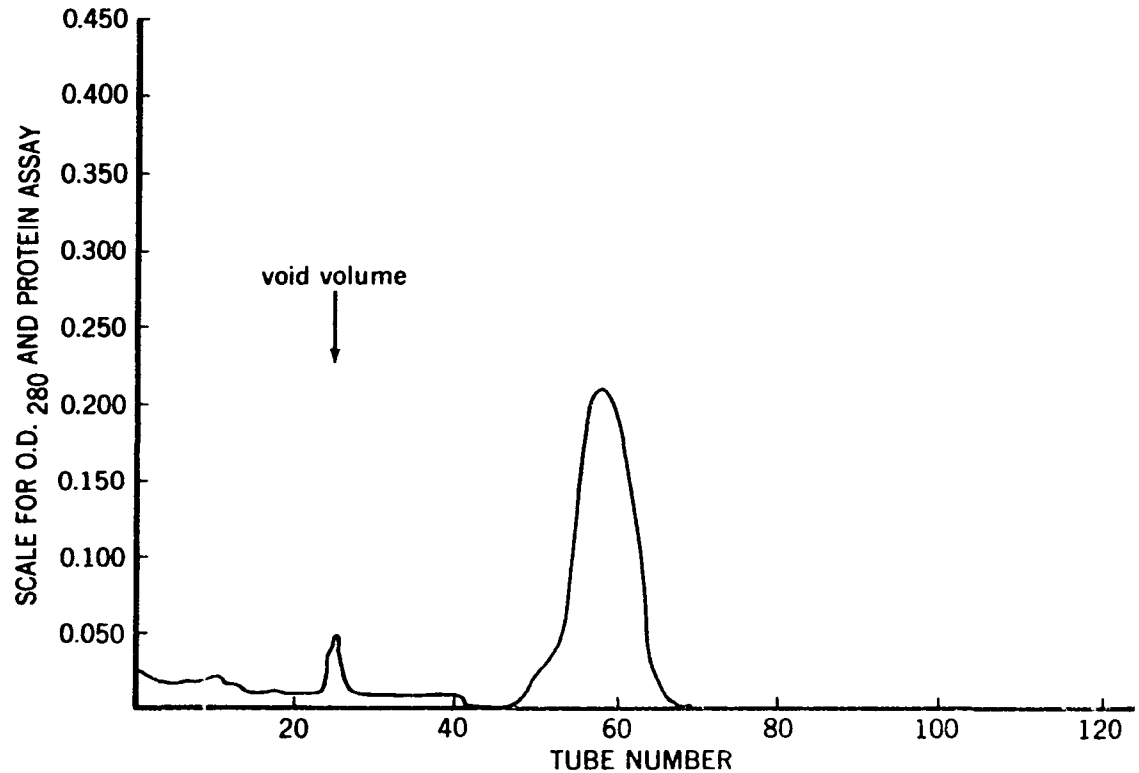

Figure 15. Sepharose 4B gel filtration of fraction III.

Fraction III was re-chromatographed as described in Figure 13.

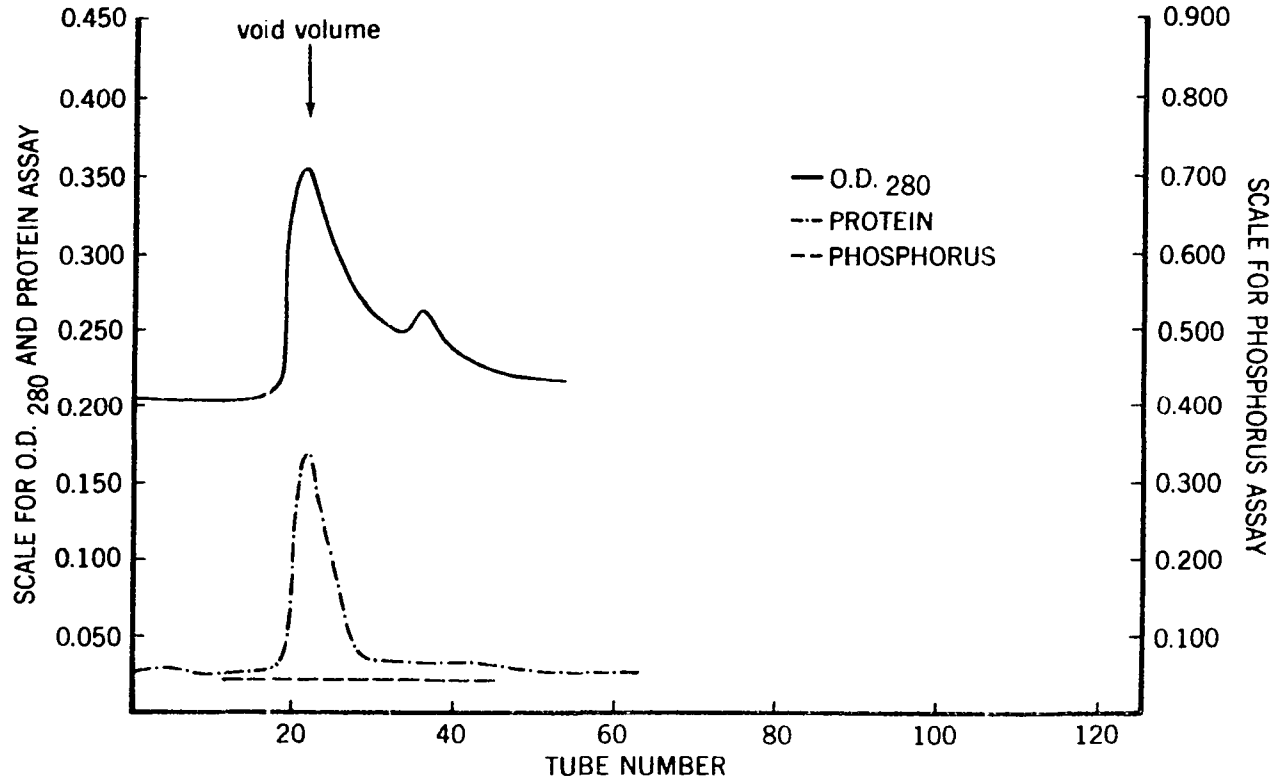

Figure 16. Sephadex G-200 gel filtration of fraction I.

Concentrated Fraction I obtained from Sepharose 4B gel chromatography was re-chromatographed on Sephadex G-200 gel. The fraction was applied to the gel and eluted with 0.05 M Tris HCl buffer (pH 7.6) at room temperature. The eluate was monitored for O.D.280 absorbance, protein content, and organic phosphorus.

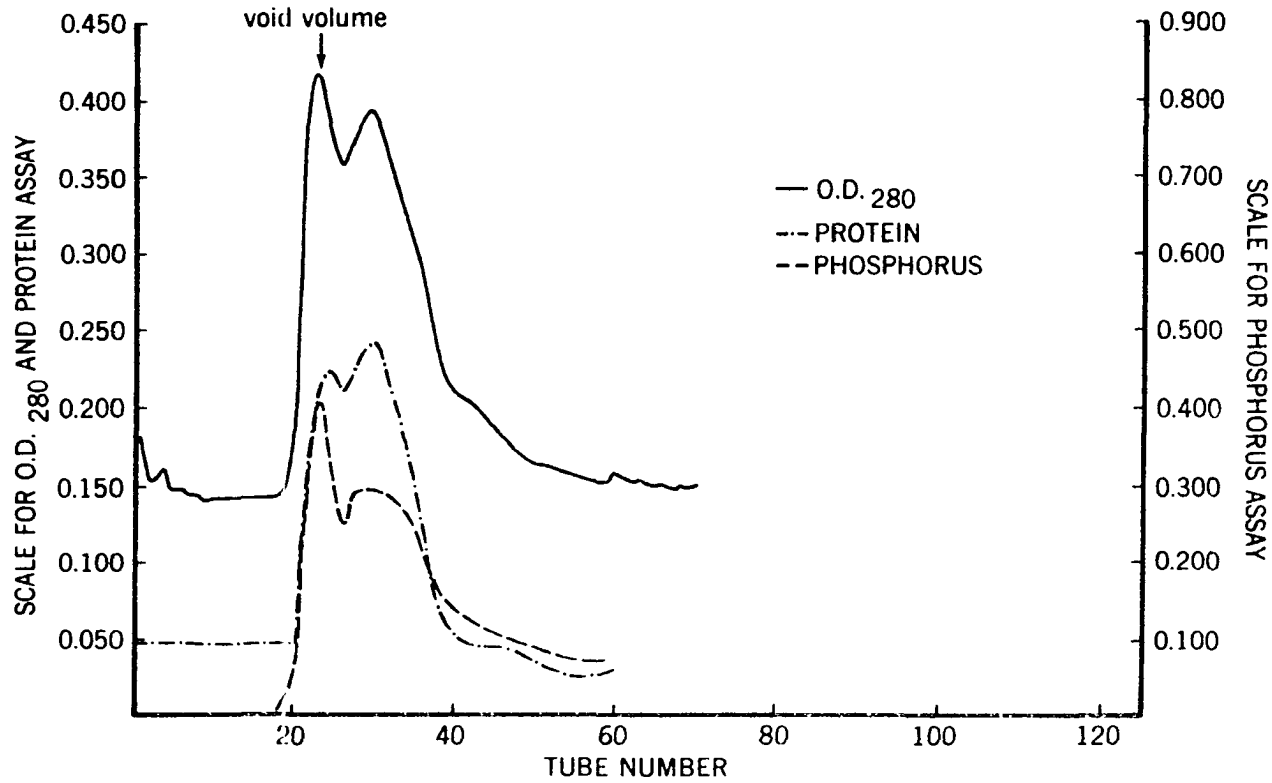

Figure 17. Sephadex G-200 gel filtration of fraction II.

Concentrated Fraction II obtained from Sepharose 4B gel chromatography was re-chromatographed as described under Figure 16.

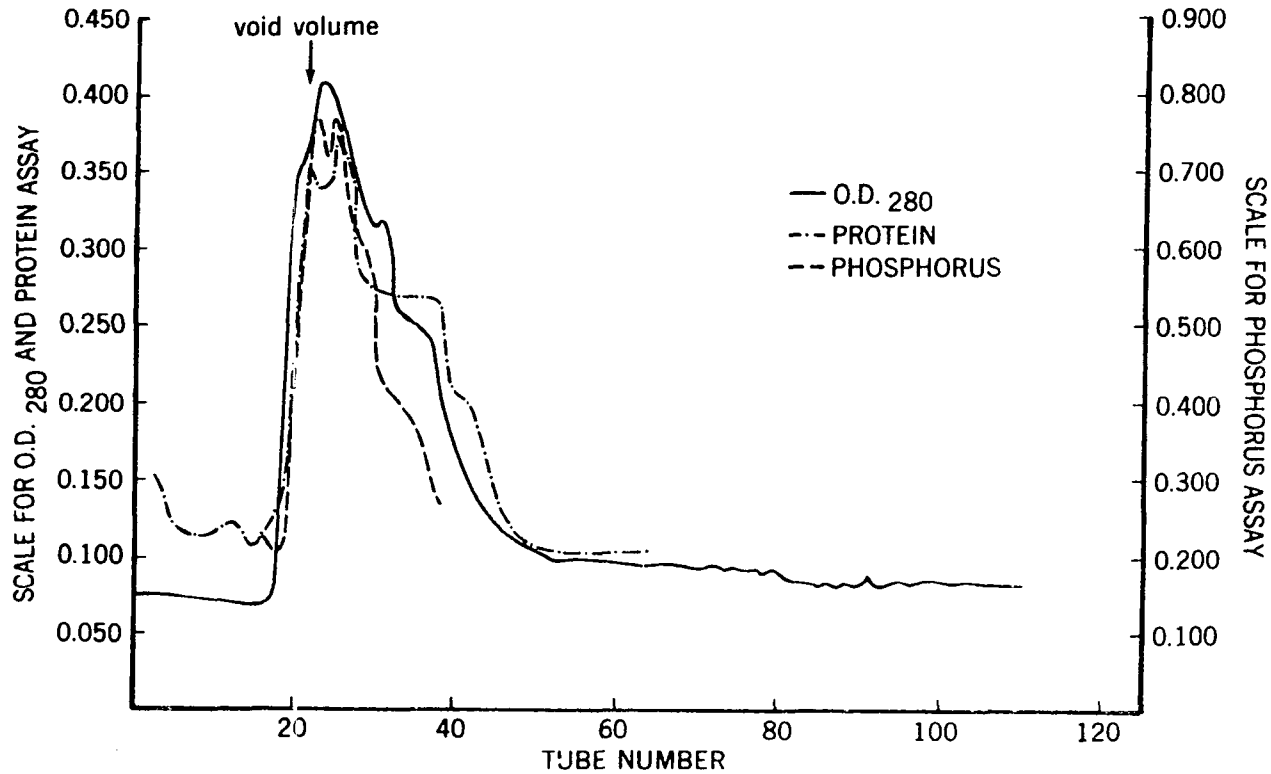

Figure 18. Sephadex G-200 gel filtration of fraction III.

Concentrated Fraction III obtained from Sepharose 4B gel chromatography was re-chromatographed as described under Figure 16.

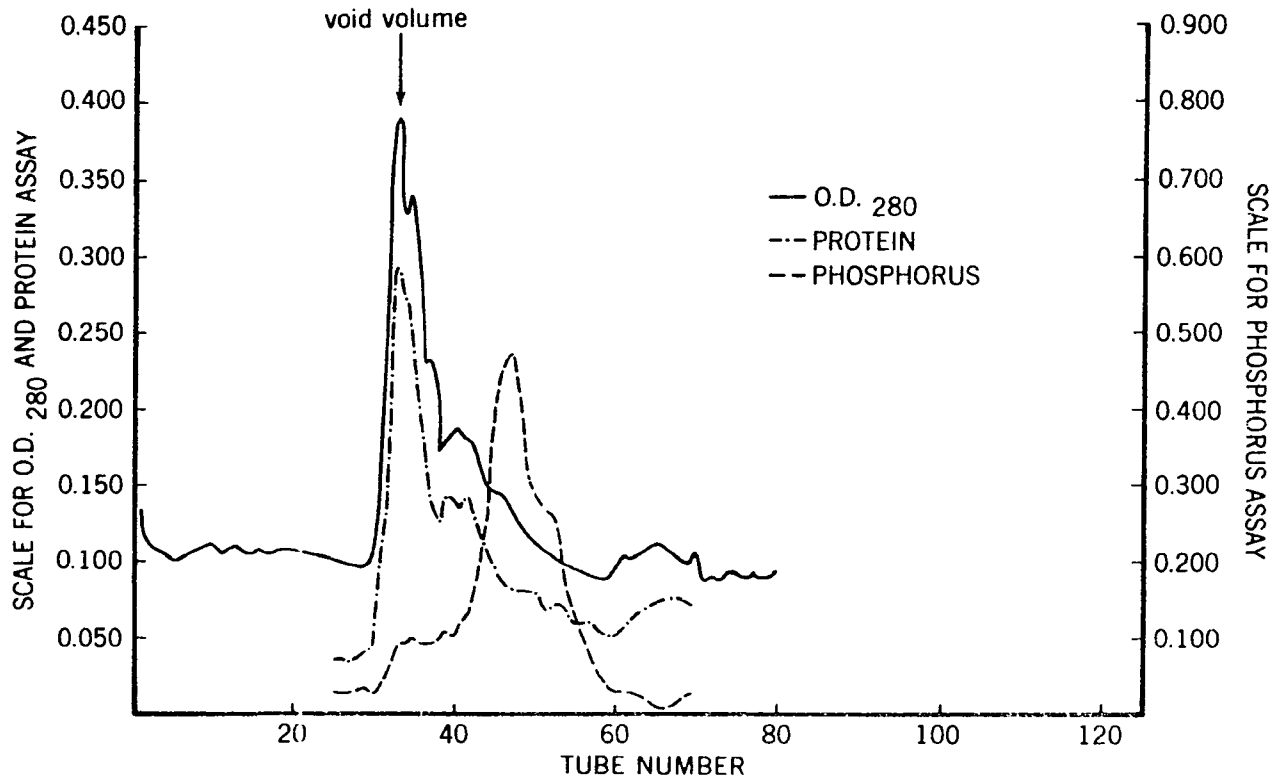

Figure 19. Sephadex G-200 gel filtration of SDS-solubilized RBC ghosts.

Erythrocytes ghosts solubilized with SDS were chromatographed as described under Figure 12, except that Sephadex G-200 gel was used and the fractions were monitored for $O.D._{280}$ absorbance, protein content, and phosphorus content.

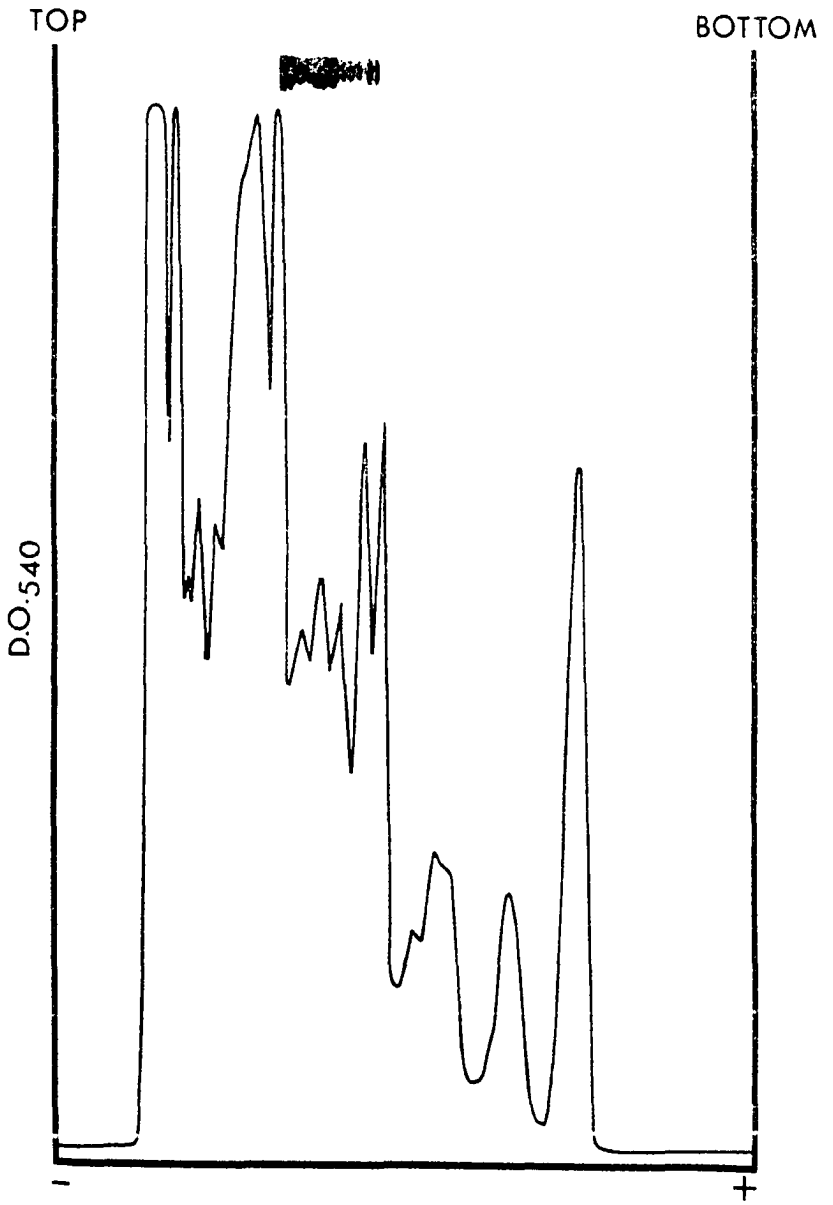

Figure 20. Polyacrylamide gel scan of RBC ghosts stained for protein.

Intact RBC ghosts were solubilized with SDS and electrophoresed on a 5% polyacrylamide gel as described in Methods. The gel was stained with Coomassie Blue dye for protein and scanned at 540 nm. The gel which was scanned is pictured at the top.

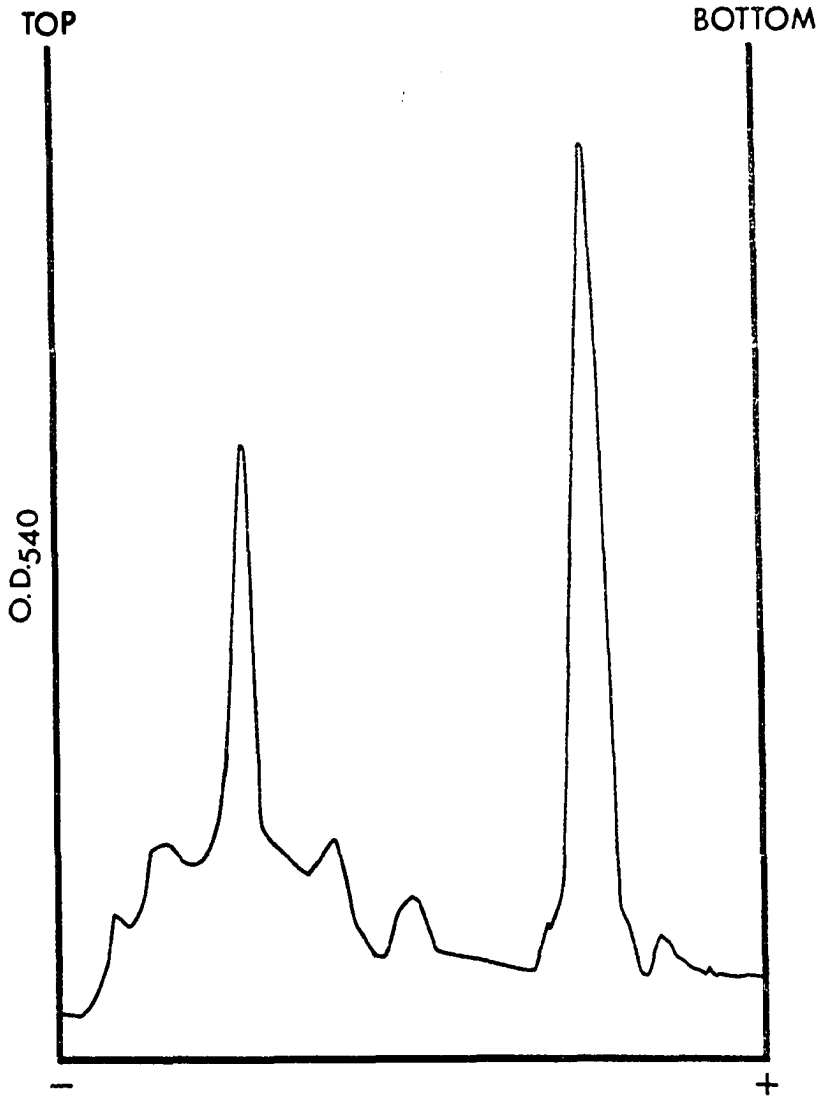

Figure 21. Polyacrylamide gel scan of RBC ghosts stained for carbohydrate.

Intact RBC ghosts were solubilized with SDS and electrophoresed on a 5% polyacrylamide gel as described in Methods. The gel was stained by the periodic acid-Schiff method for carbohydrate and scanned at 540 nm. The gel which was scanned is pictured at the top.

is the major glycoprotein and the other major peak closer to the anode is the lipid fraction containing glycolipids. There are four minor carbohydrate-positive bands. Two bands migrate more slowly and two more rapidly than the major glycoprotein.

The fractions obtained from the Sepharose 4B column (FI, FII, FIII) were electrophoresed and photographs of the gels are given in Figure 22. All three of the protein fractions are heterogeneous but their rate of migration is compatible with their elution volume on gel filtration. Some separation is obtained for the carbohydrate-positive components. FI does not contain any carbohydrate while FII has only a single band at the position of the major glycoprotein. However, FIII contains all of the four carbohydrate-positive bands characteristic for the whole ghosts.

Immunology

In Table 15, under Experiment A, hemagglutination inhibition studies are summarized for the RBC ghosts. Both blood group A activity and blood group M activity which were identified in the original intact RBC's are present in the ghosts. The inhibition is complete for both of the activities within the limits of the testing system. No attempt was made to determine if greater inhibitory activity is present.

The immunological properties of the three major fractions separated by gel filtration (FI, FII, FIII) were studied with their corresponding antibodies (a-FI, a-FII, a-FIII). The results show (Figure 23) that FI reacts with a-FI (2 precipitin lines) and with a-FII (1 precipitin line), but not with a-FIII. FII has an almost identical pattern, except that the reaction between FII and a-FII is more prominent than

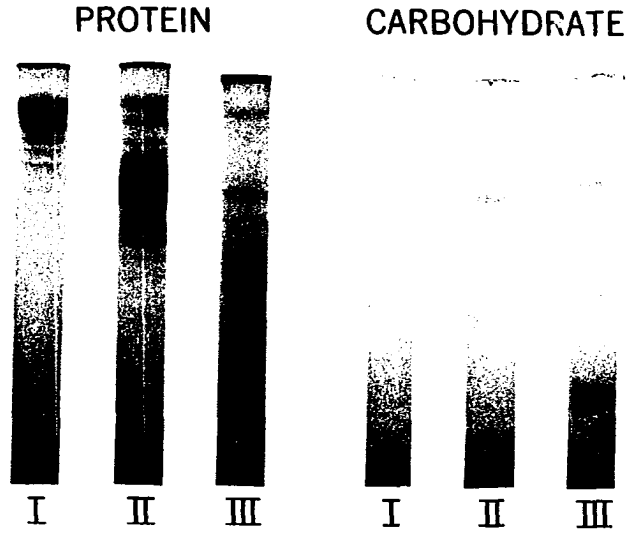

Figure 22. Polyacrylamide gels of fractions from Sepharose 4B column.

Concentrated Fractions I, II, and III were electrophoresed and stained for protein and carbohydrate as described under Figures 20 and 21, respectively.

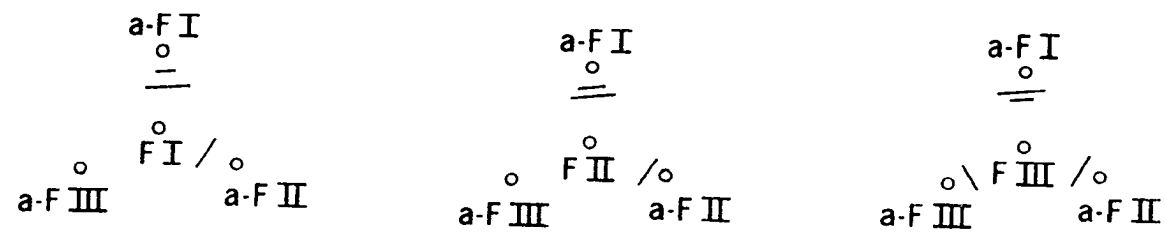

Figure 23. Immunodiffusion studies on fractions I, II, III and their antibodies.

Ouchterlony double-diffusion studies were performed using concentrated fractions, from which SDS had been removed, in the center well and antibodies against the different fractions in the outer wells. Designations starting with F and a-F indicate individual fractions and their antibodies, respectively.

between FII and a-FI. The reactions of FIII are also very similar to those of FI, except that a precipitin line forms between FIII and a-FIII. Attempts to demonstrate whether either the antibodies or the antigens are shared could not be reproduced. The only definite conclusion is that FI contains at least two antigens, neither of which reacts with a-FIII. The data also suggest that FIII contains a protein which is not present in FI and FII.

Additional studies were performed with a-FI, a-FII, and a-FIII to determine by the hemagglutination inhibition technique if RBC agglutinating activity or anti-A activity are present in the three antibody preparations. a-FII and a-FIII contain agglutinating antibodies against RBC's, although the agglutination is not inhibited by blood group A substance. a-FI does not produce RBC agglutination. These findings suggest that a-FI reacts with internal proteins while a-FII and a-FIII react with external surface proteins.

Fractionation of Ghosts by Aqueous Extraction

Several aqueous systems were used to extract proteins from the RBC ghosts as described in the Methods section. The polyacrylamide gel electrophoresis patterns of proteins extracted with water at pH 7.0 are shown in Figure 24. The gels were obtained by electrophoresis of the concentrated protein obtained at each extraction step. The extracted proteins are heterogeneous with little evidence of selectivity. The very large proteins found after extractions 1, 2, and 3 are the only group of proteins which are readily identified as being removed with water.

Extraction with tetramethylammonium bromide (TMAB) appears to have some selectivity and the results with this agent are summarized in

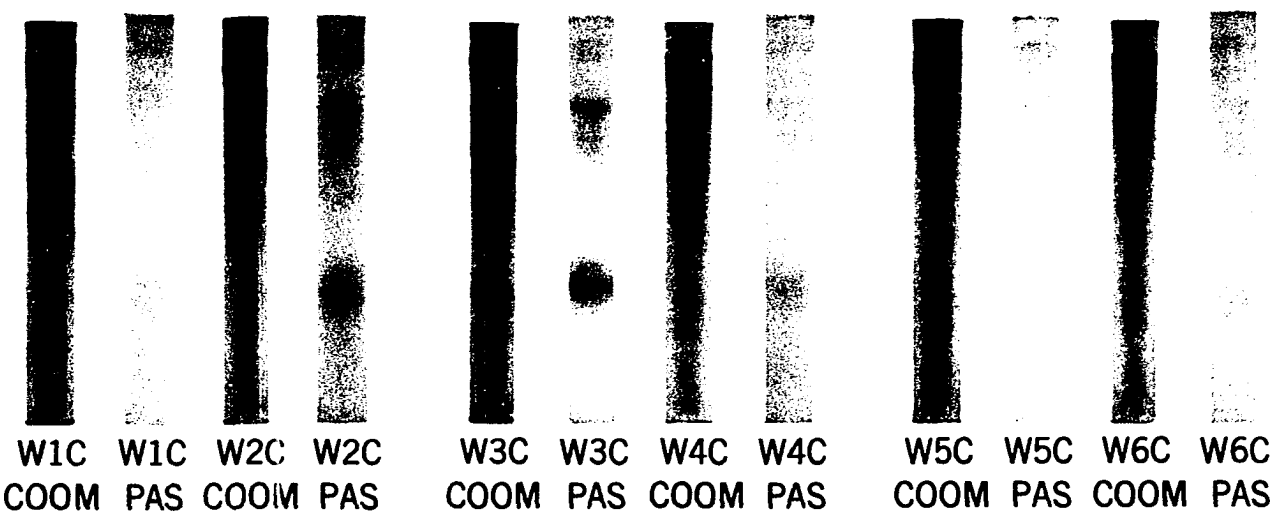

Figure 24. Polyacrylamide gels of single supernates from water extraction of RBC ghosts.

Polyacrylamide gel electrophoresis on concentrated single supernates from each extraction of RBC ghosts with distilled water was performed as described under Figure 20. Abbreviations: W, distilled water; numbers 1-6, the number of the extraction; C, supernate from single extraction; COOM, gel stained with Coomassie Blue dye; PAS, gel stained with periodic acid-Schiff technique.

Figure 25. The photographs of polyacrylamide gels reveal that with the first TMAB extraction a heterogeneous group of proteins is removed, but there are predominantly two major protein groups extracted. The first group is represented by a high molecular weight doublet which is present in relatively small amounts compared to the amount of those proteins extracted with water. The other protein group is also a doublet which falls within the middle range of molecular weights for the ghost proteins. Although they stain slightly with carbohydrate stains in this figure, repeated studies reveal that these proteins do not contain a carbohydrate moiety. Little protein was removed from the RBC ghosts after the second extraction with TMAB.

Two additional aqueous systems were used to extract proteins from the membrane and the results are shown in Figure 26. The first system was a dilute Triton X-100 solution. The extracted proteins are quite heterogeneous and include the carbohydrate-containing proteins. After the third extraction with this solution, no ghosts remain since they have been completely solubilized. The second system, a sodium chloride solution, is difficult to evaluate. Almost all extractable protein is removed on the first extraction and is clearly heterogeneous. Satisfactory polyacrylamide gels could not be obtained on the extracted protein. The high salt concentration may have aggregated the protein into a physical state in which adequate separation is not possible.

The residues remaining after these repeated extractions were also studied with polyacrylamide gel electrophoresis and the results are given in Figure 27. No residue is shown for the Triton X-100 extraction, since the membrane was completely solubilized by the procedure. However,

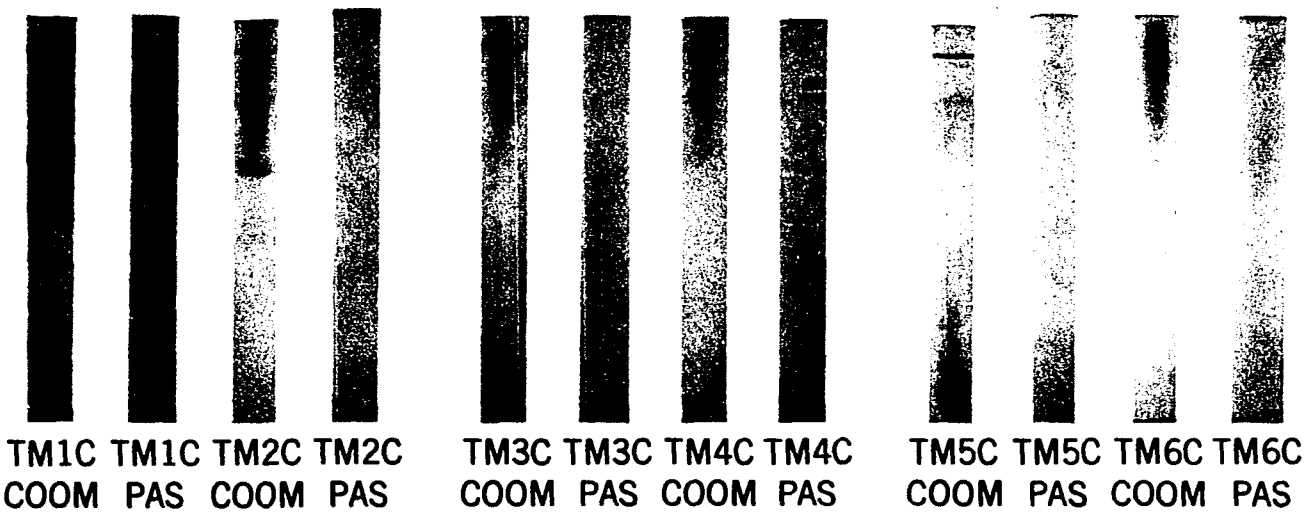

Figure 25. Polyacrylamide gels of single supernates from TMAB extraction of ghosts.

Polyacrylamide gel electrophoresis on concentrated single supernates from each extraction of RBC ghosts with 0.1 M tetramethylammonium bromide (TMAB) was performed as described under Figure 20. Abbreviations: TM, 0.1 M TMAB; numbers 1-6, the number of the extraction; C, supernate from single extraction; COOM, gel stained with Coomassie Blue dye; PAS, gel stained with periodic acid-Schiff technique.

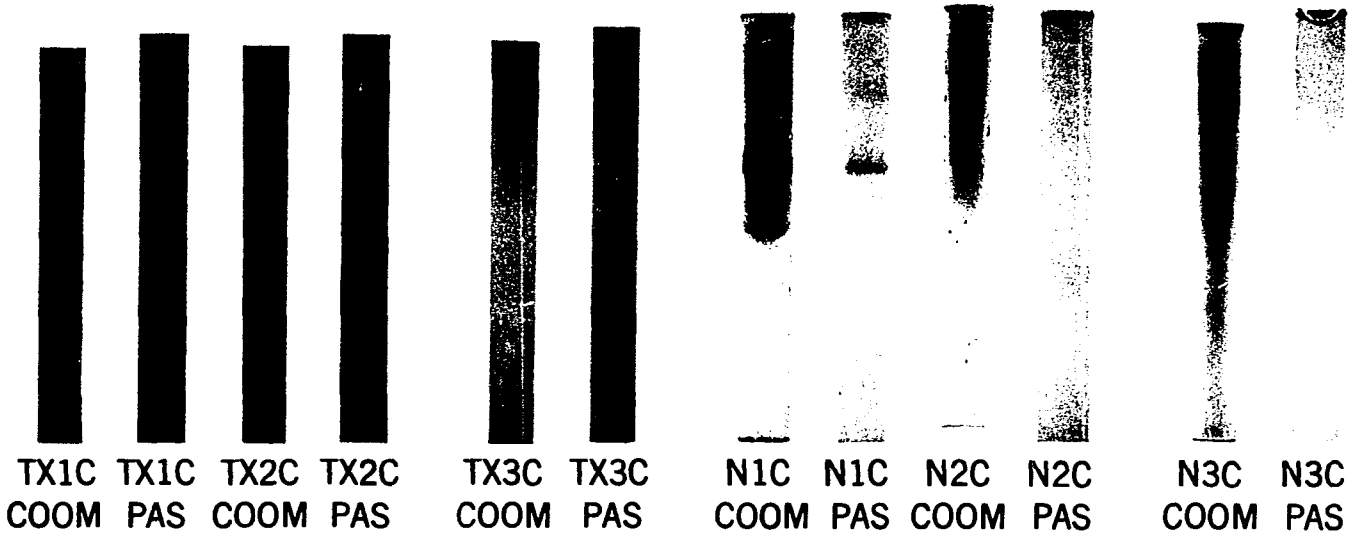

Figure 26. Polyacrylamide gels of single supernates from Triton X-100 and saline extraction of RBC ghosts.

Polyacrylamide gel electrophoresis on concentrated single supernates from each extraction of RBC ghosts with either 0.1% Triton X-100 or 0.5 M NaCl was performed as described under Figure 20. Abbreviations: TX, 0.1% Triton X-100; N, 0.5 M NaCl; numbers 1-3, the number of the extraction; COOM, gel stained with Coomassie Blue dye; PAS, gel stained with periodic acid-Schiff technique.

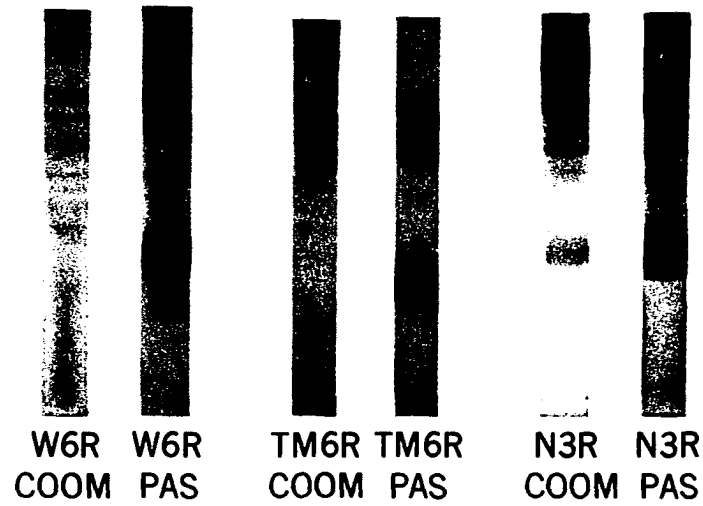

Figure 27. Polyacrylamide gels of residues after extraction with water, TMAB, or Triton X-100.

Residues of RBC ghosts extracted repeatedly with various agents were solubilized with SDS and electrophoresed on 5% polyacrylamide gels in the presence of 1% SDS. The gels were stained for protein (COOM) and carbohydrate (PAS). Abbreviations: W, after water extractions; TM, after 0.1 M TMAB extractions; N, after 0.5 M NaCl extraction; number 6, number of extractions; R, residue of extracted ghosts.

photographs of the residues after water, TMAB and sodium chloride extractions are reproduced. The proteins are still quite heterogeneous with many bands present in all molecular weight groups. In addition, the phospholipid band is demonstrated by carbohydrate staining as are some of the protein bands.

The results of extraction studies performed with 1.0 mM sodium EDTA are reported in the following sections.

Fractionation of Ghost with Hypotonic Sodium EDTA - the Supernates

Quantitation

The initial evaluation of hypotonic EDTA extractions of RBC ghosts is based on the procedure described by Juliano and Rothstein (83). The ghosts are extracted first with 1.0 mM sodium EDTA solution followed by a second extraction with a dilute base (either sodium hydroxide at pH 11.0 or ammonium hydroxide at pH 10.5). The average of triplicate determinations of the proteins and phospholipids removed by this procedure are presented in Figure 28. Approximately 55% of the protein is extracted by the EDTA-sodium hydroxide extraction sequence. No phospholipid is obtained with the EDTA solution, but approximately one-fourth is removed by the NaOH extraction.

Because the possibility existed that the type of base might be important, the experiment was repeated comparing sodium hydroxide (pH 11.0) and ammonium hydroxide (pH 10.5). The data for quadruplicate determinations are given in Figure 29. Both procedures resulted in an almost identical (50%) removal of the protein. However, ammonium hydroxide extracts approximately 5% more of the total phospholipid than does sodium

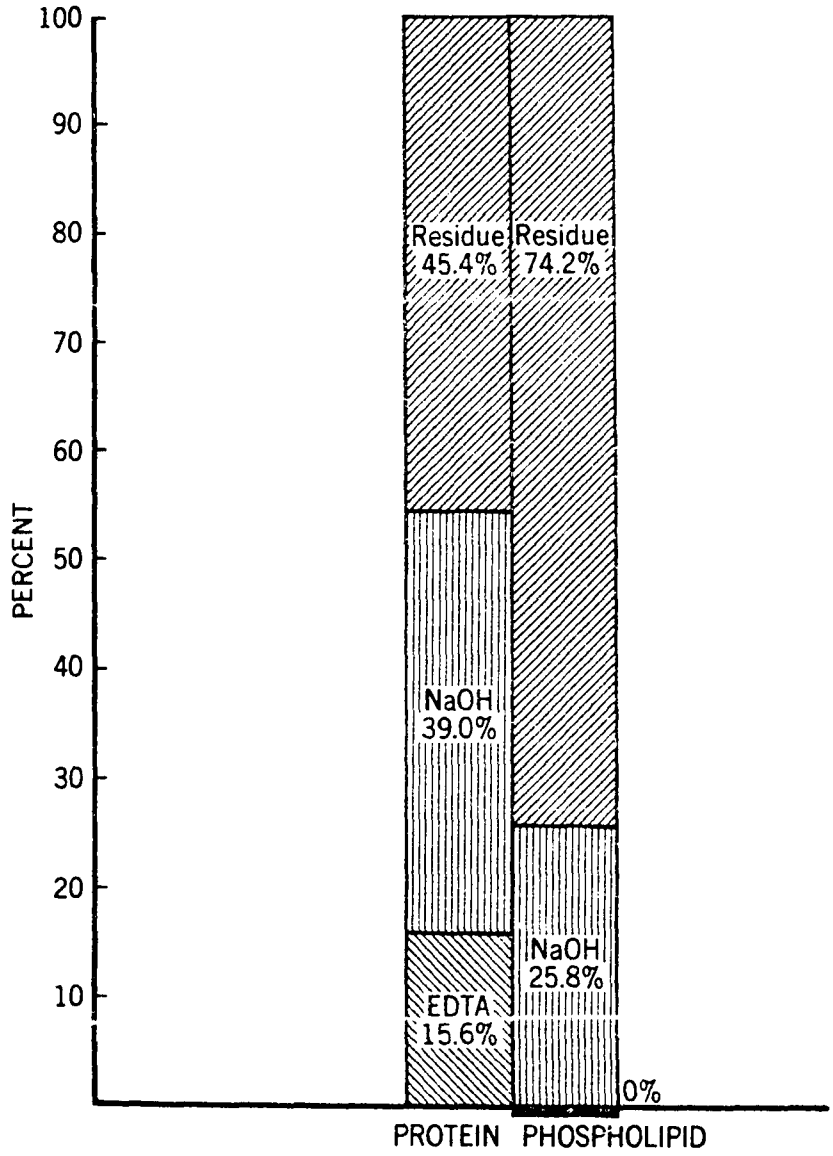

Figure 28. Quantitation of aqueous extraction.

The column on the left shows the percentages of the total protein removed by sequential extractions with EDTA and dilute sodium hydroxide (pH 11.0) and the amount of protein remaining with the residue after extraction. The column on the right shows the equivalent values for the percentage of organic phosphorus. Note that no organic phosphorus was removed by EDTA extraction in this experiment.

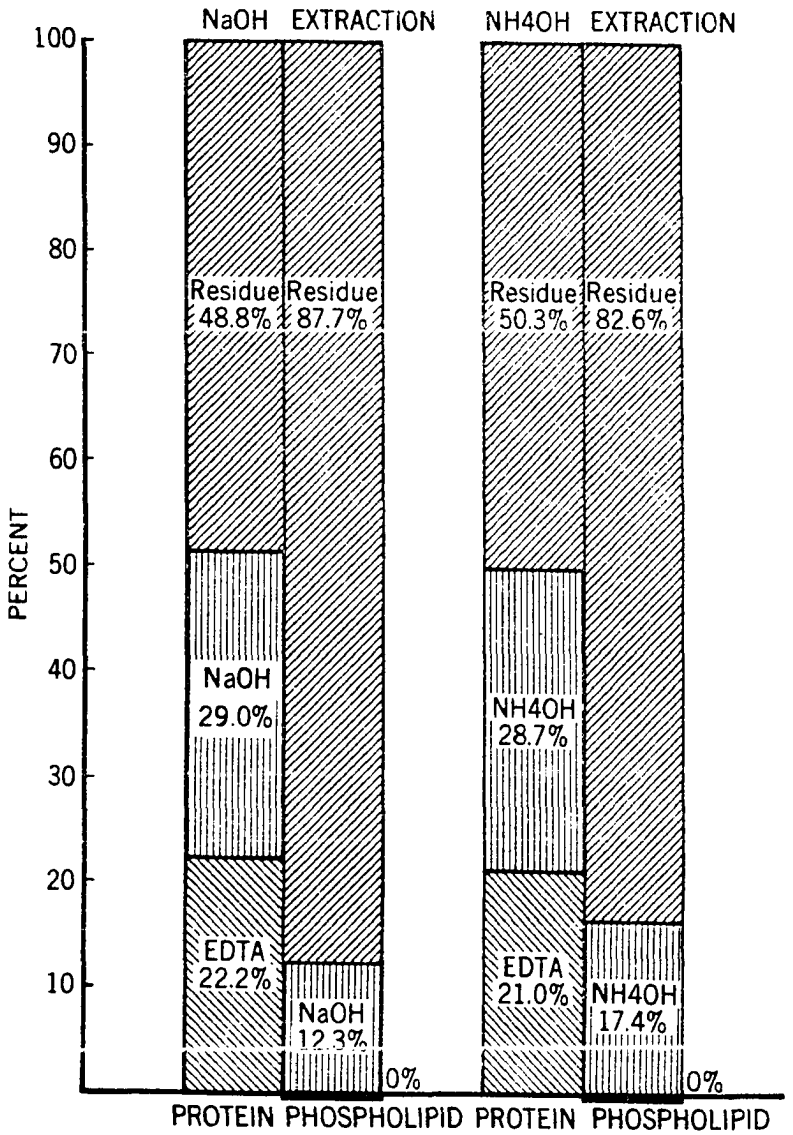

Figure 29. Quantitation of extraction with different bases.

The two columns on the left give the results for sequential EDTA-base extractions using sodium hydroxide (pH 11.0). The columns on the right give the results for equivalent extractions using ammonium hydroxide (pH 10.5). Note that ammonium hydroxide produced a five per cent greater extraction of organic phosphorus than did sodium hydroxide extraction.

hydroxide.

Since sodium hydroxide appears to remove less phospholipid than ammonium hydroxide, the extractions with EDTA and sodium hydroxide were more carefully evaluated. As shown in Figure 30, the incubation time has little effect upon the EDTA extraction of either phospholipid or protein. This contrasts with the sodium hydroxide extraction as shown in Figure 31. The removal of protein and phospholipid increases until about 40 minutes, after which little change is noted.

Since the extractions with sodium hydroxide resulted in the solubilization of more phospholipid than that with EDTA, the effects of repeated extractions with hypotonic EDTA as the only solvent were evaluated. In Figure 32 the first two extractions with EDTA solution are shown to remove approximately 25% of the protein in the ghost or residue (E1R). Thereafter, the amount removed falls off rapidly until less than 5% of the remaining protein is extracted with the fifth and sixth extractions. The data for phosphorus follow the same pattern, although in this experiment no correction was made for the presence of inorganic phosphate. Part of the change in the phosphorus content may have been the result of the removal of inorganic phosphate present in the buffer used for ghost preparation.

The experiment was repeated with two major changes. The first was to correct the value for the total phosphorus for the presence of inorganic phosphate. The second was to combine the material which was extracted at each step in order that a cumulative percentage of original RBC ghost protein and organic phosphorus removed could be calculated. The data for the percentages of protein and organic phosphorus as well

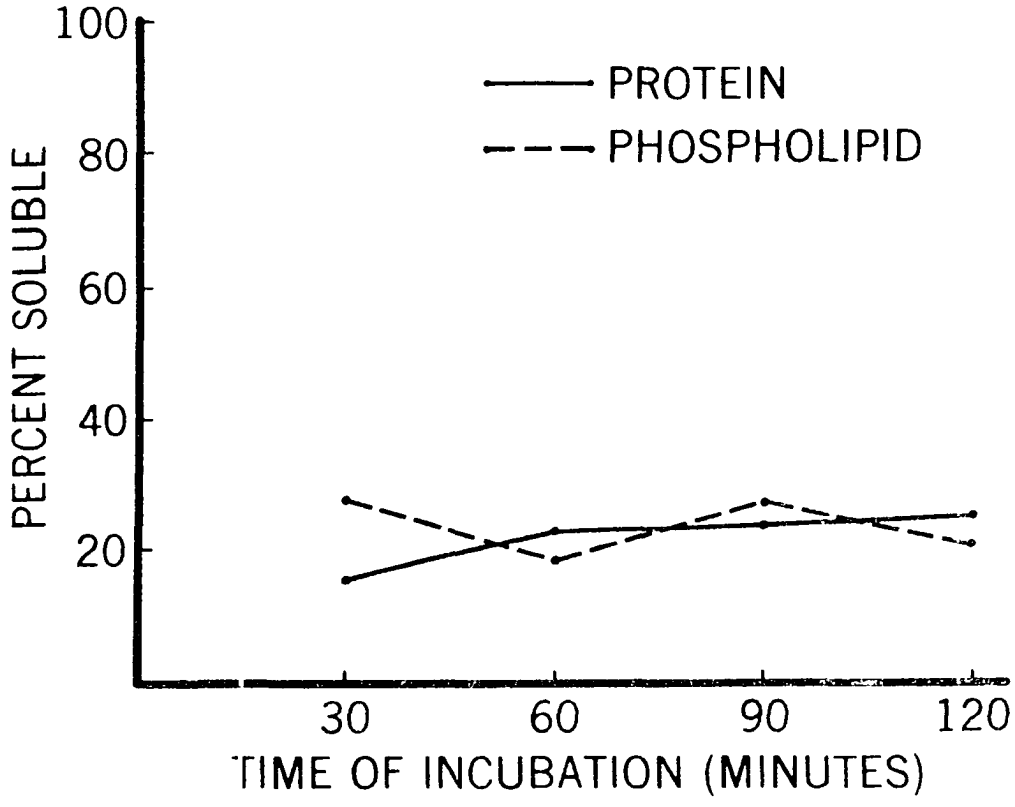

Figure 30. The effects of incubation time upon solubilization of ghost proteins.

Time of incubation of ghosts in the EDTA solution is shown on the abscissa. The per cent of protein or organic phosphorus solubilized is shown on the ordinate.

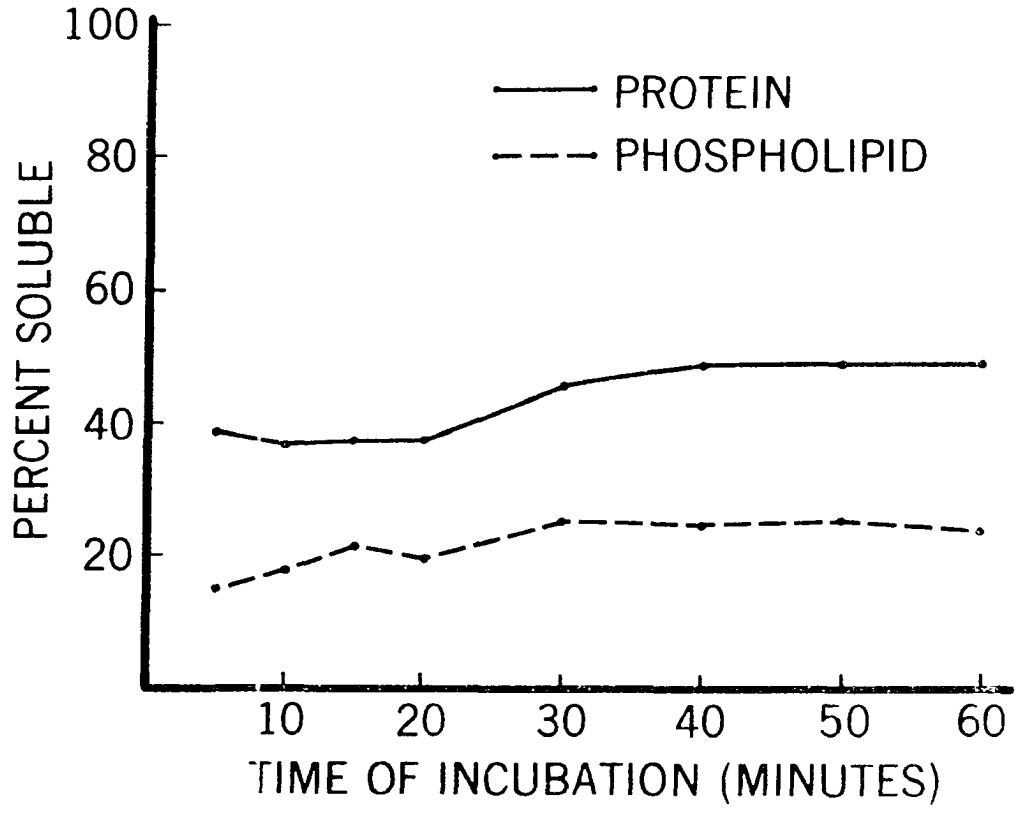

Figure 31. Effects of time upon sodium hydroxide extractions of ghost proteins.

The erythrocyte ghosts which had already been incubated with hypotonic EDTA for 60 minutes were incubated with sodium hydroxide (pH 11.0) for intervals up to 60 minutes. The percentages of proteins and organic phosphorus released are shown on the ordinate.

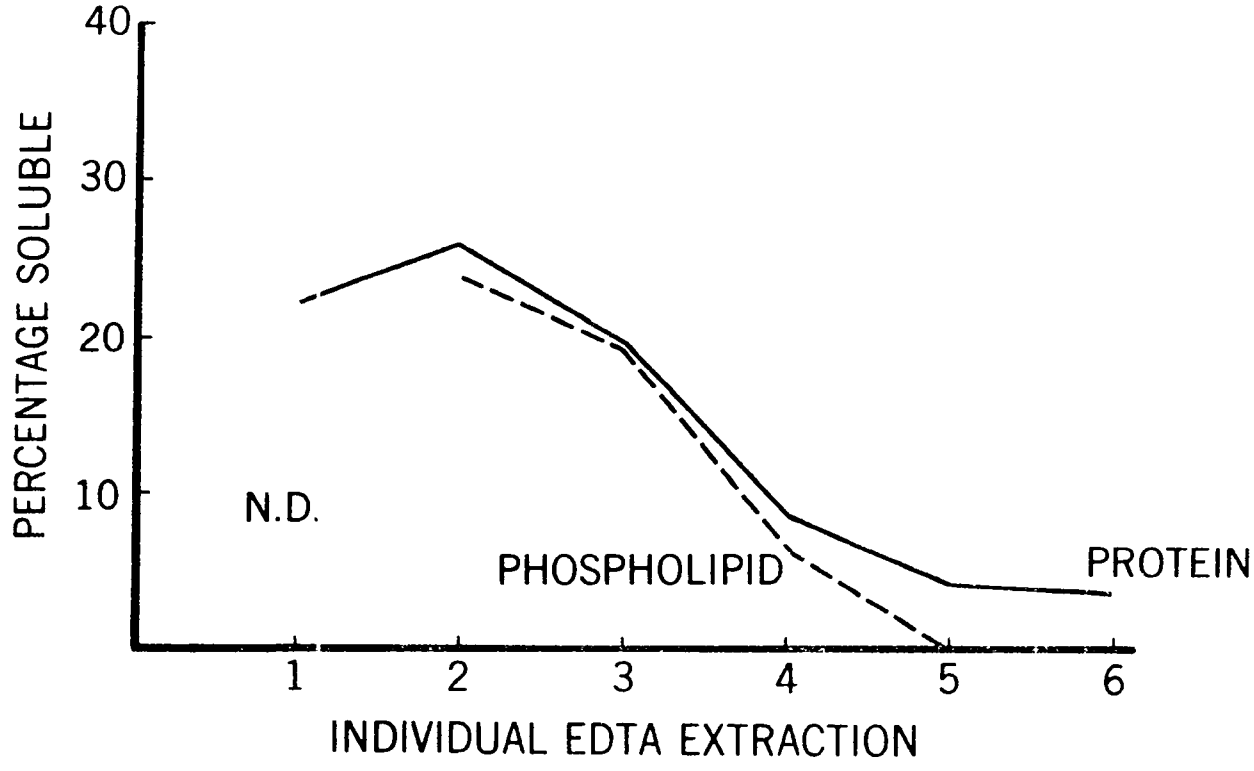

Figure 32. Protein and phospholipid removal with repeated EDTA extractions.

The amount of protein and phospholipid removed with each EDTA extraction is prevented as the percentage found in the supernatant. The percentage which is shown for each extraction represents the percentage solubilized based on the starting material for each step. For example, the second extraction removed approximately 25 per cent of the protein which remained after the first extraction with EDTA.

as the phospholipid:protein ratio of each residue are given in Figure 33. The first three EDTA extractions remove about 54% of the total membrane protein. Only 3% more of the total protein is extracted by the last three steps. Approximately 30% of the total organic phosphorus is obtained from the first two steps with an additional 4% in the subsequent four steps. The phospholipid:protein ratio of the residue after each extraction initially rose before falling to about the starting value after six extractions. This disagreement with the protein and organic phosphorus data cannot be explained and may result from an inability to correct for the presence of inorganic phosphate ions.

Equivalent results were obtained in a control experiment using distilled water (pH 7.0), rather than hypotonic sodium EDTA, to extract proteins from RBC ghosts. The first three water extractions remove 49.4% of the ghost protein. The final three steps remove an additional 10.6% for a total water-extractable protein of 60.0%. The amount of organic phosphorus removed was not determined.

Composition of Supernates

The material obtained with repeated EDTA extractions accounts for about 57% of the total membrane protein and about 34% of the total membrane organic phosphorus. Enzymatic activity present in the extracted proteins is shown in Table 13 under Experiment B. Glycolytic enzymes still present in the RBC ghosts are completely removed with the initial EDTA extraction. Lactic dehydrogenase and aldolase activity are found in the initial supernate but not in any of the subsequent supernates. This is different from the data obtained for the membrane-associated enzymes. Acetylcholinesterase (AChEase) activity is found only in the

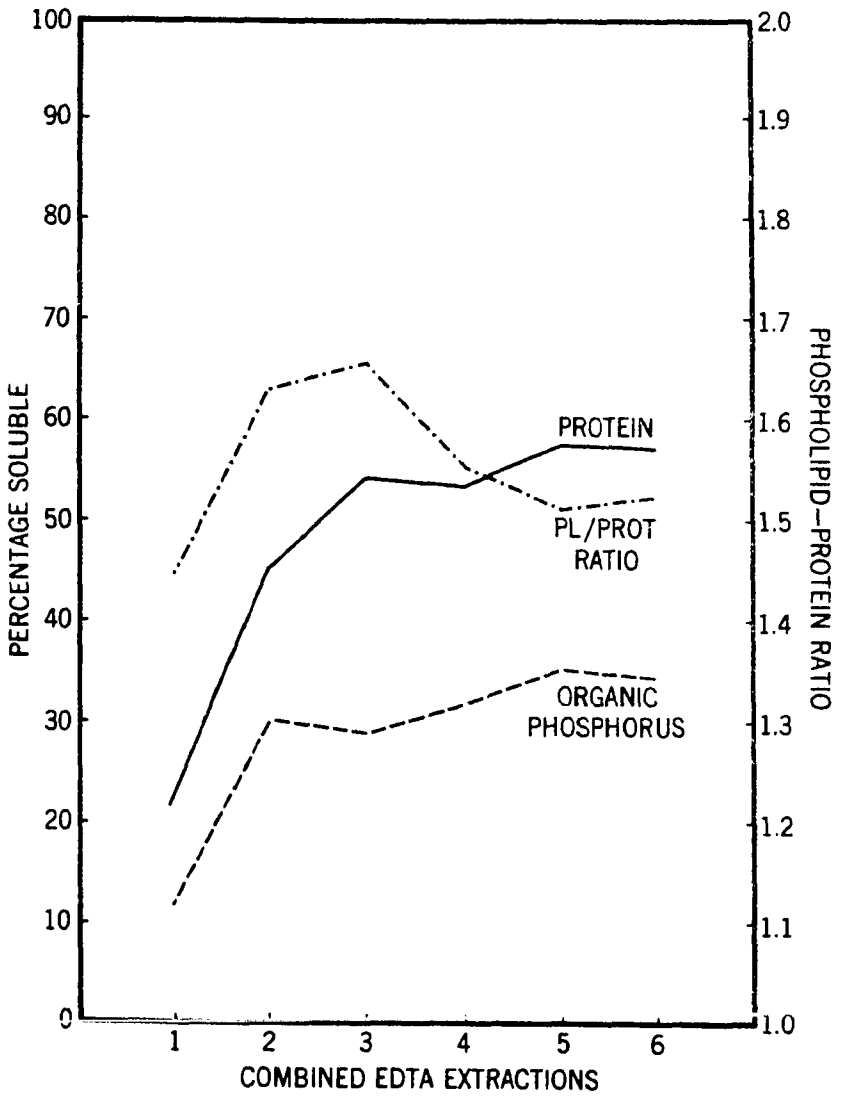

Figure 33. Protein and phospholipid removal with repeated EDTA extractions.

The protein and phospholipid removed at each step were combined so that the percentages solubilized are presented on a cumulative basis. For example, approximately 55 per cent of the original erythrocyte ghost protein was removed by 5 extractions with hypotonic EDTA.

first three supernates. The level of NADH oxidoreductase (NaOase) activity increases in the supernate with each extraction. The data for AChEase are compatible with a loss of organic phosphorus during the extraction procedure. There is no explanation for the peculiar findings obtained for the NaOase enzyme.

Polyacrylamide Gel Electrophoresis

The protein-stained polyacrylamide gel electropherogram of the combined supernates from six EDTA extractions is shown in Figure 34. The extracted proteins can be divided into three major molecular weight groups. The first group consists primarily of a doublet of two large proteins which are better demonstrated when smaller amounts of protein are applied. The second group (next largest in size) is composed of four sharp peaks. Five minor peaks of lowest molecular weight form the third group. No quantitation of the percentage of protein in each major group is possible. As demonstrated in Figure 35, little carbohydrate-containing protein is present in the supernates from six extractions.

The protein pattern of the combined supernates for each of the first five extractions is similar to the pattern obtained for the six combined EDTA supernates. In Figure 36, the combined supernates from the first through the fifth extractions are shown to have similar protein patterns, except for the combined supernate from five extractions, which was contaminated with residue.

The polyacrylamide gel electrophoresis of individual EDTA extraction steps is presented in Figure 37. As expected from the data on quantitation, most of the protein is removed in the first three steps. Although the protein is heterogeneous, there does appear to be a slight

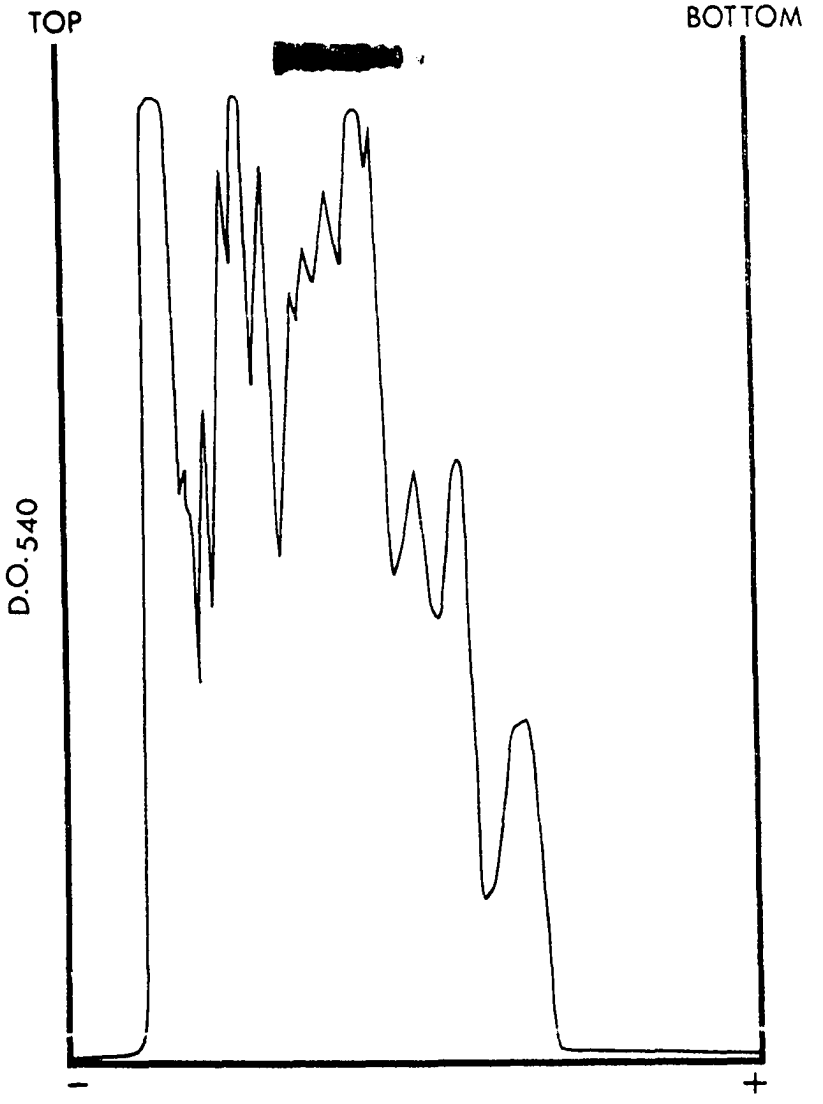

Figure 34. Polyacrylamide gel scan of combined supernate (E6S) stained for protein.

Concentrated supernatants from six extractions were combined and concentrated before electrophoresis and staining for protein as described under Figure 20.

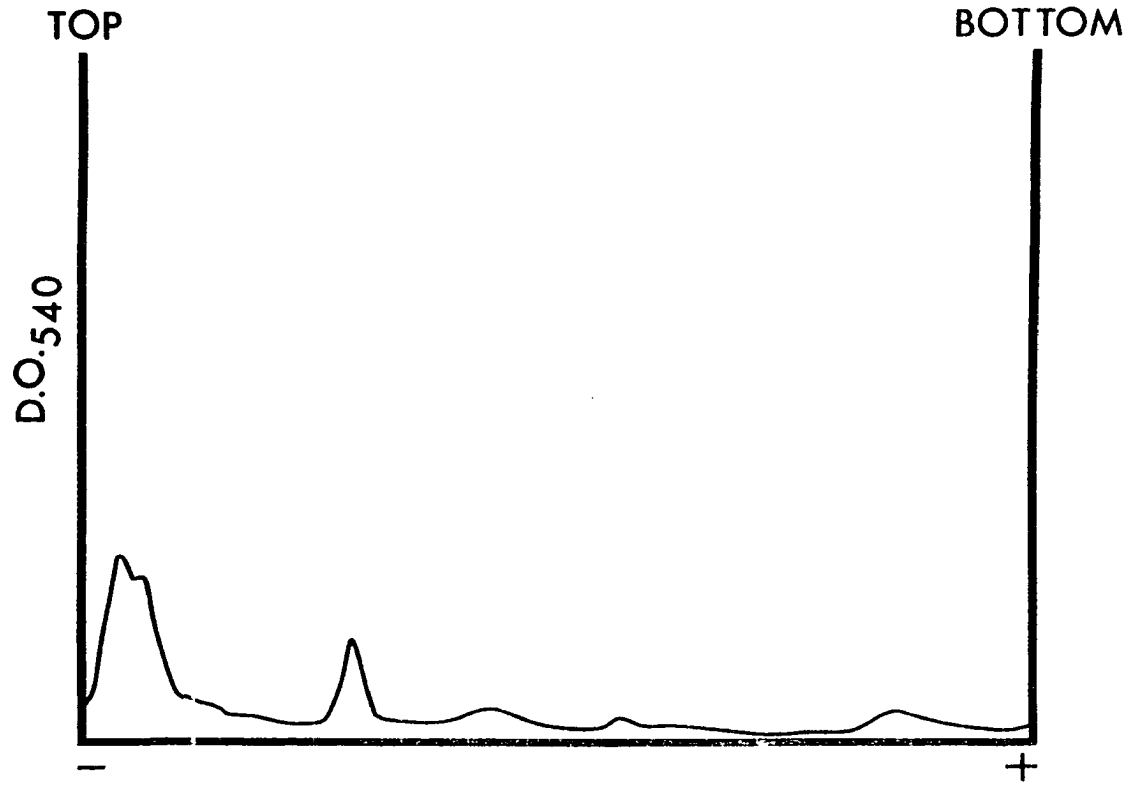

Figure 35. Polyacrylamide gel scan of combined supernate (E6S) stained for carbohydrate.

Combined supernatants from six extractions were combined and concentrated before electrophoresis and staining for carbohydrate as described under Figure 21.

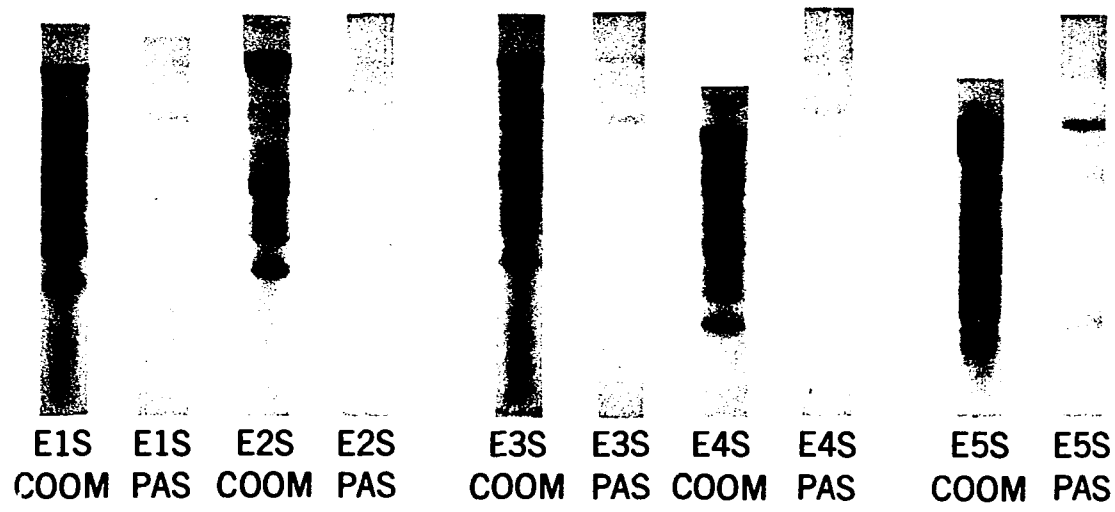

Figure 36. Polyacrylamide gel of combined supernates from EDTA extractions of RBC ghosts.

Polyacrylamide gel electrophoresis on concentrated single supernates from each extraction of RBC ghosts with hypotonic EDTA was performed as described under Figure 20. Abbreviations: E, hypotonic EDTA; numbers 1-6, the number of the extraction; S, combined supernatant from designated extractions; COOM, gel stained with Coomassie Blue dye; PAS, gel stained by periodic acid-Schiff technique.

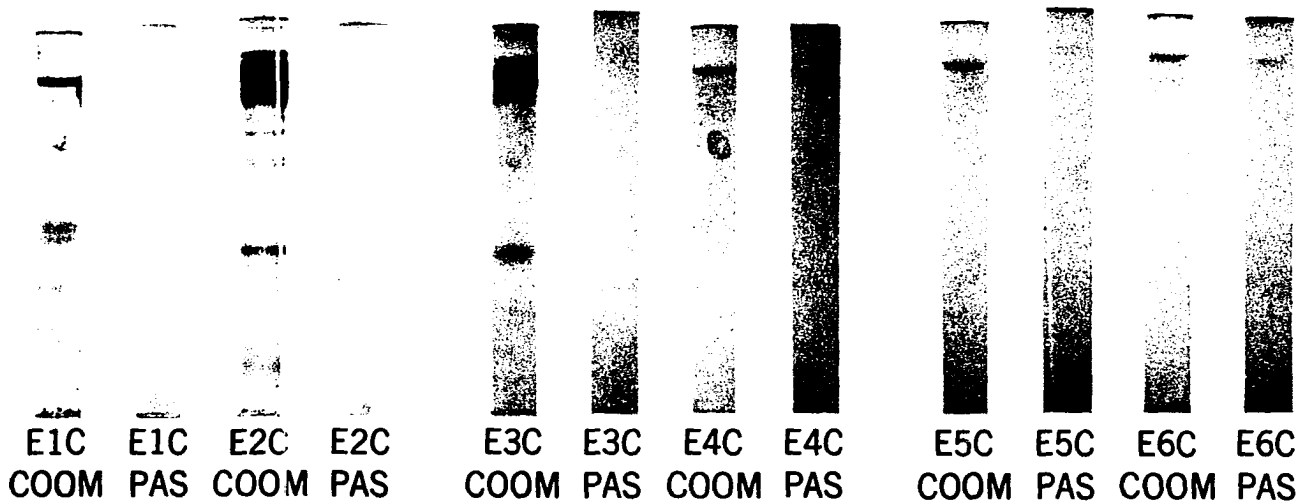

Figure 37. Polyacrylamide gels of single supernates from hypotonic EDTA extraction of RBC ghosts.

Polyacrylamide gel electrophoresis on concentrated single supernates from each extraction of RBC ghosts with hypotonic EDTA was performed as described under Figure 20. Abbreviations: E, hypotonic EDTA; numbers 1-6, the number of the extraction; C, supernate from single extraction; COOM, gel stained with Coomassie Blue dye; PAS, gel stained by periodic acid-Schiff technique.

difference in the molecular weight groups obtained with each extraction. The first EDTA step removes a small amount of high molecular weight protein with a substantial number of lower molecular weight proteins. The second EDTA step extracts the most heterogeneous group of proteins as large amounts of both high and low molecular weight proteins are present. The third EDTA step appears to be most selective, removing primarily high molecular weight proteins. The fourth, fifth, and sixth steps take out only small quantities of nigh molecular weight proteins. Little carbohydrate-containing protein is removed by the six EDTA extraction steps.

Immunology

The individual EDTA supernates were tested for their blood group activity by hemagglutination inhibition and the results are given in Table 15 under Experiment B. Significant blood group A activity is present in the first two EDTA supernates with slightly less activity present in the third. No group A activity is found in the last three supernates. A slightly different result is obtained for blood group M activity where only minimal activity is present in the first three supernates and none in the last three supernates. Both blood group A and blood group M activity are still present in the extracted residue.

Immunodiffusion data are given in Figure 38. None of the EDTA supernates react with antibodies produced against FIII. Supernates from the first three extractions share an antigen which reacts with both anti-Fraction I (a-FI) and anti-Fraction II (a-FII). In addition, a-FII contains an antibody which reacts with an antigen shared by the supernates from the third, fourth and fifth EDTA extractions. Since the two immuno-

TABLE 15

BLOOD GROUP ACTIVITY OF GHOSTS, RESIDUE AND SUPERNATES

Antigens	A Activity	M Activity
EXPERIMENT A:		
RBC ghosts	7 T+	4 T+
Residue (E6R)	7 T+	4 T+
EXPERIMENT B:		
E1C	6 T+	2 T
E2C	6 T+	2 T
E3C	4 T	1 T
E4C	1 T	0
E5C	0	0
E6C	0	0
Residue (E6R)	6 T+	4 T+

The letter T designates the number of tube dilutions which were inhibited by the given component. A plus following the letter T indicates that the component was not tested at greater dilutions and its ability to inhibit might have been more potent.

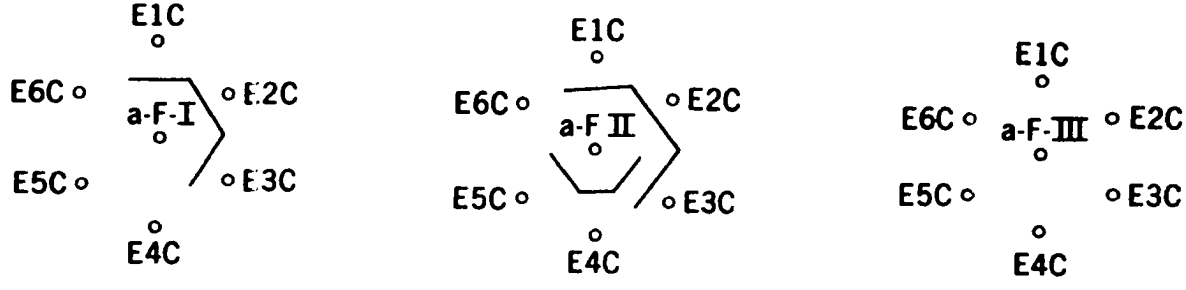

Figure 38. Immunodiffusion studies on individual EDTA supernates.

Ouchterlony double-diffusion studies were performed using concentrated individual EDTA supernates in the outer wells and antibodies against Sepharose 4B fractions (Fractions I, II, III) in the center wells. Designations starting with capital F indicate the individual fractions, while those starting with a small a indicate the antibodies.

precipitin lines with a-FII are shared by different EDTA supernates, a-FII must contain antibodies to at least two different proteins extracted by the hypotonic EDTA solution. The supernates do not react with antibodies prepared against glycoprotein-containing fractions, such as a-FIII. The immunodiffusion data support the results of polyacrylamide gel electrophoresis which demonstrate that little or no glycoprotein is extracted with EDTA solutions.

Fractionation of Ghosts with Hypotonic Sodium EDTA - the Residues

Composition

The residue after multiple EDTA extractions was extensively studied and data on its composition are given in Table 12. The protein accounts for only one-third of the total residue weight while the two lipid components, phospholipid and cholesterol, constitute over one-half of the weight. The carbohydrate content of the residue is substantially increased over that of the whole ghosts. The effect of EDTA extraction on the composition is best shown by comparing the ratios of the various components to the protein component in RBC ghosts and residue. The phospholipid:protein and cholesterol:protein ratios of residue are about 1.6 and 1.4 times greater than the corresponding values for RBC ghosts. The change is even more marked in the carbohydrate:protein ratio which doubles after extraction.

The changes in composition of individual sugars suggest that some carbohydrate may be lost during extraction in a selective manner. The glucose content increases while the content of mannose, fucose and galactosamine decreases. When these data are compared on the basis of

carbohydrate:protein ratio, the selectivity of extraction is again shown. Major increases in galactose, glucose, glucosamine and sialic acid are found. Only mannose and fucose decrease relative to the amount of protein. Since glycolipids were not measured in the EDTA supernates, it is not known whether the change in carbohydrate composition should be attributed to the loss of glycolipid or glycoprotein.

The enzyme content of the residue is given in Table 13 under Experiment A. No glycolytic enzyme activity remains in the residue. However, both AChEase and NaOase activities are present. The activities are lower than those in the RBC ghosts. A loss of these two enzyme activities into the EDTA extraction supernates does occur, although a change in enzyme conformation within the membrane might be an alternate explanation.

Amino acid analysis of the final EDTA residue is shown in Table 14. The content of basic amino acids (lysine, histidine, arginine) as well as the content of aspartic acid, glutamic acid and tyrosine are substantially reduced by the extraction procedure. In contrast to that change, the hydrophobic amino acids (valine, leucine, proline) as well as phenylalanine, glycine, serine, and threonine are increased. The increase in hexosamine content is caused by a relatively greater increase in glucosamine than galactosamine.

Electron microscopy reveals that major physical changes take place during the extraction process as shown in Figure 3. At lower magnifications the extracted residue consists of numerous microvesicles possessing a continuous, intact membrane. At the highest magnification, the typical trilaminar appearance of biological membranes is seen, al-

though the inner fuzzy coat found on RBC ghost membranes is absent. This change from the large, biconcave leaflets of RBC ghosts to many microvesicles is produced by the repeated EDTA extractions, since no physical manipulation (e.g., ultrasonication) of RBC ghosts was performed during the extraction procedure.

Gel Filtration of Solubilized Residue

Fractionation of the extracted residue was carried out by gel filtration on Sepharose 4B gel in the presence of SDS and the elution pattern is presented in Figure 39. The solubilized residue is separated into three major fractions called A, B, and C. Fraction A (F-A) is retarded the least and Fraction C (F-C) the most. The elution volumes for Fractions A, B, and C are about the same as those for Fractions I, II, and III of the RBC ghost. However, the peak for F-A is substantially smaller than that for Fraction I and the separation between Fractions B and C is better than between Fractions II and III. Rechromatography of Fractions A, B, and C is given in Figures 40, 41, and 42, respectively. Each fraction elutes at the appropriate volume, although a smaller amount of protein is present. Also, a larger void volume peak is produced than with rechromatography of Fractions I, II, and III. Gel filtration with Sephadex G-200 gel in the presence of SDS is not adequate for separation of the proteins as shown in Figure 43.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed on the residues remaining after each successive extraction. Photographs of the gels stained for protein and carbohydrate are shown in Figure 44. With each

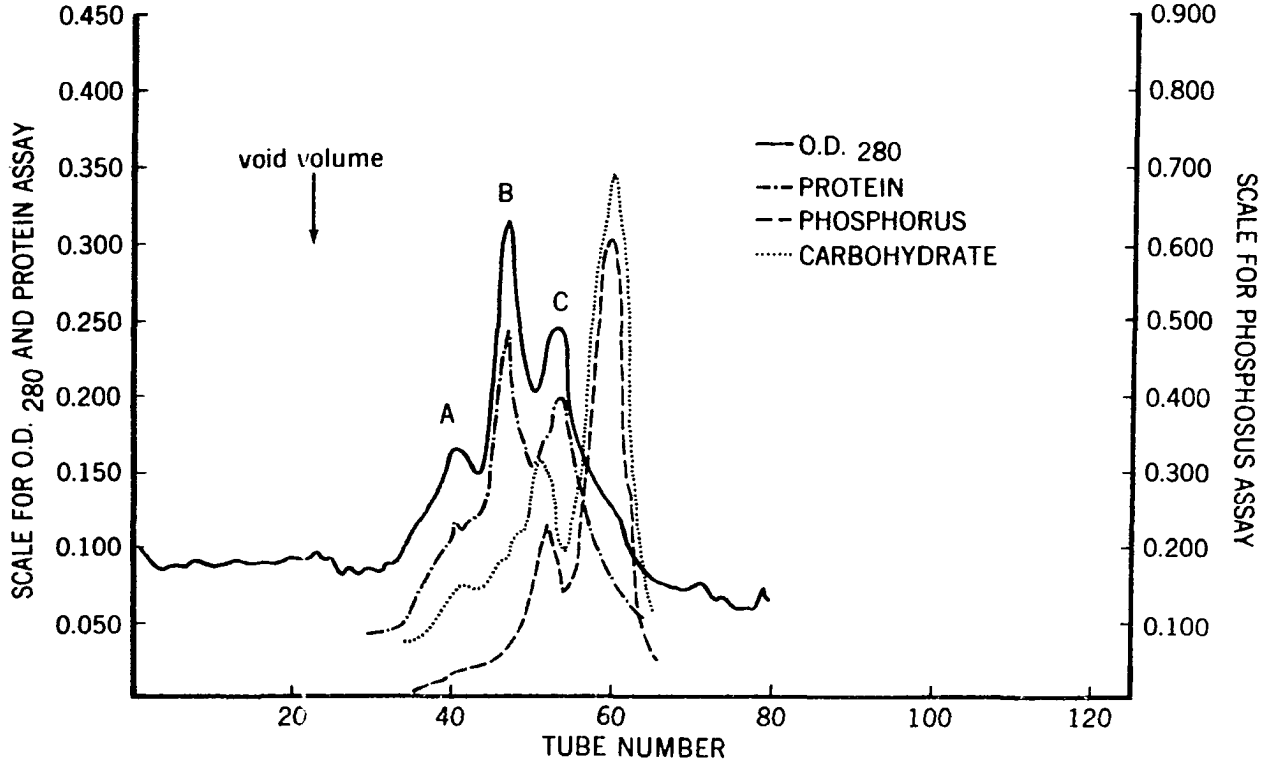

Figure 39. Sepharose 4B gel filtration of SDS-solubilized residue (E6R).

Extracted residue was solubilized and fractionated as described under Figure 12. The column was monitored for $O.D._{280}$, protein, phosphorus, and carbohydrate.

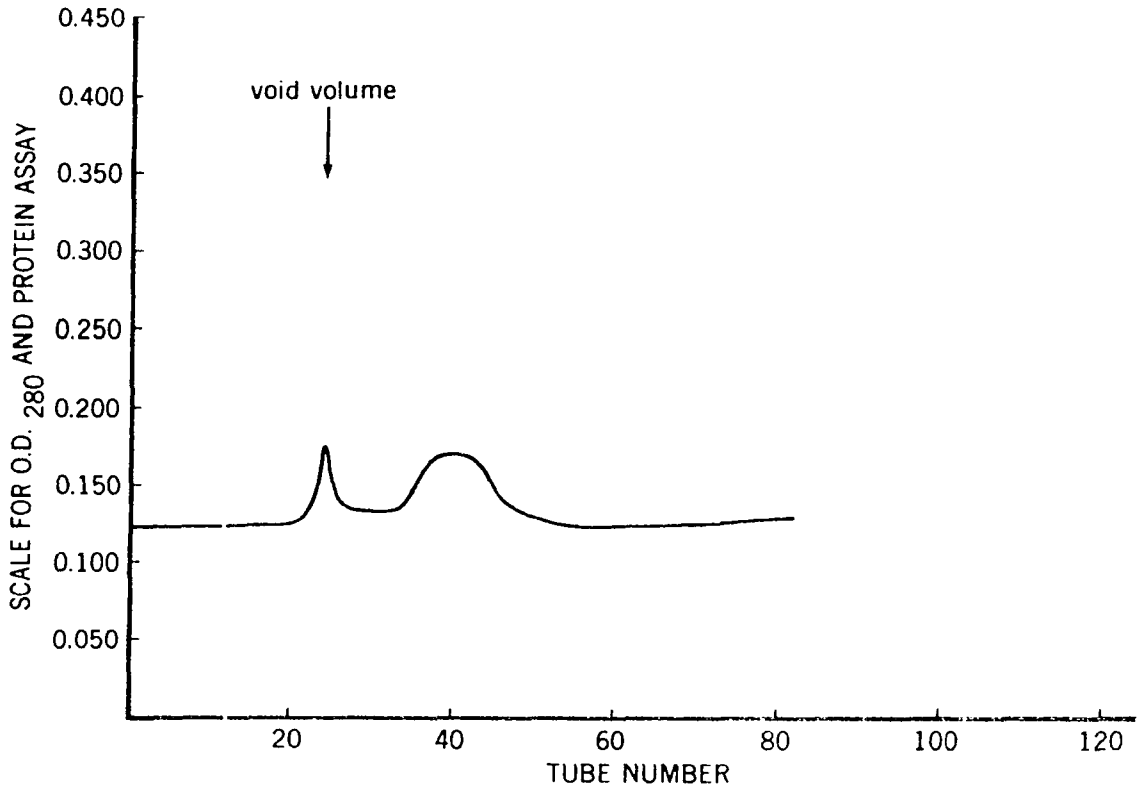

Figure 40. Sepharose 4B gel filtration of fraction A.

The eluant tubes containing Fraction A as shown in Figure 39 were combined and then concentrated by ultrafiltration. The concentrated Fraction A was re-chromatographed as described under Figure 12.

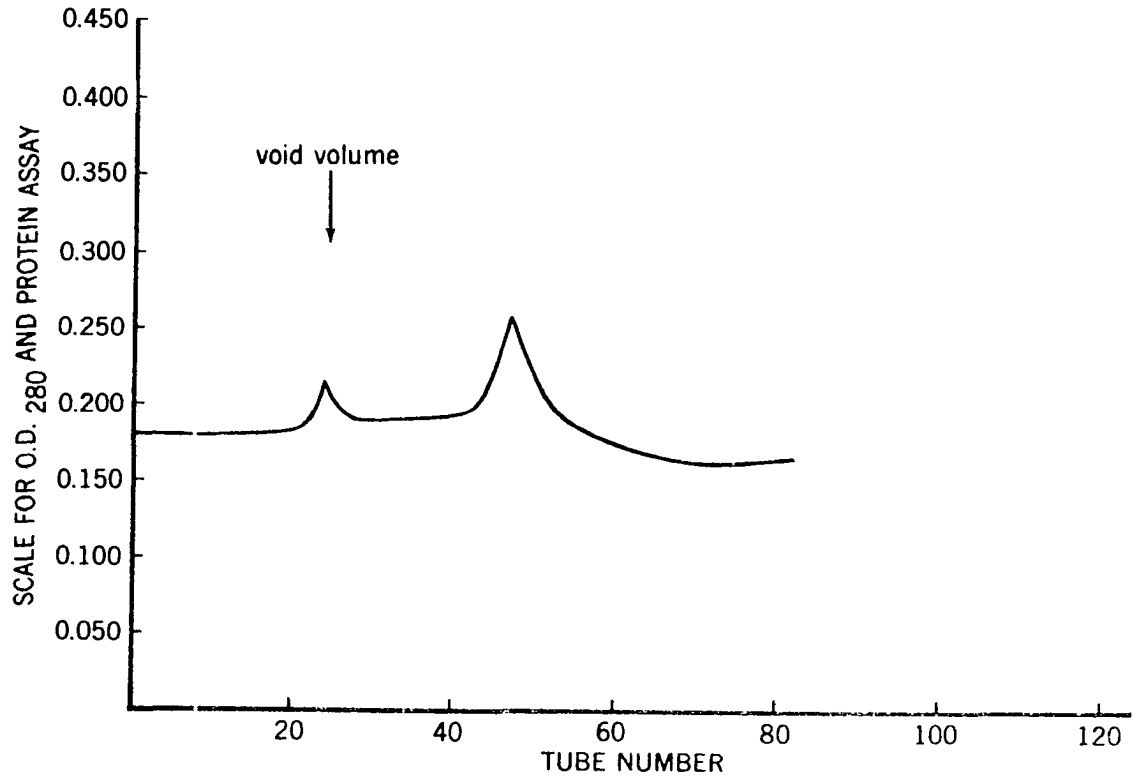

Figure 41. Sepharose 4B gel filtration of fraction B.

The eluant tubes containing Fraction B as shown in Figure 39 were combined and then concentrated by ultrafiltration. The concentrated Fraction B was re-chromatographed as described under Figure 12.

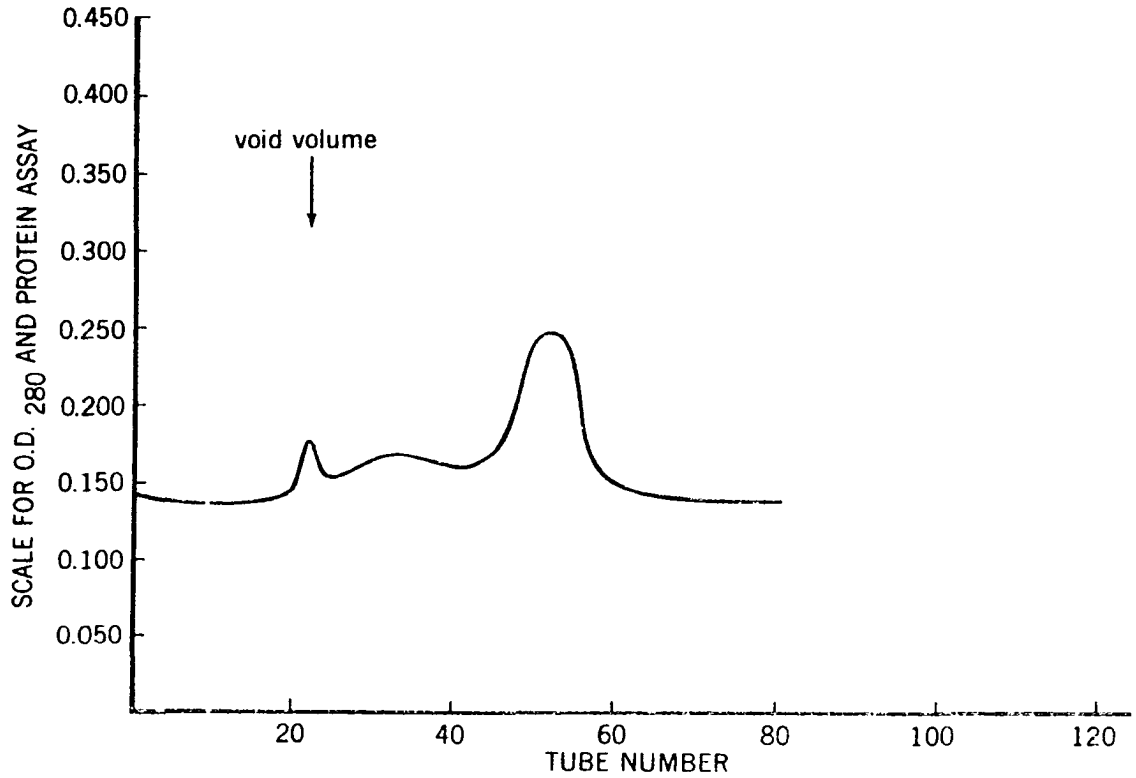

Figure 42. Sepharose 4B gel filtration of fraction C.

The eluant tubes containing Fraction C as shown in Figure 39 were combined and then concentrated by ultrafiltration. The concentrated Fraction C was re-chromatographed as described under Figure 12.

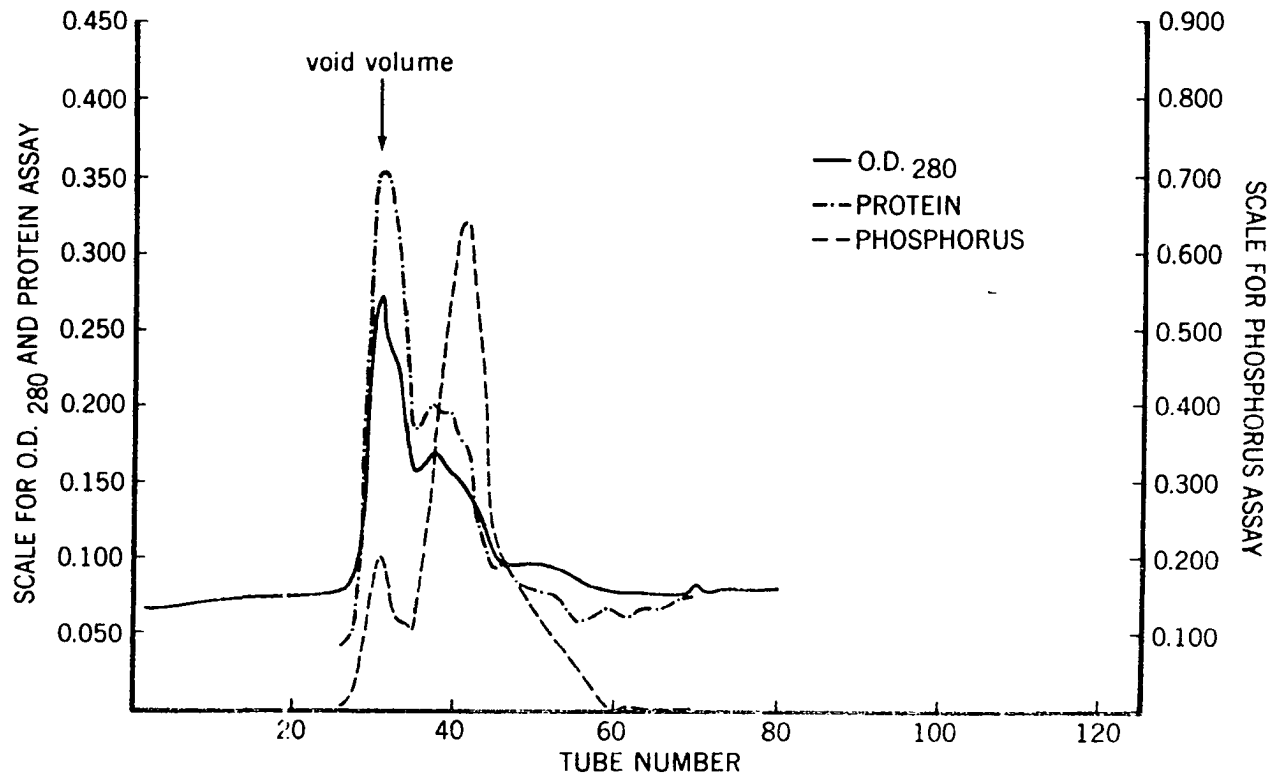

Figure 43. Sephadex G-200 filtration of SDS-solubilized residue (E6R).

Residue was solubilized and chromatographed as described under Figure 19 in the presence of SDS.

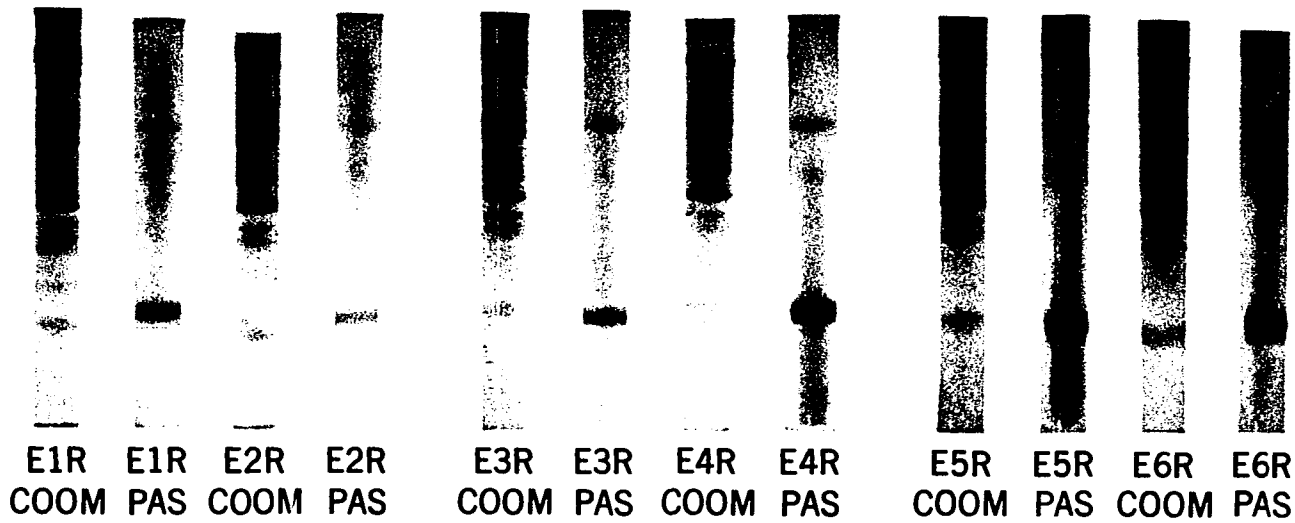

Figure 44. Polyacrylamide gels of residues remaining after repeated EDTA extractions.

Polyacrylamide gel electrophoresis on SDS-solubilized residues remaining after each extraction with EDTA was performed as described under Figure 20. Abbreviations: E, hypotonic EDTA; numbers 1-6, the number of the extraction; R, residue remaining after extraction; COOM, gel stained with Coomassie Blue dye; PAS, gel stained with periodic acid-Schiff technique.

additional extraction a progressive decrease in the number of protein bands is readily seen. There is no change in the number of carbohydrate-stained bands. In particular, the high molecular weight proteins are reduced by each extraction. The protein and glycoprotein patterns of residue after six extractions are more clearly shown in Figures 45 and 46. Six protein groups, one of which is a doublet, remain after the EDTA extractions. These protein groups are designated as CM-1 through CM-6, starting with the largest proteins as CM-1 to indicate their staining with Coomassie Blue. Four major carbohydrate bands are present, of which one is glycolipid and three are glycoproteins.

Polyacrylamide gel electrophoresis of the individual fractions A, B, and C is shown in Figure 47. Fraction A consists primarily of the large CM-1 proteins with some CM-2 proteins. Fraction B consists almost exclusively of CM-2 and CM-3 proteins. Fraction C contains no CM-1 proteins, a small amount of CM-2 and CM-3 proteins, and essentially all of CM-4, CM-5, and CM-6. The three glycoproteins (named GL-1 to GL-3) are found predominantly in Fraction C, although a small amount of the largest glycoprotein (GL-1) is also found in Fraction B.

Immunology

Hemagglutination inhbition studies for blood group activities in the residue are reported in Table 15. In both experiments A and B, the residue contained blood group A and M activities equivalent to that found in RBC ghosts.

Immunodiffusion studies indicate that at least two major proteins are present in the EDTA residue as shown in Figure 48. Under A and B, the residue (E6R) is seen to react with both a-FII and a-E6R (an

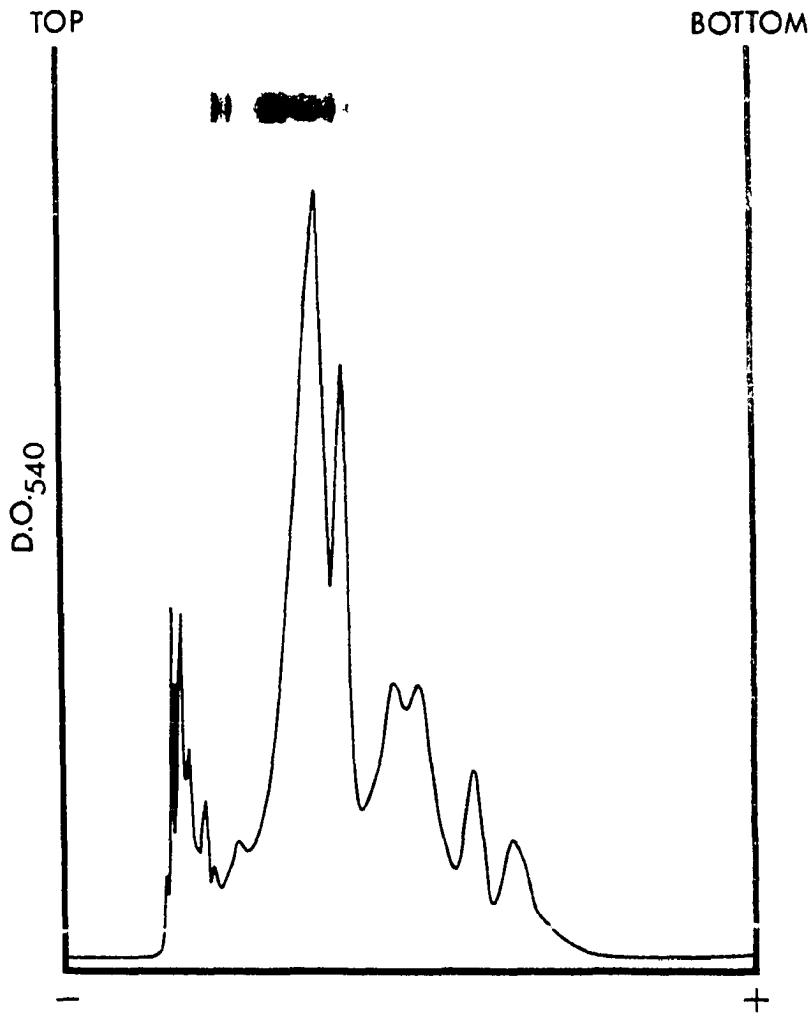

Figure 45. Polyacrylamide gel scan of residue (E6R) stained for protein.

SDS-solubilized residue (E6R) was electrophoresed, stained, and scanned as described under Figure 20.

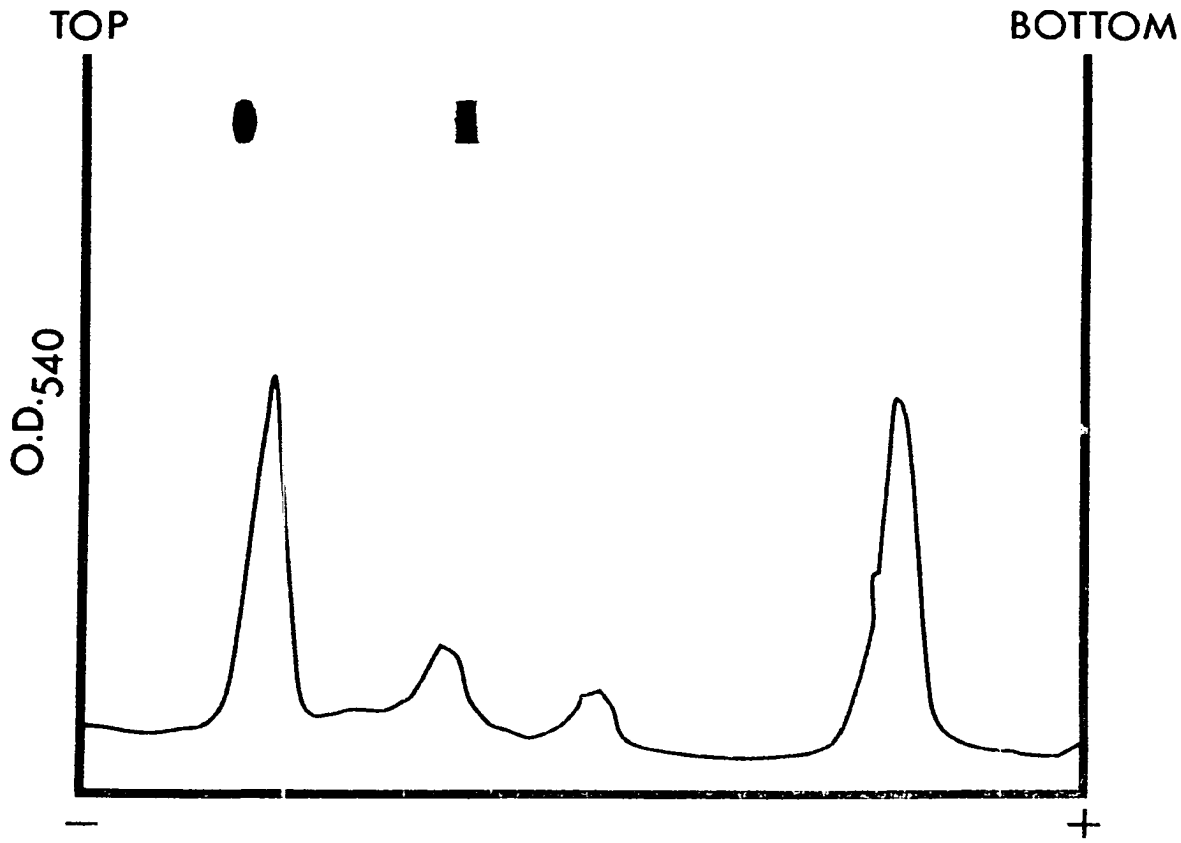

Figure 46. Polyacrylamide gel scan of residue (E6R) stained for carbohydrate.

SDS-solubilized residue (E6R) was electrophoresed, stained, and scanned as described under Figure 21.

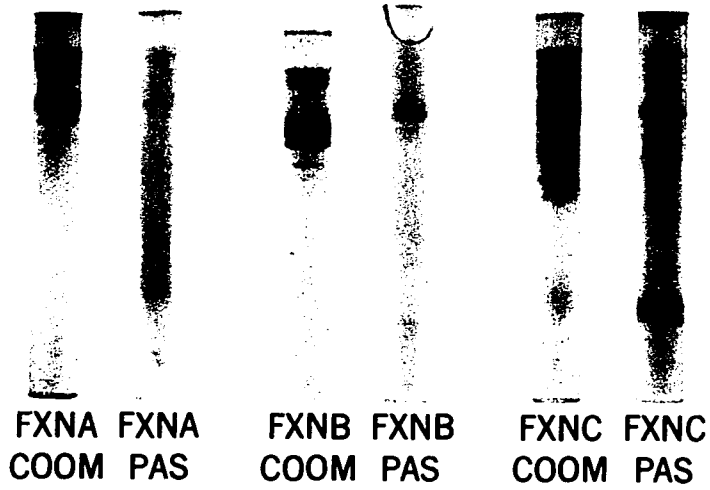

Figure 47. Polyacrylamide gels of fractions from Sepharose 4B column.

Concentrated Fractions A, B, and C were electrophoresed and stained for protein and carbohydrate as described under Figures 20 and 21.

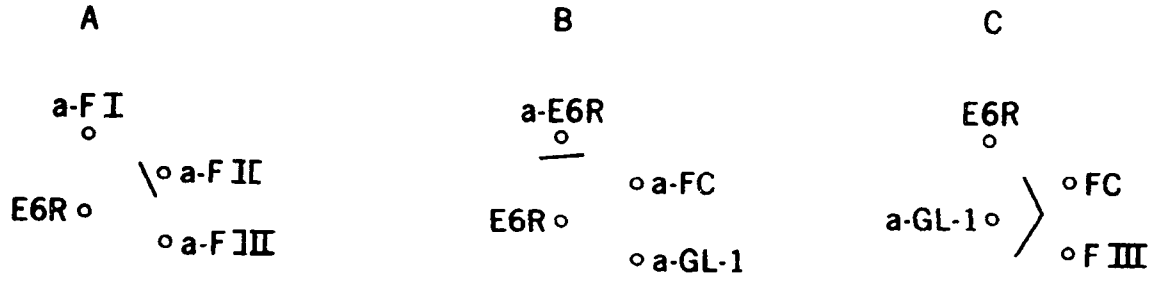

Figure 48. Immunodiffusion studies on residue (E6R) against various antibodies.

Ouchterlony double-diffusion studies were carried out using residue (E6R) against various antibodies. In the left figure, the residue was tested against antibodies to Fractions I, II, and III. In the central figure, the residue was tested with antibodies against residue (E6R), Fraction C, and the major glycoprotein (GLI). In the right figure, the activity of the antibody against the major glycoprotein was tested against residue, Fraction C, and Fraction III.

antibody against the intact residue), but not with a-GL-1 (an antibody against the major glycoprotein of the residue). Under C, a-GL-1 reacts with component shared by FIII and F-C, but not with E6R. The probable explanation is that a soluble protein diffuses from the intact residue to react with a-FII and a-F-C, while a different protein remains associated with the lipid of the residue, which must be solubilized before a precipitin reaction can occur. The lipid-associated protein is probably the major glycoprotein since it was used to stimulate the production of a-GL-1 and is present in both FIII and F-C.

Characterization of Individual Peptides

The six protein bands (CM-1 to CM-6) and the three glycoprotein bands (GL-1 to GL-3) were removed as described under Methods and analyzed for their amino acid content. The results of those analyses based on the relative proportions of the various amino acids to glutamic acid are presented in Table 16. The amino acid content varies between the different Coomassie-positive bands and between the carbohydrate-positive bands. However, the amino acid content is somewhat similar between GL-1 and CM-3, GL-2 and CM-4, and GL-3 and CM-5. The proteins with similar amino acid composition migrate about the same distance during electrophoresis. However, the carbohydrate-positive bands contain hexosamines which are not demonstrable in the Coomassie-positive bands.

TABLE 16

SUMMARY OF AMINO ACID ANALYSIS

	Ghosts	EDTA Residue	GL-1	GL-2	GL-3	CM-1	CM-2	CM-3	CM-4	CM-5	CM-6
Glutamic	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Gluc NH_2	7.5	21.2	12.4	16.5	Tr	0	0	0	0	0	
Gal NH_2	7.3	14.3	38.0	Tr	Tr	0	0	0	0	0	
Lysine	44.6	37.3	N.D.	34.7	55.8	55.9	18.3	0	Tr	63.8	
Histidine	23.6	19.5	N.D.	10.7	28.3	25.7	11.0	0	0	?	
Aspartic	63.7	66.4	62.0	71.1	87.4	77.1	43.2	74.1	62.3	89.4	
Threonine	39.2	49.6	52.4	59.1	77.5	37.6	40.5	62.4	50.0	70.2	
Serine	60.2	74.8	70.4	76.0	96.2	81.7	73.1	98.8	103.8	97.9	
Proline	47.4	62.4	61.5	61.2	37.4	48.6	59.8	62.4	59.4	51.0	
Glycine	51.9	67.9	77.6	102.5	112.4	90.8	49.8	94.1	90.7	112.8	
Alanine	58.2	62.5	68.1	92.1	106.2	69.7	44.2	94.1	83.9	95.7	
Valine	38.6	50.4	47.3	51.2	62.7	40.4	41.2	51.8	52.4	74.5	
Methionine	0	0	13.1	0	19.8	0	4.0	12.9	6.6	10.6	
Isoleucine	28.8	35.7	31.0	50	57.9	19.3	23.9	43.5	45.5	36.2	
Leucine	76.3	90.6	86.5	91	88.5	77.9	77.1	92.9	91.8	72.3	
Tyrosine	21.3	18.6	16.3	16.9	29.6	Tr	13.0	14.1	35.8	17.0	
Phenylalanine	30.3	47.6	28.2	27.8	34.4	Tr	28.6	29.4	54.7	31.9	
Arginine	44.7	38.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	

CHAPTER V

DISCUSSION

Isolation and Characterization of Erythrocyte Ghosts

The compositional studies on intact erythrocyte ghosts reported in Table 12 are similar to previously published work. They agree most closely with the work reported by Rosenberg and Guidotti in 1968 (18). They found a slightly higher content of protein and a slightly lower content of lipid, whereas the carbohydrate content was almost identical. Blumenfeld in 1968 (36) also reported a higher protein and a lower lipid content, apparently due to a smaller amount of cholesterol. The value for carbohydrate content agreed with this study. Poulik also in 1968 (34) reported similar values, although the data were not complete. The carbohydrate and phospholipid values were about the same but the protein content was lower.

The content of individual sugars did not agree as well with previous results. The percentages of the individual neutral sugars and of sialic acid, but not of the hexosamine, were similar to those reported by Winzler in 1969 (35). Winzler found twice as much galactosamine as glucosamine, whereas we found equal amounts to be present. When our values for percentages of neutral sugars, hexosamines, and sialic acid were compared with those of Blumenfeld (36), Rosenberg and Guidotti (18),

and Poulik (34), only minor differences were found. However, when the comparison was made on a microgram of carbohydrate per milligram of protein basis, little agreement was found, suggesting that variable amounts of protein were present in the starting membrane materials. Sialic acid, which is bound to membrane protein, showed the least variation.

The data in Table 13 indicate that both acetylcholinesterase (AChEase) and NADH:(acceptor) oxidoreductase (NaOase) activities are present in the ghost. The presence of small amount of glycolytic enzyme activity is not surprising since its removal may not be complete during RBC ghost preparation. Mitchell et al. (100) found AChEase activity to be comparable in both RBC ghosts and whole cell hemolysates, whereas they found almost none of the whole cell aldolase activity still present in ghosts. The osmolarity of the buffer used to prepare the ghosts had a small effect upon the AChEase activity, whereas pH had essentially no effect. Lauf and Poulik in 1968 (101) compared the AChEase activity in the human red cell before and after hemolysis and found that the RBC ghost retained 94% of the enzyme's activity. Bramley et al. in 1971 (102) studied the effect of osmolarity on AChEase activity and also found the enzyme to be present in the membrane of the RBC ghost. Thus, the RBC ghosts used for the present studies on membrane protein composition contained the enzyme activities expected for RBC ghosts prepared by hypotonic hemolysis.

The amino acid composition of RBC ghosts reported in Table 14 also agrees with previous studies (Bakerman and Wasemiller, 1967 (33); Rosenberg and Guidotti, 1968 (18); Winzler, 1969 (35); Blumenfeld, 1968 (36)). However, for specific amino acids there were substantial differ-

ences compared with previous studies. The basic amino acids, lysine, histidine and arginine were substantially higher than previous values, while the non-polar amino acids valine, isoleucine and leucine were substantially lower. The major amino acids previously found (glutamic acid, leucine, aspartic acid, serine, alanine) were the same as reported in this study.

The erythrocyte ghosts after preparation by hypotonic hemolysis showed an intact membrane structure and a fuzzy coat lining the inner surface of the ghost membrane. This electron photomicrographic appearance is in close agreement with the findings of Nicolson et al. (103) and Harris (104).

Solubilization of Erythrocyte Ghosts

Despite a limited amount of earlier data, comparisons can be made between these data and the scattered reports available on different solubilizing agents. The excellent solubilization obtained with sodium dodecyl sulfate (SDS), which at 0.5% concentration solubilized over 98% and at 1% concentration over 99% of the protein, has previously been reported to be an effective agent. Bakerman and Wasemiller in 1967 (33) found that 91.8% of the ghost protein could be solubilized at 0.5% SDS concentration. Working with lipid-extracted ghost protein Rosenberg and Guidotti in 1968 (18) were able to solubilize 98% of the ghost protein with a 1% SDS solution. In 1972, Juliano solubilized 100% of the ghost protein at a 3% SDS concentration. These data leave little doubt that SDS is an effective solubilizing agent for RBC ghost proteins.

The other agents evaluated in this study have not been as consistently effective as SDS for solubilizing ghost proteins. The 70%

solubilization at 0.5% sodium deoxycholate concentration does not agree with the 54.6% solubilization at the same concentration reported by Bakerman and Wasemiller (33). However, 85% solubility at 1% concentration agrees with the data of Philippot (106) who was able to obtain a maximal solubilization in the range of 75% to 95%. The data on guanidinium hydrochloride showed even greater variability. The 50% solubility at a 4 molar concentration in this study was obtained by Bakerman and Wasemiller (33) at a 1.5 molar concentration. The 80% solubilization at a 6 molar concentration achieved in this study contrasted with 58% solubility at the same concentration reported by Juliano (105). A minimal amount of data is available on Triton X-100, although it is shown to be an effective agent in this study. Rosenberg and Guidotti in 1968 (18) found Triton did not solubilize lipid-extracted protein. The lack of data probably results from difficulties with protein quantitation caused by Triton interaction with reagents for protein assay. Complete solubilization of the membrane has been reported with this agent, but it was performed without quantitation.

The organic solvent systems reported to be effective for solubilizing erythrocyte membrane proteins were not evaluated in this study because of the great variability reported in the literature. The original approach described by Maddy in 1966 indicated that 90 to 95% of the protein could be solubilized by a butanol-water system. This contrasts sharply with the 15.3% reported by Bakerman and Wasemiller (33) and the 20 to 35% by Poulik (34). The reasons for variability in solubilization will be discussed later in this paper.

Although no specific quantitative studies have been performed

by combining SDS solubilization and gel chromatography as carried out in this study, several reports have suggested that SDS is adequate for solubilization before gel filtration. The use of SDS as part of a gel filtration system will be discussed at length in the following section.

Gel Filtration of Erythrocyte Ghosts

The three major fractions obtained by gel filtration on a Sepharose 4B column eluted with 0.1% SDS have not been reported previously, perhaps because conditions for performing the chromatography have varied greatly from one study to another. Bakerman and Wasemiller (33), using Bio-Gel P-300, were able to separate the SDS solubilized membrane into only two peaks. The most similar data to these three fractions were reported by Rosenberg and Guidotti (18) using lipid-extracted membrane protein solubilized in SDS. They obtained three fractions, but the first appeared at the void volume and the second peak was equivalent to fraction I. The third peak corresponded to a combination of fractions II and III reported in this study. However, rechromatography resulted in a single peak and may not actually correspond to combined fractions II and III. In 1971, Gitler (107) found five fractions by gel chromatography on 4% Agarose columns in the presence of Brij 36T, a non-ionic detergent. The middle three peaks were equivalent to fractions I, II, and III. The first peak was at the void volume, and the last peak is similar to a peak found in this study when deteriorated ghosts were used for chromatography. In 1972, Kobylka *et al.* (54) reported a somewhat similar pattern for Sepharose 4B chromatography with 1% SDS for elution. The separation between fraction II and fraction III was not as good as we achieved, but a slight separation was present. Also in 1972, Carraway

and Shin (108) solubilized ghost protein from which lipid had previously been removed and separated the proteins on Sepharose 4B in the presence of SDS. Three fractions were obtained with a different configuration than in this study, primarily due to the presence of a much larger fraction II peak. Gwynne and Tanford (109) reported on the use of Sepharose 4B to separate the different proteins. A very large fraction I peak was obtained but the fraction II and fraction III peaks were much smaller. All of the protein was retarded except for a very small void volume peak. It is important to note that the protein solubilized by guanidinium hydrochloride represented only 60% of the total membrane protein. Thus, gel filtration on the gel Sepharose 4B in the presence of SDS appears to be a satisfactory technique for separating proteins into their major molecular weight classes. Failure to perform the procedure in the presence of SDS will result in a single peak at the void volume as shown by Green in 1967 (62). Most of the separations reported in the literature have not been as good as those obtained in this study. The difference is probably the result of variation in the conditions used for gel filtration.

As with this study, gels with lower exclusion limits, such as Sephadex G-200, have not been effective for gel filtration of ghost proteins. Poulik (34), using Sephadex G-200, was not able to obtain any peak other than the void volume. Lenard (50) used Sephadex G-200 in the presence of SDS to fractionate the ghost proteins. Most of the protein came off either at or closely adjacent to the void volume as was found here. No separation of the proteins into different molecular weight classes was possible. Blumenfeld *et al.* (57) used Sephadex G-200 to

separate water soluble proteins in an aqueous pyridine system. Again the bulk of the protein eluted either with or immediately adjacent to the void volume. Triplett et al. (110) found all of the protein in the void volume using a Bio-Gel P-100 column eluted with 1% sodium sulphate. Therefore, it would appear that gels with exclusion limits lower than those of Sepharose 4B are not adequate for separation of ghost proteins by gel filtration.

Electrophoretic and Immunologic Characterization of Erythrocyte Ghosts

More studies on the characterization of membrane proteins have been based on the use of polyacrylamide gel electrophoresis than any other single method. Comparing the results obtained in those studies with this study is difficult because of the many variations in technique. This study, based on the use of a 5% polyacrylamide gel with a buffer system containing relatively high concentrations of SDS and β-mercaptoethanol, permitted the identification of 11 major protein bands and three carbohydrate bands. The multiple bands obtained by polyacrylamide gel electrophoresis have been reported many times in the past. Rosenberg and Guidotti (18) using an SDS system with a 10% gel obtained anywhere from 15 to 25 protein bands. Lenard (50) reported 14 bands stained with Coomassie Blue, of which four appeared to be major components. In addition, he found two major carbohydrate bands, one of which was associated with a glycolipid fraction. Several other protein bands stained lightly for carbohydrate, but the number was not specified. Trayer et al. (111) in 1971 also found multiple bands using an SDS polyacrylamide gel system. Although there were some variations from one blood donor to another, they

found a total of 17 different bands, of which three were major components. Two of the major components had a very high molecular weight, while the other major component had a molecular weight in the middle range of the membrane proteins. Fairbanks et al. in 1971 (52), by SDS polyacrylamide gel electrophoresis, found six major protein components and three major glycoproteins. The major glycoprotein band was noted to migrate to the same area as their major components III and IV, which is similar to the results obtained in this study. Kobylka et al. (54) compared several different animal species and found multiple proteins in the erythrocyte membrane of all species including the human. Nine major protein bands could be identified on a cm gel. When a long (13 cm) 5% polyacrylamide gel was used to study human erythrocyte membranes, 11 major components could be distinguished. That number agrees exactly with the number found in this study and the patterns obtained by gel scanning are quite similar. However, their study was different in that only one glycoprotein could be found. In 1972, Hamagachi and Cleve (112), comparing several mammalian species, found over 20 different protein components with similar molecular weights in all species studied. They found only one major glycoprotein, although other minor bands also stained for carbohydrates. Consequently, on the basis of previous work and of this study, many different protein bands are present in the human erythrocyte ghost. Depending upon the individual investigator's interpretation, somewhere between six and seventeen bands are important components. In addition, at least one major glycoprotein is present and, perhaps, as many as three. Because of the complex nature of the protein components, consistent separation of the individual proteins does not

appear to be possible by polyacrylamide gel electrophoresis of all RBC membrane proteins.

Hemagglutination inhibition studies demonstrating the presence of blood group A and M activity in the RBC ghost are in agreement with previous data. Although not reported in the Results chapter on a weight basis, comparison of blood group A and blood group M activities on a weight basis agreed well with results reported by Poulik in 1968 (34). Testing only for blood group A activity, Whittemore et al. (64) always found at least a six-tube difference between the test and control assays. Thus, blood group activities in the RBC ghosts reported in this study are comparable quantitatively to previous reports.

Data on fractions I, II, and III and their reactions with corresponding antibodies has not been previously reported. In fact, almost no data on the immunochemistry of human erythrocyte ghost proteins are available, except where specific antigenic components, such as blood group M, have been studied. Howe et al. (113) studied the antibodies produced against both a total hemolysate (obtained by hemolyzing packed red cells) and the residue of the hemolysate which remained after centrifugation. Besides hemoglobin they found at least 12 antigenic components, five of which were identified as enzymes. In addition, virus receptor activity as well as blood group A, M, and N activities could be demonstrated. Howe and Lee in 1969 (114) studied not only the antigenic components of the total hemolysate and post-hemolytic residue, but also the components of hemoglobin-free erythrocyte ghosts and purified virus receptor substance. Lyophilization with subsequent aqueous extraction separated the hemoglobin-free ghost material into an insoluble fraction

and a minor soluble fraction. The soluble fraction contained at least four proteins, while the insoluble fraction contained the virus receptor substance. No further characterization of the insoluble fraction was possible. Thus, immunochemical identification of the various protein components of the erythrocyte ghost remains an area in which only tentative efforts have been made.

Fractionation of Erythrocyte Ghosts by Aqueous Extraction
Soluble Fractions ("Supernate")

To evaluate the effects of aqueous extraction on human erythrocyte ghosts, various agents in aqueous solution were used to remove proteins from ghosts. As discussed in Results, the proteins removed by the different techniques were quite heterogeneous, although one agent did show some specificity. Distilled water has been shown to extract protein from hemoglobin-free erythrocyte ghosts, such as the work of Lauf and Poulik (101) who, using urea-mercaptoethanol starch gel electrophoresis, found a heterogeneous group of high molecular weight proteins. In contrast to that, Harris (115) in 1969 could demonstrate only one major protein by polyacrylamide gel electrophoresis after water extraction. Neither system included SDS in the buffer, so aggregation may have produced the variation. Tetramethylammonium bromide at 0.1 molar concentration was used by Reynolds and Trayer (86) to solubilize over 90% of the RBC ghost protein. SDS-polyacrylamide gel electrophoresis of the solubilized material produced a pattern of protein bands identical to the results obtained with intact ghosts solubilized in SDS. This finding contrasts sharply with this study, in which a highly selective removal of two protein bands was found. The difference probably results from the use of

different techniques for solubilizing the ghost proteins. Triton X-100 was evaluated in this study because it was used in the method of Mazia and Ruby (41) for the preparation of hemoglobin-free RBC ghosts. The use of this agent could lead to partial solubiiization of ghost proteins during preparation of RBC ghosts. In fact, a study by Hamaguchi and Cleve (112) demonstrated that ghosts prepared by the method of Mazia and Ruby contained fewer proteins than ghosts prepared by the method of Dodge et al. (32). Finally, the heterogeneous protein pattern obtained with extraction of ghosts by hypertonic sodium chloride solutions is in sharp contrast with the results obtained by other workers. Fairbanks et al. (52) removed approximately 4% of the membrane protein as a single protein called component VI with hypertonic saline. Carraway and Shin (108) also were able to extract a single protein which they called component VIII. Technical differences between this study and the cited works are not enough to explain the variable results. The number and type of proteins which can be extracted with hypertonic sodium chloride remain to be determined.

As discussed, the proteins which can be extracted into aqueous media from RBC ghosts vary with the agent used. Data in this study on extraction with hypotonic sodium EDTA followed by extraction with a dilute base agree with the results obtained by Juliano and Rothstein (83) who extracted approximately one-half of the ghost protein with the same procedure. Earlier work by this same group (Hoogeveen et al. (84)) demonstrated that hypotonic base solutions followed by hypotonic EDTA (which reverses the previous sequence) extracted approximately 47% of the protein. Other reports on the use of hypotonic EDTA have shown less con-

sistent results. Marchesi et al. (45) extracted protein from the ghost membrane with low ionic-strength aqueous solutions containing EDTA but did not quantitate the amount of protein. Reynolds and Trayer (86) were able to solubilize 89% of the human RBC ghost proteins in a 5 millimolar EDTA solution after 96 hours. Prolonging incubation caused larger amounts of phospholipids to be associated with the solubilized protein. Juliano et al. (56) demonstrated that changes in pH altered the amount of protein extracted into 1 millimolar EDTA solution. The yield increased from 5% at pH 7.0 to 45% at pH 12.0. The value of about 23% at pH 9.5 agrees with the results of this study.

Quantitative studies have been performed on other aqueous solvents for both single and repeated extractions. Lauf and Poulik (101) extracted 12% of the protein from ghosts with a single exposure to distilled water at pH 7.1, although a rather large amount of lipid phosphorus was also removed. Hamaguchi and Cleve (87) removed a total of 42.8% of the ghost protein with a range of 48.1% to 52.2% by repeated extractions with distilled water. Maddy and Kelley (85) used dilute acetic acid to extract proteins and obtained from 30 to 40% of the ghost protein with three extractions. Therefore, we conclude that substantial amounts (approximately 50%) of the ghost protein can be removed by extraction with hypotonic aqueous systems. Variations in the amount removed can result from alterations in pH, osmolarity, time of incubation, and many other factors.

Compositional studies on the EDTA extracted material reported in this study are compatible with previous reports. Hoogeveen et al. (84) found primarily protein with little lipid was removed by their sys-

tem. Although the amount was small compared to the insoluble residue, A and B antigenic activity could be demonstrated in their soluble fractions. The same workers found no acetylcholinesterase activity in the supernates but total recovery was only 34%, all of which was in the residue. Other enzymes have been demonstrated in the EDTA extracts, including glyceraldhyde-3-phosphate dehydrogenase (Tanner and Gray, (79)), the only definitely identified protein of the erythrocyte ghost up to the present time.

Multiple protein bands were found to be extractable by hypotonic sodium EDTA in this study but the results reported in the literature have been less consistent. Hoogeveen et al. (84), Reynolds and Trayer (86), Hamaguchi and Cleve (112), and Maddy and Kelly (85) all found that multiple protein bands were removed from the RBC ghost by repeated aqueous extractions. The extracted proteins had the same range of molecular weights as the proteins of the complete erythrocyte ghost. The many protein bands found by these authors contrast sharply with the data of other workers who could remove only high molecular weight proteins with hypotonic EDTA. Marchesi et al. (45), Juliano et al. (56), and Fairbanks et al. (52) reported that only two very large proteins were identifiable in EDTA extracts. The variation in results is difficult to explain except as the result of variation in technique. It is of interest that Trayer et al. (111) repeated the technique of Marchesi et al. (45) as it was published and found that multiple proteins were removed by the procedure. One can conclude, therefore, that extraction of the erythrocyte ghost with hypotonic EDTA solutions removes a large number of different proteins, in particular the high molecular weight protein components.

These proteins have the same molecular weight range as the proteins of the intact erythrocyte ghost, but do not contain any glycoproteins. The significance of these proteins as a part of the erythrocyte ghost proteins will be discussed later in the text.

Little information is available on the immunologic identification of the EDTA extractable ghost proteins. Hoogeveen et al. (84) found that blood group A and B activity was removed by aqueous extraction, but over 80% of the total activity remained with the residue. The only purified or partially purified protein used to stimulate the production of antibodies has been "spectrin" by Marchesi et al. (45). A precipitating antibody was produced but the evidence presented as demonstrating its specificity was inconclusive. Consequently, immunochemical study of aqueous soluble ghost proteins has been superficial at best and offers no definitive information about the different protein components.

Fractionation of Erythrocyte Ghosts by Aqueous Extraction
Insoluble Fraction ("Residue")

Compositional studies on the residue remaining after repeated aqueous extractions with hypotonic EDTA solutions is not available since this particular approach previously has not been reported. Studies involving the use of various aqueous systems to extract the red cell ghosts have provided only a limited amount of information about the residue after aqueous extraction. For example, Rega et al. (47) found that there was a difference in carbohydrate composition between the supernatant and the residue obtained by washing red cell ghosts with distilled water. However, a difference between their study and this study is that more sialic acid was present in the supernate than in the residue. Blumenfeld

(36) found approximately equal amounts of protein in the supernate obtained by aqueous pyridine extraction and in the insoluble residue. In addition, the residue contained most of the organic phosphorus and cholesterol as did the residue in this study. However, a major difference was that she found all of the sialic acid in the supernate rather than in the residue. Lauf and Poulik (101), using a triple wash of the RBC ghost with distilled water, noted a partitioning of the sialic acid and phosphorus in the residue after the multiple treatments. Hoogeveen et al. (84) also found the lipid and sialic acid in the aqueous insoluble residue. In fact, their sialic acid values of 30 micrograms per milligram of protein and 49.2 micrograms per milligram of protein for the RBC ghosts and extracted residue, respectively, are essentially the same values as were obtained in this study for the same substrates. Similar results, although quantitatively less, were also reported in 1971 by Juliano and Rothstein (83) as well as Steck et al. (53) with the use of fewer washes of the RBC ghosts than were used in this study. Limber et al. (116), using 5 successive washes with distilled water followed by homogenization, found only a slight decrease in protein concentration compared with the ghosts and essentially no change in phospholipid and carbohydrate composition. One can conclude that the changes in membrane composition produced by repeated washings with hypotonic aqueous systems in this study are similar to previous studies using aqueous systems without homogenization. The ability to obtain consistent protein and lipid compositions for the residue after aqueous extraction suggests that the final composition may result from a specific characteristic of the RBC membrane.

The retention of acetylcholinesterase activity in the residue remaining after repeated aqueous extractions has previously been noted by several workers (Hoogeveen et al. (84), Lauf and Poulik (101), Hamaguchi and Cleve (87), Heller and Hanahan (76), Bramley et al. (102)). The other enzyme, NADH:(acceptor) oxidoreductase, retained in the residue after extraction has been less well studied. Zamudio et al. (77) found that exposure of RBC ghosts to solutions of progressively lower osmolarity increased the enzyme activity per milligram of protein. Our failure to demonstrate an increase in enzyme activity probably resulted from loss of enzyme protein at each extraction step. Complete absence of glycolytic and hexose monophosphate shunt enzymes in the residue was expected since the glycolytic enzymes are soluble components of the cytoplasm. Thus, two enzymes, acetylcholinesterase and NADH:(acceptor) oxidoreductase, previously reported to be associated with erythrocyte ghosts, were also found to be present in the residue after repeated extractions with aqueous solvents.

A limited amount of information is available on the amino acid composition of aqueous-extracted erythrocyte ghosts. Blumenfeld (36) found the amino acid composition for both her starting RBC ghosts and the insoluble protein remaining after aqueous pyridine extraction to be similar to our results for RBC ghosts and residue, respectively. In addition, the hexosamine content was almost identical to our data for ghosts. The values for the starting membranes in our study may have been a result of the fact that a total of six washes was used to remove hemoglobin before exhaustive dialysis against distilled water to remove inorganic phosphate. A substantial amount of membrane protein may have

been lost as a result of the very extensive preparation of the RBC ghost. The amino acid composition found for the glycoproteins as reported by the same group of workers (Blumenfeld (36); Blumenfeld et al. (57); Zvilichovsky et al. (58)) was substantially different than the amino acid composition of our extracted residue, especially for threonine, serine, and glutamic acid. One can conclude, therefore, that the EDTA-extracted residue has a substantially different amino acid composition than the major glycoproteins, which constitute the major group of residue proteins. Other major proteins must be present in the residue in order for a difference in the amino acid compositions for the residue and the major glycoproteins to be found.

The conversion of erythrocyte ghosts from a biconcave disc to multiple small vesicles has been reported by many different authors. Dodge et al. (32) in their study on hypotonic lysis of erythrocyte ghosts, clearly demonstrated that a reduction in the osmolarity of the lysing solution to 0 imOsm resulted in the formation of microvesicles. Reynolds and Trayer (86) found that 5 millimolar EDTA was capable of producing the same physical change. Loss of protein from the inner surface of the membrane during this process was demonstrated by Nicolson et al. in 1971 (103). The electron photomicrographic changes reported in this study are compatible with what has previously been found.

No gel filtration data are avilable on the proteins and phospholipids of EDTA-extracted residue. Rosenberg and Guidotti (49) removed 52% of the protein of the ghost by sequential extractions with 1 mM sodium EDTA containing 50 mM β-mercaptoethanol and 0.8 M sodium chloride. The remaining protein was freed of lipid by ethanol-ether extraction

and solubilized in SDS. The delipidized protein was fractionated on a Sephadex G-200 column in the presence of 1% SDS, by which three major and two minor protein peaks were found. This is substantially different from our results using Sephadex G-200 where very little separation into different protein groups could be obtained. Gel filtration data using Sepharose 4B for the EDTA-extracted ghost residue has not been previously reported.

The proteins remaining with the residue after repeated aqueous extraction of erythrocyte ghosts have been more difficult to identify. Hoogeveen et al. (84) demonstrated that after two aqueous extractions of RBC ghosts, the residue contained five protein bands of relatively low molecular weight compared to the proteins in the supernate. Juliano and Rothstein (83) found a similar loss of high molecular weight proteins with a retention of the lower molecular weight proteins in the residue after aqueous extraction. No staining for carbohydrate-containing proteins was carried out in either study. Similar results were found by Fairbanks et al. (52) using warm 0.1 mM EDTA (pH 8) for removal of RBC ghost proteins. There was a substantial reduction in the number of bands as compared to the whole ghost and protein bands CM-1, CM-2, and CM-5 (using our classification) were the most prominent protein bands. An extension of their work was reported by Steck (117) using guanidinium hydrochloride to treat erythrocyte ghosts. Protein staining of the residue revealed almost complete removal of the highest molecular weight proteins and only one major protein band (equivalent to our CM-2 and CM-3) was retained after the treatment. The lower molecular weight proteins were not well-defined. However, he did identify the four carbohydrate peaks

found in this study. The highest molecular weight carbohydrate band appeared to be identical to the major glycoprotein band of the RBC ghosts.

Limber et al. (116) used repeated distilled water extractions to remove RBC ghost proteins. When the protein band patterns of ghosts prepared from normal RBC's and from hereditary spherocytosis RBC's were compared, a band designated as C was absent frequently from the ghosts of hereditary spherocytosis, although the finding was not consistent. Also using a distilled water extraction, Hamaguchi and Cleve (87) found a reduction in the high molecular weight classes and an increase in the lower molecular weight classes for the residue proteins. Consequently, it appears to be clear that only a limited number of proteins remain after repeated aqueous extraction of the erythrocyte ghost. The proteins removed from the erythrocyte ghost during the process primarily are high molecular weight proteins, although all molecular weight classes of the ghost proteins are represented. The proteins remaining in the residue itself consist of at least five major proteins and three glycoproteins. Whether or not the bands which stain for carbohydrate also stain for protein is yet to be determined.

Immunochemical data on aqueous extracted residues are sparse. Hoogeveen et al. (84) found blood group A and B activities in the residue but performed no other immunologic studies. Lauf and Poulik (101) demonstrated that distilled water treatment of RBC ghosts did not remove blood group M activity from the ghost. Both observations are consistent with the data reported in this study. Unfortunately, immunodiffusion has not been used to elucidate the nature of the various proteins which are in the residue. The only related study was on the glycoprotein component,

which was shown by Howe et al. (118) to be a single entity immunochemically independent of the technique used to isolate the material. With the exception of that protein, the immunology of the residue proteins remains unexplored.

Implications

Several general conclusions can be drawn about the proteins of the human erythrocyte ghost. Many different proteins are present in the RBC ghosts when it is prepared by hypotonic lysis, the most common method for ghost preparation. These ghost proteins can be divided into three major groups by molecular weight as shown by the gel filtration data. Approximately one-half of the protein is aqueous soluble and is the group of proteins which is responsible for most of the ghost protein heterogeneity. The other one-half, which is not aqueous soluble, is less heterogeneous and contains almost no high molecular weight proteins. This group of proteins, which are intimately associated with the ghost lipid, seem to consist of five major proteins and three major glycoproteins. Enzymatic (for example, acetylcholinesterase) and immunologic (for example, blood group M) functions are associated with this protein group.

There is no evidence that lipoprotein units are found in the erythrocyte ghosts on the basis of this study. Much of the data supporting the presence of lipoprotein subunits assumes that almost all of the protein is associated intimately with lipid. It is hard to reconcile that concept with the clearcut evidence that one-half of the ghost protein is readily soluble in an aqueous media. The Danielli-Davson concept of a lipid bilayer covered on both sides with protein is difficult to reconcile with the aqueous removal of proteins from the ghost membrane,

especially when the removal has been shown to occur on only one side of the membrane by electron microscopy. The fluid mosaic model of Singer is more compatible with the findings reported here as well as with data previously reported on the protein composition of the erythrocyte ghost.

Consequently, a classification system for the ghost proteins can be developed to assist in the further fractionation and characterization of erythrocyte ghost proteins. A schema of the classification system is given in Figure 49. Washed human red blood cells when exposed to hypotonic aqueous buffer systems in the range of 20-40 ideal milliosmolar and at the neutral pH release both hemoglobin and the cytoplasmic enzymes, such as pyruvate kinase. Repeated exposures will remove almost all of these two types of proteins. The end product of the washing procedure is the erythrocyte ghost, a stable membranous structure consisting of lipids and protein. Exposure of RBC ghosts to hypotonic aqueous buffer systems containing a chelating agent, but at an osmolarity less than 10 ideal milliosmoles and a basic pH, extracts a different group of proteins which are the aqueous soluble membrane proteins and are normally located on the inner surface of the ghost membrane. This group of proteins has been designated as Group I membrane proteins and consist of the high molecular weight proteins called "spectrin" and multiple enzymes which could not be removed during osmotic lysis. Two possible mechanisms exist to explain why these proteins are now soluble in aqueous media when they had previously not been soluble. The first possibility is that the proteins are bound to the membrane by a calcium bridge which can be removed by a chelating agent, thereby releasing the protein. The other possibility is that the process of endocytosis is involved. This

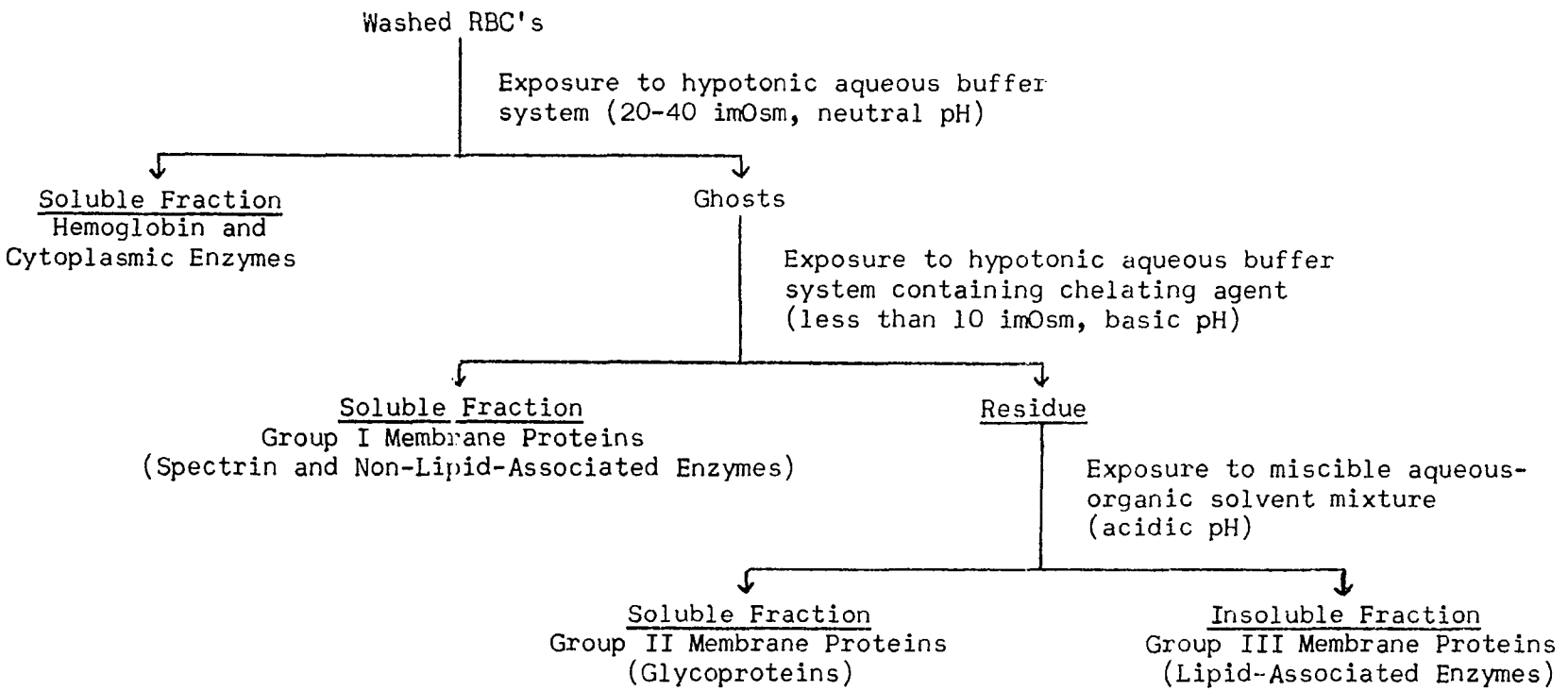

Figure 49. Classification system for erythrocyte ghost proteins.

process involves invagination of the outer membrane surface into the cell interior. During the process the membrane reseals to form an "inside-out" vesicle and exposes the inner surface to the external environment. The proteins are then free to be released directly into the fluid environment. Neither mechanism appears to be solely responsible for the protein release and a combination of the processes is probably the explanation for the phenomenon.

The insoluble product of the process is a residue containing the lipid-associated proteins which are fewer in number. These proteins can be further broken down by exposing the residue to miscible aqueous organic solvent mixtures. This process will solubilize the membrane glycoproteins leaving the other proteins such as the enzyme proteins in an insoluble lipid-protein mixture. The glycoproteins contain the blood group activities and are designated as Group II membrane proteins. The proteins still associated with the lipid are called the Group III membrane proteins and probably contain the enzymes intrinsic to the membrane, such as acetylcholinesterase and Na^+, K^+ adenosine triphosphatase.

Thus, the proteins of the erythrocyte ghosts appear to fall into several characteristic groups based on their fractionation. However, these same groups also relate to specific cellular functions. The aqueous soluble membrane proteins appear to be involved in maintaining the biconcave disc shape of the erythrocyte, probably through a closely linked enzyme system. The membrane glycoproteins are of major importance in that they are the immunologic markers for the erythrocyte and play a major role in cell recognition phenomenona. Finally, the enzymes which are most intimately associated with the lipid appear to play a

major role in the transport of ions, especially cations, across the membrane. Much remains to be done before a complete understanding of all ghost proteins and their function can be defined.

CHAPTER VI

SUMMARY

The proteins of the human erythrocyte ghost were completely solubilized by the addition of sodium dodecyl sulfate (SDS), an anionic detergent. Eleven major proteins and a variable number of minor proteins were found in the ghost by SDS-polyacrylamide gel electrophoresis (PAGE). The solubilized proteins were fractionated by gel filtration on Sepharose 4B, using buffered 0.1% SDS for elution, and three major peaks (Fractions I, II, III) were obtained, indicating that three major molecular weight classes of proteins are present in the ghost. Each class of proteins was heterogeneous by PAGE, although the distance of migration for the major proteins of each fraction corresponded to their approximate molecular weights by gel filtration. Immunodiffusion studies also demonstrated their heterogeneity.

By repeatedly extracting ghosts with aqueous hypotonic EDTA, over one-half of their protein could be removed. The extracted protein contained proteins, which did not stain for carbohydrate, having a range of molecular weights the same as that for all ghost proteins. However, the major aqueous-soluble proteins consisted primarily of the high molecular weight ghost proteins. Glycolytic enzymes and blood group A activity were found in this fraction. From immunodiffusion studies, these

proteins were shown to elute in Fractions I and II by gel filtration.

The proteins remaining after aqueous extraction were associated with the ghost lipid as microvesicles. Gel filtration on Sepharose 4B separated these proteins into two peaks (Fractions B and C) with almost no high molecular weight protein (Fraction A) present. Five bands which stained for protein and three bands which stained for carbohydrate were demonstrated by PAGE. Enzyme activity (e.g., acetylcholinesterase) and blood group activity (e.g., blood group M) previously shown to be intimately associated with membrane lipid were found in this fraction. Amino acid analysis suggested that the five major protein bands had different amino acid compositions.

These findings led to the conclusion that three major protein groups are present in the human erythrocyte ghost. The first group (Group I) is water soluble, accounts for about one-half of the ghost protein, and contains most of the high molecular weight proteins. The second group (Group II) is associated with the ghost lipids and contains all of the protein-bound carbohydrates. The third group (Group III) is also associated with the ghost lipids, contains no carbohydrate, but is responsible for those enzymatic activities which are an integral part of the ghost membrane.

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