LOCALIZATION OF ERYTHROCYTE MEMBRANE PROTEINS

By

RICHARD BLACK TRIPLETT

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

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Thesis Approved:

Kermit Carraway
Thesis Adviser

George Odell

Kurt E. Ebner

E. Gruden

K. D. Berlin

D. Durham
Dean of the Graduate College

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

NOMENCLATURE

ATP - Adenosine triphosphate

BD - Blue dextran

CD - Circular dichroism

CTAB - Cetyltrimethylammonium bromide

cyt c - Cytochrome c

EDTA - Ethylenediamine tetraacetic acid

EGTA - Ethyleneglycol-bis(β-amino-ethylester)N,N'-tetraacetic acid

ESR - Electron spin resonance

Hb - Haemoglobin

HS - Hereditary spherocytosis

IR - Infrared

LIS - Lithium diiodosalicylate

ma - Milliamp

mC - Millicurie

mOsM - Ideal milliosmolar

MW - Molecular weight

NMR - Nuclear magnetic resonance

ORD - Optical rotatory dispersion

PMSF - Phenylmethylsulfonyl fluoride (benzylsulfonyl fluoride)

POP - 2,5 Diphenyloxazole

POPOP - 1,4-bis-[2-(5-Phenyloxazolyl)]-benzene

SDS - Sodium dodecyl sulfate

CHAPTER I

INTRODUCTION

To describe cellular control, one must have a thorough understanding of the structure of plasma membranes. The surface of cells is particularly interesting because of its importance in cell-cell and cell-molecule interactions. Membranes have been shown to be composed of carbohydrates, proteins and lipids, with the carbohydrate being covalently linked to either protein or lipid. The membrane may then be considered to be composed of protein, glycoprotein, lipid and glycolipid. The question to be answered is: "How are these components organized in the cell membrane such that a cell possesses the properties that it does?" A number of electron microscopic studies of membrane structure, physical studies of the properties of membrane components within the membrane, immunological localization and chemical studies have led to a variety of membrane models. These range from the lipid bilayer unit membrane (1), in which lipids interact by hydrophobic interaction with an equal distribution of protein present at the interior and exterior of the cell, to the lipoprotein subunit membrane (2), in which the membrane is composed of repeating subunits of lipid and protein. Most of the models presented are combinations or extensions of these two basic ideas. Virtually all of the models that have been presented to date show a large portion of the membrane protein associated with the exterior surface of the membrane (3). An

excellent source of material for studying the organization of membrane components is the erythrocyte membrane. Erythrocyte membranes can be obtained in large quantities, are easily isolated and can be obtained essentially free of any contaminating intracellular material.

In order to obtain a better understanding of membranes, the individual components comprising a membrane must be fractionated in a single step or they must be isolated for further characterization studies. One of the most useful methods that has been developed for the fractionation of membrane proteins is polyacrylamide gel electrophoresis. Electrophoreses of a variety of membranes in sodium dodecyl sulfate (SDS) or acetic acid-urea solutions have been performed. Electrophoresis of erythrocyte membranes in SDS solutions has resulted in the fractionation of the membrane proteins into a large number of species according to their molecular weights (4, 5). Using this method, it was shown that erythrocyte membranes are composed of 12-14 different classes of proteins. One problem which may be responsible for this heterogeneous pattern is aggregation of small protein components to form aggregates differing in molecular weight. To insure that aggregation is not leading to this heterogeneous pattern, Lenard (4) disaggregates membranes during solubilization by heating at 100° in 2-3% SDS for three minutes before electrophoresis in 1% SDS. He observes a number of bands with molecular weights ranging from 22,000 to 255,000. He also observes these same types of electrophoretic patterns after heat denaturation in urea and piperidine. The possibility of reaggregation upon cooling is not ruled out, nor is the formation of disulfide bridges. He has also indicated that the erythrocyte membranes contain two carbohydrate staining species. The species occurring in

the 108,000 molecular weight region was identified as the glycoprotein, while the other species occurring at a much lower molecular weight was identified as the glycolipid. Electrophoresis in SDS solutions shows that the erythrocyte membrane is composed of four major components with molecular weights of 255,000, 240,000, 108,000 and 86,000. Another electrophoretic system which has been used quite extensively is the acetic acid-urea procedure developed by Takayama et al. (6). This procedure involves disaggregating the membrane in phenol-acetic acid-water before subjecting it to electrophoresis. Demus and Mehl (7) have solubilized pig erythrocytes with phenol-formic acid-water and have observed 14-16 bands after polyacrylamide gel electrophoresis. They observed that the major components comprising about 30% and 13% of the total membrane protein had molecular weights of 48,000 and 27,000, respectively, with very little of the protein having a molecular weight above 100,000. Demus and Mehl (8) have also developed a procedure which renders possible the identification of water insoluble membrane proteins by immunoelectrophoresis. This procedure involves extracting erythrocytes with butanol followed by solubilization of the proteins in Tris buffer containing urea and Triton X-100. Schnaitman (9) has used an electrophoresis system in SDS-urea to fractionate the protein components of mitochondrial and microsomal membranes. A large number of protein components have been shown in other systems such as envelopes of gram-negative (10) bacteria and Mycoplasma membranes (11). These systems support the concept that membranes are composed of a heterogeneous collection of proteins instead of a single protein component.

Approximately 20% of the membrane protein can be isolated by dialysis against adenosine triphosphate (ATP) and mercaptoethanol (12),

and has been given the name spectrin. This component has been shown to be the largest molecular weight component seen in SDS electrophoresis. Salt-induced disaggregation (13), protein distribution in phenol-formic acid-water (8) and immunological cross reaction studies (14) have been presented which suggest that spectrin is an aggregate. Laico et al. (15) have claimed that about 95% of the membrane protein is composed of a "miniprotein" of molecular weight 5,000. Gwynne and Tanford (16) have presented evidence for the heterogeneity of membrane proteins. They have extracted human erythrocyte proteins with 6 M guanidine hydrochloride and chromatographed them on a 4% Agarose column and have found the molecular weights of membrane proteins to be in the range of 14,000 to 200,000. These proteins were studied by optical rotatory dispersion and shown to be in the random coil conformation. This type of study would seem to confirm the heterogeneity of membrane proteins with a major portion being of high molecular weight instead of low molecular weight.

The distribution of membrane proteins can be affected by the isolation technique used to prepare membranes. Lenard (17) has shown that there is a considerable similarity between the erythrocyte membranes of different species. Some differences between dog and cat membranes when compared to horse and cow membranes have been noted (5, 18). However, this difference appears to be due to proteolytic digestion of the membranes during isolation (19, 20). This proteolysis results in part from contamination of the membrane preparation by white cells.

A number of other methods have been used to extract erythrocyte membranes for further characterization of specific proteins. Extraction with high salt concentrations (0.1-1.4 M NaCl) partially solubi-

lizes membrane proteins (21), but this results in a mixture of proteins. This procedure also gives partial solubilization of stromal lipids and the membrane bound enzyme, acetylcholinesterase, but does not give any gross disruption of the membrane structure. Fairbanks et al. (20) report that incubation of ghosts at low ionic strength gives the complete and selective release of three components of the membrane. The eluted material comprised 24% of the ghost protein and contained no sialic acid or carbohydrate-containing lipids. At high ionic strength, only one major component was extracted, which comprised 4% of the total ghost protein. It contained negligible amounts of sialic acid and no carbohydrate-containing lipids. Rosenberg and Guidotti (22) have removed approximately 50% of the proteins of the erythrocyte membrane by sequential extraction first with a solution of chelating agents, then with concentrated salt solutions. They also performed an alcohol-ether extraction of partially fractionated membranes. The remaining proteins were solubilized in SDS and fractionated by gel filtration into different but still heterogeneous fractions containing at least twelve membrane proteins with molecular weights ranging from 10,000 to 150,000. Acetic acid solutions have been used to solubilize ghost proteins, but this yields a heterogeneous mixture which may contain aggregates (23). Other solvents such as butanol (24, 25, 26), pentanol (18), and dilute aqueous solutions (27, 28) have been used to solubilize certain protein components of erythrocyte membranes. Various detergents have also been used to fractionate bacterial (29) and mitochondrial (30) membranes.

The glycoprotein of the erythrocyte has been extracted in purified form by phenol (31, 32, 33) and by lithuim diiodosalicylate (LIS) (34,

35). Blumenfeld (36) and co-workers (37) have used aqueous pyridine to separate proteins of the erythrocyte into two fractions: a water soluble, lipid-free fraction containing the glycoprotein and a water insoluble lipoprotein. Both fractions showed multiple bands on electrophoresis and were further fractionated by chromatography in 33% aqueous pyridine. The human glycoprotein extracted from erythrocyte membranes by LIS (35) has a reported molecular weight of 60,000, while a molecular weight of 31,000 has been reported for the disaggregated monomer as isolated by phenol extraction. Studies by SDS electrophoresis have shown that the molecular weight is dependent upon acrylamide percentage in the gel used (19, 38, 39). This may be due to the decreased binding of SDS to glycoprotein when compared to other proteins. The presence of sialic acid has also been shown to influence the mobility of the glycoprotein during electrophoresis (19, 38).

A glycopeptide can also be isolated from human erythrocytes following cleavage by trypsin (40). The glycopeptide can be purified by gel filtration and has been shown to have phytohaemagglutinin receptor site activity (41). There is evidence that the glycoprotein also contains the blood group activities, ABH, after removal of lipids (42, 43). There is still considerable controversy concerning the nature of these blood group substances. Hakamori (44) has extracted complex glycolipids from the erythrocyte membranes which have ABO and Lewis blood group activity. The glycoprotein isolated by LIS or by pyridine have been shown to possess ABH activity (35, 45) and MN blood group activity (40).

Because of the limited studies on individual components, there is little evidence for the molecular homogeneity of any of the proteins.

The protein spectrin, as isolated by Marchesi et al. (46), shows two or more bands in SDS electrophoresis. Spectrin tends to form a gel in the presence of divalent cations and fibrous elements can be demonstrated by electron microscopy (47). Spectrin seems to be similar to the muscle protein actin, but early studies showed it contains no detectable ATPase activity (47); however Rosenthal et al. (48) have reported a Ca^{++}-dependent ATPase activity associated with EDTA extracts of human erythrocyte membranes. It has been suggested (48, 49) that because of its ability to form fibrous elements, spectrin may play an important role in maintaining erythrocyte shape and deformability. Antisera have been prepared against spectrin (50) and soluble proteins from bovine stroma (14) and used to indicate the absence of spectrin at the cell surface (50).

By determining the localization of membrane components, one can obtain more information concerning the structure-function relationship of membranes. These involve such things as cellular control and cell-cell, cell-molecule interactions. There are four different methods which are used to obtain information about the localization of membrane components. These are: 1) electron microscopy studies of membrane structure, 2) physical studies of the properties of membrane components within the membrane, 3) immunological localization, and 4) chemical studies. A great deal of work has been done with the electron microscope; however structural artifacts are produced when samples are prepared for analysis. Staining membrane preparations with osmium tetroxide or permanganate shows the typical trilaminar structure which has been proposed to represent the lipid bilayer unit membrane (1). Sjöstrand and Barajas (51), using similar staining conditions, have been

able to show particular structures in the membrane and have suggested that these are lipoprotein subunits. A more recent technique to be used in membrane investigations is freeze cleaving. This procedure allows the membrane to be fractured along its natural cleavage planes in the frozen state (52). Using this technique, one is able to etch away the surrounding ice structure by sublimation and observe the exterior surface of membranes. Evidence has been presented that the erythrocyte membrane fractures in a plane within the structure of the membrane (53). Particulate surfaces have been observed on both sides of the fracture, but the number of particles was greater for the surface nearer the inside of the cell. It has been postulated that these particles contain carbohydrate (54, 55). The possibility of these particles containing lipid is not ruled out. This same type of cleavage pattern resembled those found for fatty acid bilayers (56). To date, this is the strongest evidence for the presence of large amounts of lipid bilayer structure in the membrane.

A number of attempts have been made to ascertain the conformational state of membrane proteins. With the use of infrared (IR) studies, Maddy and Malcolm (57) showed that there was little β-structure in the erythrocyte membrane. However, using this technique, some β-structure was found in mitochondrial membranes (58), suggesting that there is a difference between the two types of membranes. This difference observed may be due to differences in functions of the two membranes. Optical rotatory dispersion (ORD) and circular dichroism (CD) measurements from several laboratories (59, 60, 61, 62) have shown the presence of considerable α-helix and the absence of β-structure. Physical studies with CD have shown some of the features characteristic of proteins in

a partially α-helical conformation with a red shift in the CD spectra (63, 64, 65). This red shift was shown to be due to scattering effects and after necessary corrections were made, the membrane proteins were still about 40% in the right-hand α-helix. A large fraction of red cell membrane proteins can be converted to β-structure by incubation with ATP or D_2O as shown by IR studies (66). The presence of lipid-protein or lipid-sensitive protein-protein interactions have been suggested by a shift in the CD spectra after pertubation of membranes with lipolytic enzymes and detergents (67). Chapman et al. (68) have presented evidence for lipid-protein hydrophobic interactions. They showed a broadening of the NMR signals of lipid methyl and methlyene groups of lipid hydrocarbon chains. Hubbell and McConnell (69), using electron spin resonance (ESR), have shown that the preferred orientation of phospholipids is one in which the long amphiphilic axis is perpendicular to the membrane surface. Steim et al. (70) have shown that membranes of Mycoplasma laidlawii and water dispersions of protein free membrane lipids exhibit thermal phase transitions. The identity of membrane and lipid transition temperatures suggests that in membranes, lipids are in the bilayer conformation in which the hydrocarbon chains associate with each other rather than with proteins. Mycoplasma membranes have also been studied by ESR after spin labeling of fatty acids (71). The signals obtained indicate that a major portion of membrane lipids is in a semiviscous hydrocarbon environment. The authors suggest that the membrane consists of a lipid bilayer. Freeze-etch techniques have revealed particulate components in the hydrophobic region of the membrane (71). The mobility of lipids in intact cells may be influenced by their association with these particles. X-ray

diffraction studies also indicate that the bulk of the membrane lipid is in a bilayer arrangement (72).

The importance of the interaction between membrane proteins and lipids has brought about numerous chemical studies on membranes. The subunit model of membranes was based on the isolation of a "structural protein" in membranes (73). This substance was proposed to be a protein which comprises a large fraction of the membrane protein and was shown to have the ability to bind lipids by hydrophobic interactions. Senior and MacLennon (74) have developed new methods for preparing "structural protein" and were able to show that the preparation was heterogeneous and that it contained two major components. They concluded that at least one major component is inactivated ATPase and that there is no justification for stating the preparation fulfills a structural role.

A large number of experiments have been performed with erythrocyte membranes in order to obtain more information regarding the localization of membrane components. The experiments have suggested that the distribution of membrane proteins is not as uniform as most models show. Localization of membrane components relative to the permeability barrier has been performed by chemical and enzymatic studies. Eylar et al. (75) have used neuraminidase to show the presence of sialic acid at the surface of erythrocyte membranes. Trypsin has been used to show the presence of glycoprotein at the cell surface (76). Bender et al. (77), using a different proteolytic enzyme, Pronase, have shown that two protein species are present at the exterior surface. These have been identified as the glycoprotein and another species with a calculated molecular weight of 108,000, which is cleaved to yield a 70,000 molec-

ular weight species. Carraway et al. (78) have used diazotized sulfanilic acid, a non-penetrating agent, also to show that two protein species are present at the surface of the erythrocyte. These two were also identified as the glycoprotein and the 108,000 molecular weight species. The enzyme lactoperoxidase has been used by Phillips and Morrison (79, 80) to iodinate the exterior proteins of the erythrocytes. By using this enzyme, it has been shown that only two protein species are specifically labeled. Using inside-out and rightside-out vesicles, obtained from erythrocyte membranes, and proteolytic enzymes, Steck et al. (81) have reached a different conclusion concerning the orientation of the erythrocyte membrane proteins. They have concluded that a majority of the membrane proteins are present at the exterior surface. However, studies have been performed in which ferritin-labeled antibodies to spectrin (50) and ferritin conjugated plant agglutinins (82) have been attached to the membrane. Localization of the ferritin by electron microscopy is consistent with glycoproteins on the exterior surface and spectrin on the interior surface of the cell membrane.

Exchange of erythrocyte cholesterol and phospholipids with lipid dispersions (83) suggests that there are lipids at the surface of the membrane. This has also been suggested by phospholipase haemolysis of red blood cells (84). However, Zwaal et al. (85) have chromatographically purified phospholipase and have shown that this purified enzyme does not haemolyze red blood cells. This does not prove that lipids are not present at the surface. They were able to show that the phospholipids of ghost membranes are more susceptible to phospholipase than those of the intact red blood cells. They concluded that this was due to structural differences between the two types of membranes or to the

permeability. Casu et al. (86) have also been able to show a difference in phospholipid accessibility to phospholipase in sheep erythrocytes before and after haemolysis. Carraway et al. (87) have also obtained evidence of a change in phospholipid interactions in intact red blood cells and ghost membranes by using acetic anhydride to label the membranes. Nanni et al. (88) have used immunological techniques to demonstrate the presence of lipids at the surface of sheep erythrocytes. Recombination of lipids and proteins can lead to more information regarding the interaction between membrane lipids and proteins. Zwaal and van Deenen (89) have studied the recombination of proteins and lipids and have shown that recombination requires opposite charges on lipids and proteins and is pH dependent. Zahler and Weibel (90) separated lipids and proteins by column chromatography after the membranes were solubilized in 90% 2-chloroethanol. Recombination experiments were performed and the typical trilaminar structure was observed with the recombinant by electron microscopy. Density gradient studies of original and reconstituted membranes showed binding of lipid to only about 60% of the protein after recombination.

There are a number of enzymes associated with the erythrocyte membrane, most notably those of the glycolytic pathway (91, 92). These enzymes are not known to play any role in membrane structure because they are loosely held to the membrane. Other enzymes such as acetylcholinesterase and ATPase may play a role in membrane structure since they are dissociated from the membrane with difficulty. Acetylcholinesterase from bovine erythrocytes can be removed from the membrane during haemolysis (93). This process requires the absence of divalent cations in the medium. The release of acetylcholinesterase is pre-

vented by Ca^{++} or Mg^{++} in 1-5 mM concentrations in the haemolyzing buffer. Membranes prepared by this method maintain their morphological shape and total acetylcholinesterase and lipid levels. The membrane fragment solubilized in the absence of divalent cations behaves as a lipoprotein. Acetylcholinesterase is apparently localized at the exterior of the erythrocyte since it can be inactivated by trypsin treatment of erythrocytes that does not cause haemolysis (78, 94). Three different ATPase activities are associated with the erythrocyte membrane, Ca^{++}-ATPase (95), Mg^{++}-ATPase, and Na^{+}, K^{+}-ATPase (91). These three enzymes may be associated with only one protein in the membrane and may not represent three different proteins. Rosenthal et al. (48) have reported a Ca^{++} dependent activity associated with a protein that can be extracted by EDTA. This protein may be spectrin but it is unlikely that a protein as abundant as spectrin would have such a low enzyme activity unless this enzyme must be associated with lipids to exhibit full activity. Using electron microscopic cytochemical techniques, Marchesi and Palade (96) have shown that the Na^{+}, K^{+}-ATPase and Mg^{++}-ATPase enzymes are located at the inner surface of erythrocyte membranes. Osmotic lysis of red cells has also been used to elucidate the localization of membrane bound enzymes and to observe alterations in these enzymes during membrane preparation. Bramley et al. (97) reported that human erythrocyte membranes prepared at low osmolarities (20 osmolar) showed a high Na^{+}, K^{+}, Mg^{++}-ATPase activity, whereas membranes prepared at high osmolarities (80 osmolar) exhibited a low Na^{+}, K^{+}, Mg^{++}-ATPase activity. This suggests that this enzyme may be located on the interior side of the membrane and that low osmolarity buffers are required to increase the permeability of the membrane to ATP. More

recently (98) this was shown to be true for human erythrocytes. It was shown that membranes prepared by osmotic lysis in 20 osmolar buffer exhibited a decrease in total ATPase activity with an increase in Na^+, K^+, Mg^{++}-ATPase activity during "opening" of the membrane. Other enzymes associated with erythrocyte membranes include the acid phosphatase (99) and a protease (100).

The effect of Ca^{++} on membrane stability may be of importance in understanding the organization of membranes. Calcium may play a role in binding lipids and proteins together resulting in the formation of differentiated regions within the membrane. Recent studies have indicated that several characteristics of red cells or ghosts are dependent on cellular Ca^{++} and ATP content. The depletion of ATP has been shown to be accompanied by intracellular accumulation of Ca^{++} (101). As a result of this Ca^{++} accumulation, it has been shown that the cell decreases in elasticity (101), volume (102, 103) and deformability (101), and increases in density (102). Chau-Wong and Seeman (106) have presented evidence that ATP could control membrane deformability by chelating the intracellular Ca^{++} and thus reducing the amount of Ca^{++} bound to the interior side of the cell membrane. This suggests the role of an ATPase in maintaining the energized state of the erythrocyte membrane. Palek et al. (103) have suggested that ghost configurations are controlled by membrane contractile proteins possessing Ca^{++}-ATPase activity. They showed that the addition of ATP and low concentrations of Ca^{++} stimulated membrane-bound ATPase, but there was no effect on membrane deformability. However, with higher Ca^{++} concentrations, such that ionic Ca^{++} is present, repression of deformability is observed. The loss of biconcavity was also shown to occur when the cell was de-

pleted of ATP (49, 107). It was found that as normal cells are depleted of ATP, membrane Ca^{++} increases (49). Also residual haemoglobin and non-haemoglobin proteins of the isolated membranes increase 200% and 60%, respectively. Regeneration of ATP, upon incubation in adenosine solutions, produced a reversal in both shape and deformability. EDTA (49, 107) and externally added ATP (49) block the effect of Ca^{++} on fresh cells. Other nucleotides had no effect in reversing the Ca^{++} effect.

Szász (108) observed that trypsin hinders the contraction of membranes induced by Ca^{++} only in the presence of trace amounts of ATP. This treatment also enhances binding of ATP to the membrane. It is not known whether it is the enzymatic utilization of the high energy stored in the ATP molecule, or the potent Ca^{++} chelating ability of this nucleotide, which plays a role in the maintenance of the biconcave shape. Szász (108) has also suggested that trypsin modification may decrease the number of Ca^{++} binding sites. This may result from modification of spectrin, since it is known that Ca^{++} causes this protein to aggregate (47, 48, 49). It has been suggested (109) that Ca^{++} is causing a conformational change in spectrin thereby affecting the shape of the membrane.

The loss of deformability and elasticity (101) during ATP depletion and Ca^{++} accumulation appears to be associated with the aging process (49). As a result of these losses, spherical rigid cells are produced which are more subject to fragmentation during passage through the capillary circulation. This fragmentation process has been suggested as a mechanism for determining, in part, erythrocyte life span of normal cells, and particularly in Heinz body anemias and microangi-

opathic haemolytic anemia (49). Erythrocytes from individuals with hereditary spherocytosis (HS), the most prevalent congenital haemolytic anemia of man, have been shown to be less deformable than normal erythrocytes. The HS membrane is qualitatively similar to normal, but it has a quantitatively different ATP requirement to prevent Ca^{++} interaction with the membrane. From this, LaCelle (49) has postulated that HS cells differ in their membrane affinity for Ca^{++} and that increased Ca^{++}-membrane interaction leads to decreased deformability as well as increased cation fluxes, which are a characteristic of haemolytic anemias.

The objective of this study is to obtain more information about the number, distribution and organization of polypeptide chains of the erythrocyte membrane and to determine the extent and the effects of calcium binding to erythrocyte membranes.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

Human blood was obtained from the Dallas Community Blood Bank and was used within one week of the withdrawal date. Animal blood samples were collected by the staff of the College of Veterinary Medicine, Oklahoma State University, in citrate or acid-citrate-dextrose solution. The samples were used within two days of collection. Chemicals for electrophoresis were obtained from Eastman (highest purity grade) or Canalco. Other materials and their sources are: sodium dodecyl sulfate, guanidine hydrochloride, cetyltrimethylammonium bromide (CTAB), Triton X-100, sodium azide, mercaptoethanol, bovine serum albumin, cytochrome c, soybean trypsin inhibitor, trypsin, Pronase, chymotrypsinogen A, phenylmethanesulfonyl fluoride (PMSF, [benzylsulfonyl fluoride]), adenosine triphosphate (ATP), ethylenediamine tetraacetic acid (EDTA), ethyleneglycol-bis (β-amino-ethylester) N,N'-tetraacetic acid (EGTA), Trizma base and Trizma acid from Sigma; 1,4-bis-[2-(5-Phenyloxazolyl)]-benzene (POPOP) and 2,5-Diphenyloxazole (POP) from Packard; α-chymotrypsin from Worthington; Enzite-trypsin from Miles Laboratories; ^{3}H-acetic anhydride and calcium-45 from Amersham/Searle; ^{14}C-cholesterol from New England Nuclear; Blue Dextran from Pharmacia; Bio-Gel P-100, Bio-Gel A-15M, Dowex 50 X-4 and Dowex 1 X-8 from Bio-Rad. All other reagents were of reagent grade. SDS, guanidine hydro-

chloride and CTAB were recrystallized from hot ethanol before use.

Methods

Procedure for Washing Blood

Whole blood was centrifuged at 1,100 x g for twenty minutes. The plasma and buffy coat were removed by aspiration. The red cells were washed four to five times in 310 milliosmolar (mOsM)[1] phosphate or Tris, pH 7.4, or 0.15 M sodium chloride-10 mM Tris, pH 7.4. The cells were centrifuged at 1,100 x g after each washing and special precaution was taken to remove all white cells. After the final washing the cells were packed at 3,000 x g for twenty minutes before use.

Preparation of Erythrocyte Membranes

Erythrocyte membranes were prepared by the procedure of Dodge et al. (110). This involves haemolyzing one volume of washed red cells with fourteen volumes of 10 mOsM Tris, 20 mOsM phosphate or 10 mM Tris, all at pH 7.4. Haemolysis was allowed to occur for one hour at 4°. The cells were centrifuged at 20,000 x g for twenty minutes and washed free of haemoglobin with haemolyzing buffer. After each centrifugation run, the tightly packed pellet at the bottom of the tube was removed by aspiration. After the final washing, the ghosts were packed at 48,000 x g for one to two hours and the ghosts were taken up to 4.8 mg protein per ml.

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[1] mOsM is ideal milliosmolar and is defined as the total concentration of all ionizable species in the solution.

Preparation of Resealed Ghosts

Resealed ghosts were prepared according to the procedure of Redman (111). To one volume of washed red cells was added ten volumes of 10 mM Tris, pH 7.4, or 10 mM Tris containing 1.0-20.0 mM Mg^{++}, 0.1-5.0 mM Ca^{++} or 1 mM Ca^{++} with a two-fold excess of ATP, EDTA or EGTA, at pH 7.4. After ten minutes incubation at 4^{o}, the haemolysate was taken to isotonic conditions by the addition of either 1.42 M KCl-0.18 M NaCl, 3 M NaCl, 3 M NaCl containing 0.1-5.0 mM Ca^{++} or 3 M NaCl containing 2 mM ATP, EDTA or EGTA. The suspension was incubated at 37^{o} for thirty minutes. The cells were centrifuged at 5,900 x g for twenty minutes and the supernatant was decanted. The cells were washed free of haemoglobin with 0.15 M NaCl-10 mM Tris, pH 7.4. Ghosts were prepared by haemolysis with 10 mM Tris as described earlier. In some cases, after haemolysis with Ca^{++}-Tris, the haemolysate was not taken to isotonic conditions, but was incubated at 37^{o} for thirty minutes directly after haemolysis, or at 4^{o} for thirty minutes.

Preparation of Closed Membranes

Closed membranes were prepared by suspending one volume of freshly prepared ghosts in one volume of Krebs-Ringer salt solution (112) and then incubating for two hours at 37^{o}. The closed membranes were washed one time with Krebs-Ringer salt solution before use.

Procedure for ^{3}H-Acetic Anhydride Labeling of Ghosts

Five ml of ghosts in isotonic phosphate, pH 7.4, were allowed to react with 2 mC of ^{3}H-acetic anhydride (40 mM) for thirty minutes at

room temperature (87). The ghosts were dialyzed overnight against 0.02% sodium azide at 4° to remove unreacted ^{3}H-acetic anhydride. The labeled ghosts were collected after centrifugation at 48,000 x g for one hour and stored at -20°.

Procedure for ^{3}H-Acetic Anhydride Labeling of α-Chymotrypsin and Chymotrypsinogen A

Inactive α-chymotrypsin (2.5 mg/ml) was prepared by treating with PMSF (5 mM) (113). Inactive chymotrypsin or chymotrypsinogen A at 2 mg/ml was reacted with 10 mM ^{3}H-acetic anhydride (0.5 mC) in 0.15 M NaCl-10 mM Tris, pH 8.0, or Krebs-Ringer solution at room temperature for one hour. The labeled proteins were dialyzed against 1 mM HCl or distilled deionized water for 24 hours with three changes of dialyzing media. The samples were lyophilized, then taken up in 1 ml of NaCl-Tris buffer or Krebs-Ringer salt solution.

Amino Acid Analysis

Amino acid analysis was performed on a Beckman Model 120 C amino acid analyzer after hydrolysis of the protein in 6 N HCl at 110° for 22 hours (114).

Determination of Individual Neutral Sugars and Hexosamines

Carbohydrate samples were hydrolyzed, in sealed tubes, with 2 N H_2SO_4 in a boiling water bath for four to six hours. The hydrolysate was passed through a 1.35 x 12 cm column of Dowex 50 X-4 cation exchange resin over a 1.35 x 5 cm column of Dowex 1 X-8 anion exchange

resin (115). The columns were washed four times with 10 ml of water used at each washing. The Dowex 1 X-8 column was washed an additional two times with 12 ml of water each. These washings, containing neutral sugars, were lyophilized and then taken up in 5 ml of water. The individual neutral sugars were determined with the use of a Technicon-auto carbohydrate analyzer using buffer system as described by Hudson et al. (116). The hexosamines were collected after eluting the Dowex 50 column with three washes of 6 ml each using 2 N HCl. The hexosamines were determined with the amino acid analyzer.

Assays

Protein was determined by the method of Lowry et al. (117), using bovine serum albumin as a standard. Sialic acid was determined either by the procedure of Warren (118) or by Jourdian et al. (119). Total carbohydrate was determined by the procedure of Chen et al. (121) and cholesterol by the procedure of Zlatkis et al. (122).

Procedure for Solubilization and Chromatography in SDS

One volume of packed ^{3}H-labeled ghosts was added to one volume of 6% SDS and 0.02% sodium azide in 0.1 M phosphate at pH 7.8. The sample was made 1% in mercaptoethanol and allowed to stand 18-20 hours at room temperature under a nitrogen atmosphere. Cholesterol-4-^{14}C (2 μl) was added and the sample was chromatographed on a 2.5 x 90 cm P-100 column, previously calibrated with blue dextran and cytochrome c, and eluted with 1% SDS and 0.02% sodium azide in either 0.05 M Tris, pH 8.0, or 0.05 M phosphate, pH 7.0. Fractions (3.0 ml) were collected with an

Isco fraction collector. The radioactivity profile for all columns was determined by counting aliquots in Bray's solution (123) on a Packard Tri-Carb Liquid Scintillation counter. The protein profiles were determined from the absorbance at 280 nm on a Hitachi Perkin-Elmer Double Beam Spectrophotometer.

Procedure for Solubilization and Chromatography in CTAB-Guanidine Hydrochloride

To 0.4 ml of ^{3}H-labeled ghosts was added 0.1 ml of 10% CTAB. The sample was mixed vigorously, 2.5 ml of 6 M guanidine hydrochloride was added and the mixing repeated until complete clarification of the solution was obtained. After adjustment of the pH to 8.5, the mixture was made 1% in mercaptoethanol and allowed to stand overnight at room temperature under a nitrogen atmosphere. The mixture was then taken to pH 6.5 and a 0.5 ml aliquot was chromatographed on a 1.5 x 90 cm P-100 column or a 1.5 x 75 cm A-15 M column. The columns were eluted with 5.5 M guanidine hydrochloride which contained 0.1% mercaptoethanol. The profile was obtained as described above.

Procedure for Rechromatography of Fraction from Guanidine Hydrochloride Column on SDS Column

The total fraction obtained from chromatography in guanidine hydrochloride was dialyzed against 0.02% sodium azide at room temperature for two days with four changes of dialyzing media. The sample was then dialyzed one day against 1% SDS at room temperature, lyophilized and redissolved in 0.5 ml water. To this was added 0.5 ml of 6% SDS and 0.02% sodium azide in 0.1 M phosphate, pH 7.8, and the mixture

was incubated with 1% mercaptoethanol overnight at room temperature under a nitrogen atmosphere. The sample was chromatographed on a P-100 column with an elution buffer of 1% SDS and 0.02% sodium azide in 0.05 M Tris, pH 8.0.

Procedure for Reaggregation of Fractions from SDS Column

Aliquots of fractions obtained from SDS chromatography on P-100 columns were dialyzed, individually or combined, overnight against 0.02% sodium azide at room temperature. The samples were then transferred to the cold room and dialyzed against 20 mM Mg^{++} in 20 mM Tris, pH 7.4, for three days with two buffer changes. The contents of the dialysis bags were transferred to small centrifuge tubes and centrifuged at 48,000 x g for thirty minutes. The supernatant solution was decanted from the pellet and both were saved for analysis.

Electrophoresis

Samples for electrophoresis were prepared by adding 200 µl of ghosts (2.5 mg protein per ml) to 100 µl of 4% SDS in 0.1 M phosphate, pH 8.0. The samples were made 1% in mercaptoethanol and incubated at 100° for three minutes or overnight at room temperature before being subjected to electrophoresis. The samples were made 20% in glycerol before being applied to gels.

SDS polyacrylamide electrophoresis was performed according to the procedure of Kobylka *et al.* (19) on 10 cm 5% gels containing 0.1% SDS. Electrophoresis was carried out at 8 ma/gel for three and one half hours. The gels were stained with coomassie blue according to the

procedure of Fairbanks et al. (20) with the elimination of the third staining step and were destained in 7% acetic acid. Gels were stained for carbohydrates by the procedure of Glossmann and Neville (124). Stained gels were scanned at 550 nm on a Gilford 2000 spectrophotometer equipped with a Model 2410 linear transport assembly. Band designations are given by Kobylka et al. (19) and by Carraway and Shin (125).

Proteolysis of Intact Red Cells

Washed red cells (25% suspension) from human, bovine, horse and sheep were digested for one hour at room temperature with trypsin or chymotrypsin in isotonic phosphate, Tris or sodium chloride-Tris at pH 7.4. Trypsin concentration was varied from 0-5 mg enzyme per ml of reaction media, and in some cases 0.1-5.0 mM Ca^{++} was present. Chymotrypsin concentration was varied from 0-2 mg per ml in the presence and absence of 1.0-5.0 mM Ca^{++}. Trypsin was inhibited by the addition of a two fold excess of soybean trypsin inhibitor or 2 μmoles PMSF (in dimethylsulfoxide) per mg enzyme. Chymotrypsin was inhibited by the addition of 2 μmoles PMSF per mg enzyme. After the addition of inhibitor, incubation was continued for an additional thirty minutes. The cells were centrifuged at 3,000 x g for twenty minutes, and the supernatant solution saved for carbohydrate and sialic acid determinations. The cells were washed four times with isotonic buffer. Ghosts were prepared as described earlier, and after the last washing they were taken to a volume of 2 ml with hypotonic buffer. An aliquot of ghosts was solubilized in SDS and subjected to electrophoresis. The ghosts obtained from trypsin digested human red cells were analyzed for cholesterol, sialic acid and acetylcholinesterase activity (126).

Pronase digestion of intact red cells was performed according to the procedure of Bender et al. (77). This involved washing the red cells three times in 0.15 M NaCl and two times in Tris total buffer, pH 7.4 (Tris, 17.3 mM; NaCl, 130 mM; KCl, 3.6 mM; $CaCl_2$, 1.2 mM; $MgSO_4$, 1.2 mM). A 25% suspension of red cells from human, bovine, horse and dog was digested with 0.05-2.0 mg Pronase per ml for one hour at 37° in Tris total buffer, pH 7.4. Pronase was inhibited by the addition of 3 μmoles PMSF per mg enzyme followed by incubation for thirty minutes at room temperature. Twenty ml of cold 15 mM EDTA in Tris total buffer was added and the cells were centrifuged at 3,000 x g for twenty minutes. The supernatants were saved for carbohydrate and sialic acid determinations. The cells were washed two times with Tris total buffer. In one instance, PMSF was not added to inhibit Pronase digestion on human red cells and the red cells were washed five times instead of two. Ghosts were prepared in 10 mOsM Tris, pH 7.4, or in Ca^{++}-veronal buffer, pH 7.4 (19, 93), to insure membrane integrity. The membranes were taken to 2 ml and an aliquot was solubilized in SDS and subjected to electrophoresis.

Proteolysis of Resealed Ghosts

A 25% suspension of resealed ghosts was digested in isotonic Tris or sodium chloride-Tris, pH 7.4, for one hour at room temperature with 0.1-2.0 mg trypsin per ml. Digestion was carried out in the presence and absence of 1 mM Ca^{++}. Trypsin was inhibited with PMSF, followed by incubation for thirty minutes at room temperature. Ghosts were prepared in 10 mOsM Tris, pH 7.4. Samples of digested resealed ghosts were subjected to electrophoresis.

Proteolysis of Isolated Membranes

Ghosts were isolated as described earlier and were subjected to digestion by trypsin and chymotrypsin by a number of different procedures. These are described below:

Procedure A - Ghosts, at a membrane protein concentration of 1.25 mg/ml, were digested with trypsin at 0-5 mg/ml in isotonic phosphate, Tris or sodium chloride-Tris at pH 7.4. Digestion was carried out for one hour at room temperature. Trypsin was inhibited by soybean trypsin inhibitor or PMSF as described in the section on proteolysis of intact red cells. After inhibition the ghosts were washed four times with hypotonic buffer. After the last washing the volume of ghosts was taken to 2 ml with buffer. Aliquots of digested ghosts were solubilized in SDS and subjected to electrophoresis. The remaining ghosts were analyzed for cholesterol, sialic acid and acetylcholinesterase activity.

Procedure B - Ghosts, at a protein concentration of 1.25 mg per ml, were digested in isotonic Tris with 0.05 mg/ml trypsin in the presence of 20 mM Mg^{++}, Ca^{++}, ATP, EDTA, MgATP or CaATP. Inhibition of trypsin was by soybean trypsin inhibitor as described earlier. The ghosts were washed five times with hypotonic Tris, pH 7.4, and the volume of ghosts was taken to 2 ml after washing. Aliquots of ghosts were subjected to electrophoresis, and the Ca^{++}-ATPase activity was determined on the remaining ghosts according to the procedure of Cha *et al.* (127).

Procedure C - Ghosts (2.5 mg protein per ml) were digested with trypsin and chymotrypsin at 10 μg/ml for 0-20 minutes in isotonic and hypotonic phosphate, pH 7.4. Digestion was stopped at the times desired

by adding aliquots of digestion media to SDS at 100°. The samples were then prepared for electrophoresis.

Procedure D - Ghosts (2.5 mg protein per ml) were digested with 0-10 μg trypsin per ml for one hour at room temperature. Aliquots were added to SDS at 100° and subjected to electrophoresis.

Procedure E - Ghosts (2.5 mg protein per ml) were digested with trypsin and chymotrypsin at 0.5 μg/ml in the presence of 1 and 5 mM Ca^{++}. Digestion was carried out in isotonic Tris, pH 7.4, for one hour at room temperature. Aliquots were added to SDS at 100° and subjected to electrophoresis.

Procedure F - Enzite-trypsin was suspended in sodium chloride-Tris, pH 7.4, and washed five times with this buffer. Each washing was preceded with a 15 minute incubation at room temperature. Ghosts were suspended to 2.5 mg protein per ml and incubated with washed and unwashed Enzite-trypsin at 5 mg/ml for two hours at room temperature. The reaction was stopped by incubating an aliquot of reaction media in SDS at 100° for three minutes. The suspension was centrifuged to remove the resin. The solubilized membranes were prepared for electrophoresis.

Proteolysis of Closed Membranes

Closed membranes, prepared from ghosts obtained by haemolysis of red cells in 20, 40, 60 and 80 mOsM phosphate, were suspended to 2.5 mg/ml and subjected to trypsin digestion. Digestion was performed at room temperature for 30 minutes with trypsin at 0-200 μg/ml. Digestion was terminated by adding aliquots of ghosts to SDS at 100°. These samples were subjected to electrophoresis.

Permeability of Membranes to α-Chymotrypsin or Chymotrypsinogen A

Labeled α-chymotrypsin or chymotrypsinogen A at concentrations of 0.05, 0.10 and 1.0 mg/ml were incubated with 30, 50 and 70% suspensions of packed erythrocytes, ghosts or closed ghosts for fifteen minutes at room temperature. The suspensions were centrifuged and aliquots of 0.1 ml were taken for counting in Bray's solution (123). Control samples of labeled proteins were prepared which contained no membranes.

Procedure for Studying the Effects of Calcium on Membranes

To one volume of ghosts (4.8 mg protein per ml) was added ten volumes of 10 mM Tris, pH 7.4, containing 0.1-5.0 mM Ca^{++}. Sufficient 3 M NaCl was added to bring the suspension to isotonic conditions and incubation was carried out at 37° for thirty minutes. The cells were washed three times with 10 mM Tris and packed at 48,000 x g for one hour. The ghosts were resuspended to 2.4 mg protein per ml, then prepared for electrophoresis.

Procedure for Calcium-45 Binding Studies

One volume of red blood cells or ghosts was added to ten volumes of 10 mM Tris, pH 7.4, containing 0-15 mM Ca^{++} with approximately 1.0 μC of ^{45}Ca (128). In some cases the haemolyzing buffer contained 0-15 mM ATP or EDTA. The suspension was brought to isotonic conditions by the addition of 3 M NaCl and incubated at 37° for thirty minutes. The cells were washed two times in 0.15 M NaCl in 10 mM Tris, pH 7.4, then rehaemolyzed with 10 mM Tris in the presence and absence of 0-15 mM ATP

or EDTA and washed with 10 mM Tris. The isolated membranes along with ^{45}Ca quench standards were counted according to the procedure of Forstner and Manery (128). The remaining membranes were saved for cholesterol determination. Membranes were subjected to SDS acrylamide electrophoresis. Gels were sliced and counted as previously described (78) to determine the ^{45}Ca localization.

CHAPTER III

COLUMN CHROMATOGRAPHY OF ERYTHROCYTE MEMBRANES

Chromatography in SDS Solutions

Erythrocyte membranes can be separated into two different fractions by chromatography on Bio-Gel P-100 in 1% SDS. Figure 1 shows the profile for SDS-solubilized membranes to which ^{14}C-cholesterol had been added as a marker. In SDS solutions, membrane proteins are disaggregated from the membrane lipids. The second peak, containing ^{14}C-cholesterol, contains the lipid fraction of the membrane, since it is known that externally added cholesterol exchanges with membrane cholesterol (87). That the lipid exists as a micelle of SDS, phospholipid, glycolipid and cholesterol has been shown by carbohydrate and phosphorous analyses (129). Prelabeling membranes with radioactive acetic anhydride offers a very sensitive method of assay which can be used to monitor the elution profile from chromatographic columns. Membrane proteins and lipids can be labeled, since both contain free amino groups. Figure 2 shows the elution profile of a sample of labeled human erythrocyte membranes. The absorbance profile indicates that there is little protein present in the lipid peak, while a majority of the protein is eluted at the void volume. The lipid peak elutes well before the sucrose marker, indicating that the lipid exists as an aggregate rather than as a molecularly dispersed species. To determine the distribution of protein, carbohydrate and phospholipid in each peak,

Figure 1. Chromatographic Profile of Human Erythrocyte Membranes Solubilized in SDS

One volume of packed ghosts was solubilized in one volume of 6% SDS and 0.02% sodium azide in 0.1 M phosphate at pH 7.8. ^{14}C-cholesterol was added and the sample was chromatographed on a 2.5 x 90 cm P-100 column and eluted with 1% SDS and 0.02% sodium azide in 0.05 M phosphate, pH 7.0. Protein (——) and ^{14}C-cholesterol (----). 3.0 ml fractions were collected.

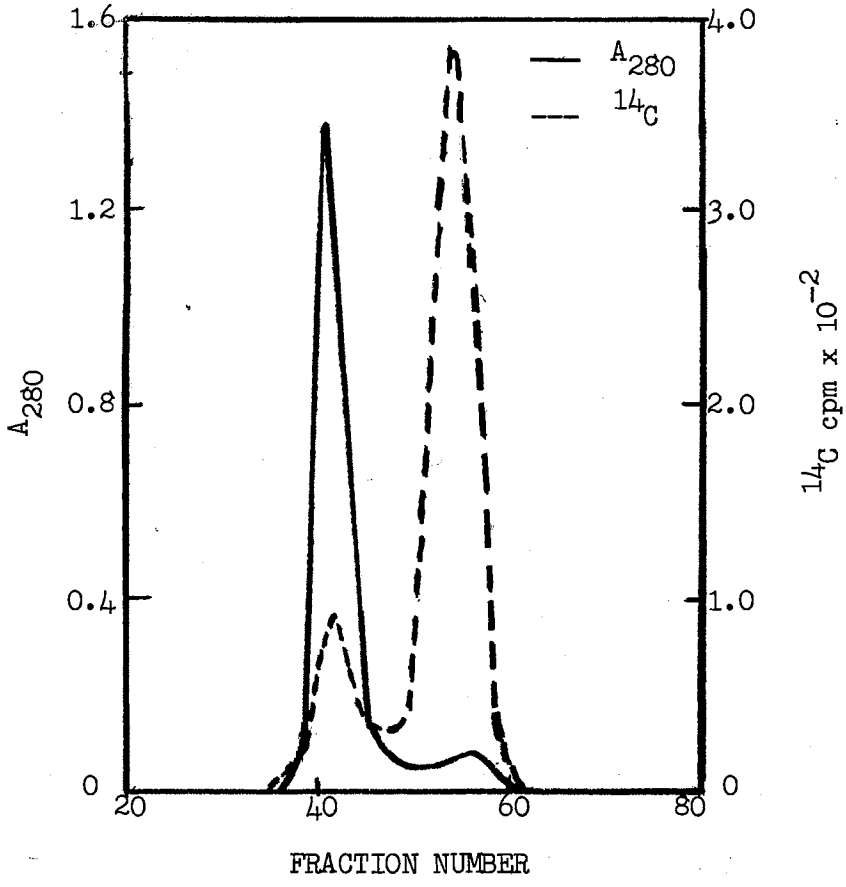
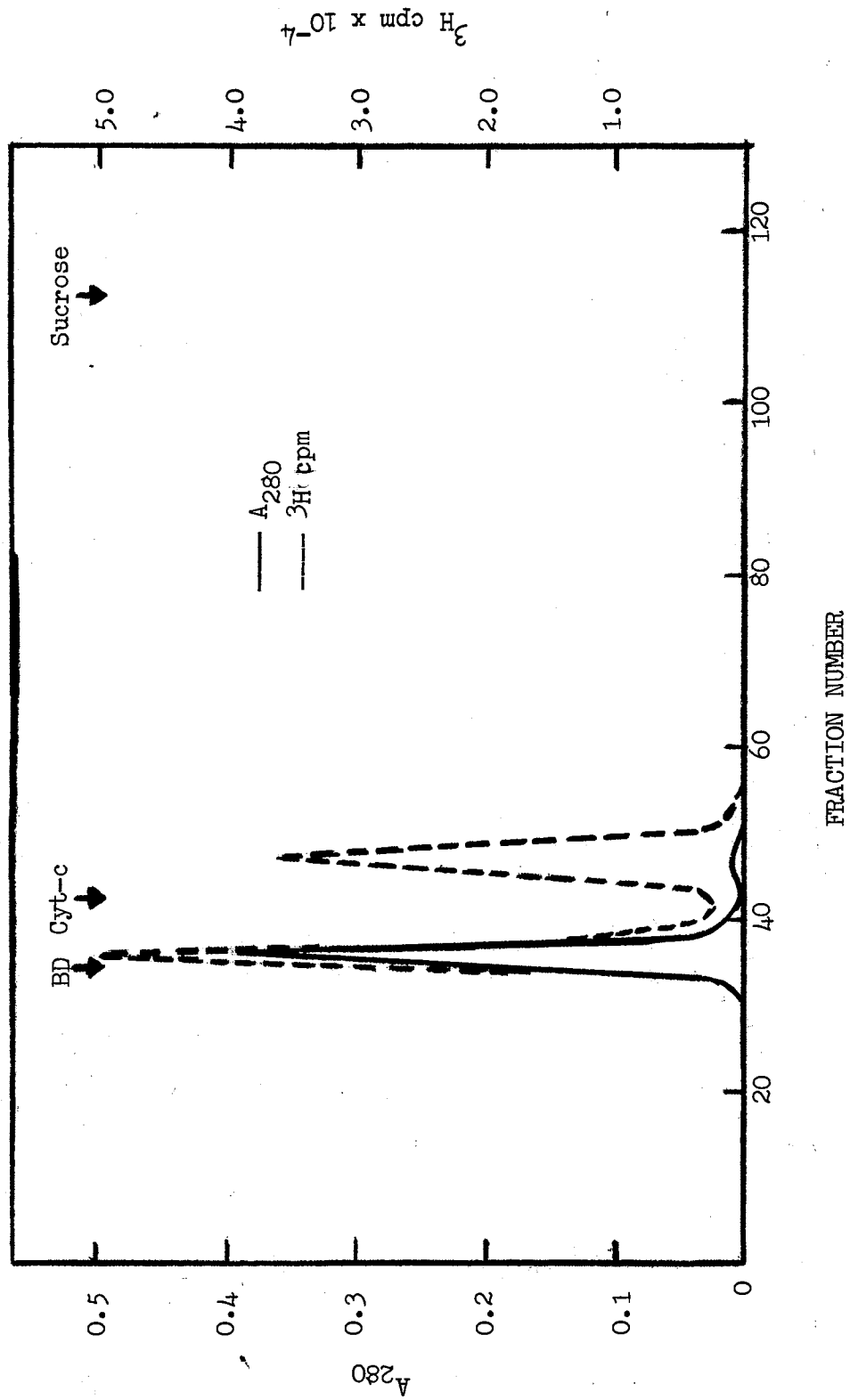

1.6
1.2
0.8
0.4
0
A_{280}
4.0
3.0
2.0
1.0
0
^{14}C cpm x 10^{-2}
20
40
60
80
FRACTION NUMBER
A_{280}
^{14}C

Figure 2. Chromatographic Profile of ^{3}H-labeled Human Erythrocyte Membranes Solubilized in SDS

One volume of ghosts labeled with ^{3}H-acetic anhydride was solubilized in one volume of 6% SDS and 0.02% sodium azide in 0.1 M phosphate at pH 7.8. The sample was chromatographed on a 3.5 x 90 cm P-100 column and eluted with 1% SDS and 0.02% sodium azide in 0.05 M Tris, pH 8.0. Protein (——) and radioactivity (----). BD, blue dextran; cyt c, cytochrome c. 3.0 ml fractions were collected.


A_{280}
^3H cpm x 10^{-4}
FRACTION NUMBER
Sucrose
BD Cyt-c
A_{280}
^3H cpm
0
0.1
0.2
0.3
0.4
0.5
1.0
2.0
3.0
4.0
5.0
20
40
60
80
100
120

all fractions containing radioactivity for each peak were combined. Samples of each peak were analyzed for protein by the Lowry procedure, or were hydrolyzed and subjected to amino acid analysis. Phospholipid was determined as total phosphorous. Table I shows the composition of the separated fractions for a typical chromatographic separation. Amino acid analysis of the chromatographic fractions indicates that no significant amount of the total membrane protein exists in the lipid fraction. Less than 5% of the total protein was eluted after cytochrome c, suggesting that the bulk of membrane protein is of high molecular weight. Attempts to measure protein by the Lowry method gave higher protein values for the lipid fraction, indicating the poor reliability of this technique for measuring protein in the presence of lipid. Table II shows the amino acid analysis of the two fractions obtained during chromatography. Hydrolysis of the protein was performed in the presence of SDS to prevent loss of peptides during SDS removal, but this procedure does not permit analysis of amino acids which are susceptible to oxidation. No ethanolamine was observed in the protein peak suggesting that lipid is disaggregated from protein. Separation of protein and lipid by SDS chromatography allows one to identify the individual carbohydrates associated with each component. Table III shows the neutral sugar and hexosamine content of the two fractions. The lipid fraction contains no mannose or fucose and approximately one-half as much galactose and twice as much glucose as the protein fraction. The protein fraction contains the bulk of glucosamine and both fractions contain an equal amount of galactosamine.

Laico *et al.* (15) have suggested on the basis of SDS electrophoresis and chromatography that the protein of erythrocyte membranes is

TABLE I

COMPOSITIONS OF P-100 FRACTIONS

Fraction	Proteins (%)		Carbohydrates (%)	Phosphorous (%)
	Lowry	Amino Acid		
1	79.4	95.1	52.4	15.0
2	20.6	4.9	47.6	85.0

TABLE II

AMINO ACID ANALYSIS OF PROTEIN AND LIPID FRACTIONS FROM SDS CHROMATOGRAPHY[a]

Amino Acid	Protein Fraction[b]	Lipid Fraction[b]
Lys	3.2	---
His	1.5	---
Arg	3.4	---
Asp	5.3	Tr
Thr	3.4	Tr
Ser	4.3	0.9
Glu	7.8	Tr
Pro	2.9	---
Gly	3.6	Tr
Ala	4.2	Tr
Val	4.4	---
Met	---	---
Ile	3.0	---
Leu	7.1	---
Tyr	---	---
Phe	2.0	---
Eth-NH_2[c]	---	2.0

[a]Values expressed as μmoles eluted in the total fraction for each peak.

[b]Values substituted by dash indicate the amino acid was not detectable; Tr indicates trace amount present.

[c]Ethanolamine

TABLE III

NEUTRAL SUGAR AND HEXOSAMINE COMPOSITION OF PROTEIN AND LIPID FRACTIONS FROM SDS CHROMATOGRAPHY[a]

Sugar	Protein Fraction	Lipid Fraction[b]
Mannose	0.9	---
Fucose	0.3	---
Galactose	4.4	2.8
Glucose	0.9	1.9
Glucosamine	13.5	2.0
Galactosamine	3.5	3.2

[a]Values expressed as μmoles eluted in the total fraction for each peak.

[b]Values substituted by dash indicate the sugar was not detectable.

an aggregate of low molecular weight species. The amino acid data from Tables I and II indicate that this is not the case. To see if further disaggregation of the protein could be achieved, the protein fraction from the experiment of Figure 2 was rechromatographed on the same P-100 column. Figure 3 shows the profile obtained from this rechromatography procedure. No further disaggregation could be achieved, as shown by the single radioactive peak eluted at the void volume of the column.

Since chromatography in SDS solutions appeared to be a good method of separating glycoprotein from glycolipid, it also appeared that this may be a useful method for studying antigenic activities associated with one or both of these components. The objective was to remove the disaggregating agent from each fraction, separately or recombined, and to determine if the components express antigenic activity in their disaggregated or aggregated states. This involved reaggregation experiments in which equal amounts of the two fractions were dialyzed, separately or recombined, to remove SDS. Table IV shows the protein and phosphorous distribution between the two fractions and between the pellets and supernatants after exhaustive dialysis to remove SDS. The pellets obtained from the dialyzed samples of protein (F1P) and lipid (F2P) fractions and from the combined fractions (F1,2P) were washed with 0.9% NaCl then tested for the presence of SDS. This involved adding a small amount of washed red cells to an aliquot of washed pellet and determining the extent of haemolysis. In all reaggregation experiments, the SDS could not be completely removed, as evidenced by 100% haemolysis of red cells. This made it impossible to determine the antigenic activity of the fractions.

Figure 3. Rechromatography of Protein Fraction from Figure 2

The protein fractions from Figure 2 were pooled and a one milliliter aliquot was taken to 3% in SDS. The sample was reduced with mercaptoethanol, then placed on a P-100 column, 3.5 x 90 cm, and eluted with 1% SDS and 0.02% sodium azide in 0.05 M Tris, pH 8.0. 3.0 ml fractions were collected.

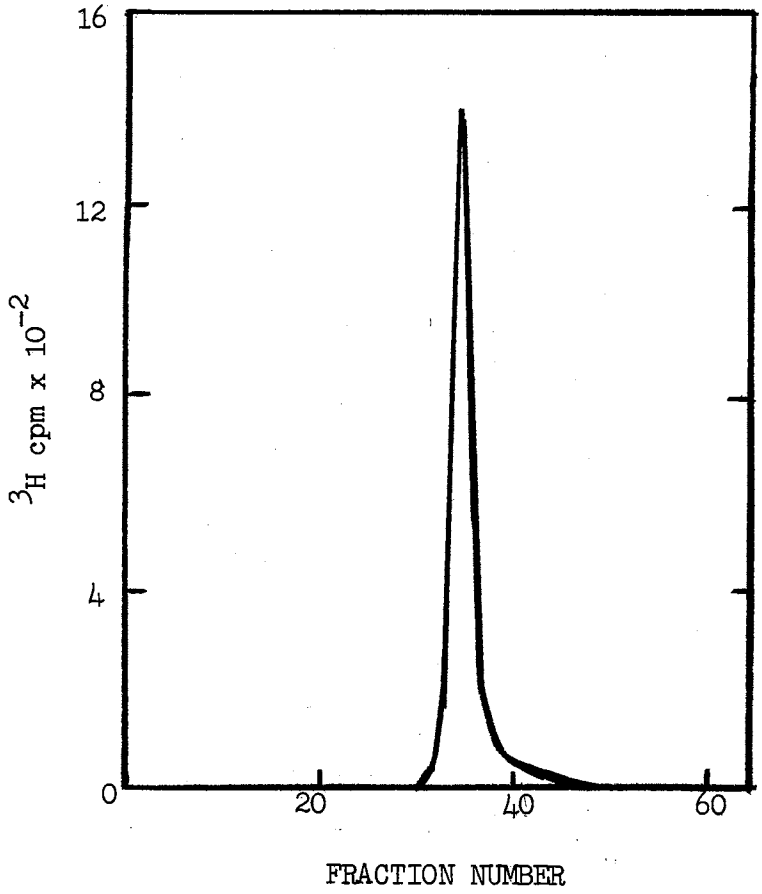

16
12
8
4
0
20
40
60
^{3}H cpm x 10^{-2}
FRACTION NUMBER

TABLE IV

COMPOSITION OF PROTEIN AND LIPID FRACTIONS
FROM RECOMBINATION EXPERIMENT

Samples of protein and lipid fractions were dialyzed, separately or recombined, against 0.02% sodium azide overnight at room temperature. The samples were transferred to the cold room and dialyzed against 20 mM Mg^{++} in 20 mM Tris, pH 7.4, for three days. The samples were centrifuged at 48,000 x g for thirty minutes. The pellets and supernatant solutions were separated and analyzed.

Fractions[a]	Protein[b]	Phosphorous[c]
F1	0.36	3.0
F2	0.09	17.0
F1P	0.18	—
F1S	0.29	—
F2P	—	12.5
F2S	—	2.9
F1,2P	0.29	5.2
F1,2S	0.12	18.0

[a] F1 - Total Fraction 1
F2 - Total Fraction 2
F1P - Fraction 1 pellet
F1S - Fraction 1 supernatant
F2P - Fraction 2 pellet
F2S - Fraction 2 supernatant
F1,2P - Fractions 1 & 2 pellet, recombined
F1,2S - Fractions 1 & 2 supernatant, recombined

[b] Values expressed as mg of protein; dash indicates amount not detectable.

[c] Values expressed as μg of phosphorous; dash indicates amount not detectable.

Chromatography in CTAB-Guanidine Hydrochloride Solutions

Gwynne and Tanford (16) were able to show that about 60% of the erythrocyte membrane was solubilized and fractionated in 6 M guanidine hydrochloride. As a result of this, a system was developed for the total solubilization of membranes at high concentrations of guanidine. To obtain total solubilization, the membranes were first treated with 10% CTAB followed by vigorous mixing and the addition of 6 M guanidine hydrochloride. Total solubilization was measured by turbidity. An attempt was made to fractionate the membrane components by chromatography on P-100 in 5.5 M guanidine hydrochloride. As seen in Figure 4, only one peak of radioactivity at the void volume of the column was obtained. This indicated that no separation of the protein and lipid components had been achieved as in the SDS system. The fractions comprising the peak were pooled and the guanidine was removed by dialysis. The sample was lyophilized, then hydrolyzed and subjected to amino acid analysis. Table V shows the amino acid analysis of the peak obtained by chromatography in guanidine. These values are compared to those of the intact membrane (130). These data show that both protein and phospholipid are still present in the one peak. Failure of component separation could be due to an overlap of the protein peak with an aggregate of phospholipid. This could be a result of the low resolving power of the P-100 column. Gwynne and Tanford (16) used a 4% Agarose column to fractionate guanidine hydrochloride solubilized membranes; therefore this type of column was used to attempt to fractionate the CTAB-guanidine solubilized membranes. As seen in Figure 5, only one peak at the column void volume was obtained, and it contained both the

Figure 4. Chromatographic Profile of ^{3}H-labeled Human Erythrocyte Membranes Solubilized in CTAB-Guanidine Hydrochloride from P-100 Column

To 0.4 ml of ^{3}H-labeled ghosts were added 0.1 ml of 10% CTAB and 2.5 ml of 6 M guanidine hydrochloride. The sample was reduced with mercaptoethanol and a 0.05 ml aliquot was eluted from a P-100 column (1.5 x 90 cm) with 5.5 M guanidine hydrochloride. 1.8 ml fractions were collected.

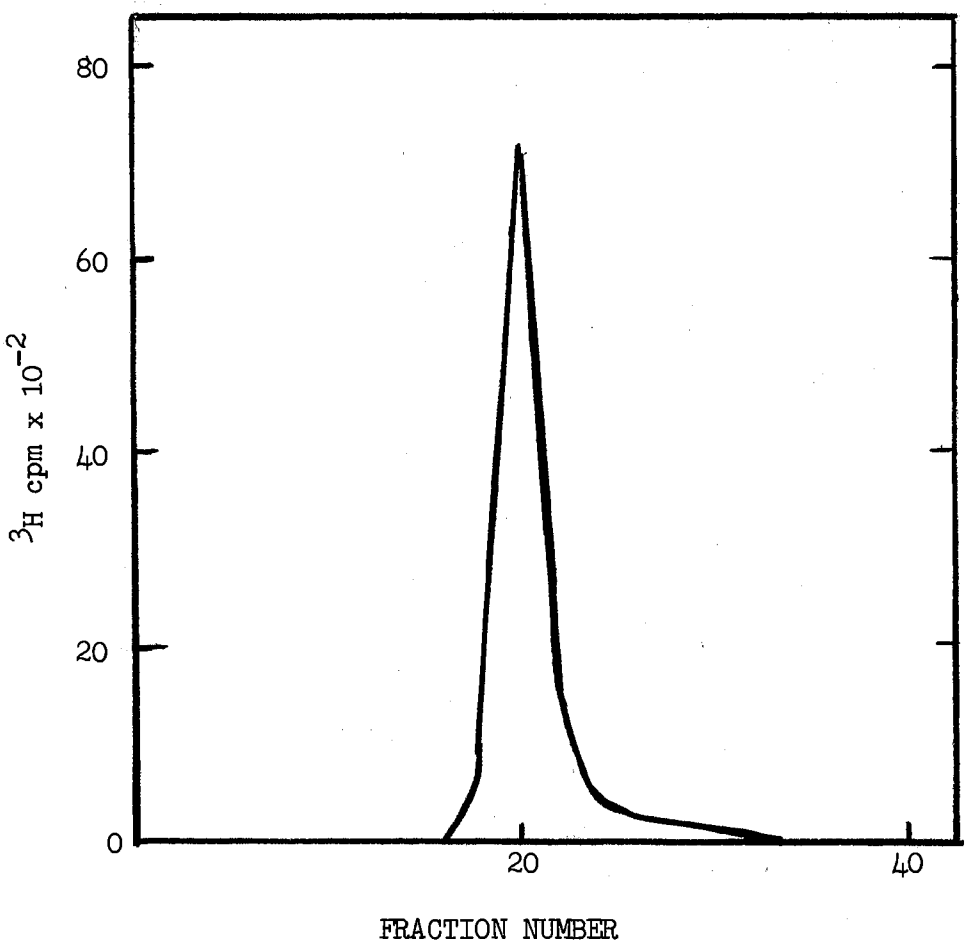

80
60
40
20
0
^{3}H cpm x 10^{-2}
20
40
FRACTION NUMBER

TABLE V

AMINO ACID ANALYSIS OF GUANIDINE HYDROCHLORIDE FRACTION AND ORIGINAL RED CELL MEMBRANE[a]

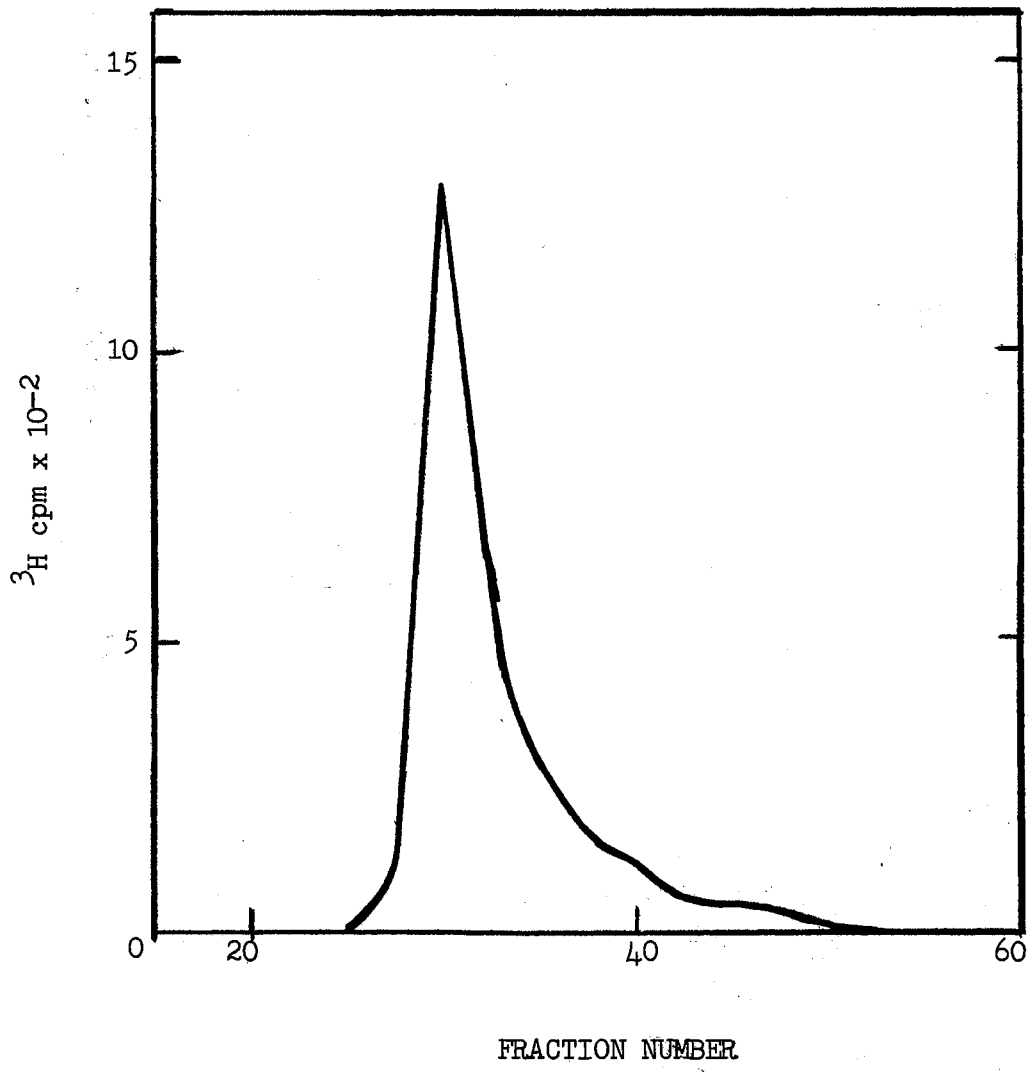
Amino Acid	Guanidine Hydrochloride Fraction	Ghost[b]
Lys	5.4	5.0
His	2.5	2.2
Arg	4.6	5.1
Asp	11.6	9.4
Thr	5.0	5.7
Ser	11.2	7.9
Glu	12.9	12.8
Gly	9.1	6.3
Ala	8.7	8.8
Val	5.8	6.4
Met	2.1	2.0
Ile	4.2	4.5
Leu	10.4	12.4
Tyr	2.5	2.3
Phe	4.2	4.0

[a]Values expressed as mole percent

[b]Values obtained from D. Kobylka, Ph. D. dissertation (130)

Figure 5. Chromatographic Profile of ^{3}H-labeled Human Erythrocyte Membranes Solubilized in CTAB-Guanidine Hydrochloride from A-15M Column

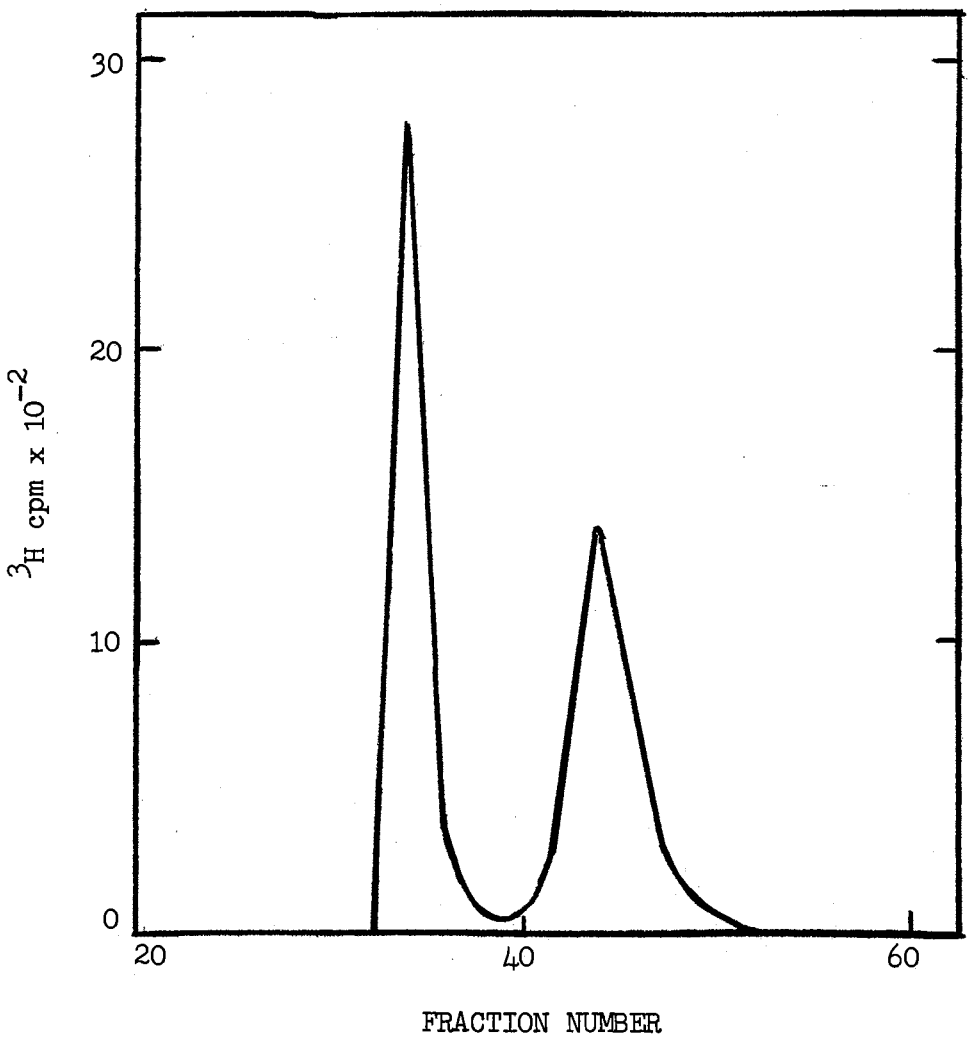
^{3}H-labeled ghosts were solubilized in CTAB-guanidine hydrochloride as described in Figure 4. A 0.5 ml aliquot was eluted from an A-15 M column (2.5 x 75 cm) with 6.0 M guanidine hydrochloride. 1.8 ml fractions were collected.


15
10
5
0
20
40
60
^{3}H cpm x 10^{-2}
FRACTION NUMBER

protein and phospholipid of the membrane. This suggests that a soluble aggregate of membrane components must be formed in the presence of CTAB and guanidine hydrochloride. To show that this aggregate can be fractionated into protein and lipid components, the guanidine and CTAB of the peak fraction from a P-100 column were removed by exhaustive dialysis. The material was lyophilized and the residue was taken up in SDS and chromatography of the material was performed on the P-100 column in 1% SDS. As seen in Figure 6, the usual pattern of separated protein and lipid was obtained and is similar to that seen in Figure 2. This supports the idea that a soluble aggregate of membrane components is formed in the presence of CTAB and guanidine hydrochloride.

Figure 6. Rechromatography of Peak from Figure 4

The total peak from Figure 4 was dialyzed to remove the CTAB and guanidine hydrochloride. The sample was lyophilized, then taken up in 3% SDS and reduced with mercaptoethanol. The sample was placed on a P-100 column (3.5 x 90 cm) and eluted with 1% SDS and 0.02% sodium azide in 0.05 M Tris, pH 8.0. 3.0 ml fractions were collected.


30
20
10
0
^{3}H cpm x 10^{-2}
20
40
60
FRACTION NUMBER

CHAPTER IV

PROTEOLYTIC DIGESTION OF ERYTHROCYTES, RESEALED GHOSTS AND ISOLATED MEMBRANES

Proteolytic Digestion of Intact Human Erythrocytes

Proteolytic enzymes provide one of the most useful methods of investigating the distribution of proteins in the erythrocyte membrane. These enzymes do not penetrate membranes of intact cells; therefore any modification of the membrane is a result of modification of exterior membrane proteins. Proteolytic enzymes are known to release glycopeptides from erythrocyte membranes (76); however the accessibility of other membrane proteins to proteolytic digestion cannot be ascertained in this manner, since their cleavage products may not be released from the membrane. Polyacrylamide gel electrophoresis in SDS solutions has proven to be the most useful method to determine the effect of proteolytic enzymes on the membrane. If any protein is cleaved, but not released, a shift will result in its position on the gel toward the area of lower molecular weight.

Intact human erythrocytes were treated with a range of trypsin concentrations in isotonic phosphate-saline, and the membranes were isolated by hypotonic haemolysis. Changes in the components staining for protein and glycoprotein were monitored by SDS-polyacrylamide electrophoresis. Glycoprotein content was estimated by densitometric

analysis of periodate-Schiff stained gels from the areas under the curves. Only the major glycoprotein peak was included in the area calculations, so that staining of cleavage products does not contribute to the glycoprotein determination results. Figure 7 shows the loss of glycoprotein, acetylcholinesterase activity and sialic acid of isolated ghosts from trypsin treated human erythrocytes as a function of trypsin concentration. Approximately 60% of both the sialic acid and acetylcholinesterase activity were lost and virtually all of the glycoprotein was released from the membrane. Figure 8 shows the carbohydrate gel scans from trypsin treated erythrocytes. The glycoprotein which appears in the 100,000 MW region appears to be completely lost even at the lowest enzyme concentration. Figure 9 shows the protein patterns obtained from trypsin treated erythrocytes. The protein patterns are not drastically changed, even by trypsin at 5 mg/ml (not shown in figure). This indicates that none of the major membrane proteins are readily accessible to trypsin except the glycoprotein. It also shows that the glycoprotein cannot be identical to any of the major proteins which stain with coomassie blue. Digestion of erythrocytes was also performed with trypsin in the presence of 0.1-5 mM Ca^{++}, since it is known that Ca^{++} has an effect on both trypsin stability (130) and the deformability of the membrane (49). The addition of Ca^{++} had no effect on the digestibility of erythrocytes when subjected to trypsin. Gel patterns typical for erythrocyte membranes were obtained from Ca^{++}-trypsin treated erythrocytes.

Different proteases were used to compare the digestibility of membrane proteins with the digestibility obtained with trypsin. Bender et al. (77) have shown that the nonspecific protease, Pronase, cleaves

Figure 7. Alteration of Acetylcholinesterase Activity, Sialic Acid and Glycoprotein Content of Membranes Isolated from Trypsin-treated Human Erythrocytes

Washed human erythrocytes (25% suspension) were treated with 0-5 mg/ml of trypsin in isotonic phosphate-saline at pH 7.4 for one hour at room temperature. Trypsin inhibitor was added to stop the reaction, cells were washed in cold isotonic phosphate and membranes were isolated. Membranes were assayed for acetylcholinesterase, sialic acid and cholesterol and were subjected to gel electrophoresis. Glycoprotein was estimated from gel scans using Gilford Model 2000 after periodate-Schiff staining. 100% glycoprotein represents 62.4 cm^2 of chart paper. 100% acetylcholinesterase activity represents 2.5×10^{-3} moles of substrate hydrolyzed per minute per mg membrane cholesterol.

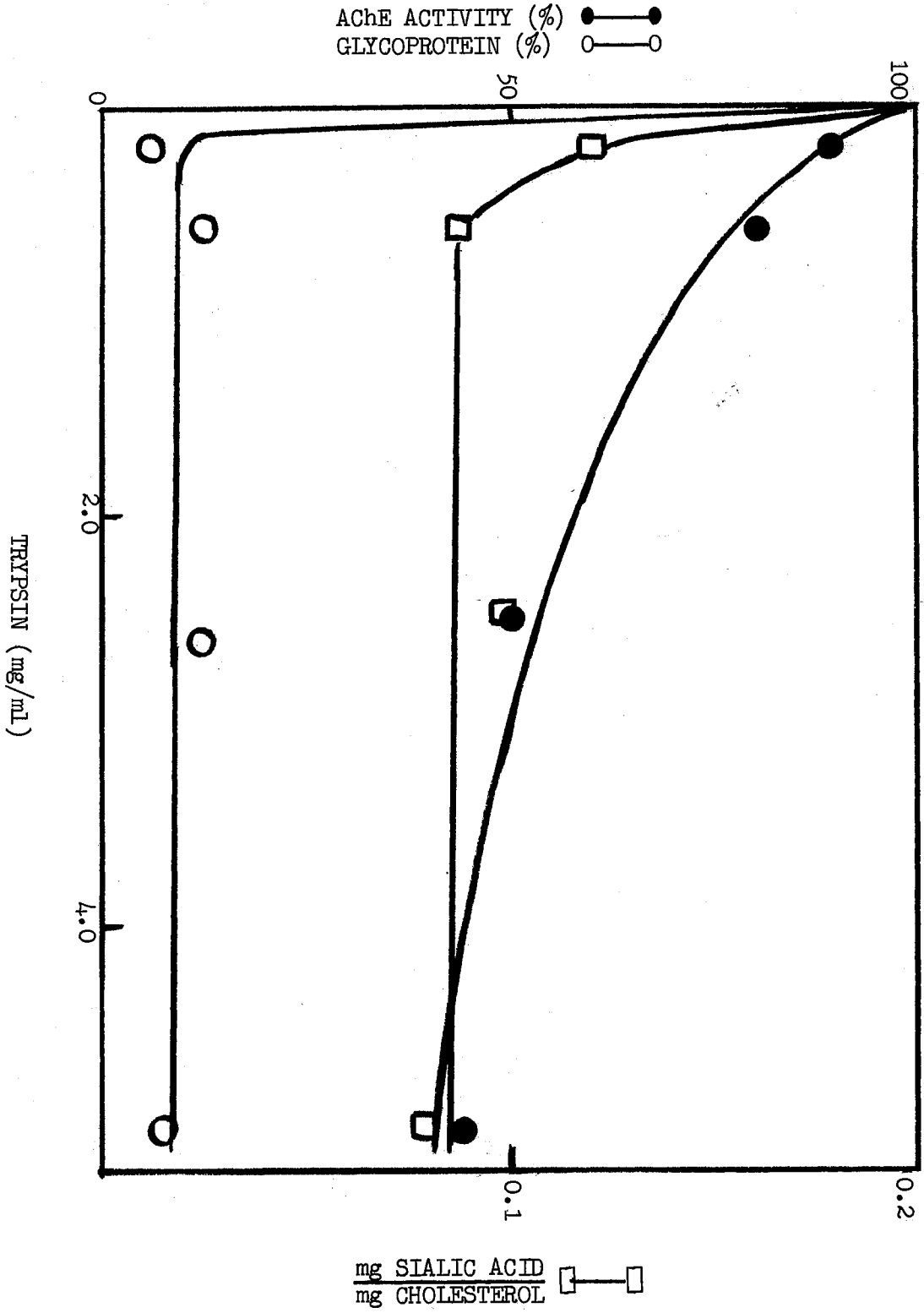

AChE ACTIVITY (%)
GLYCOPROTEIN (%)
0
50
100
2.0
4.0
TRYPSIN (mg/ml)
0.1
0.2
mg SIALIC ACID / mg CHOLESTEROL

Figure 8. Effect of Trypsin Digestion on Glycoprotein of Human Erythrocytes

Washed human erythrocytes (25% suspension) were incubated with trypsin as described in Figure 7. Trypsin concentrations were: Scan A, untreated control; Scan B, 0.25 mg/ml; Scan C, 5.0 mg/ml. Gels were run and stained as described in Methods and scanned at wavelength 550 nm. Peak at left is glycoprotein. Peak at far right is glycolipid.

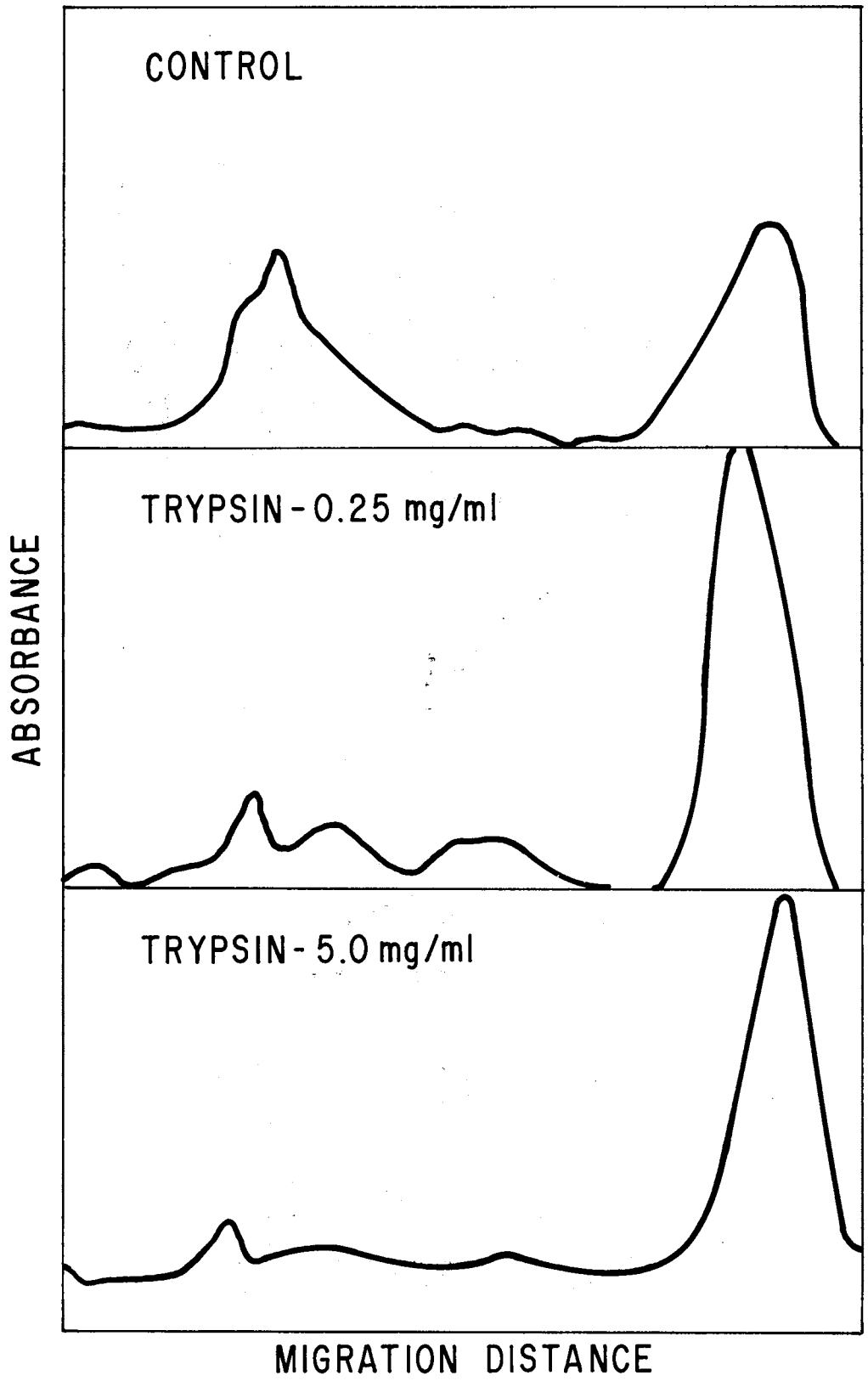
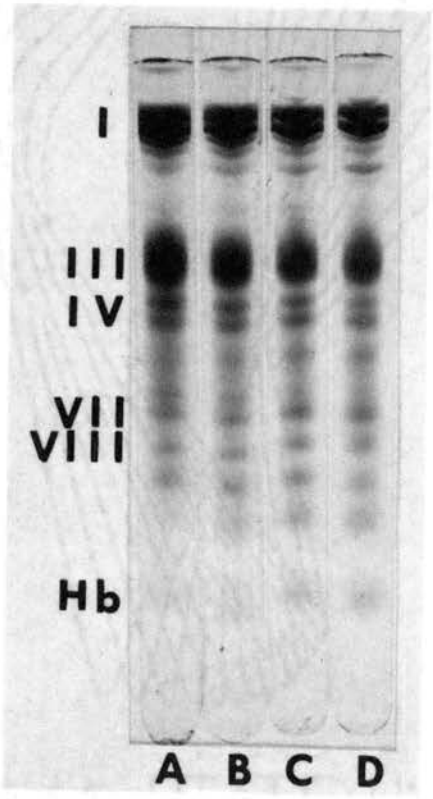

CONTROL
TRYPSIN - 0.25 mg/ml
TRYPSIN - 5.0 mg/ml
ABSORBANCE
MIGRATION DISTANCE

Figure 9. Concentration Dependence of Trypsin Digestion of Human Erythrocytes

Experimental conditions are described in Figure 7. Trypsin concentrations were: Gel A, untreated control; Gel B, 0.05 mg/ml; Gel C, 0.5 mg/ml; Gel D, 2.0 mg/ml. Gels were run, stained for protein and destained as described in Methods. Hb, haemoglobin.


I
III
IV
VII
VIII
Hb
A B C D

component III (see Figure 9) to yield a fragment, whose molecular weight was reported as 73,000. Steck et al. (81) have also shown that the protein patterns derived from trypsin and chymotrypsin digestion of inside-out and rightside-out vesicles were different, suggesting a different specificity of digestion by the two enzymes.

Erythrocytes were digested with chymotrypsin at 0-2 mg per ml under the same conditions as those used for trypsin. Figure 10 shows the concentration dependence of chymotrypsin digestion. There is a large decrease in the amount of component III (MW 108,000), paralleled by an increase in a proteolytic fragment (III-P) with a molecular weight of 70,000. Component III is quantitatively converted to III-P, since the protein present in bands III-P plus III is equivalent to that of III for membranes of undigested cells. In addition to cleavage of component III, there is a loss of glycoprotein as seen in the gel scans in Figure 11. Digestion of red cells with chymotrypsin in the presence of 1.0-5.0 mM Ca^{++} had no effect on digestibility of membrane proteins. Human erythrocytes were also digested with Pronase. Figure 12 shows the protein patterns obtained from membranes of Pronase digested cells. Pronase and chymotrypsin give the same type of digestion patterns. This suggests that component III must be a single polypeptide chain with a very limited number of sites that are accessible to proteolytic attack and that it may be partially buried within the membrane. An experiment which supports this hypothesis was performed in which Pronase was not completely inactivated from the cell preparation before preparation of the membranes. As seen in Figure 13, the pattern of membrane proteins is drastically altered by active Pronase which is still present during the haemolysis and washing steps. Component III-P

Figure 10. Concentration Dependence of α-Chymotrypsin Digestion of Human Erythrocytes

Experimental conditions are identical to those described in Figure 7, except that chymotrypsin was used in place of trypsin. Chymotrypsin concentration: Gel A, untreated control; Gel B, 0.05 mg/ml; Gel C, 0.5 mg/ml; Gel D, 2.0 mg/ml. Hb, haemoglobin.

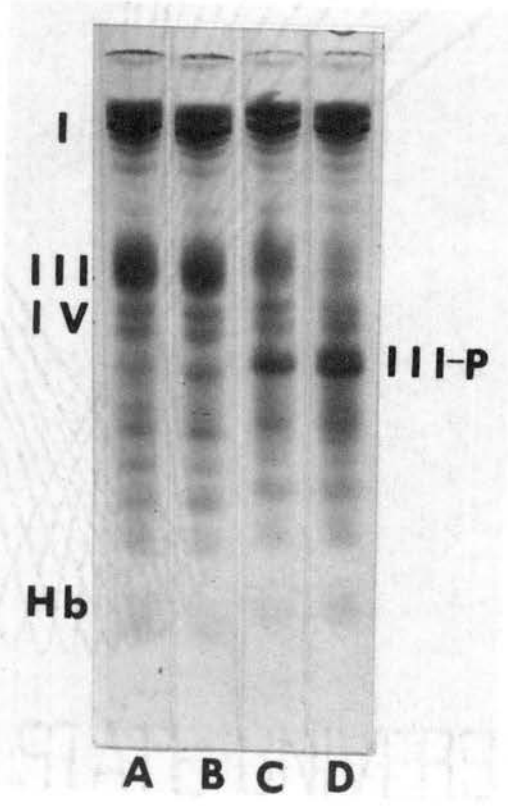

I
III
IV
III-P
Hb
A
B
C
D

Figure 11. Effect of Chymotrypsin Digestion on Glycoprotein of Human Erythrocytes

Experimental conditions are described in Figure 7. Chymotrypsin concentrations are: Scan A, untreated control; Scan B, 0.05 mg/ml; Scan C, 0.1 mg/ml. Gels were scanned at wavelength 550 nm.

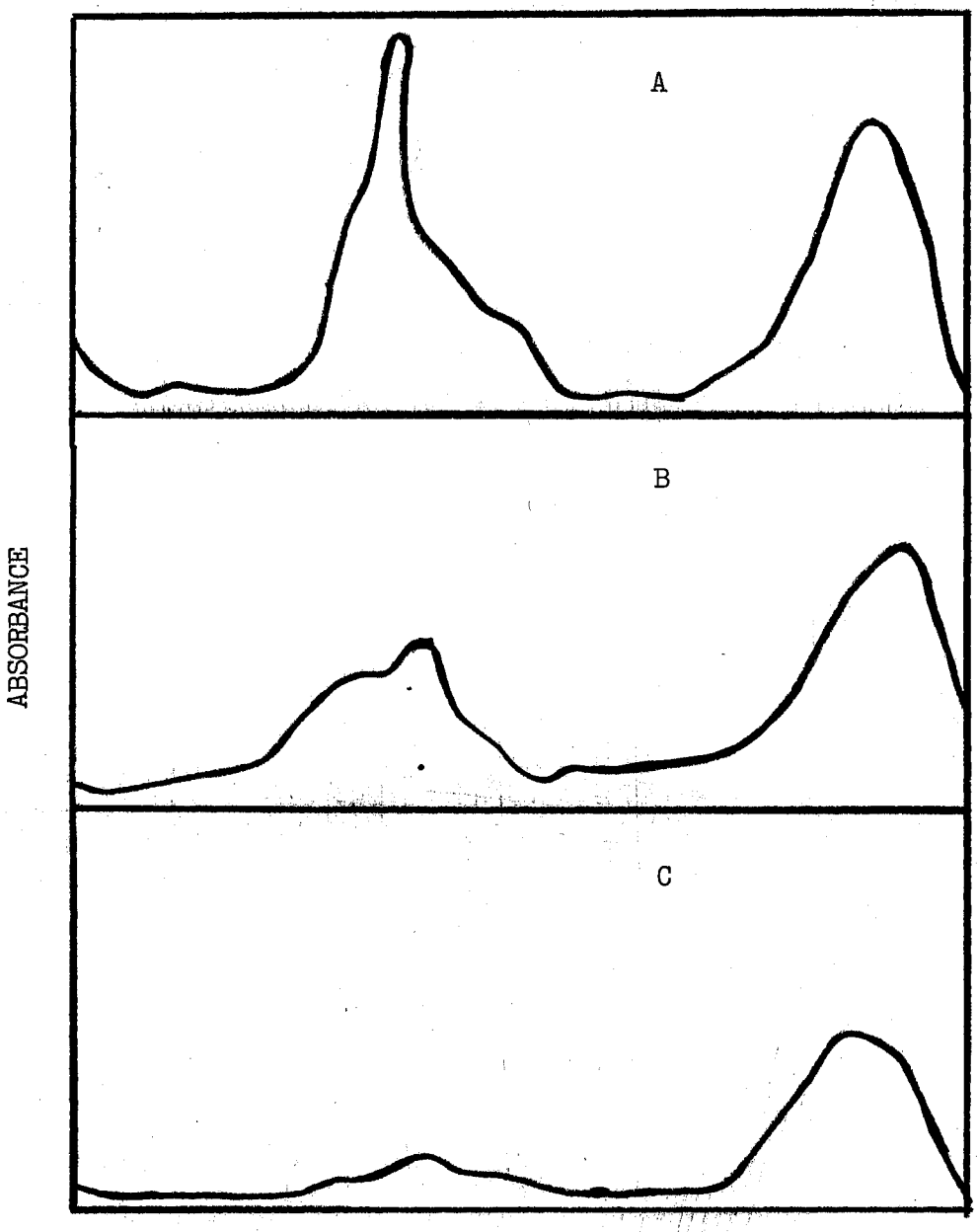

A
B
C
ABSORBANCE
MIGRATION DISTANCE

Figure 12. Concentration Dependence of Pronase Digestion of Human Erythrocytes

Washed human erythrocytes (25% suspension) were incubated in Tris total buffer, pH 7.4, at 37° for one hour with Pronase. The enzyme was inhibited with PMSF and the cells were washed two times before preparation of membranes. Pronase concentrations were: Gel A, untreated control; Gel B, 0.05 mg/ml; Gels C and D, 0.1 mg/ml. Hb, haemoglobin.

Figure 13. SDS Electrophoretic Patterns of Pronase-treated Human Erythrocytes Which Contained Proteolytic Activity During Membrane Preparation

Washed red cells were incubated at 37° for one hour with Pronase in Tris total buffer. The enzyme was inhibited with 0.15 M EDTA in Tris total buffer and cells were washed five times. Ghosts were prepared and subjected to electrophoresis. Gel A, untreated control; Gel B, 0.15 mg/ml; Gel C, 0.5 mg/ml.

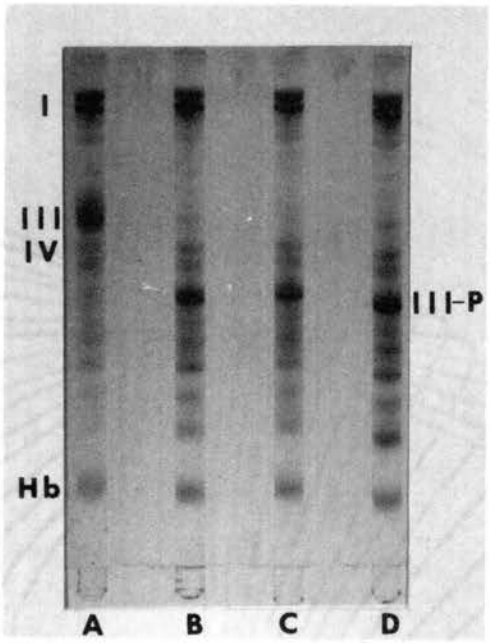


Figure 12

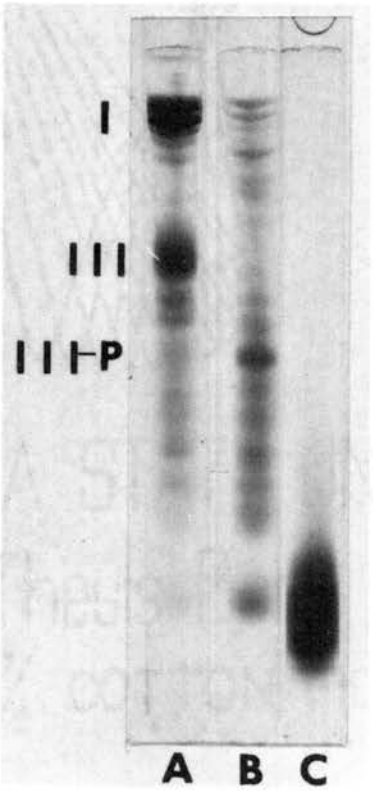


Figure 13

is still present even though most of the other protein species have been digested or washed into the medium. This result suggests that a part of component III corresponding to its digestion product III-P is protected by its orientation within the membrane.

Two possibilities may account for this digestibility of component III in erythrocyte membranes: 1) The polypeptide chain contains no basic groups (lysine or arginine) in the accessible area. This seems unlikely, since it has been shown (81) that a slight cleavage of component III of trypsin treated inside-out vesicles does occur, and yields a product of molecular weight 70,000. 2) The attack by trypsin is blocked in the intact cell by groups which can be removed by proteolytic digestion with the other enzymes. The glycoprotein which extends from the surface of the membrane may be the blocking group. Four different experiments were performed to see if the glycoprotein could possibly be the blocking group. 1) Cells were treated with trypsin (0-0.1 mg/ml) and washed to remove glycopeptide fragments which might adsorb to the membrane. The cells were then treated a second time with trypsin at the same concentration, washed and haemolyzed. 2) Cells were subjected to a double digestion with trypsin (0-0.1 mg/ml) followed by chymotrypsin (0-0.1 mg/ml). 3) Cells were subjected to a pre-treatment with chymotrypsin (0-0.1 mg/ml), which does not cleave component III extensively, followed by trypsin (0-0.1 mg/ml) treatment. Figures 14, 15 and 16 show the gel patterns obtained from experiments 1, 2 and 3, respectively. In each case, there is no significant increase in digestibility of component III or other membrane proteins after double digestion. The fourth experiment involved determining the amount of neutral sugar and sialic acid re-

Figure 14. Double Trypsin Digestion of Human Erythrocytes

Washed human erythrocytes (25% suspension) were digested with trypsin in isotonic phosphate-saline, pH 7.4, for one hour at room temperature. Trypsin was inhibited with soybean trypsin inhibitor and the cells were washed to remove trypsin and inhibitor. The cells were treated a second time with trypsin. Membranes were isolated in hypotonic phosphate, pH 7.4. Trypsin concentrations for double digestion were: Gel A, untreated control; Gel B, 0.05 mg/ml; Gel C, 0.1 mg/ml. Hb, haemoglobin.

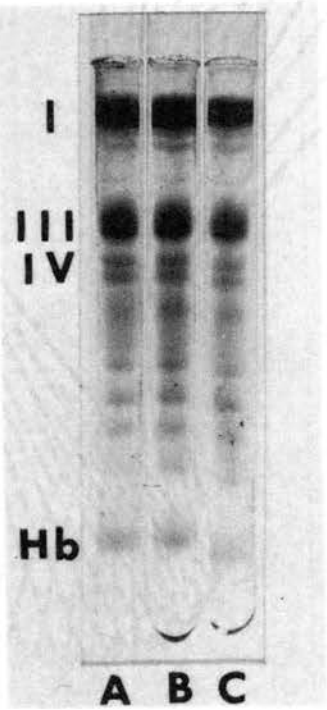

I
III
IV
Hb
A B C

Figure 15. Effect of Double Digestion of Human Erythrocytes with Trypsin Followed by Chymotrypsin

Washed human erythrocytes (25% suspension) were subjected to trypsin digestion as described in Figure 14. After washing the cells to remove trypsin and inhibitor, the cells were subjected to a second digestion with chymotrypsin, followed by inhibition of the enzyme with PMSF. Membranes were isolated and subjected to electrophoresis. Trypsin and chymotrypsin concentrations for double digestion were: Gel A, untreated control; Gel B, 0.05 mg/ml chymotrypsin; Gel C, 0.1 mg/ml chymotrypsin; Gel D, 0.05 mg/ml trypsin; Gel E, 0.05 mg/ml trypsin, 0.05 mg/ml chymotrypsin; Gel F, 0.1 mg/ml trypsin; Gel G, 0.1 mg/ml trypsin, 0.1 mg/ml chymotrypsin.

Figure 16. Effect of Double Digestion of Human Erythrocytes with Chymotrypsin Followed by Trypsin

Experimental conditions are described in Figure 15, except digestion was performed with chymotrypsin followed by trypsin. Gel A, untreated control; Gel B, 0.05 mg/ml trypsin; Gel C, 0.1 mg/ml trypsin; Gel D, 0.05 mg/ml chymotrypsin; Gel E, 0.05 mg/ml chymotrypsin, 0.05 mg/ml trypsin; Gel F, 0.1 mg/ml chymotrypsin; Gel G, 0.1 mg/ml chymotrypsin, 0.1 mg/ml trypsin.

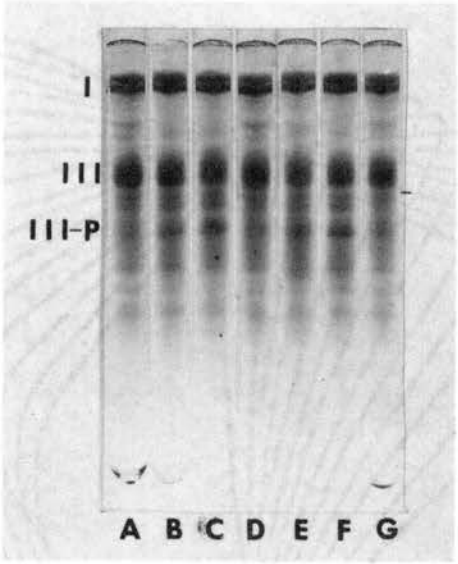


Figure 15

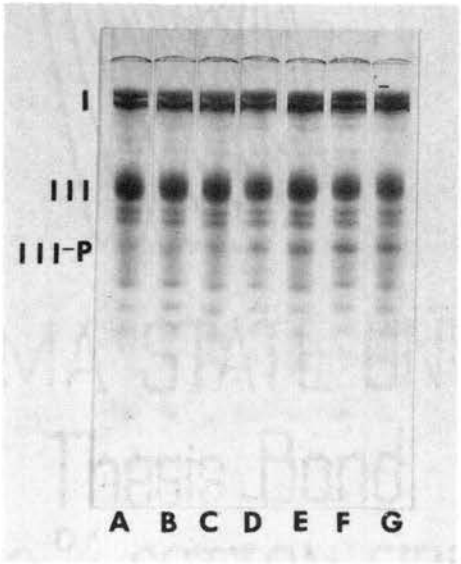


Figure 16

leased from the membrane after digestion of erythrocytes with different concentrations of trypsin, chymotrypsin and Pronase. If the glycoprotein is protecting component III, there would be a relationship between the amount of carbohydrate released and the amount of cleavage of component III. In this case, chymotrypsin and Pronase would be expected to release a greater amount of carbohydrate than trypsin. The results in Table VI do not indicate such a relationship. These experiments do not show any evidence for glycoprotein protection of component III from trypsin digestion.

Proteolytic Digestion of Erythrocytes from Other Species

Erythrocytes from other species were subjected to proteolytic enzymes to determine if there are any differences in the surface properties of these erythrocytes as compared to the human erythrocyte. The glycoprotein is lost from bovine erythrocytes when digested with trypsin but a double digestion of these erythrocytes with trypsin does not affect the protein pattern as seen in Figure 17. There is no change in the number or intensity of protein species upon trypsin digestion. The results obtained by chymotrypsin digestion of human erythrocytes differed from those obtained with all other species examined. Figure 18 shows the gel patterns obtained from chymotrypsin digestion (0.5-2.0 mg/ml) of human, bovine, horse and sheep erythrocytes. Under these conditions, only component III of human erythrocytes was completely cleaved, while component III of the other species remained intact.

The accessibility of component III in erythrocytes from bovine,

TABLE VI

CARBOHYDRATE AND SIALIC ACID RELEASE FROM HUMAN ERYTHROCYTES BY PROTEOLYSIS

Enzyme	Enzyme Conc. mg/ml	Carbohydrates Released[a] Expt 1 µg/ml	Carbohydrates Released[a] Expt 2 µg/ml	Sialic Acid Released[b] Expt 1 µg/ml	Sialic Acid Released[b] Expt 2 µg/ml
Trypsin	0.05	18	29	62	60
	0.50	33	41	96	100
	2.00	42	47	122	98
Chymotrypsin	0.05	10	23	34	31
	0.50	19	22	44	36
	2.00	24	38	56	53
Pronase	0.05	18	53	52	30
	0.50	62	84	60	41
	2.00	168	142	68	59

[a]Expressed as carbohydrates released per ml of packed red blood cells.

[b]Expressed as sialic acid released per ml of packed red blood cells.

Figure 17. Double Trypsin Digestion of Bovine Erythrocytes

Experimental conditions are described in Figure 14. Trypsin concentrations for double digestion were: Gel A, untreated control; Gel B, 0.05 mg/ml; Gel C, 0.1 mg/ml.

Figure 18. Effect of Chymotrypsin Digestion on Erythrocytes from Different Species

Digestion was performed as described in Figure 7. Gel A of each set is untreated control; Gel B, 0.5 mg/ml chymotrypsin; Gel C, 2.0 mg/ml chymotrypsin.

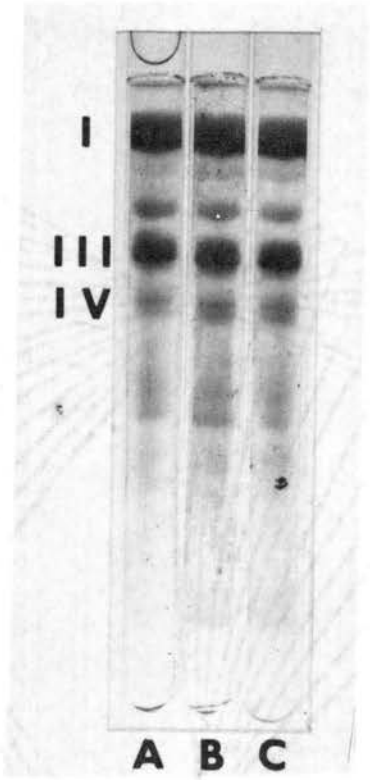


Figure 17

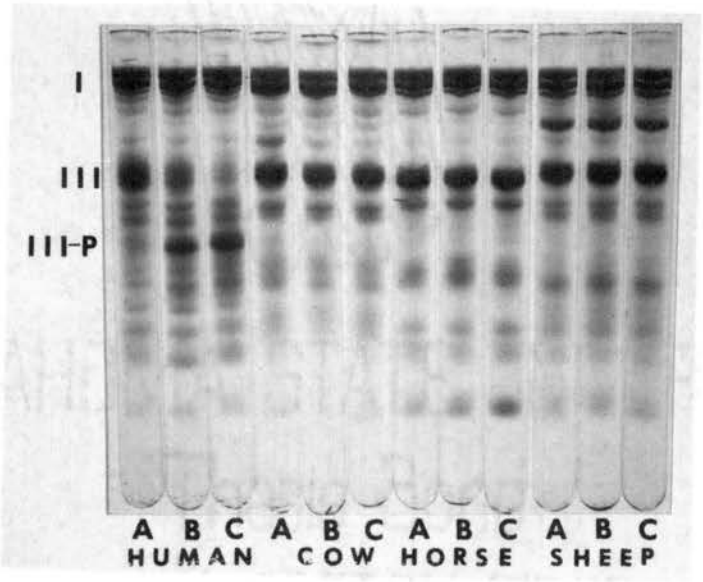


Figure 18

horse and dog was studied by Pronase digestion. Figure 19 shows the gel patterns obtained when erythrocytes from these three species were digested with Pronase at 0.05 and 0.1 mg/ml. In bovine erythrocytes, component III appears to be resistant to Pronase; however there is evidence that some digestion of the membrane is occurring by the formation of a new minor band, labeled P, with a molecular weight of about 60,000. Pronase digestion of horse erythrocytes appears to have a more drastic effect as evidenced by extensive digestion of components I and III. This may be due to incomplete inhibition of Pronase by PMSF, resulting in digestion of membrane proteins during membrane isolation. Pronase digestion of dog erythrocytes results in the loss of component III and the production of three new bands with molecular weights 80,000, 72,000 and 49,000 (labeled as P1, P2 and P3).

Proteolysis of Isolated Erythrocyte Membranes

To see if erythrocyte membrane proteins are inherently resistant to proteolysis, isolated erythrocyte ghosts were subjected to trypsin digestion under the same conditions used for digestion of the intact membrane. Figure 20 shows the protein patterns obtained from such digestion. It appears that all protein species are either susceptible to trypsin digestion or are released in soluble form during digestion. Figure 21 shows the loss of acetylcholinesterase activity and sialic acid as a function of trypsin concentration. Loss of sialic acid from ghost membranes during trypsin digestion is superimposable with loss of acetylcholinesterase activity. The acetylcholinesterase activity falls off at a much faster rate in the case of trypsin digestion of ghosts than with the intact cells. This suggests that there may be

Figure 19. Effect of Pronase Digestion on Erythrocytes from Different Species

Digestion was performed as described in Figure 12. Gel A of each set is untreated control; Gel B, 0.05 mg/ml Pronase; Gel C, 0.1 mg/ml Pronase.

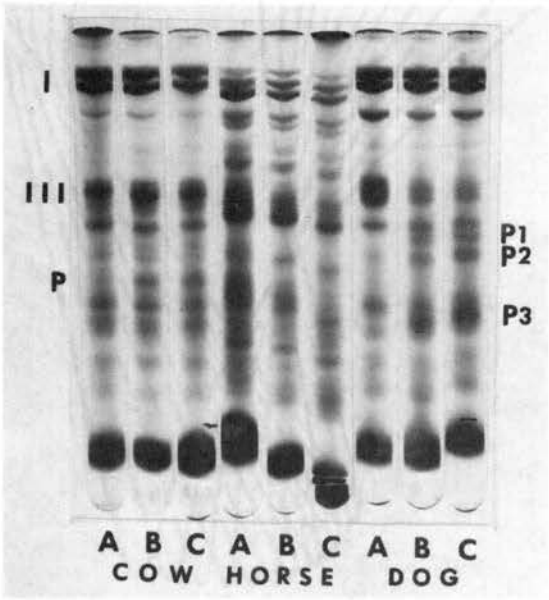

I
III
P
P1
P2
P3
A B C A B C A B C
COW HORSE DOG

Figure 20. Concentration Dependence of Trypsin Digestion on Human Erythrocyte Membranes

Membranes (1.2 mg/ml protein) were digested with trypsin at room temperature for one hour in isotonic phosphate-saline, pH 7.4. Digestion was stopped by the addition of SDS at 100°. Trypsin concentrations were: Gel A, untreated control; Gel B, 0.25 mg/ml; Gel C, 1.25 mg/ml; Gel D, 2.5 mg/ml; Gel E, 3.75 mg/ml; Gel F, 5.0 mg/ml.

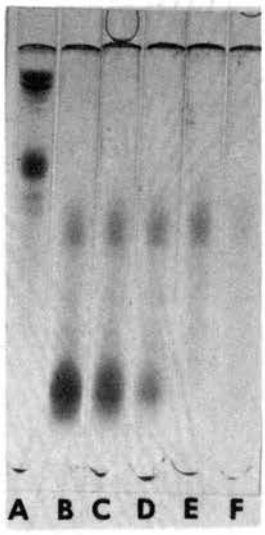
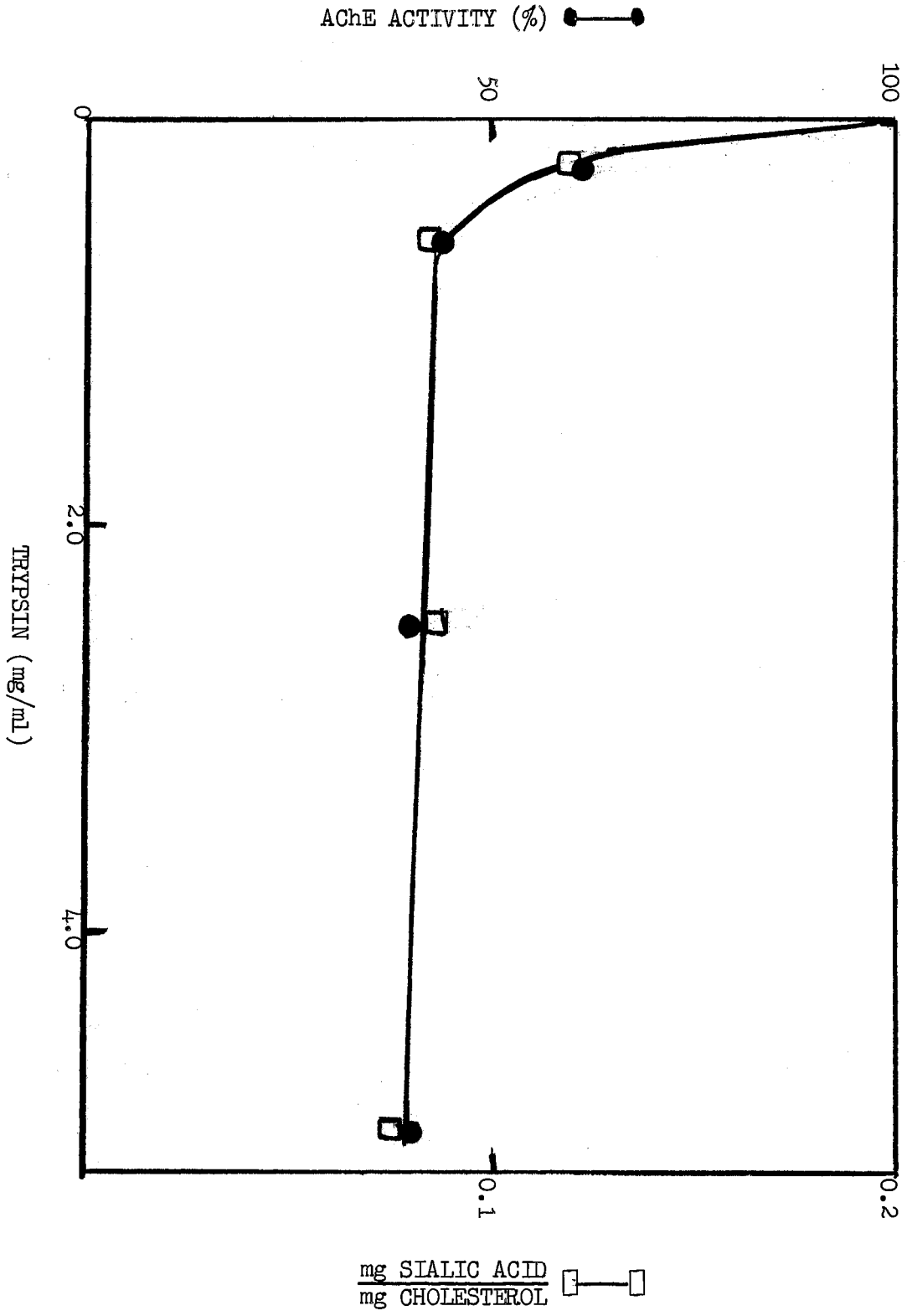

A B C D E F

Figure 21. Alteration of Acetylcholinesterase Activity and Sialic Acid Content of Trypsin Digested Human Erythrocyte Membranes

Digestion was performed as described in Figure 7. Membranes were assayed for acetylcholinesterase, sialic acid and cholesterol. 100% acetylcholinesterase activity represents 2.3×10^{-3} moles of substrate hydrolyzed per minute per mg membrane cholesterol.


AChE ACTIVITY (%)
0
50
100
TRYPSIN (mg/ml)
2.0
4.0
0.1
0.2
mg SIALIC ACID / mg CHOLESTEROL

some rearrangement or loss of membrane components during the haemolysis procedure.

Marchesi and Palade (132) have stated that the presence of ATP and Mg^{++} during trypsin digestion of ghosts results in the prevention of ghosts' structural breakdown and inactivation of membrane bound ATPase. Ghosts were treated with 50 μg/ml of trypsin in the presence of Mg^{++}, Ca^{++}, ATP, EDTA MgATP and CaATP. Examination by phase microscopy of digested cells in the presence of these effectors did not show any protection from structural breakdown, and the gels obtained from electrophoresis were virtually blank. Table VII shows the Ca^{++}-ATPase activity remaining in these effector treated, trypsin digested ghosts. In the presence of MgATP, 34% of the ATPase remains.

Digestion of ghosts at low protease concentrations was performed to see if an order of accessibility of protein digestion could be established, which might reflect their organization in the membrane. Figures 22 and 23 show the gel scans for the proteins from human erythrocyte ghosts which have been treated with chymotrypsin and trypsin, respectively, at concentrations of 10 μg/ml for 0-20 minutes. Several observations were noted from this study: 1) Band IVa is the first to be completely removed from the pattern by each enzyme. 2) There is a specificity of digestion for the two enzymes. Trypsin appears to act more on component I than III, while chymotrypsin performs in the opposite manner. 3) Component III appears as a doublet after a short term digestion by chymotrypsin. 4) There is no product of molecular weight 70,000 which appears during the course of digestion of component III by chymotrypsin. The above observations were made after proteolysis in isotonic phosphate buffer; however, if digestion

TABLE VII

CaATPase ACTIVITY OF DIGESTED GHOSTS IN THE PRESENCE OF EFFECTORS

Incubation Conditions	CaATPase Activity[a]
No trypsin, no effectors	0.41
Trypsin, no effectors	0
Trypsin + Mg^{++}	0
Trypsin + Ca^{++}	0
Trypsin + EDTA	0
Trypsin + ATP	0.03
Trypsin + Mg^{++} + ATP	0.14
Trypsin + Ca^{++} + ATP	0.05

[a]Values expressed as μmoles phosphorous released per hr per mg protein.

Figure 22. Time Course of Chymotrypsin Digestion of Human Erythrocyte Membranes in Isotonic Buffer

Membranes (2.4 mg/ml protein) were digested with 10 μg/ml chymotrypsin in isotonic phosphate-saline, pH 7.4. Reactions were stopped at indicated intervals by the addition of SDS at 100°. Gels were scanned at wavelength 550 nm.

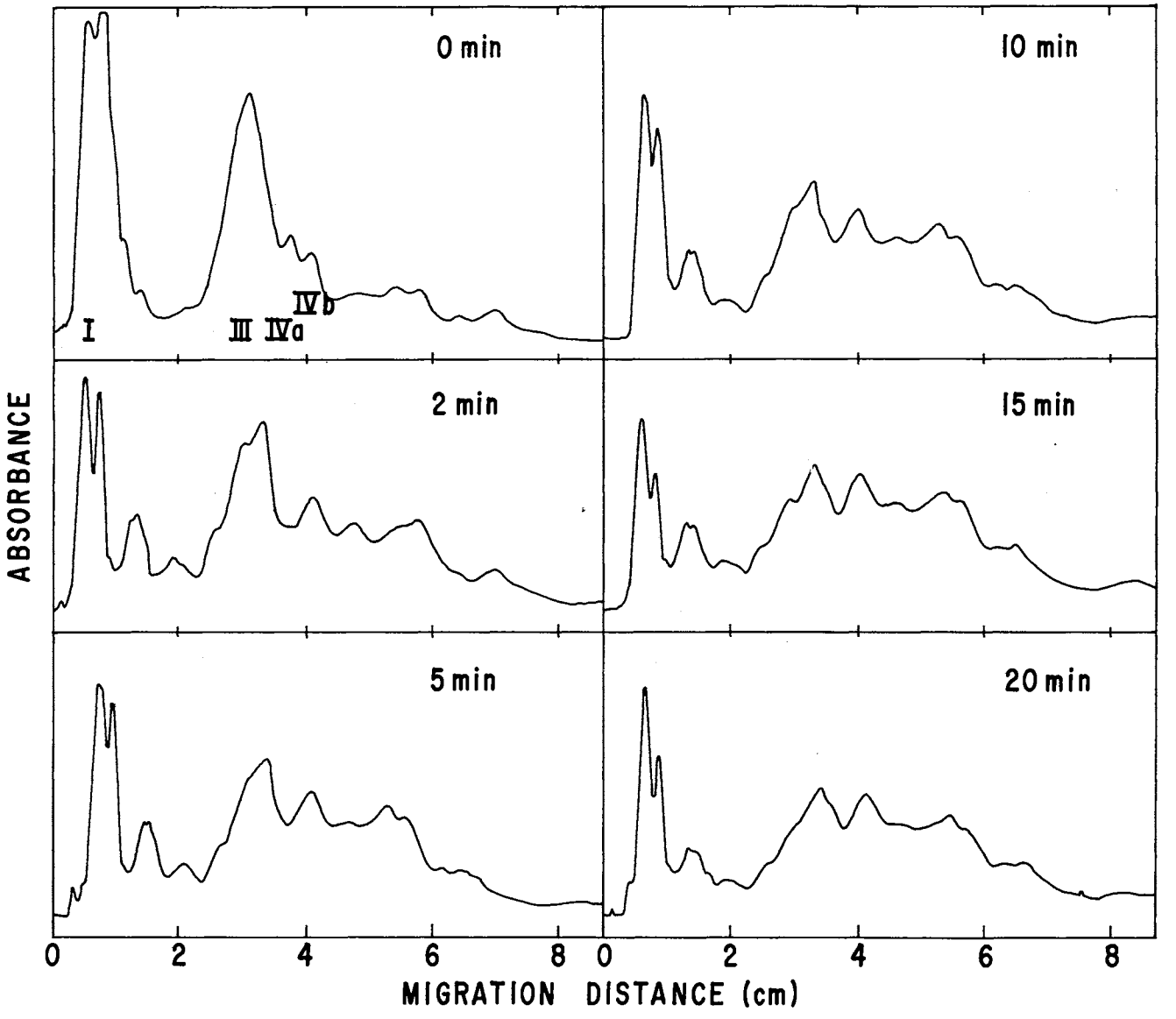
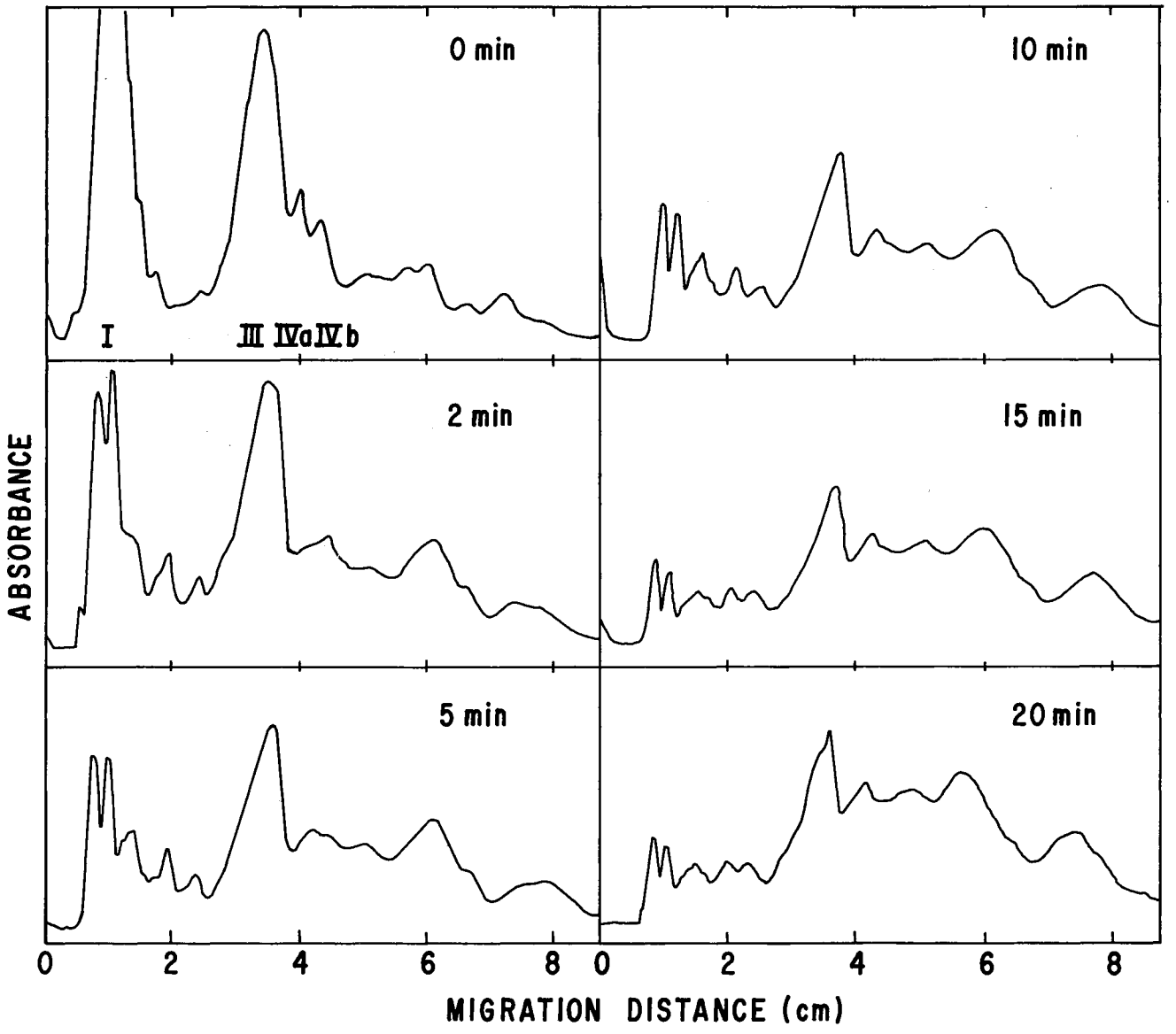

0 min
10 min
2 min
15 min
5 min
20 min
I
III
IVa
IVb
ABSORBANCE
0
2
4
6
8
MIGRATION DISTANCE (cm)

Figure 23. Time Course of Trypsin Digestion of Human Erythrocyte Ghosts in Isotonic Buffer

Experimental conditions are described in Figure 22. Gels were scanned at wavelength 550 nm.


0 min
10 min
I
III IVa IVb
2 min
15 min
5 min
20 min
ABSORBANCE
0
2
4
6
8
0
2
4
6
8
MIGRATION DISTANCE (cm)

is performed in hypotonic buffer, slightly different results are obtained, as seen in Figures 24 and 25, chymotrypsin and trypsin, respectively. Band IVa appears to be one of the first to disappear with both enzymes. With trypsin, component III is the next species which is completely digested, followed by component I. This also occurs with chymotrypsin. There is more digestion of membranes in hypotonic buffer compared to isotonic buffer. Ghosts were also subjected to 0-10 μg/ml of trypsin in isotonic buffer to obtain the order of accessibility to proteolysis. Figure 26 shows that band IVa disappears at trypsin concentrations as low as 0.1 μg/ml, and that component I is completely digested before complete digestion of component III is achieved. Digestion of ghosts with 0.5 μg/ml of trypsin and chymotrypsin in the presence of 1-5 mM Ca^{++} was performed to see if Ca^{++} increased proteolysis. Figure 27 shows ghosts that have been incubated with 1-5 mM Ca^{++} in the presence and absence of these enzymes. The addition of Ca^{++} had no effect on the digestibility of membrane proteins under these conditions.

Proteolysis of Resealed Ghosts

The proteins of the erythrocyte membrane are more susceptible to proteolytic digestion in the isolated membrane than in the intact cell. This suggests that the ghosts must be permeable to the protease or that the protein components of the membrane must be reorganized during haemolysis and washing. Erythrocytes were haemolyzed, then resealed in the presence and absence of Mg^{++}. The resealed ghosts were treated with trypsin at 0.1 mg/ml. Figure 28 shows the gel patterns obtained from trypsin digestion of resealed ghosts plus the pattern from a

Figure 24. Time Course of Chymotrypsin Digestion of Human Erythrocyte Membranes in Hypotonic Buffer

Membranes (2.4 mg/ml protein) were digested with 10 μg/ml chymotrypsin. Reactions were stopped at desired intervals by the addition of SDS at 100°. Digestion times were: Gel A, 0 minutes; Gel B, 2 minutes; Gel C, 5 minutes; Gel D, 15 minutes; Gel E, 20 minutes.

Figure 25. Time Course of Trypsin Digestion of Human Erythrocyte Membranes in Hypotonic Buffer

Experimental conditions described in Figure 24 above. Digestion times were: Gel A, 0 minutes; Gel B, 2 minutes; Gel C, 5 minutes; Gel D, 15 minutes; Gel E, 20 minutes.

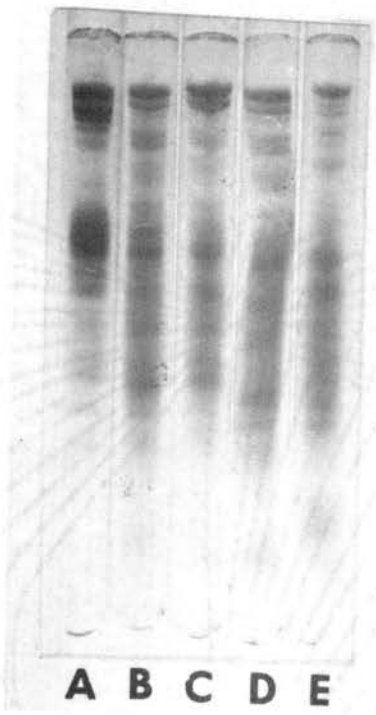


Figure 24

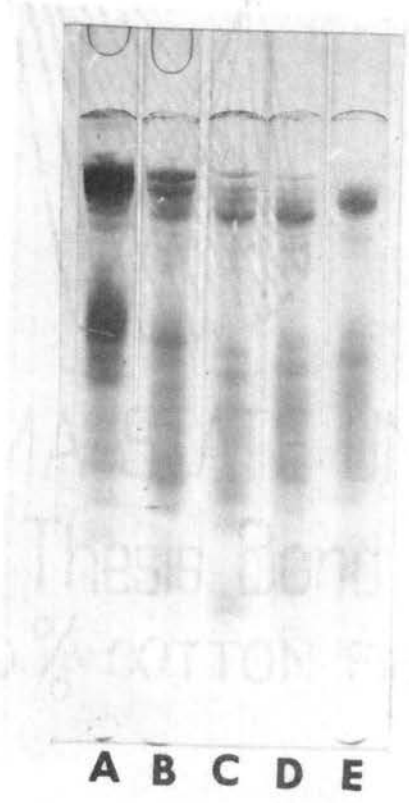


Figure 25

Figure 26. Concentration Dependence of Trypsin Digestion of Human Erythrocyte Membranes

Membranes (2.4 mg/ml) were treated with 0–10 μg/ml of trypsin in isotonic phosphate at room temperature for one hour. Digestion was stopped by the addition of SDS at 100°. Trypsin concentrations were: Gel A, untreated control; Gel B, 0.1 μg/ml; Gel C, 0.5 μg/ml; Gel D, 1.0 μg/ml; Gel E, 3.0 μg/ml; Gel F, 5.0 μg/ml; Gel G, 7.0 μg/ml; Gel H, 10 μg/ml.

Figure 27. Effect of Trypsin and Chymotrypsin on Human Erythrocyte Membranes in the Presence of 1 and 5 mM Ca^{++}

Membranes (2.4 mg/ml protein) were digested with trypsin or chymotrypsin in isotonic Tris for one hour at room temperature in the presence and absence of Ca^{++}. Digestion was stopped by the addition of SDS at 100°. Gel A, untreated control; Gel B, 1 mM Ca^{++}; Gel C, 5 mM Ca^{++}; Gel D, 0.5 μg/ml trypsin; Gel G, 0.5 μg/ml chymotrypsin; Gel H, 1 mM Ca^{++}; 0.5 μg/ml chymotrypsin; Gel I, 5 mM Ca^{++}, 0.5 μg/ml chymotrypsin.

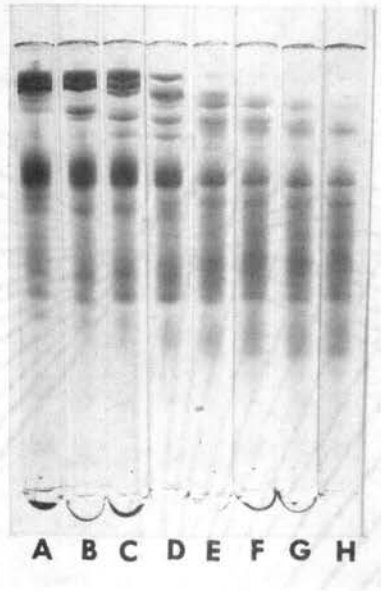


Figure 26

Figure 27

sample of isolated membranes which were treated with trypsin. None of the major proteins were cleaved in the resealed ghosts. Resealed ghosts were subjected to trypsin at 0-2.0 mg/ml in the presence of 1 mM Ca^{++} and no differences were observed in the electrophoretic gel patterns. Also, the protein profile of human erythrocyte ghosts resealed in the presence of Ca^{++} were not altered by trypsin treatment.

Proteolysis of Closed Membranes

An attempt was made to prepare closed membranes from haemoglobin free ghosts and to determine the accessibility of membrane proteins of closed ghosts to trypsin. Figure 29 shows the gel patterns obtained from trypsin digestion (50 μg/ml) of closed membranes prepared by haemolysis of red cells in 20, 40, 60 and 80 mOsM phosphate followed by incubation in Krebs-Ringer salt solution. It appears that these membranes are partially resistant to proteolysis. To determine if these membranes are completely resistant, closed membranes were prepared from ghosts prepared with 20 mOsM phosphate. Figure 30 shows the gel patterns obtained from closed and open ghosts when digested with 0-200 μg/ml of trypsin in Krebs-Ringer salt solution. These closed membranes do not appear to be completely resistant to proteolysis, but the experiments show that haemoglobin free ghosts can be made to reseal partially.

Permeability of Open and Closed Ghosts to Proteases

Trypsin bound to an insoluble resin was used to try to digest the exterior proteins of the membrane. If the difference between the in-

Figure 28. Effect of Trypsin Digestion on Proteins of Resealed Ghosts

Preparation of resealed ghosts is described in Methods. Gels A, C and E are untreated control samples of isolated washed ghosts, resealed ghosts and resealed ghosts prepared in the presence of 1.0 mM Mg^{++}, respectively. Gels B, D and F are samples treated with 0.1 mg/ml trypsin. Gel B, washed ghosts; Gel D, resealed ghosts; Gel F, resealed ghosts prepared in the presence of 1.0 mM Mg^{++}.

Figure 29. Effect of Trypsin Digestion on Closed Membranes Prepared in Increasing Concentrations of Hypotonic Buffer

Red cells were haemolyzed in increasing concentrations of hypotonic buffer and membranes were isolated by washing in appropriate buffers. Closed membranes were prepared as described in Methods. Gels A, C, E and G are untreated closed membranes prepared by haemolysis of red cells in 20, 40, 60 and 80 mOsM phosphate, respectively. Gels B, D, F and H are closed membranes prepared by haemolysis of red cells in 20, 40, 60 and 80 mOsM phosphate and treated with 0.05 mg/ml trypsin.

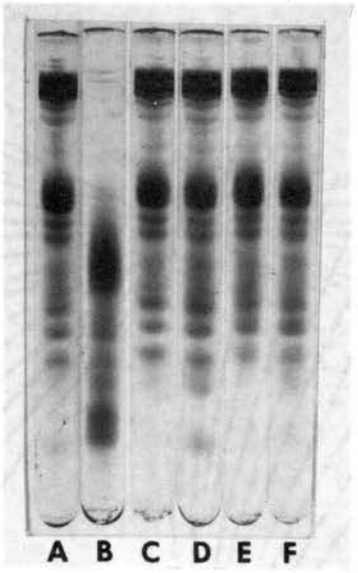


Figure 28

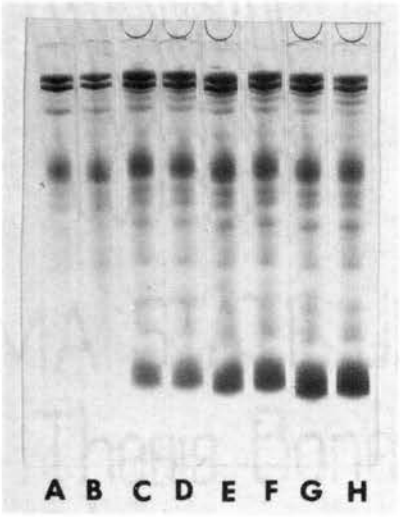
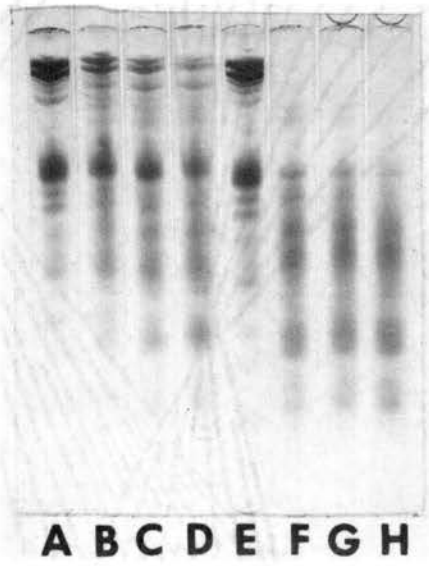


Figure 29

Figure 30. Effect of Trypsin Digestion on Closed and Open Ghosts

Membranes were prepared with 20 mOsM phosphate, pH 7.4. Closed ghosts were prepared as described in Methods. Gels A-D are closed ghosts and Gels E-H are open ghosts. Gel A, untreated control of closed ghosts; Gel B, 0.05 mg/ml; Gel C, 0.1 mg/ml; Gel D, 0.2 mg/ml; Gel E, untreated control of open ghosts; Gel F, 0.05 mg/ml; Gel G, 0.1 mg/ml; Gel H, 0.2 mg/ml.


A B C D E F G H

tact cell and ghosts is one of permeability, then there should be little digestion of the membrane protein by insoluble trypsin. The digestion studies were conducted with unwashed Enzite-trypsin and with a sample of the insoluble enzyme which had been washed five times with incubation buffer before addition of the ghosts. Figure 31 shows the gel scans of ghosts which were treated with Enzite-trypsin. The unwashed enzyme caused extensive degradation of component I and IVa, while the washed resin caused only the loss of IVa with some minor degradation of I. The digestion observed with Enzite-trypsin can be best explained by digestion by enzyme released from the resin, since it was shown that the Enzite-trypsin washings contained trypsin activity.

Evidence of the permeability of closed and open membranes to proteolytic enzymes was obtained by measuring the exclusion of a sample of labeled chymotrypsinogen A or inactivated labeled chymotrypsin from erythrocytes, haemoglobin-free ghosts and closed ghosts. Samples of the labeled proteins at three concentrations were incubated with suspensions containing 30, 50 and 70% (v/v) packed erythrocytes, ghosts or closed ghosts. The suspensions were centrifuged and aliquots of the supernatants were counted to determine the amount of enzyme excluded. The results presented in Tables VIII and IX show that intact red cells are impermeable to enzymes while ghosts are permeable. Closed ghosts are partially impermeable but the results do not indicate total impermeability of these membranes to proteolytic enzymes. These values were not corrected for the volume of space between the packed membrane or the adsorption of protein to the membrane. From these results, proteases are

Figure 31. Enzite-Trypsin Treatment of Human Erythrocyte Membranes

Membranes (2.4 mg/ml protein) were treated with washed and unwashed Enzite-trypsin as described in Methods. Digestion was stopped by the addition of SDS at 100°. Scan A, untreated control; Scan B, washed Enzite-trypsin; Scan C, unwashed Enzite-trypsin. Gels were scanned at wavelength 550 nm.

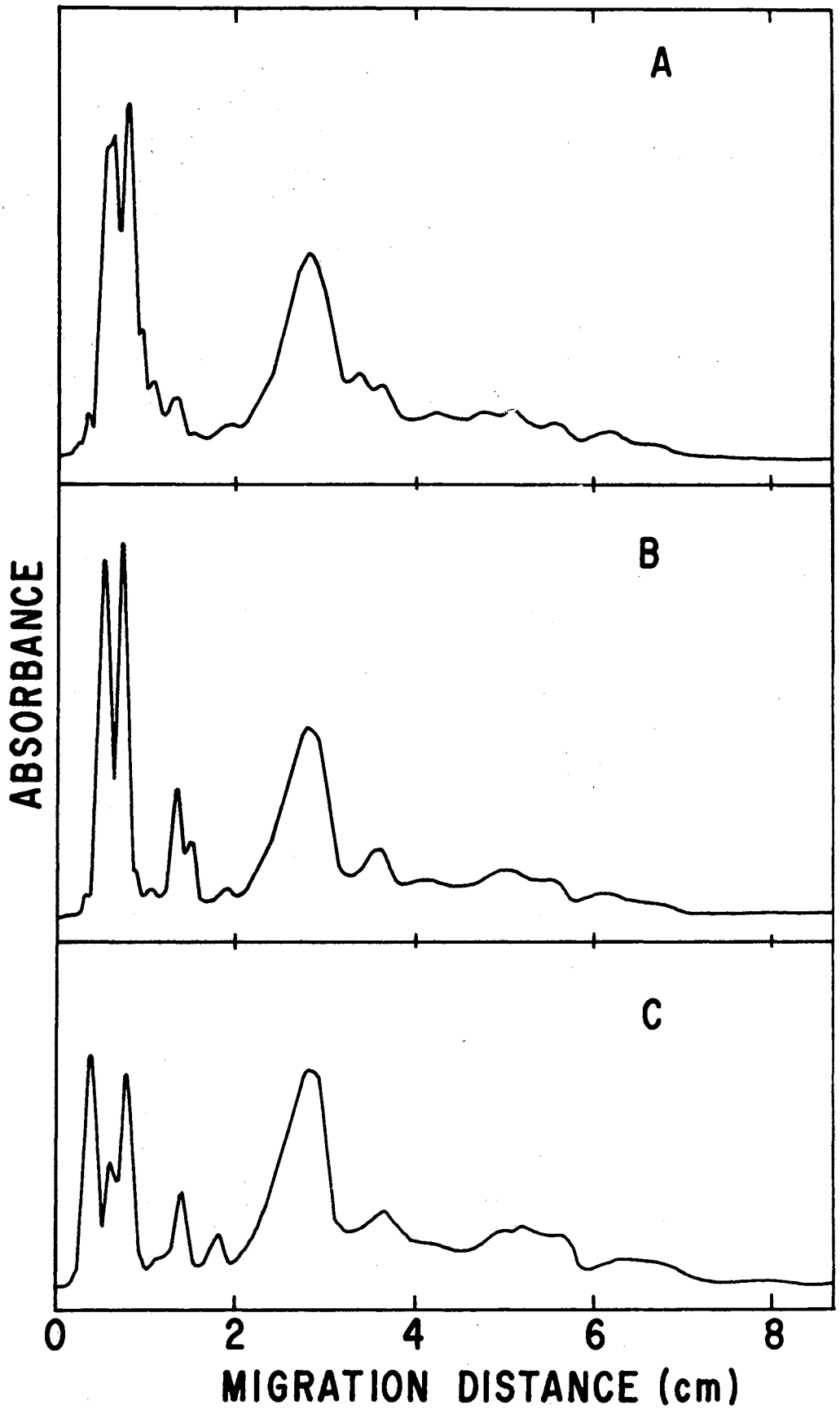

A
B
C
ABSORBANCE
0
2
4
6
8
MIGRATION DISTANCE (cm)

TABLE VIII

PERMEABILITY OF ERYTHROCYTES AND GHOSTS
TO LABELED CHYMOTRYPSIN

% Suspension	Chymotrypsin conc. mg/ml	CPM Determined		CPM Predicted	
		RBC	Ghosts	Impermeable[a]	Permeable[b]
30	0.05	950	650	1000	740
	0.10	1880	1420	1730	1210
	1.00	17600	12900	19200	13500
50	0.05	1150	530	1480	740
	0.10	2410	1200	2420	1210
	1.00	22200	11900	16900	13500
70	0.05	1600	510	2460	740
	0.10	3150	1130	4030	1210
	1.00	28800	9690	44500	13500

[a]Calculated from value for total permeability by assuming that 30, 50 and 70% of the volume of the suspension is inaccessible.

[b]Determined experimentally by adding aliquots of labeled chymotrypsin to control buffer samples containing no cells or ghosts.

TABLE IX

PERMEABILITY OF ERYTHROCYTES AND CLOSED GHOSTS TO LABELED CHYMOTRYPSINOGEN A

Chymotrypsinogen A conc. mg/ml	CPM Determined		CPM Predicted	
	RBC	Closed Ghost	Impermeable[a]	Permeable[b]
0.05	5520	4110	6100	3050
0.10	9850	7710	13000	6520
1.00	87300	82300	133400	66710

[a]Calculated from value for total permeability by assuming that 50% of the volume of the suspension is inaccessible.

[b]Determined experimentally by adding aliquots of labeled chymotrypsinogen A to control buffer samples containing no cells or ghosts.

apparently able to penetrate into the ghosts and digest proteins inside as well as outside of the membrane.

CHAPTER V

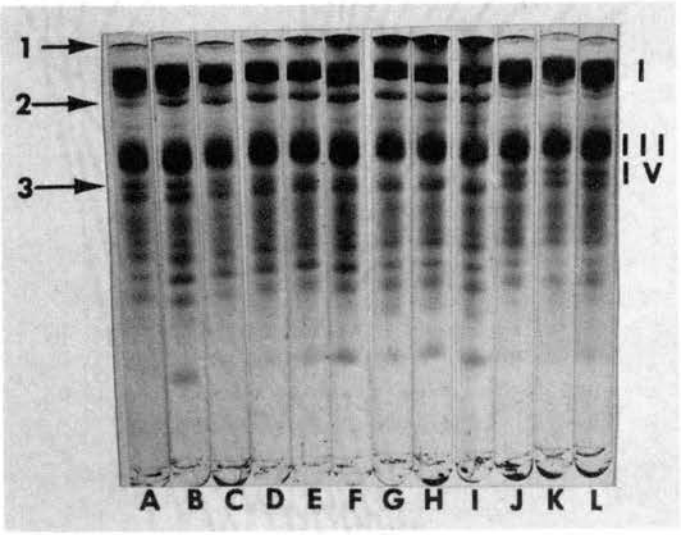
CALCIUM EFFECTS ON ERYTHROCYTE MEMBRANE PROTEINS

Effects of Adding Calcium or Magnesium to Intact Erythrocytes

When erythrocytes are haemolyzed in the presence of calcium, an alteration in the proteins occurs as seen on SDS acrylamide gel electrophoresis. Figure 32 shows the membrane protein electrophoretic patterns of erythrocytes haemolyzed in the presence of Ca^{++} or Mg^{++}. Gels A-I represent erythrocytes haemolyzed in the presence of 0-5 mM Ca^{++} in 10 mM Tris, pH 7.4, and gels J-L represent erythrocytes haemolyzed in 5-15 mM Mg^{++} in 10 mM Tris, pH 7.4. After haemolysis, the haemolysate was taken to isotonic conditions with 3 M NaCl, then the suspensions were incubated at 37^{o} for thirty minutes. As noted in Figure 32, there are three areas of significant changes in the protein patterns in the case of Ca^{++} treated erythrocytes. These are: 1) aggregation at the top of the gels which appears to be dependent upon Ca^{++} concentration; 2) an increase in a band just below component I; and 3) a loss of component IVa, which appears to be dependent upon Ca^{++} concentration. These three changes are not seen when erythrocytes are haemolyzed in the presence of Mg^{++}, indicating that this is a Ca^{++} specific alteration of membrane proteins. To show that these effects were not caused by an imbalance of salts during haemolysis, the haemolysates of Ca^{++} and Mg^{++} haemolyzed erythrocytes were taken to isotonic

Figure 32. Effect of Increasing Amounts of Calcium and Magnesium on Erythrocyte Proteins with Resealing with 3 M NaCl

Human erythrocytes washed in 0.15 M NaCl in 10 mM Tris, pH 7.4, were haemolyzed for ten minutes at 4° in 10 mM Tris, pH 7.4, containing 0-5 mM Ca^{++} or 5-20 mM Mg^{++}. The haemolysate was taken to isotonic conditions by the addition of 3 M NaCl followed by incubation at 37° for thirty minutes. The membranes were washed two times with NaCl-Tris buffer and membranes were isolated with 10 mM Tris. Electrophoresis and staining of gels were performed as described in Methods. Gel A, untreated control; Gel B, 0.1 mM Ca^{++}; Gel C, 0.3 mM Ca^{++}; Gel D, 0.5 mM Ca^{++}; Gel E, 0.7 mM Ca^{++}; Gel F, 1.0 mM Ca^{++}; Gel G, 2.0 mM Ca^{++}; Gel H, 3.0 mM Ca^{++}; Gel I, 5.0 mM Ca^{++}; Gel J, 5.0 mM Mg^{++}; Gel K, 10.0 mM Mg^{++}; Gel L, 20.0 mM Mg^{++}.


1
2
3
I
III
IV
A B C D E F G H I J K L

conditions by the addition of a balanced salt solution of 1.42 M KCl in 0.28 M NaCl. Sodium and potassium ions are required for stimulation of ATPase which has been postulated to be a requirement to keep membranes in an energized state (106, 138), i.e., to move ions across the membrane. Figure 33 shows the protein patterns obtained from membranes prepared in this manner. The same Ca^{++} specific alterations are obtained as described above. The loss of IVa and the appearance of II can be explained on the basis of the results obtained from trypsin digestion on erythrocyte ghosts. As seen in Figure 26, page 93, treatment of membranes with trypsin at 0.1 μg/ml and 0.5 μg/ml causes a loss of IVa and an appearance of II. It appears that the changes in these two species may be a result of an internal membrane bound protease which is activated or released by high Ca^{++} concentrations. These effects might also be caused in part from contamination of the membrane preparation by white cells, since it is known that white cells do cause proteolysis of membrane proteins (19, 20). Figure 34 shows the extent of proteolysis of membrane proteins when white cells are added to the preparation. Gels A-C are normal patterns seen when erythrocytes are haemolyzed in the presence and absence of 1 mM and 5 mM Ca^{++}. Gels D-F represent those membranes in which an aliquot of a 1:10 dilution of white cells has been added to erythrocytes during haemolysis in the presence and absence of Ca^{++}. There is extensive degradation of proteins in the absence of Ca^{++} as well as in the presence of 1 mM Ca^{++}; however in the presence of 5 mM Ca^{++} there appears to be some inhibition of white cell protease digestion on erythrocyte membrane proteins. Gels G-I represent those membranes in which an aliquot of a 1:100 dilution of white cells has been added

Figure 33. Effect of Increasing Amounts of Calcium and Magnesium on Erythrocyte Proteins with Resealing with 1.42 M KCl in 0.28 M NaCl

Experimental conditions were the same as those described in Figure 32, with the exception that after haemolysis, the haemolysate was taken to isotonic condition by the addition of 1.42 M KCl and 0.28 M NaCl. Gel A, untreated control; Gel B, 0.3 mM Ca^{++}; Gel C, 0.5 mM Ca^{++}; Gel D, 0.7 mM Ca^{++}; Gel E, 1.0 mM Ca^{++}; Gel F, 3.0 mM Ca^{++}; Gel G, 5.0 mM Ca^{++}; Gel H, 5.0 mM Mg^{++}; Gel I, 10.0 mM Mg^{++}; Gel J, 20.0 mM Mg^{++}.

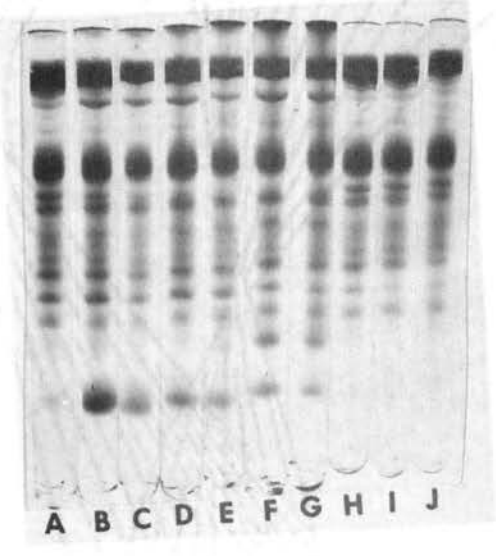
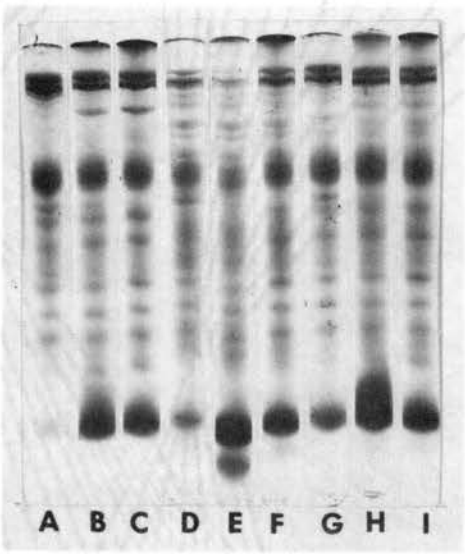

A B C D E F G H I J

Figure 34. Effect of Adding White Cells to Calcium Treated Erythrocytes

Human erythrocytes washed in 0.15 M NaCl in 10 mM Tris, pH 7.4, were haemolyzed for ten minutes at 4° in 10 mM Tris, pH 7.4, containing 0-5 mM Ca^{++}. White cells collected from the first washing of erythrocytes were diluted 1:10 and 1:100 and an aliquot of the white cells was added to the erythrocytes at the haemolysis step. The haemolysate was taken to isotonic conditions by the addition of 3 M NaCl followed by incubation at 37° for thirty minutes. The membranes were washed two times with NaCl-Tris buffer and membranes were isolated with 10 mM Tris. Gels A-C represent those membranes in which no white cells were added; Gels D-F represent those membranes in which an aliquot of a 1:10 dilution of white cells was added; Gels G-I represent those membranes in which an aliquot of a 1:100 dilution of white cells was added. Gel A, untreated control; Gel B, 1.0 mM Ca^{++}; Gel C, 5.0 mM Ca^{++}; Gel D, 1:10 white cell control; Gel E, 1:10 white cell with 1.0 mM Ca^{++}; Gel F, 1:10 white cell with 5.0 mM Ca^{++}; Gel G, 1:100 white cell control; Gel H, 1:100 white cell with 1.0 mM Ca^{++}; Gel I, 1:100 white cell with 5.0 mM Ca^{++}.


A B C D E F G H I

to erythrocytes. In each case where white cells are added, there is the usual aggregation occurring at the top of the gel, as well as the loss of IVa, when Ca^{++} is present. The failure to see an increase in the band below component I could be caused from more extensive degradation of component I, thereby suggesting a higher concentration of protease present in Gels D-I as compared to Gels B and C. This is seen by the multiple bands occurring between components I and III of Gels D-I. If this is the case, then it would seem unlikely that the membrane preparations are contaminated with white cells to the extent that they are causing the effects seen with Ca^{++}. There does not appear to be any potentiation of the white cell effects by Ca^{++} or vice versa.

When erythrocytes were treated with Ca^{++} as described above, but the haemolysate was not taken to isotonic conditions, the same results as those seen in Figures 32 and 33 were obtained. Since this resealing step was not required to see the Ca^{++} effects, it was of interest to see if incubation at 37^{o} was essential. Identical experiments were performed as those described above except that after resealing, the membranes were incubated at 4^{o} for thirty minutes. Figure 35 shows that the Ca^{++} effect is altered somewhat by incubation at 4^{o}. Only at a Ca^{++} concentration of 5 mM did any significant aggregation of protein appear at the top of the gel. This is also the only concentration where IVa disappeared. The addition of Ca^{++} during the resealing process did not bring about any changes in the protein patterns, as seen in Figure 36. However, if Ca^{++} was added to cells during the second haemolysis step the usual effects were noted. Thus the apparent proteolysis and aggregation are dependent upon the pres-

Figure 35. Effect of Incubation at 4^{o} on Calcium Treated Erythrocytes

Experimental conditions are the same as those described in Figure 32, with the exception that incubation was performed at 4^{o} for thirty minutes after salt addition. Gel A, untreated control; Gel B, 0.1 mM Ca^{++}; Gel C, 0.5 mM Ca^{++}; Gel D, 1.0 mM Ca^{++}; Gel E, 2.0 mM Ca^{++}; Gel F, 5.0 mM Ca^{++}.

Figure 36. Effects of Addition of Calcium During Resealing of Haemolyzed Erythrocytes

Human erythrocytes washed in 0.15 M NaCl in 10 mM Tris, pH 7.4, were haemolyzed ten minutes at 4^{o} in 10 mM Tris, pH 7.4. The haemolysate was taken to isotonic conditions by the addition of 3 M NaCl containing 0-5 mM Ca^{++} followed by incubation at 37^{o} for thirty minutes. The membranes were washed two times with NaCl-Tris buffer and membranes were isolated with 10 mM Tris, pH 7.4. Gel A, 0.1 mM Ca^{++}; Gel B, 0.5 mM Ca^{++}; Gel C, 1.0 mM Ca^{++}; Gel D, 2.0 mM Ca^{++}; Gel E, 5.0 mM Ca^{++}.

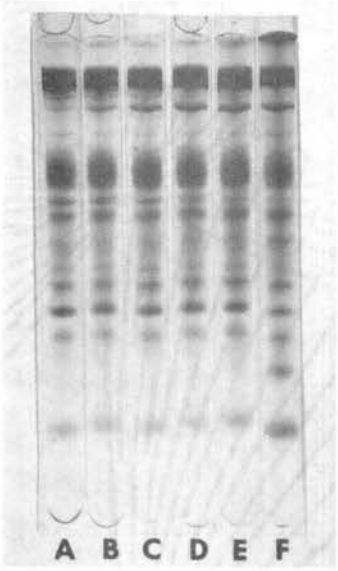


Figure 35

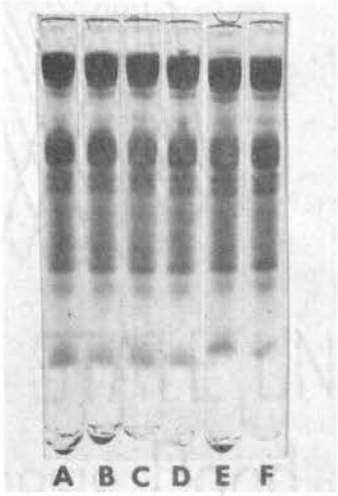


Figure 36

ence of Ca^{++} during a haemolysis procedure.

Addition of Calcium Chelators During Haemolysis with Calcium

Calcium chelators, such as ATP and EGTA, have been shown to be necessary for the regeneration of the biconcave shape of ghosts prepared from fresh, ATP depleted erythrocytes (108). Calcium chelators were tested to see what effect they had on the protein patterns of erythrocyte membranes. When a two-fold excess of ATP, EDTA and EGTA were added to the haemolyzing buffer containing Ca^{++}, a reversal of the Ca^{++} effect was seen. It was of interest to see if the aggregate seen at the top of the gels could be removed by chelators added after formation of this aggregate. Erythrocytes were haemolyzed with Ca^{++} and resealed with 3 M NaCl containing ATP, EDTA or EGTA, or haemolyzed with Ca^{++}, resealed with NaCl, then haemolyzed a second time with ATP, EDTA and EGTA in 10 mM Tris, pH 7.4. These Ca^{++} chelators failed to cause reversal of the effects seen with Ca^{++} under these conditions.

Addition of Calcium to Isolated Membranes

Isolated membranes were treated with increasing Ca^{++} concentration to see if the same effects could be seen with this type of membrane that were seen with erythrocytes. The membranes were treated under identical conditions as for the haemolysis of erythrocytes. Upon electrophoresis, no effect on the protein patterns could be seen. The patterns were identical to Gel A, Figure 32, which is an untreated control. This experiment also shows that Ca^{++} is required during a haemolysis step of intact erythrocytes. However, the failure to see

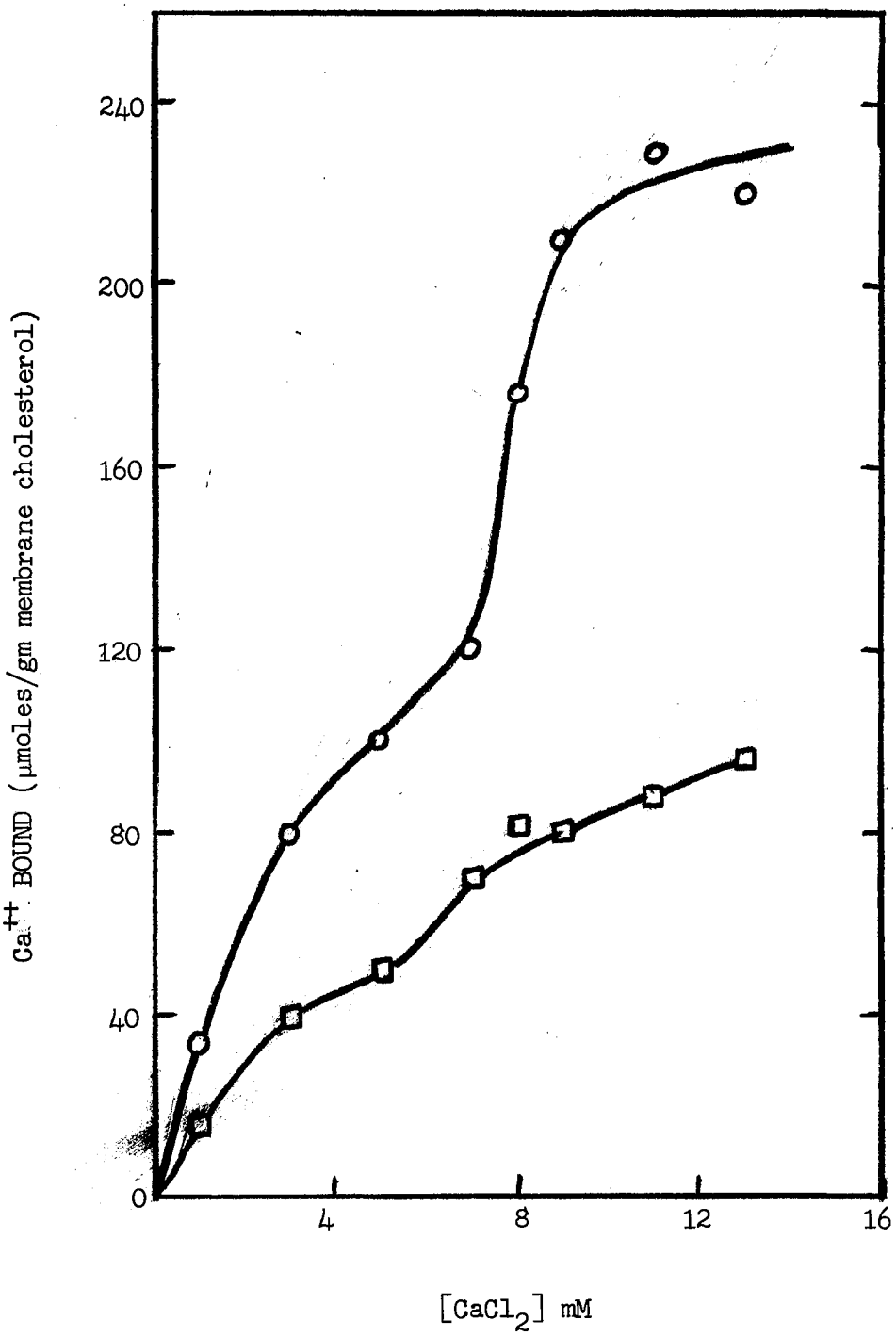
the Ca^{++} effects with isolated ghosts could be due to an organizational difference between the components of the intact and isolated membranes. Also, if a membrane bound protease is involved, it could be released by the extensive washings during membrane isolation. The transition in going from erythrocytes to ghosts could result in the masking or loss of Ca^{++} binding sites on the membrane. If this is the case, then one would expect the isolated membranes to bind less Ca^{++} than the intact membrane.

Binding of Calcium by Erythrocytes and Isolated Membranes

To determine if there is a relationship between calcium binding and the effects seen with calcium on intact erythrocyte membranes, the characteristics of Ca^{++} binding to erythrocytes and ghosts were investigated by using ^{45}Ca. Forstner and Manery (128) were able to show that ghosts bound a maximum of 283 μmoles of Ca^{++} per gram of protein. Their procedures for Ca^{++} binding studies were followed in this study. However, the data will be presented as μmoles of Ca^{++} bound per gram of cholesterol because, with increasing Ca^{++} concentrations on erythrocytes, there is an increase in retention of haemoglobin. When erythrocytes or ghosts are incubated with 0–15 mM Ca^{++} containing 1.0 μC of ^{45}Ca, then resealed with 3 M NaCl, biphasic binding curves are obtained. The binding curves seen in Figure 37 show that ghosts bind more Ca^{++} than erythrocytes; however, this may be due to removal of Ca^{++} from the erythrocytes during haemolysis and the extensive washing during membrane preparation. It has been suggested (109) that upon binding of Ca^{++}, spectrin undergoes a confor-

Figure 37. Effect of External $CaCl_2$ Concentration on Calcium Binding by Erythrocytes and Ghosts

Washed erythrocytes and ghosts were suspended in 10 mM Tris, pH 7.4, containing 0–15 mM Ca^{++} with 1.0 μC ^{45}Ca. The haemolysate was taken to isotonic conditions by the addition of 3 M NaCl and incubated at 37° for thirty minutes. The cells were washed two times and membranes were isolated with 10 mM Tris, pH 7.4. ^{45}Ca counting was performed according to Forstner and Manery (128). O—O ghost; □—□ RBC.


240
200
160
120
80
40
0
4
8
12
16
Ca++ BOUND (μmoles/gm membrane cholesterol)
[CaCl2] mM

mational change. This might result in unmasking of Ca^{++} binding sites on other proteins or phospholipids, giving a biphasic binding curve. Alternatively, two binding sites with very different affinities may be present. This conformational change could be more pronounced in the case of ghosts as compared to erythrocytes thereby giving the ghosts a better possibility of binding Ca^{++}. Also, Ca^{++} causes the retention of haemoglobin in erythrocytes which then could reduce the amount of conformational change occurring in spectrin, thereby preventing the unmasking of hidden Ca^{++} binding sites. One must also consider the possibility of protein organizational differences between intact cells and ghosts and the loss of endogenous Ca^{++} during haemolysis and washing. Electrophoresis of these Ca^{++} treated membranes shows the three significant effects seen in Figure 32 only on those membranes that were isolated from Ca^{++} treated erythrocytes. Electrophoresis of the Ca^{++} treated membranes did not show any change in the protein patterns.

The extent of Ca^{++} binding was determined after haemolysis in 10 mM Tris containing 1, 5 and 15 mM Ca^{++} with equal amounts of ATP or EDTA. Table X shows that EDTA has a more drastic effect upon Ca^{++} binding than does ATP. However, if these two chelators are added during the second haemolysis step, the effect exerted by them is approximately the same. It appears from these Ca^{++} binding studies that in the presence of chelators, the aggregation product seen at the top of the gels (Figure 32) would have to be held together rather tightly with a small fraction of the bound Ca^{++} if Ca^{++} is involved in maintaining the aggregation. ^{45}Ca treated erythrocytes were subjected to SDS electrophoresis, followed by gel slicing to

TABLE X

EFFECT OF ATP AND EDTA ON CALCIUM BINDING BY ERYTHROCYTES

Calcium Conc. (mM)	Calcium Bound (μmoles per gram membrane cholesterol) First Haemolysis[1] Control	ATP	EDTA	Second Haemolysis[2] Control	ATP	EDTA
1	22	13	3	16	7	2
5	59	47	9	62	19	7
15	143	110	35	112	32	26

[1]Erythrocytes were suspended in 10 mM Tris, pH 7.4, containing 1, 5 and 15 mM Ca^{++} and 1 μC ^{45}Ca. ATP or EDTA was present at 1, 5 and 15 mM. The haemolysate was taken to isotonic conditions by the addition of 3 M NaCl. The suspensions were incubated at 37° for thirty minutes. The cells were washed two times with 0.15 M NaCl-10 mM Tris, pH 7.4, and membranes were isolated with 10 mM Tris, pH 7.4.

[2]Erythrocytes were suspended in 10 mM Tris, pH 7.4, containing 1, 5 and 15 mM Ca^{++} and 1.0 μC ^{45}Ca. The haemolysate was taken to isotonic conditions by the addition of 3 M NaCl. The suspensions were incubated at 37° for thirty minutes. The cells were washed two times with 0.15 M NaCl in 10 mM Tris, pH 7.4. The second haemolysis was performed in 10 mM Tris, pH 7.4, containing 1, 5 and 15 mM ATP or EDTA.

determine the location of radioactivity in the gel. All of the radioactivity was found about two-thirds of the distance down the gel and did not correspond to any protein band. A similar experiment was performed with SDS and ^{45}Ca and the radioactivity in this case was also located about two-thirds of the distance down the gel. No detectable ^{45}Ca was shown to be associated with the aggregate at the top of the gel or any other protein; instead, it is apparently incorporated into an SDS-^{45}Ca complex upon solubilization of the membranes by SDS.

CHAPTER VI

DISCUSSION

The polypeptide chains in the erythrocyte membrane, which vary in molecular weight from about 25,000 to over 200,000 (4, 5, 16, 20), have received considerable attention in recent years. Some of the more interesting observations reported are those which state that an abundance of these polypeptides are of low molecular weight. Laico et al. (15) have presented evidence that a majority of the membrane protein of human and bovine erythrocytes is made up of a "miniprotein" of molecular weight 5,000. Kiehn and Holland (133) have presented chemical labeling studies combined with electrophoresis and chromatography to show that the bulk of the erythrocyte membrane protein is of low molecular weight. However, Fairbanks et al. (20) were not able to demonstrate any low molecular weight polypeptide components of the erythrocyte membrane by electrophoresis. Electrophoresis alone does not allow for the complete description of membrane proteins, since detection of these proteins depends upon particular staining techniques. This is evidenced by the fact that membrane glycoprotein does not stain with common protein stains (20, 78). Chromatographic fractionation of membranes provides a good method for examination of membrane components; however, a complete chromatographic analysis requires that all components of the membrane be solubilized and disaggregated.

Erythrocyte membranes have been solubilized in two different systems: 1% SDS, and CTAB-guanidine hydrochloride. The objective was to develop a suitable system for the complete disaggregation of the protein and lipid components so that separation of these components could be achieved by chromatography. Once this was achieved, then quantitative data on the distribution of protein, carbohydrates and phosphorous between these fractions could be collected. This would lead to a better understanding on the nature of the low molecular weight polypeptides. When membranes solubilized by these two different systems were subjected to chromatography, disaggregation of the protein and lipid occurred only in SDS. Amino acid analysis of the two chromatographic fractions obtained by SDS chromatography of human erythrocyte membranes indicate that no significant fraction of the total membrane protein exists as a low molecular weight polypeptide. Less than 5% of the protein was eluted at a molecular weight below 12,000. Protein values of the lipid fraction, obtained by the Lowry method (117), gave higher readings (20-25% of total), indicating the poor reliability of this technique for measuring protein in the presence of lipid. To check for further evidence of low molecular weight polypeptides, the protein peak was rechromatographed on the same P-100 column. Only one peak was obtained at the void volume, indicating no further disaggregation of the protein fraction. Separation of lipid from protein is not complete as evidenced by the high phosphorous content (15%) of the protein fraction. This value is too high to represent the phosphoprotein component of the membrane (134). In SDS the lipid exists as a mixed aggregate of SDS, phospholipid, cholesterol and glycolipid. This is supported by the addition

of ^{14}C-cholesterol to solubilized ghosts (Figure 1) and the phosphorous and carbohydrate data in Tables I and III. It is this aggregate which appears to correspond to the "miniprotein" of Laico et al. (15). After electrophoresis of membranes, they obtain a low molecular weight band which stains for carbohydrate and protein. This staining area corresponds to a band identified by periodate-Schiff stain and by an exchange experiment in which ^{14}C-cholesterol was incorporated into human red cell membranes (5, 87). The protein staining characteristics of this species has been explained by Carraway et al. (135) by showing that various phospholipids can be stained with the common protein stain coomassie blue after electrophoresis. It may also be this lipid aggregate which Kiehn and Holland (133) have mistakenly identified as a low molecular weight polypeptide. They apparently failed to recognize that aminophospholipids as well as proteins can be labeled by most reagents that are attacked by nucleophiles. Reagents such as acetic anhydride have been used to label the protein and lipid components of erythrocyte membranes (87) and to show the relative reactivity of these components to acetic anhydride in the intact cell and isolated membranes.

Solubilization of the erythrocyte membrane in CTAB-guanidine hydrochloride failed to cause disaggregation of the membrane protein and lipid. This is quite evident from the amino acid analysis data presented from chromatography of CTAB-guanidine solubilized membranes on P-100. This failure of component separation could be due to an overlap of the protein peak with an aggregate of phospholipid. Gwynne and Tanford (16) solubilized erythrocyte membranes in 6 M guanidine and, upon chromatography of such membranes on a 4% Agarose column,

they were able to show disaggregation of a significant portion of the membrane protein. However, this type of column was unsuccessful in separation of membrane components after the membrane was solubilized in CTAB-guanidine. It appears from the results obtained that a soluble aggregate of membrane components is formed in the presence of CTAB and guanidine hydrochloride. This is suggested since the aggregate from the 4% Agarose column can be fractionated upon solubilization in 1% SDS and chromatography on a P-100 column.

The organization of polypeptide chains in the erythrocyte membrane has been another source of controversy in recent years. This controversy centers around the difference in accessibility between the proteins of the intact cell when compared to the isolated ghosts. The results presented indicate that this difference can be explained by the permeability of isolated membranes to proteolytic enzymes. However, these results do not rule out the possibility that organizational differences may exist between the isolated membrane and the intact erythrocyte membrane. Organizational differences have been suggested for the lipid components of the two types of membranes. These differences have been based upon lipolytic enzyme (85) studies and on chemical modification studies with acetic anhydride (87).

The results with proteolysis of intact erythrocytes and resealed ghosts are very similar to those reported by Steck et al. (81) for inside-out vesicles. The proteins of this type of membranes are similar in their extent of resistance to digestion by trypsin. Trypsin does cleave the glycoprotein of erythrocyte membranes which is at the surface of these membranes (40). Chymotrypsin digestion of intact erythrocytes and inside-out vesicles give similar results, in that

the glycoprotein is digested and component III is digested to yield III-P, the 70,000 molecular weight fragment. Pronase digestion of erythrocytes also cleaves III to yield III-P as does papain digestion upon inside-out vesicles (81). A similar parallel has been noted between the rightside-out vesicles (81) and the isolated ghosts in that both of these show extensive degradation by proteases. These similarities suggest that the difference between the inside-out and rightside-out vesicles may be the same as between intact erythrocytes and ghosts, i.e., a difference in permeability to the enzyme. The evidence certainly does not support an inverted structure. One possibility which should not be ignored is the possibility of protein organizational or conformational changes occurring in the transition from erythrocytes to washed ghosts. The results also suggest that the inside-out vesicles may be similar or identical to resealed ghosts which closely resemble intact erythrocytes in their digestibility to proteolytic enzymes.

Steck et al. (81) also performed proteolysis of isolated membranes at low protease concentrations. However, their study was not detailed enough to determine an order of accessibility. The results presented in this report show a more detailed study in that band IVa is the first protein species to be removed by low concentrations of trypsin and chymotrypsin and that there is an order of specificity for the two enzymes. Trypsin appears to act more on component I than III, while chymotrypsin performs in the opposite manner. With trypsin, component I must be completely digested before any appreciable loss of III occurs.

The localization of several of the proteins can be predicted

from the results presented in this study. Two major proteins are present at the surface of the human erythrocyte, the glycoprotein and component III. A significant portion of III is protected in some manner within the membrane, since it is undigested under conditions where the other proteins are lost from the membrane. Bretscher (136), using an impermeable reagent to label the membrane, has suggested that both the glycoprotein and component III extend through the membrane. His studies make two assumptions: 1) the isolated membrane does not differ from the membrane of the intact cell in its protein organization; and 2) the reagent can react only at the outside and inside surfaces of the membrane. Since the reagent must travel through channels within the membrane in order to get inside, it seems equally likely that reaction might occur within the membrane. Any protein which bounded this channel would be available for attack by this reagent in the ghost even if it were not present at either membrane surface. The question of whether these components extend through the membrane is left unanswered; however, the fact that they are on the surface has been established. The other major protein components must be located at the inside surface of the erythrocyte membrane or within the membrane structure. Component I, which has been called spectrin (12) or tektin A (137), has been localized at the inner surface of the ghost membrane by the ferritin-labeled antibody technique (82). Component IVa must also be located at the inner surface, since it is not digestible in the intact erythrocyte. The similarity in the accessibility to digestion of components I and IVa suggests that they may be proximally located at the membrane inner surface. One may also visualize these two components to be located next to that

portion of component III which may be exposed to the inner surface. This is supported by the fact that both I and IVa must be digested by trypsin before digestion of III occurs. Components IVa and IVb do not appear to be related functionally or in location, but the two polypeptides of I apparently are related. The localization of the other polypeptide chains is more difficult since they are only lost during digestion of ghosts at high enzyme concentrations.

The digestibility of component III varies for cells of different species. This variation may arise from differences in cell surface properties. This may reflect a difference in the membrane glycoprotein, but this seems unlikely since no association between the glycoprotein and III for human and bovine erythrocytes was demonstrated. Component IV also varies between species from a widely spaced doublet (human) to a single band (dog).

The membrane bound enzyme, acetylcholinesterase, is also present at the surface of the membrane. Approximately 60% of the activity of this enzyme is lost during trypsin digestion of intact erythrocytes and ghosts. This protein represents only a small fraction of the total membrane protein, since no major protein which may represent this enzyme is lost during trypsin digestion of erythrocytes. The activity loss of this enzyme during trypsin digestion of ghosts is more sharply dependent on protease concentration than in erythrocytes, again suggesting protein organizational difference in the transition from erythrocytes to ghosts.

The organization of proteins within the membrane may also be of importance in understanding what effect Ca^{++} plays in membrane stability. Several characteristics of red cells or ghosts have been

shown to be dependent upon Ca^{++} content. These involve ATP depleted cells in which there is an intracellular accumulation of Ca^{++} (101). With an increase of Ca^{++} in erythrocytes, there is a decrease in elasticity, deformability (101) and volume (102, 103) and an increase in density (102) of the membrane. Ca^{++} accumulation in erythrocytes has been implicated to be the cause of the membrane defect seen in erythrocytes of individuals suffering from hereditary spherocytosis (139), the most prevalent congenital haemolytic anemia of man. LaCelle (49) has stated that ATP depletion and Ca^{++} accumulation results during aging of erythrocytes, changing their ability to deform. In this case, the erythrocyte would become rigid and fragment when trying to pass through the capillary circulation (140). This would then be a possible mechanism for red cell destruction.

If the effects of Ca^{++} on erythrocytes seen in this investigation are a result of activating or releasing a membrane bound protease, then a mechanism of membrane destruction could be proposed. This might then aid in predicting the localization of some membrane components with respect to each other. This protease, possibly the one isolated by Moore et al. (100), could be released or activated in the intact cell by high Ca^{++} concentrations. This might be triggered by a spectrin conformational change (109), which also altered the shape of the cell. When the cell becomes rigid and fragments in an attempt to pass through the capillary circulation, the protease may be more active, initiating destruction of membrane proteins. This fragmentation process has been proposed as a mechanism for determining the life span of normal erythrocytes, as well as cells from individuals with Heinz body anemia and microangiopathic haemolytic anemia

(49).

The proteins undergoing proteolysis would have to be in close proximity to the enzyme. By examination of the protein patterns by gel electrophoresis after the cells have been exposed to excess Ca^{++}, one could get some indication of the localization of components that have undergone proteolysis. From the results of this investigation, components I and IVa are the only components significantly affected by high Ca^{++} concentrations, other than the aggregation phenomenon. These results can be partially explained on the basis of the results obtained from trypsin digestion of erythrocyte ghosts. As seen in Figure 26, page 93, the treatment of membranes with trypsin causes the same changes in components I and IVa that occur when erythrocytes are exposed to high Ca^{++} concentrations. From this, it appears that components I and IVa are in close proximity to each other and to the protease. Component I has been given the name spectrin (12) and has been localized at the interior of the membrane (50). This also suggests the protease is located inside the membrane. This scheme is highly speculative, but it may be useful in explaining red cell destruction.

The formation of the aggregate seen at the top of the gels after electrophoresis requires the Ca^{++} to be present during the haemolysis step and cannot be reversed by Ca^{++} chelators once it is formed. This could be due to the Ca^{++} being tightly bound to the aggregate or to a process which does not require Ca^{++} for aggregate stability. Since the residual haemoglobin and non-haemoglobin proteins have been shown to increase 200% and 60%, respectively (49), this aggregate may contain haemoglobin or glycolytic enzymes tightly bound

to some membrane protein.

The failure to see these Ca^{++} effects with isolated membranes could be due to loss of the protease by excessive washing during isolation of these membranes, or to a reorganization of the membrane which affects protease release or activation. The lack of aggregate formation is also suggestive of a reorganization of the membrane components in the ghost.

Calcium-45 was used to see if there was a relationship between the amount of Ca^{++} bound and the formation of the aggregate. If this were true, one would expect binding of Ca^{++} by erythrocytes to be higher than for ghosts, since only with Ca^{++} treated erythrocytes did any aggregation occur. Since this was not the case, no correlation appears to exist between the amount of aggregation and amount of Ca^{++} bound; however, the fact that ghosts bind more Ca^{++} than erythrocytes could be due to removal of endogenous Ca^{++} from the erythrocytes during the isolation of membranes, freeing more Ca^{++} binding sites. The differences in the binding curves between erythrocytes and ghosts could also be due to an organizational difference between the two types of membranes. Also, since Ca^{++} treated erythrocytes retain more haemoglobin, the Ca^{++} binding sites could be hidden by this bound haemoglobin.

The fact that no ^{45}Ca could be demonstrated to be associated with the aggregate of Ca^{++} treated erythrocytes suggests that this aggregate may be held together by forces other than Ca^{++}. These forces may be hydrophobic in nature, or may be a result of disulfide formation between proteins, although it is difficult to see why Ca^{++} should trigger disulfide formation. Further studies are obviously

needed to understand the nature of the aggregation and its importance to red cell behavior.

CHAPTER VII

SUMMARY

Solubilization of erythrocyte membranes has been achieved in two different systems, 1% SDS and CTAB-guanidine hydrochloride. Upon chromatography of SDS solubilized membranes, on Bio-Gel P-100, disaggregation of the protein and lipid components occurred. Only 5% of the total membrane protein, as indicated by amino acid analysis, was present at low molecular weights. Rechromatography of the protein fraction failed to give any further disaggregation, thereby providing evidence against the "miniprotein." Chromatography of the CTAB-guanidine hydrochloride solubilized membrane on Bio-Gel P-100 or Bio-Gel A-15M failed to give any separation of the protein and lipid components.

Localization of erythrocyte membrane proteins was studied with the use of proteolytic digestion on intact cells, isolated membranes, resealed ghosts and closed ghosts, followed by electrophoretic analysis on polyacrylamide gel electrophoresis in SDS. Trypsin digestion of human and bovine erythrocytes and resealed ghosts cleaves only the glycoprotein of the major membrane proteins. About 60% of both the sialic acid and acetylcholinesterase activity was lost during digestion of human erythrocytes with trypsin. Digestion of human erythrocytes with Pronase or chymotrypsin results in the cleavage of the glycoprotein as well as component III to yield a second component with a molec-

ular weight of 70,000. Chymotrypsin digestion of erythrocytes from bovine, horse and sheep did not show any discernible cleavage of any of the major membrane proteins, in contrast to the situation with human cells. Pronase digestion of bovine erythrocytes did not show any noticeable loss of component III or other membrane proteins, but some change in the membrane apparently occurred as evidenced by the formation of a new band in the 60,000 molecular weight region of a polyacrylamide gel. Component III of dog erythrocytes is cleaved by Pronase, and three new bands are noted with molecular weights of 49,000, 72,000 and 80,000.

Digestion of isolated erythrocyte membranes with trypsin results in extensive degradation of the membrane proteins. Eighty percent of the sialic acid and acetylcholinesterase activity is lost; however the activity loss is more rapid in the case of digestion of ghosts than with intact cells. Digestion of ghosts at low trypsin and chymotrypsin concentrations in isotonic buffer shows component IVa is the most readily digested. The difference in the digestibility between the membrane of the intact cell and that of the isolated ghosts results from the ability of the protease to penetrate into the ghost. The ghosts have been shown to be permeable to inactivated labeled chymotrypsin, while intact cells are impermeable to this enzyme. Closed ghosts are only partially permeable to labeled chymotrypsinogen A with intact cells showing impermeability to this protein.

High concentrations of Ca^{++} (0-5 mM) on erythrocyte membrane proteins show three significant effects: 1) aggregation appearing at the top of electrophoresis gels which appears to be dependent upon Ca^{++} concentration. These are Ca^{++} specific effects as evidenced by the

absence of them in the presence of high concentrations of Mg^{++} (5–20 mM). The apparent proteolysis effects seen with Ca^{++} can be best explained by the activation or release of a membrane bound protease within the red cell. Treatment of isolated membranes with Ca^{++} did not result in any significant change that occurred when erythrocytes were treated with Ca^{++}, suggesting a reorganization of proteins in the ghost.

The Ca^{++} binding curves for erythrocytes and ghosts were determined with the aid of ^{45}Ca. A biphasic binding curve was obtained with both types of membranes. ATP and EDTA reduced the amount of Ca^{++} bound to erythrocytes when added during the first haemolysis step with Ca^{++}. ^{45}Ca could not be demonstrated to be associated with the aggregation at the top of the gels by gel slicing experiments. The mechanism of the Ca^{++}-induced aggregation remains unclear, but Ca^{++} does not appear to be necessary for maintenance of the stability of the aggregate.

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VITA

Richard Black Triplett

Candidate for the Degree of

Doctor of Philosophy

Thesis: LOCALIZATION OF ERYTHROCYTE MEMBRANE PROTEINS

Major Field: Biochemistry

Biographical:

Personal Data: Born in Brownsville, Texas, January 25, 1943, the son of Mr. and Mrs. Jack D. Triplett; married to Donna Hensley on May 25, 1968.

Education: Graduated from Lenapah High School, Lenapah, Oklahoma, in 1961; received the Bachelor of Science degree in biochemistry from Oklahoma State University, in 1966; completed requirements for the Doctor of Philosophy degree on July 28, 1972.

Professional Experience: Served as Laboratory Assistant at Oklahoma State University from 1964 to 1966; served as Amino Acid Analyzer Operator at Oklahoma State University from June 1968 to September 1968; served as a teaching assistant at Oklahoma State University from August 1970 to December 1970; served as a research assistant at Oklahoma State University from 1968 to 1972.

Professional Organizations: Member of the American Chemical Society, and Division of Biological Chemistry.

Honarary Societies: Alpha Zeta, Phi Sigma and Phi Lambda Upsilon.