CALCIUM EFFECTS ON HUMAN ERYTHROCYTE MEMBRANES

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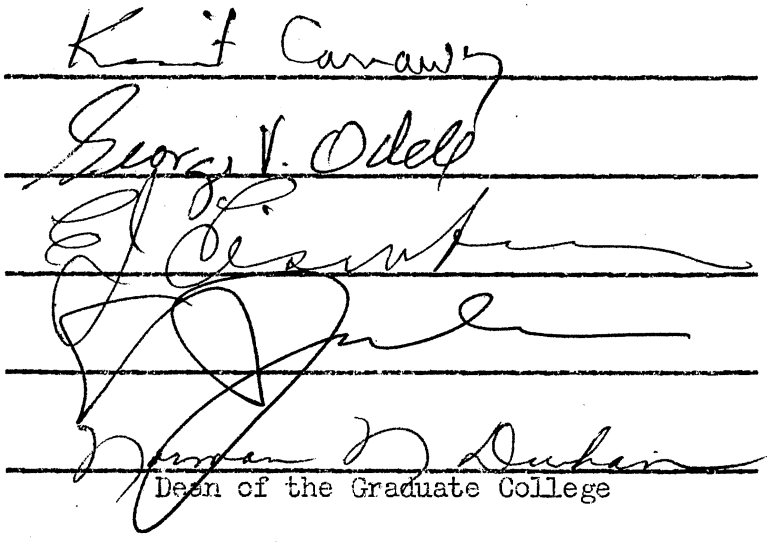

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NOMENCLATURE

RBC	red blood cell
vol	volume
ATP	adenosine triphosphate
EDTA	Ethylenediamine tetraacetic acid
SDS	Sodium Dodecylsulfate
ISC	irreversibly sickled cells
m.w.	molecular wieght
ANS	1-anilinonaphthalene-8-sulfonic acid
UV	Ultra Violet
Tris	Trishydroxymethyl amino methane

CHAPTER I

INTRODUCTION

To circulate and transport oxygen in the blood stream, a red blood cell (RBC) must maintain membrane flexibility. The maintenance of membrane deformability requires energy in the form of ATP. ATP-depleted RBC's become rigid and echinocytic (1), leak cations (2), accumulate calcium (3) and are eventually destroyed in the micro-circulation. If cells are repleted with ATP the rigidity effects are reversible (1). Certain processes do occur, however, in which the cell permanently loses its deformability. These membrane alterations are believed due to a change in membrane protein structure. The mechanism by which a cell undergoes disc to sphere changes accompained by reversible and irreversible deformability loss, is of considerable interest. A number of disease states including sickle cell anemia and heriditary spherocytosis involve increased membrane rigidity (1), as well as the normal <u>in vivo</u> aging process.

Human RBC membranes contain at least 7 major proteins. One of the most abundant species is spectrin, which is a large protein containing two high molecular weight chains (13). Spectrin is defined as a peripheral protein (37) because of its ease of extraction (8). Current evidence suggests that spectrin and RBC actin are disposed in a submembrane microfibrillar meshwork, which interacts with the

cytoplasmic end of certain transmembrane proteins and inhibits their lateral mobility (9, 95).

The effect of Ca^{++} on erythrocyte membranes is of interest because of its possible involvement in the disease states of sickle cell anemia (11) and hereditary spherocytosis (12), in which high concentrations of intracellular calcium are found. Calcium may have several roles of importance in membrane structure and organization, e.g. stabilizing protein conformations, binding lipids and proteins together, altering protein-protein interactions or changing lipid organization. Recent studies have shown that several shape and morphology characteristics of red cells and ghosts are dependent on cellular Ca^{++} and their ATP content. The depeletion of ATP is accompanied by accumulation of Ca^{++} (3). As a result of this accumulation the cell undergoes a decrease in volume (4) and deformability (5). One line of evidence (6) suggests that ATP controls membrane deformability by chelating the intracellular Ca^{++} bound to the interior side of the cell membrane. This would justify a role for ATPase in regulating the energized state of the membrane. Palek et al. (7) suggest that membrane configurations are controlled by membrane contractile proteins possessing Ca^{++}-ATPase activity. They showed that the addition of ATP and low concentrations of Ca^{++} could stimulate membrane-bound ATPase with no effect on deformability. However, at 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 depleted of ATP (15, 9). It was found that as normal cells are depleted of ATP,

membrane Ca^{++} increases (1). Also residual hemoglobin and non-hemoglobin proteins of the isolated membranes, increase 200% and 60% respectively. Regeneration of ATP, upon incubation in adenosine solutions, produced reversal in both shape and deformability. EDTA and externally added ATP (1, 15) block the effect of Ca^{++} on fresh cells.

Studies involving proteases such as trypsin offer further evidence for the involvement of membrane proteins in maintenance of cell shape (94). Trypsin treatment reportedly hinders the contraction of membranes induced by Ca^{++}, but only in the presence of trace amounts of ATP (2). This treatment also enhances binding of ATP to the membrane. It remains to be conclusively shown 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 the key role in maintenance of biconcave shape. It should be noted that other nucleotides have no effect on reversal of the Ca^{++} effect. It has also been suggested that trypsin modification may decrease the number of Ca^{++} binding sites (2). This could result from modification of spectrin, since Ca^{++} does cause aggregation of this protein (1, 16, 17). It has been suggested that Ca^{++} is causing a conformational change in spectrin there by affecting the shape of the membrane (18).

The loss of deformability during ATP depletion and Ca^{++} accumulation appears to be associated with the normal <u>in vivo</u> aging process (1). As a result of their losses, spherical and rigid cells are produced which are more subject to fragmentation during passage through the microcapillaries of the spleen. This fragmentation

process has been suggested as a mechanism for determining the erythrocyte life span in normal cells, as well as the events occuring under certain pathological conditions. It is well known that irreversibly sickled cells (ISCs) have rigid membrane alterations which eventually fragment and destroy the cell (56). It is also evident that abnormally high Ca^{++} levels are found in the membranes of ISCs (11). Erythrocytes from individuals with heriditary spherocytosis (HS), the most prevalent congenital hemolytic anemia of man, have been shown to be less deformable than normal erythrocytes. The HS membrane is qualitatively similar to normal, except for one missing protein (55), but leaks cations due to its altered membrane permeability (12). LaCelle (1) 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.

Introduction of Ca^{++} (>1 mM) into erythrocytes during hemolysis causes a number of distinct changes in membrane protein composition of the isolated erythrocyte ghosts. The most prominent change appears to be the formation of a protein aggregate which is highly resistant to disruption by sodium dodecylsulfate and other denaturing agents (19). A concomitant apparent proteolysis is also observed resulting in a new band of 180,000 molecular weight as determined by dodecyl-sulfate electrophoresis. These effects are observed when Ca^{++} is added in sufficient concentration during the initial hemolysis step. Since the mode of action of Ca^{++} was of interest, the effects on addition of Ca^{++} at subsequent membrane preparation steps were also investigated. Addition of Ca^{++} up to 5 mM with resealing buffer

(isotonic conditions) causes no effect. When resealed ghosts are rehemolyzed in presence of Ca^{++} the results are identical to addition at the first step. This suggests that the ghosts must be permeable to the high concentrations of Ca^{++} for the process to be effective.

The effects of Ca^{++} were tested under conditions where isotonicity was not restored (20). The mixture when incubated at 37°C under hypotonic conditions gives essentially identical effects. When tested under hypotonic conditions at 4°C aggregation was present but required higher concentrations of Ca^{++} for a significant amount to occur. The proteolyic processes were also temperature responsive (20). Addition of chelating agents such as EDTA with Ca^{++} during initial hemolysis prevents both aggregation and proteolysis. If Ca^{++} is present at the initial hemolysis step and chelators are added at a later step, neither aggregation, nor proteolysis is prevented or reversed (20). The membrane glycoprotein(s) do not appear to be present in the aggregate. Periodate-Schiff staining of electrophoretic gels of Ca^{++}-treated samples showed no evidence of carbohydrate in the aggregate band (20). It is unlikely that less highly glycosylated proteins can be detected by this procedure.

If Ca^{++} is involved in stabilizing the aggregate in the membrane, an enhanced binding of Ca^{++} to the membranes might be expected in the membranes containing aggregates. However, if radioactive Ca^{++} is added during hemolysis, the amount which remains bound to the membrane is actually less than that which is bound to isolated untreated membranes free of aggregate (20). Electrophoresis in dodecylsulfate solution of membranes treated with $^{45}Ca^{++}$ shows the radioactivity migrating independently of any protein band. This

indicates that Ca^{++} is not involved in stabilizing the aggregate since the aggregate is still present in dodecylsulfate.

Whatever the mechanism for loss of deformability may be, it most likely involves some of the major erythrocyte membrane proteins. As mentioned earlier, human RBC membranes contain at least 7 major proteins. The only high molecular weight protein which has been characterized to a significant degree is spectrin. This protein was originally isolated by Marchesi and Steers (13). They showed that it forms filaments in presence of ATP and Ca^{++} or Mg^{++}. The protein is present in all species of erythrocyte ghosts that were examined (21, 22). In addition it can be solubilized and extracted from the membrane by low ionic strength buffers, in presence of chelating agents. The protein contains two polypeptide chains with molecular weights of about 210,000 and 220,000 as determined by SDS electrophoresis (23) or by chromatography in guanidine hydrochloride (13). Some speculations exist about the exact nature of these polypeptides. Dunn and Maddy (24) using electrophoresis and chromatography, report evidence for a 40,000 molecular weight subunit comprising the larger chains. Physical measurements indicate that the protein has a rod like shape with limited solubility under conditions of high ionic strength, low pH or with divalent cations (21). The 210,000 and 220,000 units are indicated as protein dimers by crosslinking agents (21). These units apparently can exist in the membrane in higher molecular weight forms (25), although this point has been disputed by Tilney and Detmers (86).

In most cases when erythrocyte membranes are extracted with EDTA, a lower molecular weight species ($\sim$45,000 m.w.) copurifies with

spectrin. The size of this polypeptide is very similiar to muscle actin. Guidotti (26) has suggested the possibility of spectrin and the smaller molecule as being an "erythrocyte actomyosin", which provides an underlying network of structural support. The physical properties studied thus far, however, indicate no similarity to those of muscle actomyosin. Spectrin does contain a weak ATPase activity (17), but nothing similiar to muscle myosin. In addition the lower molecular weight spectrin polypeptide is specifically phosphorylated by a endogenous protein kinase (27).

Protein kinases are a widely known group of enzymes which regulate a number of cell processes by protein phosphorylation. This type of phosphorylation is distinct from that of ATPase reactions. Many of these are cyclic AMP dependent (27, 28, 29) which affords a second degree of regulation. Since one of the two chains of spectrin is phosphorylated it is possible that phosphorylation could be involved in the maintenance or control of mechanical properties or shape of the membrane through alteration in spectrin-membrane interactions (14). Phosphorylation of the spectrin chain by added $^{32}PO_4$ is enhanced in intact turkey erythrocytes by isoproterenol, a β-adrenergic agonist (30). The effect is coincident with increased cyclic AMP levels and enhanced sodium transport, suggesting that phosphorylation of this protein may be involved in the mechanism by which cation permeability of avian erythrocytes is increased by β-adrenergic reagents. Roses and Appel (31) have presented evidence from studies on the phosphorylation of endogenous membrane proteins of human erythrocyte membranes, suggesting that myotonic muscular dystrophy involves a change in ability of protein kinase to phosphorylate erythrocyte membrane

proteins. The exact meaning of all phosphorylation patterns found on membrane components, induced by cyclic AMP or monovalent cations is difficult to interpret at this time. Whatever the regulatory mechanism may be, it is clear that all components of the cytoskeleton must be able to interact in a cooperative manner. The number of types of protein components involved in the erythrocyte cytoskeleton are not yet well defined. At least two other components may be involved by interacting with cytoskeletal elements. One is a major membrane spanning component of about 90,000 daltons, commonly designated as Component III.

Component III is a slightly glycosylated protein and constitutes about 30-35% of the total membrane protein mass. It has recently been isolated by Furthmayr et al. (32). It has been suggested that this protein may be involved in several distinct functions including anion permeability (33, 34) and sugar transport (35). It appears that this molecule carries a receptor for Concanavalin A (36, 93). It is still not possible to determine how many different polypeptides are in this band and thus how many different polypeptide chains contribute to these functions.

In freeze-fracture studies on enzymatically modified membranes, Tillack, Scott and Marchesi (38) suggested that a link may exist between the intramembranous particles (component III) and the major sialoglycoprotein (glycophorin). It is not absolutely certain that these two components are associated but they appear to be the major components of intramembraneous particles obtained from membrane vesicles prepared by treatment with NaOH (39, 93). The studies of Wang and Richards (40) using crosslinking reagents show no evidence

for association between Component III polypeptides and the major sialoglycoproteins. However, these studies did suggest that the Component III polypeptide is a subunit of a larger protein of 165,000 daltons. It still remains to be determined, which of the various integral proteins can ultimately be correlated with the morphological structure of the intramembraneous particles. At least five components detected on the external surface can span the membrane (89).

The fluid mosaic model of membrane structure proposed by Singer and Nicolson (37) depicts the two intramembraneous components as a point of attachment for membrane peripheral proteins. In this way they serve as an anchor for the cytoskelatal structures of the cell and regulate the mobility of the lipids. The evidence that transmembrane proteins are involved in inner membrane surface structural organization was given by Ji and Nicolson (41). They showed that lectins which bind to and perturb the outer surface of membranes can induce organizational alterations at the inner surface. The role of transmembrane linkages in the structure and function of cellular membranes is largely unknown. They may provide the means by which a cell can control its outer surface and the display and topography of outer-surface components. Alternatively, transmembrane aggregation initiated at the outer surface may bring catalytic units together at the inner membrane surface causing activation of enzymes such as adenylate cyclase, and/or transport components.

It is conceivable that erythrocyte cytoskeletal proteins can induce shape changes, maintain biconcavity and deformability. In order to do this they must interact with identical molecules and molecules of other components of the network. The types of forces

involved and interactions which occur are important to the understanding of the morphological transformations. It is also important to understand what types of changes can occur which cause irreversible effects on shape and deformability. Two possibilities are by covalent association of protein units and proteolysis. Both types of changes may occur in the calcium perturbation effect (20). These modifications are probably enzymatically linked and the endogenous activities of the enzymes should be apparent if adequate assays can be developed. In the case of the Ca^{++} effects, one should look for an enzyme which is activated by Ca^{++}. The evidence suggests that cytoskeletal proteins are being covalently crosslinked into an aggregate which can not be disrupted by denaturation. A perfect candidate for the crosslinking agent is the Ca^{++} stimulated transglutaminase (96).

Transglutaminases are enzymes which catalyze a Ca^{++}-dependent acyl-transfer reaction in which the γ-carboxamide groups of peptide-bound glutamine residues are the acyl donors. Primary amino groups in a variety of compounds may function as acyl acceptors with the subsequent formation of mono-substituted γ-amides of peptide bound glutamic acid. This Ca^{++}-dependent enzymatic activity was discovered in the livers of a number of mammals (42). Studies leading to this discovery were stimulated by earlier reports of a Ca^{++}-dependent system of guinea pig liver that promoted the covalent attachment of L-lysine, through its ϵ-amino group, to protein (43). The activity of transglutaminases should not be confused with the activity of other enzymes that affect hydrolysis or transfer at the carboxamide group of free glutamine.

The physiological role of liver transglutaminase was the source

of some early speculation (44). To date, however, there is no concrete evidence to support the assignment of any specific biological function for this enzyme. In contrast two other transglutaminases found in guinea pigs and humans appear to participate in important biological reactions. The first of these, blood coagulation factor XIII, exists in blood plasma (45, 46), platelets (47) and other tissues in zymogen form. The transglutaminase formed from the inactive proenzymes by thrombin catalyzes the formation of ϵ (γ-glutamyl) lysine crosslinks between fibrin monomers, thus causing the establishment of an insoluble fibrin lattice (48). The second enzyme has been isolated from hair follicles (49) and is probably responsible for crosslinks found in the proteins of hair.

Proteases have a wide variety of functions in many different cells. They are found in some cells in degradative roles for the catabolic processes. In plasma, proteases function to activate zymogens such as factor XIII and convert other factors from inactive forms to active forms in the plasma clotting system (50, 54). Many proteases themselves occur in inactive forms that require a specific proteolyic cleavage for activation (52). This is one way in which the activity of proteases can be regulated. A number of unspecified endogenous protease activities have been reported to be present in intact erythrocytes (53). The isolation of these enzymes has not been presently accomplished and no functional role may yet be assigned.

CHAPTER II

METHODS

Changes in Membrane-Associated Proteins Resulting from Calcium Treatments of Human Erythrocytes

Calcium Treatment

Fresh human blood was obtained from Stillwater Municipal Hospital and washed 3 times. Calcium treatments were performed by a modification of the procedures of Triplett, et al. (19). One volume of packed red cells was hemolyzed in 10 vol. of 10 mM Tris (pH 7.4) containing varying amounts of $CaCl_2$. The hemolysate was incubated at 37° for 30 minutes. Where resealing was necessary, the isotonicity was restored to 0.174 M with 3 M NaCl prior to the incubation step. Ghost membranes were obtained by washing in 10 mM Tris (pH 7.4) with centrifugation at 17,000 x g for 20 minutes.

Analytical Procedures

Polyacrylamide gel electrophoresis of membranes was performed on 5% gels by previously reported procedures (51, 57). Gels were stained for protein by Coomasie Blue and for carbohydrate with periodate-Schiff (56). Gels containing radioactive samples were sliced and counted by previously reported procedures (59). Column chromatography

of membrane samples was performed in dodecylsulfate Tris buffer on Sepharose 4B.

Membrane Extractions

Membranes obtained from erythrocytes hemolyzed in presence of increasing concentrations of $CaCl_2$ were extracted in 5 vol. of 5 mM glycine, 5 mM mercaptoethanol, 1 mM EDTA buffer (pH 9.5) at 4° for 24 hours. Aliquots of extracts were taken for electrophoresis in dodecylsulfate solution along with aliquots of extracted residual membranes. Protein concentrations were determined by the procedure of Lowry et al. (60). The extracted soluble material was concentrated by ultrafiltration or precipitation with 50% $(NH_4)_2SO_4$. Protein components found in Ca^{++}-treated extracts were compared to those from untreated extracts after separation by Sepharose 4B gel chromatography eluted with glycine-EDTA-Mercaptoethanol buffer at pH 8.6. The residual extracted membranes were compared from treated and untreated samples by polyacrylamide electrophoresis in dodecylsulfate.

Isolation and Partial Characterization of Protein Components

The protein components associated with two membrane Ca^{++}-related changes were isolated and purified by Sepharose 4B gel column chromatography. Component II was isolated from extracted Ca^{++}-treated membranes after solubilization in 1% dodecylsulfate - 50 mM Tris (pH 7.4) and separation on a Sepharose 4B column (2.5 x 90 cm) eluted with 50 mM Tris, 1% SDS (pH 7.4). The partially purified component II was concentrated by ultrafiltration and rechromatographed over the

same column. The protein was further purified by separation on a polyacrylamide gel in presence of dodecylsulfate. The band corresponding to component II was then excised and hydrolyzed in 6N HCl. The amino acid composition was obtained on a microanalyzer designed and built by Dr. T.H. Liao (61, 69).

Component Va was easily obtained from the EDTA extracted portion of the membranes. The extract was concentrated by ultrafiltration and dialyzed against 10 mM Tris (pH 7.4) to remove mercaptoethanol from the sample. The sample was then placed in a solution of 1% dodecylsulfate solution and chromatographed on a Sepharose 4B column containing 1% dodecylsulfate, 50 mM Tris (pH 7.4) as eluting buffer. Under these conditions component Va forms an aggregate which elutes in the void vol. fraction. This aggregation is reversible by reduction with mercaptoethanol. The void vol. peak was concentrated and treated with a ten fold excess of the sulfhydryl reagent. The sample was then rechromatographed over a Biogel A-5M (1.5 x 90 cm) column in presence of 1 mM mercaptoethanol and 50 mM Tris in 1% dodecylsulfate (pH 7.4). The reduced component Va separates from other higher molecular weight contaminants and may be isolated in pure form from the Biogel column peak. After removal of salts and detergent, the sample was hydrolyzed in 6N HCl. The amino acid composition was obtained on a Beckman 121C amino acid analyzer.

Iodoacetate ^{3}H-labeling of Ca^{++}-treated Membranes

Human erythrocytes were washed in pH 7.4 Krebs-Ringer buffer. A 1.0 ml solution of Krebs-Ringer containing 0.3 ml of packed washed

cells was incubated with 500 μCi of ^{3}H-iodoacetic acid for 60 minutes at 37°. Treated samples were washed 3 times in 10 mM Tris, 150 mM NaCl (pH 7.4) and hemolyzed at 4°C in presence and absence of 5 mM Tris (pH 7.4). The samples were incubated at 37° for an additional half hour and washed at 4° in 10 mM Tris until free of hemoglobin. The ghost membranes were immediately solubilized by addition of 4% SDS and boiling for 5 minutes. The samples were placed on 5% polyacrylamide gels in dodecylsulfate and run for 4 hours at 8 ma/gel. Gels were sliced into 2 mm slices and counted. Another sample was hemolyzed in the presence of Ca^{++} and washed prior to incubation with ^{3}H-iodoacetate (790 μCi/mg protein) for comparison of components labeled after Ca^{++} changes have occured.

Inhibition of Aggregation Process by Chemical Modification

Fresh human blood was washed 3 times in Krebs-Ringer (pH 7.4). One ml of packed cells in 9 ml of Krebs-Ringer buffer was reacted with 0.0 to 5.0 mM iodoacetic acid for 60 minutes at 37° and washed 3 times in isotonic saline containing 10 mM Tris buffer (pH 7.4). Half of each cell suspension was hemolyzed in 10 mM Tris containing 5 mM $CaCl_2$. The remaining samples were hemolyzed in the same buffer without $CaCl_2$ for controls. The suspensions were incubated for 30 minutes at 37° and washed 3 additional times in 10 mM Tris to remove hemoglobin. The washed ghosts were immediately solubilized in 4% SDS and placed on 5% polyacrylamide gels in presence of dodecylsulfate. Gels were run for 4 hours at 8 ma/gel.

Localization of an Aggregating Factor from Erythrocyte Hemolysates

Fresh whole human blood was washed in 10 mM Tris, 154 mM NaCl (pH 7.4). One volume of packed cells was hemolyzed in 10 volumes of 10 mM Tris (pH 7.4) for 10 minutes at 4°C. The membranes were sedimented from the soluble portion by centrifugation at 1500 x g for 5 minutes. The supernatant was removed and saved. The pelleted membranes were resuspended in 10 mM Tris (pH 7.4) and the washing process repeated until free of hemoglobin. The washed, packed, hemoglobin free ghosts were resuspended in the original hemolysate and the suspension was made 5 mM in $CaCl_2$. The mixture was incubated at 37° as in previous calcium treatments. An identical sample of membranes was mixed with hemolysate in absence of added Ca^{++} and incubated as a control. The membranes were again washed free of hemoglobin and dissolved in 4% sodium dodecylsulfate solution. The membrane patterns were analyzed for aggregation and other changes by SDS gel electrophoresis.

^{14}C-Putrescine Assay for Transglutaminase Activity

The assay procedure for guinea pig liver transglutaminase (62) was modified for use in studying the red blood cell enzyme. ^{14}C-putrescine was used as a radioactive substrate for incorporation into endogenous red cell protein. A total of 0.2 μCi of radioactivity was added to each assay containing varying amounts of calcium (0.2-5.0 mM) or the enzyme inhibitor iodoacetamide (1-1000 μM). A fixed amount of fresh hemolysate was added in a Tris buffer system (pH 8.0) and the

suspensions were incubated at 37°C for 2 hours. The reactions were rapidly terminated by addition of an equal volume of ice cold 10% Trichloroacetic acid (TCA). Samples were washed 3 times by centrifugation at 1500 x g for 3 minutes and resuspension in cold 5% TCA. Blank samples containing enzyme but no Ca^{++} or no inhibitor were included. TCA precipitates were solubilized in 0.5 ml of NCS tissue solubilizer (Amersham Searle Corp.) and counted in a liquid scintillation counter.

Activation of Erythrocyte Endogenous Transglutaminase by Ca^{++}

Freshly drawn human blood was washed in isotonic Hepes buffered saline (pH 7.4). One volume of washed packed cells was hemolyzed in 10 volumes of 10 mM Tris (pH 8.0). A 0.1 ml volume of hemolysate was added to the assay mixture containing various concentrations of calcium ranging from 0.0 to 5.0 mM. The samples were incubated, washed and counted. The activity in counts per minute (cpm) was converted to disintegrations per minute (dpm) from a standard quench correction curve. Activity was compared to the amount of calcium present in the assay medium and the minimal activating concentration of the divalent cation was determined.

Inactivation of Erythrocyte Transglutaminase by Iodoacetamide

Fresh hemolysate (0.1 ml) was added to assay tubes containing 5.0 mM $CaCl_2$ and all other necessary assay reagents. The assay mixtures contained varying concentrations of the potent inhibitor iodoacetamide

(62) ranging from 1 to 1000 μM. Incubation times and counting procedures were identical to previous assays. Radioactivity incorporated into endogenous protein was compared with the amount of inhibitor present. A plot of Activity vs. Inhibitor provided the approximate concentration of which complete inhibition was obtained.

Prevention of Aggregation Process in Ca^{++}-treated Cells by Transglutaminase Inhibitor

Washed intact cells in 0.5 ml volumes were suspended to 5 ml in 135 mM CaCl, 4.5 mM KCl, 15 mM Choline, 10 mM Tris (pH 7.4), containing the inhibitor iodoacetamide in concentrations ranging from 1 to 1000 μM. Calcium (5 mM) was introduced by addition of the calcium ionophore A23187 (Eli Lilly and Co.), followed by incubation at room temperature for 30 minutes. The cells were subsequently isolated, hemolyzed and washed until free of hemoglobin. The washed membranes were solubilized in sodium dodecylsulfate (SDS) and subjected to SDS polyacrylamide electrophoresis. Gels were stained with Coomassie blue and aggregation observed as a function of inhibitor.

Biophysical Properties of Human Erythrocyte Spectrin

Isolation and Purification of Spectrin

Large quantities of Spectrin were partially purified by extraction according to the procedure of Marchesi et al. (63). To remove a 43,000 m.w. contaminant, it was necessary to separate the concentrated extracts over a 2.5 x 90 cm column containing Sepharose 4B Agarose gel. The resulting peak was partially resolved into two main species.

Individual column fractions were analyzed for their content by polyacylamide electrophoresis in dodecylsulfate. The fractions from the first half of the peak contained no contaminating components. It was these fractions which were routinely isolated and used extensively for the studies of the physical properties under low ionic strength conditions.

Fluorescence Measurments

ANS fluorescence measurments were taken under steady state using an AVCO EVERETT model C-400 Pulsed nitrogen laser as a source. The signal was averaged on a model 162 Boxcar Averager from Princeton Applied Research fed in from a Spex 1704 spectrometer. Fluorescence titrations were performed on the purified protein using the procedure of Rubalcava et al. (64). The magnesium salt of 1-anilinonaphthalene-8-sulfonic acid was recrystallized several times from practical grade material obtained from Sigma Chemical Company (65).

Effects of pH were monitored in 20 mM Tris-Acetate buffers. The pH was checked prior to and following each measurment. Scans of fluorescence emission were obtained from 400 to 600 nm on samples containing 3 mg/ml and 1 mg/ml protein. Samples of both protein concentrations at five pH values were scanned in presence and absence of 5 mM $CaCl_2$.

Intrinsic fluorescence was measured using 0.25 mg/ml protein concentrations. Tryptophan residues on the molecule were excited at 280 nm using a 300 W. Xenon lamp with a Spex minimate monochromator. The emission signal was amplified by use of a Model 128 Lock in Amplifier (Princeton Applied Research). The solution was scanned from

320 to 520 nm. A protein solution containing 10^{-5} M ANS was scanned over the same range to assay fluorescence energy transfer from Tryptophan to ANS.

Circular Dicroism

Circular Dicroism studies were performed in the near UV range at a protein concentration of 1.5 mg/ml in 1 cm pathlength cells. Measurments in the far UV range were taken in a 1 mm pathlength cell at a protein concentration of 53 μg/ml. Mean residue ellipticity was obtained using a mean residue molecular weight of 133 g/mole. Measurments were taken on a Cary 61 recording spectropolarimeter. The scan rate was 0.2 nm/s with a period of 10s (66). The instrument was calibrated by the method of Cassim and Yang (67). Far UV scans (range 275-190 nm) were obtained at pH values from pH 7.4 through the range of insolubility (pH 4.5). Scans in presence and absence of 3.5 mM $CaCl_2$ were obtained. Near UV scans (range 310 to 250 nm) were obtained at pH 7.4 and pH 8.6 in presence and absence of 5.0 mM $CaCl_2$.

pH-Solubility Studies

The effects of Ca^{++} on the solubility of Spectrin were observed over a pH range of 5.0 to 6.0. Insoluble protein was separated by centrifugation and protein remaining in solution was determined from the supernatants by the method of Lowry et al. (60).

Ultracentrifugation Studies

Samples of the highly purified Spectrin at a concentration of 3.61 mg/ml were dialyzed against 6 M Guan HCl and against Tris buffer

(10 mM pH 8.6) containing 0.1 M KCl both in presence and absence of 5 mM $CaCl_2$. A volume of 0.4 ml for each sample was placed on a Beckman Model E Analytical Ultracentrifuge. Running speed was 59,780 rpm. Sedimentation coefficients were determined and compared to previously published values (68).

Amino Acid Analysis of Ca^{++}-treated Spectrin and Spectrin Subunits

Protein bands from SDS polyacrylamide gels corresponding to spectrin isolated from EDTA extracts (extractable spectrin) and membrane residues (non extracted spectrin) were excised and hydrolyzed according to previously stated procedures (61). In addition the 220,000 and 210,000 molecular weight polypeptides were separated and excised independently for individual comparative analysis. The lower molecular weight "actin" like polypeptide was also hydrolyzed from these preparations for comparison to the other components. The hydrolyzed samples were analyzed on a microanalyzer (69).

CHAPTER III

CHANGES IN MEMBRANE-ASSOCIATED PROTEINS RESULTING FROM CALCIUM TREATMENTS OF HUMAN ERYTHROCYTES

Ca^{++} Promoted Aggregation of Membrane Proteins

The preparation of erythrocyte membranes for these experiments involved a four-step process: 1) hypotonic hemolysis; 2) resealing by incubation in isotonic salt solution; 3) re-hemolysis; and 4) washing of the membranes to remove hemoglobin (19). Addition of Ca^{++} in sufficient concentrations during the initial hemolysis causes an apparent protein aggregation (Figure 1, Arrow 1), determined by dodecylsulfate-acrylamide electrophoresis. A concomitant apparent proteolysis (20) is also shown by Arrows 2 and 3.

Isolation of the Aggregate and Dissociation Attempts

Because of its large size in dodecylsulfate solution the aggregate can be isolated by chromatography on Sepharose 4B in the detergent. Figure 2 shows elution profiles of membranes prepared from Ca^{++}-treated and untreated cells. The profiles differ primarily by the presence of an excluded peak (Fractions 40-45) in the treated samples and by changes which occur in the areas of the proteolyzed

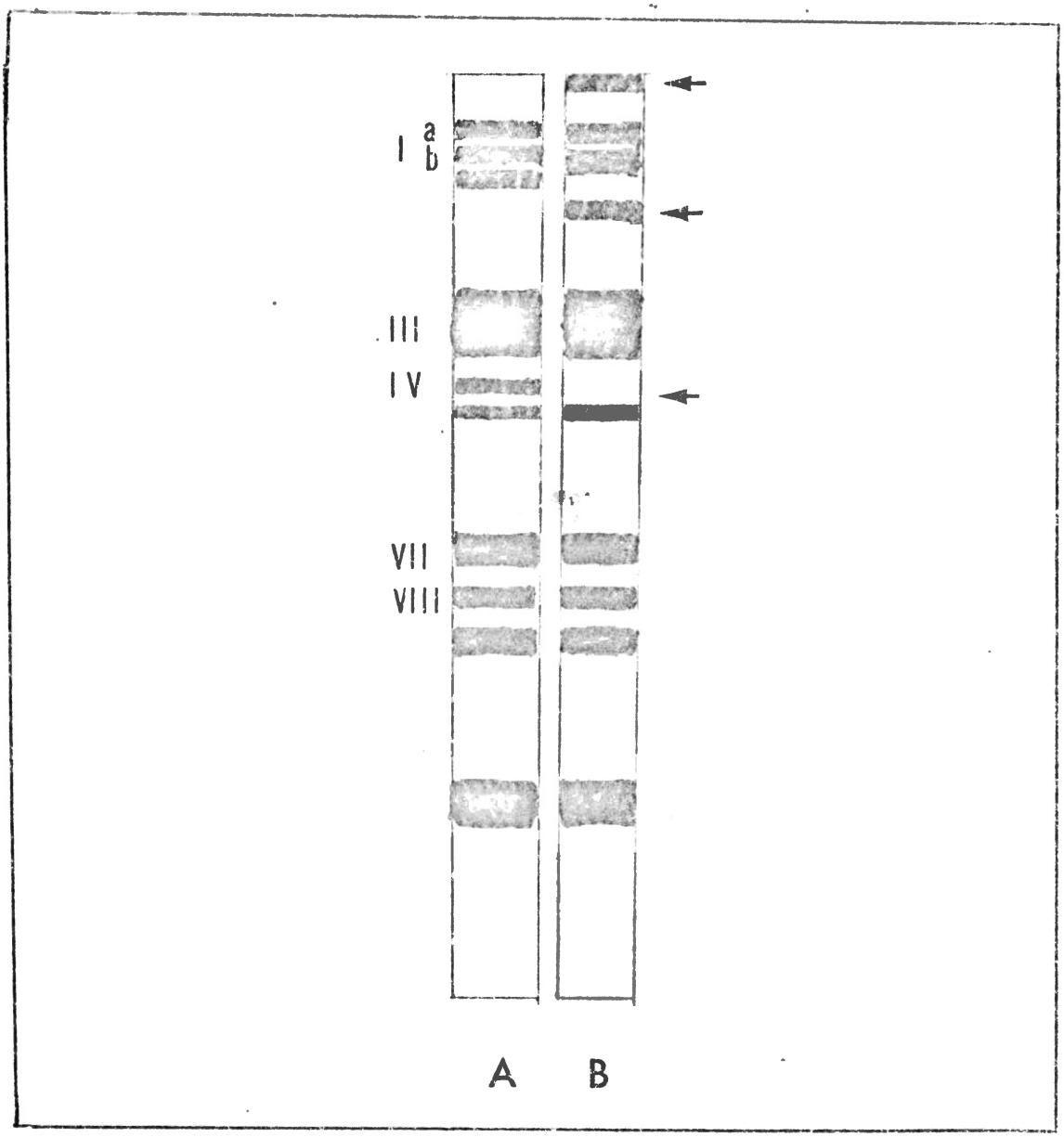


Figure 1. Ca^{++}-Promoted Aggregation of Erythrocyte Membrane Proteins

Erythrocytes were hemolyzed in Ca^{++} solutions in 10 mM Tris (pH 7.4), and membranes were prepared as described previously. Membrane samples were subjected to electrophoresis on 5% polyacrylamide gels in dodecylsulfate. Bands are numbered as in previous publications (19,20). Ca^{++} concentrations are 0 and 5.0 mM for Gels A and B respectively.

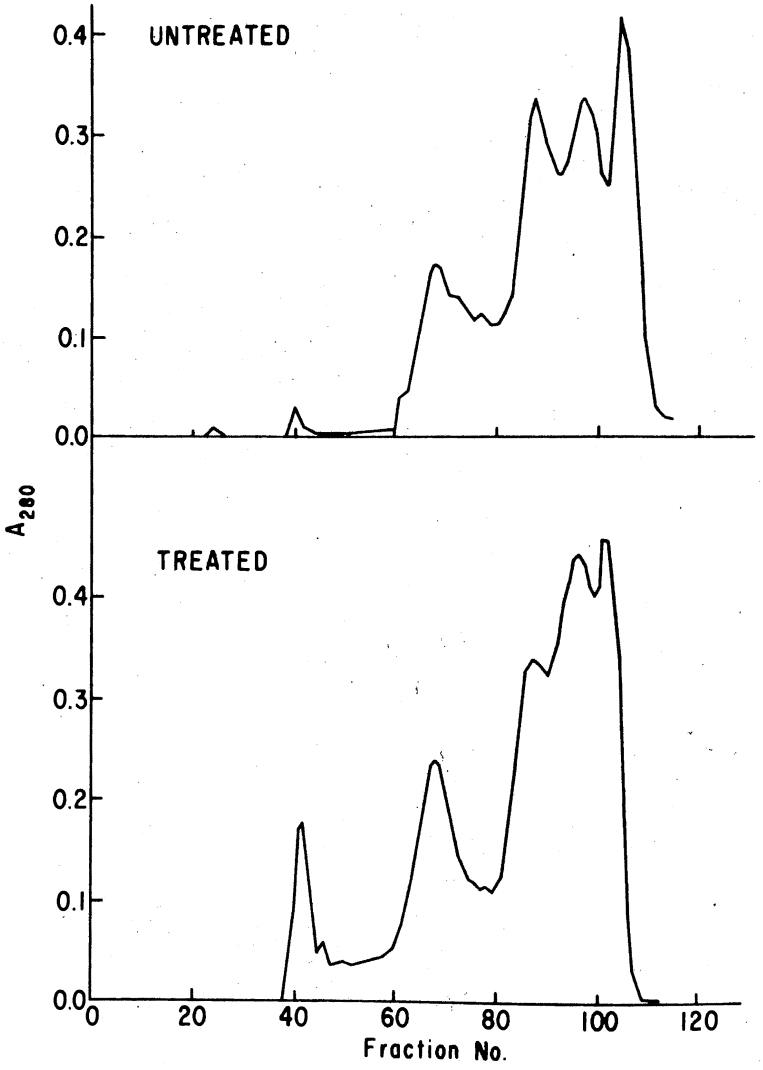


Figure 2. Chromatography of Membranes from Ca^{++}-treated and Untreated Erythrocytes

Membranes were prepared from cells treated with 5 mM Ca^{++} during hemolysis and from control cells. The membranes were dissolved in 1% sodium dodecylsulfate in Tris (pH 7.4) and chromatographed on Sepharose 4B as described previously.

components. Electrophoresis of the excluded peak in dodecylsulfate indicates that it bands near the top of the electrophoresis gels, as does the aggregate. The fractions containing the aggregate were pooled, dialyzed to remove detergent and hydrolyzed for amino acid analysis. The amino acid composition is shown in Table I, together with the composition for spectrin, the high-molecular-weight erythrocyte membrane protein which is denoted as Band 1 in Figure 1. Comparison of these amino acid analyses with similar analyses of the other column chromatography fractions indicate that spectrin comprises a major fraction of the aggregate. The key observation is the high glutamic acid content of both spectrin and the aggregate. None of the other membrane proteins which might contribute have such a high glutamic acid content. It is also obvious, however, that spectrin cannot account for the analytical data completely. There must be at least one additional protein present.

The conformation of the aggregate isolated by chromatography in detergent was examined by circular dichroism to determine if the apparent high molecular weight might be due to conformational restrictions which prevent the protein from assuming the appropriate shape in the detergent solution (71). The spectrum is typical of that of other globular proteins in this detergent (72). This suggests that a true aggregation of polypeptides rather than a conformational anomaly is responsible for the high molecular weight.

A number of attempts have been made to disrupt the aggregate in the membrane before the membranes were subjected to dodecylsulfate electrophoresis. The dissociating agents included 3% dodecylsulfate ($\pm$ heat), 6 M guanidine hydrochloride, 8 M urea ($\pm$ 0.2% EDTA),

TABLE I

AMINO ACID ANALYSES OF AGGREGATE AND SPECTRIN

Amino acid	Aggregate (mole %)	Spectrin (mole %)
Lysine	6.1	6.8
Histidine	2.5	2.8
Arginine	5.2	6.1
Aspartic acid	8.9	10.7
Threonine	4.9	4.0
Serine	6.8	6.0
Glutamic acid	16.2	19.5
Proline	5.2	2.6
Glycine	7.4	5.9
Alanine	7.7	7.3
½-Cystine	0.5	0.5
Valine	5.1	4.9
Methionine	2.0	2.3
Isoleucine	3.4	3.0
Leucine	12.2	11.6
Tyrosine	2.2	2.3
Phenylalanine	3.8	3.8

Values for aggregate are average of three separate preparations. A single preparation was analyzed for the spectrin data but the data compare well to previous samples analyzed in this laboratory and the results of Marchesi et al. (63). Tryptophan was not analyzed. No amino sugars were detected.

phenol-acetic acid-water (1:1:1, by vol.), 90% 2-chloroethanol and chloroform methanol (2:1, v/v). Samples were dialyzed against distilled water and solubilized in dodecylsulfate for electrophoresis. In no case was any dissociation of the aggregate detected. If any disaggregation occured during these treatments, it must have been reversed during the removal of the dissociating agent.

Membrane Extractions

Extraction of normal erythrocyte membranes with glycine-EDTA-mercaptoethanol causes release of spectrin (13) (two polypeptide chains) and a smaller polypeptide with a mol. wt. similar to that of actin (58, 73). Extracts of membranes from cells hemolyzed in presence of 1.0 mM Ca^{++} show significant differences. Figure 3 shows electrophoresis patterns of membranes and extracts from cells hemolyzed in buffers containing 0, 2.0, 3.0, 4.0 and 5.0 mM $CaCl_2$. Gels A-E represent the material remaining in the membrane following extraction in pH 9.5 EDTA buffer. Gels F-J are the corresponding patterns of soluble material extracted from the membranes. Several interesting features can be noted from these gels. First, neither the Ca^{++}-promoted aggregate nor component II is extracted from the membrane. Membranes from the calcium-treated cells show an increased spectrin content after extraction even through there is no apparent decrease in the amount of spectrin extracted. Extraction of spectrin also increases the visibility of component Ic which has a slightly higher mobility than the lower band of spectrin (Gel A). However, it is quite note-worthy that Ic is absent from Ca^{++}-treated samples. The disapperance of Ic and the concomitant appearance of component II

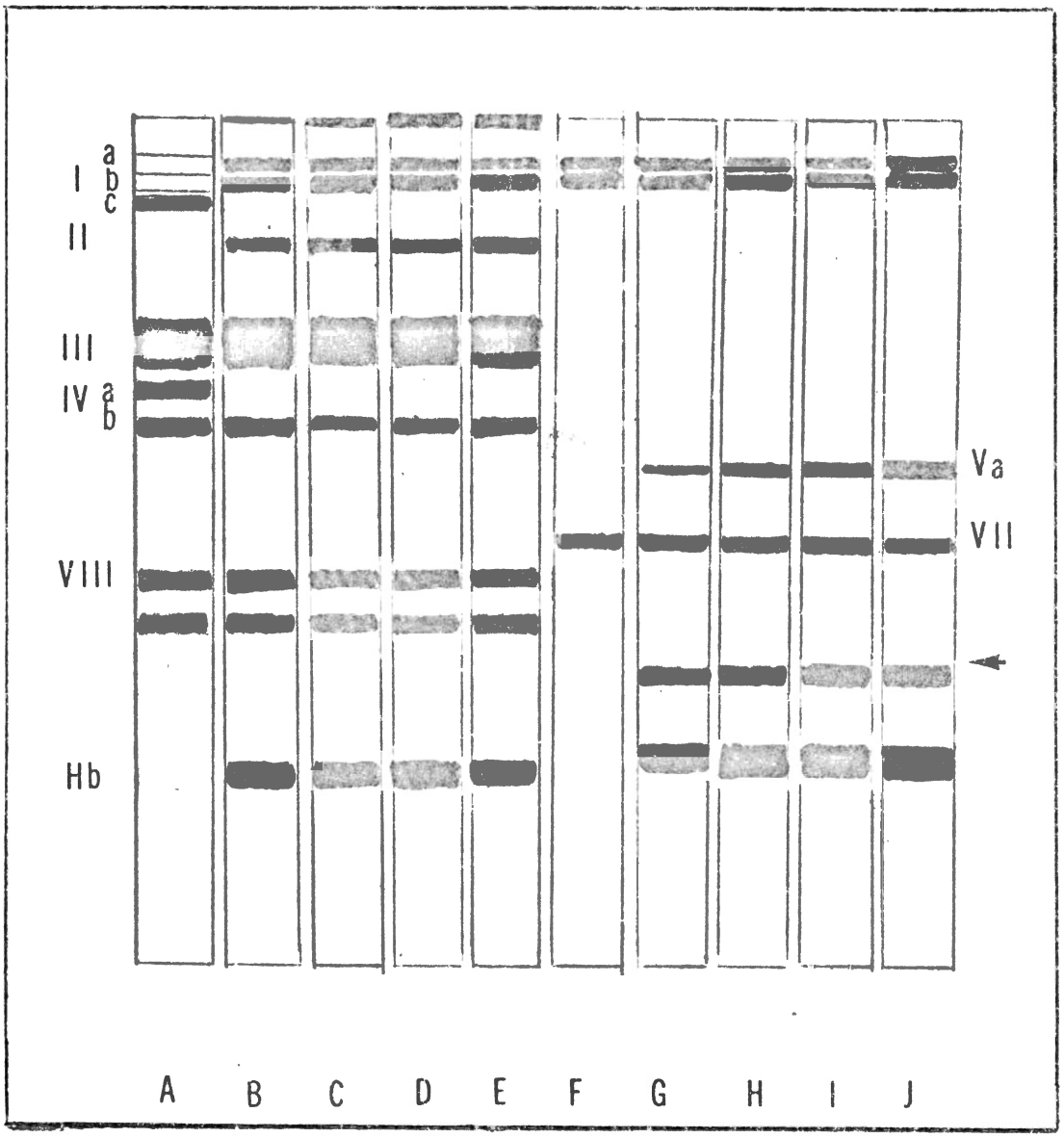


Figure 3. Effects of Calcium Addition During Hemolysis on Proteins of the Human Erythrocyte Membrane

Patterns are shown for extracted residual membranes and EDTA extracts from cells hemolyzed in presence of 0.0, 2.0, 3.0, 4.0, and 5.0 mM $CaCl_2$, 10 mM Tris pH (7.4). Residual membrane samples (gels A-E) and extracts (gels F-J) were subjected to electrophoresis on 5% acrylamide gels. Concentrations are 0, 2.0, 3.0, 4.0 and 5.0 mM Ca^{++} for gels A-E and gels F-J, respectively.

suggests that the component II arises from Ic rather than spectrin, presumably by the action of a protease activated by calcium. The extracts from the membranes of Ca^{++}-treated cells show 3 significant changes, the appearance of bands with approx. mol. wts. of 60,000, 25,000 and 16,000. The last of these is the hemoglobin subunit. Table II shows the amounts of protein present is extracted membranes and extracts as a function of Ca^{++} concentrations during hemolysis. Increased amounts of nonextractable protein are found in the membranes with increased calcium concentrations. After correction for hemoglobin the protein content of the extract appears to peak at 3 mM Ca^{++}.

The differences seen in components found in Ca^{++}-treated membrane extracts can be further illustrated by separation on a Sepharose 4B gel column. Figure 4 shows the chromatographic profiles of extracts obtained from membranes hemolyzed in presence (A) and in absence (B) of 5.0 mM $CaCl_2$. At least five distinct peaks are partially resolved from Ca^{++}-treated extracts, but only two are seen in control extracts. Peaks I-IV were pooled and analyzed for polypeptide components by dodecylsulfate gel electrophoresis. The composition of each peak is shown in Figure 5. Gel A contains whole extracts from Ca^{++}-treated membranes showing the presence and relative proportions of all components found. Gels B-E display the composition of Seph 4B fractions I-IV. Fraction IV contains large amounts of components Va, VII and VIII. Only components Ia, Ib and VII (actin) are found to any extent in membrane extracts of untreated cells.

Isolation of Component Va

Component Va can be isolated by taking advantage of the fact

TABLE II

PROTEIN CONTENTS OF RESIDUAL MEMBRANES
FOLLOWING EXTRACTION IN EDTA BUFFER
AND THE CORRESPONDING EXTRACTS

	Ca^{++} mM	Total Protein (mg)	%	Total Memb. Prot. (mg) *	%
Residual Membranes	0.0	6.2	55.4	5.2	59.1
	2.0	9.8	65.3	9.0	70.6
	3.0	10.6	61.6	9.2	66.2
	4.0	10.4	65.9	9.6	70.6
	5.0	12.0	67.0	11.2	77.2
Extracts	0.0	4.6	44.6	3.6	40.9
	2.0	5.8	34.7	4.0	29.4
	3.0	6.6	38.4	4.7	33.8
	4.0	5.4	34.1	4.0	29.4
	5.0	5.9	33.0	3.3	22.8

Protein values were determined by Lowry et al. (60). Hemoglobin contents were subtracted to give total membrane protein in both membranes and extracts.

* Values obtained after subtraction of Hemoglobin.

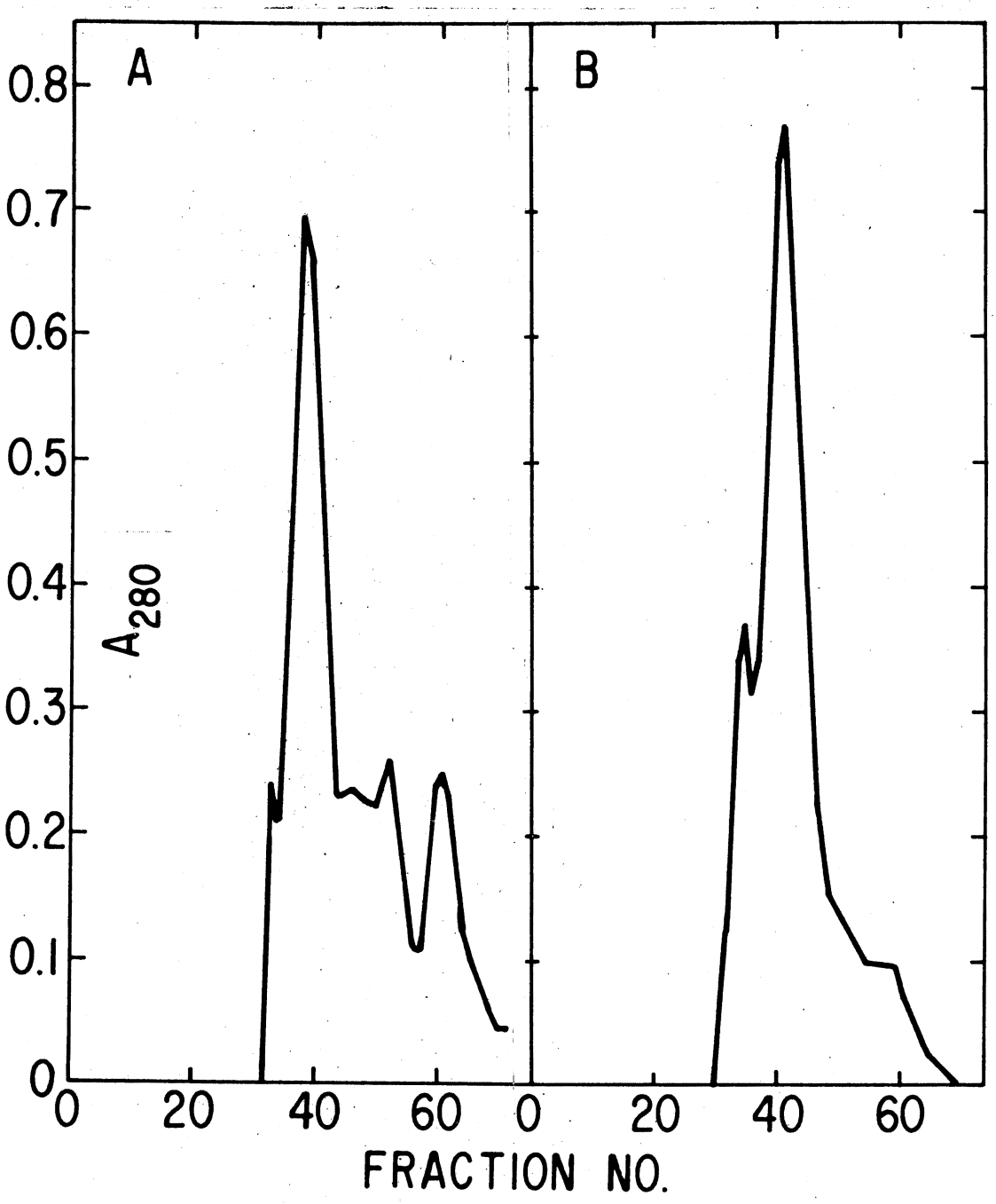


Figure 4. Chromatography of EDTA Extracts From Membranes Prepared From Cells Hemolyzed in Presence and Absence of 5.0 mM $CaCl_2$

Extracts were chromatographed on a sepharose 4B column eluted with glycine-EDTA-Mercaptoethanol buffer (pH 8.6). (A) Chromatography of extracts from cells hemolyzed in presence of Ca^{++} (5.0 mM). (B) Chromatography of extracts from cells hemolyzed in absence of calcium.

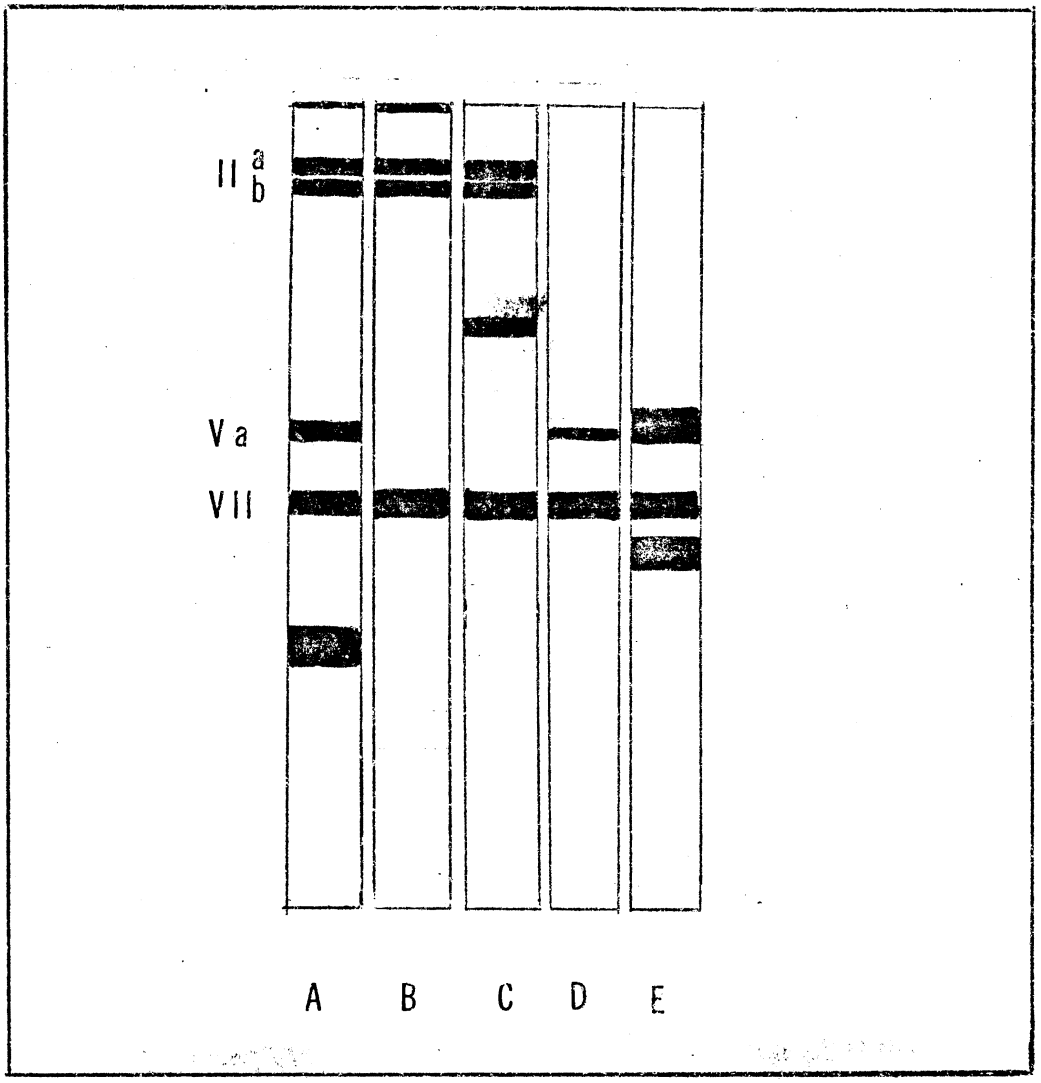


Figure 5. Effect of Ca^{++}-treatment on Protein Composition in EDTA Extracts

Erythrocytes were hemolyzed in the presence of 10 mM Tris (pH 7.4) and 5.0 mM Ca^{++}. The extract was chromatographed on Sepharose 4B eluted with glycine-EDTA-Mercaptoethanol buffer (pH 8.6). Fractions from peaks I-IV were pooled and subjected to electrophoresis in dodecylsulfate. Gel A, whole extract (control). Gels B-E fractions I,II,III,IV respectively.

that it forms an aggregate if EDTA extracts of membranes of Ca^{++}-treated cells are dialyzed against 10 mM Tris without mercaptoethanol. Figures 6 and 7 show the column profiles and electrophoretic analyses of the column peaks, respectively, for samples dialyzed in the presence and absence of mercaptoethanol before solubilization in dodecylsulfate. These patterns show clearly that Va is eluted in the void volume of the column if the sample is not treated with mercaptoethanol, but it is well within the retarded volume if the sample is so treated. If the void volume peak of the unreduced sample is reduced and subsequently rechromatographed, it can be isolated free of contaminating proteins (Figure 8). Its amino acid analysis is shown in Table III.

Isolation of Component II

Isolation of component II by column chromatography was not possible. The component can be partially separated from residual membrane spectrin and component II on dodecylsulfate-Sepharose 4B columns. The column fraction containing the highest concentration of component II was subjected to electrophoresis on an acrylamide gel for final purification. The smaller concentrations purified in this manner required the use of a microanalyzer column (see Chapter II). Approximately 40 μg of material are required for this method. The amino acid compositions for the isolated component is displayed in Table III.

^{3}H-Iodoacetate Labeling

The suggestion that component II is derived from Ic is consistent with labeling studies with iodoacetate. Membranes isolated from

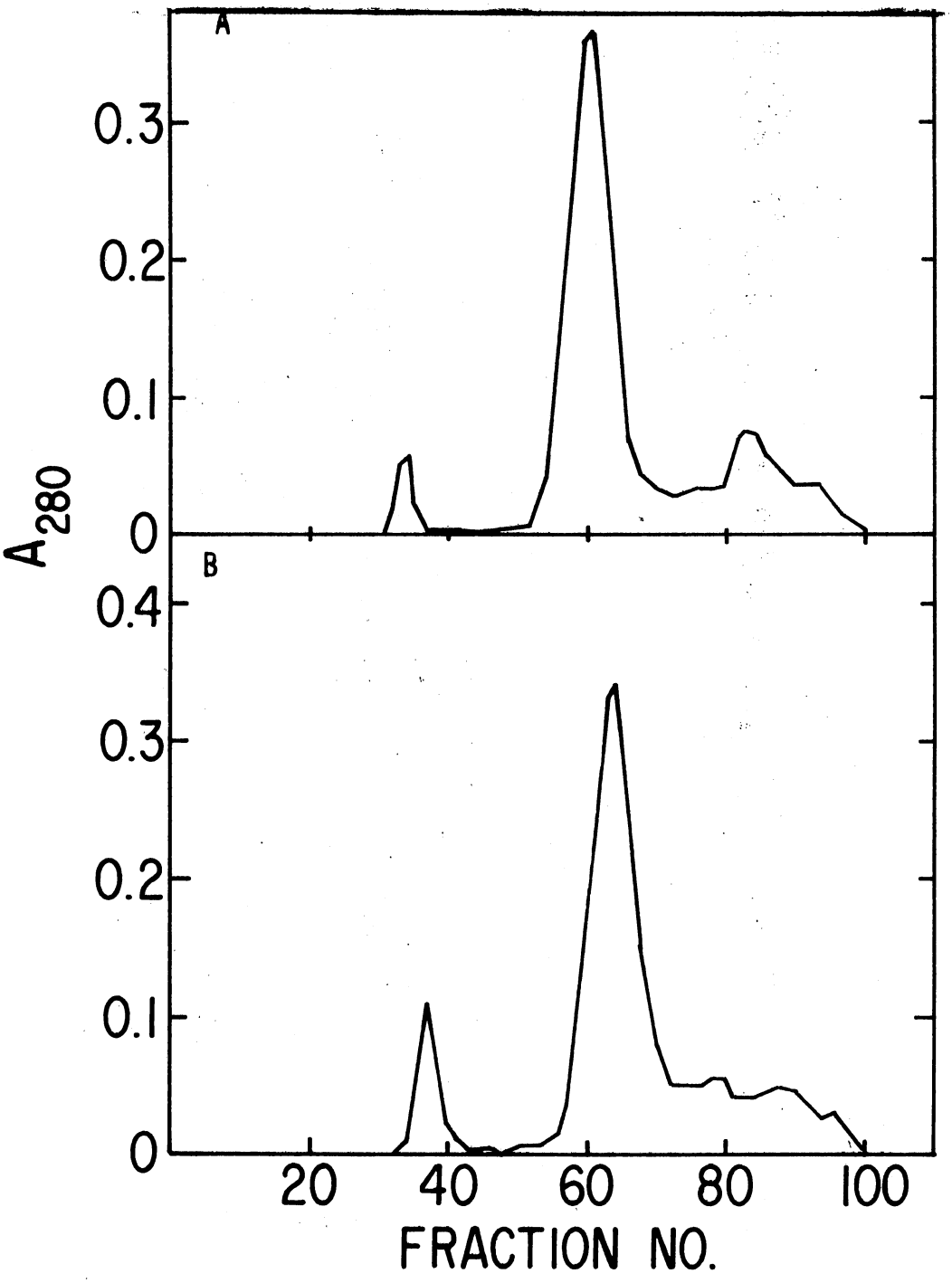


Figure 6. Sepharose 4B Gel Chromatography of EDTA Extracts in Presence and Absence of Sulfhydryl Reducing Reagents

Extracts were eluted in 1% dodecylsulfate in 50 mM Tris (pH 7.4) following dialysis in presence or absence of Mercaptoethanol. (A) Chromatography of extract following reduction with mercaptoethanol. (B) Chromatography of extract in absence of mercaptoethanol.

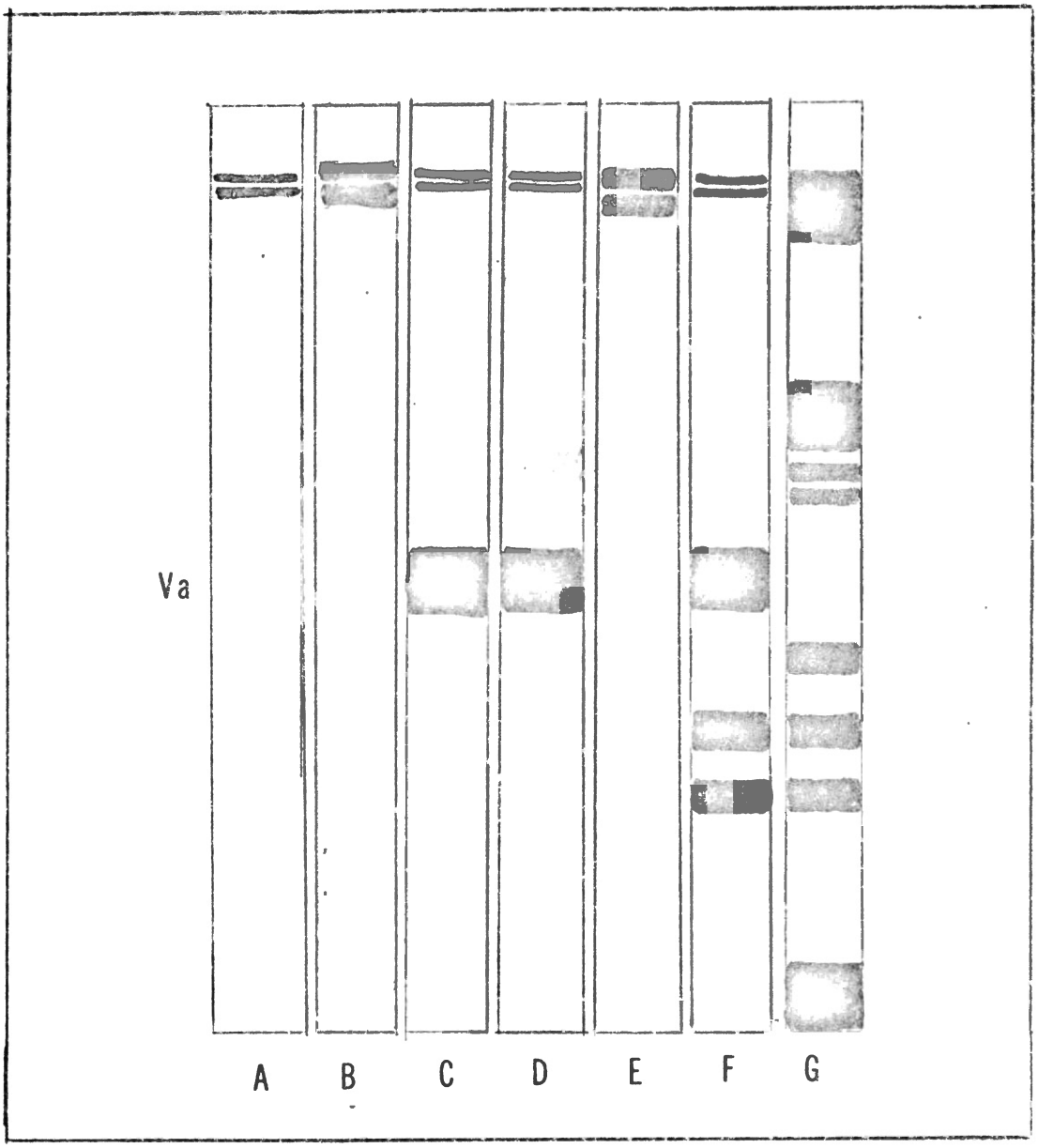


Figure 7. Effect of the Sulfhydryl Reducing Reagent Mercaptoethanol on the Molecular Weight Properties of Component Va

Gels ABC are electrophoresis patterns of sepharose 4B chromatography profiles in presence of mercaptoethanol. Gels DEF are the corresponding patterns from chromatography in absence of mercaptoethanol. Gels A and D represent the void volume peaks of both samples. Gel G is a standard whole membrane untreated control.

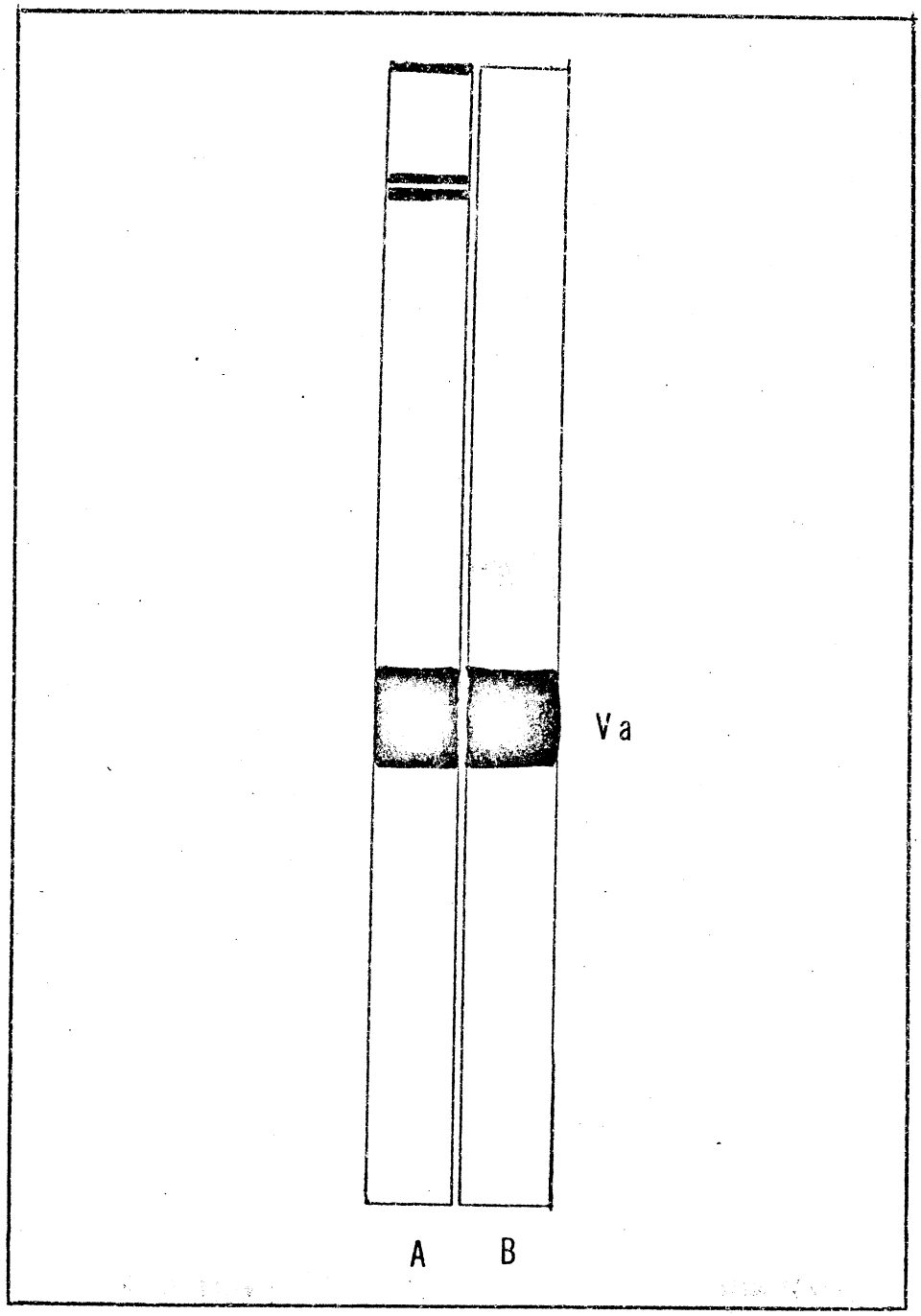


Figure 8. Purification of Component Va by Agarose Gel Chromatography

Gel A, polyacrylamide electrophoresis of Sepharose 4B void volume isolated in absence of mercaptoethanol. Gel B, electrophoresis of peak isolated after separation on a Biogel A 5-m column eluted with 1 mM mercaptoethanol in 50 mM Tris (pH 7.4).

TABLE III

AMINO ACID COMPOSITIONS OF ISOLATED CA^{++} RELATED COMPONENTS II AND Va

Amino Acid	Mole % Comp. II	Mole % Comp. Va
Lysine	5.7	7.2
Histidine	1.5	1.6
Arginine	5.1	4.5
Aspartic acid	9.0	8.8
Threonine	7.4	5.9
Serine	6.2	9.9
Glutamic acid	12.3	12.8
Proline	5.3	5.2
Glycine	5.7	11.4
Alanine	8.1	8.6
Valine	6.0	5.9
Methionine	4.0	0.7
Isoleucine	7.0	5.6
Leucine	11.0	7.9
Tyrosine	2.8	0.7
Phenylalanine	3.0	3.3

Ca^{++}-treated cells are extensively labeled, as shown previously (59) for membranes from untreated cells (Figure 9). Two additional peaks are present in the position of the aggregate and component II, indicating that these can be labeled. Labeling of intact red cells yields a simpler pattern (Figure 9). If the membranes are isolated after hemolysis in the presence of Ca^{++}, three additional peaks are present, corresponding to the aggregate, component II and the 25,000 mol. wt. component. All of these must arise from either proteins in the soluble cell contents or from the labeled membrane components I and VIII. The size of component II indicates that it must arise from one of the bands of I. The iodoacetate reaction has additional interest because at higher concentrations it also prevents aggregate formation. However, there is no effect on the formation of component II. This clearly shows that these processes are not directly related.

Inhibition of Aggregation Process by Modification with Iodoacetate

The iodoacetate labeling reaction has additional interest because at higher concentration of the modifying reagent the aggregate formation is inhibited. Figure 10 shows the effects of iodoacetate at varying concentrations, ranging from 0 to 5 mM. The aggregation is clearly inhibited at concentrations greater than 2 mM. Although aggregation is prevented there is no effect on the formation of component II. This strongly indicates that the two processes are not directly related.

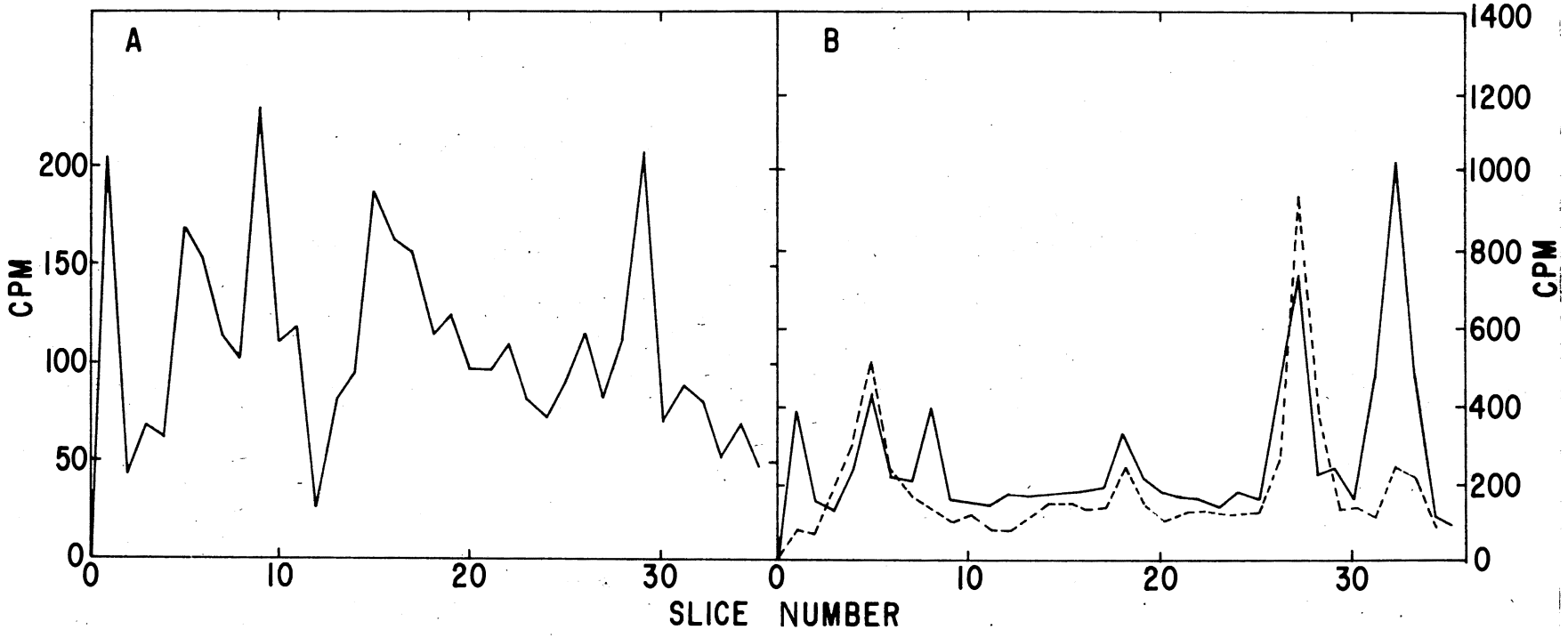


Figure 9. ^{3}H-Labeling of Erythrocyte Membranes with Radioactive Iodoacetic Acid.

Labeling of washed ghost membranes from cells previously hemolyzed in presence of 5 mM $CaCl_2$, 10 mM Tris pH 7.4 (A). Labeling patterns of washed membranes (B) from cells hemolyzed in presence (———) and absence (- - - - -) of 5 mM $CaCl_2$ in pH 7.4, 10 mM Tris. Intact cells were labeled by incubation at 37° for 60 minutes and washed prior to hemolysis step.

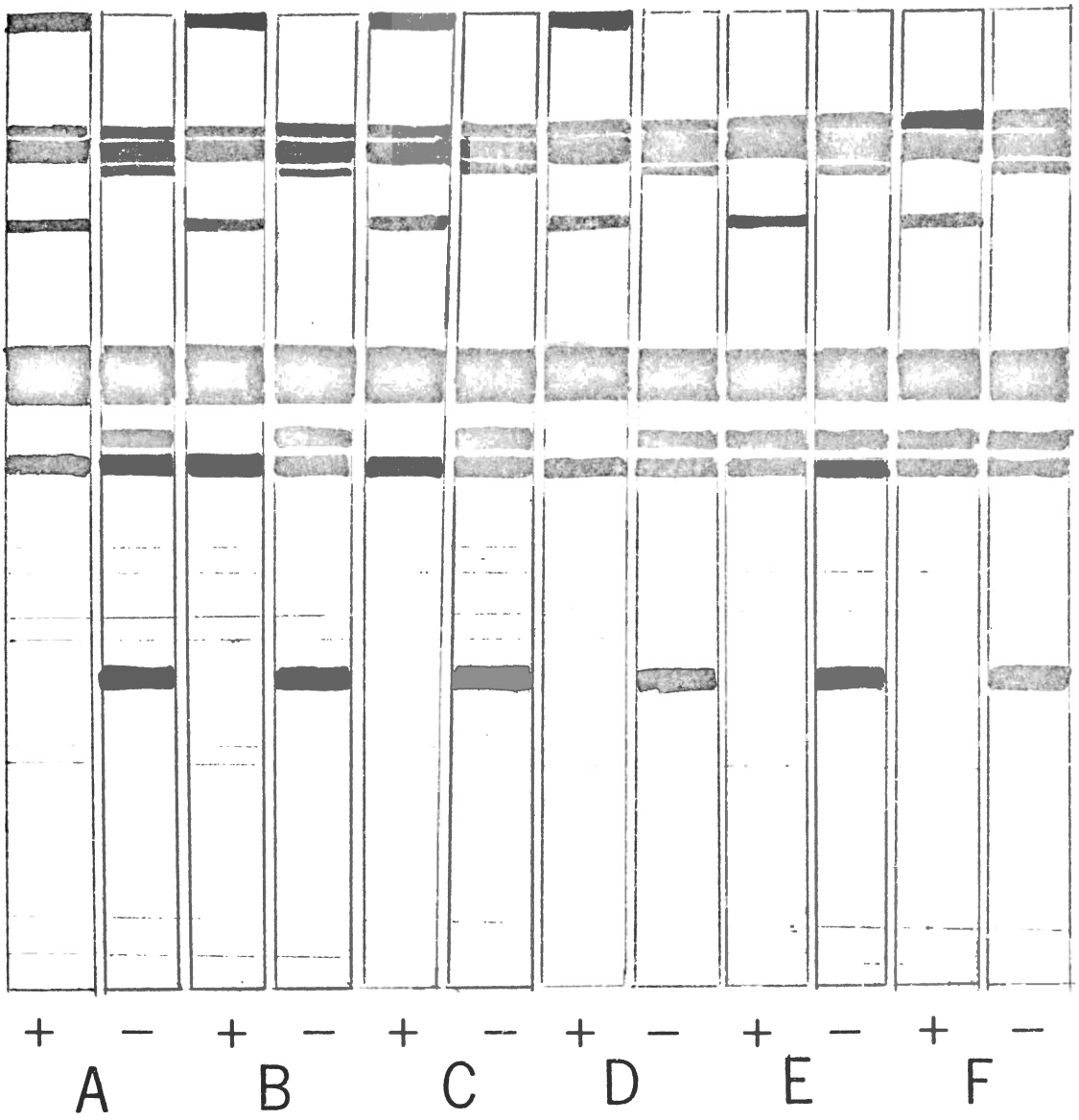


Figure 10. Inhibition of Aggregation by Modification of Protein with Iodoacetic Acid

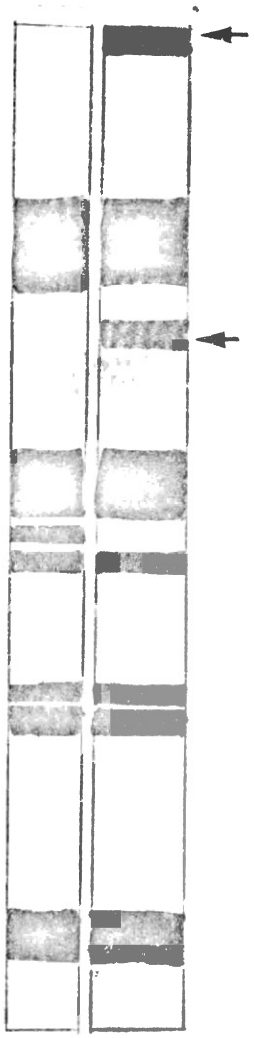
Intact cells were incubated in presence of 0, 0.5, 1.0, 2.0, 3.0 and 5.0 mM iodoacetic acid for 60 minutes at 37° (gels A-F). Electrophoresis patterns from ghosts were obtained following hemolysis in presence (+) and in absence (-) of 5 mM $CaCl_2$.

Localization of an Aggregating Factor from Erythrocyte Hemolysates

It has been previously shown (19) that incubation of washed hemoglobin free membranes with Ca^{++} produced none of the effects seen at the hemolysis step. There are no apparent signs of aggregation or proteolysis when analyzed by SDS acrylamide electrophoresis. This indicates that an aggregating factor and possibly a proteolytic factor exists in the soluble cytoplasmic portion of the intact cell. This portion was isolated from fresh hemolysates of intact cells and separated by centrifugation from the membranes. Addition of this material back to washed hemoglobin free cell membranes can produce identical effects when 5 mM $CaCl_2$ is present and the suspension is incubated for 30 minutes at 37°C. Figure 11 shows the effect where washed membranes were incubated in presence and in absence of 5 mM $CaCl_2$. Both aggregation and proteolyic effects are readily apparent in the mixture incubated with Ca^{++}. This supports the idea that protein factors sensitive to Ca^{++} are present in the soluble portion of the cell and are primarily responsible for the alterations observed in the membrane proteins.

Ca^{++} Activation of Erythrocyte Endogenous Transglutaminase

Utilizing the assumption that the aggregating factor is a soluble enzyme, an assay was developed for determining the activity of a Ca^{++} stimulated transglutaminase. If a endogenous transglutaminase is responsible for the aggregation it should be possible to



A B

Figure 11. Localization of an Aggregating Factor in Erythrocyte Hemolysates

Washed hemoglobin free erythrocyte membranes were incubated with cell free hemolysates in Absence (A) and in presence (B) of 5 mM $CaCl_2$.

incorporate a radioactively labeled amine substrate into the endogenous protein substrates found within the cell. The amounts of label incorporated should be proportional to the amount of Ca^{++} present. The quantities of radioactivity covalently incorporated into endogenous red cell protein in presence of varied amounts of Ca^{++} are seen in Figure 12. The results suggest that the minimum activation level is on the order of 0.2 mM Ca^{++}. A significant increase in activity is observed from this point forward and approaches maximum incorporation at approximately 5 mM $CaCl_2$. This also is the concentration at which maximum aggregation in calcium incubated cell suspensions occurs (19). The data are consistent with the idea that Ca^{++} brings about irreversible changes in the membrane due to crosslinking of membrane proteins by transglutaminase.

Analysis of membranes by SDS polyacrylamide electrophoresis indicates that the first traces of aggregation are seen at approximately 0.5 mM Ca^{++} (Figure 13). Aggregation is strongly visible at 1.0 mM Ca^{++}. The aggregation observed on these gels correlate well with the radioactivity incorporation assay. Although significant activity is detectable at Ca^{++} concentrations as low as 0.3 mM this probably represents incorporation into other proteins not associated with the aggregate.

Inactivation of Erythrocyte Transglutaminase by Iodoacetamide

The effects of the chemical modifying reagent iodoacetic acid were previously shown. They indicate that aggregation can be

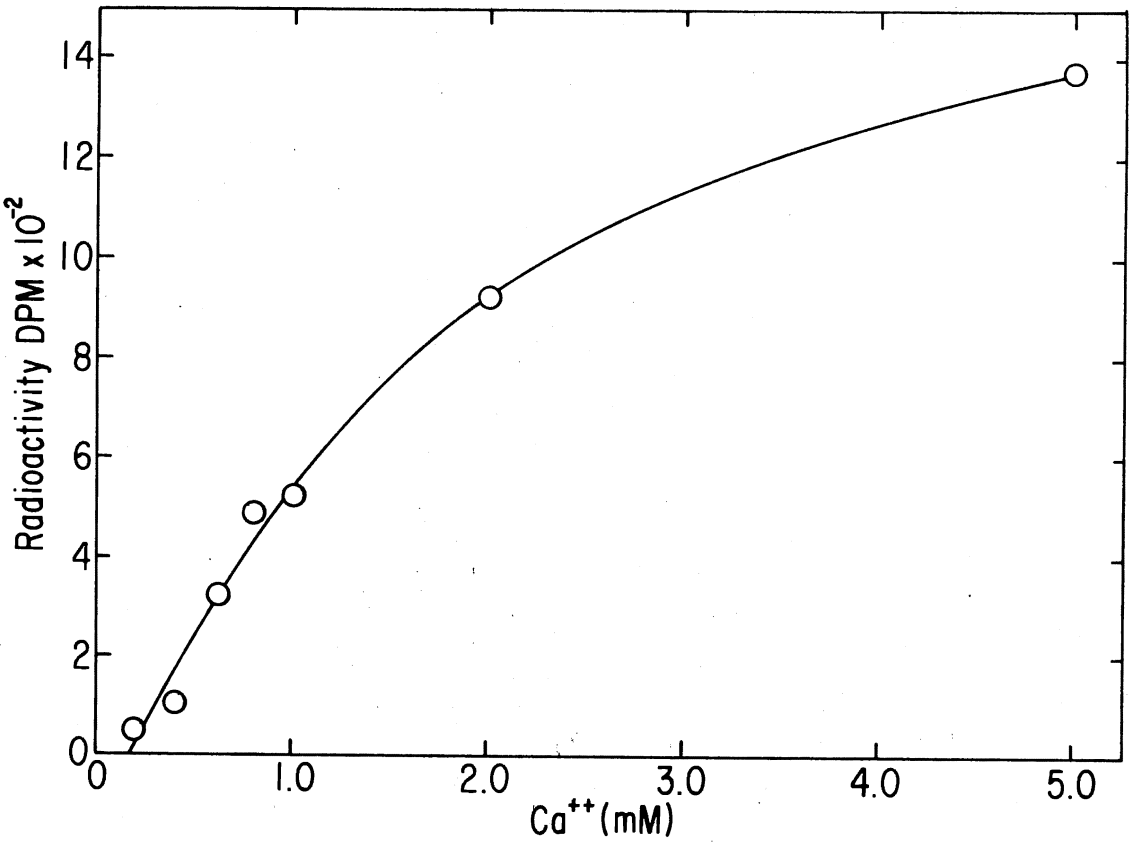


Figure 12. Incorporation of ^{14}C-Putrescine Into Endogenous Red Cell Protein as a Function of Ca^{++}

Red Cell hemolysates were incubated for 2 hours with 0.2 μCi of ^{14}C-putrescine. Radioactivity was covalently bound to endogenous protein by the action of Ca^{++}-stimulated Transglutaminase.

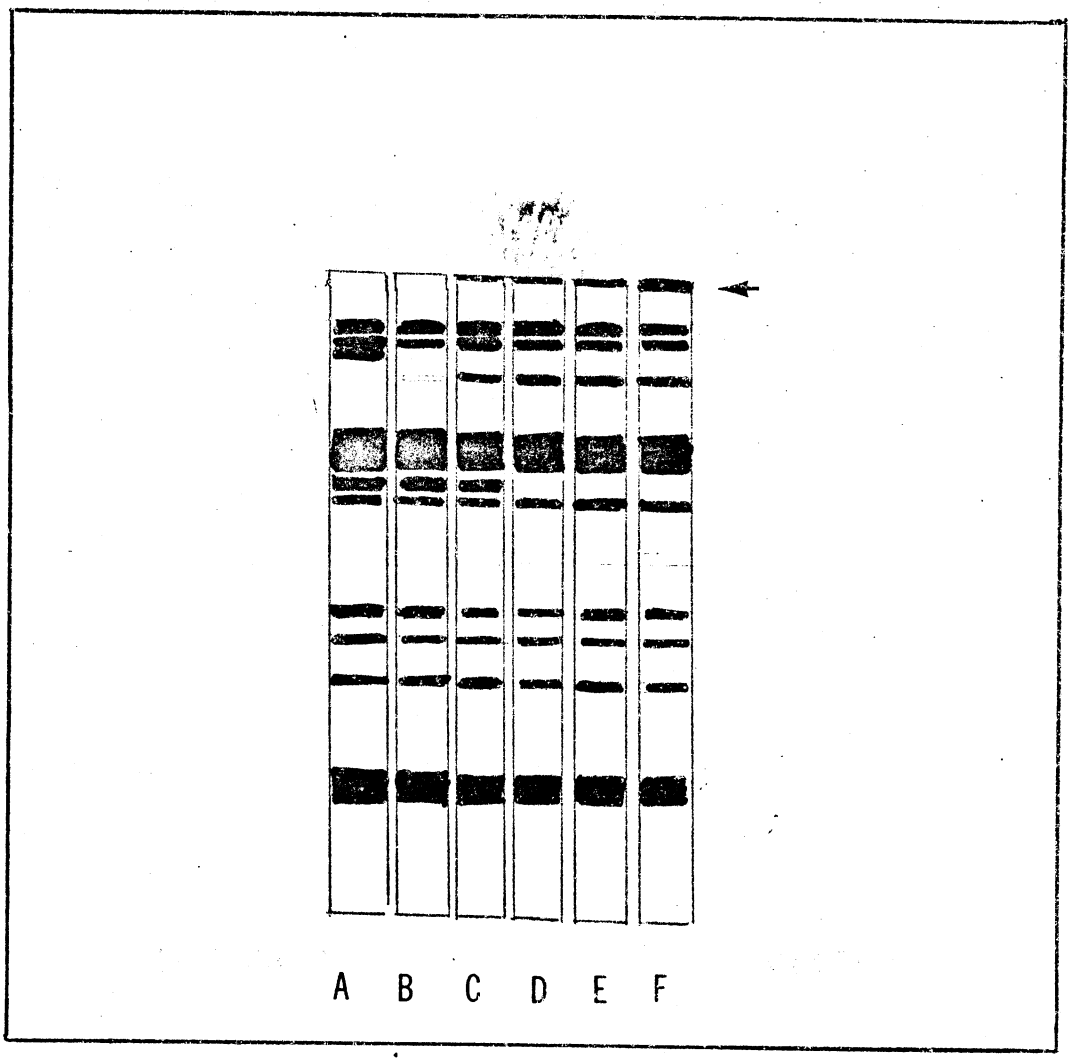


Figure 13. Correlation of Aggregation Process with Activation of Transglutaminase by Ca^{++}

Activation of transglutaminase by Ca^{++} (fig. 12) corresponds to the formation of aggregate as detected by polyacrylamide electrophoresis in dodecylsulfate. Ca^{++} concentrations for gels A-F are 0.0, 0.1, 0.5, 1.0, 2.0, 5.0 mM.

inhibited with sufficient quantities of the reagent (~2-3 mM). Another very similiar compound with apparently a much higher specificity and affinity for Transglutaminases is the acid amide (iodoacetamide). This compound has been previously shown to be a potent inhibitor of the transglutaminase from guinea pigs (62). When hemolysates of red cells are incubated with Ca^{++} (5 mM) in presence of varied amounts of iodoacetamide using the ^{14}C-putrescine incorporation assay, the inhibition pattern is much more pronounced than with iodoacetic acid (Figure 14). The amounts of radioactivity incorporated into endogenous protein are insignificant with inhibitor concentrations of 0.1 mM or greater. Inhibition effects are seen as low as 10μM.

Inhibition of Protein Aggregation by Inactivation with Iodoacetamide

Analysis of membrane proteins by SDS polyacrylamide electrophoresis over the same inhibitor range indicates that aggregation is markedly inhibited at concentrations that correlate well with the loss of radioactivity incorporation (Figure 15). Indeed, little or no aggregation is seen even at concentrations as low as 1μM iodoacetamide. Although substantial activity is seen at a tenfold greater inhibitor concentration by radioactive assay, this could be activity bound to other soluble substrates and not to aggregated material. It is difficult to quantitate aggregation based on visual inspection for comparison with radioactive data since the aggregate represents only a specific limited number of substrates as compared to the nonspecific and numerous substrates labeled with radioactivity.

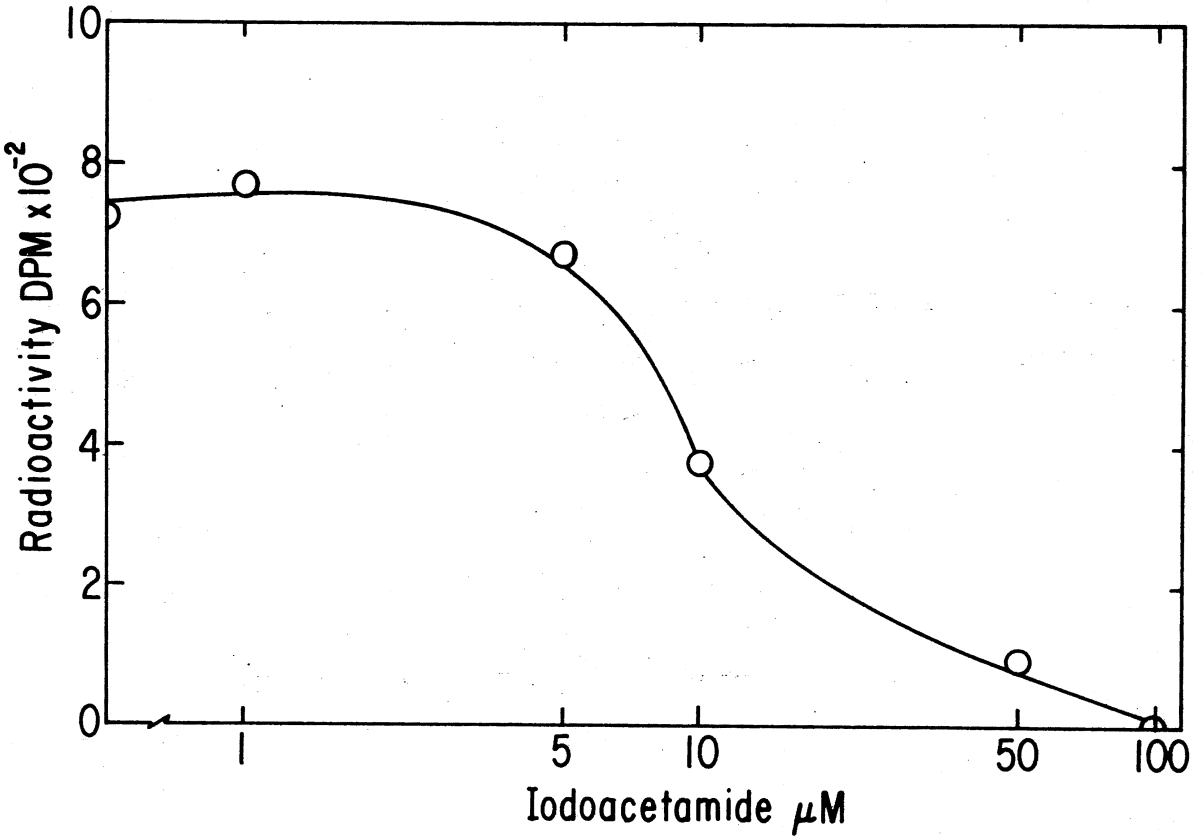


Figure 14. Inhibition of ^{14}C-Putrescine Incorporation with Iodoacetamide

Red cell hemolysates were incubated for 2 hours with 0.2 μCi of ^{14}C-putrescine. Each assay contained 5.0 mM $CaCl_2$ with increasing concentrations of the inhibitor iodoacetamide ranging from 1 to 100 μM. No activity was detectable above 100 μM.

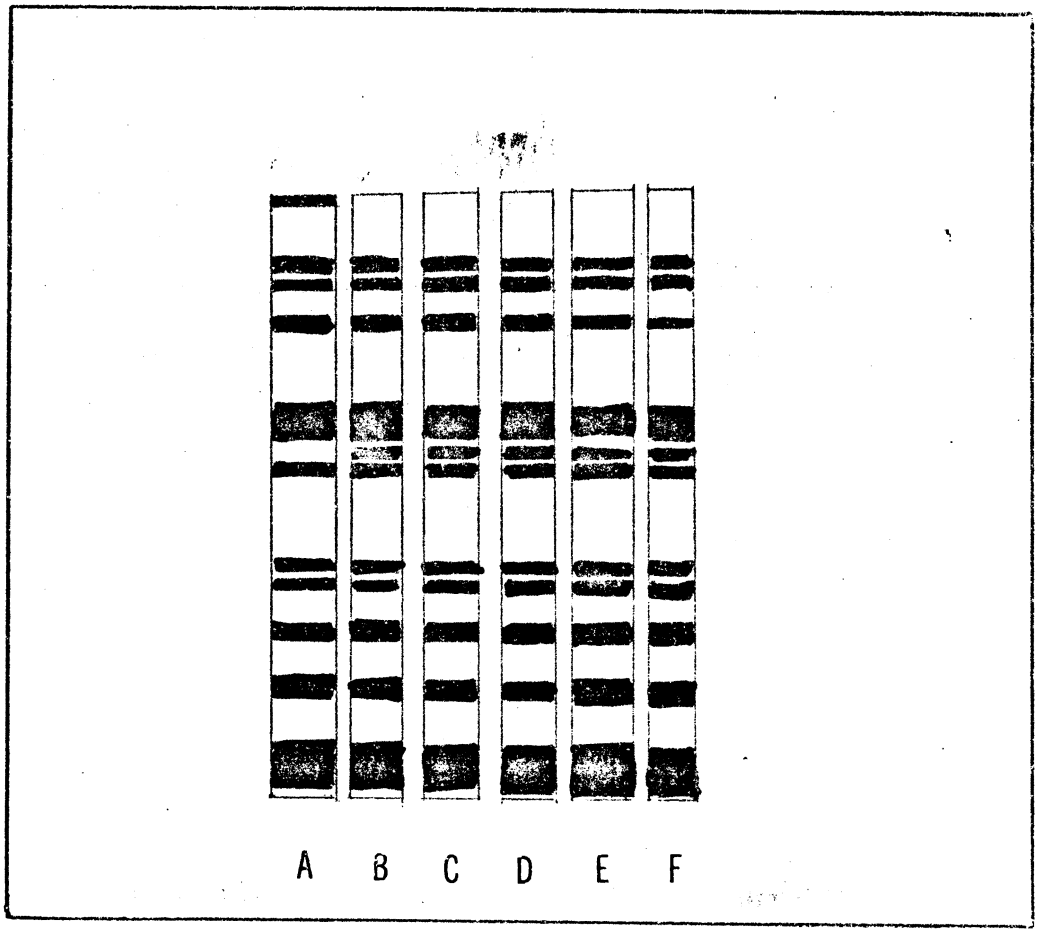


Figure 15. Correlation of Aggregation Loss with the Inhibition of Transglutaminase by Iodoacetamide

Hemolysates were incubated in various concentrations of iodoacetamide, washed and solubilized for polyacrylamide electrophoresis in dodecylsulfate. Concentrations for inhibitor for Gels A-F are 0, 1, 5, 10, 50, 100 μM.

In addition one cannot discount the possibility that the inhibitor has modified the substrates rendering them less susceptable to crosslinking.

The data also indicate again that no prevention of proteolysis occurs in the presence of the inhibitor. This refers only to the formation of component II from the higher molecular weight component Ic. The other event described earlier as a possible proteolytic effect was the dissappearence of band IVa. This band still appears to be present under the effect of the inhibitor. This suggests that the loss of IVa is linked with the transglutaminase activity while the formation of component II is not. The formation of component II appears to be initiated at a much lower Ca^{++} level than aggregation, possibly within the physiological Ca^{++} range under certain conditions. Loss is IVa occurs only when aggregation occurs, suggesting that it may be crosslinked into the aggregated material. If this is the case, then proteolysis would be responsible for only one change in the membrane and at a much lower Ca^{++} level.

CHAPTER IV

BIOPHYSICAL PROPERTIES OF HUMAN ERYTHROCYTE SPECTRIN

Isolation and Purification of Spectrin by Column Chromatography

To evaluate chemical and physical properties of a macromolecule, it must first be purified to homogeneity. Gel column chromatography provides a means by which a large quanitity of material may be easily separated into components. In the case of spectrin, however, some problems are inherent. This protein behaves differently under various conditions and thus causes difficulty in its isolation. In low ionic strength, high pH buffers, the protein is freely soluble and extracts easily from the membrane. Extracts eluted from a sepharose 4B gel column using a low ionic strength buffer, gives two peaks if the extracts come from ghosts made from outdated blood. This blood is depleted of ATP and the spectrin extracts contain large quantities of aggregated spectrin. Under low ionic strength this aggregated material elutes as a peak in the void volume (Figure 16a) with a second peak eluting in a "monomer" state. The monomer peak usually contains a shoulder which is composed of the component VII "actin" molecules. Extracts from membranes obtained from fresh blood contain only one peak, or diminished amounts of the aggregated material possibly

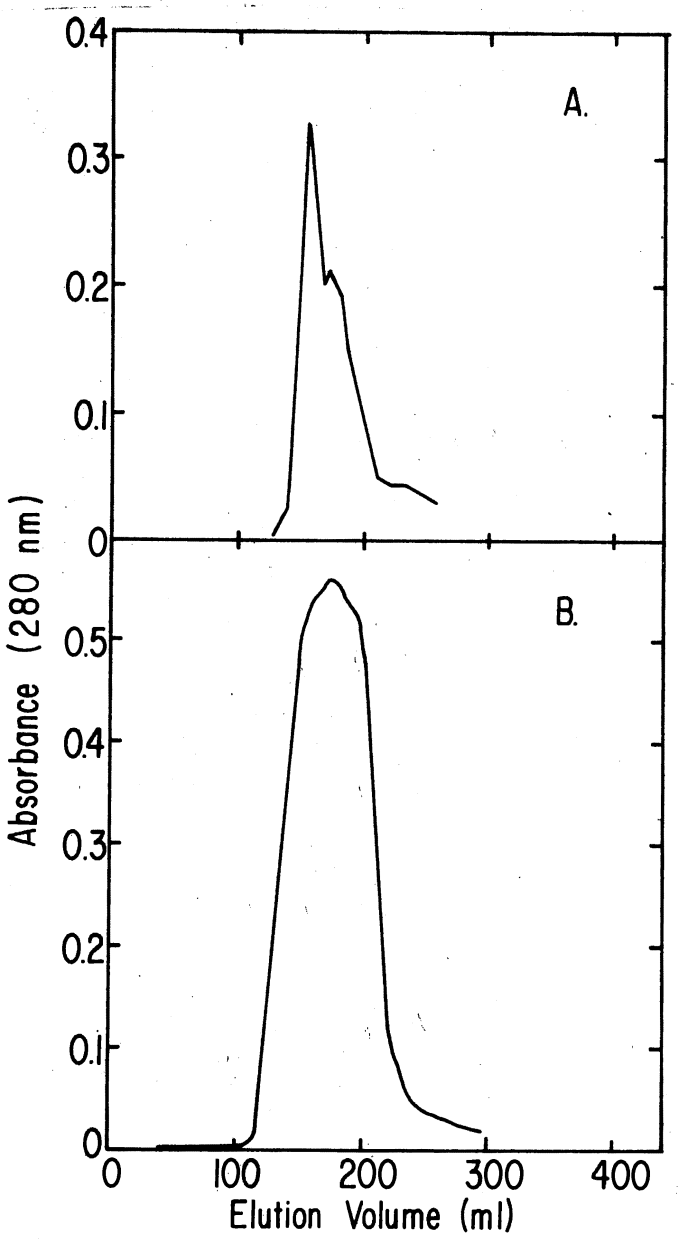


Figure 16. Sepharose 4B Column Chromatography of Extracts from Membranes Isolated from Fresh and Outdated Blood

(A) Chromatography of extracts obtained from membranes of aged blood. Elution under low ionicsstrength 5 mM Glycine, 5 mM Mercaptoethanol, 1 mM EDTA buffer pH 8.6. (B) Chromatography of extracts obtained from membranes of fresh blood. Elution in 5 mM Glycine, 5 mM Mercaptoethanol, 1 mM EDTA, pH 8.6.

dependent upon the state of ATP depletion (Figure 16b). The ratio of the two peaks seems to vary with the age of the blood from which the extracts are obtained. Extracts of fresh blood show the composition of the monomeric peak (Figure 17) on polyacrylamide gels in dodecylsulfate. The shoulder of the peak contains a large amount of the component VII (actin) which is partially resolved from the monomer of spectrin. This indicates that the two components are similiar in molecular weight but not identical under the conditions of low ionic strength. If extracts are chromatographed under isotonic conditions (140 mM KCl, pH 7.4, 10 mM Tris) the spectrin peaks are shifted. The monomer peak from fresh blood extracts shifts to a lower apparent molecular weight while the dimer (when present) shifts out of the void volume (Figure 18A). The actin-like component appears to separate more cleanly under these conditions (Figure 19). Extracts made from aged cells depleted of ATP show larger amounts of dimeric material (Figure 18B). Various fractions of spectrin were isolated from isotonic and hypotonic column chromatography. The fractions used for study of various biophysical properties contained no actin as judged by analysis of the isolates by polyacrylamide electrophoresis in dodecylsulfate.

Binding of 1-Anilinoaphthalene-8-Sulfonic Acid to Spectrin

Protein fractions containing both the 43,000 m.w. component and the spectrin dimer bind considerably more of the fluorescent probe (ANS) than do equivalent amounts of pure spectrin. Pure fractions of spectrin, however, do possess sites of hydrophobic nature and are

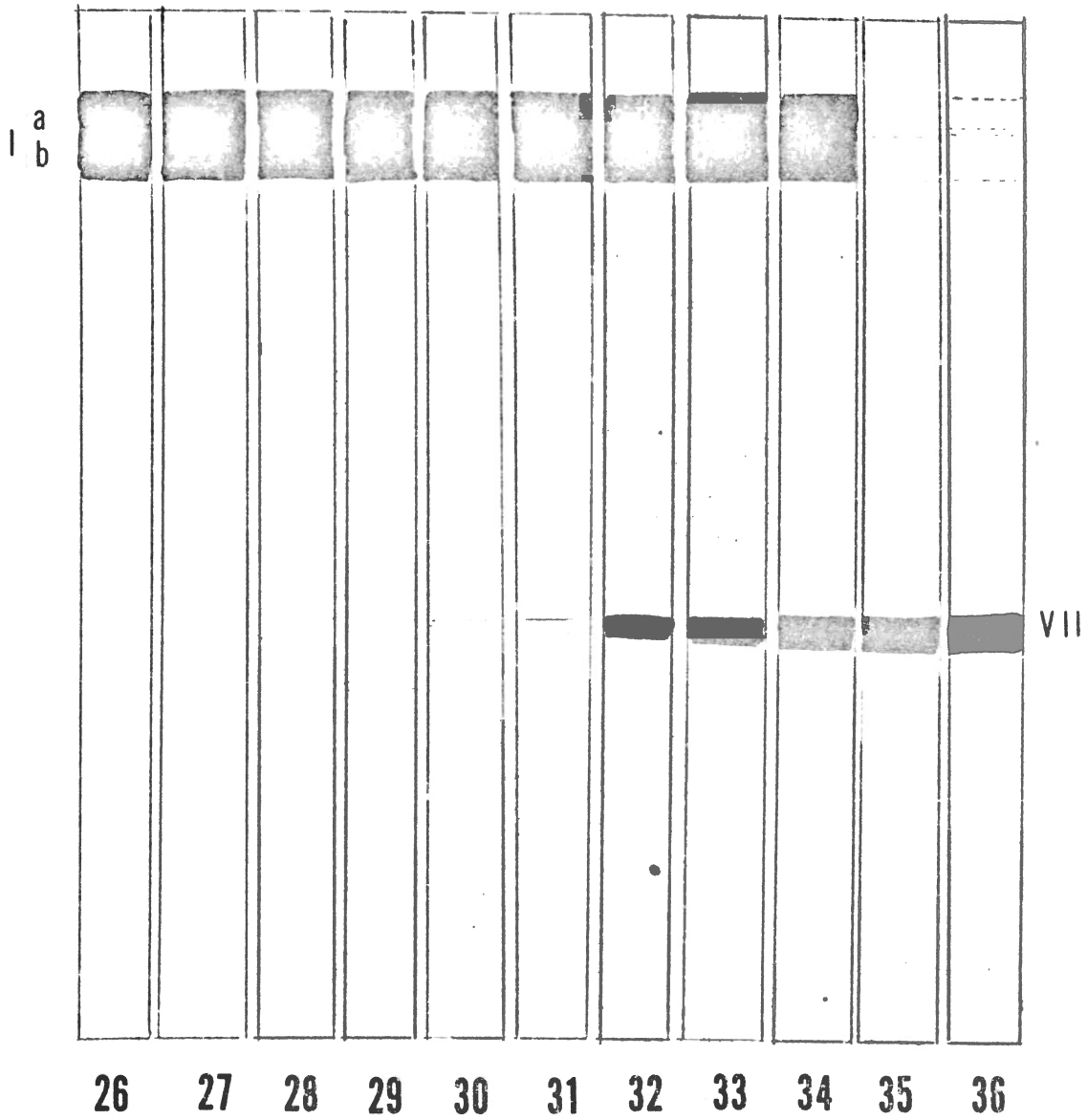


Figure 17. Sepharose 4B Column Chromatography Fractions of Extracts from Fresh Blood Analyzed by Dodecylsulfate Acrylamide Electrophoresis

EDTA extracts of erythrocyte membranes were chromatographed in 5 mM glycine, 1 mM EDTA, 5 mM Mercaptoethanol (pH 8.6). Two major components are present, component Ia,b (Spectrin) and component VII (43,000 mw). Numbers indicate the column fractions from which aliquots were analyzed.

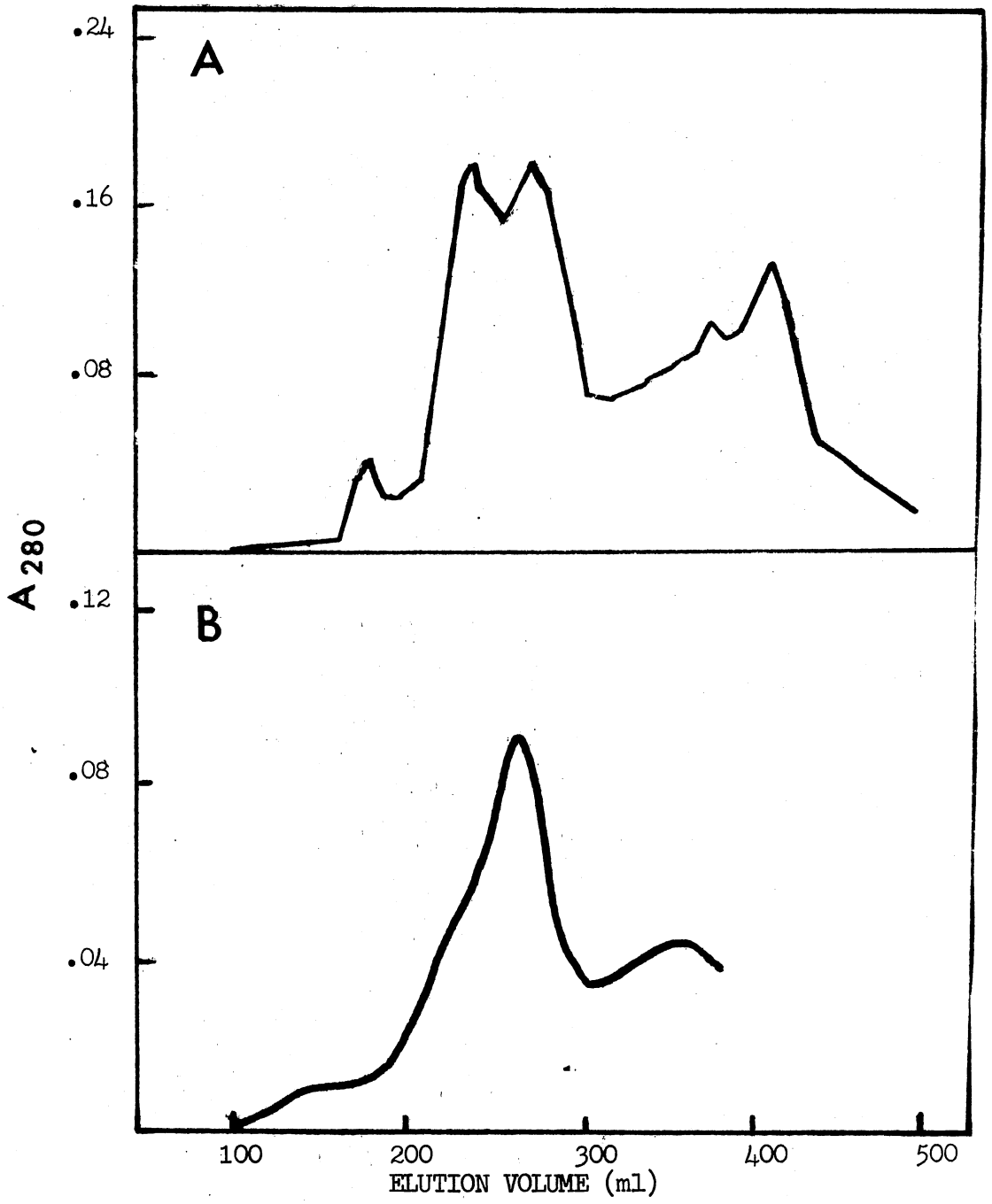


Figure 18. Sepharose 4B Column Chromatography of Extracts from Membranes Isolated from Fresh and Outdated Blood Eluted Under Isotonic Conditions

(A) Chromatography of extracts obtained from membranes of aged blood. Elution buffer is 140 mM KCl, 10 mM Tris, .1 mM EDTA (pH 7.4). (B) Chromatography of extracts obtained from membranes of fresh blood. Elution buffer is 140 mM KCl, 10 mM Tris, .1 mM EDTA (pH 7.4).

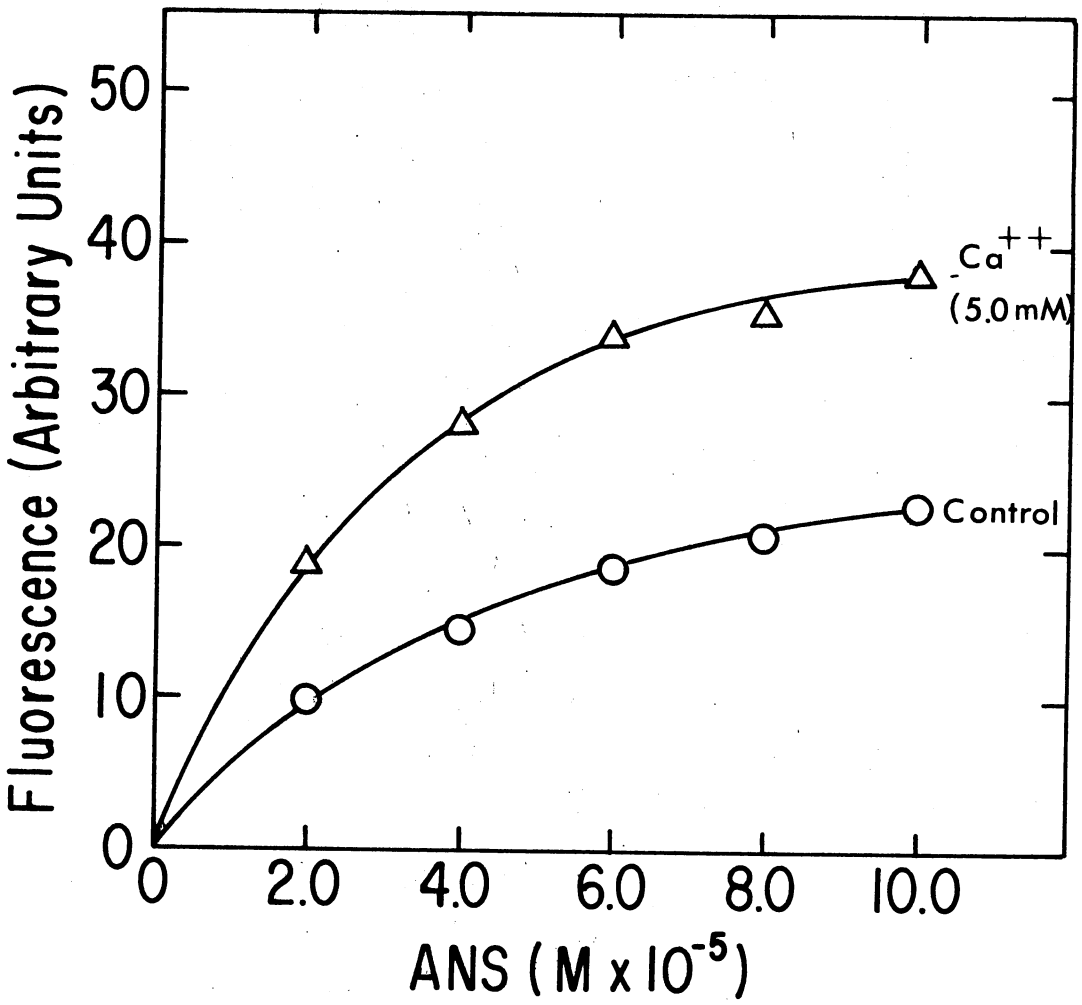


Figure 19. Binding of the Fluorescent Probe 1-anilinonaphtalene-8-sulfonic Acid (ANS) to Purified Spectrin Preparation

Aliquots of 2 x 10^{-5} M ANS were added to a protein concentration of 0.33 mg/ml. Relative intensity was measured as arbitrary units in presence -Δ-Δ- and absence -O-O- of 5 mM $CaCl_2$.

capable of binding the probe. Binding is enhanced in solutions where 5 mM $CaCl_2$ is present (Figure 19). The expression

$$P_o/xD_o = \frac{1}{n} + \frac{Kd}{n} \frac{1}{(1-x)D_o} \qquad (1)$$

was used to determine the apparent average dissociation constant (Kd) and number of binding sites n (64). In the above expression P_o is the concentration, D_o is the concentration of ANS and x is the mole fraction of the bound dye. The mole fraction is determined by:

$$x = \frac{F}{F_o} \qquad (2)$$

where F is the fluorescent intensity observed when a given amount of protein is present with a fixed concentration of dye. The term F_o is obtained from the intercept $(1/F_o)$ of a double reciprocal plot of fluorescent intensities observed when a fixed dye concentration is titrated with a range of protein concentrations (Figure 20). Extrapolation to zero gives $1/F_o$ which is the reciprocal of the intensity that would be expected if all the dye in the solution were bound to protein.

The dissociation constants (Kd) and number of binding sites (n) were obtained from the slopes and intercepts respectively of a graphical representation of the binding expression (equation 1) (Figure 21). The computed values are listed in Table IV.

Effects of pH on Binding of ANS

Previous studies have shown that ANS fluorescence in erythrocyte membranes is increased at lower pH values (74). Large increases in intensity are generally seen below (pH 3.5). Similiar effects with solutions of spectrin are apparent. A several fold increase in intensity is found even at pH values of 4.5 which decreased rapidly

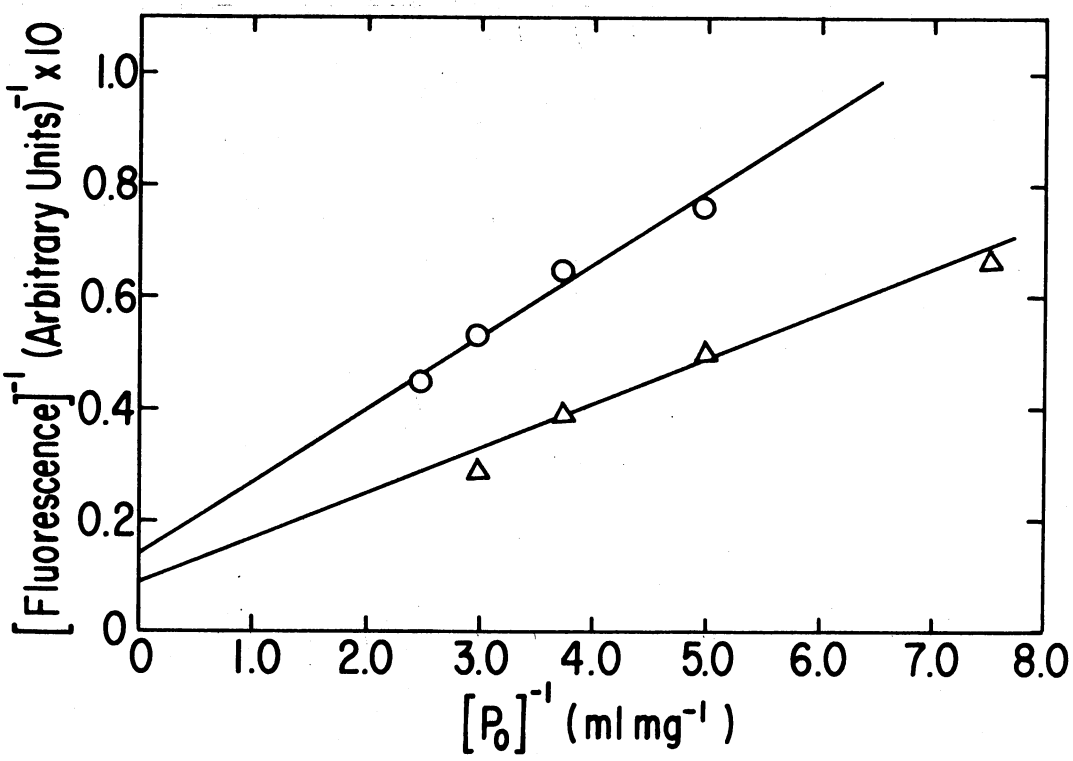


Figure 20. The Binding of 1-anilinonaphthalene-8-sulfonate (ANS) to Purified Spectrin as a Function of Protein Concentration

Protein in 25 mM glycine buffer (pH 8.6) (O); with added 5.0 mM $CaCl_2$ (Δ). Protein concentration varied from 0.13 mg/ml to 0.40 mg/ml. Concentration of ANS was 2 x 10^{-5} M.

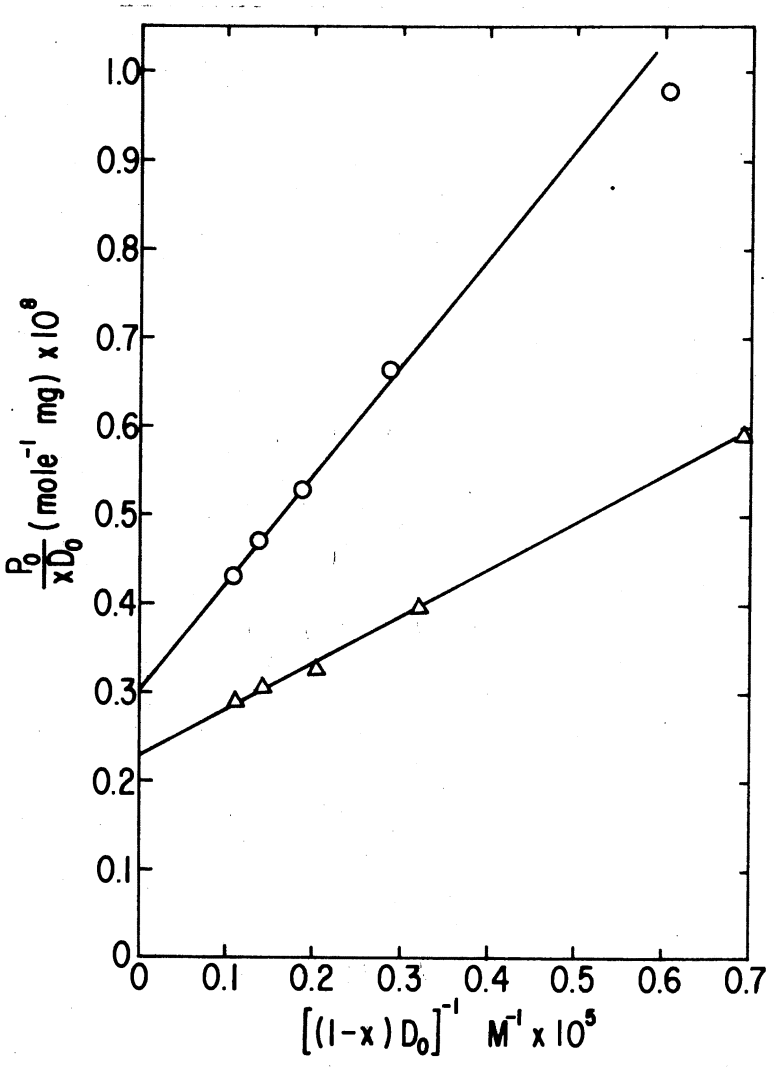


Figure 21. The Binding of 1-anilinonaphthalene-8-sulfonate to Purified Spectrin as a Function as ANS Concentration

Data of figure 19 plotted according to equation 1. Spectrin in 25 mM Glycine buffer (pH 8.6) (O); and in presence of 5.0 mM $CaCl_2$ (△).

TABLE IV

EFFECT OF ADDED CALCIUM ON THE BINDING OF 1-ANILINONAPHTHALENE-8-SULFONATE TO PURIFIED HUMAN ERYTHROCYTE SPECTRIN

	kd($M \times 10^{-5}$)	n(10^{-8} mole mg^{-1})	moles ANS per mole 220,000 daltons
Spectrin + Ca^{++} (5 mM)	2.2	4.3	9
Spectrin - Ca^{++}	6.6	3.3	7

Conditions as in Figure 21. Derived parameters are from Figure 21 and equation 1.

above neutral pH (Figure 22). Although there is increased intensity, Ca^{++} effects are reduced at both ends of the pH range. Calcium has significant effects at neutral values and below except at pH 4.5 where no effects are observed (Figure 23). The Ca^{++} effects seem to vary with another parameter as well. Table V displays the values for intensities measured at two different protein concentrations. No effects by Ca^{++} are seen at either 1 or 3 mg/ml protein concentrations for pH 4.6. Higher protein concentrations are more greatly affected by Ca^{++} at lower pH ranges in the area of pH 5.5 while the neutral values are more greatly affected at lower protein concentrations. It should be noted that turbidity at pH 4.6 was extremely high due to the insolubility of the protein and quenching may be masking any Ca^{++} effects which might be apparent. The data suggests a possible interaction of hydrophobic sites among individual polypeptide chains with the possible creation of an even larger number of sites due to protein-protein interactions.

Intrinsic Fluorescence and Energy Transfer

When spectrin is excited at 280 nm a strong emission band is seen to peak at approximately 357 nm. This is predominately due to tryptophan residues and indicates that they are in a highly polar environment. The emission maximum was identical to that of a calibration standard in an identical aqueous buffer solution. The residues exhibited absolutely no energy transfer when 10^{-5} M ANS was present in the solution. This implies that the fluorophores are situated in an area away from the proximity of the ANS binding sites or are restricted in the orientation of their transition moments.

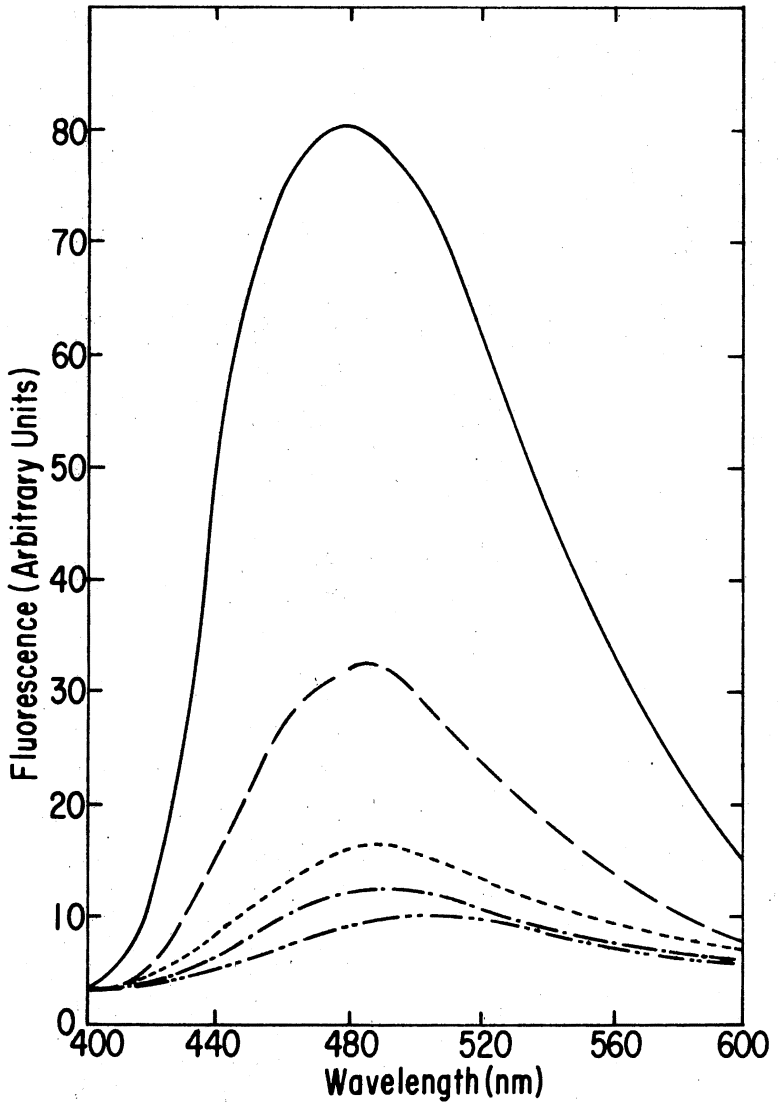


Figure 22. Intensity of 1-anilinonaphthalene-8-sulfonic Acid Binding to Spectrin at Various pH Values

Binding and fluorescence are enhanced as pH decreases. Intensities shown are at pH 8.2 (— - - —), pH 7.2 (— • —), pH 6.5 (- - - -), pH 5.5 (— —) and pH 4.6 (——).

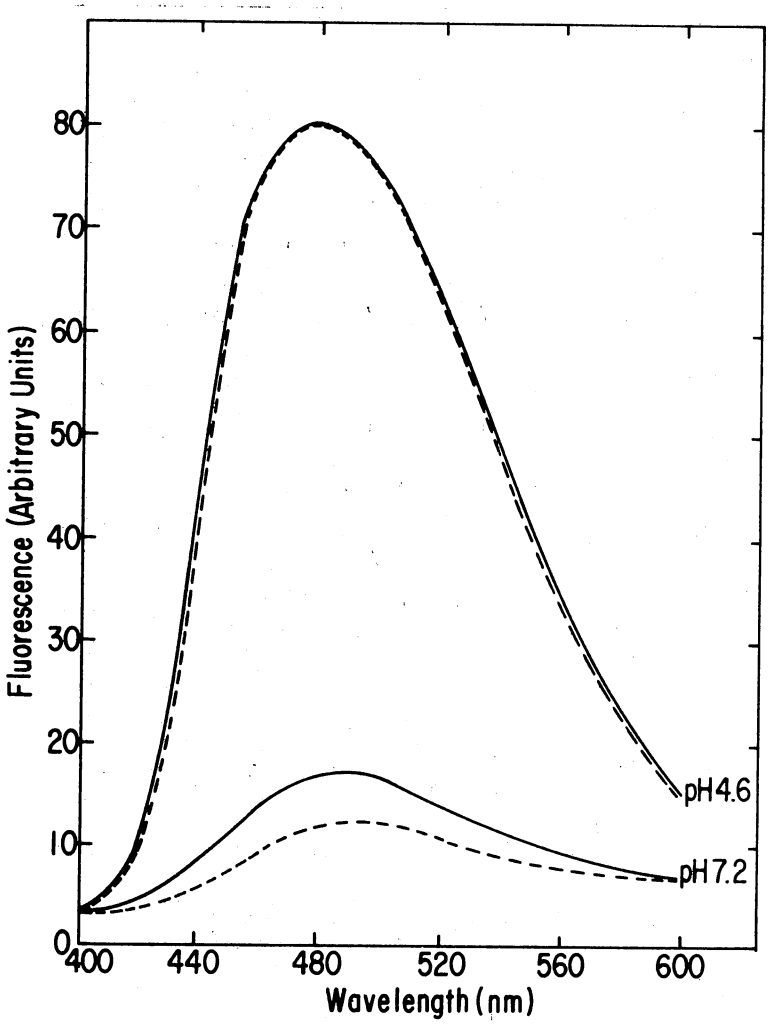


Figure 23. Intensity of 1-anilinonaphthalene-8-sulfonic Acid at Neutral pH and at Acidic pH Bound to Spectrin

Protein solutions were measured in absence (- - - -) and presence (——) of 5 mM $CaCl_2$.

TABLE V

EFFECT OF ADDED CALCIUM ON THE OBSERVED FLUORESCENT INTENSITY OF 1-ANILINONAPHTHALENE-8-SULFONATE BOUND TO HUMAN SPECTRIN

	+5.0 mM Ca^{++}		No Ca^{++}	
pH	3 mg/ml	1 mg/ml	3 mg/ml	1 mg/ml
4.6	87	80	87	80
5.5	50	36	28	33
6.5	-	21	-	16
7.2	19	17	14	12
8.2	17	17	12	10

Circular Dicroism of Spectrin

The effect of Ca^{++} on the secondary and tertiary structure of the macromolecule was studied using circular dicroism. Results indicate that Ca^{++} has little or no effect on secondary structure alone at neutral pH or above. The effects below neutral pH to the point of insolubility are readily apparent in Table V and very obvious at pH values of 5.8 and 6.0 (Figure 24). These measurements are above the point at which the protein completely precipitates from solution. The values were corrected to mean residue ellipticity based on the amount of protein measured in the supernatant after centrifugation at 27,000 x g for 30 minutes. The spectra are definitely not free of optical artifacts (75) since a strong red shift of ellipticity is seen due to increased light scattering. For this reason one may not accurately interpret what effects are occuring to the secondary structure. Perhaps the most obvious and significant observations are that the molecular weight is in fact rapidly changing over a very narrow pH range. Over this range Ca^{++} seems to have a pronounced effect on the spectra as a red shift of several n.m. is seen. The protein is still small enough to remain in solution but large enough to produce light scattering. Comparison of Ca^{++} effects in the near UV range (310-250 nm) in Figure 25, indicates that some subtle changes in tertiary structure at neutral and higher pH values are occuring but the changes involved are difficult to interpret.

pH Solubility Studies

The effects of calcium on circular dicroism spectra correlate

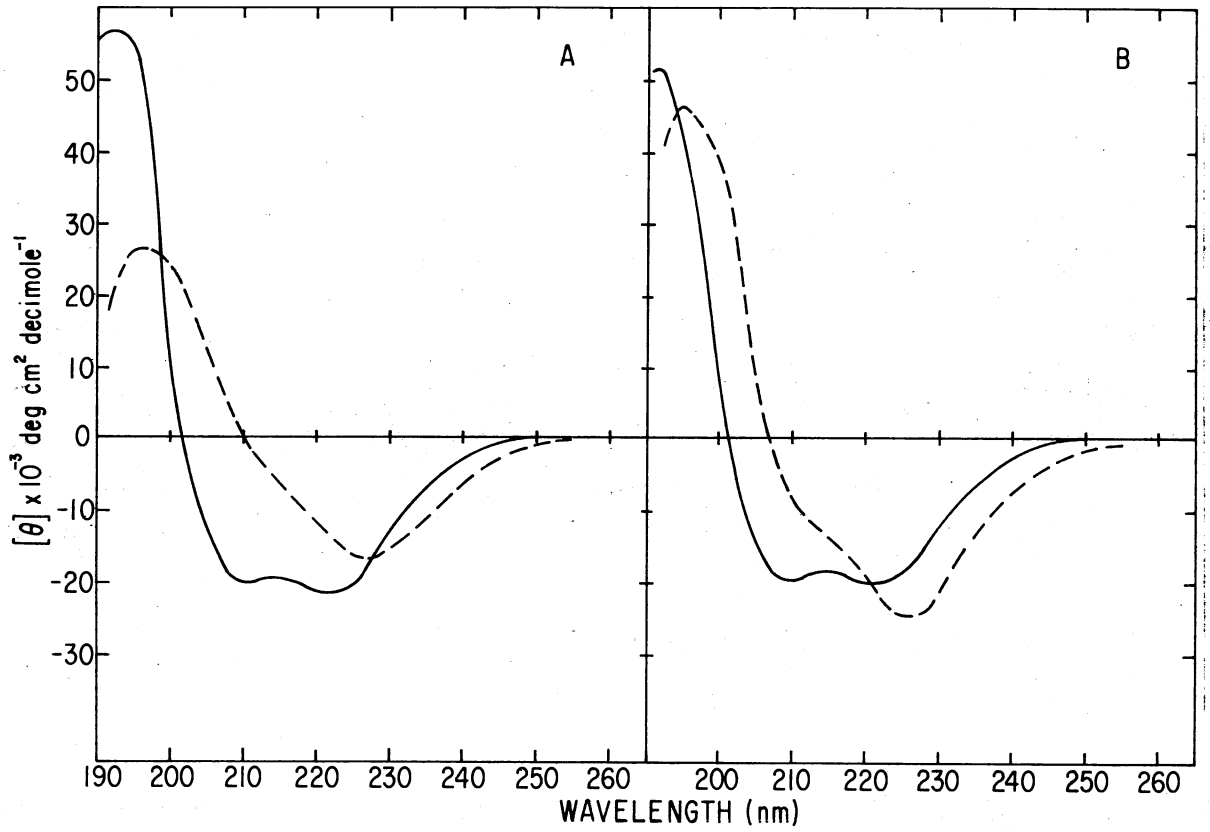


Figure 24. Circular Dicroism Spectra of Purified Spectrin as a Function of (H^+)

Comparison of soluble protein as it approaches a point of insolubility. Lowering of pH causes an observed red shift over a very narrow range of 0.2 pH units. Comparison of Ca^{++} effects at pH 6.0 (B). Protein concentration is 53 g/ml in presence (- - - -) and absence (———) of 3.5 mM $CaCl_2$. The observed effects indicate rapid changes in molecular weight to very large particle sizes when the divalent cation is present.

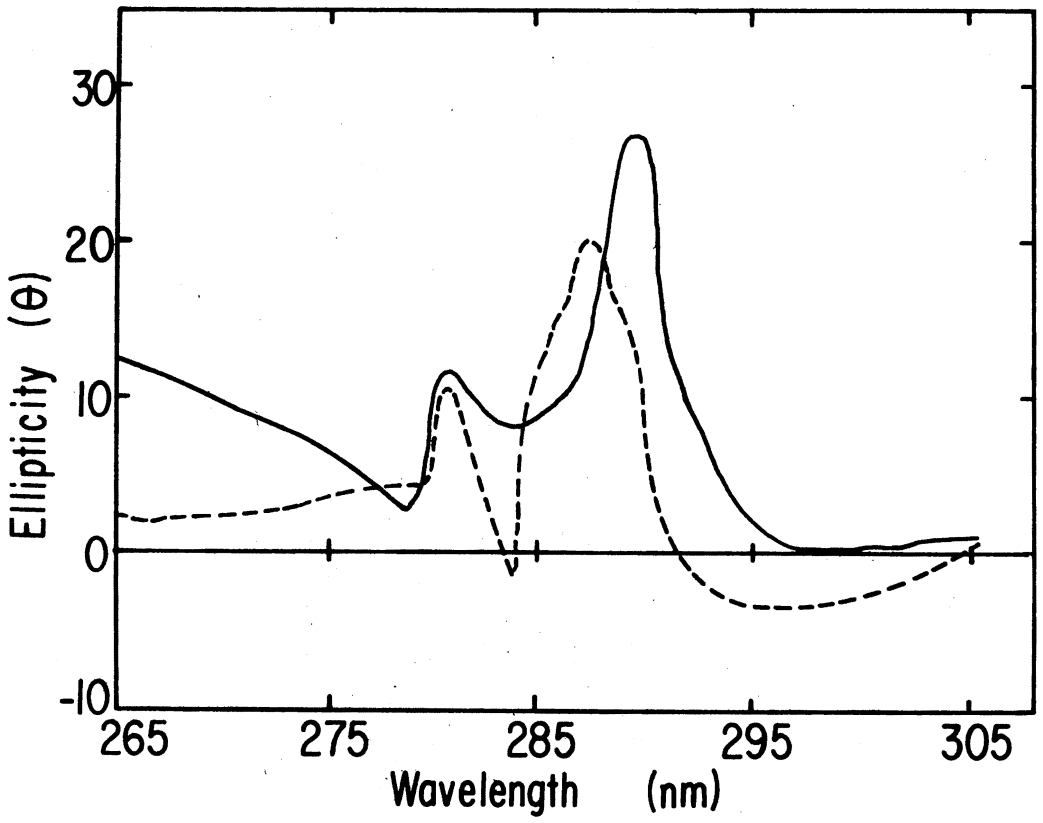


Figure 25. Near Ultraviolet Circular Dicroism of Purified Spectrin in Presence and Absence of Ca^{++}

Samples were run in presence of 5 mM $CaCl_2$ (- - - -) and absence (——). Conditions were at pH 7.4 in 10 mM Tris buffer.

with the solubility of the spectrin. A profile of solubility based on protein remaining in solution after centrifugation at 27,000 x g for 30 minutes is shown in Figure 26. This agrees to a certain extent with the information reported by Gratzer et al. (68) except no peak is seen in solubility over the pH range 5.0 to 6.0 in our studies. These studies were done under conditions where the protein concentration was fairly dilute ($\sim$100 μg/ml) using spectrin extracted from membranes derived from fresh blood.

Sedimentation Velocity of Spectrin

Sedimentation coefficients obtained for spectrin in 6 M guanidine HCl and in Tris-KCl buffers agree with previously reported values. Table VI shows the sedimentation coefficients obtained when purified samples of spectrin were sedimented in 10 mM Tris buffer in presence of 100 mM KCl. A sample containing 5 mM $CaCl_2$ under nondenaturing conditions separates into two peaks. These peaks appear to be slightly heterogeneous, and an accurate sedimentation coefficient was difficult to obtain for the lighter molecular weight peak. An approximate sedimentation coefficient ($S_{20,w}$) of 8.7 was obtained for the heavier peak. A sample containing no calcium under identical solvent and temperature sediments into two peaks. These two peaks appear to be a monomer and dimer form of the molecule. $S_{20,w}$ values for the two peaks were computed as 5.6 and 10.74 for the lighter and heavier species respectively. This correlates well with the observations made from gel chromatography experiments concerning the presence of a monomeric and dimeric species which vary in proportion as a result of the energetic state of the cells from which

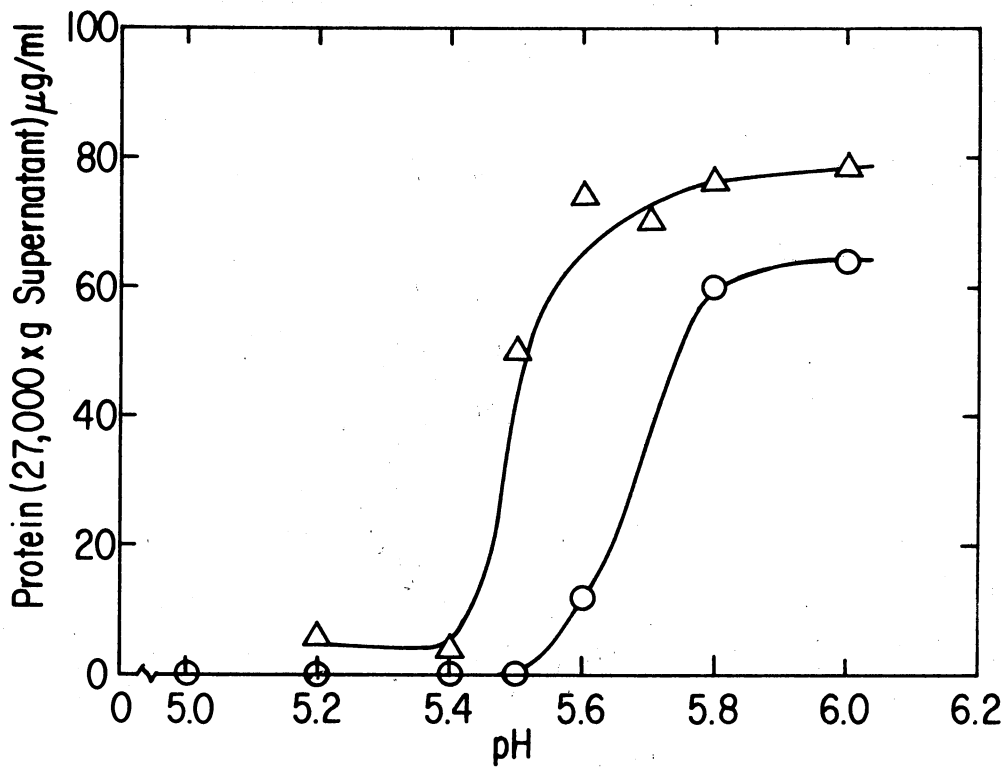


Figure 26. Solubility of Spectrin as a Function of pH

Protein was measured at each pH value after centrifugation at 27,000 xg for 30 minutes. Soluble protein remaining in the supernatant was measured according to the method of Lowry et al. (60). Spectrin solubility behavior in presence of 5 mM $CaCl_2$ -O-O- and in absnece of calcium -Δ-Δ- ranging from pH 5.0 to pH 6.0.

TABLE VI

SEDIMENTATION VELOCITY OF PURIFIED SPECTRIN WITH AND WITHOUT CA^{++} AND IN GUANIDINE HYDROCHLORIDE

	Sedimentation value ($S_{20,w}$)	Buffer
Spectrin - Ca^{++}	1.32	6 M Guanidine HCl
Spectrin + Ca^{++}	1.37	6 M Guanidine HCl
Spectrin - Ca^{++}	8.7 (*)	.1 M KCl 10 mM Tris pH 8.6
Spectrin + Ca^{++}	10.7 (5.6)	.1 M KCl 10 mM Tris pH 8.6

* denotes presence of a second peak but unable to evaluate accurately.

the extract was obtained. When the protein is denatured into 6 M guanidine HCl and centrifuged, a single peak exists which has a 1.3 sedimentation value and is apparently unaffected by the presence of Ca^{++}.

Amino Acid Analysis of Ghost Membrane Extract Components

Membrane extracts containing spectrin and component VII (actin) were separated on polyacrylamide gels and excised for amino acid analysis. The two polypeptide chains of spectrin were analyzed for comparison of compositions between the two chains. Their compositions are listed in Table VII. The data indicate that the compositions are very much the same even though there is a slight difference in molecular weight between the two chains.

Treatment of cells with Ca^{++} produces an altered extractability pattern (Figure 3). A large portion of protein remains unextracted from the membrane when treated with calcium. It is of interest to know if any differences exist in the spectrin remaining and that which is removed by low ionic strength extraction. The results shown in Table VIII indicate that the extracted material is very similar to that which remains behind. This suggests that the membrane or the spectrin has altered its affinity for the other. Lux et al. (76) have noted that spectrin from ATP depleted cells has altered extractability patterns. These results indicate that the physical state is altered in similar ways and the depletion of ATP may have the same effect as that observed in presence of Ca^{++}.

The amino acid composition of the erythrocyte component VII

TABLE VII

AMINO ACID ANALYSIS OF SPECTRIN INDIVIDUAL POLYPEPTIDE CHAINS

Amino Acid	Composition (Mole %)	
	Band Ia	Band Ib
Aspartic acid	10.6	10.4
Threonine	4.5	4.1
Serine	6.9	6.0
Glutamic acid	19.0	18.8
Proline	-	1.2
Glycine	6.8	6.3
Alanine	9.1	8.8
½-Cystine	-	-
Valine	5.3	5.4
Methionine	1.2	1.6
Isoleucine	4.2	4.0
Leucine	13.9	14.2
Tyrosine	2.0	1.4
Phenylalanine	2.9	2.6
Lysine	6.3	7.0
Histidine	2.5	2.5
Arginine	4.9	6.1

TABLE VIII

AMINO ACID COMPOSITION OF SPECTRIN EXTRACTED FROM Ca^{++}-TREATED MEMBRANES AND NON-EXTRACTABLE RESIDUAL SPECTRIN

Amino Acid	Composition (Mole %) extracted	residual
Aspartic acid	11.3	10.4
Threonine	5.0	5.2
Serine	5.8	5.3
Glutamic acid	20.5	19.0
Proline	-	-
Glycine	4.5	4.0
Alanine	10.9	10.0
$\frac{1}{2}$ Cystine	-	-
Valine	3.3	5.8
Methionine	0.7	1.2
Isoleucine	4.6	4.3
Leucine	15.3	16.2
Tyrosine	0.7	1.6
Phenylalanine	3.3	2.8
Lysine	6.6	6.2
Histidine	1.4	1.8
Arginine	5.9	6.2

(actin) is reported in Table IX. The protein analysis is compared with spectrin. There is a considerable difference in many residues from that of spectrin with which it normally extracts from the membrane.

When calcium treated ghost membranes are extracted with EDTA, it is noted that the aggregated material does not extract. This is not entirely true, as a small amount does extract from the membrane and is found in the void volume of sepharose 4B column eluants. This material can be detected if large quantities of membranes are extracted and concentrated. The amount of material extracted is equal to only a few milligrams compared to several hundred milligrams of total extracted material. It was first postulated to be crosslinked spectrin which was not covalently bound to integral proteins and thus more easily extracted. The amino acid composition is shown in Table X and compared to the composition of unextracted aggregate. The two compositions are almost identical, indicating that the material extracted is the same material as that which remains unextracted. This material must contain the intramembraneous particle or whatever is the other component present in the aggregate.

TABLE IX

AMINO ACID ANALYSIS OF SPECTRIN AND COMPONENT VII (ERYTHROCYTE ACTIN)

Amino acid	Composition (Mole %) component VII	spectrin
Aspartic acid	9.0	10.7
Threonine	7.4	4.0
Serine	6.2	6.0
Glutamic acid	12.3	19.5
Proline	5.3	2.6
Glycine	5.7	5.9
Alanine	8.1	7.3
½-Cystine	-	-
Valine	6.0	4.9
Methionine	4.0	2.3
Isoleucine	7.0	3.0
Leucine	11.0	11.6
Tyrosine	2.8	2.3
Phenylalanine	3.0	3.8
Lysine	5.7	6.8
Histidine	1.5	2.8
Arginine	5.1	6.1

TABLE X

AMINO ACID ANALYSIS OF AGGREGATE, EXTRACTABLE AND NON-EXTRACTABLE

Amino Acid	Composition (Mole %) extracted	non-extracted
Aspartic acid	9.7	9.2
Threonine	4.9	5.4
Serine	8.5	7.0
Glutamic acid	13.7	14.3
Proline	5.3	6.0
Glycine	9.3	8.0
Alanine	7.5	8.0
½-Cystine	-	-
Valine	5.3	5.6
Methionine	1.3	1.7
Isoleucine	4.2	3.4
Leucine	12.4	13.6
Tyrosine	2.4	2.0
Phenylalanine	3.0	2.9
Lysine	5.7	5.6
Histidine	1.8	2.1
Arginine	5.0	5.4

CHAPTER V

DISCUSSION

Addition of Ca^{++} to erythrocytes undergoing hemolysis results in the formation of a protein aggregate which is resistant to disaggregation by sodium dodecylsulfate and a variety of other denaturing agents. The aggregation is sensitive to temperature and Ca^{++} concentration and is prevented by chelating agents, if they are added prior to or simultaneously with the Ca^{++}. The aggregate can be isolated by chromatography of solubilized membranes on Sepharose 4B in dodecylsulfate. Amino acid analysis comparisons indicate that it contains spectrin as a major, but not exclusive, component. Previous studies (20) have shown that Ca^{++} is important for aggregate formation, but not in its stabilization once it is formed.

These facts suggest a model for the initiation of aggregate formation based on current concepts of erythrocyte membrane-protein organization (77). The model depicts Ca^{++} as the initial means by which spectrin alters its conformation or changes its association patterns. Ca^{++} in low to moderate amounts could alter conformations of large proteins by alterations in their net charge. This could cause an enhanced association of the spectrin molecules. This in turn could cause any associated component III molecules to interact to form a more stable complex with the spectrin. This model assumes a prior interaction of spectrin and component III which would

restrict the movement of the latter in the membrane. This is consistent with the limited mobility of the intramembrane particles, which presumably contain component III, in the intact erythrocyte (77). The evidence for the involvement of component III in aggregate formation is still somewhat circumstantial. Riggs and Ingram (78) have reported radioactivity from external lactoperoxidase labeling present in the aggregated material. This suggests an external protein or transmembrane protein. The inclusion of component III into the model is used as a representative of the other protein, or proteins which must be present in the aggregate in order to explain the events which might take place during aggregate formation.

The question of the physiological implications of protein aggregation must be raised at this time. Since the red cell will become rigid at a Ca^{++} concentration 10-fold less than required for aggregation, the aggregation process probably does not occur in vivo. However, the course of events that occur in red cells with increasing internal Ca^{++} concentrations suggests that the process at higher concentrations may be an extension of events that occur during cell rigidification (1). The Ca^{++} studies are also of interest as a perturbation method for studying erythrocyte membrane structure-function relationships. Enzyme and protein net retention (79) and enzyme activities (80) are all affected by Ca^{++} concentrations in the range studied here. The results of this study permit one to elucidate several facets of the protein changes that occur as a result of the Ca^{++} perturbation of erythrocyte membranes.

The extraction and labeling experiments indicate that component II arises from Ic. This is more reasonable than the previous idea

(19, 20) that it was derived from one of the bands of spectrin, since both Ic and II fail to extract from the membrane with EDTA whereas spectrin is more readily extracted. The proteolytic digestion to produce the Ic → II transition must result from endogenous protease activity at the inner surface of the erythrocyte membrane since it is observed when Ca^{++} is introduced into the erythrocyte by the ionophore A23187 (unpublished observation) under conditions where external protease would be excluded. An alternate possibility is that Ca^{++}-induced changes in the membrane cause exposure of Ic to external protease. This appears unlikely because no cleavage of the protease-sensitive cell surface glycoproteins (57) are observed during Ca^{++} treatments to suggest the presence of external protease.

Avruch and Fairbanks (28) have shown that Ic (labeled 2.1 by their nomenclature) is phosphorylated by cyclic AMP dependent protein kinase. This may be the same polypeptide whose catecholamine sensitive phosphorylation appears to regulate ion movements in avian erythrocytes (30). Additional studies are needed in this area.

In addition to the proteolysis and aggregate formation the Ca^{++} treatment also increases the amount of protein isolated with the membrane after hemolysis and the amount which cannot be extracted with EDTA. The latter results primarily from a fraction of spectrin which is not released from the membranes of Ca^{++}-treated cells by extraction. Amino acid analysis does not indicate any compositional differences in the residual and extracted spectrin.

The Ca^{++}treatment also results in the binding of two polypeptides of molecular weight 60,000 and 23,000 to the erythrocyte membranes. The former can be isolated because of its tendency to

aggregate, presumably by disulfide bond formation, when dialyzed in the absence of mercaptoethanol. The function of this protein is unknown, but it is presumably an intracellular erythrocyte constituent, possibly an enzyme.

The relationship of the protein changes to physiologically important events is still unclear. Riggs and Ingram (78) have shown the presence of polypeptides of molecular weight 25,000 and 63,000 in membranes of erythrocytes from sickle cell anemia homozygotes. The aggregate does not appear to occur in these cells. The 23,000 molecular weight peptide observed in normal cells treated with Ca^{++} could be the fragment from a monospecific cleavage of Ic since the difference between Ic and II is similar in magnitude. This peptide appears to be very highly labeled by ^{3}H-iodoacetate labeling. This could be due to a small strand of Ic which extends into the periphery of the membrane within easy access of the labeling reagent either at a membrane channel or within the interior. This type of arrangement would also explain a monospecific proteolytic cleavage. The large fragment II would be less extractable and less highly labeled while the smaller fragment would be highly labeled and more easily extracted. In untreated membranes labeled with iodoacetate this fragment is not highly labeled due to the lack of its presence. Tentative results indicate that one does not see the presence of band II without the other.

Another important consideration of the aggregation process is the stabilizing force. This phenomenon is important because it could represent a manifestation of a process which causes irreversible

deformability loss in membranes due to covalent crosslinking of key structural elements. The experiments involving incorporation of ^{14}C-putrescine together with inhibition of aggregation by iodoacetamide and iodoacetic acid indicate that aggregation is due to an enzymic reaction involving erythrocyte transglutaminase (42, 82). This would provide a mechanism for the irreversible deformability changes of erythrocytes based on experimental observations. At low concentrations of Ca^{++} erythrocytes lose their deformability, presumably as a result of conformational changes or polypeptide associations induced by Ca^{++}. The changes are reversible if sufficient chelating agent can be added or if the calcium pump can be activated and supplied with energy to pump out excess Ca^{++} (10, 81). At higher calcium concentrations the transglutaminase in the erythrocyte interior is activated and crosslinks the proteins into their new organizational state so that deformability loss is irreversible. If this model is correct, the transglutaminase is actually responsible for the irreversible deformability changes. The enzyme could be the key factor in aging and in premature destruction in disease states involving membrane rigidification. The exact state of the enzyme prior to the advent of elevated calcium levels is not known. It has yet to be determined whether the enzyme exists as a zymogen and is activated by proteolysis via a thrombin-like reaction, or is simply activated by the increased level of divalent cation. The involvement of the protease in the deformability events can only be speculated on at this time. It is apparent that the proteolytic events that do occur and are detectable by electrophoresis, occur at lower calcium levels than the aggregation. It is readily apparent

that the formation of band II is independent of transglutaminase activity since the event occurs even in presence of 100 percent inhibition of the enzyme. However, the loss of band IVa does not occur unless aggregation occurs. This strongly suggests that band IVa is not proteolyzed as originally thought (19) but could be quantitativley crosslinked with spectrin and perhaps band III in the aggregate.

The data point to the fact that certain erythrocyte membrane proteins (particulary spectrin) may be altered in their conformation, resulting in dramatic effects on erythrocyte membrane shape and deformability. These effects provide an example of cooperative behavior in protein-protein and protein-lipid interactions. The reversible shape changes in erythrocytes which occur as a result of energy depletion or cation accumulation provide a good system for study of structural protein behavior. It is of interest to know what types of forces can cause proteins to interact and what types of interactions can occur. Membrane proteins are subject to many types of effectors such as hormones and ions in maintaining viability of the cell. The reversible calcium effects and the spectrin protein in the red cell provide an interesting model system for studying protein interactions.

The information obtained from studies concerning the biophysical properties of spectrin may be applicable to the mechanism for reversible deformability loss in erythrocytes. Column chromatography techniques ultilized during isolation and purification of the protein indicate that the age of the blood has significant effects on molecular state of the spectrin, which is extracted under low ionic

strength conditions. The column chromatographic profiles from extracts indicate the presence of two species. A lower molecular weight species predominates in extracts from fresh membranes. As the age of the blood increases the higher molecular form begins to predominate. Lux et al. (76) have suggested that these observations can be directly correlated to the extent of ATP depletion of the whole cell from which the membrane extracts were obtained. Sedimentation velocity ($S_{20,w}$) values on spectrin in presence of salt show a correlation with column chromatographic information. There are apparently two molecular weight species which sediment at different rates. The larger species has a S_{20w} value equal to twice that of the smaller species. This change in molecular weight states may be the earliest observed change in ATP-depleted membranes. These observations provide the first direct evidence for a change in the physical state of spectrin.

Perturbations of the purified molecule with high Ca^{++} levels indicate that spectrin is further susceptable to alterations in the presence of the cation. Titration of spectrin with a hydrophobic specific fluorescent probe indicates the presence of low polarity binding sites within the molecule by an increased fluorescence intensity and a shift in the emission maximum to 480 nm. Approximately 14 molecules of ANS are bound per monomeric unit of 450,000 daltons (Band Ia and Ib). Calculation of the apparent dissociation constant provides a value of 6.6×10^{-5} M. In the presence of Ca^{++} (5 mM) at pH 7.4 the number of bound ANS molecules increases to 18 and the Kd is decreased to 2.2×10^{-5} M. In addition large increases in intensity are observed in the binding of ANS as pH decreases. Circular dicroism

indicates that Ca^{++} acting in conjuction with these pH changes causes rapid, reversible alterations in aggregation states. Solubility studies of protein in presence and absence of Ca^{++} at various pH values confirm that Ca^{++} and pH act in concert causing spectrin to fall out of solution as pH drops. Fluorescence binding of ANS indicates that protein concentration, pH and Ca^{++} have definite effects on the physical state of spectrin. This provides the first evidence for the involvement of calcium in the alteration of the physical state, which could be applied to membrane morphological transformations.

These observations are consistent with the postulated role of the spectrin-actin lattice in the control of red blood cell shape and deformability. To modulate this control some lattice protein(s) would have to exist in at least two physical states. One state would be associated with membrane flexibility and the other with membrane rigidity. These studies expand the concept that such membrane proteins could exist <u>in vivo</u> under conditions which allow extreme sensitivity to Ca^{++} and pH. If such conditions do exist it could be possible for local concentrations of cations to accumulate to an extent that protein physical state alterations would occur. Locally high concentrations of these cations could seemingly occur since a high concentration of impermeant molecules in the cell causes unbalanced osmotic forces. The cell balances this force by active transport of sodium out of the cell at the expense of energy in the form of ATP. Under conditions of low energy a local imbalance of ions would be possible. The appearence and disappearence of localized membrane morphology changes, such as crenations or spicules might be a result of locally high levels of cations. This would alter the physical

state of the cytoskeleton over a limited area. Such effects would be reversible due to delocalization of the cations by pumping or redistribution. Larger effects such as spherocytes would by the same mechanism be reversible if sufficient energy were available.

Other investigators have shown that Ca^{++} and spectrin can act in concert to regulate permeability of phospholipid vesicles (18). The precise nature of the interplay between protein and Ca^{++} in this phenomenon remains unclear. Ca^{++} may cause a conformational change in the protein which favors penetration. Use of spin labels indicates that fluidity changes in the lipid regions occur as a result of perturbations caused by Ca^{++}. These changes appear to be linked to the gross alteration of protein structure (84) brought about by aggregation of proteins in Ca^{++}-treated cells.

Other reports provide support for the interaction of spectrin with well known microfilament cytoskeletal elements (85, 86). This type of interaction may be of great importance, particularly since proteins similar to spectrin are not restricted to mammalian erythrocytes. The existance of microfilament structures in mammalian erythrocytes in the manner described in previously presented models is greatly supported by the fact that certain microfilament inhibitors reproduce the heriditary spherocytosis syndrome in normal intact cells (87). These ideas could lead to a basic understanding of membrane behavior in disease states of red cells as well as to membranes in general from other non-muscle cell systems.

CHAPTER VI

SUMMARY

Introduction of Ca^{++} (> 1.0 mM) causes at least four major changes and three minor changes in human erythrocyte membrane proteins based on their analysis by polyacrylamide gel electrophoresis in dodecyl-sulfate. These changes appear to be irreversible. The four major changes involve the formation of a protein aggregate, formation of a new component (component II) loss of one membrane component (component IVa) and alteration of the extractability of spectrin. Three minor changes seen are the association with the membrane of a 60,000 molecular weight component designated as Va and a smaller (22,000 m.w.) component plus the enhanced binding of hemoglobin (16,000 m.w.).

The Ca^{++}-dependent protein aggregation is undissociable in various denaturing agents, suggesting a covalent protein crosslinking. The aggregate was isolated by gel chromatography and amino acid analyses indicate it contains spectrin as its major constituent but must contain some other component(s) as well.

The formation of component II appears to be the result of a Ca^{++} activated protease which cleaves a protion of membrane component (mol. wt. = 205,000) and forms a new band which migrates with an approximate molecular weight of 180,000. Both components Ic and II are not extractable from the membrane.

Loss of protein band IVa from Ca^{++}-treated membranes does not

appear to be linked with the protease as initially thought. The disappearence of this band occurs at a higher Ca^{++} concentration than the other proteolytic effect and is retained after inhibition with iodoacetamide. The protease, on the other hand, is not affected by the inhibitor. The loss of IVa occurs only when aggregation is apparent which suggests that it may be a constituent of the aggregate.

The appearence of a new component (Va) associated with Ca^{++}-treated membranes is probably a result of the altered structure of the membrane thereby increasing its affinity for cytoplasmic components. Component Va was isolated and purified by aggregation in absence of sulfhydril reducing agents. Its amino acid analysis is typical of cytoplasmic proteins. Its function and significance as to the Ca^{++} response is unknown. It is possibly a cytoplasmic enzyme.

The significance of the adherence of hemoglobin to Ca^{++}-treated membranes is not clear. It possibly reflects the same phenomenon as Va and the 23,000 molecular weight band. All of these could be associating due to changes in surface charge brought about by alteration and restructuring of membrane proteins. The 23,000 m.w. band labels very heavily with ^{3}H-iodoacetic acid. It is not present in untreated membranes where component II is not formed. Circumstantial evidence suggests that it is the product of a monospecific cleavage of the Ic $\longrightarrow$ II proteolytic transition. Spectrin extractability is diminished in membranes treated with Ca^{++}. A possible explanation could be a trapping of the uncrosslinked protein among the aggregated material which is not released until total solubilization occurs in dodecylsulfate.

Irreversible aggregation apparently is the result of a covalent γ-glutamyl ϵ-amino crosslinkage catalyzed by the Ca^{++}-sensitive enzyme transglutaminase. Calcium concentrations as low as 0.2 mM are capable of activation with maximum activation occuring at 5 mM Ca^{++}. Aggregation can be detected at concentrations as low as 0.5 mM and can be directly correlated with incorporation of ^{14}C-putrescine substrate in the radioactive transglutaminase assay. Additional conclusive evidence was obtained by correlation of inhibition of aggregation with the increase in enzyme inhibitor iodoacetamide. Complete inhibition of incorporation of radioactivity is obtained at a concentration of 0.1 mM iodoacetamide. Inhibition of aggregation is achieved at concentrations as low as 1 μM inhibitor.

The physical properties of spectrin are of interest both because of its functions in the erythrocyte and as a model for cytoskeletal elements of more complex cells. The mode of binding to the membrane is of particular interest, especially before crosslinking occurs and deformability effects are still reversible. Fluorescence titration of spectrin with 1-anilinonaphthalene-8-sulfonate (ANS) indicates the presence of low polarity binding sites by an increased fluorescence intensity and a shift in the emission maximum to 480 nm. Approximately 14 molecules of ANS are bound per dimeric unit of 450,000 daltons with an apparent dissociation constant (Kd) of 6.6 x 10^{-5} M. In the presence of Ca^{++} (5 mM) at pH 7.4 the number of bound ANS molecules increases to 18 and the Kd is decreased to 2.2 x 10^{-5} M. No energy transfer from tryptophan was observed in the presence or absence of Ca^{++}. The tryptophan emission maximum is identical to that found in water. Circular dicroism observations

in the far ultraviolet region produce light scattering artifacts which increase in red shifts as pH is lowered suggesting rapid transition to higher molecular weight forms. Ultracentrifugation of spectrin in buffered solution shows two higher molecular weight forms in solution with or without Ca^{++}. The presence of two peaks are observed in gel column chromatography with the higher molecular weight species predominating in extracts from membranes of aged blood. The results are consistant with a model for a polymerizing system sensitive to calcium. The presence of hydrophobic sites may provide a mode of enhancing binding to the membrane. Amino acid analyses were obtained for spectrin extracted from Ca^{++}-treated cells and for that which remains behind. No differences in the compositions were detected. Individual polypeptide chains of spectrin were analyzed and appear to be identical in composition although exhibiting slightly different mobilities in dodecylsulfate acrylamide electrophoresis. Extractable aggregate also appears identical in composition to the bulk of the material which does not extract.

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VITA 2

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