ASSOCIATION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE WITH THE HUMAN ERYTHROCYTE MEMBRANE

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NOMENCLATURE

ACHE	acetylcholinesterase
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
EDTA	ethylenediamine tetraacetic acid
GAPD	glyceraldehyde-3-phosphate dehydrogenase
GTP	guanosine 5'-triphosphate
Hb	hemoglobin
IAA	iodoacetic acid
LDH	lactic dehydrogenase
μCi	microcurie
μM	micromolar
mCi	millicurie
mM	millimolar
NEM	N-ethylmaleimide
NAD	nicotinamide adenine dinucleotide
POPOP	P-bis 2-(5-phenyloxazolyl)-benzene
PPO	2,5-diphenyloxazole
SDS	sodium dodecyl sulfate
Tris	tris-(hydroxymethyl)-aminomethane
UTP	uridine 5'-triphosphate

CHAPTER I

INTRODUCTION

Biological membranes have many functions; they act as a permeability barrier separating two kinds of milieu and regulating the transport of materials across them, and they are also the surface or interface upon which functional molecules can be assembled for optimal kinetic and thermodynamic performance. These membrane functions can be studied and quantitated by observing parameters such as coupled enzyme functions, active transport, electrical properties and cellular interactions. Biological membranes can be described chemically as lipoprotein complexes containing about 40-75 percent protein and 25-60 percent lipid, with some carbohydrates being covalently linked to either protein or lipid (1). The various proteins and lipids making up the membrane are held together in the proper architectural arrangement by non-covalent forces.

The study of membrane proteins generally proceeds by one of two different routes. Either the proteins are fractionated in a single step (or a small number of steps) or individual protein components are extracted by specific techniques. The most successful procedures for single step fractionation use acrylamide gel electrophoresis, either in sodium dodecyl sulfate (SDS) or in acid-urea solutions. The former has been developed by Lenard (2) and by Carraway and Kobylka (3) into a procedure which clearly separates erythrocyte membrane proteins and lipid, and fractionates the proteins into a large number of bands according to

their molecular sizes in the SDS solutions.

Marchesi and Steers (4) first extracted two polypeptides with masses of about 250,000 daltons from human erythrocyte membranes by washing them with the hypotonic EDTA solutions, and named them spectrin. Spectrin, in the presence of divalent cation, has the appearance of a fibrous protein by electron microscopy and is located on the inner surface of the membranes. It is believed to function as a supporting unit of the erythrocyte membrane (5). There are two proteins on the exterior surface of the erythrocyte membrane. One is the major sialoglycoprotein of the membrane. About 60 percent of the mass of the molecule is carbohydrate, and it contains most of the sialic acid and half of the hexose and hexosamine in the human erythrocyte (6). It carries multiple blood group antigens, the receptors for influenza viruses and various plant agglutinins (6,7, 8). The other surface protein is the molecule with a mass of approximately 100,000 daltons which reacts in intact cells with labeling reagents impermeable through the membrane, suggesting that the 100,000 dalton glycoprotein is exposed to the outside of the intact erythrocyte (9,10,11).

Recently Tanner and Boxer (12) isolated two other glycoproteins with smaller amounts of carbohydrate from human erythrocyte membranes by sequential extractions followed by gel filtration in SDS and preparative electrophoresis. By labeling the glycoprotein receptors over the intact erythrocyte surface with the ferritin-conjugated phytohemagglutinin, and by the freeze-etching technique plus electron microscopy, Marchesi, et al. (8) observed that the label localizes to sites on the membrane that overlie the intramembraneous particles, suggesting that the sialoglycoprotein is oriented at the cell surface with its oligosaccharide-rich

N-terminal end exposed to the exterior, while its C-terminal segment interacts with other components in the interior of the membrane to form intramembraneous particles.

The current data suggest that the erythrocyte membrane is a remarkably asymmetric structure with respect to the two surfaces of the membrane; there is asymmetry in the distribution of proteins (5) and glycolipids, and there may be asymmetry in the distribution of cholesterol and phospholipid. On the other hand, in each surface the distribution of components may be random at times (13) and governed by translational diffusion (14).

Although some information exists on the structure of the erythrocyte membrane proteins, very little is known about their function except for the presence of antigenic determinants on the main sialoglycoprotein (6). The nature of the proteins of biological membranes has been a subject of considerable interest in recent years because of the controversies concerning their roles in membrane structure and functions (15,61). Most of membrane functions have not yet been assigned to specific components of the membrane. The understanding of the roles of these proteins requires information both at the level of the intact membrane and at the level of the isolated proteins.

One method of obtaining such information is by chemical modification studies of the membrane. A number of such investigations of erythrocyte membranes have shown that sulfhydryl groups of proteins are important to maintenance of different membrane functions: (a) the uptake of glucose is reduced so that substrate supply is diminished (17); (b) the active transports of Na^+ and K^+ are diminished, and the cells lose K^+ and gain Na^+. The cells swell and ultimately undergo colloidal osmotic lysis,

because the colloidal osmotic pressure can no longer be compensated (18).

Shapiro, et al. (19) have classified sulfhydryl groups of erythrocyte membranes on the basis of modification of cell permeability by sulfhydryl reagents and of their binding patterns to cells. They showed that p-chloromercuribenzoate (PCMB), chlormerodrin and p-chloromercuribenzene sulfonate (PCMBS) react with at least three classes of sulfhydryls, two of which are associated with the Na^+,K^+ barrier and, when altered, result in K^+ loss, Na^+ accumulation and hemolysis. 1-Bromo-mercuri-2-hydroxy propane (BMHP) reacts with at least two classes of sulfhydryls, one of which is associated with permeability and, when altered, results in hemolysis in isotonic solution of choline chloride or lactose.

There have been only limited attempts to relate reactive sulfhydryl groups to specific membrane proteins. Lenard (20) has described the distribution of N-ethylmaleimide (NEM) labeling among the erythrocyte membrane proteins, showing that the major high molecular weight proteins have the greatest number of reactive groups toward this reagent in the isolated erythrocyte membrane. In the present study the reactivity of the various proteins in the erythrocyte membrane toward sulfhydryl reagents was studied at three levels: the intact erythrocyte, the resealed ghost and the isolated membrane. The relative reactivities of the individual membrane proteins can be determined by fractionating the membrane proteins by polyacrylamide gel electrophoresis in SDS and determining the radioactivity of the various protein bands by slicing and counting the gel slices. Virtually all of the label, iodoacetate, is incorporated into a single protein band (component VIII). Previous studies from this laboratory (11) have shown that component VIII can be extracted from the

isolated membrane by dialysis against a mixture of 0.3 M NaCl, 0.5 mM EDTA and 5 mM mercaptoethanol at pH 9.5. Even more specific procedures, using a brief exposure to 0.5 M NaCl (21) or to 0.1 M EDTA (22) have been reported. Comparison of electrophoresis patterns suggested that these procedures extracted the same protein, component VIII.

Specific labeling of a single protein, coupled with isolation and partial characterization of this protein offers the possiblity of a dual approach to understanding some of the problems of membrane protein chemistry. On the basis of the enzyme activity of the purified component, specific inhibition of the enzyme activity by iodoacetate and comparison with the results of Tanner and Gray (22), who identified the same membrane component by a partial amino acid sequence analysis, this component (VIII) is identified as a subunit of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.23; D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating)).

The composition and properties of the erythrocyte membranes may vary enormously according to the methods of preparation and subsequent treatment. For example, the hemoglobin content of human erythrocyte membranes has been variously reported as from 54 percent of the dry weight (23) to 0.2 percent (24). The glycolytic enzymes have been previously considered as primarily cytoplasmic, but Green, et al. (25) claimed that the complete glycolytic sequence was operative in membranes from bovine erythrocytes. However, modification of the methods used in preparation of membranes markedly altered the activities retained by the membrane. Furthermore, the glycolytic complex bound to the membrane could be resolved, and, reassembled by a simple cycle of pH changes. Baum, et al. (26), in a continuation of these investigations, concluded that the

glycolytic complexes of enzymes, together possibly with hemoglobin, may be attached to the interior surface of the erythrocyte stroma as "head-piece" subunits.

Mitchell, et al. (27) demonstrated that the retention of hemoglobin, aldolase and glyceraldehyde phosphate dehydrogenase by human erythrocyte membranes was dependent upon pH and ionic strength. These parameters had no effect upon the acetylcholinesterase, which has been shown by many workers to be incorporated in the membrane. Zamudio, et al. (28) investigated the Na^+,K^+ ATPase and NADH oxidoreductase activities of human erythrocyte membranes. The oxidoreductase activity of sonicated fragments was much greater than that of membranes, which led them to the concept of the "crypticity".

Duchon and Collier (29) classified the erythrocyte membrane enzymes into three classes: (a) firmly bound (eg., aldolase and glyceraldehyde phosphate dehydrogenase); (b) intermediate (eg., phosphoglycerate kinase); and (c) loosely bound (eg., triose isomerase, pyruvate kinase, lactic dehydrogenase and glutathione peroxidase and reductase). They observed that the pH of the medium had little effect upon the firmly bound enzymes but it markedly affected the retention of hemoglobin and the activities of the loosely bound enzymes, and that the presence of Mg^{++} or Ca^{++} enhanced the retention of hemoglobin and the activity of the loosely bound enzyme, with little effect on aldolase and glyceraldehyde phosphate dehydrogenase.

The role and importance of enzyme-membrane associations have received considerable attention in recent years. Membrane-bound proteins have also been dividied into two categories, integral and peripheral, according to the definitions of Singer and Nicolson (30). Integral

enzymes, such as ATPase and acetylcholinesterase of the erythrocyte membrane, are very difficult to dissociate from the membrane without inactivation of the enzyme and gross membrane distortion or destruction. These enzymes often require lipid for activity. The peripheral enzymes are most loosely bound and can be dissociated by relatively mild treatments, eg., extraction with the salt solutions, which do not destroy the membrane structure. The categorization can not be defined rigorously, however, as evidenced by the extraction of human erythrocyte acetylcholinesterase by a high salt concentration as a lipoprotein particle without the destruction of gross membrane morphology (31). The peripheral enzymes also exhibit a wide range of behaviors in their associations with membranes, as shown by studies of Green, et al. (25) and of Duchon and Collier (29), on the association of a series of enzymes with erythrocyte membranes.

During the course of experiments on the activity of glyceraldehyde-phosphate dehydrogenase (GAPD) in the erythrocyte membrane, it was observed that the enzyme activity decreased dramatically under certain conditions of incubation. The causes of the activity loss were studied and procedures were sought to recover the lost activity in order to insure an accurate assay for the enzyme. Also, distributions of enzyme activities and hemoglobin were studied in the human erythrocyte hemolyzed under different conditions. It seems clear that enzymes can associate with membranes in a variety of modes, and that a variety of such associations will have to be studied to understand the factors which might be involved for different enzymes. Two factors relating to the association of glyceraldehyde phosphate dehydrogenase with the erythrocyte membrane are of particular interest. First, glyceraldehyde

phosphate dehydrogenase (GAPD) of isolated membranes was not attacked readily by proteolytic enzymes, but was released into the supernatant solution during the course of digestion (32,33). Second, the addition of adenosine triphosphate to the hemolyzing solution during hypotonic hemolysis resulted in a dramatic decrease in the amount of glyceraldehyde phosphate dehydrogenase (GAPD) isolated with the membrane as measured by either iodoacetate labeling or the enzyme activity, as observed in the present study. The latter discovery is of particular interest because of the known effects of adenosine triphosphate on the activity and quaternary structure of glyceraldehyde phosphate dehydrogenase (GAPD) from rabbit muscle and yeast. Deal and his coworkers (34,35,36) observed that adenosine triphosphate caused a dissociation of the native, tetrameric, soluble glyceraldehyde phosphate dehydrogenase from rabbit muscle and yeast into dimers or monomers and destabilized the native enzyme toward proteolytic inactivation, and that NAD or cyclic AMP prevents completely the effects of ATP on the enzyme. From these observations they deduced that rabbit muscle and yeast GAPD enzyme exists as an equilibrium mixture of tetramers, dimers and monomers, and postulated that glyceraldehyde phosphate dehydrogenase (GAPD) serves as an important metabolic control point in glycolysis. The effects of nucleotides and trypsin on the association of the enzyme with the human erythrocyte membranes were studied in order to gain some insight about the nature of association of this enzyme to the erythrocyte membrane.

The objectives of this work were to study the modification of

erythrocyte membranes with sulfhydryl reagents, to characterize the selectively modified protein(s) isolated from membranes, and to study the association of the protein with erythrocyte membranes under various conditions.

CHAPTER II

EXPERIMENTAL PROCEDURE

A. Materials

ACD whole human blood was obtained from the Dallas Communit Blood Bank and used within one week of the withdrawal date. Radioactive iodoacetic acid (iodoacetic acid-1-^{14}C, specific activity 13.9 mCi per mmole; iodoacetic acid-^{3}H, 81.5 mCi per mmole) was obtained from New England Nuclear and N-ethyl [maleimide-2,3-^{14}C] (2.1 mCi per mmole) was obtained from Amersham/Searle. NCS solubilizer was a product of Amersham/Searle. All enzymes and chemicals for enzyme assays were products of Sigma Chemical Company. Chemicals for electrophoresis were obtained from Eastman (highest purity grade) or Canalco. Columns and chromatographic supports were obtained from Pharmacia or Bio-Rad. Other chemicals were reagent grade or highest purity available.

B. Methods

B.1 Preparation of Erythrocytes, Resealed Ghosts and Membranes

The intact erythrocytes were isolated by centrifugation of whole blood for 10 minutes at 1600 x g at 4^{o} C. The plasma and buffy coat were removed by aspiration. The cells were washed three times with isotonic saline (166 mM NaCl). Red cells were washed with isotonic saline to remove plasma and buffy coat. For preparation of resealed ghosts (37) one

volume of packed red cells was hemolyzed in 10 volumes of ice-cold 10 mM Tris buffer (pH 7.4) at 4° C for 10 minutes. This suspension was then treated with one volume of a mixture of 1.42 M KCl and 0.28 M NaCl, kept at 25° C for 15 minutes, and centrifuged for 10 minutes at 35,000 x g, 4° C. The resealed ghosts were washed at 4° C with 10 volumes of isotonic saline, then with Krebs-Ringer solution. For the preparation of erythrocyte membranes a modification of the procedure of Dodge, et al. (24) was used. One volume of packed red cells was hemolyzed in ten volumes of 7 mM phosphate (pH 7.4) at 4° C centrifuged for 20 minutes at 35,000 x g, 4° C, and washed three times with ten volumes of the same buffer. The ghosts prepared by this procedure contain less than 1% of the original cellular hemoglobin. Some variations in the hemoglobin content of modified membranes were noted as described in later sections.

B.2 Labeling of Erythrocyte Membranes of Intact Cells, Resealed Ghosts or Isolated Membranes

Washed cells, resealed ghosts or isolated ghosts were allowed to react for 90-120 minutes at 37° C with ^{14}C-iodoacetate (0.25 mM, 4 μCi) or ^{14}C-N-ethylmaleimide (1.65 mM, 10 μCi) in Krebs-Ringer buffer (pH 7.4) at a cell or ghost concentration equivalent to a red cell hematocrit of 30 (1.6 mg/ml of membrane protein). The intact cells and resealed ghosts were washed with Krebs-Ringer buffer, then hemolyzed as described above. The isolated ghosts were washed with Krebs-Ringer buffer, then with 7 mM phosphate (pH 7.4) before being prepared for electrophoresis.

For the double labeling experiment 1.0 ml of packed red cells was suspended in 2.0 ml of Krebs-Ringer buffer (pH 7.4) and allowed to react with ^{3}H-iodoacetate (0.59 mM, 80 μCi) at 35° C for 60 minutes. The

labeled cells were washed and hemolyzed as described previously. The isolated ghosts were then reacted with ^{14}C-iodoacetate (0.75 mM, 10 μCi) in Krebs-Ringer buffer at 35° C for 60 minutes. After being washed with 7 mM phosphate buffer (pH 7.4), the ghosts were prepared for electrophoresis.

B.3 SDS Acrylamide Electrophoresis

For electrophoresis 200 μl of packed membranes (0.7-1.0 mg of protein) were dissolved by adding 100 μl of a freshly prepared solution of 4% SDS and 2.5% mercaptoethanol and incubating overnight at room temperature. Glycerol (to 15-20%) was added to the sample before loading directly onto the gels. For lyophilized samples weighed amounts were dissolved in 2% SDS and 2.5% mercaptoethanol in 20 mM phosphate at pH 7.4 by incubating overnight at room temperature. The gels were prepared and run according to previously described procedures (3,38) using a 5% acrylamide concentration and 10-13 cm gels. For radioactive samples one gel was stained overnight at room temperature with 0.05% Coomassie blue in 10% methanol and 7% acetic acid. The gel was destained in 7% acetic acid in a Hoefer destainer. The second gel was sliced immediately after the electrophoresis run with a homemade gel slicer (39). The slices were extracted and counted by a modification (40) of the procedure of Basch (41). The gel patterns observed by staining did not differ significantly from those reported previously for human erythrocyte membranes (2,21,40, 41), except for the higher resolution of bands achieved by using the longer gels and a lower SDS concentration in the gel (11). The molecular weights of the membrane proteins were estimated from a plot of the logarithm of molecular weight vs. migration distance for a series of standard

proteins: hemoglobin, chymotrypsinogen, catalase, bovine serum albumin and β-galactosidase. Proteins were dissolved in SDS and reduced as described for membranes.

B.4 Gel Filtration in SDS or in Guanidine Hydrochloride

Membrane samples or lyophilized fractions were dissolved in a solution of 1% SDS, 0.05 M phosphate and 0.02% sodium azide (pH 7.0) and reduced with 1% mercaptoethanol. Chromatography was performed on Bio-Gel P-100 (3.5 x 80 cm), Sephadex G-200 (2.5 x 90 cm) or Sepharose 4B (2.5 x 90 cm) at room temperature using the SDS-phosphate-azide system as the elution buffer. Fractions for electrophoretic or amino acid analysis were dialyzed at room temperature for 2-3 days against 40% methanol to remove SDS, against distilled water at 4^{o} C overnight, and lyophilized. The molecular weight of the isolated membrane protein was estimated from its elution position on G-200 in comparison to a standard curve prepared using catalase, bovine serum albumin, ovalbumin, α-chymotrypsinogen, and cytochrome c. The purified, lyophilized ^{14}C-iodoacetate (IAA) labeled component VIII was dissolved in a mixture of 6 M guanidine HCl and 1% mercaptoethanol, and subjected to Sepharose 4B gel filtration (column dimension 90 x 1.5 cm) with 6 M guanidine HCl as an eluant, for the molecular weight estimation of component VIII. Myosin, BSA, ovalbumin, chymotrypsinogen and cytochrome c were used as molecular weight standards.

B.5 Chemical and Enzymatic Assays

Hemoglobin (43), protein (44), phosphorus (45) and cholesterol (46) were determined by established procedures. The enzymes glyceraldehyde-3-phosphate dehydrogenase (47,48), lactic dehydrogenase (49),

acetylcholinesterase (50) and aldolase (47,48) were assayed by previously described procedures.

B.6 Measurement of Radioactivity

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (Model 3320, Packard Instrument Co., Downers Grove, Ill.) by counting in either Bray's solution (51) or in toluene-ethanol-PPO-POPOP (1200:800:8:0.4, V:V:W:W) solution. The membranes, supernates of hemolysate or gel slices were placed in a counting vial with 0.5 ml NCS solubilizer, capped with a polyethylene lined cap and heated at 50° C for 2 hours. Ten milliliters of toluene ethanol scintillation solution were added to the digested material and the radioactivity was determined. Samples containing hemoglobin were corrected for quenching using a standard quenching curve for known hemoglobin concentrations. Fractions (0.1 ml or 0.5 ml) from the gel filtration or from an amino acid analyzer column were added to 10 ml of Bray's solution and assayed for the radioactivity. Radioactivity measurements were corrected for machine efficiency and quenching by external standardization.

B.7 Amino Acid Analysis and Identification of the Labeled Amino Acid

Samples for amino acid analysis were hydrolyzed 22 hours at 110° C (52). Amino acid analyses were performed on a Beckman 120 C amino acid analyzer using a three buffer, single column elution system on the long column. Cysteic acid analyses were performed by the method of Hirs (53), and trypotophan was determined after hydrolyses with p-toluene-sulfonic acid (54). Samples of the labeled material (membranes or purified protein) were hydrolyzed at 110° C for 22 hours and applied to the long

column of a Beckman 120 C amino acid analyzer. The column was eluted using a three buffer system, and fractions were collected. Samples of 0.5 ml were counted in Bray's solution (51). This elution system was calibrated by observing the elution positions of ^{14}C-labeled serine, alanine and tyrosine. The elution position of any amino acid could then be calculated from its known elution volume in the standard ninhydrin analytical system.

B.8 Molecular Weight Determination of Extracted Glyceraldehyde-3-phosphate Dehydrogenase

The enzyme was extracted from isolated membranes with NaCl (11) or EDTA (22). The crude extracts were dialyzed against 50 mM glycine, 10 mM Tris and 5 mM mercaptoethanol (pH 9.2) at 4^{o} C overnight. After concentration by Aquacide III the enzyme was dialyzed into 250 mM glycine, 5 mM mercaptoethanol and 1 mM EDTA at 4^{o} C and loaded onto a 2.5 x 90 cm Sephadex G-200 column equilibrated with the same buffer. Fractions of 3.8 ml were collected and assayed for protein, hemoglobin, acetylcholinesterase and glyceraldehyde-3-phosphate dehydorgenase. Fractions containing the latter two activities were combined and assayed for phosphorus and cholesterol. The molecular weight of the dehydrogenase was estimated from a calibration curve for the column using catalase, aldolase, lactic dehydrogenase, ovalbumin, chymotrypsinogen, lysozyme and cytochrome c as molecular weight standards. For sucrose density gradient centrifugation the crude EDTA extract was precipitated in a 60-90% ammonium sulfate fraction and dialyzed into 50 mM Tris and 2 mM mercaptoethanol (pH 7.4). Samples of 200 µl plus 50 µl of molecular weight standard were mixed, and 100 µl of the mixture were applied to 5 to 20%

buffered sucrose gradients. These were centrifuged 17 hours at 4° C and 114,000 x g in a Beckman L-2 Ultracentrifuge with an SW 50.1 head. The molecular weight was estimated by the procedure of Martin and Ames (55), using catalase, lysozyme and lactic dehydrogenase as standard proteins.

B.9 Detergent Effects on Membrane Glyceraldehyde-3-phosphate Dehydrogenase

One volume of detergent (Triton X-100 or SDS) in an appropriate medium was mixed with one volume of packed ghosts. At timed intervals 50 µl of the mixture were taken and immediately assayed for glyceraldehyde-3-phosphate dehydrogenase activity. Similar procedures were used to determine the detergent effects on aldolase and acetylcholinesterase activities. The molecular weight of Triton X-100 was taken as 680.

B.10 Trypsin Digestion of Erythrocyte Membranes

Isolated erythrocyte membranes were digested with trypsin at an appropriate concentration at room temperature in 7 mM phosphate (pH 7.4). At timed intervals three samples were removed: (a) an aliquot was assayed directly for glyceraldehyde-3-phosphate dehydrogenase activity; (b) an aliquot was solubilized by mixing with one-fourth volume of 10% SDS-5% mercaptoethanol and used for SDS electrophoresis; and (c) an aliquot was treated with soybean trypsin inhibitor and centrifuged. The supernatant solution and pellet fractions of this sample were assayed for enzyme activities. In some experiments pellets were subjected to a freeze-thaw or SDS treatment before assay. The mixture from the freeze-thaw disruption was centrifuged, and the pellet and supernatant solution

were assayed again for glyceraldehyde-3-phosphate dehydrogenase.

B.11 Hemolysis and Enzyme Distribution of Erythrocytes

B.11.1 Hypotonic Hemolysis. One volume of washed, packed erythrocytes was hemolyzed in 12.5 volumes of ice-cold 10 mM Tris (pH 7.4), 7 mM phosphate buffer (pH 7.4) or distilled water at 0° C for 30 minutes.

B.11.2 Hemolysis by Freeze-Thaw. One volume of washed, packed erythrocytes was suspended in 12.5 volumes of isotonic media, i.e., 166 mM Tris (pH 7.4), 114 mM phosphate buffer (pH 7.4), 150 mM NaCl-10 mM Tris (pH 7.4), 150 mM KCl-10 mM Tris (pH 7.4) or 250 mM sucrose-10 mM Tris (pH 7.4), frozen in a dry ice-isopropanol bath and thawed in a water bath at room temperature. The freeze-thaw procedure was repeated once.

B.11.3 Hemolysis by Digitonin. Digitonin was solubilized in 5 N NaOH and then its pH was adjusted to 7.0 with 1 N HCl and the concentration to 20 mg per ml (56). Two ml of washed, packed erythrocytes were hemolyzed in 25 ml of 250 mM sucrose-10 mM Tris (pH 7.4) or of 150 mM NaCl-10 mM Tris (pH 7.4) with digitonin (0.1-2.7 mg/ml) at 0° C for 30 minutes.

The hemolysate was centrifuged for 20 minutes at 35,000 g, 4° C, and the supernatant solution and the pellet were separated. The supernatant solution and the pellet after hemolysis were weighed. The pellets were suspended in 2-4 ml of 10 mM Tris (pH 7.4). An aliquot of the supernatant solution and the pellet suspension were dialyzed against 10 mM Tris (pH 7.4)-2 mM mercaptoethanol. The dialyzed pellet suspension was diluted with one volume of 2 mM SDS in distilled water. The pellet suspension in 1 mM SDS and the dialyzed supernatant solution were assayed for GAPD, LDH, acetylcholinesterase and hemoglobin.

B.12 Addition of Membrane Effectors During Hemolysis

One volume of washed, packed erythrocytes was hemolyzed in 10-12 volumes of 10 mM Tris (pH 7.4) containing appropriate concentrations of ATP, EDTA, 2,3-diphosphoglycerate, other nucleotides or divalent cations at 0° C for 20 minutes. The mixture was centrifuged at 35,000 x g for 20 minutes at 4° C and samples of the supernatant solution and pellet were dialyzed against 10 mM Tris (pH 7.4) and 2 mM mercaptoethanol at 4° C overnight. Supernatant solution and pellet samples were assayed for hemoglobin and enzymes. The dialyzed pellets were pretreated with SDS before assay. Activities released into the supernatant solution are expressed as a percentage of the total activity (supernatant solution + pellet). These total activities did not deviate significantly from untreated controls as a result of treatment with the various effectors.

B.13 Effect of ATP or NAD on Solubilized Glyceraldehyde-3-phosphate Dehydrogenase

ATP or NAD was added to samples of the crude EDTA extract of the erythrocyte membrane at 0° C or 25° C to give 1 mM ATP or 2 mM NAD, respectively. After 10 minutes this was followed by addition to the appropriate samples of NAD or 2 mM or ATP to 1 mM. After an additional 30 minute incubation aliquots were assayed for glyceraldehyde-3-phosphate dehydrogenase.

To determine effects of chymotrypsin on erythrocyte glyceraldehyde-3-phosphate dehydrogenase, a 60-80% ammonium sulfate fraction of the crude EDTA extract was used. The crude dehydrogenase preparation (0.17 mg protein/ml) was incubated with chymotrypsin (10 µg/ml) in 60 mM Tris

(pH 7.4) and 1.5 mM mercaptoethanol in the presence or absence of 5 mM ATP, 5 mM NAD or both at 25° C. Aliquots were removed at timed intervals for dehydrogenase assay.

CHAPTER III

RESULTS

A. Specificity of the Reactions of Sulfhydryl Reagents

To study the reactivity of the various proteins in the membrane toward sulfhydryl reagents, erythrocyte membranes in three forms (the intact cell, the resealed ghost and the isolated membrane) were subjected to reaction with radioactive iodoacetic acid (IAA) or N-ethylmaleimide (NEM). Both reagents penetrated the membrane, as shown by radioactivity incorporated into the hemolysates from the red cells and resealed ghosts. The amounts of radioactivity incorporated into the hemolysate and membrane fractions were dependent on the ratio of reagent to cells and the efficiency of removal of the intracellular proteins from the membrane. Prior labeling of cells and resealed ghosts caused a slightly increased retention of the intracellular proteins after hemolysis and washing of the membranes. This effect was variable for different blood samples, but it did not affect the results except as noted. The specific activities of the iodoacetate-labeled hemolysate protein and membrane protein were 7.0 x 10^4 dpm/mg hemoglobin and 2.0 x 10^5 dpm/mg membrane protein, respectively, when the intact cells were labeled with ^{3}H-iodoacetate in a typical experiment (Table I). Since the carboxymethyl groups are not equally distributed among all of the membrane proteins, this shows that there is considerably higher incorporation into the labeled membrane proteins than into hemoglobin. This suggests a higher reactivity for

TABLE I

INCORPORATION OF ^{3}H-IODOACETATE INTO ERYTHROCYTE MEMBRANE AND INTRACELLULAR PROTEIN FRACTIONS[1]

Species Labeled	Radioactivity Incorporated %		Specific Activity dpm/mg protein	
	Hemolysate	Membrane	Hemolysate	Membrane
Intact cell	19	0.94	7.0×10^4	2.0×10^5
Resealed ghost	2.2	1.59	1.0×10^5	3.7×10^5
Hemoglobin-free ghost	---	1.89	---	4.2×10^5

[1]One ml of the packed intact erythrocytes, the resealed ghosts or the erythrocyte membranes were suspended in 3 ml of Krebs-Ringer with ^{3}H-iodoacetate (specific activity, 81.5 mCi per mmole) at the concentration of 0.26 mM and incubated at 37° C for 90 minutes. The labeled intact erythrocytes and the resealed ghosts were pelleted, twice washed with Krebs-Ringer, hemolyzed with 10 ml of 7 mM phosphate buffer (pH 7.4) and centrifuged. The pellets were washed 3 times with the same buffer. The supernatant of hemolysates were dialyzed against distilled water at 4° C for one day. The labeled membranes were washed 3 times with 7 ml of the same buffer. Samples were prepared and counted for radioactivities as described in Experimental Procedure.

the membrane protein reactive functional groups, since both membrane groups and hemoglobin groups are in competition for the reagent.

The relative reactivities of the individual membrane proteins can be determined by fractionating the membrane by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and determining the radioactivity of the various protein bands by slicing and counting the gel. The results of a typical iodoacetate labeling experiment are shown in Figure 1. Surprisingly, only a single band of the fractionated membrane contains a significant amount of the radioactivity incorporated into the membrane when the intact red cell is labeled. The resealed ghost and ghost, which have a higher incorporation of label into the membrane (Figure 1 and Table I) because of the absence of intracellular proteins to compete for reagent, also show this band as the major labeled species, but other proteins have been labeled more heavily in these cases. At the top of Figure 1 is shown a schematic representation of the protein distribution as determined by staining with Coomassie blue. The superimposed molecular weight scale permits estimating a value of about 40,000 for the labeled band. This band corresponds to component VIII by the previous system of labeling the erythrocyte membrane proteins shown at the top of Figure 2 (11).

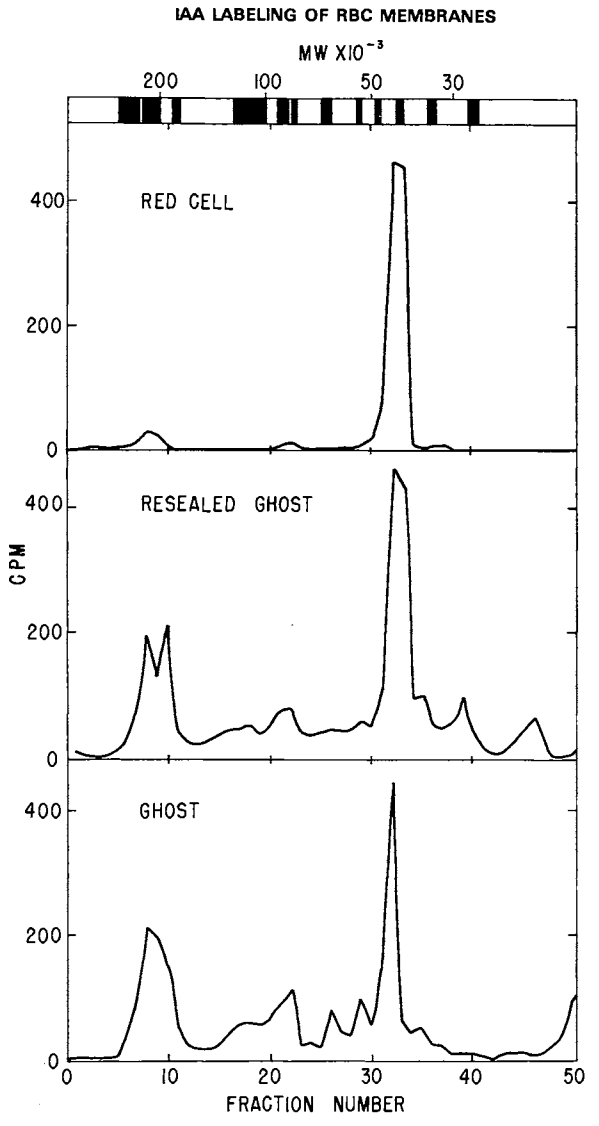
The specificity of the reaction is not nearly so great for N-ethylmaleimide labeling, as shown in Figure 2. This reagent labels all of the major proteins of the membrane. For the ghost the amount of label incorporated into each band roughly parallels the staining intensity of the band with Coomassie blue. Component VIII, which is heavily labeled in the iodoacetate experiment, is not specifically labeled by N-ethylmaleimide under these conditions. Figure 2 also shows the increased

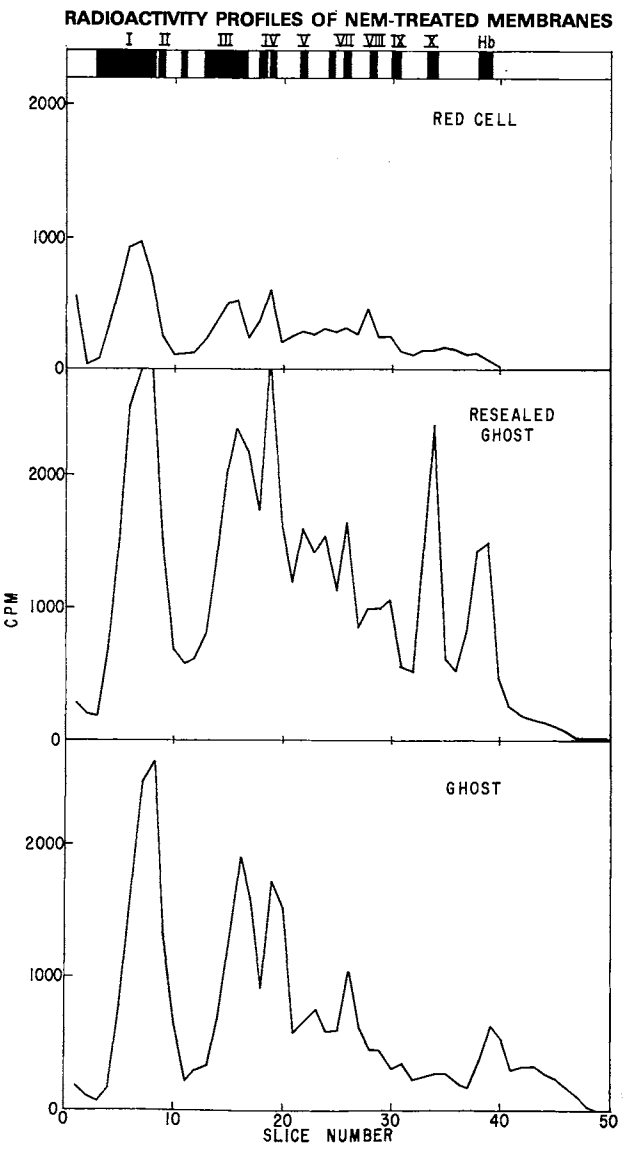
ure 1. Radioactivity Profile of ^{3}H-iodoacetate-labeled Membranes From Treated Erythrocytes, Resealed Ghosts and Ghosts

The three types of samples were labeled at a membrane protein con-tration of 1.6 mg/ml with 0.26 mM ^{3}H-iodoacetate (specific activity, 6 mCi per mmole) in Krebs-Ringer phosphate (pH 7.4). Membranes were lated by the procedure of Dodge, et al. (24), solubilized in SDS and ctionated by electrophoresis in 0.1% SDS on 5% acrylamide gels. Du-cate gels were sliced and counted for radioactivity and stained with massie blue. The diagram at the top of the figure shows the staining tern observed, which was essentially identical for all of the gels. molecular weight distribution for the protein bands is also shown at top of the figure.

ure 2. Radioactivity Profile for ^{14}C-N-ethylmaleimide Labeled Mem-branes From Treated Erythrocytes, Resealed Ghosts and Ghosts

The samples were labeled as described in Figure 1, at an NEM concen-tion of 1.65 mM (specific activity, 2.1 mCi per mmole). Membrane iso-ion and electrophoretic analyses were performed as described in Figure At the top of the figure is shown the Coomassie blue staining pattern a numerical assignment of band areas described previously (11). Com-ent X and hemoglobin were present to a significant extent only in the branes from labeled resealed ghosts.


IAA LABELING OF RBC MEMBRANES
MW X10⁻³
200
100
50
30
RED CELL
RESEALED GHOST
GHOST
CPM
400
200
0
0
10
20
30
40
50
FRACTION NUMBER


RADIOACTIVITY PROFILES OF NEM-TREATED MEMBRANES
I
II
III
IV
V
VII
VIII
IX
X
Hb
RED CELL
RESEALED GHOST
GHOST
CPM
2000
1000
0
0
10
20
30
40
50
SLICE NUMBER

retention of intracellular proteins observed after labeling the resealed ghost. Both hemoglobin and component X were observed in significant quantities by staining in the case of the labeled resealed ghost, even though they were virtually absent from the staining patterns in the cases of the intact cell, which was less heavily labeled, and the ghost, which was washed before labeling.

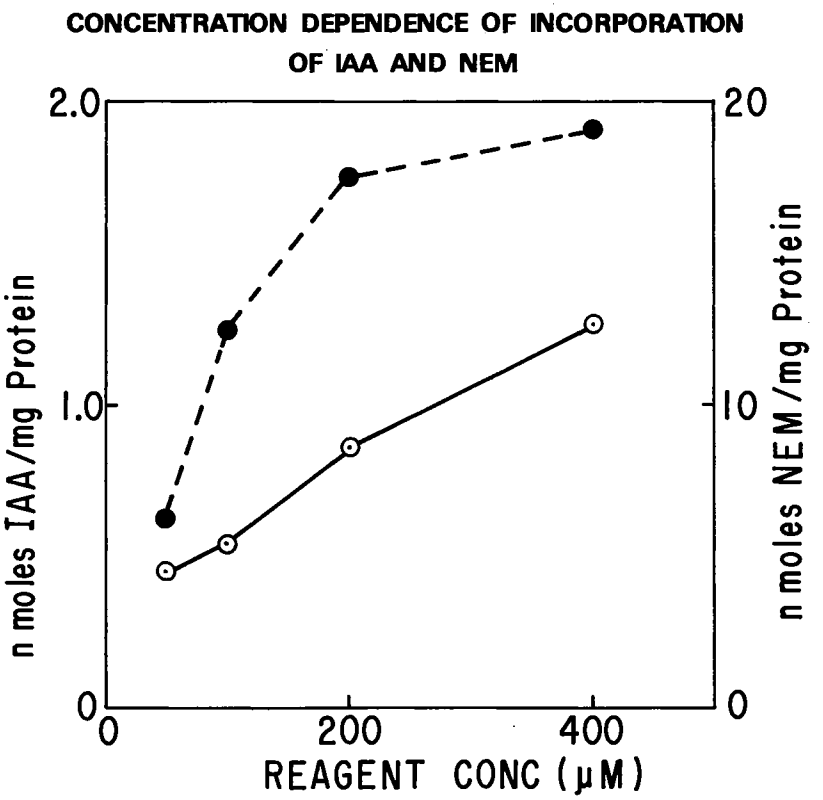
The foregoing results suggest that a single protein of the erythrocyte membrane can be specifically labeled by iodoacetate in the red blood cell. The reaction specificity is decreased in the ghost and abolished by changing the reagent to N-ethylmaleimide. To investigate the reaction specificity in more detail, the concentration dependence of the reactions with the two reagents was studied with isolated ghosts (Figure 3). Several interesting features of the reaction system were found. (a) The number of N-ethylmaleimide groups incorporated was considerably greater (15-20 fold) than the number of carboxymethyl groups for equivalent reagent concentrations. (b) Incorporation of NEM groups approached saturation at 0.5 mM, but IAA incorporation was still increasing in a linear fashion. (c) The specificity of the IAA reaction was dependent on reagent concentrations (Figure 4). At the lowest reagent concentration used component VIII was labeled almost exclusively, indicating that the specificity difference observed between the ghost and red cell was a function of effective reagent concentration and not due to a difference in membrane properties. (d) The specificity of the NEM reaction was not dependent on reagent concentration over the range used. However, since the number of NEM groups incorporated at the lowest concentration of this reagent used for these experiments was still about four-fold greater than the IAA groups incorporated at its highest concentration, a further

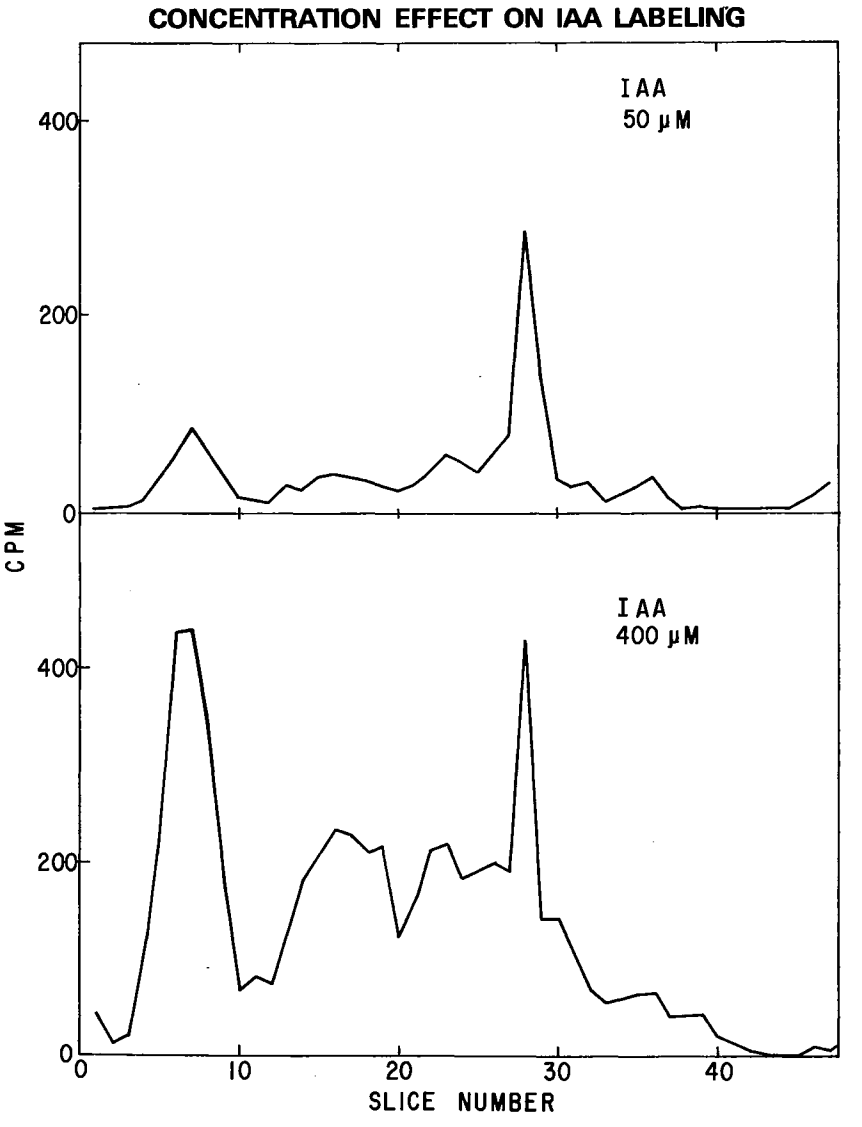
gure 3. Concentration Dependence of Iodoacetate and N-ethylmaleimide Reactions With Isolated Erythrocyte Membranes

Membranes were labeled as described in Figures 1 and 2 with reagent ıcentrations from 50-400 μM. Labeled membranes were washed, counted l analyzed for protein by the Lowry method (44). Iodoacetate (O———O) l N-ethylmaleimide (●— — —●).

gure 4. Concentration Dependence of Specificity of Iodoacetate Labeling of Erythrocyte Membranes

Conditions for labeling and electrophoresis are described in Figures and 2.


CONCENTRATION DEPENDENCE OF INCORPORATION
OF IAA AND NEM
2.0
1.0
0
20
10
0
0
200
400
n moles IAA/mg Protein
n moles NEM/mg Protein
REAGENT CONC (μM)


CONCENTRATION EFFECT ON IAA LABELING
IAA
50 μM
IAA
400 μM
400
200
0
400
200
0
CPM
0
10
20
30
40
SLICE NUMBER

experiment was performed at a lower NEM concentration (10 μM). In this case only 0.5 nmole of NEM was incorporated per mg of membrane protein. Since the level of radioactivity precluded radioactivity analysis by acrylamide gel electrophoresis, the membranes were fractionated by column chromatography in SDS on Sepharose 4B. Comparison of the radioactivity and absorbance profiles gave no indication of an enhanced specificity of reaction of low molecular weight components even at these low concentrations (Figure 5). Therefore, the reaction of N-ethylmaleimide must be considerably less specific than the reaction with iodoacetate and does not show a strong concentration dependence of specificity.

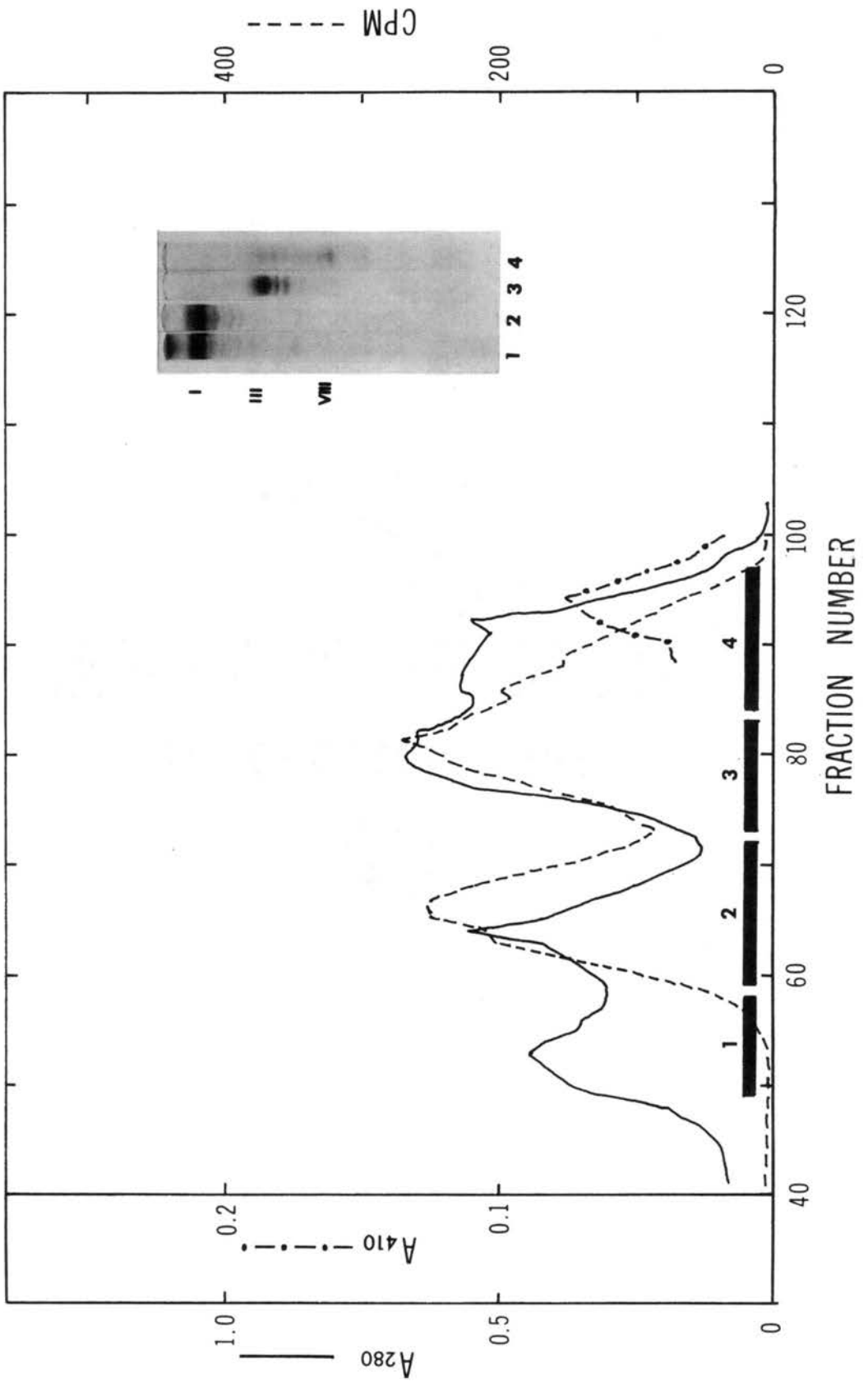
In order to show clearly that band VIII corresponded to the labeled component in IAA-labeling, a sample of membranes from labeled resealed ghosts was subjected to electrophoresis. The gels were stained with Coomassie blue and the individual bands were sliced from the gels and counted. Bands I and VIII contained the bulk of the radioactivity, in agreement with the radioactivity profile of the membranes from labeled resealed ghosts (Figure 1).

B. Partial Characterization of the Labeled Band

The labeling and electrophoresis studies indicate that a single protein of the erythrocyte membrane can be heavily labeled with iodoacetate. However, because of possible anomalies which could arise in the labeling or electrophoresis experiments, it was desirable to use other methods to verify the nature of the labeled component. Several experiments were performed to confirm that the labeled component was not loosely bound to the membrane. Labeled membranes were subjected in separate experiments to repeated washings with 7 mM phosphate (pH 7.4),

gure 5. Sepharose 4B Fractionation of ^{14}C-N-ethylmaleimide Labeled Erythrocyte Membranes and SDS Acrylamide Gel Electrophoresis of Fractions From Sepharose 4B Column

Erythrocyte membranes (2.3 mg protein/ml) were labeled with ^{14}C-NEM pecific activity, 2.1 mCi per mmole) at a concentration of 10 μM at ° C for 2 hours. The membranes in which 0.5 nmoles NEM was incorpo-ted was subjected to SDS-Sepharose gel filtration as described in Ex-rimental Procedure. Protein (A_{280} ———), radioactivity (CPM — — —) d hemoglobin (A_{410} — · —) were monitored on 3 ml fractions. Fractions noted by the black bars were collected and subjected to SDS acrylamide l electrophoresis. Protein bands separated by SDS acrylamide gel ectrophoresis are shown on the right upper region of the figure.


CPM
400
200
0
A280
A410
1.0
0.5
0
0.2
0.1
40
60
80
100
120
FRACTION NUMBER
1
2
3
4
I
III
VIII
1 2 3 4

dialysis at 4° C against distilled water (24 hours) and extraction with 8% mercaptoethanol at pH 8.0 at room temperature (18 hours). In none of these cases was the radioactive profile of the membranes altered.

Additional characterization experiments to show the protein nature of the labeled resealed ghosts were performed on membranes isolated from IAA-labeled resealed ghosts. Membranes were solubilized in 1% SDS and chromatographed on a Bio-Gel P-100 column, a procedure which separates the membrane protein from the lipid (57). Essentially all of the radioactivity (> 95%) was eluted in the protein peak at the excluded volume of the column (Figure 6). Combined fractions of the protein peak were subjected to a second chromatographic analysis on a Sepharose 4B column in 1% SDS. The protein and radioactivity profiles of this peak are shown in Figure 7. Fractions were collected from the major radioactive peak for electrophoretic analysis and identification of the labeled amino acid. SDS electrophoresis of this material showed the presence of several protein bands stained by Coomassie blue. One of these (corresponding to the migration position of the component VIII), contained most of the radioactivity (Figure 8).

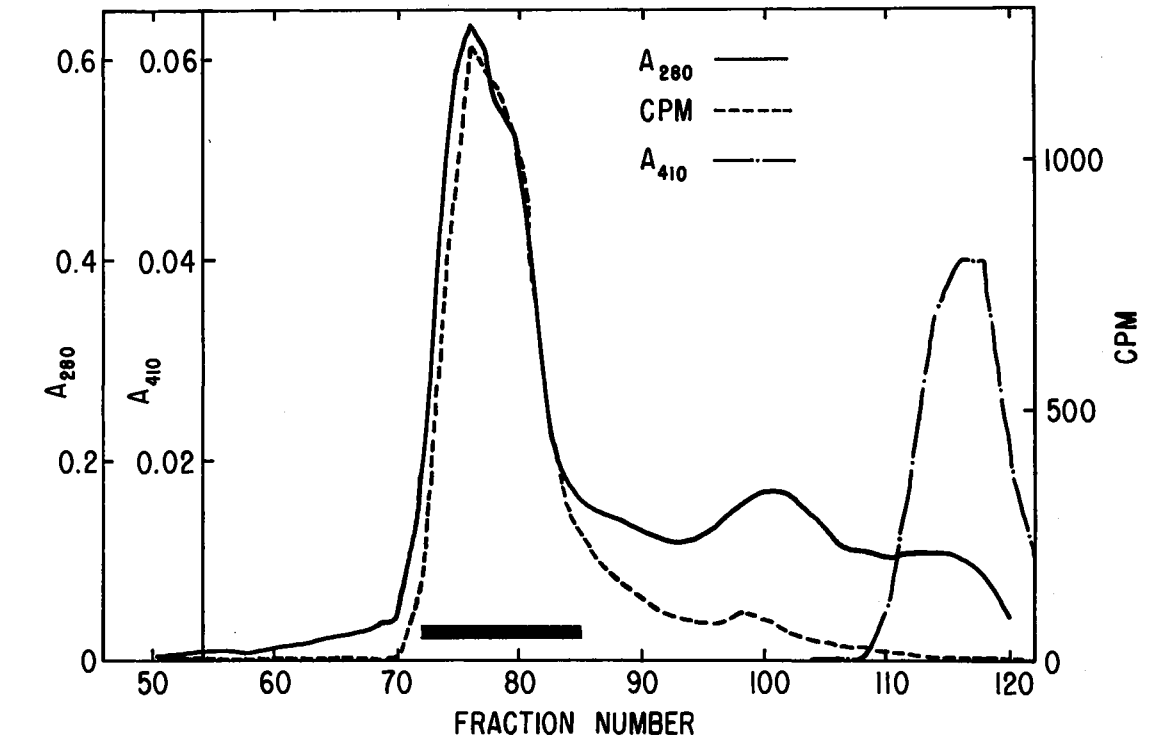
Identification of the labeled amino acid was achieved by chromatography on the long column of a Beckman 120 C amino acid analyzer. Two radioactive peaks were eluted (Figure 9). The major peak (90%) corresponded in position to carboxymethylcysteine. The minor peak eluted slightly after the position of cysteic acid and did not correspond to any known amino acid or known carboxymethyl derivative. Samples of unfractionated membrane protein from labeled ghosts or resealed ghosts were also analyzed for their labeled amino acids. These showed a peak corresponding to the position of carboxymethylhistidine as well as

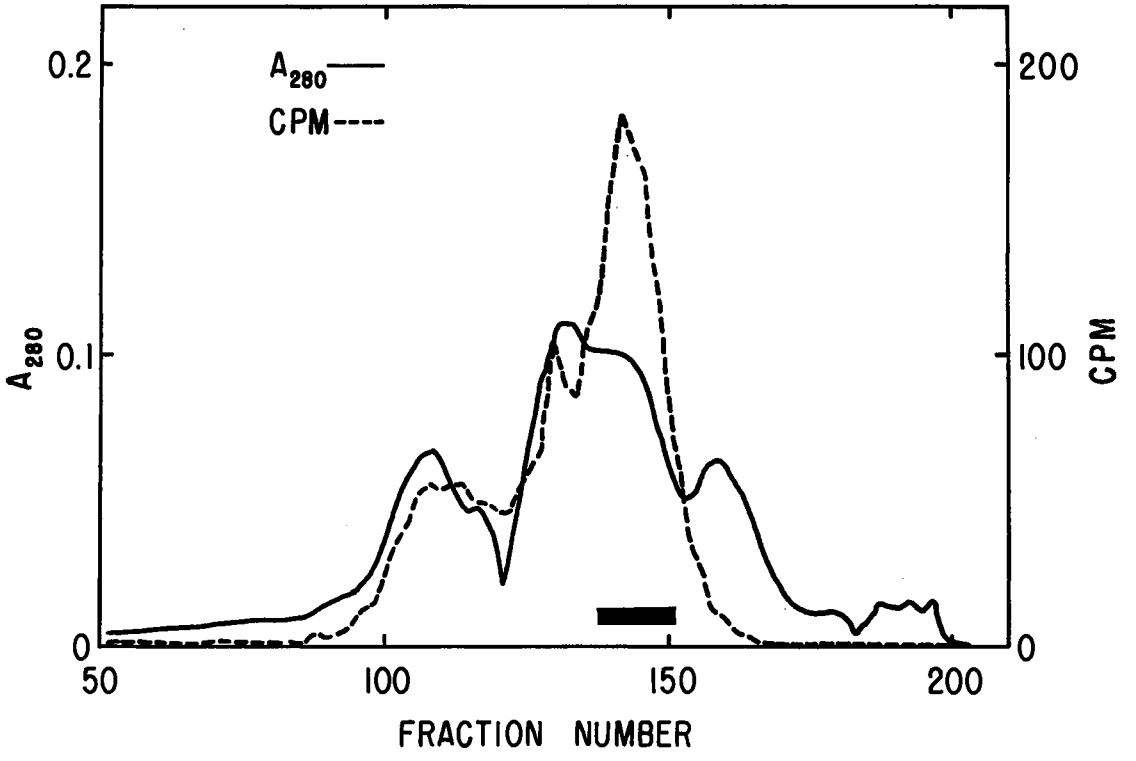
gure 6. Bio Gel P-100 Fractionation of ^{14}C-iodoacetate Labeled Erythrocyte Membranes

Membranes from ^{14}C-labeled resealed ghosts were separated into pro-in and lipid fractions on Bio Gel P-100 gel filtration in 1% SDS. The otein fraction denoted by a black bar was collected, prepared and sub-cted to the Sepharose 4B gel filtration of Figure 7 as described in perimental Procedure.

gure 7. Sepharose 4B Fractionation of Protein From Labeled Resealed Ghosts

The protein fraction from Figure 6 was collected, prepared and romatographed on Sepharose 4B column as described in Experimental Pro-dure. Protein (A_{280} ———) and radioactivity (cpm — — —) were moni-red on 3 ml fractions. Fractions denoted by the black bar were llected for SDS acrylamide electrophoresis and identification of the beled amino acid.


A280
CPM
A410
0.6
0.4
0.2
0
0.06
0.04
0.02
1000
500
0
50
60
70
80
90
100
110
120
FRACTION NUMBER

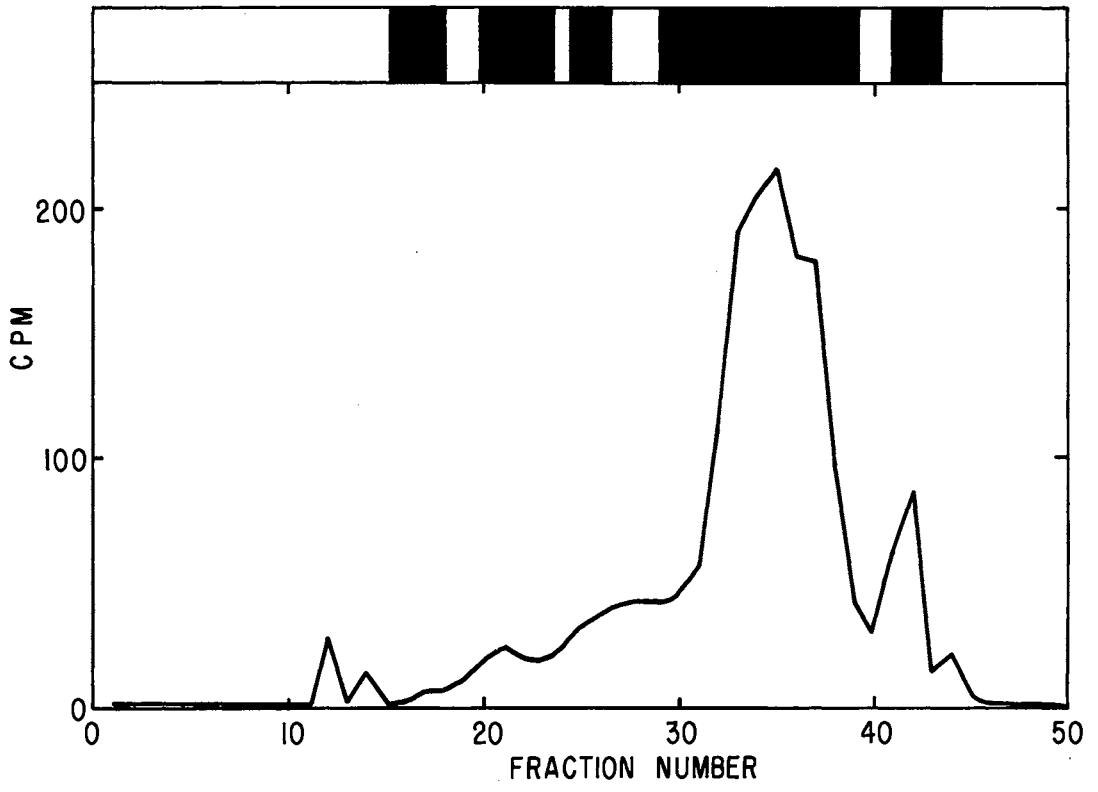

A280
CPM
0.2
0.1
0
200
100
0
50
100
150
200
FRACTION NUMBER

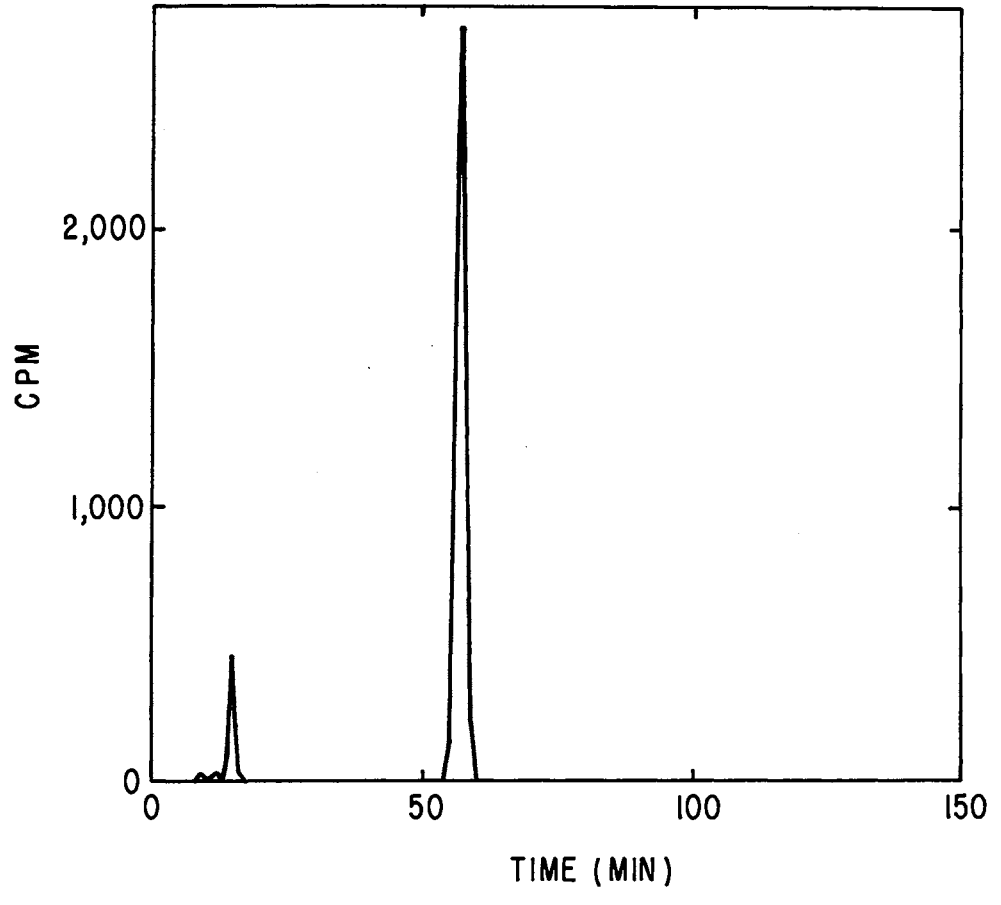
ɜure 8. Radioactive Profile and Protein Bands Separated by SDS Acrylamide Gel Electrophoresis of a Major Radioactive Peak From Figure 7

The major radioactive peak of Figure 7 was pooled, prepared and sub-ɔcted to SDS acrylamide gel electrophoresis as described in Experimental ɔcedure. The major radioactive peak of this figure corresponded to the ɜration position of the component VIII.

ɜure 9. Identification of the Labeled Amino Acid by Chromatography on the Long Column of a Beckman 120 C Amino Acid Analyzer

The major radioactive peak of Figure 7 was dialyzed to remove SDS ɨ lyophilized. The lyophilized sample was acid-hydrolyzed and loaded the column of Beckman amino acid analyzer. The fractions from a ɑlyzer column were collected and counted for the radioactivity as des-ɨbed in Experimental Procedure. The analyzer column was calibrated th radioactive standard amino acids including carboxymethyl cysteine fore and after the sample run. The major peak corresponded to the ɹtion position of carboxymethyl cysteine.


CPM
200
100
0
0
10
20
30
40
50
FRACTION NUMBER


CPM
2,000
1,000
0
0
50
100
150
TIME (MIN)

carboxymethylcysteine.

C. Purification of the Labeled Protein

Previous studies (11) from this laboratory have shown that component VIII can be extracted from the isolated membrane by dialysis against 0.3 M sodium chloride, 0.5 mM EDTA and 5 mM mercaptoethanol at pH 9.5. An even more specific procedure, using a brief exposure to 0.5 M sodium chloride, has been reported by Fairbanks, et al. (21). Comparison of electrophoretic patterns suggested that the two procedures extracted the same protein. Therefore the sodium chloride extraction procedure was investigated as a means of purifying the labeled component. Membranes from IAA-labeled red cells were extracted according to a modified procedure of Fairbanks, et al. (21). About 16 percent of protein and 54 percent of ^{14}C radioactivity was extracted into the supernatant from the ^{14}C-IAA labeled membranes by 1 M NaCl (Table II). The supernatant was found to have about 3 times higher ^{14}C-specific activity than that of the original membranes. Because the hemolysis did not remove all of hemoglobin from the membranes and the extraction with NaCl effectively removed hemoglobin from the membranes, the ^{14}C-specific activity of the labeled protein should be higher than the figure shown in Table II, and it was not possible to quantitate the percentage of membrane protein extracted. However, it was shown that 54% of the membrane-bound radioactivity was extracted. SDS acrylamide electrophoresis showed two protein bands (Figure 10) corresponding to hemoglobin and component VIII, with essentially all of the radioactivity present in the latter. For purification of sufficient quantities of protein for analytical purposes samples of labeled and unlabeled sodium chloride extracts were mixed,

TABLE II

DISTRIBUTION OF PROTEIN AND RADIOACTIVITY OF ^{14}C-IODOACETATE LABELED ERYTHROCYTE MEMBRANES AFTER EXTRACTION WITH 1.0 M NaCl[1]

Components	Whole Ghosts	Supernatant Solution	Pellet
Protein (mg)	31.9 (100)	5.1 (16)	26.8 (84)
^{14}C Activity (cpm)	404,000 (100)	218,000 (54)	187,000 (46)
Specific Activity (cpm/mg protein)	12,700	42,700	7,000

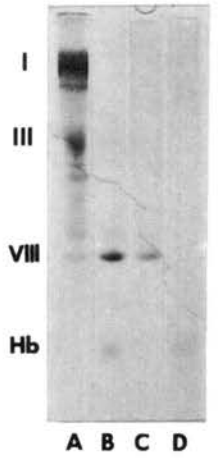
[1]Five ml of intact erythrocytes was labeled with 98 μM ^{14}C-IAA (specific activity 13.9 mCi per mmole) in 10 ml of Krebs Ringer at 37° C for 90 minutes. Membranes were prepared from the ^{14}C-IAA-labeled erythrocytes as described in Experimental Procedure. The packed, ^{14}C-IAA labeled membranes were extracted with two volumes of 1 M NaCl - 1 mM phosphate buffer (pH 8.0) for 20 minutes at 4° C. The supernatant and the pellet were dialyzed against distilled water 4° C and assayed for protein and radioactivity. Numbers in brackets represent percentage of the total amounts.

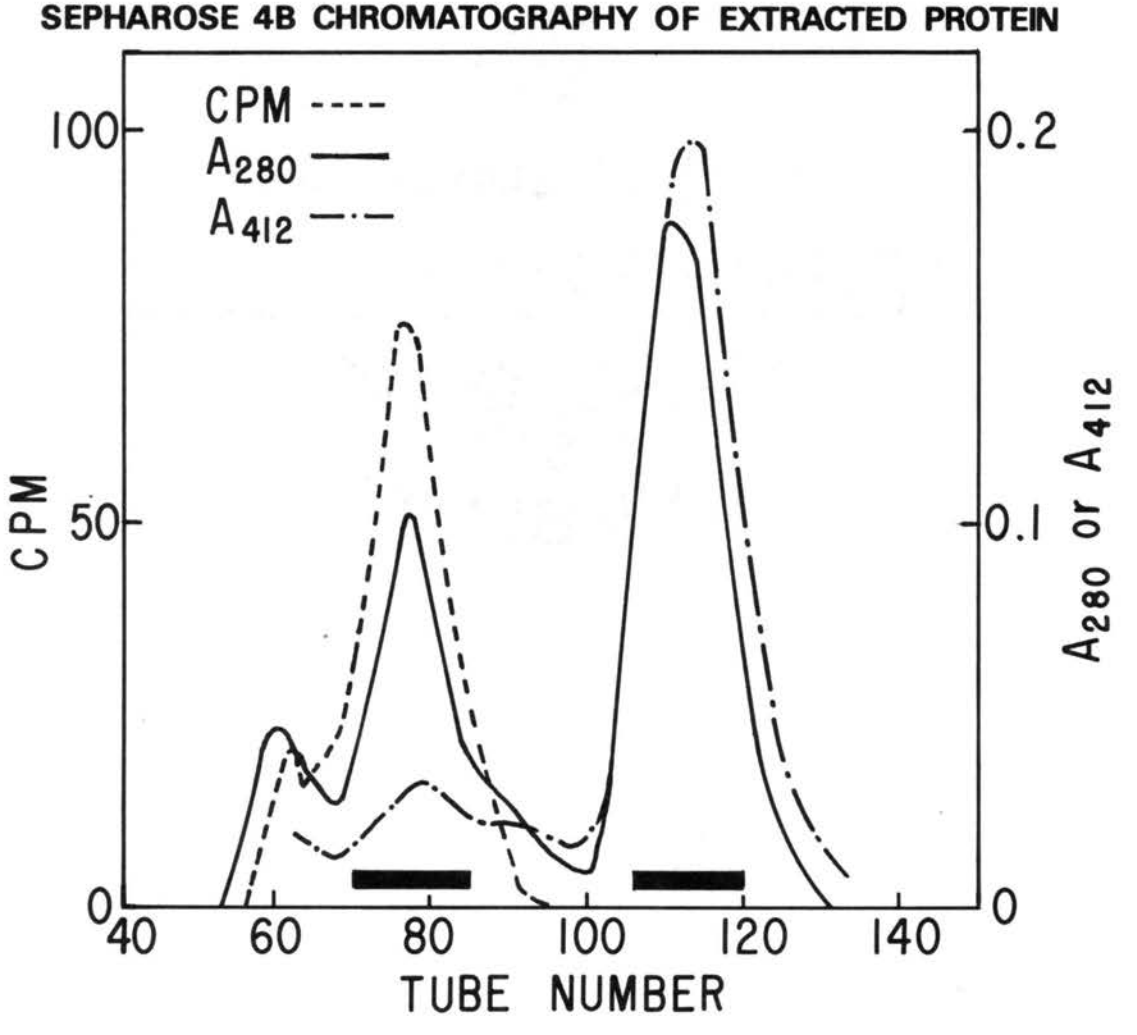
ɜure 10. SDS Acrylamide Gel Electrophoresis of Purified Fractions of Membrane Protein

Gels contain (left to right): A, isolated erythrocyte membranes; B, lium chloride extract; C, purified component VIII after Sephadex G-200 romatography; and D, fraction II (globin) from G-200 chromatograph.

ɜure 11. Purification of Labeled Component VIII by Chromatography on G-200 in 1% SDS

The labeled component was extracted from erythrocyte membranes with 5 M sodium chloride. The extracts were dialyzed at 4° C against distlled water to remove salt and lyophilized. The sample was dissolved in ffered 1% SDS and chromatographed on G-200. Fractions of 3 ml were llected and analyzed for protein (A_{280} ———), heme (A_{410} — · —) and lioactivity (cpm — — —). Fractions denoted by the black bars were comıed for electrophoresis and amino acid analysis.


I
III
VIII
Hb
A B C D


SEPHAROSE 4B CHROMATOGRAPHY OF EXTRACTED PROTEIN
CPM ----
A280 ——
A412 —·—
100
50
0
CPM
0.2
0.1
0
A280 or A412
40
60
80
100
120
140
TUBE NUMBER

solubilized in SDS and subjected to chromatography in 1% SDS on G-200. The elution profiles for protein (A_{280}), heme (A_{410}) and radioactivity are shown in Figure 11. The elution position of the labeled protein corresponds to a molecular weight of 36,000. Because heme and globin were dissociated in the SDS solution, their peaks do not coincide, although they fortuitously overlap because of the aggregate size of the heme-SDS complex. It is interesting to note that the globin was almost completely devoid of radioactivity. The fractions indicated were combined for further analysis. SDS electrophoresis of the labeled protein showed only a single band for both protein stain (gel "C" of Figure 10) and for radioactivity, indicating that the polypeptide was pure by this criterion. The amino acid analysis of the purified component VIII is shown in Table III. It is significantly different from the analyses for whole erythrocyte membranes (11) and for spectrin, the erythrocyte membrane protein fraction which is extracted from the membranes with EDTA (58). Of particular interest was the half-cystine content of component VIII. Performic acid oxidation and analysis for cysteic acid gave a value of 1.0 mole percent, corresponding to 2.9 moles of half-cystine per mole of protein. The combined fractions of the second peak from Sephadex G-200 were also subjected to amino acid analysis to confirm its identification as globin. A sample of the purified component VIII was hydrolyzed for identification of the labeled amino acid. Carboxymethylcysteine was identified by radioactivity analysis of the eluent from a Beckman 120 C long column.

In order to estimate the molecular weight of a purified, labeled membrane protein (component VIII), the purified protein was subjected to SDS-acrylamide gel electrophoresis, SDS-Sephadex G-200 gel filtration and

TABLE III

AMINO ACID COMPOSITION OF COMPONENT VIII, SPECTRIN AND HUMAN ERYTHROCYTE MEMBRANES[1]

Amino Acid	Component VIII	Spectrin	Membranes[5]
	as mole % (residues/molecule)[2]		
Lysine	8.36 (23.5)	6.66	5.12
Histidine	3.41 (9.6)	2.83	2.57
Arginine	3.32 (9.3)	5.92	4.00
Aspartic Acid	10.98 (30.9)	10.43	8.98
Threonine[3]	5.87 (16.5)	4.29	5.24
Serine[3]	6.31 (17.8)	5.87	7.88
Glutamic Acid	7.66 (21.6)	18.03	13.41
Proline	3.95 (11.1)	2.31	4.78
Glycine	10.36 (29.2)	4.70	6.31
Alanine	10.29 (29.0)	9.12	8.29
Half-cystine[4]	1.04 (2.9)	0.89	0.34
Valine[5]	8.27 (23.3)	4.45	5.40
Methionine	2.07 (5.8)	1.82	1.96
Isoleucine[5]	5.52 (15.5)	3.65	3.77
Leucine	7.12 (20.0)	12.01	12.28
Tyrosine	1.90 (5.3)	2.04	2.13
Phenylalanine	3.77 (10.6)	3.25	3.37
Tryptophan[3]	--- (---)	1.73	n.d.

[1]Except where noted, all figures are the average of one 22 hour hydrolysis in 6 N HCl and one 22 hour hydrolysis in 3 N p-toluenesulfonic acid.

[2]Calculations based on molecular weight of 35,000.

[3]Values obtained from 22 hour hydrolysis with 3 N p-toluenesulfonic acid.

[4]Values obtained as cysteic acid by method of Hirs and corrected for oxidation losses by comparison with ribonuclease samples hydrolyzed under identical conditions.

[5]Values obtained from 22 hour hydrolysis in 6 N HCl.

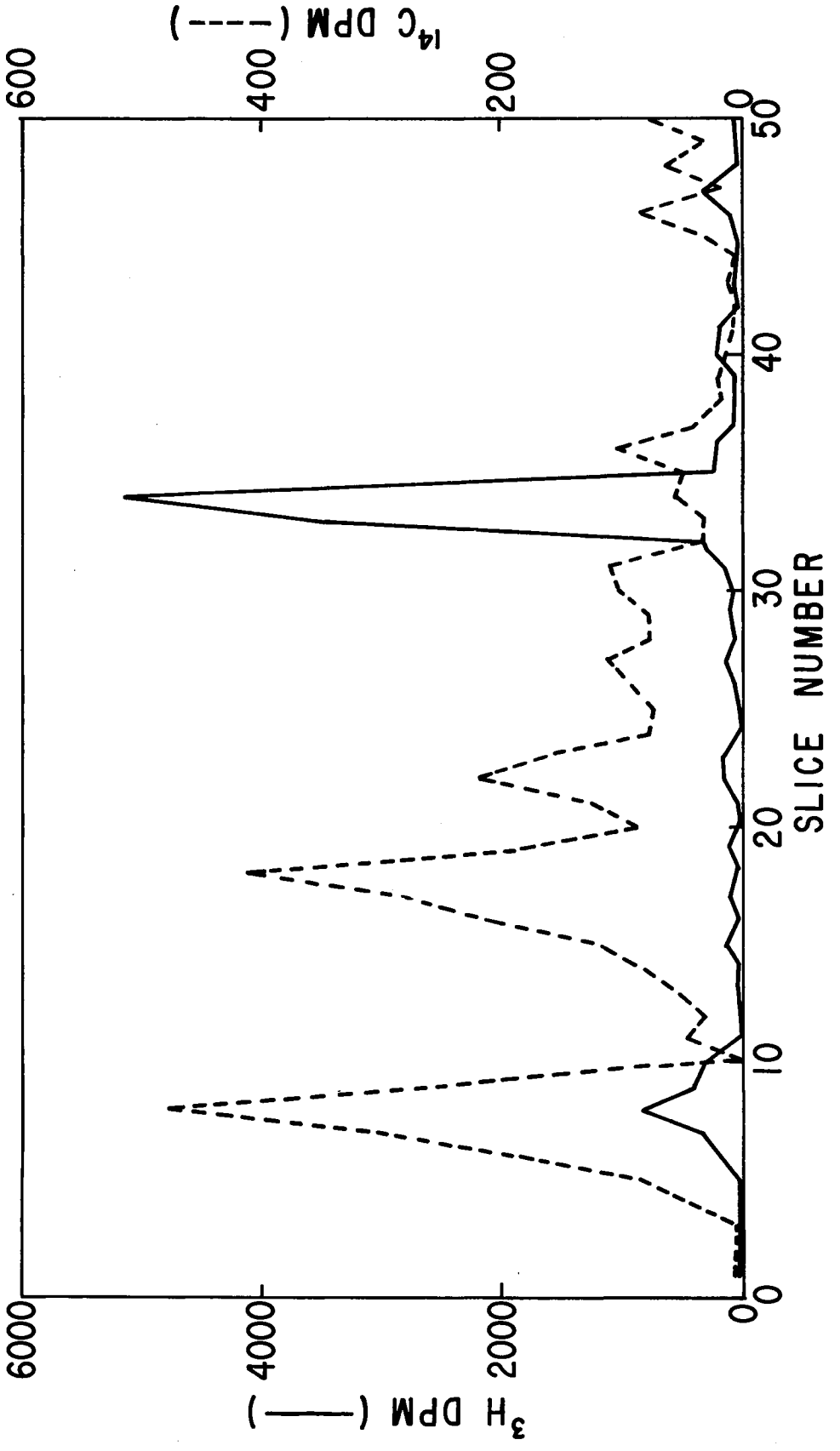
6 M guanidine HCl-Sepharose 4B gel filtration, using proteins of known molecular weights as molecular weight standards. The molecular weight of the purified protein (component VIII) was estimated as 35,000 to 40,000 by SDS-acrylamide gel electrophoresis, SDS-Sephadex G-200 gel filtration or 6 M guanidine HCl-Sepharose 4B gel filtration.

D. Reactivity of Component VIII in the Erythrocyte Membrane

A consideration of the concentration dependence of specificity of iodoacetate labeling of erythrocyte membrane (Figure 4) suggested that component VIII was almost completely saturated with label before any other membrane protein begins to react. In order to check this possibility a double labeling experiment (40) was performed using ^{14}C- and ^{3}H-labeled iodoacetic acid. Intact erythrocytes were reacted with ^{3}H-iodoacetate as previously described, and the labeled membranes were isolated. These membranes were then labeled with ^{14}C-iodoacetate at a higher reagent concentration than usually used. After being washed to remove unreacted reagent, the membranes were analyzed by SDS acrylamide electrophoresis. The radioactivity profile (Figure 12) of the isolated membrane shows that only component VIII was labeled to a significant extent in the reaction of the intact cell with ^{3}H-iodoacetate. This component is not labeled in the second reaction even though the other membrane proteins are heavily labeled. Thus, essentially all of the reactive groups of component VIII were blocked before there was significant reaction with other protein groups.

gure 12. Double Labeling of Erythrocyte Membranes With Radioactive Iodoacetate

Washed erythrocytes (33% hematocrit) were labeled with ^{3}H-doacetate (0.59 mM), washed and hemolyzed. The isolated membranes were beled a second time with ^{14}C-iodoacetate (0.75 mM). The washed membranes were fractionated by SDS acrylamide electrophoresis; ^{3}H, (———) d ^{14}C (— — —).


^{14}C DPM (----)
600
400
200
0
50
40
30
20
10
0
SLICE NUMBER
6000
4000
2000
0
^{3}H DPM (——)

E. Characterization of the Glyceraldehyde-3-phosphate Dehydrogenase

In order to characterize an erythrocyte membrane protein, component VIII, several experiments were performed. The supernatant solution of the NaCl-extracted erythrocyte membranes was assayed for enzyme activities, along with the pellet and the original membranes. About 86 percent of glyceraldehyde phosphate dehydrogenase (GAPD) and aldolase, and 20 percent of acetlcholinesterase were extracted into the supernatant solution from the membranes by 1 M NaCl (Table IV). The relationship between enzyme activities and ^{14}C-IAA incorporation was studied with erythrocyte membranes labeled with ^{14}C-IAA of different concentrations. Among membrane enzymes studied only GAPD activity was inversely proportional to the amount of ^{14}C-IAA incorporated into the membranes (Figure 13), suggesting that IAA can react with SH groups of GAPD enzyme to inactivate the enzyme activity. Activities of acetylcholinesterase, aldolase and lactic dehydrogenase were not inactivated, but often slightly increased.

In the iodoacetate labeling studies only a single protein of the human erythrocyte membranes was shown to be labeled by iodoacetate (Figure 4). This labeled protein (Table II) and the GAPD activity (Table IV) could be extracted from the membranes with 1 M NaCl. The molecular weight of component VIII was 35,000 to 40,000 which corresponds to the molecular weight of tetrameric subunits of glyceraldehyde-3-phosphate dehydrogenase (59). The GAPD activity of membranes was inversely proportional to amounts of ^{14}C-IAA incorporated into the membranes. All these results indicated that the protein (component VIII) was the enzyme glyceraldehyde-3-phosphate dehydrogenase.

This same protein was extracted with 0.1 M EDTA from human

TABLE IV

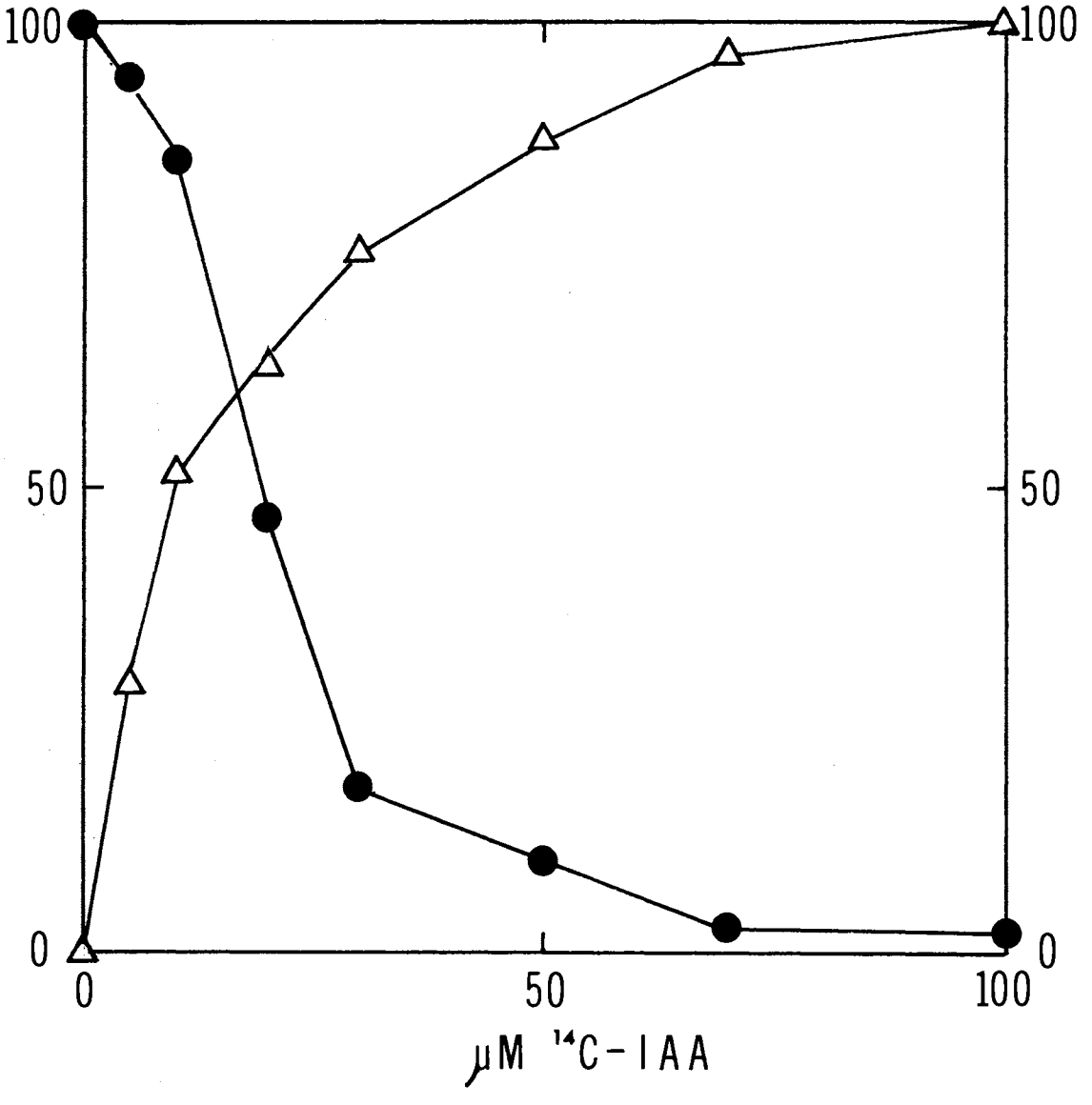
PERCENT DISTIRUBTION OF ENZYME ACTIVITIES OF ERYTHROCYTE MEMBRANES AFTER EXTRACTION WITH 1.0 M NaCl[1]

Components	Supernatant Solution	Pellet
Protein	16.6	83.4
GAPD	86.2	13.8
Aldolase	86.0	14.0
ACHE	19.5	80.5

[1]Packed erythrocyte membranes were extracted with one volume of 1 M NaCl - 7 mM phosphate buffer (pH 8.0) for 20 minutes at 4° C. The supernatant solution and the pellet of the NaCl extraction were dialyzed against 10 mM Tris buffer (pH 7.4), 4° C overnight, concentrated by Aquacide II, assayed for protein and enzyme activities. Enzyme activities of the erythrocyte membranes were 0.76 µmoles per min per mg protein for GAPD, 0.03 µmoles for aldolase, and 1.14 µmoles for acetylcholinesterase.

gure 13. Relationship Between GAPD Activity of and ^{14}C-IAA Incorporation Into Erythrocyte Membranes

One milliliter of erythrocytes was labeled with ^{14}C-IAA of different ncentrations in 2 ml of Krebs-Ringer at 25° C, 30 minutes. The memanes were prepared from the incubated erythrocytes as described in Exrimental Procedure. The isolated membranes were assayed for GAPD tivity and ^{14}C radioactivity. The GAPD (●———●) was expressed as per nt of the GAPD activity (0.836 µmoles per min per mg protein) of the ntrol membranes. The ^{14}C specific activity (Δ———Δ) was expressed as r cent of the ^{14}C specific activity (8,638 dpm per mg protein) of memanes labeled with 100 µM ^{14}C-IAA.


100
50
0
100
50
0
0
50
100
μM ¹⁴C-IAA

erythrocyte membranes by Tanner and Gray (22), who established its identity by partial amino acid sequence analysis and activity studies. As a further characterization of the erythrocyte enzyme, some other parameters of the membrane extracts have been investigated using both sodium chloride and EDTA as extractants. Table V shows analytical data on extracts obtained from isolated membranes by the two different procedures. Both methods have shown considerable variation in the amounts of protein and lipid extracted from different membrane preparations. This was due in part to the variation in the membrane preparations themselves, which contain variable amounts of hemoglobin and other extractable proteins. The bulk of the protein extracted by either method was the hemoglobin and glyceraldehyde dehydrogenase, as shown by electrophoresis (Figure 10). Smaller amounts of other proteins were also extracted. The EDTA extraction routinely yielded more consistent results in that smaller amounts of lipid and other proteins were extracted with the glyceraldehyde-3-phosphate dehydrogenase. As a result the specific activity of the enzyme was higher.

The large amount of lipid associated with the NaCl extract of Table V suggested that the enzyme glyceraldehyde-3-phosphate dehydrogenase may also interact with lipid to some extent in binding to the membrane. Therefore a sample of the extract was chromatographed on Sephadex G-200 to determine the size of the enzyme isolated and the presence of lipid. Figure 14 shows that the glyceraldehyde-3-phosphate dehydrogenase activity was eluted as a broad peak. The molecular weight, calculated from a column calibration curve, was 150,000, consistent with the monomeric weight of 35-37,000 found by either SDS electrophoresis or SDS-gel filtration and a tetrameric native structure. The broadness of the peak

TABLE V

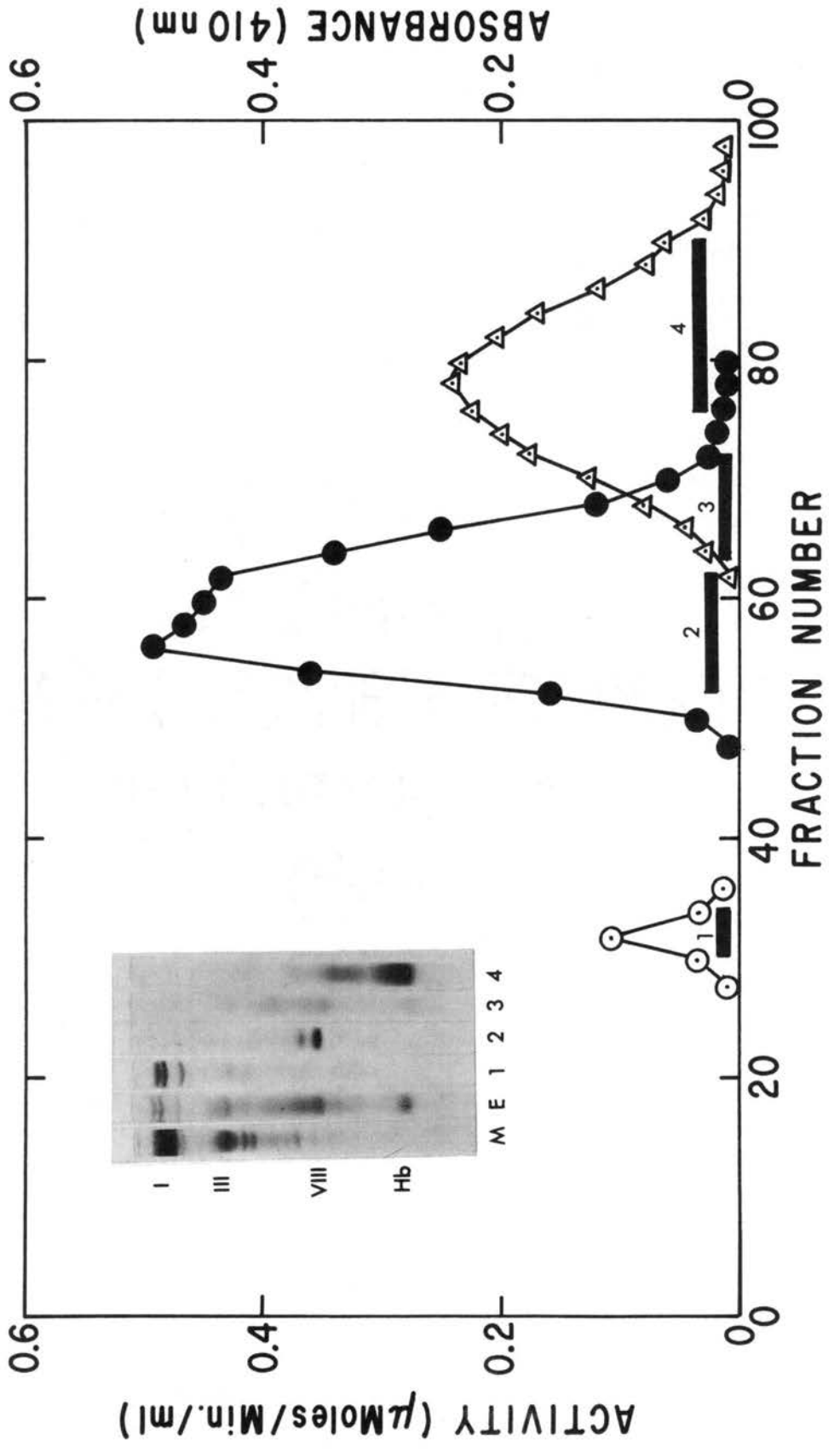
DISTRIBUTION OF ENZYMES AND CHEMICAL COMPONENTS OF ERYTHROCYTE MEMBRANE AFTER EXTRACTION WITH NaCl OR EDTA[1]

Component	NaCl Extract			EDTA Extract	
	1st Supernatant Solution	2nd Supernatant Solution	Pellet	Supernatant Solution	Pellet
Protein	28	1.5	70 (82.6)	18	82 (74.8)
Cholesterol	11	2.2	84 (17.7)	0.3	100 (14.8)
Phosphorus	14	6.8	74 (9.9)	2.9	97 (5.4)
Glyceraldehyde-3-phosphate Dehydrogenase	97	2.8	0 (59.7)	85	15 (42.5)
Lactic Dehydrogenase	98	2.4	0 (4.6)	---	---
Acetylcholin-esterase	20	5.7	75 (82.1)	4.2	96 (85.0)

[1]Membranes were extracted as described in Experimental Procedure. Enzyme assays were determined directly on extracts or pellets. Chemical analyses were performed after dialysis against distilled water. All assays were done in duplicate. Values are expressed as a percentage of the total amount recovered. The values in parentheses are the absolute total quantities for each analysis. These are expressed in mg for protein and cholesterol, μmoles for phosphorus and μmoles/min for each enzyme.

gure 14. Column Chromatography of NaCl-extract of Erythrocyte Membranes on Sephadex G-200

Isolation and chromatography are described in the Experimental Procedure. Combined fractions from the column were analyzed for phosphrous, d cholesterol and subjected to SDS acrylamide electrophoresis. Acetylolinesterase (O———O), glyceraldehyde-3-phosphate dehydrogenase ———●) and hemoglobin (Δ———Δ). Fractions denoted by the black bars re collected and subjected to SDS-acrylamide gel electrophoresis. Proin bands separated by SDS-electrophoresis are shown on the left upper gion of the figure. Gel M, original membranes, and gel E, the supertant solution of NaCl extracts of membranes.

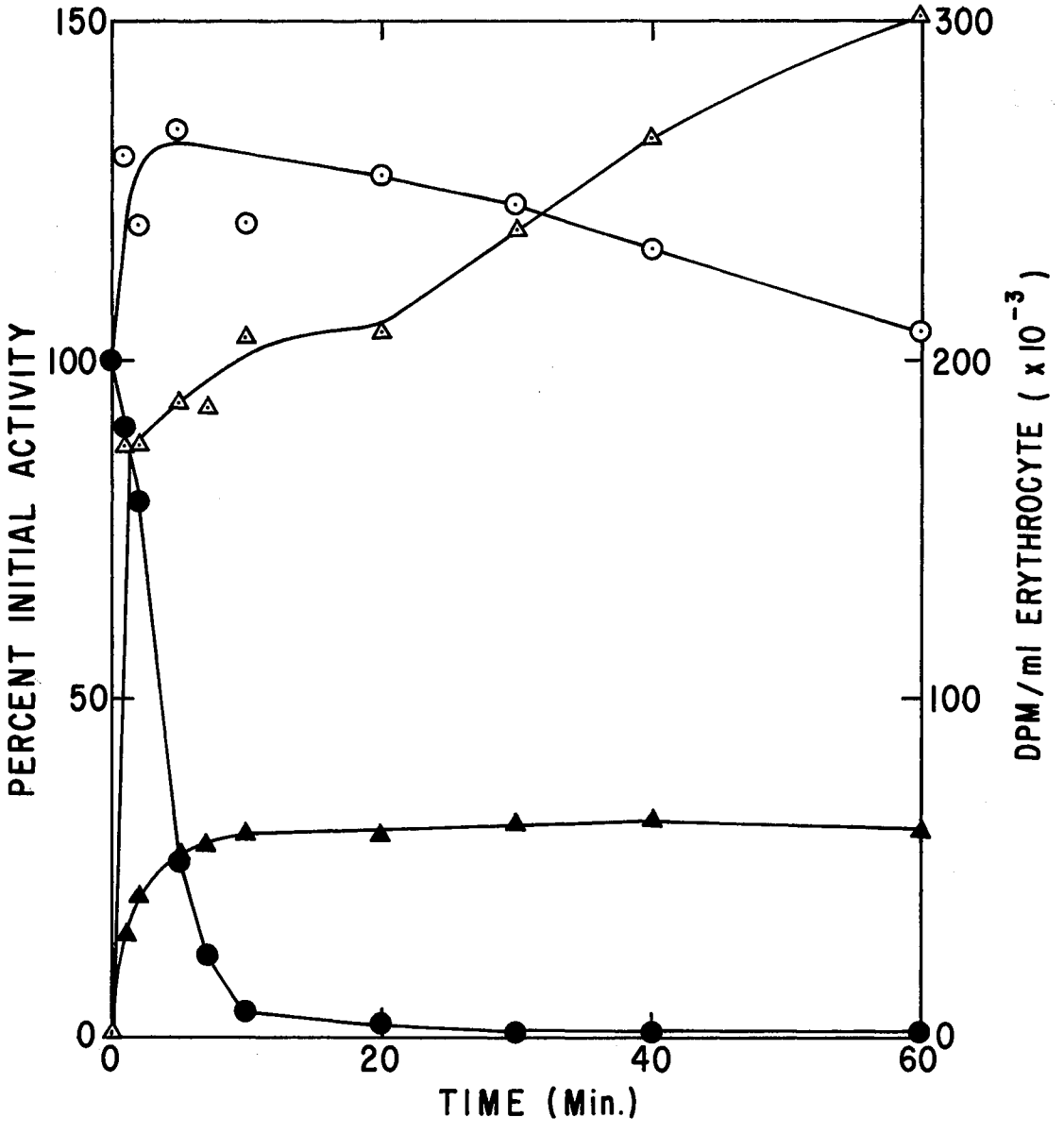

ACTIVITY (μMoles/Min./ml)
ABSORBANCE (410 nm)
FRACTION NUMBER
0.6
0.4
0.2
0
20
40
60
80
100
1
2
3
4
I
III
VIII
Hb
M E 1 2 3 4

and the skew toward lower molecular weight suggest that some dissociation may occur in this system. The native molecular weight (150,000) was confirmed by sucrose density gradient centrifugation, which gave a sharper, more symmetrical peak. Acetylcholinesterase activity was eluted at the void volume of the column. All of the detectable lipid eluted from the column was present in this fraction. SDS electrophoresis indicated that the major protein eluted in this excluded fraction was component I (spectrin), the large polypeptide component of the erythrocyte membrane (4). This does not imply that spectrin associates with lipid or acetylcholinesterase, but merely that both are aggregated under the conditions of chromatography. The association of acetylcholinesterase and lipid does seem likely, since it has been previously shown by Burger, et al. (60) for bovine erythrocyte membrane acetylcholinesterase. It should be noted that increased salt extraction of acetylcholinesterase was paralleled by increased lipid release from the membrane. This is behavior which would be expected of a membrane enzyme which is associated with the membrane lipid. Both column chromatography and sucrose density gradient centrifugation indicated that the glyceraldehyde-3-phosphate dehydrogenase was extracted from the membrane in a nonaggregated form with a native molecular weight similar to that observed for this enzyme from other sources, while the acetylcholinesterase was released in an aggregated form with lipid.

The reaction of iodoacetate with erythrocyte membranes of intact erythrocytes was strongly directed toward a single protein. That this specificity was due to a highly specific reation of glyceraldehyde-3-phosphate dehydrogenase can be seen from Figure 15. The incorporation of the label into the membrane closely paralleled glyceraldehyde-3-phosphate

gure 15. Incorporation of Iodoacetate Into Membrane and Hemoglobin of Intact Erythrocyte and its Effect on Glyceraldehyde-3-phosphate Dehydrogenase and Acetylcholinesterase

Reaction was performed with 100 μM ^{14}C-iodoacetate (specific activ-y, 6.45 mCi per mmole) at 25° C as previously described (4). Acetyl-olinesterase activity (O———O), glyceraldehyde-3-phosphate hydrogenase activity (●———●), hemoglobin labeling (Δ———Δ) and mem-ane labeling (▲———▲). The initial activity of acetylcholinesterase s 0.698 μmole per min per mg membrane protein and that of GAPD was 0.72 oles per min per mg protein.


150
100
50
0
300
200
100
0
0
20
40
60
PERCENT INITIAL ACTIVITY
DPM/ml ERYTHROCYTE (x 10⁻³)
TIME (Min.)

dehydrogenase inactivation. Two other interesting facets of the iodoacetate reaction with erythrocytes were noted. The reaction with the intracellular soluble proteins (primarily hemoglobin) was multiphasic and the activity of membrane acetylcholinesterase was enhanced by the iodoacetate treatment. A similar enhancement of membrane-bound aldolase activity was also noted under similar conditions. The high reactivity of glyceraldehyde-3-phosphate dehydrogenase relative to the other membrane proteins and hemoglobin suggested that it might occupy a unique location along or near an anion permeation pathway in the membrane. Therefore labeling experiments were conducted on cells which had been broken by freezing and thawing to eliminate the membrane barrier. No differences were noted in iodoacetate incorporation into either hemoglobin or glyceraldehyde-3-phosphate dehydrogenase between the intact and broken cells. Therefore, it appears that the specificity of the reaction was due to the high affinity of the iodoacetate for the glyceraldehyde-3-phosphate dehydrogenase, with the reagent acting as an affinity label because of the presence of positively charged NAD at the active site, as indicated by Fenselau (61).

F. Detergent Effects on Glyceraldehyde-3-phosphate Dehydrogenase

During the course of experiments on the activity of glyceraldehyde-3-phosphate dehydrogenase in erythrocyte membranes, it was found that the enzyme activity decreased dramatically under certain conditions of incubation. This activity loss was shown to be due to resealing of the ghosts such that they were impermeable to the enzyme substrate. Proteolysis of the ghosts is a good indicator of whether resealing has

occurred, since the proteins accessible to proteolysis are different in the sealed and unsealed ghosts (33). With this prior knowledge it is possible to use glyceraldehyde-3-phosphate dehydrogenase activity as a monitor for the sealing of the membranes, although it is probably not an absolute marker because of some leakage of substrates into the membranes.

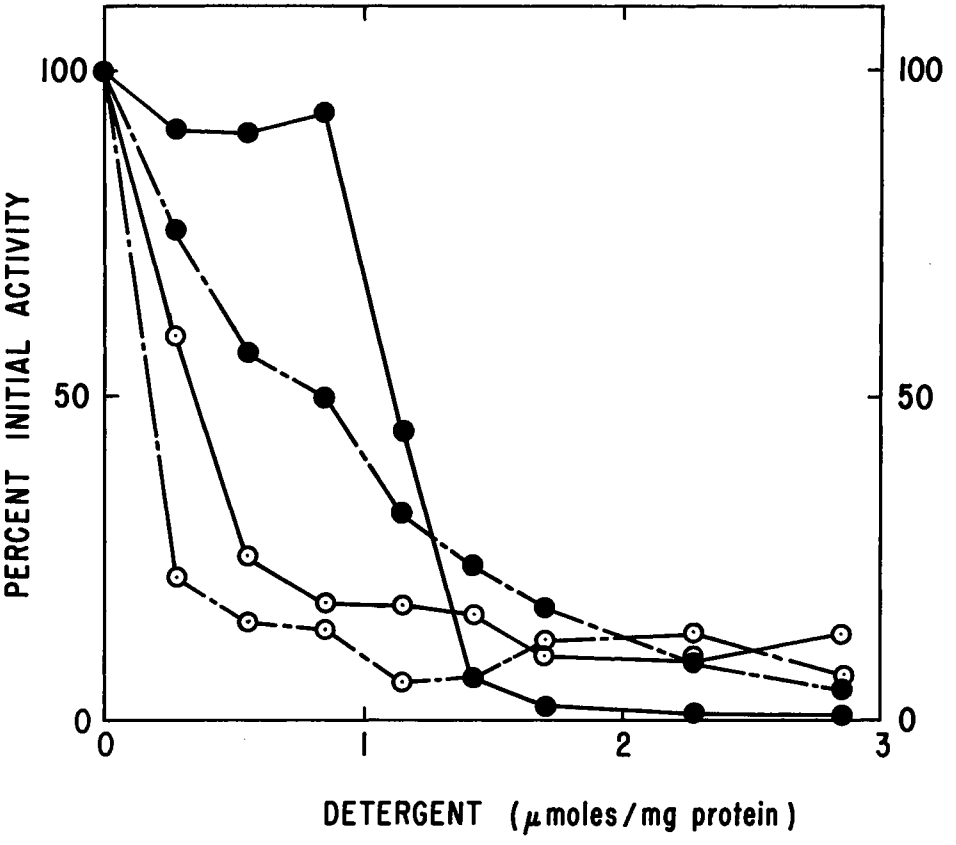
In order to investigate the association of the dehydrogenase with the membrane, it was necessary to eliminate the effect of membrane re-sealing on the activity. Since this was most readily accomplished by detergent treatment, the effects of two different common detergents on the dehydrogenase activity were investigated. The effects of sodium dodecyl sulfate and Triton X-100 on the glyceraldehyde-3-phosphate dehydrogenase activity of freshly prepared erythrocyte ghosts in distilled water are shown in Figure 16. The activity remained fairly constant up to an SDS concentration of 0.8 μmole SDS per mg protein, then dropped off at higher concentrations. Solubilized GAPD of erythrocyte membranes was also inactivated almost to the same extent by SDS as the membrane GAPD (Figure 17). The activity of the erythrocyte GAPD may not be dependent upon the association with the membrane. Triton X-100 caused some inactivation of the enzyme at all concentrations tested, exhibiting a much stronger inhibitory effect than SDS. The time course of the Triton inactivation is shown in Figure 18, indicating that there was no lag period. The effects of the two detergents were also dependent on the incubation medium. Ghosts incubated in Krebs-Ringer phosphate buffer appeared to be completely resealed (Figure 19). Addition of detergent (either SDS or Triton X-100) to ghosts in this medium was not as efficient in solubilizing the ghosts when compared to ghosts in distilled water (Figure 16). The comparisons between the two detergents are

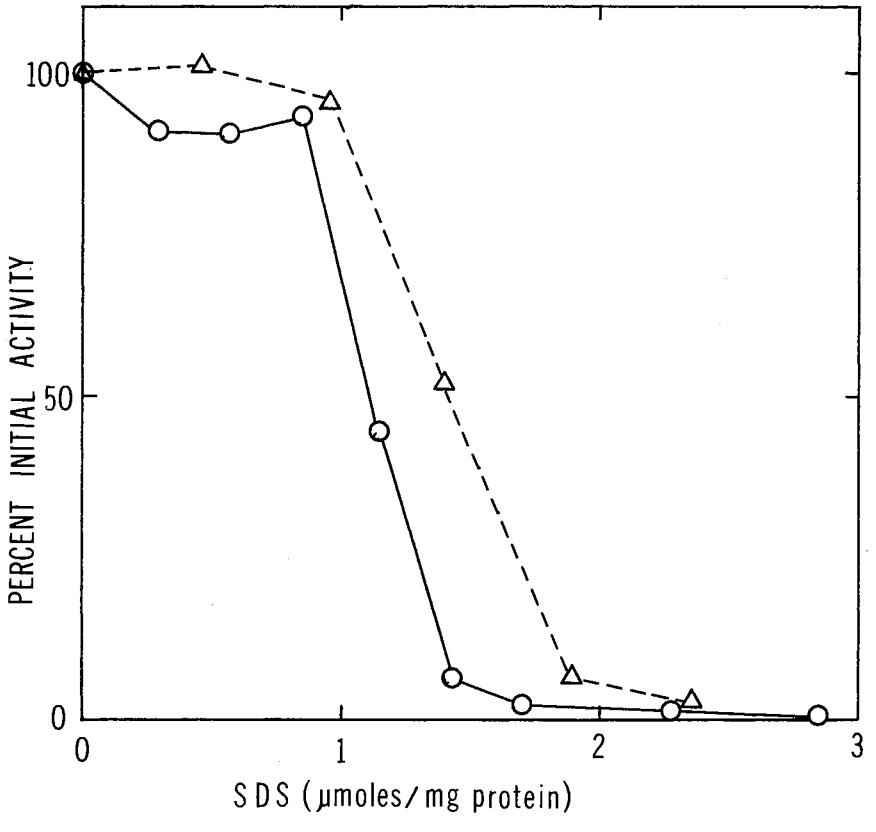
gure 16. Effect of SDS and Triton X-100 on Erythrocyte Membrane Glyceraldehyde-3-phosphate Dehydrogenase Activity in Distilled Water

One volume of fresh membranes was mixed with one volume of detergent distilled water to give final concentration of 3.5 mg/ml protein. er incubating 30 minutes, samples were assayed for glyceraldehyde-3-sphate dehydrogenase and turbidity (at 600 nm after 1:10 dilution in tilled water). Turbidity (O) and glyceraldehyde-3-phosphate dehydrase activity (●). SDS (———) and Triton X-100 (— · —). The control ivity (100%) for GAPD was 0.763 µmoles per min per mg membrane pron. The control absorbance (100%) for turbidity measurements was 67 absorbance unit for 0.35 mg membrane protein in 1 ml distilled er.

ure 17. Effect of SDS on the Solubilized and the Insoluble GAPD Activity of Erythrocyte Membranes

One volume of the packed erythrocyte membranes or the solubilized D activity was mixed with one volume of SDS of various concentrations distilled water, incubated at 25° C for 60 minutes, and then assayed GAPD activity. The erythrocyte membranes, (O———O) and the soluized enzyme (Δ— — —Δ).


100
50
0
PERCENT INITIAL ACTIVITY
0
1
2
3
DETERGENT (μmoles/mg protein)

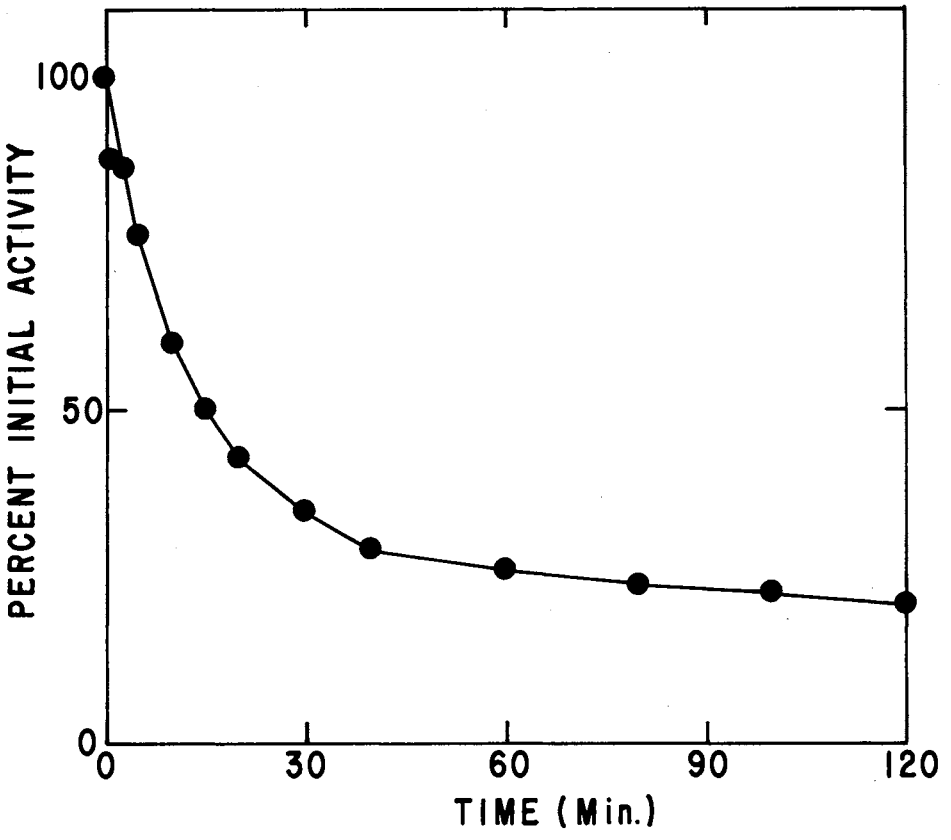

100
50
0
PERCENT INITIAL ACTIVITY
0
1
2
3
SDS (μmoles/mg protein)

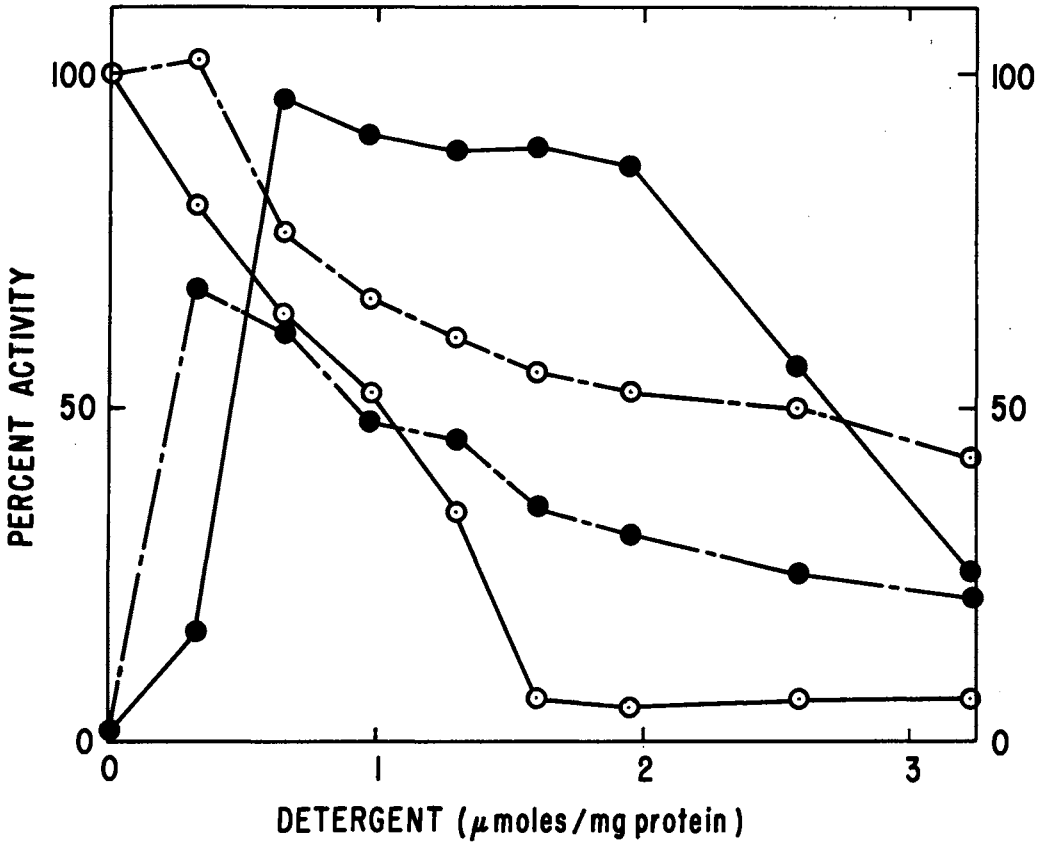
gure 18. Time Course of Triton Inactivation of Erythrocyte Membrane Glyceraldehyde-3-phosphate Dehydrogenase

Erythrocyte membranes (2.1 mg/ml protein) and 5 mM Triton X-100 were cubated at 25° C. At intervals aliquots were withdrawn for assay.

gure 19. Effect of Incubation in Krebs-Ringer on Detergent Action on Membranes

Ghosts (3.1 mg/ml protein) and detergent in Krebs-Ringer phosphate ffer (pH 7.4) were incubated at 25° C and assayed for turbidity and ıydrogenase activity. Turbidity (O), glyceraldehyde-3-phosphate ıydrogenase (●), SDS (———) and Triton (– – –). The control activity)0%) for GAPD was 0.763 μmoles per min per mg membrane protein. The ıtrol absorbance (100%) for turbidity measurements was 0.220 absorbance it for 0.31 mg membrane protein in 1 ml Krebs-Ringer phosphate.


100
50
0
PERCENT INITIAL ACTIVITY
0
30
60
90
120
TIME (Min.)


100
50
0
PERCENT ACTIVITY
100
50
0
0
1
2
3
DETERGENT (μmoles/mg protein)

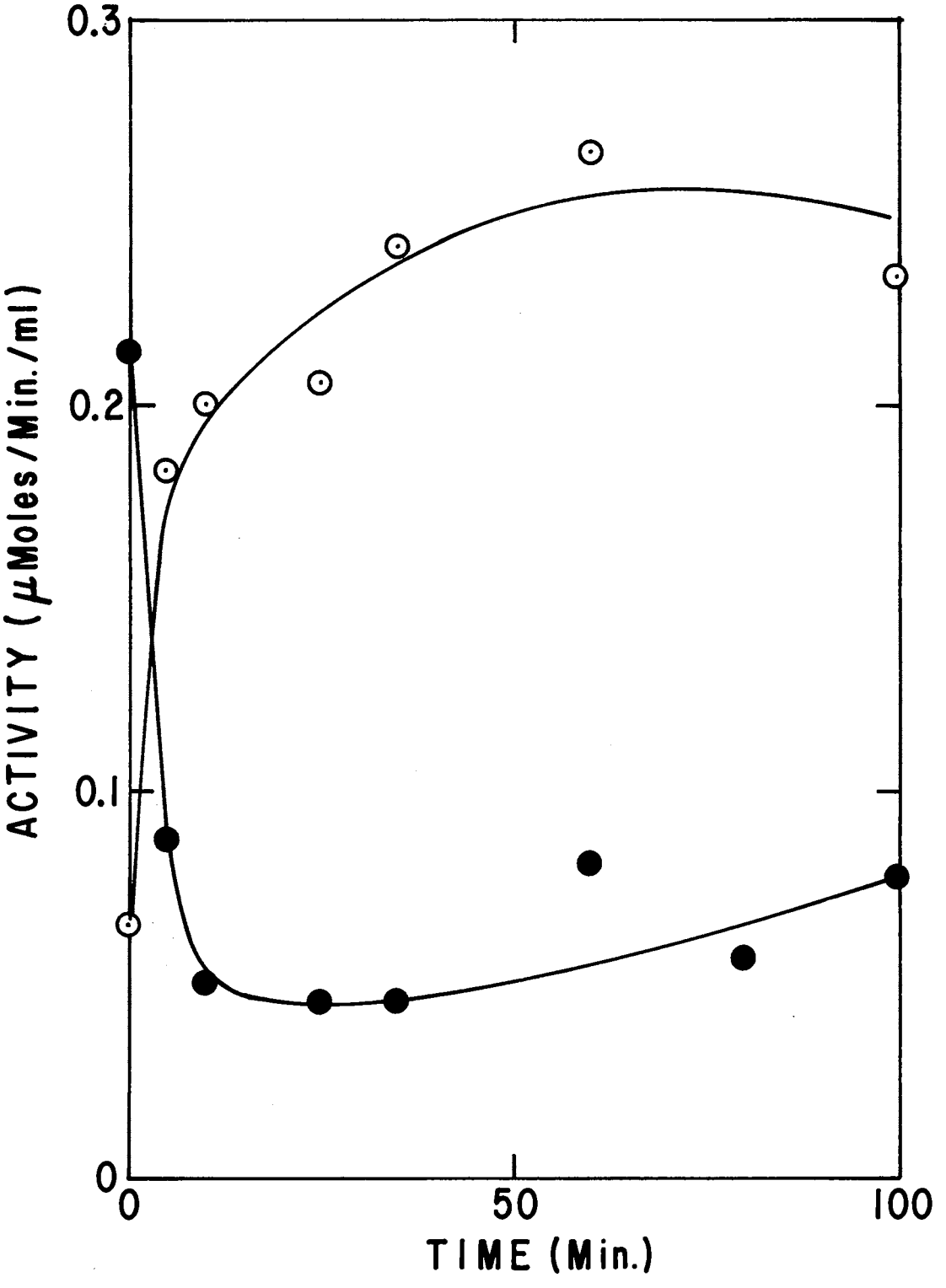
somewhat surprising, since Triton X-100 is usually considered to be the milder detergent in terms of protein denaturation and is often used to solubilize membranes for enzyme assays (29). In view of the results, further measurements of enzyme activities were performed after incubation in SDS in distilled water or other media of low ionic strength. The activities of aldolase, acetylcholinesterase and the other enzymes assayed were inhibited only slightly or not at all under these conditions. The present results also indicate the care which must be taken in the use of detergents to study membrane-associated enzymes.

G. Effect of Trypsin Treatment on Association of Glyceraldehyde-3-phosphate Dehydrogenase With Erythrocyte Membranes

Previous experiments from this laboratory (33) and by Steck, et al. (32) have shown that the polypeptide (component VIII) corresponding to the subunit of glyceraldehyde-3-phosphate dehydrogenase is very resistant to proteolysis by tripsin or chymotrypsin. This can be demonstrated by either staining of SDS acrylamide gels or iodoacetate labeling. Steck, et al. (32) have also noted that this protein was released from the erythrocyte ghost during proteolysis. In order to examine the association of the dehydrogenase with the erythrocyte membrane, the proteolysis effects were investigated further. Isolated human erythrocyte membranes were treated with trypsin and centrifuged. The pellet and supernatant samples were assayed for glyceraldehyde-3-phosphate dehydrogenase and showed a time-dependent release of the enzyme (Figure 20). However, subsequent investigations showed several discrepancies with this type of experiment. First, the release of enzyme was highly variable between

gure 20. Trypsin Release of Glyceraldehyde-3-phosphate Dehydrogenase From Erythrocyte Membranes

Ghosts (2.7 mg/ml protein) were incubated at 25° C with trypsin (10 /ml) in 7 mM phosphate (pH 7.4). At timed intervals soybean trypsin nibitor was added to a concentration of 20 µg/ml and the mixture was ntrifuged. Supernatant solution and pellet samples were assayed for yceraldehyde-3-phosphate dehydrogenase without detergent incubation. pernatant solution (O———O) and pellet (●———●).


0.3
0.2
0.1
0
ACTIVITY (μMoles/Min./ml)
0
50
100
TIME (Min.)

different membrane preparations. Second, assays of the trypsin-treated whole ghost samples (before centrifugation) showed a dramatic decrease of dehydrogenase activity, which was roughly inversely correlated with the release of the enzyme. Since the enzyme had not been removed from the suspension in this case or proteolytically cleaved, the loss of activity must be due to an effect at the membrane level rather than on the enzyme. Table VI illustrates the effect of trypsin treatment on the glyceraldehyde-3-phosphate dehydrogenase and aldolase activities of the membrane fractions. In this experiment the treated ghost sample showed a loss of about 50% of its glyceraldehyde-3-phosphate dehydrogenase activity before centrifugation. Both enzymes were released into the supernatant in the treated samples but not in the controls, and the residual treated pellet showed very little activity. In terms of the total glyceraldehyde-3-phosphate dehydrogenase activity of these samples (as opposed to the specific activities) only 42% of the activity was present in the treated sample and 28% was released into the supernatant solution.

The most logical explanation for the results of Table VI is that the ghosts reseal upon trypsin treatment and thus show an apparent loss of enzyme activity. In order to test this proposal trypsin-treated and control membranes were assayed in the presence of SDS. The membranes were also centrifuged to yield supernatant solution and pellet fractions. This pellet (pellet I) was assayed in the presence of SDS or subjected to a freeze-thaw procedure. The frozen-thawed membranes were also centrifuged to yield supernatant solution and pellet fractions which were assayed in the presence or absence of SDS. The results of these determinations are shown in Table VII and are consistent with the resealing hypothesis. Trypsin treatment of the ghosts caused an apparent loss

TABLE VI

EFFECT OF TRYPSIN ON ERYTHROCYTE MEMBRANE ENZYME ACTIVITIES[1]

Trypsin Treatment	Fraction	Protein (mg)	Enzyme Activities (μmoles/min)	
			GAPD	Aldolase
			%	%
None	Whole Ghost	27.0	19.9 (100)	0.49 (100)
	Supernatant Solution	1.2	0.0 (0)	0.0 (0)
	Pellet	25.4	16.2 (81.2)	0.28 (57.6)
Trypsin	Whole Ghost	27.0	8.4 (42.4)	0.30 (61.1)
	Supernatant Solution	8.4	5.6 (28.0)	0.21 (43.2)
	Pellet	18.4	1.8 (9.3)	0.0 (0)

[1]Erythrocyte membranes (2.7 mg protein per ml in volume of 10 ml) were incubated with or without trypsin (10 μg/ml) at 25° C for 60 minutes. Aliquots were taken directly for enzyme assays (whole ghost). Samples were also centrifuged at 4° C after addition of soybean trypsin inhibitor (50 μg/ml). Pellet and supernatant solution samples were assayed without detergent incubation.

TABLE VII

EFFECT OF TRYPSINIZATION ON ERYTHROCYTE MEMBRANE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ACTIVITY[1,2]

Trypsin Treatment	1	2	3	4	5	6	7	8
	Ghost	Ghost + SDS	Supernatant Solution	Pellet	Pellet + SDS	Freeze-thaw supernate	Freeze-thaw pellet	Freeze-thaw pellet + SDS
None	78	100	0	100	95	0.5	64	60
Trypsin	21	107	1.3	1.6	81	24	5	33

[1]Values are expressed as percentage of control ghosts incubated in detergent.

[2]Incubation conditions are as in Table VI except ghost concentration was 2.1 mg protein/ml and trypsin concentration was 2 µg/ml. The pellet from the initial incubation (pellet 1) was frozen and thawed in 7 mM phosphate and centrifuged to yield a second supernatant solution and pellet fraction. Incubation with SDS was at 25° C for 30 minutes at the concentration of about 0.8 µmole SDS/mg protein. The control ghosts had GAPD activity (100%) of 0.827 µmole/min/mg protein.

of activity that was not observed if the samples were incubated with detergent (column 1 vs. 2). Almost no enzyme was released into the supernatant solution in the trypsin treatment (column 3), and the activity in the pellet was virtually all in a cryptic form, i.e., inaccessible to substrate (column 4 vs. 5). The 20% loss of activity in the treated pellet was unexplained. Perhaps release of the enzyme inside the ghost may subject it to other modes of inactivation. This enzyme is rather susceptible to oxidative inactivation. Freezing and thawing the pellet fraction resulted in a partial release of dehydrogenase from the treated sample but not the control sample (column 6). The activity of the enzyme in the pellet from the freeze-thaw procedure was still in the cryptic form for the treated sample (column 7 vs. 8), suggesting that the freeze-thaw treatment had not opened these vesicles completely. The control sample showed no significant cryptic activity at any stage, although it, as well as the treated sample, had lost 40% of its activity after freezing and thawing.

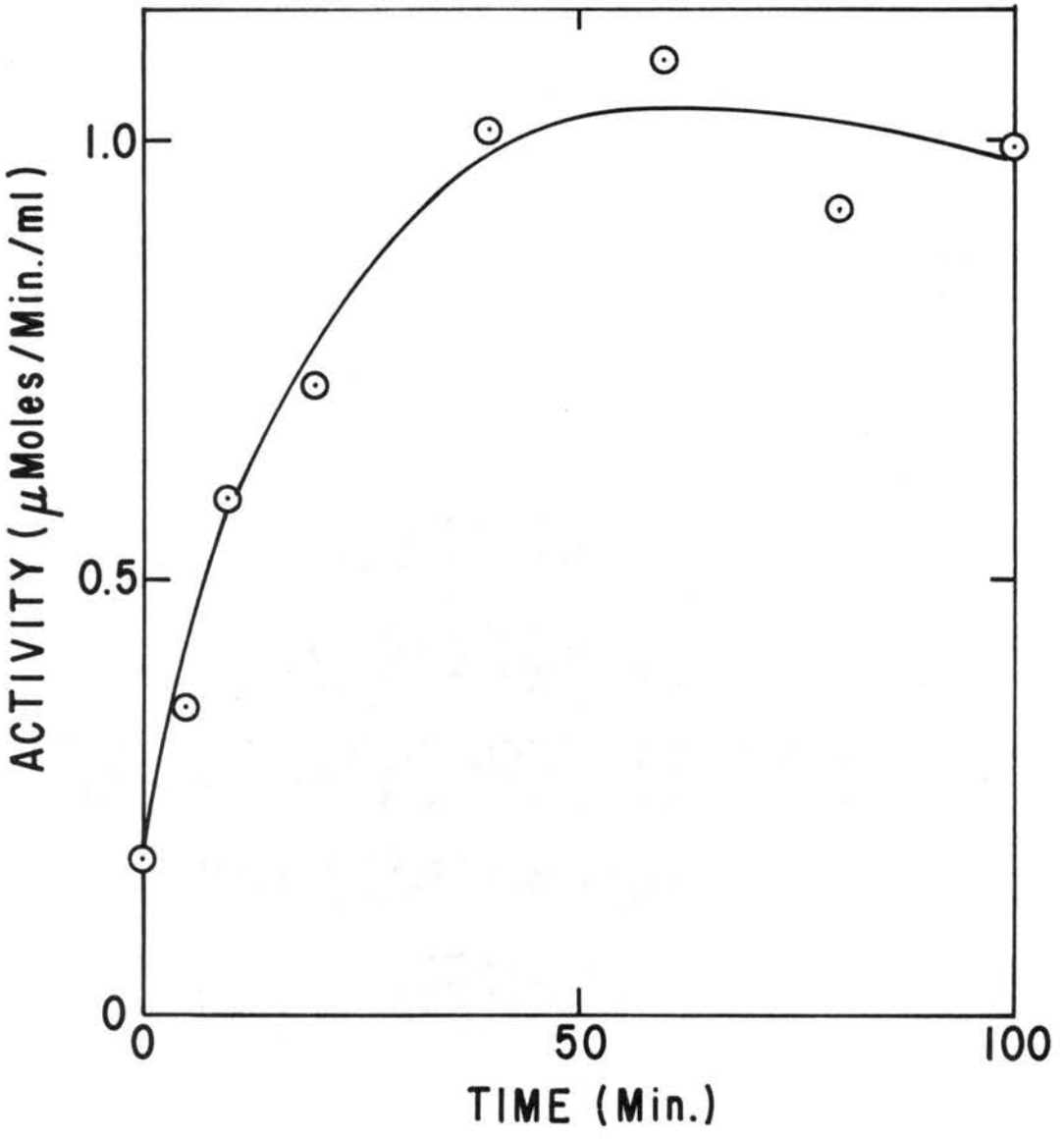
To gain some information about the proteins destroyed during the process of glyceraldehyde-3-phosphate dehydrogenase release, it was necessary to observe protein patterns on a membrane sample from which a significant quantity of the enzyme was released. Figures 21 and 22 show the enzyme release and SDS acrylamide gel protein patterns, respectively, for membranes treated at a trypsin concentration low enough to prevent extensive protein degradation. A maximum of about 25% of the dehydrogenase activity was released into the supernatant solution during the digestion. This probably represents only part of the enzyme released from the membranes, since a portion of that released from the membrane was probably trapped within the sealed ghosts. The most interesting

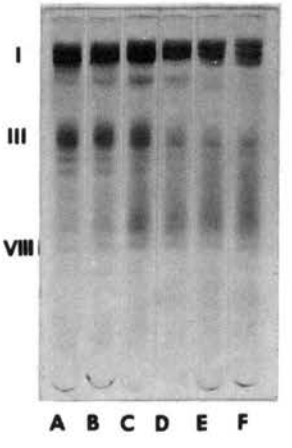
ure 21. Release of Glyceraldehyde-3-phosphate Dehydrogenase at Low Trypsin Concentration

Ghosts (2 mg/ml protein) and trypsin (1 μg/ml) were incubated at C. Aliquots for centrifugation were treated with a 10-fold excess of bean trypsin inhibitor. Supernatant solutions were assayed for ydrogenase. Aliquots of the reaction mixture were also solubilized ectly in SDS for electrophoresis.

ure 22. SDS Acrylamide Electrophoresis of Trypsin Treated Membranes

Conditions for trypsinization are given in Figure 20. Gels A and B, trols; Gel C, 5 minutes; Gel D, 10 minutes; Gel E, 20 minutes; Gel F, minutes.


ACTIVITY (μMoles/Min./ml)
1.0
0.5
0
0
50
100
TIME (Min.)


I
III
VIII
A B C D E F

observation of this experiment is that the release of the dehydrogenase reached a maximum value without significant proteolysis of component I (spectrin) of the membrane (Figure 20).

H. Effect of ATP and Other Membrane Perturbing Agents on Binding of Glyceraldehyde-3-phosphate Dehydrogenase to the Erythrocyte Membrane

In the present study experiments with iodoacetate were designed to use this reagent as a probe for membrane changes resulting from addition of membrane effector molecules such as ATP or divalent cations. It is known that Ca^{++} causes loss of deformability and shrinkage of the resealed ghost when introduced during hemolysis (62). ATP causes an apparent conformational change of proteins of the isolated erythrocyte membrane to yield more β structure, as determined by infrared spectroscopy (63). Therefore, experiments were conducted in which erythrocytes were hemolyzed in the presence of low concentrations of effectors, resealed by addition of salt, labeled with radioactive iodoacetate, re-hemolyzed and washed to obtain hemoglobin-free ghosts. In these experiments a double labeling procedure was used. The control (no effector) was labeled with ^{14}C-iodoacetate, and the effector-treated sample was labeled with ^{3}H-iodoacetate. Under the labeling conditions used only two membrane proteins were significantly labeled, component I (spectrin) and component VIII (glyceraldehyde-3-phosphate dehydrogenase). Only one of the four effectors tested (Mg^{++}, Ca^{++}, ATP and EDTA) caused a significant alteration in the labeling pattern. ATP greatly decreased the labeling of the second band (glyceraldehyde-3-phosphate dehydrogenase), as shown in

Figure 23. Two possible explanations for this decrease can be postulated: (a) there was a loss of the enzyme from the membrane as a result of the ATP treatment; or (b) there was a direct effect of ATP on the enzyme. By scanning SDS acrylamide gels of the control and treated samples, it was possible to show that the former proposition was correct. The identification of the labeled component as glyceraldehyde-3-phosphate dehydrogenase then indicated that the binding of this key glycolytic enzyme to the erythrocyte membrane was influenced by the presence of ATP. This finding was particularly interesting in view of earlier work (34,35, 36) which showed that yeast and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase is subject to inactivation, conformational changes and dissociation in the presence of ATP.

Table VIII shows the distribution of GAPD, LDH and hemoglobin in the supernatant solution and the pellet of erythrocytes after hypotonic hemolysis in different media. When the erythrocytes are osmotically hemolyzed in 10 mM Tris (pH 7.4), only 15 percent of GAPD was released into the supernate. While about half of GAPD was released into the supernate, when hemolyzed in 7 mM phosphate buffer (pH 7.4) or in distilled water. However, almost all of lactic dehydrogenase and hemoglobin were released into the supternate in all three media (Table VIII). The release of GAPD during the hypotonic hemolysis was dependent on the hemolysis media. Hemolysis in hypotonic Tris buffer yielded a higher retention of GAPD than hemolysis in phosphate buffer or distilled water.

Table IX shows the release of above components into the supernate during hypotonic hemolysis in the presence of various effector substances. The effects of various substances added on the release of glyceraldehyde-3-phosphate dehydrogenase activity were consistent with

ıre 23. Double Labeling of Resealed Human Erythrocyte Ghosts Prepared in the Presence and Absence of ATP

Two batches of resealed ghosts were prepared, except in one batch (2 mM) was included in the hemolysis mixture. After resealing, the ts prepared in the presence of ATP were treated with ^{3}H-iodoacetate those prepared in absence of ATP were treated with ^{14}C-iodoacetate. membranes were washed with hypotonic phosphate to remove hemoglobin. two membrane samples were combined, solubilized in SDS and subjected DS acrylamide electrophoresis. The protein pattern by Coomassie blue ning did not differ significantly from that of membranes not treated iodoacetate. Samples of 4,700 and 40,000 dpm of ^{14}C and ^{3}H, re- tively, were applied to the gels. Fifty percent of the two labels recovered from the gels. No attempts were made to increase recovery these particular experiments. ^{14}C, (———), ^{3}H, (– – –).

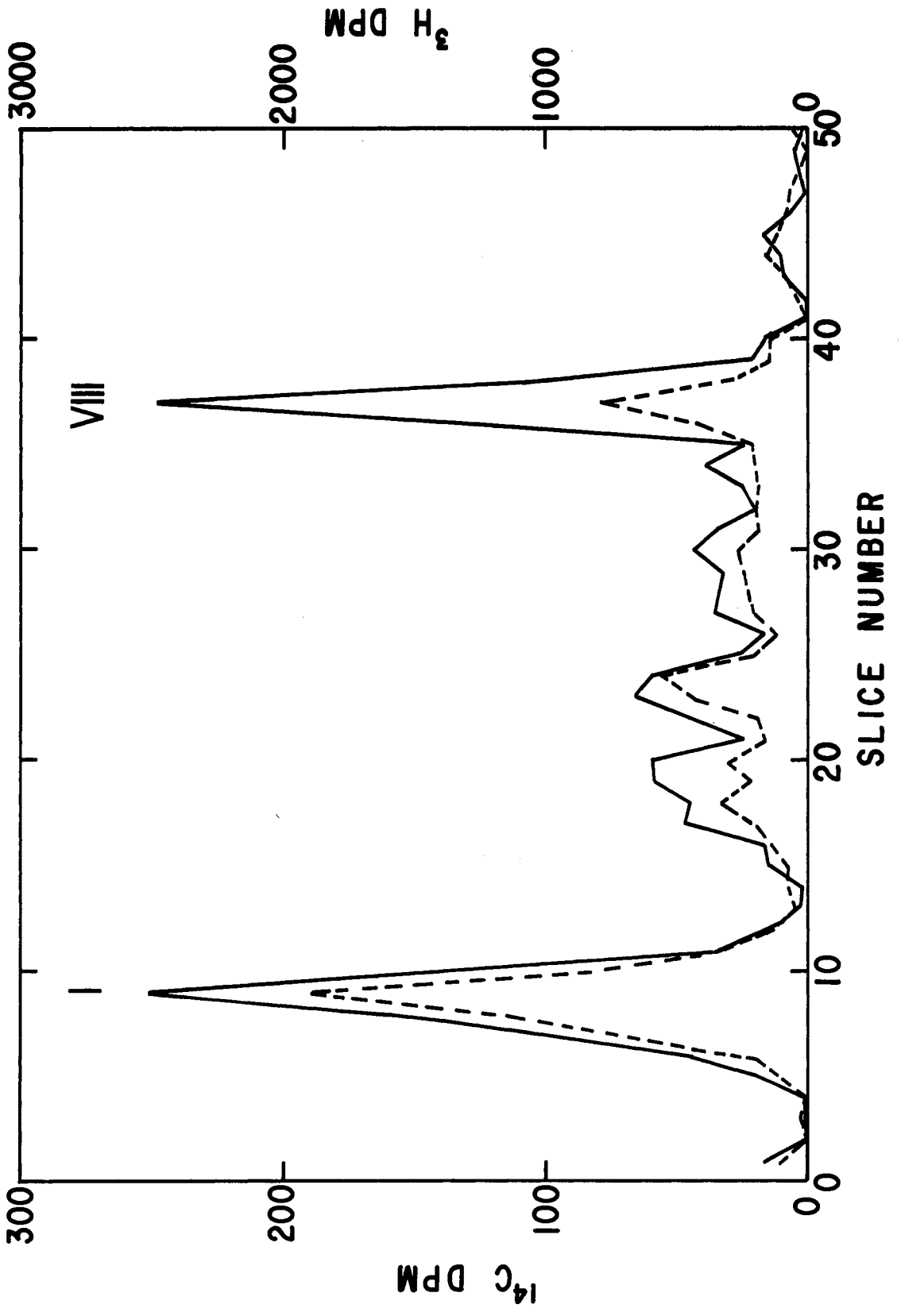

^{3}H DPM
3000
2000
1000
0
50
40
30
20
10
0
VIII
SLICE NUMBER
300
200
100
0
^{14}C DPM

TABLE VIII

DISTRIBUTION OF ENZYME ACTIVITIES AND HEMOGLOBIN OF ERYTHROCYTES AFTER HYPOTONIC HEMOLYSIS IN DIFFERENT MEDIA[1]

Components	Fractions	Hemolytic Media					
		Tris Buffer 10 mM (pH 7.4)		Phosphate Buffer 7 mM (pH 7.4)		Distilled Water	
			(%)		(%)		(%)
GAPD Activity (μmoles/min 2 ml erythrocytes)	Supernate	5.45	(14.5)	16.62	(49.7)	18.39	(52.9)
	Pellet	32.16	(85.5)	16.82	(50.3)	16.36	(47.1)
	Sum	37.61	(100)	33.44	(100)	37.75	(100)
LDH Activity (μmoles/min 2 ml erythrocytes)	Supernate	46.86	(90.6)	46.04	(90.6)	40.09	(85.7)
	Pellet	4.85	(9.4)	4.81	(9.4)	6.72	(14.3)
	Sum	51.71	(100)	50.84	(100)	46.81	(100)
Hemoglobin (mg/2 ml erythrocytes)	Supernate	577.9	(91.3)	558.8	(91.3)	633.8	(94.2)
	Pellet	54.8	(8.7)	53.4	(8.7)	39.3	(5.8)
	Sum	632.7	(100)	612.2	(100)	673.1	(100)

[1]Conditions for hypotonic hemolysis are given in Experimental Procedure.

TABLE IX

RELEASE OF ENZYME ACTIVITIES AND HEMOGLOBIN FROM ERYTHROCYTES DURING HEMOLYSIS IN PRESENCE OF MEMBRANE EFFECTORS[1]

Substance Released	Percent of Total Activity in Supernatant					
	Effector Added					
	None	EDTA 2 mM	Mg^{++} 2 mM	Ca^{++} 0.4 mM	ATP 1 mM	ATP $+Mg^{++}$
Glyceraldehyde-3-phosphate Dehydrogenase	39	45	36	37	80	61
Lactic Dehydrogenase	83	87	61	56	82	59
Hemoglobin	86	85	81	81	84	76

[1]Conditions for hypotonic hemolysis are given in Experimental Procedure. Activities released into the supernatant are expressed as a percentage of the total activity (supernatant plus pellet). Values in this experiment were not corrected for soluble enzyme in the pellet fraction. Total activity values (100%) were 13.8 and 32.1 μmoles/min/ml erythrocytes for GAPD and LDH, respectively. Hemoglobin values were 294 mg/ml erythrocytes.

the results of the earlier labeling study. Only ATP caused significantly enhanced release. The other substances were relatively ineffective at the concentrations used. The presence of Mg^{++} inhibited the ATP effect. Lactic dehydrogenase and hemoglobin release were essentially complete, if correction was made for the volume of the pellet. ATP had no effect on these but divalent cations slightly enhanced retention to the membrane. In a number of experiments of this type the amount of glyceraldehyde-3-phosphate dehydrogenase associated with the erythrocyte membrane was 60-80% after hemolysis in Tris buffer. When ATP was present in the hemolyzing medium, this value dropped to 5-20%. Figure 24 shows the concentration dependence of the ATP effect on glyceraldehyde-3-phosphate dehydrogenase release at 0^{o} C. It was also shown that Ca^{++} can cause a release of the enzyme, although it requires a higher concentration than ATP (Figure 24).

Because of the pronounced effects of ATP during hypotonic hemolysis it was of interest to examine the effects of other nucleotides. Table X shows the effects of different nucleotides on GAPD release during hypotonic hemolysis. ATP was the most effective of the substances tested. Other nucleotides were considerably less effective, although other di- and trinucleotides did show some ability to enhance release. The concentration dependence of the release of the enzyme by 2,3-diphosphoglycerate was quite similar to that observed with Ca^{++} (Figure 25). 2,3-diphosphoglycerate in the hemolyzing medium did not affect the GAPD releasing effect of ATP.

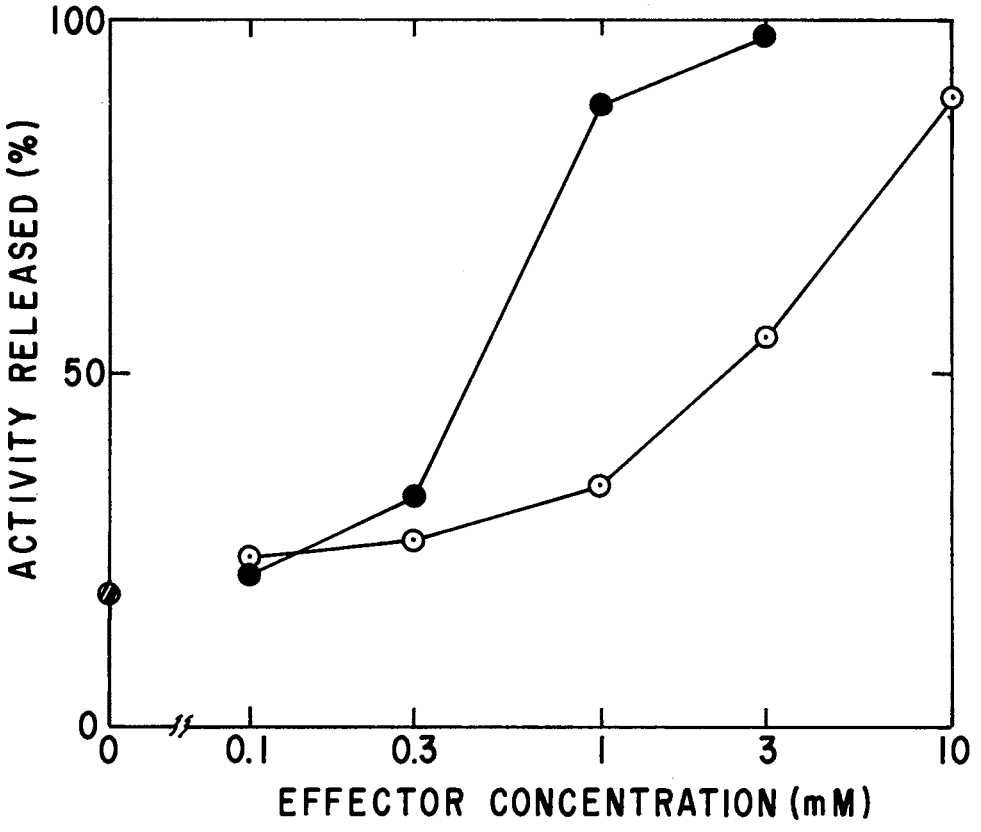
The effects of ATP on release of erythrocyte glyceraldehyde-3-phosphate dehydrogenase from the membrane were of particular interest in view of the previously demonstrated effects of ATP on the soluble rabbit

ıre 24. Concentration Dependence of ATP and Ca^{++} Effects on the Release of GAPD During Hemolysis

Conditions for hemolysis are described in Experimental Procedure. control GAPD activity (100%) was 18.7 µmoles per min per ml :hrocytes. ATP (●———●) and Ca^{++} (O———O).

ıre 25. Concentration Dependence of 2,3-Diphosphoglycerate on the Release of GAPD Activity From Erythrocyte Membranes

Conditions for hemolysis are described in Experimental Procedure. hemolyzing medium had no ATP (O———O) or 1 mM ATP (Δ— — —Δ).


100
50
0
ACTIVITY RELEASED (%)
0
0.1
0.3
1
3
10
EFFECTOR CONCENTRATION (mM)

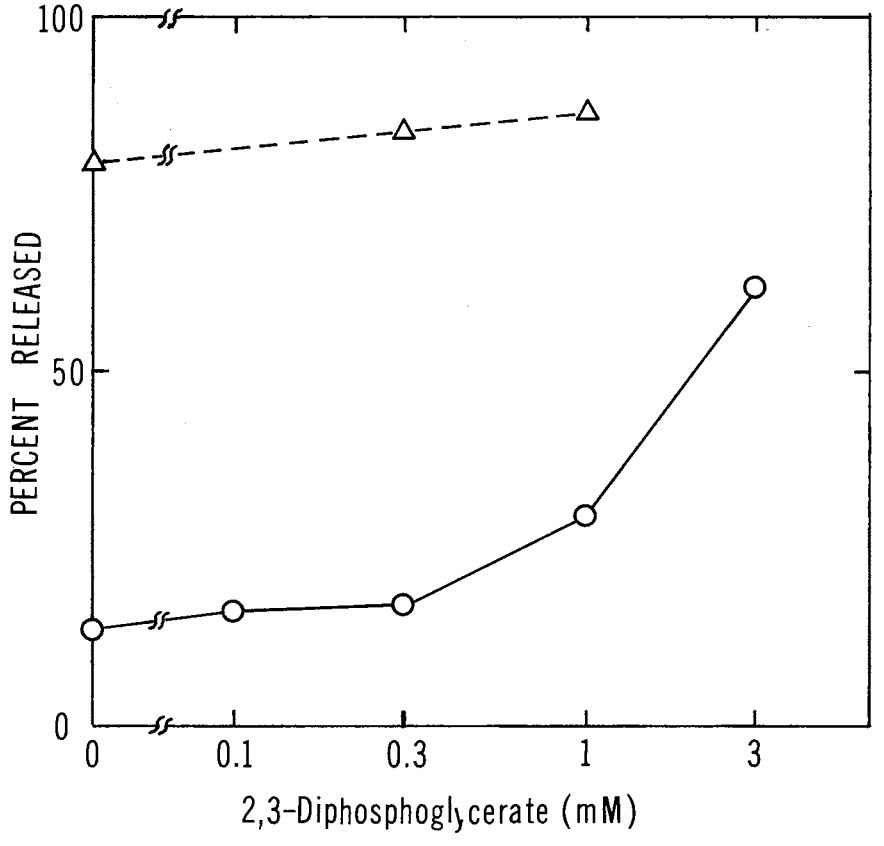

100
50
0
PERCENT RELEASED
0
0.1
0.3
1
3
2,3-Diphosphoglycerate (mM)

TABLE X

EFFECTS OF DIFFERENT NUCLEOTIDES ON RELEASE OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE DURING HYPOTONIC HEMOLYSIS[1]

Nucleotide Added (conc.)	Percent GAPD in Supernatant Solution
None	21
Adenosine (1 mM)	15
AMP (0.9 mM)	24
ADP (0.9 mM)	37
ATP (0.9 mM)	61
GTP (1 mM)	44
UTP (1 mM)	32

[1]Conditions for hemolysis are given in Experimental Procedure. The GAPD activity released into the supernatant solution are expressed as a percentage of the total activity (supernatant plus pellet).

muscle and yeast enzymes (34,35,36). To determine if similar behavior was exhibited by the erythrocyte enzyme, the solubilized enzyme was treated under conditions similar to those used previously. As shown in Table XI, the erythrocyte enzyme was also inhibited by ATP at 0° but not at 25° C. The inhibition could be prevented by preincubation with NAD, but it was only partially reversed if NAD was added after incubation with ATP. In addition ATP has been shown to destabilize the enzyme toward proteolytic inactivation with chymotrypsin (Figure 26). Again, NAD preincubation prevented this ATP effect. To determine if the effects of ATP on the soluble enzyme were correlated with the release of the enzyme from the membrane, two different experiments were performed. In the first the effect of NAD on the enzyme release during hemolysis was determined. As shown in Figure 27, NAD effected a somewhat enhanced release of the dehydrogenase in the absence of ATP, but it did not inhibit the ATP-promoted release. In addition the effect of hemolysis at 25° C in the presence of ATP was studied. Figure 28 shows the ATP concentration dependence of the glyceraldehyde-3-phosphate dehydrogenase release at 25° C. It was essentially the same as at lower temperature (0° C). Thus there does not appear to be any direct correlation between the inactivation or conformation changes of the enzyme and the release of the enzyme during hemolysis. The enzyme release was not dependent on either temperature or NAD preincubation, while the inactivation was dependent on both.

I. Freeze-thaw Treatment of Erythrocytes

Hemolysis of human erythrocytes by the freeze-thaw technique can be contrasted quite sharply to hypotonic lysis in terms of the release of GAPD from the membrane (Table XII). Essentially all of the hemoglobin

TABLE XI

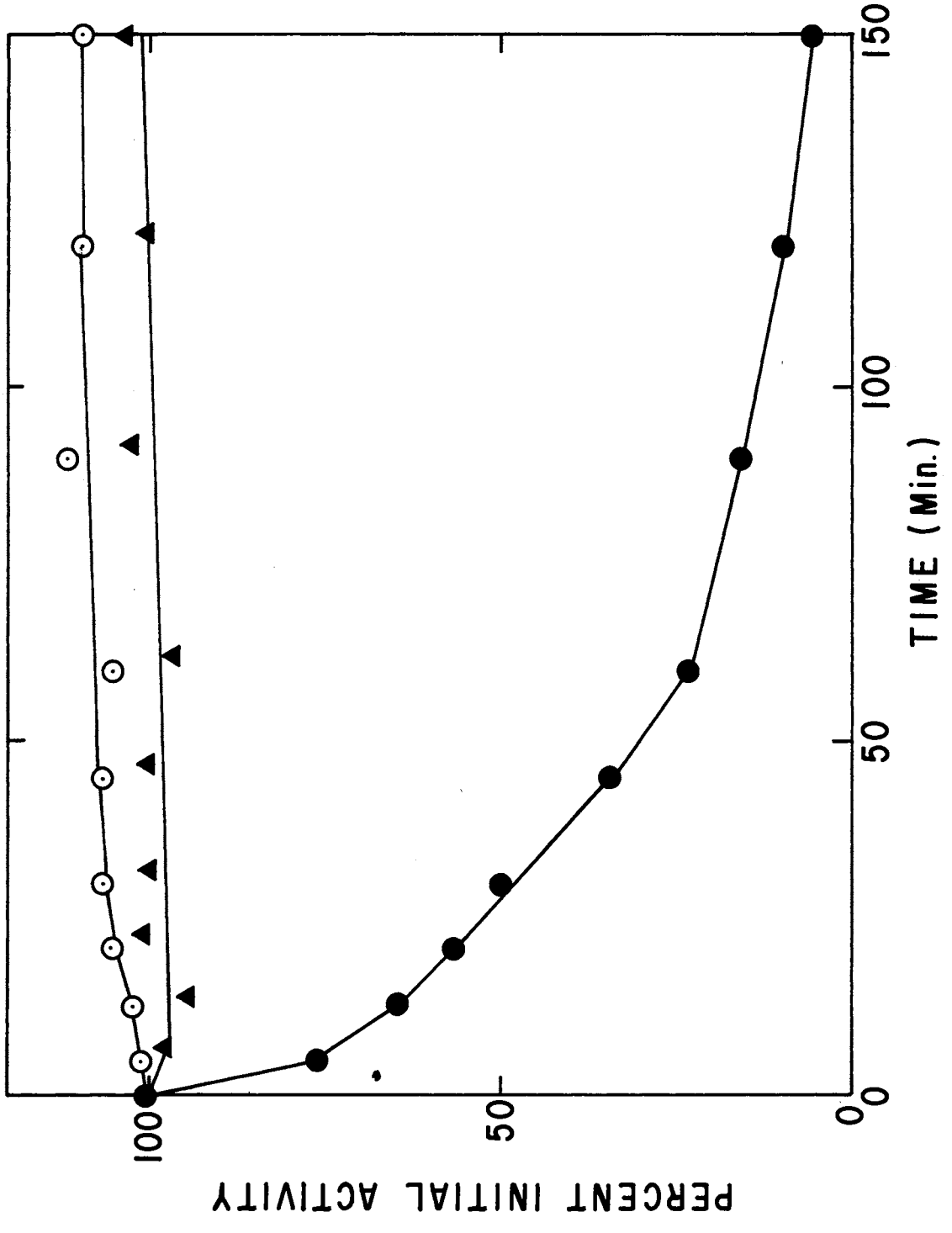
EFFECT OF ATP AND NAD ON SOLUBILIZED ERYTHROCYTE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE[1]

Temperature	0°	0°	0°	0°	0°	25°	25°
1st Addition	-	1mM ATP	2mM NAD	1mM ATP	2mM NAD	1mM ATP	2mM NAD
2nd Addition	-	-	-	2mM NAD	1mM ATP	-	1mM ATP
Activity (%)	100	31.4	124	58.8	120	109	119

[1]Crude enzyme was incubated at the stated temperature with either ATP or NAD. After 10 minutes, a second addition of either NAD or ATP was made. The samples were assayed for glyceraldehyde-3-phosphate dehydrogenase after 30 minutes. For control samples additions contained only buffer (50 mM Tris, 2 mM mercaptoethanol, pH 7.4) and no effectors (indicated by dashes). Control activity (100%) was 2.9 μmoles/min/mg protein.

ure 26. Effect of ATP and NAD on Chymotrypsin Inactivation of Solubilized Glyceraldehyde-3-phosphate Dehydrogenase

Incubation conditions are given in Experimental Procedure. Activis are expressed as percentage of initial activity for each sample. control GAPD activity (100%) was 2.20 μmoles per min per mg protein. trol (O———O), plus ATP (●———●) and plus ATP and NAD (▲———▲).

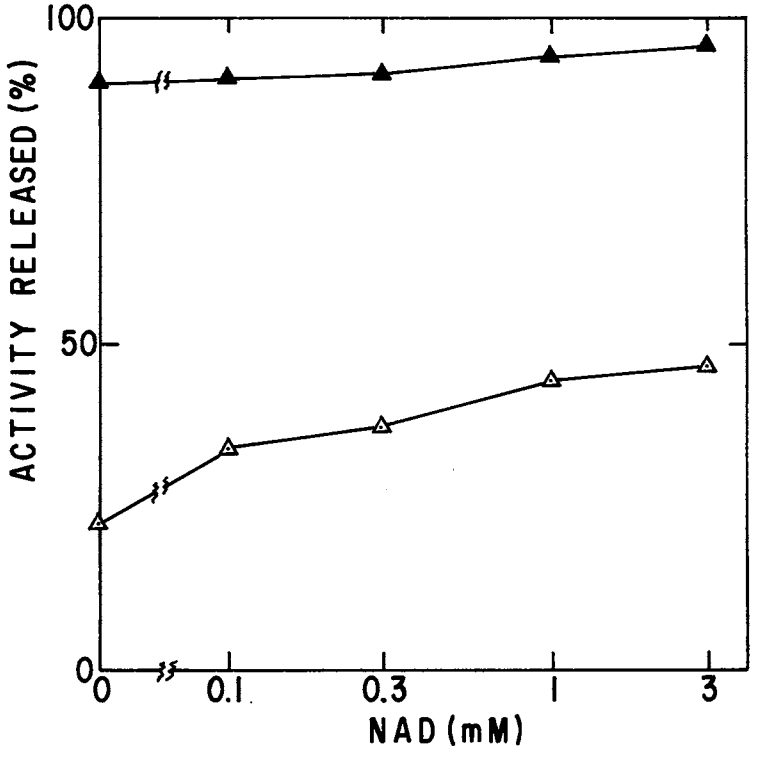

PERCENT INITIAL ACTIVITY
100
50
0
0
50
100
150
TIME (Min.)

ıre 27. Effect of NAD on Release of Glyceraldehyde-3-phosphate Dehydrogenase From Erythrocyte Membranes During Hemolysis in Presence of ATP

Hemolysis was performed with increasing concentrations of NAD in 10 'ris (pH 7.4) at 0° C for 30 minutes in the presence and absence of 1 .TP. NAD (Δ———Δ) and NAD plus ATP (▲———▲).

.re 28. Concentration Dependence of ATP-promoted Release of Glyceraldehyde-3-phosphate Dehydrogenase at 25° C

Conditions are identical to those of Figure 24 with exception of erature. Glyceraldehyde-3-phosphate dehydrogenase (●———●), lactic drogenase (O———O) and hemoglobin (Δ———Δ).


100
50
0
ACTIVITY RELEASED (%)
0
0.1
0.3
1
3
NAD (mM)

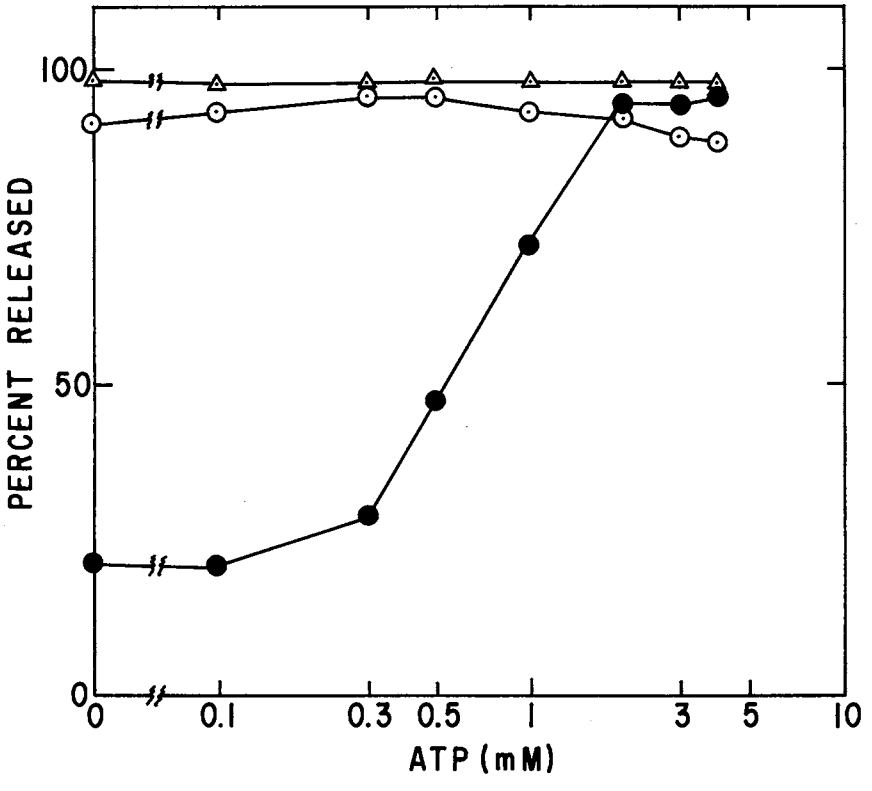

100
50
0
PERCENT RELEASED
0
0.1
0.3
0.5
1
3
5
10
ATP (mM)

TABLE XII

RELEASE OF ENZYMES AND HEMOGLOBIN FROM ERYTHROCYTES BY FREEZE-THAW HEMOLYSIS[1]

Hemolysis Method	Percent of Total in Supernatant Solution		
	Hemoglobin	LDH	GAPD
Osmotic, Tris	100	96	17
Freeze-thaw			
Tris-NaCl	97	96	94
+ 1 mM Mg^{++}	98	96	94
+ 1 mM Ca^{++}	97	95	94
+ 1 mM ATP	97	98	98
Tris-Sucrose	91	88	85
+ 1 mM Mg^{++}	94	75	75
+ 1 mM Ca^{++}	73	78	73
+ 1 mM ATP	82	90	84

[1]Conditions for hemolysis are given in Experimental Procedure. Activities released into the supernatant solution are expressed as a percentage of the total activity (supernatant plus pellet).

and LDH were released during both hypotonic and freeze-thaw lysis, but the bulk of the GAPD was released only by the freeze-thaw procedure. This release was dependent on the medium. In the presence of isotonic sucrose or sucrose plus divalent cations the GAPD release was somewhat inhibited. However, this may have been due, in part, to incomplete lysis, since hemoglobin and LDH were partially retained. ATP had little effect on the GAPD release by freeze-thaw hemolysis, particularly when compared to the large effect shown during hypotonic lysis.

Table XIII shows the effect of the variation of the medium upon the release of hemoglobin, LDH and GAPD by freeze-thaw. Freeze-thaw procedures released essentially all of the GAPD except in sucrose, in which about 30% of the enzyme was retained with the membrane. Retention of hemoglobin and LDH was enhanced in sucrose, although the effects were not as great as with GAPD. The variations in release of hemoglobin and the enzymes between Tables XII and XIII are indicative of the differences observed between different experiments. In all of the hemolysis experiments in Table XIII acetylcholinesterase activities were monitored to determine if membrane fragmentation might be a source of the variations in enzyme retention. No significant losses of acetylcholinesterase activity into the supernatant solutions were noted.

The effect of the freeze-thaw procedure on erythrocyte ghosts was also investigated, using several different suspending media. Freeze-thaw treatments in hypotonic Tris or phosphate or in 0.25 M sucrose did not cause release of GAPD, while treatment in isotonic NaCl or KCl caused virtually complete release (Table XIV). Protein release was most pronounced with the treatments in isotonic salt solutions. Cholesterol assays of the pellet fractions of the treated ghosts indicate that

TABLE XIII

VARIATION IN RELEASE OF ENZYMES AND HEMOGLOBIN BY FREEZE-THAW IN DIFFERENT MEDIA[1]

Hemolysis Method	Percent of Total in Supernatant Solution		
	Hemoglobin	LDH	GAPD
Freeze-thaw			
Isotonic Tris	100	99	99
Isotonic phosphate	96	100	100
Tris-NaCl	99	100	100
Tris-KCl	98	100	100
Tris-sucrose	98	92	70

[1]Conditions for hemolysis are given in Experimental Procedure.

TABLE XIV

RELEASE OF PROTEIN, GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND ACETYLCHOLINESTERASE FROM ISOLATED ERYTHROCYTE MEMBRANES BY FREEZE-THAW PROCEDURE[1]

Medium	Percent of Total in Supernatant Solution		
	Protein	GAPD	ACHE
Tris, 10 mM	22.1	1.2	24.1
Phosphate, 7 mM	9.9	1.1	22.3
Tris-NaCl	41.1	97.0	77.5
Tris-KCl	38.7	94.6	41.2
Tris-sucrose	25.6	2.0	22.2
Tris, 100 mM	36.1	33.5	23.1

[1]Two ml of the washed, packed erythrocyte membranes were suspended in 20 ml of different media, frozen in a dry ice-isopropanol bath and thawed in a water bath at 25° C. The freeze-thawing once more repeated. The membrane suspension was centrifuged (35,000 g, 20 minutes). The supernatant solution and the pellet were separated and assayed for protein and enzyme activity.

fragmentation was not a significant factor in the release of GAPD and other proteins. Electrophoretic analysis of the pellets on dodecyl sulfate acrylamide gels showed no evidence of proteolysis during the treatments (11).

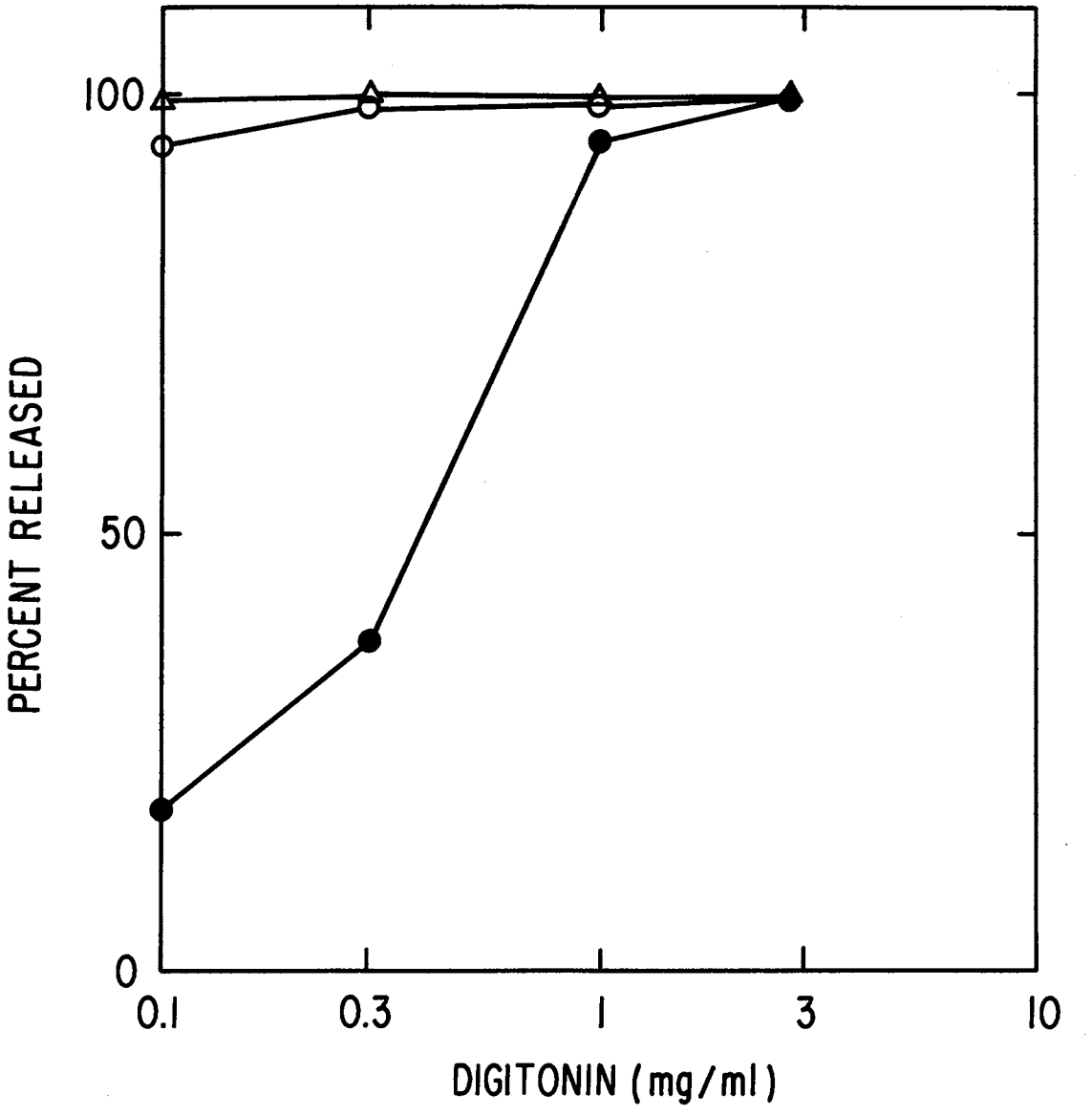
J. Digitonin Lysis of Erythrocytes

Erythrocytes could be hemolyzed by digitonin with a release of hemoglobin and LDH similar to that observed by hypotonic lysis. At low digitonin concentrations (1.25 mg digitonin per ml erythrocyte) release of GAPD from the membrane was low, comparable to that observed with hypotonic lysis. With increasing digitonin concentrations the release of GAPD was more pronounced and had nearly reached its maximum value at 12.5 mg digitonin per ml erythrocytes (Figure 29). The release of GAPD during digitonin lysis is also medium dependent. Addition of NaCl resulted in a pronounced enhancement of GAPD release (Figure 30). ATP will also promote the release of GAPD (Figure 31), in a manner similar to that observed during hypotonic lysis (Figure 24). The effect of ATP was essentially the same regardless of whether added before or after hemolysis of the cells. NAD (2 mM) also released GAPD from the erythrocyte membrane to approximately the same extent as ATP (1 mM) if added either before or after digitonin lysis. This behavior was different from that during hypotonic lysis, in which NAD was far less effective than ATP in promoting the release of GAPD.

ıre 29. Concentration Dependence of GAPD Release During Digitonin Hemolysis of Human Erythrocytes

Two ml packed erythrocytes in 25 ml of 250 mM sucrose-10 mM Tris 7.4) were hemolyzed with increasing concentrations of digitonin. The ›onents released into the supernatant are expressed as percentage of total (supernatant plus pellet). GAPD (●———●), LDH (O———O) and ›globin (Δ———Δ).

GAPD RELEASE DURING DIGITONIN HEMOLYSIS



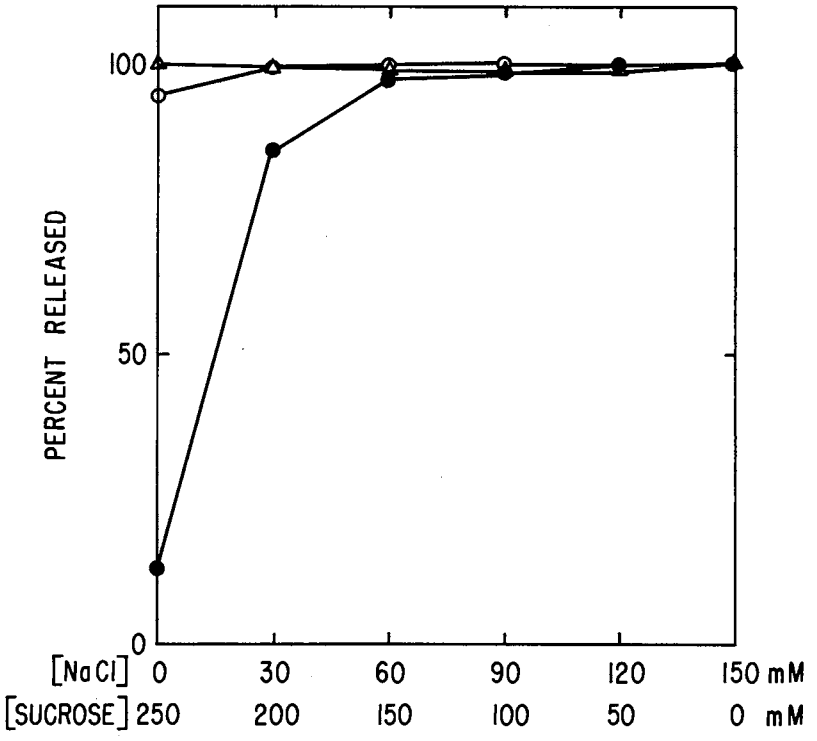
ıre 30. Effect of Salt Concentration on GAPD Release During Digitonin Hemolysis

Two ml of packed erythrocytes were hemolyzed in 25 ml of digitonin ıtion (0.1 mg per ml) with varying concentrations of NaCl and sucrose)° C for 30 minutes. The components released into the supernatant are ressed as percentage of the total (supernatant plus pellet). GAPD ——●), LDH (O——O) and hemoglobin (Δ——Δ).

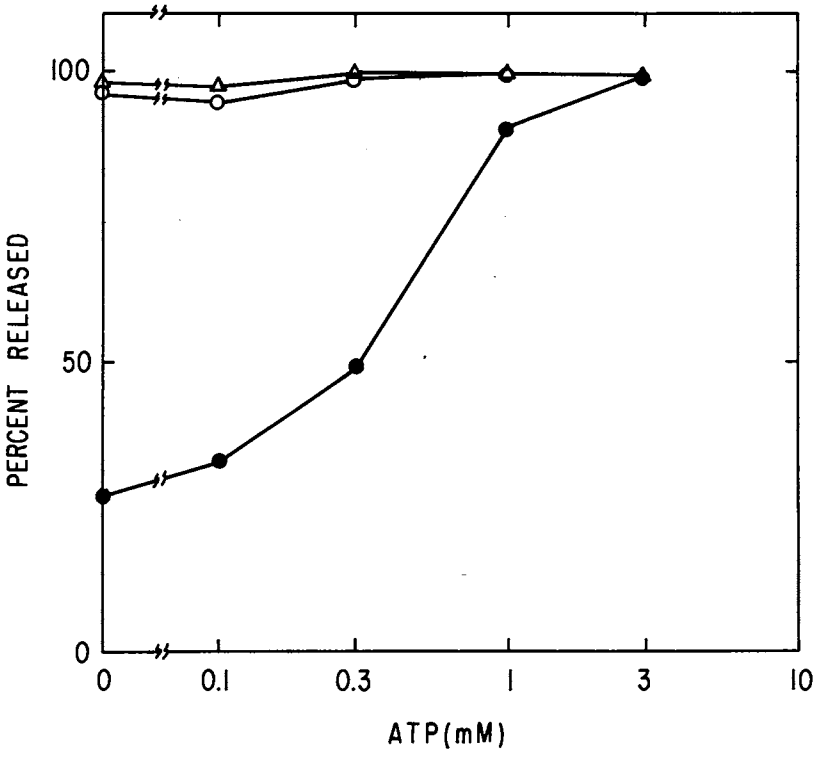
ıre 31. Effect of ATP Concentration on GAPD Release During Digitonin Hemolysis

Two ml of packed erythrocytes were hemolyzed in 25 ml of a mixture ligitonin (0.1 mg per ml)-250 mM sucrose-10 mM Tris (pH 7.4) with ying concentrations of ATP at 0° C for 30 minutes. The components eased into the supernatant are expressed as percentage of the total ernatant plus pellet). GAPD (●——●), LDH (O——O) and hemoglobin ——Δ).

EFFECT OF SALT CONCENTRATION
ON GAPD RELEASE BY DIGITONIN



EFFECT OF ATP ON GAPD RELEASE BY DIGITONIN



CHAPTER IV

DISCUSSION

The reaction of iodoacetate with the erythrocyte membrane showed an unusual specificity, in which one (component VIII) of the 11-12 major membrane proteins reacted to give virtual saturation of its reactive groups before any of the other proteins reacted to a significant extent. This specific reaction occurred almost completely at the sulfhydryl groups of this protein, although other protein side chains were shown to react at higher reagent concentrations. A second sulfhydryl reagent, N-ethylmaleimide, showed completely different behavior, labeling the membrane proteins rather nonspecifically, under the conditions used, in approximate proportion to the quantity of protein in the membrane. Thus the reactivity observed was reagent-specific as well as protein-specific. The labeling patterns with N-ethylmaleimide observed in the current study are similar to those of Lenard (20) in that the high molecular weight protein spectrin was the most heavily labeled. However, it was also observed heavier labeling of the proteins in the 100,000 molecular weight region than was reported previously. The discrepancy between these results may derive from differences in reaction conditions or in prior treatments of the membranes. Bellhorn, et al. (64) have noted that the sulfhydryl content of the erythrocyte membrane decreased under certain conditions.

The observation that the number of N-ethylmaleimide groups

incorporated into the membrane approached the saturation condition at the highest reagent concentrations permitted an estimate of the number of reactive sulfhydryl groups in the isolated membrane at 20 nmoles per mg of membrane protein. This corresponds to a total of 2.2 reactive cysteines per 100 amino acid residues (assuming an average molecular weight of 110 for an amino acid), which compares favorably with a value reported previously from modification studies with high concentrations of iodoacetamide (64). A value of 10^{-17} moles of reactive cysteine residues per cell membrane was calculated from these data, based on the recovery of 5.0 mg of membrane protein per ml of packed erythrocytes and the assumption of 10^{10} packed cells per ml.

The identification of the iodoacetate-labeled protein as component VIII permitted a simple purification, based on a sodium chloride extraction reported earlier by Fairbanks, et al. (21). The efficiency of extraction in the present work was lower than that obtained earlier, but may have resulted from variations in the treatment of the membranes during the labeling and hemolysis. Alternatively, the modification of the protein sulfhydryl groups may have affected its extractability. Taking advantage of properties of component VIII, i.e., specific labeling with iodoacetate and the extractability from membranes with concentrated salt solutions, an erythrocyte membrane protein, component VIII, could be purified by successive procedures of extraction, SDS-gel filtration and SDS-acrylamide gel electrophoresis. The purification of this protein represented one of the few examples of a membrane protein which has been purified to this state of homogeneity. However, although the protein was homogeneous by SDS-acrylamide electrophoresis, the possibility that more than one polypeptide chain was present in this preparation cannot be

completely ruled out. The specificity of the extraction and of the labeling does suggest that all of the isolated components must be closely related, if multiple components indeed exist. The purification of component VIII presented an excellent opportunity to study some of the properties of a membrane protein which might be relevant to its function. Consideration of the amino acid composition is not particularly revealing, since it does not show an unusually high content of hydrophobic residues or any other anomalous features. The composition is also quite different from spectrin and other "tectin-type structural proteins" (65). In addition to its unique extraction and reaction behavior this protein has been shown to be resistant to proteolysis (21,33) and to be released from isolated membranes by proteolysis (21). Incorporation of ATP into resealed ghosts before labeling with iodoacetate also resulted in a marked decrease in the retnetion of component VIII upon washing the membranes in hypotonic buffers. The ease of removal of this protein from the membrane under various conditions may indicate a peripheral location at one of the membrane surfaces, probably the interior surface, since labeling studies with non-penetrating membrane reagents do not show any incorporation into this component (10,66). The variations in extractability, digestibility, and reactivity observed for the various erythrocyte membrane proteins can be associated with the membrane. It is interesting to note that the hemoglobin which was not removed from the membrane during hemolysis and washing does not contain any iodoacetate, suggesting that its reactivity was affected by association with the membrane.

From behavior and properties of component VIII, such as (a) extractability of component VIII and of GAPD activity from erythrocyte

membranes by 1 M NaCl- or 0.1 M EDTA-solution; (b) specific labeling of component VIII with radioactive iodoacetate; (c) the reciprocal relationship between amounts of iodoacetate incorporated into membranes and the GAPD activity of membranes; and (d) molecular weight of component VIII which corresponded to that of subunits of native GAPD enzyme, component VIII of erythrocyte membranes which was separated by SDS-acrylamide gel electrophoresis (11) can be identified as subunits of GAPD enzyme associated with erythrocyte membranes. This discovery is important in that it gives us the first identification of a functional activity which is associated with the erythrocyte membrane in significant quantity, permits an estimation of the quantity of a particular enzyme associated with the membranes in terms of the amounts of protein present, and establishes a known standard to which iodoacetate inhibition of other membrane activities can be compared.

The identification of component VIII as GAPD clearly showed that this enzyme was only partially released from the erythrocyte during hypotonic hemolysis; the bulk of the enzyme was usually associated with the membrane. Further studies indicated that release could be effected by treatment of the isolated membranes with salt solutions at moderately high ionic strengths. This effect suggests ionic interactions as the primary mode of binding the enzyme to the membrane. Such a proposition must be advanced cautiously, since it is known that increased ionic strengths cause significant changes in erythrocyte membrane structural parameters (67). A salt effect of the membrane could alter the membrane-enzyme association in the absence of specific ionic interactions between the two. The converse argument, i.e., that the failure to release a membrane-bound protein by high salt extraction is evidence against ionic

association, must also be considered suspect in some cases. For example, the erythrocyte membrane protein spectrin is released from the membrane at low ionic strengths and high pH in the presence of EDTA suggesting that its association with the membrane may involve ionic interaction. However, spectrin is not released at high ionic strengths (11) because it aggregates and becomes insoluble under these conditions. Thus a protein ionically bound to the membrane might not be detected by salt treatment. Three major components, spectrin (component I), glyceraldehyde-3-phosphate dehydrogenase (component VIII) and component VII, representing 30-50% of the membrane protein, can be released in disaggregated form by mild procedures which do not destroy the membrane structure, although they do fragment the membranes. All of these can thus be considered peripheral proteins by most definitions. The observation by Reynolds and Trayer (68) that 90% of the erythrocyte membrane protein can be solubilized by 5 mM EDTA suggests an even higher proportion of peripheral protein. However, it was not demonstrated in this case that all of this protein was disaggregated from lipids or from other proteins.

The ability of erythrocyte ghosts to seal must be considered in any studies on the structure of the isolated membranes. This sealing has been monitored by glyceraldehyde-3-phosphate dehydrogenase activity and proteolysis, but measurement of the exclusion of membrane-impermeable molecules can also be used (33,69). Considerable variability in the ability to reseal has been noted between different membrane preparations. Aging at low temperature and freezing and thawing both reduce the ability of ghosts to reseal. It was interesting to note that trypsinization of the ghosts effected resealing. The proportion sealed by trypsin is also dependent on prior treatment. The process is quite rapid, which suggests

that cleavage of a limited number of polypeptides may be involved. Component IVa of the erythrocyte membrane is the most rapidly cleaved of the major erythrocyte polpeptides (33), but there is no direct evidence that this cleavage is involved in resealing. Resealing of the ghost does not prevent further digestion of the membrane polypeptides, indicating that trypsin is present on both sides of the membrane before resealing. Digestion cannot be halted completely by addition of protein inhibitors of the protease since the protease on the inside is inaccessible to external proteins. Whether these observations might be pertinent to other membrane types is unknown, but appropriate cautions should be exercised in interpreting studies on protease digestion of membranes of all types.

Because proteolytic resealing of the erythrocyte membrane is also dependent on the condition and prior treatments of the membrane sample, it is possible to observe release of glyceraldehyde-3-phosphate dehydrogenase by trypsinization in some case. The maximal release of the enzyme was observed with virtually no digestion of spectrin (Figure 22). This suggests that the dehydrogenase is not bound to the spectrin, although proteolysis of other membrane components might affect the enzyme-spectrin interaction and cause release of the dehydrogenase. These observations are pertinent in the light of previous reports of the binding of aldolase and glyceraldehyde-3-phosphate dehydrogenase to actin (70), which is quite similar to spectrin in many of its properties (71).

The hypotonic hemolysis of erythrocytes in Tris buffer causes the release of only 20-40% of the glyceraldehyde-3-phosphate dehydrogenase from the erythrocyte membrane under conditions where 85-95% of the hemoglobin and lactic dehydrogenase are released. The addition of ATP (1 mM) causes a marked enhancement of the release of the glyceraldehyde-3-

phosphate dehydrogenase activity without affecting hemoglobin release. EDTA, 2,3-diphosphoglycerate and divalent cations show little effect on the dehydrogenase release at comparable concentrations, although enhanced release can be caused by higher concentrations of these substances. Thus the ATP effect does not appear to be related to its ability to chelate ions.

Most of the previous studies have concentrated on the effects of variations in hypotonic lysis conditions on the enzymes associated with the membrane. In the present study it was also sought to determine how variations in the lysis procedure might affect enzyme association with the membrane. Freeze-thaw lysis in the presence of isotonic salt caused virtually complete release of the GAPD. In isotonic sucrose the release was not complete, but it was still considerably more extensive than by hypotonic hemolysis. Digitonin will also cause release of GAPD from the erythrocyte membrane, although considerably higher digitonin concentrations were required than for hemolysis. ATP and salt increased GAPD release during digitonin hemolysis.

The effect of increased salt concentration on the release of GAPD from the membrane suggests that ionic forces may be involved in its binding. However, the release of GAPD by ATP and digitonin does not appear to support this hypothesis, although it is possible that these agents could affect ionic binding indirectly. It seems more plausible that a number of agents which perturb membrane structure are capable of effecting GAPD release. Certainly the sites and modes of action of salt, ATP and digitonin should be different. Thus it appears that the association of the enzyme with the membrane is sensitive to a number of different membrane perturbing agents and can be readily broken by these.

Unfortunately this does not permit us to specify the nature of the enzyme-membrane interaction.

Soluble erythrocyte glyceraldehyde-3-phosphate dehydrogenase was affected by ATP in virtually the same manner as the rabbit muscle and yeast enzymes (34,35,36). The erythrocyte enzyme was inactivated at low temperatures in the presence of ATP by a process that was dependent on the concentration of the enzyme. At 25^{o} C no inactivation was noted. Preincubation with NAD prevented the ATP inhibition. ATP also rendered the dehydrogenase sensitive to proteolysis by chymotrypsin at 25^{o} C. By analogy with previous work on the rabbit muscle and yeast enzymes (34, 35,36) it appears that in the presence of ATP the erythrocyte enzyme undergoes a conformational change which leads to an eventual dissociation into subunits at low temperatures. These changes are apparently not involved in the release of the dehydrogenase from the membrane, since the ATP-promoted release is not temperature dependent and is not affected by NAD.

The ATP effect on the enzyme release may result from a direct effect on the erythrocyte membrane. Graham and Wallach (64) have shown evidence of ATP-induced conformational changes on erythrocyte membrane proteins by infrared spectroscopy. Calcium can also alter membrane structure, as indicated by fluorescent probes (70) and by Ca^{++}-induced proteolysis and aggregation of erythrocyte membrane proteins (72). This latter effect occurs over the same range of Ca^{++} concentrations which causes the dehydrogenase release. The difference between the effect of ATP on the inactivation of the soluble enzyme and on enzyme release is not too unexpected, since the concentration of free ATP which is available for binding to the dehydrogenase is probably not very great in the hemolysis

system because of the number of ATP binding sites on the membrane or hemoglobin.

The physiological significance of the ATP-promoted release of the dehydrogenase from the membrane is still a matter for conjecture. Any attempt to answer this question must first consider whether the dehydrogenase (and other glycolytic enzymes) is bound to the membrane in the intact cell or whether it becomes associated during hemolysis. One can envision four different possibilities for the arrangement of the dehydrogenase and other glycolytic enzymes of the intact erythrocyte: (a) the enzymes are all soluble in the cell; (b) some of the enzymes are soluble and some are membrane bound; (c) all of the enzymes are bound to the membrane; or (d) the enzymes are situated between the interior surface of the membrane and the fibrous network at or near the interior surface. The last organization would be similar to the periplasmic space of Gram-negative bacteria. In this case the bulk of the enzymes would be released by hypotonic shock (hemolysis), which could partially free the fibrous material from its association with the membrane. Only those enzymes most closely associated with the membrane would not be released. This type of explanation might account for the large variations noted in the tendencies of different erythrocyte enzymes to associate with the membrane during hemolysis (29). In any of the cases in which the enzymes are bound directly to the membrane, there is a potential for some control of the enzyme activities by substances which act on the membrane. In the latter example an additional perturbation of activities might be achieved through the fibrous protein. Experimental results are currently too limited to suggest particular examples of this type of control, but such mechanisms should be considered along with substrate levels and

allosteric effects as a mode of controlling metabolic fluxes. The effects of ATP are particularly significant because of the role of GAPD in ATP production in the erythrocyte. It is possible that ATP control of GAPD binding to the membrane could exert a regulating effect on ATP production in the intact erythrocyte. However, it is questionable if GAPD exerts a key role in the control of erythrocyte glycolysis (73). Further investigations are needed to understand both the forces involved in enzyme-membrane associations and the role of these associations in enzyme functions and control.

CHAPTER V

SUMMARY

Treatment of erythrocytes or their isolated membranes with radioactive iodoacetate resulted in the specific modification of one (component VIII) of the membrane proteins, as determined by acrylamide gel electrophoresis in sodium dodecyl sulfate. The reactive groups of this protein were almost completely blocked before any of the other major membrane proteins reacted. The reaction with N-ethylmaleimide did not show a similar specificity of labeling, indicating a considerable degree of reagent specificity for the iodoacetate reaction. The specifically labeled protein was extracted from the membrane with sodium chloride and further purified by chromatography on Sephadex G-200 in sodium dodecyl sulfate. This preparation showed a single band on acrylamide gel electrophoresis in sodium dodecyl sulfate with a molecular weight of 35,000-40,000. The labeled amino acid was identified as cysteine from its elution position on ion exchange chromatography.

By enzyme activity studies of specific membrane extracts and the specificity of iodoacetate inactivation and labeling of the enzyme, component VIII of erythrocyte membrane proteins was identified as subunits of glyceraldehyde-3-phosphate dehydrogenase (GAPD) associated with erythrocyte membranes.

The association of enzymes with membranes is of interest because of the possibility for the control of enzyme activities or metabolic fluxes

by enzyme-membrane interaction. Studies on the association of the enzyme with the membrane are complicated by the tendency of the ghosts to seal under certain conditions, thus rendering the enzyme inaccessible to its substrates. This crypticity of the enzyme activity could be eliminated by detergent treatment. Sodium dodecyl sulfate was preferred, since at low concentrations it caused only minor decreases in the enzyme activity, while Triton X-100 gave more extensive inactivation at all concentrations tested. Trypsin digestion could release glyceraldehyde-3-phosphate dehydrogenase from the membrane, but it also caused a fraction of the ghosts to seal. The fraction which resealed was somewhat variable between different membrane preparations and was related to the prior treatments of the membranes.

Under standard hypotonic hemolysis conditions (10 mM Tris) 60-80% of the glyceraldehyde-3-phosphate dehydrogenase remained associated with the membrane. Aldolase exhibited similar behavior, while lactic dehydrogenase and hemoglobin were almost completely released. In the presence of ATP (1 mM) the amount of glyceraldehyde-3-phosphate dehydrogenase associated with the membrane was reduced to 5-20%. Other nucleotides were not as effective as ATP. EDTA, Ca^{++}, Mg^{++} and 2,3-diphosphoglycerate showed little effect at a concentration of 1 mM, although release of the enzyme was promoted by these agents at higher concentrations. Hemolysis of erythrocytes by a freeze-thaw procedure in isotonic buffer or sucrose caused virtually complete release of glyceraldehyde-3-phosphate dehydrogenase from the membrane. However, release of glyceraldehyde-3-phosphate dehydrogenase from isolated membranes by freeze-thaw treatment was strongly dependent on the medium used for the treatment. Lysis by digitonin at low concentrations (1.25 mg

digitonin per ml of erythrocytes) did not result in appreciable enzyme release, but higher concentrations of digitonin resulted in almost complete release. ATP and salt enhanced the dissociation of the glyceraldehyde-3-phosphate dehydrogenase from the membrane at low digitonin concentrations. Thus it appears that the enzyme-membrane association is very susceptible to effects which result in a perturbation of membrane structure.

At 0^{o} C solubilized glyceraldehyde-3-phosphate dehydrogenase was inactivated by ATP, a process that can be prevented if the enzyme was preincubated with NAD or if the incubation with ATP was performed at 25^{o} C. The ATP-promoted release of glyceraldehyde-3-phosphate dehydrogenase during hemolysis was not altered by preincubation with NAD or by raising the temperature to 25^{o} C. Therefore the two phenomena (enzyme inactivation and release from the membrane) appeared to be unrelated. It is postulated that the ATP effect on the enzyme release is due to a direct action of ATP on the membrane.

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