IDENTIFICATION AND CHARACTERIZATION OF ABNORMAL ERYTHROCYTE MEMBRANE PROTEINS IN HEREDITARY SPHEROCYTOSIS

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ABSTRACT

Several lines of evidence point to an inherent membrane disorder associated with erythrocytes in hereditary spherocytosis (HS). Membrane proteins have been studied to elucidate an abnormality.

Erythrocyte membranes isolated from affected and unaffected members of a large kindred have been partially solubilized with a zwitterionic detergent which can be employed in gel isoelectric-focusing (IEF). Members affected with HS frequently exhibit an absence of protein bands which focus between pH 6.5 and 7.5 when compared to unaffected relatives and other normals.

Separations of normal membrane proteins using gel filtration chromatography have been employed in order to identify these particular proteins. Subsequent fraction analysis by IEF and SDS electrophoresis indicates that these proteins correspond to the isozymes of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This finding was confirmed when GAPDH isolated directly from normal erythrocyte membranes was similarly analyzed.

Elution of GAPDH from erythrocyte membranes as a function of ionic strength has been studied. Analysis of the membranes by quantitative densitometry of SDS electrophoresis gels indicates that half as much GAPDH (band 6) remains on HS membranes in solutions with sodium chloride concentrations at or near 0.15 M when compared to normal membranes. Further, a two-fold increase in release of GAPDH enzyme activity from erythrocyte membranes was observed at these sodium chloride concentrations for HS membranes compared to normals. These results indicate an altered attachment.

An abnormality involving GAPDH binding to erythrocyte membranes of affected members of this kindred may lead to an understanding of the molecular basis of HS and of autosomal dominant disorders.

IDENTIFICATION AND CHARACTERIZATION OF ABNORMAL ERYTHROCYTE MEMBRANE PROTEINS IN HEREDITARY SPHEROCYTOSIS

CHAPTER I

GENERAL INTRODUCTION

Hereditary spherocytosis is a congenital hemolytic disorder associated with an unknown defect in the red blood cell. It is inherited in an autosomal dominant fashion and is therefore expressed in heterozygotes. The prevalence of hereditary spherocytosis in the general population is approximately 1 per 5000.

A. Distinguishing Characteristics

Microscopic examination of a sample of red blood cells from hereditary spherocytosis patients reveals that a portion of the cells are more spheroidal in shape when compared to the biconcave disk shaped cells from normal individuals. These abnormally shaped red cells are called spherocytes.

Spherocytes may be distinguished from normal erythrocytes by their dark, rounded appearance, their lack of a central pale area, and their smaller diameter and greater thickness. They may be quantitatively measured by performing osmotic fragility tests in which the red cells are suspended in aqueous solutions of various sodium chloride concentrations. The hypotonic solutions cause the cells to absorb water in order to relieve the osmotic pressure and to swell. As the red cells swell they become leaky and lose intracellular components including hemoglobin. The amount of hemoglobin which escapes can be quantitatively measured and the results are usually plotted as percent

hemolysis versus gram percent sodium chloride (% NaCl w/v). Typical osmotic fragility curves of normal red blood cells and hereditary spherocytosis cells clearly indicate the presence of spherocytes in patients affected with the disorder, as they are more susceptible to hypotonic hemolysis (1). When normal and abnormal red cells are subjected to sterile incubations for 24 hours at 37°C, they are found to be more osmotically fragile than the corresponding unincubated cells. Further, spherocytes which have been incubated for the 24 hour period are much more osmotically fragile when compared to normal, incubated red blood cells. Presumably this difference is due to structural instability of the spherocyte as a result of metabolic deprivation since supplementing the incubating cells with glucose suppresses the instability (2,3).

Spherocytes have other characteristics which distinguish them from normal erythrocytes. Membranes of spherocytes are inherently more permeable to sodium, a fact which has far-reaching consequences (4). Spherocytes are deficient in surface area (5) and have diminished lifespans in both patients and normal subjects (6).

The direct consequence of increased sodium permeability is a rise in intracellular sodium levels. As this begins to occur, the sodium-potassium pump is increased, involving activation of ATPase. This enzyme catalyzes the reaction $ATP \rightarrow ADP + P_i$, the products of which cause energy production via glycolysis to increase. Several workers have demonstrated overall elevated rates of glycolysis by measuring increased glucose consumption or lactic acid formation in the red cells of hereditary spherocytosis patients (4,7,8).

The spleen is the principle site of abnormal red blood cell destruction. It serves to filter out abnormal cells from normal ones, to damage them, and eventually to destroy them. At some point the spleen becomes engorged with spherocytes often resulting in splenomegaly. As a result of the spleen's filtering capacity, the lifespan of the spherocyte is considerably less than the 120 day average lifespan of the normal erythrocyte. The stem cells of the bone marrow must produce red blood cells at elevated rates to relieve the affects of the resulting anemia; the immature red cell or reticulocyte count is therefore frequently elevated in patients affected by this disorder. Occasionally the stem cells cease to produce red blood cells altogether, a condition known as an aplastic crisis. The present clinical solution for minimizing the manifesting symptoms is to splenectomize the patient. Although the cells themselves remain abnormal after splenectomy, their lifespan is greatly increased in vivo.

B. Normal Erythrocyte Membrane Protein Nomenclature

Many lines of evidence lead eminent investigators to suspect that the primary defect associated with red blood cells from hereditary spherocytosis patients is membrane-related (9). Studies of erythrocyte membrane proteins and lipids are therefore fields under intense investigation. A major contribution in this field has been made by Fairbanks *et al* (10) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) as the protein separating technique. When hemoglobin-free, pearly white erythrocyte membranes ("ghosts") are solubilized in SDS and subsequently analyzed by SDS PAGE, seven

major protein-stained bands result. These bands may correspond to major erythrocyte membrane proteins or to molecular weight classes of proteins. The nomenclature for the protein bands established by Fairbanks et al (10) is extensively cited in the literature and will be used in forthcoming discussions (Figure 4, left gel).

The protein bands of least mobility, bands 1 and 2, correspond to fibrous proteins collectively referred to as "spectrin" (11). They have molecular weights of approximately 240,000 and 215,000 daltons, respectively (12) and have been implicated in membrane shape regulation. Band 3 has a greater relative mobility on SDS PAGE and is thought to exist in dimeric form in vivo within the erythrocyte membrane. It extends entirely through the bilayer, having ends lying in both intracellular and extracellular spaces. Band 3 has been associated with the anion channel which leads into the erythrocyte (13). Depending upon the electrophoretic conditions, band 4 can be resolved into bands 4.1, 4.2 and a multiplet designated as 4.5. Little is known about the proteins which comprise band 4, but some investigators feel that at least some of the proteins of band 4 may be associated with band 3 (14). The protein corresponding to band 5 has been labeled the "actin-like" protein since it interacts with spectrin in a way comparable to the actomyosin complex which exists in muscles (13). Data supporting this comparison, however, are scarce. Band 6 has been identified as the monomeric form of the enzyme glyceraldehyde-3-phosphate dehydrogenase (15,16). This is an enzyme which specifically regulates the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, a reaction in the Embden

Meyerhoff Parnas pathway. Band 7 is another of the stainable bands on SDS PAGE, but very little is known about it.

By using periodic acid schiff base (PAS) staining techniques, glycoproteins of erythrocyte membranes may be visualized on SDS gels. These have been designated PAS bands 1, 2, 3 and 4.

Other peptide species of the erythrocyte membrane which migrate into SDS gels are difficult to detect by staining methods, even though they may have major functional roles associated with erythrocytes (e.g. ATPase).

C. Literature Survey of Abnormalities Associated with Hereditary Spherocytosis

Many studies of erythrocyte membranes have been made in order to elucidate the nature of a molecular defect in hereditary spherocytosis. Four broad categories of abnormalities associated with hereditary spherocytosis have been reported.

1. Abnormal Fibrous Membrane Proteins. Jacob has studied erythrocyte membranes of hereditary spherocytosis patients specifically with regard to fibrous membrane proteins, of which spectrin is at least a constituent (9). Extractions of these proteins from normal membranes are accomplished in ion-free media. Subsequent increases in the potassium or calcium ion concentrations bring about the formation of protein polymers because the sedimentation coefficients for the proteins increase coincident with cation addition. Similar studies with membranes from hereditary spherocytosis patients indicate that in some cases some and in other cases none of the fibrous protein polymerizes. Jacob interprets these findings by

postulating two forms of hereditary spherocytosis, both of which involve mutant protein interaction sites.

Boivin and Garland have studied the erythrocyte's fibrous proteins by isolating and purifying spectrin from normal and hereditary spherocytosis affected membranes (17). Isoelectric-focusing of the purified spectrin in 8 M urea resulted in identical staining patterns for affected and unaffected subjects with the exception of the lack of one pH 8.7 component in two patients. The authors interpret these results by inferring that spectrin is probably not altered in the membranes of the hereditary spherocytosis affected patients which they examined.

2. Abnormal Membrane Phosphorylation. Feig and Guidotti have studied the Ca^{2+}-dependent ATPase of hereditary spherocytosis membranes (18). They find its activity to be similar for hereditary spherocytosis and normal erythrocytes. Since hereditary spherocytosis red blood cell populations contain relatively elevated reticulocyte counts compared to normal red blood cell populations, separations of the cells according to age were made. The result reported is that when hereditary spherocytosis red cells are age matched with normals, the spherocytic red cells are relatively deficient in Ca^{2+}-ATPase activity.

Kirkpatrick, Wood, and LaCelle have studied Ca^{2+} and Mg^{2+} stimulated ATPase activity associated with water soluble (WSP contain spectrin) and insoluble proteins (residual vesicles) from normal and hereditary spherocytosis membranes (19). The ATPase activity of the WSP isolated from hereditary spherocytosis red cell membranes is

absent at low levels of Ca^{2+} (< 1 mM). This is not the case for normal WSP. Although the WSP Ca^{2+}-ATPase activity is absent in hereditary spherocytosis cells, the Mg^{2+}- ATPase activity is elevated in the corresponding residual vesicles when compared to the activity of normal residual vesicles.

Zail and Van Den Hoek (20) sought to confirm the finding of decreased Ca^{2+}-ATPase as initially reported by Feig and Guidotti (18). Intact erythrocytes from hereditary spherocytosis affected and unaffected patients were preloaded with Ca^{2+}. The efficiency of the calcium pump operating within the cell was subsequently determined by measuring the Ca^{2+} efflux from the cells. The results indicate both normal Ca^{2+} efflux and intracellular erythrocyte Ca^{2+} concentrations for hereditary spherocytosis cells, contrary to the two earlier studies. These investigators conclude that calcium does not accumulate in hereditary spherocytosis affected erythrocytes.

Greenquist and Shohet have examined membrane phosphorylation by incubating erythrocyte membranes in γ-^{32}P ATP reaction mixtures (21). Treated membranes were solubilized in SDS and subjected to SDS PAGE. Membrane incorporated ^{32}P was measured by slicing SDS gels and counting the ^{32}P in each slice. Although variable absolute levels of ^{32}P incorporation were reported between experiments with the same preparations, average ^{32}P labeling was determined. Twenty-two of the twenty-five hereditary spherocytosis affected patients examined in this study exhibited reduced average ^{32}P labeling in bands 2, 3, and 4.5. The authors conclude that the hereditary spherocytosis disorder involving cell shape and deformability may be mediated by

a defect in erythrocyte membrane phosphorylation.

Matsumoto, Yawata, and Jacob also find decreased membrane protein phosphorylation associated with red cells from hereditary spherocytosis patients (22). Erythrocytes in the presence of elevated temperatures (50°C) or sulfhydryl inhibitors also manifest deficient membrane phosphorylation, phenomena which the authors report correspond exactly with the onset of red cell sphering. The conclusion reached is that phosphorylation of certain membrane proteins may modulate the shape of the red cell, although the mechanism of control is unknown.

The studies of Zail and Van Den Hoek indicate that Ca^{2+}-dependent membrane phosphorylation is not deficient in the hereditary spherocytosis affected patients examined (20).

Protein kinases are enzymes which catalyze the phosphorylation of specific proteins. Beutler, Guinto, and Johnson have studied erythrocyte protein kinase activity in order to identify abnormalities in phosphorylation of erythrocyte membranes isolated from hereditary spherocytosis patients (23). Protein kinase activity of the abnormal membranes was found to be normal when the enzyme assays were limited to short incubation times of 5 - 10 minutes. Incubation for 60 minutes, however, resulted in an average decreased phosphorylation of the membranes of hereditary spherocytosis affected patients. The conclusion reached was that the mild phosphorylation abnormality which was observed in hereditary spherocytosis afflicted membranes could not be shown to be due to decreased protein kinase activity or altered kinetic properties, as both parameters were normal.

3. Abnormal Charge of Membrane Proteins. Engelhardt has devised a method for studying and quantifying the charge of erythrocyte membrane proteins (24). The theory behind the method assumes that the normal arrangement of membrane components is partly controlled by electrostatic forces; this arrangement in turn controls the erythrocyte's structure and permeability. The technique involves first, the disintegration of red blood cell membranes and second, their reassemblance within two-phase systems in the presence of ionic solutions. The results were that more cations (Ca^{2+} and less so Na^{+} and K^{+}) were required to bring about the reassembly of proteins isolated from hereditary spherocytosis affected membranes when compared to normal controls at protein concentrations $\leq$ 0.4 mg/ml. The conclusion is that membranes from hereditary spherocytes consist of components which have an excess of negative charges when compared with the components of normal controls.

4. Abnormal Quantities of Membrane Proteins. Hayashi et al (25) have done SDS electrophoresis studies of the SDS solubilized red cell membranes from 15 patients affected with hereditary spherocytosis. In four of the hereditary spherocytosis patients almost complete deficiency of band 4.2 was observed and in the eleven remaining patients a small but significant decrease in band 4.2 was reported. The conclusion made was that heterogeneity of the clinical defect of hereditary spherocytosis exists, since two forms of the hemolytic disorder were observed.

McCann et al have studied a large kindred which manifests hereditary spherocytosis (26). SDS PAGE of the proband's erythrocyte

membranes revealed diminished quantities of stainable band 6 (glyceraldehyde-3-phosphate dehydrogenase, GAPDH), a finding which was confirmed by specific enzyme assay. Determinations of whole red blood cell GAPDH for the proband resulted in values approximately half the normal value. Further examination of other family members led the authors to conclude that hereditary spherocytosis and GAPDH deficiency genetic traits separate independently, since several hereditary spherocytosis unaffected members exhibited GAPDH deficiency and several affected members exhibited no such deficiency. Membranes of family members affected by both traits, however, contained elevated quantities of band 4.5. In light of this and other data, the conclusion reached was that the increase in band 4.5 is a consequence of elevated erythrocyte metabolic stress.

As a follow-up study Allen et al (27) has identified the protein corresponding to band 4.5, the band (elevated) associated with metabolically stressed hereditary spherocytosis affected, GAPDH deficient patients in the previous study. Fourteen enzyme assays were performed on the affected ghosts and only catalase and lactic dehydrogenase activities were increased. Only catalase had the correct subunit molecular weight on SDS electrophoresis gels. The authors conclude that catalase accounts for an appreciable portion of the increased membrane bound band 4.5.

The many examples of abnormalities which have been observed in the membranes of hereditary spherocytosis patients demonstrate that a molecular defect of this disorder remains undetermined.

D. Preliminary Isoelectric-focusing Studies of Erythrocyte Membrane Proteins From a Kindred With Hereditary Spherocytosis

In an effort to develop new methods for the analysis of membranes and membrane proteins, Rowe (28) has devised a technique involving isoelectric-focusing (IEF) of erythrocyte membranes. This technique involves solubilization of erythrocyte membranes in buffered solutions which contain 8 M urea and 0.5% (w/v) sulfobetaine. Unlike many charged detergents, such as sodium dodecyl sulfate (SDS), the zwitterionic detergent sulfobetaine may be employed in IEF procedures because it is effectively neutral, yet charged, over the pH 3 - 10 range normally studied (29). The charged character of sulfobetaine provides an advantage over uncharged detergents, such as Triton X-100 or NP-40, because it more effectively solubilizes the hydrophobic portions of proteins which may be embedded within the lipid bilayer of the membrane (Chapter 2).

After membranes are solubilized according to this technique, they are applied to polyacrylamide gels which contain 8 M urea, 0.5% sulfobetaine, and 1.5% ampholytes. Ampholytes are small molecules which migrate to their isoelectric point within the gel when a current is applied. A pH gradient is formed across the gel in this way. Solubilized proteins are separated according to their intrinsic charge upon application of an electric field by also migrating to their isoelectric point. The proteins remain at this point and become sharply focused because IEF is an equilibrium chromatographic technique. After the proteins have focused, the gel is stained for protein with Coomassie brilliant blue.

With this technique, Rowe has examined a kindred which manifests the hemolytic disorder hereditary spherocytosis. In these studies alterations in the protein patterns were observed for family members who had been clinically diagnosed as having hereditary spherocytosis when compared to the protein patterns of unaffected relatives and controls. Specifically, a series of three to five bands which focus in the upper pH range of a pH 3.5 - 10 gel, was missing or diffusely focused in family members affected by hereditary spherocytosis when compared to the normal pattern (Figure 1). Corresponding densitometric scans of normal and hereditary spherocytosis gel protein patterns are given in Figure 2. As can be seen, several peaks are missing in the scan of the hereditary spherocytosis protein pattern in the pH range 6.5 to 7.5. Figure 3 is a pedigree chart of the kindred examined by Rowe in which the biochemical marker seen upon isoelectric-focusing of erythrocyte membranes was observed. All family members studied who were hereditary spherocytosis affected exhibited altered banding patterns when compared to those of unaffected relatives and other unrelated normals.

E. Thesis Goals

The goal of this thesis project involves identification of the proteins, from normal erythrocyte membranes, which focus in the upper pH range of a pH 3.5 - 10 isoelectric-focusing gel. Further, characterization of their properties when isolated from hereditary spherocytosis erythrocyte membranes will be studied. The elucidation of an abnormality involving one or several proteins may lead to an understanding of the molecular basis of hereditary spherocytosis and a better understanding of autosomal dominant disorders.

Figure 1. Comparison of Isoelectrically - Focused Erythrocyte Membrane Proteins from Normal Individual and Hereditary Spherocytosis Patient. Isolated membranes were dissolved in 0.5% sulfobetaine and 8 M urea, and applied to a 1 mm thick horizontal slab gel containing ampholytes to form a pH gradient from pH 3.5 to 10. Proteins were stained with Coomassie brilliant blue. Bands focusing at cathodal end (top) of gel are present in the normal individual (left) and absent in the hereditary stpheocytosis patient (right).

FIGURE 1

COMPARISON OF ISOELECTRICALLY - FOCUSED ERYTHROCYTE MEMBRANE PROTEINS FROM NORMAL INDIVIDUAL AND HEREDITARY SPHEROCYTOSIS PATIENT

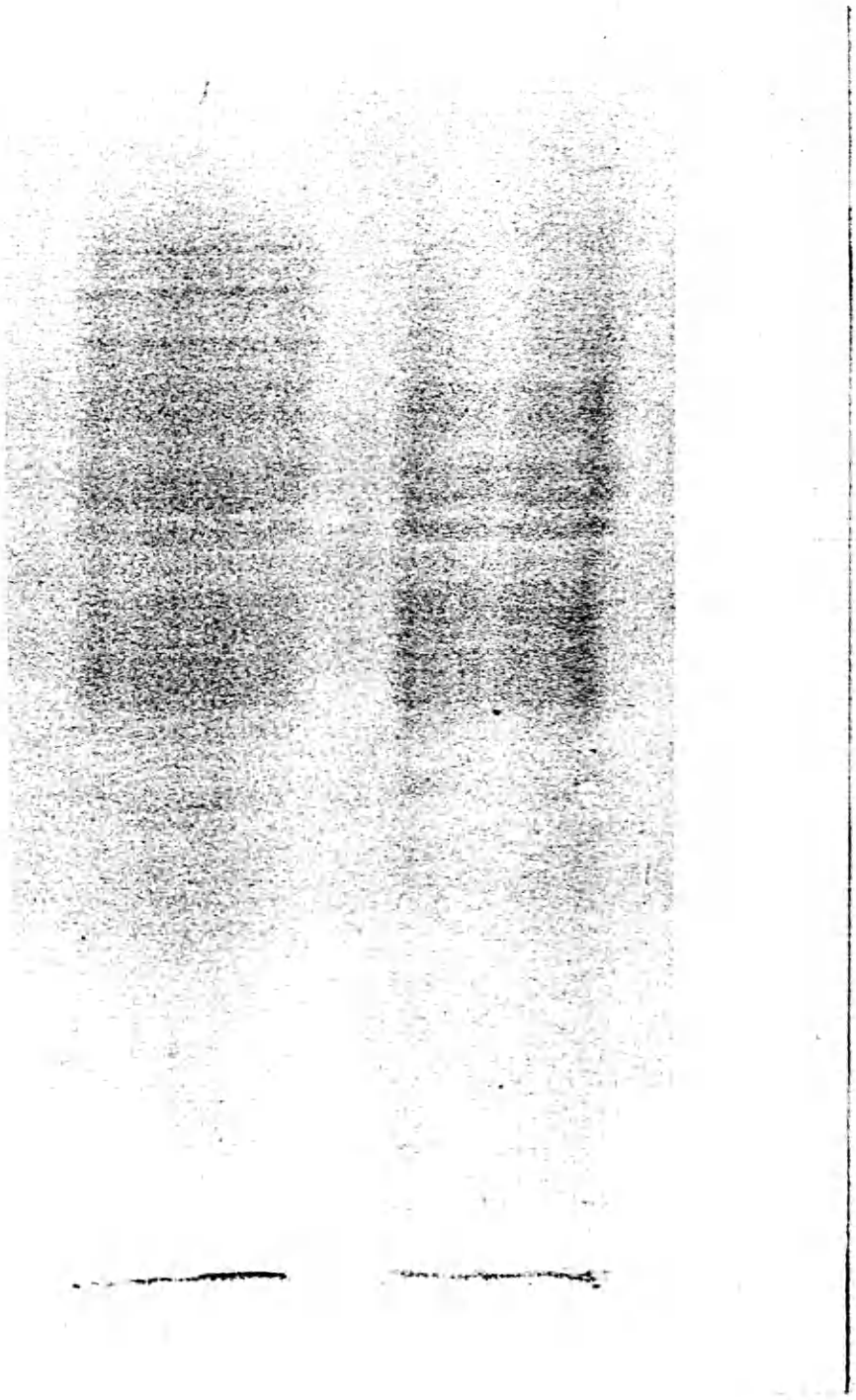
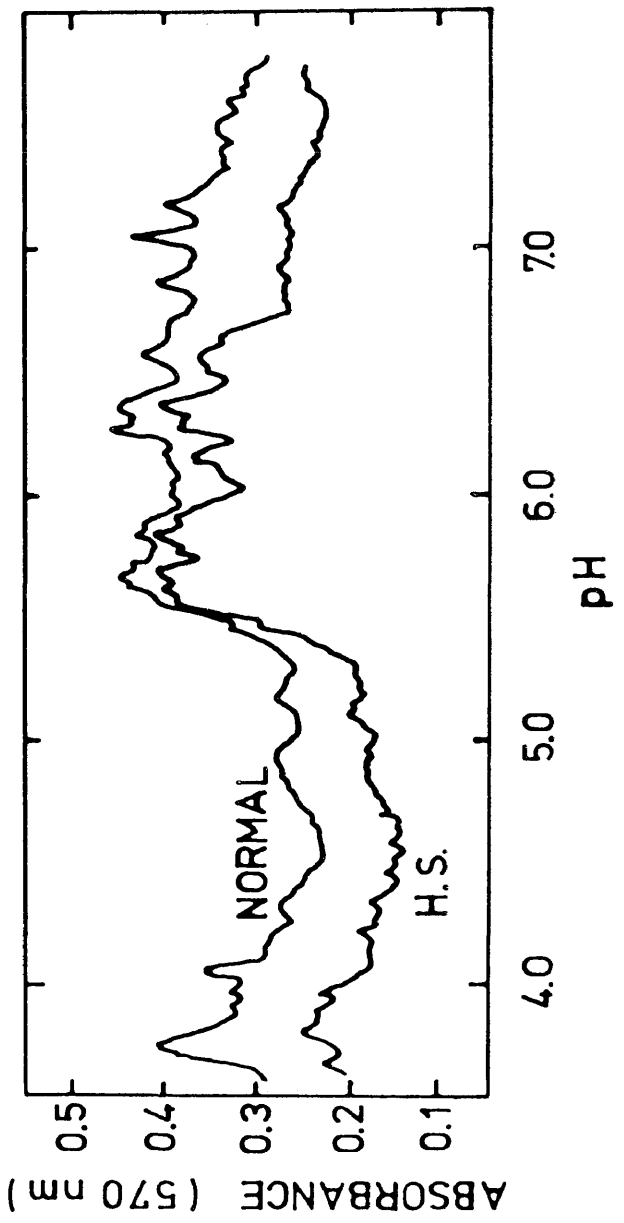


FIGURE 2

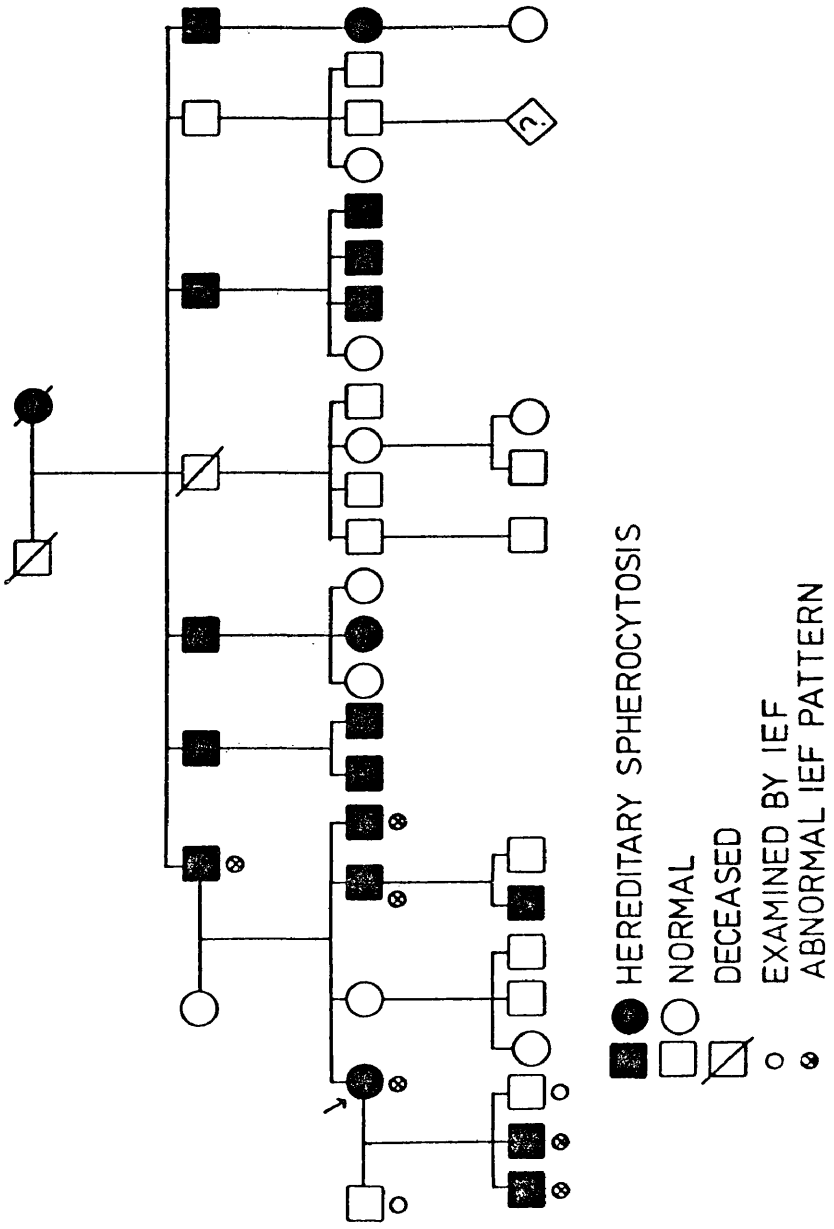
DENSITOMETRIC SCANS OF NORMAL INDIVIDUAL
AND HEREDITARY SPHEROCYTOSIS PATIENT



Membranes were prepared, focused, and stained as described. Protein bands were scanned at 570 nm. Proteins focusing between pH 6.5 and 7.5 in the normal profile were either missing or have an altered pI in hereditary spherocytosis patient.

FIGURE 3

PEDIGREE CHART OF KINDRED AFFECTED BY HEREDITARY SPHEROCYTOSIS



The biochemical marker seen upon isoelectric-focusing of erythrocyte membranes has been observed.

CHAPTER II

IDENTIFICATION OF ALTERED ERYTHROCYTE MEMBRANE PROTEINS IN HEREDITARY SPHEROCYTOSIS PATIENTS

INTRODUCTION

A method of analyzing erythrocyte membrane proteins using isoelectric-focusing in the presence of sulfobetaine and urea has been developed by Rowe (28; see Chapter I). Using this method, erythrocyte membrane proteins of a kindred manifesting hereditary spherocytosis have been examined. Comparisons of the hereditary spherocytosis membrane protein staining profiles with those from normal individuals led Rowe to conclude that a series of three to five protein bands, which focus in the upper pH range of a pH 3.5 to 10 gel, was either missing of altered in a way which affects the ionic charge of the proteins.

Identification of these affected proteins by analyzing normal erythrocyte membranes has been accomplished as described below.

EXPERIMENTAL

A. Materials

1. Blood. Samples of blood were obtained from subjects with hereditary spherocytosis, from their unaffected relatives, from other unaffected individuals, and from 21 day old packed red cells obtained at the Red Cross.

2. Chemicals. The following chemicals were obtained from:

Mallinckrodt Chemical Co.
Ammonium sulfate (pyridine-free)
Sodium hydroxide
Acetic acid
N,N,N',N'-Tetramethylethylenediamine (TEMED)
Tricarboxylic acid
Urea

Sigma Chemical Co.
Ethylenediamine tetraacetic acid, disodium salt (EDTA)
Trizma base, reagent grade (Tris)
Phenylmethylsulfonylfluoride (PMSF)
Sodium lauryl sulfate (SDS)
Dextran-2000
Coomassie brilliant blue, R-250

Fisher Scientific Co.
Ammonium persulfate
Sodium phosphate, monobasic
Sodium chloride
Sodium acetate
Sucrose

Eastman Kodak
Acrylamide
N,N'-methylene bisacrylamide
Ethanolamine

Matheson Coleman and Bell
Pyronin Y

U.S. Industrial Chemical, Co.
Ethyl alcohol, reagent grade

New England Nuclear
Triton X-100

Lonza, Inc.
Lonzaine, C.S. (sulfobetaine, 50% w/v)

Pharmacia
Sephadex G-200

Aldrich Chemical Co.
Methanol

LKB Instruments, Ltd.
Ampholine carrier ampholytes, pH 3.5 - 10 (40% w/v)

Calbiochem
Cleland's reagent (dithiothreitol)

3. Solutions.

a. 5P8
5 mM NaH_2PO_4
pH 8.0 with NaOH

b. PBS
5 mM NaH_2PO_4
150 mM NaCl
pH 8.0 with NaOH

c. PMSF
0.5 M PMSF in absolute ethanol

d. 2X Sulfobetaine-Urea Solution
40 mM Tris sulfate, pH 7.1
0.2 mM EDTA
16 mM dithiothreitol
1% (w/v) sulfobetaine
16 M urea

e. ConAcBis
40% (w/v) acrylamide
1.5% (w/v) N,N'-methylenebisacrylamide

f. IEF Stain Solution
45% (v/v) absolute ethanol
10% (v/v) glacial acetic acid
9% (w/v) Coomassie brilliant blue R-250

g. IEF Destain Solution
25% (v/v) absolute ethanol
10% (v/v) glacial acetic acid

h. 10X Buffer
0.4 M Tris
0.2 M sodium acetate
0.2 M EDTA

i. Tracking Dye Solution
2 mM Tris-Cl, pH 7.4
2 mM EDTA
8 mM dithiothreitol
0.6 M sucrose
0.07 mM pyronin Y

j. Electrophoresis Buffer
40 mM Tris
20 mM sodium acetate
2 mM EDTA
acetic acid to pH 7.4
0.1% (w/v) SDS

k. SDS Electrophoresis Stain Solution
50% (v/v) methanol
9% (v/v) glacial acetic acid
0.05% (w/v) Coomassie brilliant blue R-250

l. SDS Electrophoresis Destain Solution
5% (v/v) methanol
7.5% (v/v) glacial acetic acid

B. Methods

1. Erythrocyte Membrane Preparation. Erythrocyte membranes (ghosts) were prepared by the method of Steck et al (30). With 10 ml of heparinized blood, 1.1 ml of 0.1 M EDTA was mixed. Samples were subsequently centrifuged at 1000 x g at 4°C for 15 minutes. Plasma and buffy coat were removed by aspiration. Red cells were mixed with one volume of PBS and subsequently centrifuged as described. After aspiration of the supernatant buffer, this step was repeated twice, with final centrifugation for 20 minutes at 4°C. Packed red cells were then mixed with 40 volumes of 5P8, centrifuged at 20,000 x g at 4°C for 15 minutes, and the resulting supernatant buffer was removed by aspiration. Unlysed red cells which pack tightly to the bottom of the pellet were removed at this point. Washings in 5P8 buffer were repeated as before until a pearly-white pellet of ghosts remained. Membranes were washed in 25 volumes of 5P8, centrifuged at 100,000 x g at 4°C for 30 minutes, and the supernatant buffer was removed.

2. Isoelectric-focusing. The method of Rowe (28) was followed for isoelectric-focusing. Erythrocyte membranes were dissolved in one volume of 2X sulfobetaine-urea solution and 2 µℓ of PMSF were added. Gels 1 mm thick cast between glass plates 26 x 12.5 cm were of the following composition: 5.9% acrylamide, 0.2% N,N'-methylene

bisacrylamide, 0.04% ammonium persulfate, 8 M urea, 0.5% sulfobetaine, 1.5% ampholytes (pH 3.5 - 10). Electrofocusing strips (LKB Instruments, Ltd.) 24 cm long were soaked in 0.1 M HCl for the anode wick and in 0.15 M ethanolamine for the cathode wick. Solubilized membrane samples were applied to 10 x 2 mm strips. These were placed 0.5 cm from the anode wick so that focusing would occur from anode to cathode. Focusing was accomplished on an LKB Multiphor apparatus at 10°C by maintaining 2.5 watts across the gel for 30 minutes, 5 watts for 45 minutes, and 12.5 watts for the remaining 5 hour run. After focusing was complete, the gel was placed in 200 ml of 12.5% (w/v) trichloroacetic acid for 20 minutes while constantly being agitated. The gel was then rinsed with distilled H_2O and subsequently placed in 100 ml of IEF stain solution at 56°C, while being agitated for 20 minutes. The gel was then placed in 50 ml of IEF destain solution at room temperature while being agitated for 5 minutes. This step was then repeated one time. Storage of the gel was in 10% glacial acetic acid.

3. SDS Electrophoresis. The method of Fairbanks et al (10) was followed for SDS polyacrylamide gel electrophoresis. Gels cast in tubes 7.5 x 0.5 cm were of the following composition: 5.6% acrylamide, 0.2% N,N'-methylene bisacrylamide, 40 mM Tris acetate, pH 7.4, 20 mM sodium acetate, 2 mM EDTA, 0.1% ammonium persulfate, 0.1% SDS, and 0.02% TEMED. Layering solution of the following composition: 0.1% ammonium persulfate, 0.1% SDS, 0.02% TEMED was dispensed on top of the gel solution prior to polymerization in order to obtain a flat gel surface. Layering solution was replaced with electrophoresis buffer

prior to electrophoresis. Erythrocyte membranes dissolved in 1 volume of 4% (w/v) SDS were mixed with 1 volume of tracking dye solution (final concentration of SDS is 1%) and incubated at 37°C for 30 minutes. The mixture was then applied to the top of the gel by underlayering the electrophoresis buffer. Electrophoresis in a Buchler Polyanalyst was accomplished, from cathode to anode, by applying a current of 1 m amp per gel until the tracking solution moved into the gel, followed by 4 m amps/gel until the tracking solution was about 0.5 cm from the bottom of the gel. Gels were then removed from the tubes, placed in about 100 ml of SDS electrophoresis stain solution, and agitated overnight. Gels were subsequently placed in two changes of 100 ml of SDS electrophoresis destain solution and agitated overnight. Storage of the gels was in 10% glacial acetic acid.

4. Determination of Erythrocyte Membrane Proteins Solubilized in Sulfobetaine-Urea Solution. Erythrocyte membranes were solubilized in 1 volume of 2X sulfobetaine-urea solution. The mixture was centrifuged at 20,000 x g for 15 minutes at 4°C. The resulting supernatant solution was extensively dialyzed in 2% (w/v) SDS and subsequently analyzed by SDS electrophoresis.

5. Gel Filtration Chromatography. Sephadex G-200 was equilibrated in 50% (v/v) 2X sulfobetaine-urea solution in a 20 x 1.6 cm column. Erythrocyte membrane proteins soluble in 1 volume of 2X sulfobetaine-urea solution were made 10% in sucrose and one ml of this solution was layered onto the column which was eluted with 50% 2X sulfobetaine-urea solution at a flow rate of 13 ml/hr. Fractions corresponding to absorption peaks at 280 nm were analyzed by isoelectric-focusing

without further preparation and by SDS electrophoresis after extensive dialysis in 2% SDS in the presence of 0.25 mM PMSF (final concentrations).

6. Glyceraldehyde-3-Phosphate Dehydrogenase Isolation From Erythrocyte Membranes. The method of Kant and Steck (31) was used to isolate glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from erythrocyte membranes. Erythrocyte membranes were incubated for 20 minutes at 4°C in 20 volumes of PBS. The mixture was then centrifuged at 20,000 x g for 10 minutes at 4°C. The supernatant solution was then concentrated to 1 ml in an ultrafiltration cell (Diaflo model 12, plus PM-10 membrane, Amicon Corp.) and subsequently diluted to 10 ml with 5P8. Reconcentration of the supernatant to 1 ml was made and the process was repeated one time. Concentrated GAPDH was analyzed by IEF and SDS electrophoresis.

RESULTS

In an attempt to characterize the difference in isoelectric-focusing protein patterns of sulfobetaine-urea solubilized erythrocyte membranes between affected members of this kindred and unaffected relatives, solubilized proteins were analyzed by isoelectric-focusing in the previous set of experiments. Subsequent comparisons to the better characterized SDS electrophoresis system were made.

Erythrocyte Membrane Proteins Solubilized in Sulfobetaine-Urea Solution. As determined by direct observation, erythrocyte membranes are only partially solubilized in 1 volume of 2X sulfobetaine-urea solution. To determine which membrane proteins are soluble and therefore capable of being separated by the isoelectric-focusing

technique previously described, a fully solubilized sample was dialyzed in 2% SDS and subsequently analyzed by SDS electrophoresis. The gels in Figure 4 were obtained from such an experiment. As can be observed from the gels, the presence of spectrin (bands 1 and 2) is indicated in the sulfebetaine-urea soluble protein preparation, although some molecular weight differences seem evident. Bands 2.1, 2.2, and 2.3 are present, but only the shoulder of band 3 is observable in this gel. The presence of bands 4.1 and 4.2 may only be inferred since a diffuse series of bands appears in this region, as compared to the normal. A high concentration of the composite band 4.5 is present, while lesser relative quantities of band 5 are observable. Band 6 is enriched relative to the other bands. Some band 7 and elevated amounts of hemoglobin (bottom band) are also observable in this preparation.

Gel Filtration Chromatography. Separations of the sulfobetaine-urea soluble erythrocyte membrane proteins were accomplished using Sephadex G-200 in a gel filtration chromatography system. Figure 5 shows the elution profile of a column monitored at 280 nm. As can be seen, the peak of greatest intensity includes fraction 17. When this fraction was analyzed by isoelectric-focusing in the presence of sulfobetaine and urea and compared to the normal isoelectric-focusing staining profile, it was enriched in the bands which were missing in the staining profiles of hereditary spherocytosis affected kindred members (gel not shown). A portion of fraction 17 was extensively dialyzed in 2% SDS and analyzed by SDS electrophoresis (Figure 6). It may be observed to contain primarily band 6 when compared to the

Figure 4. Normal Erythrocyte Membrane Proteins and Those Soluble in Sulfobetaine-Urea Solution. Isolated membranes were dissolved in 1 volume of 2X sulfobetaine-urea solution, the soluble proteins were extensively dialyzed in 2% SDS, and subsequently electrophoresed in 0.1% SDS polyacrylamide gels. Normal erythrocyte membrane proteins soluble in SDS appear left and those soluble in sulfobetaine-urea appear right. Bands are designated according to the nomenclature of Fairbanks *et al* (10).

FIGURE 4

NORMAL ERYTHROCYTE MEMBRANE PROTEINS
AND THOSE SOLUBLE IN SULFOBETAINE-UREA SOLUTION

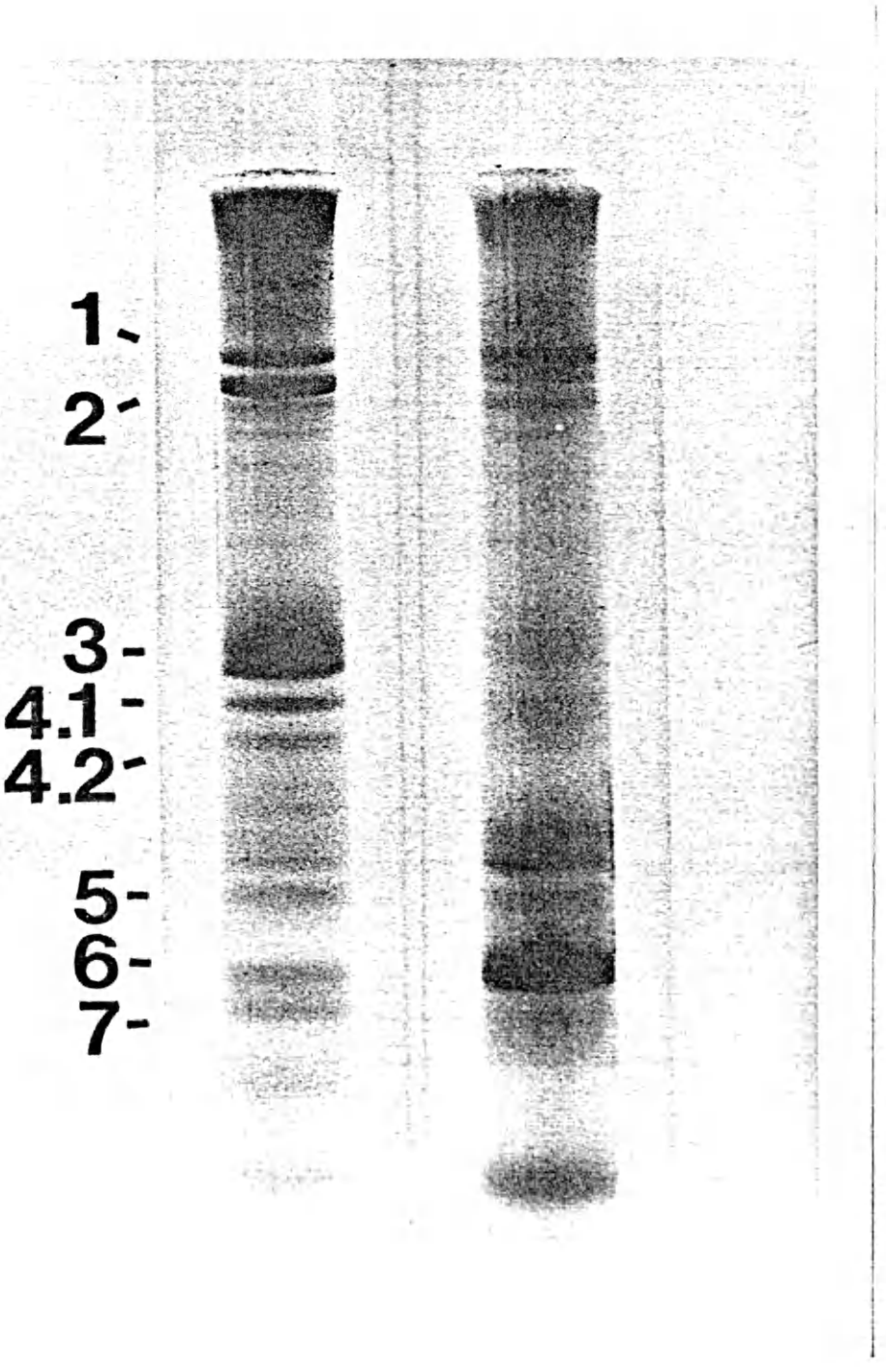


Figure 5. Gel Filtration Chromatography Elution Profile of Proteins Soluble in Sulfobetaine-Urea. Sulfobetaine-urea soluble proteins were separated using Sephadex G-200 in a column 20 x 1.6 cm, which was eluted with 50% 2X sulfobetaine-urea solution at a flow rate of 13 ml/hour. Absorbance of eluant was monitored at 280 nm.

FIGURE 5

GEL FILTRATION CHROMATOGRAPHY ELUTION PROFILE
OF PROTEINS SOLUBLE IN SULFOBETAINE-UREA

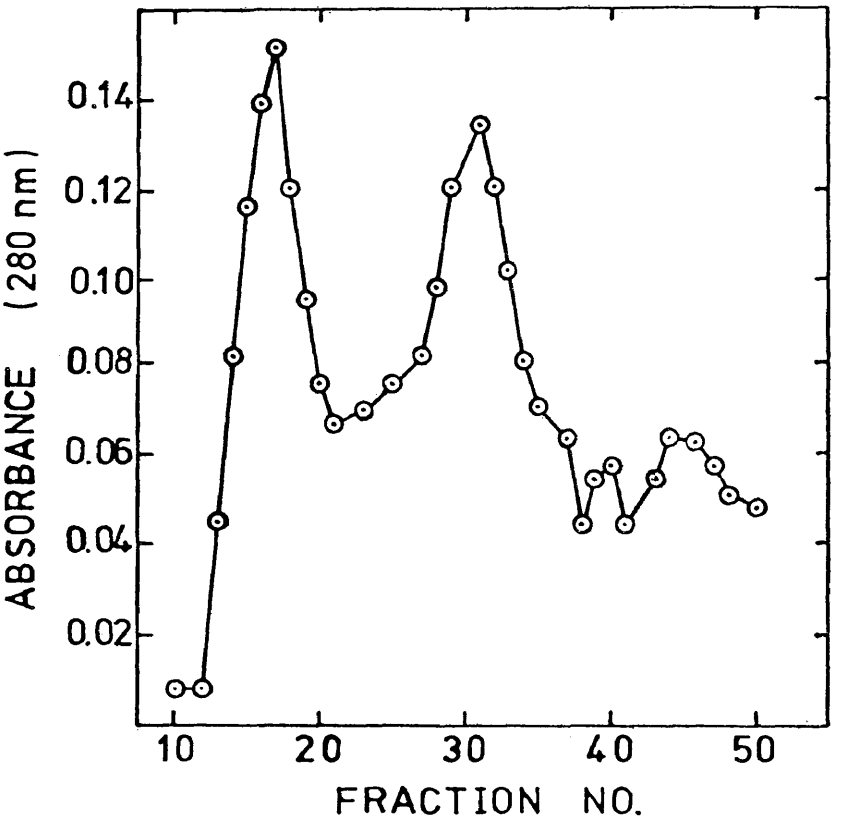
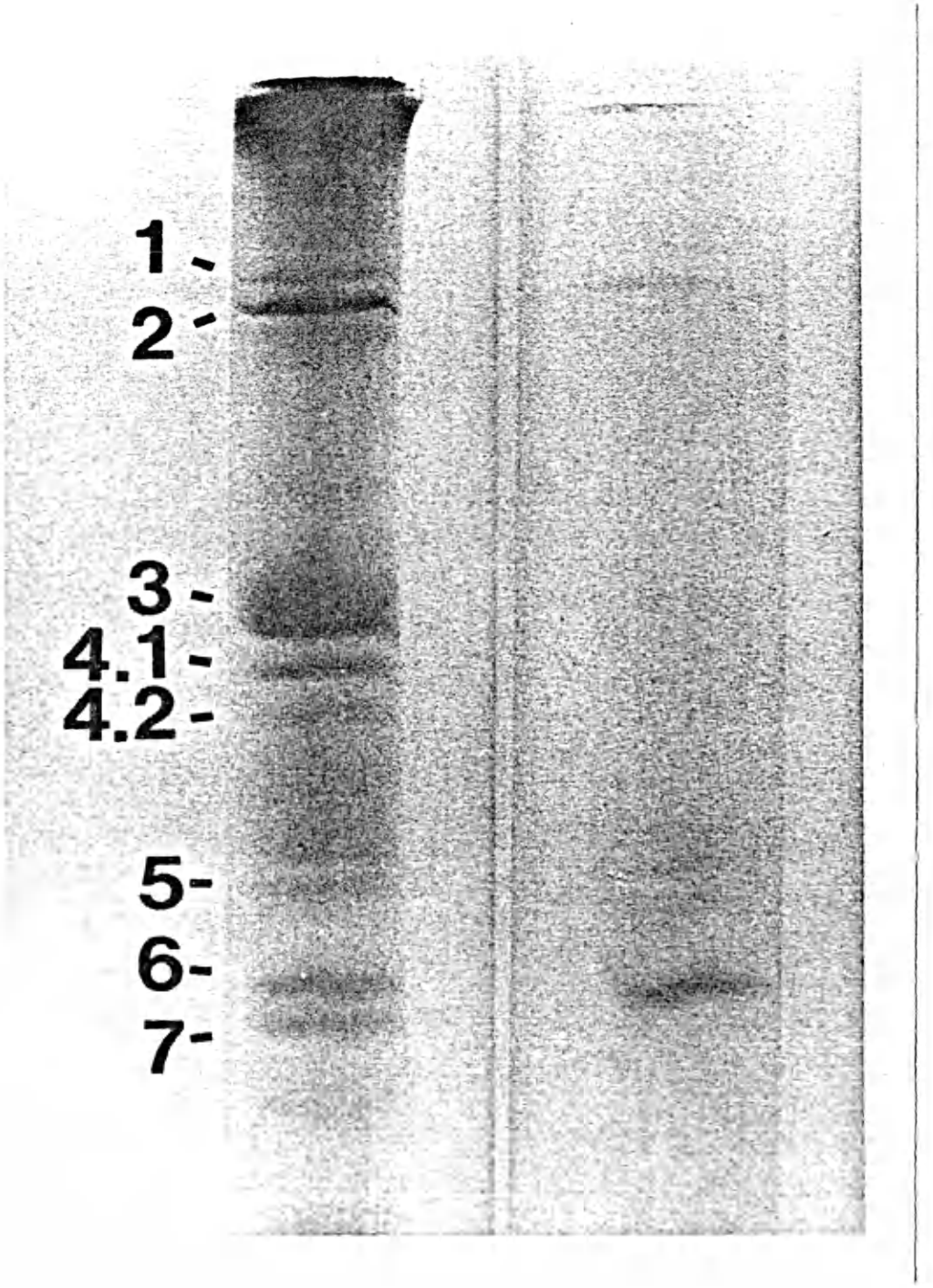


Figure 6. SDS Polyacrylamide Gel Electrophoresis of Normal Erythrocyte Membrane Proteins and of Fraction 17. Erythrocyte membrane proteins soluble in sulfobetaine-urea were separated using Sephadex G-200 gel filtration chromatography as described and fraction 17 (see Figure 5) was dialyzed in 2% SDS prior to SDS electrophoresis. Normal erythrocyte membrane proteins appear left and proteins contained in fraction 17 appear right.

FIGURE 6

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS
OF NORMAL ERYTHROCYTE MEMBRANE PROTEINS AND OF FRACTION 17



normal SDS electrophoresis staining profile. The working hypothesis was that band 6 corresponded to the bands (perhaps isozymes) which were missing in the isoelectric-focusing staining profile of the erythrocyte membranes from the kindred with hereditary spherocytosis.

GAPDH Isolation from Erythrocyte Membranes. Since band 6 of erythrocyte membranes has been shown to correspond to GAPDH (15,16), it was of interest to examine a preparation of GAPDH by isoelectric-focusing. GAPDH was isolated from normal erythrocyte membranes by the method of Kant and Steck (31), solubilized in 1 volume of 2X sulfobetaine-urea solution, and separated by isoelectric-focusing (Figure 7). The isoelectric-focusing staining pattern shows that the preparation contains high concentrations of the proteins missing in the hereditary spherocytosis affected kindred members. SDS electrophoresis of a sample of this same GAPDH preparation resulted in the gels shown in Figure 8. Although minute amounts of bands 4.2 and 4.5 are observed, the procedure is highly selective for removal of band 6 from the erythrocyte membrane. Densitometric scans (at 570 nm) of the gels in Figure 8 confirm the visual observation which identifies GAPDH as band 6 of normal erythrocyte membranes (Figure 9). Band 6, and therefore GAPDH, corresponds to the bands which were missing in the isoelectric-focusing staining profiles of the erythrocyte membranes from hereditary spherocytosis affected kindred members.

DISCUSSION

Erythrocyte membrane proteins have been classified by Singer (32) as extrinsic or intrinsic. Extrinsic proteins are those which can be

Figure 7. Isoelectric-Focusing Pattern of GAPDH Isolated from Normal Erythrocyte Membranes. GAPDH was isolated from normal erythrocyte membranes by washing them in 20 volumes of PBS for 20 minutes at 4°C (31), centrifuging the suspended membranes at 20,000 x g for 10 minutes at 4°C, and concentrating and dialyzing the supernatant. The concentrated preparation was solubilized in sulfobetaine-urea and analyzed by isoelectric focusing as described.

FIGURE 7

ISOELECTRIC-FOCUSING PATTERN OF GAPDH
ISOLATED FROM NORMAL ERYTHROCYTE MEMBRANES

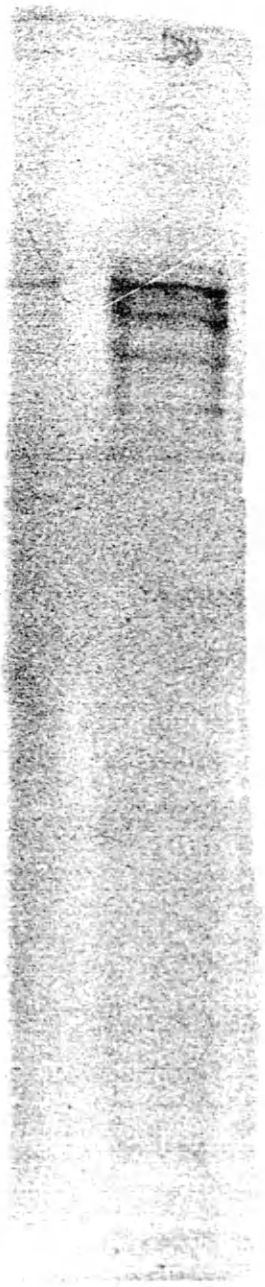


Figure 8. SDS Polyacrylamide Gel Electrophoresis of Normal Erythrocyte Membrane Proteins and of GAPDH Isolated from Normal Erythrocyte Membranes. GAPDH was isolated from normal erythrocyte membranes as described, dissolved in 2% SDS, and analyzed by SDS electrophoresis. Normal erythrocyte membrane proteins appear left and GAPDH preparation appears right.

FIGURE 8

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS OF NORMAL ERYTHROCYTE MEMBRANE PROTEINS AND OF GAPDH ISOLATED FROM NORMAL ERYTHROCYTE MEMBRANES

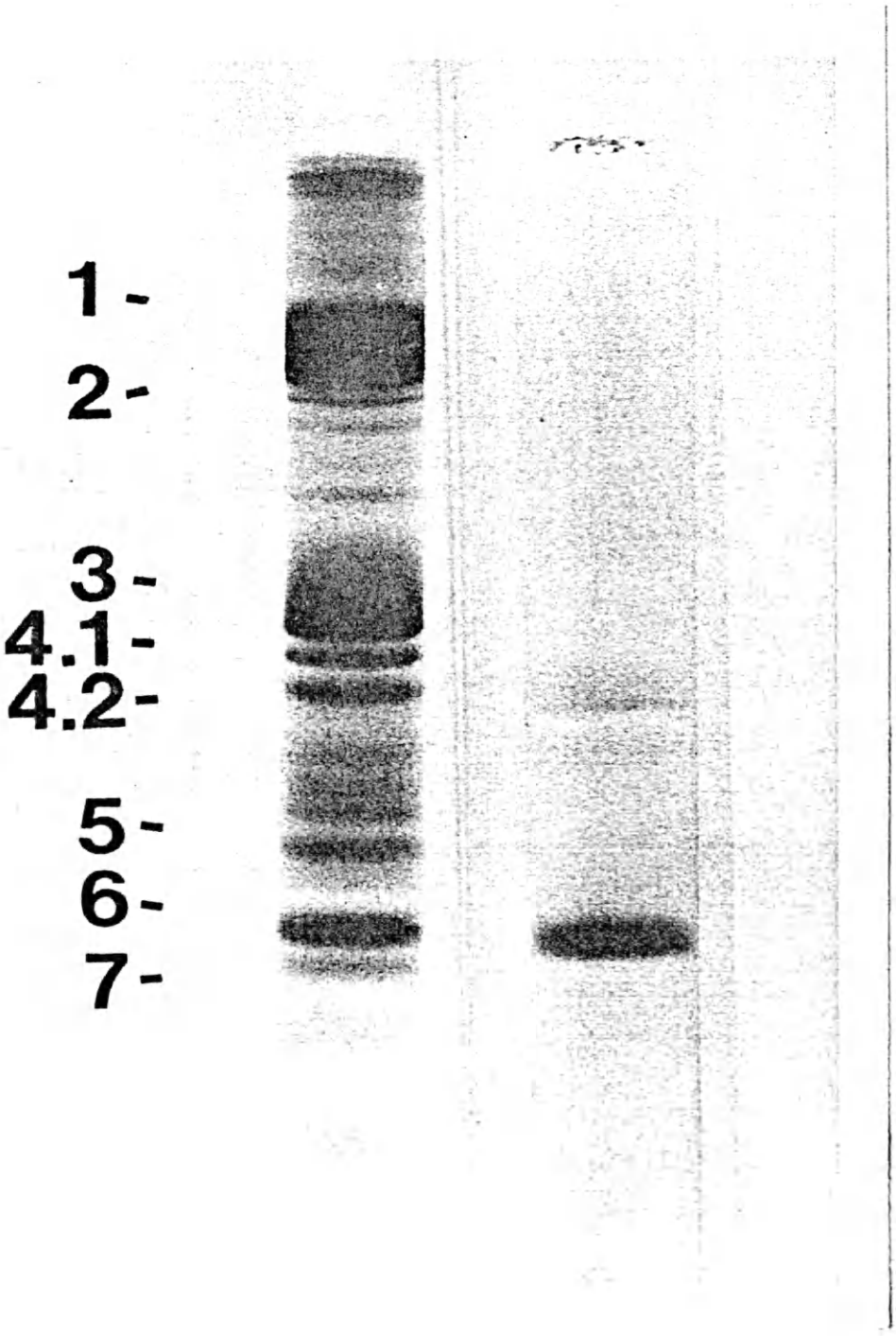
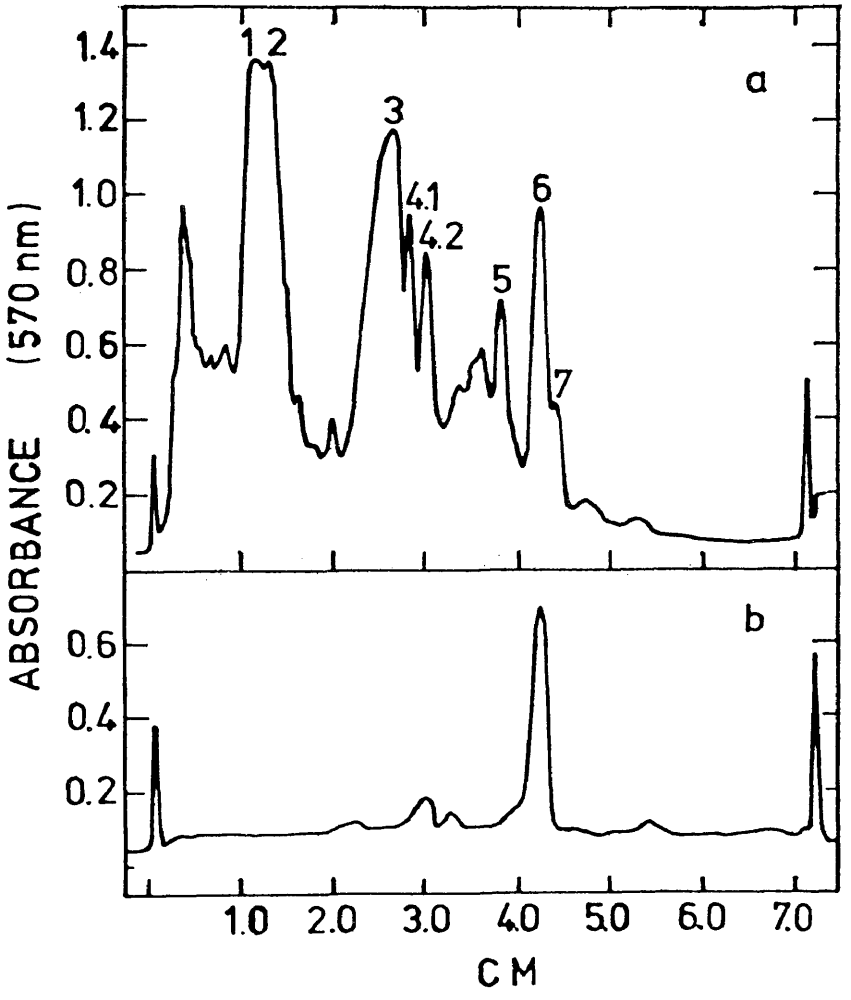


Figure 9. Densitometric Scans of Normal Erythrocyte Membrane Proteins and of GAPDH Isolated from Normal Erythrocyte Membranes. Normal erythrocyte membranes and GAPDH isolated from normal erythrocyte membranes were solubilized in 2% SDS and subjected to SDS electrophoresis. Stained gels were scanned at 570 nm.
(a) scan of normal erythrocyte membrane proteins
(b) scan of GAPDH isolated from normal erythrocyte membranes.

FIGURE 9

DENSITOMETRIC SCANS OF NORMAL ERYTHROCYTE MEMBRANE PROTEINS AND OF GAPDH ISOLATED FROM NORMAL ERYTHROCYTE MEMBRANES



isolated from the erythrocyte membrane by gentle means, usually involving manipulations of ionic strength or pH. Intrinsic proteins require harsher isolation techniques which make use of detergents or chaotropic agents. The gel in Figure 4 corresponding to the proteins which are soluble in sulfobetaine-urea, indicates that the extrinsic proteins, bands 1, 2, 4.1, 4.2, 5, and 6, are readily soluble in this detergent system. The intrinsic proteins corresponding to bands 3 and 7 are partially soluble as well. These findings indicate that sulfobetaine, when used in conjunction with high concentrations of urea, is an effective membrane solubilizing detergent.

Band 6 is enriched, relative to all the other SDS electrophoresis bands, in the preparation of sulfobetaine-urea soluble erythrocyte membranes (see Figure 4). Similarly, the bands which were missing in the isoelectric-focusing staining profiles of hereditary spherocytosis affected patients often seem to be of highest concentration in sulfobetaine-urea solubilized normal membranes (see Figure 1). These observations support the gel filtration chromatography and GAPDH isolation evidence which identified the missing isoelectric-focusing bands as band 6 on the SDS electrophoresis staining profile. The method used for isolating GAPDH from erythrocyte membranes (31) was found to be facile and selective. Few contaminants were isolated along with the enzyme (see Figures 8 and 9).

As shown in Figure 7, a series of 4 or 5 isoelectric-focusing bands is associated with the GAPDH isolation preparation, with the more concentrated bands being least anodal in mobility. Edwards, Clark,

and Harris (33) have studied the isozymes of GAPDH in human red blood cells. They report similar findings of at least five distinct GAPDH isozymes and feel that the more anodal isozymes may be analogous to those associated with human nucleoside phosphorylase.

The fact that GAPDH isozyme bands are missing or diffusely focused in erythrocyte membranes isolated from hereditary spherocytosis affected kindred members when compared to those from unaffected relatives and other unrelated normals indicates that the isoelectric-focusing technique used may have clinical diagnostic potential. Clearly, characterization of the specific abnormality affecting these isozymes is an important prerequisite.

CHAPTER III

BINDING SENSITIVITY OF BAND 6 TO ERYTHROCYTE MEMBRANES

INTRODUCTION

Band 6 of the erythrocyte membrane has been shown to correspond to the enzyme GAPDH (15, 16) and to bands which were missing in isoelectric-focusing staining profiles obtained from erythrocyte membranes of hereditary spherocytosis affected kindred members (see Chapter II).

Upon isolating erythrocyte membranes from an affected member of the kindred under study on more than five occasions, it became apparent that the abnormal protein staining profile obtained by isoelectric-focusing (see Figure 1) was not consistently reproducible; occasionally the GAPDH isozyme bands were observable. This finding indicated that the binding of GAPDH was sensitive to either the condition of the patient or to something in the isolation technique. Since it has been shown that the binding of GAPDH to the membrane is sensitive to ionic strength (31), it was of interest to compare normal and hereditary spherocytosis band 6 erythrocyte membrane binding sensitivity to sodium chloride concentration.

EXPERIMENTAL

A. Materials

Refer to Chapter II.

B. Methods

1. Band 6 Elution From Erythrocyte Membranes. Erythrocyte membranes were prepared as previously outlined. Each of six fractions of these membranes was washed at 4°C in 5P8 containing one of the following concentrations of NaCl: 0, 0.034, 0.068, 0.102, 0.136, or 0.150 M. Membranes were then centrifuged at 20,000 x g for 15 minutes, the buffer was aspirated, and the washing was repeated. The pellet was then washed twice in 5P8 to remove the sodium chloride. Each fraction was subsequently analyzed by SDS electrophoresis as previously described, with the following modification: the tracking dye solution was allowed to migrate the additional 0.5 cm to the bottom of the gel in order to enhance separation of band 6 from band 7. SDS gels were scanned for protein at 570 nm on a Beckman ACTA CIII Spectrophotometer and the amount of band 6 remaining in each preparation was determined as the area under the peak, using the area under the peak corresponding to band 3 as an internal comparison.

Each of the membrane fractions was also analyzed by isoelectric-focusing to insure that GAPDH isozymes were being selectively eluted from the membranes as a function of sodium chloride concentration.

RESULTS

Band 6 Elution From Erythrocyte Membranes. Erythrocyte membranes isolated from a hereditary spherocytosis affected kindred member (patient AG) and from normal controls were washed in 5P8 solutions containing varying concentrations of sodium chloride. After the sodium chloride was removed by several washings in 5P8, the

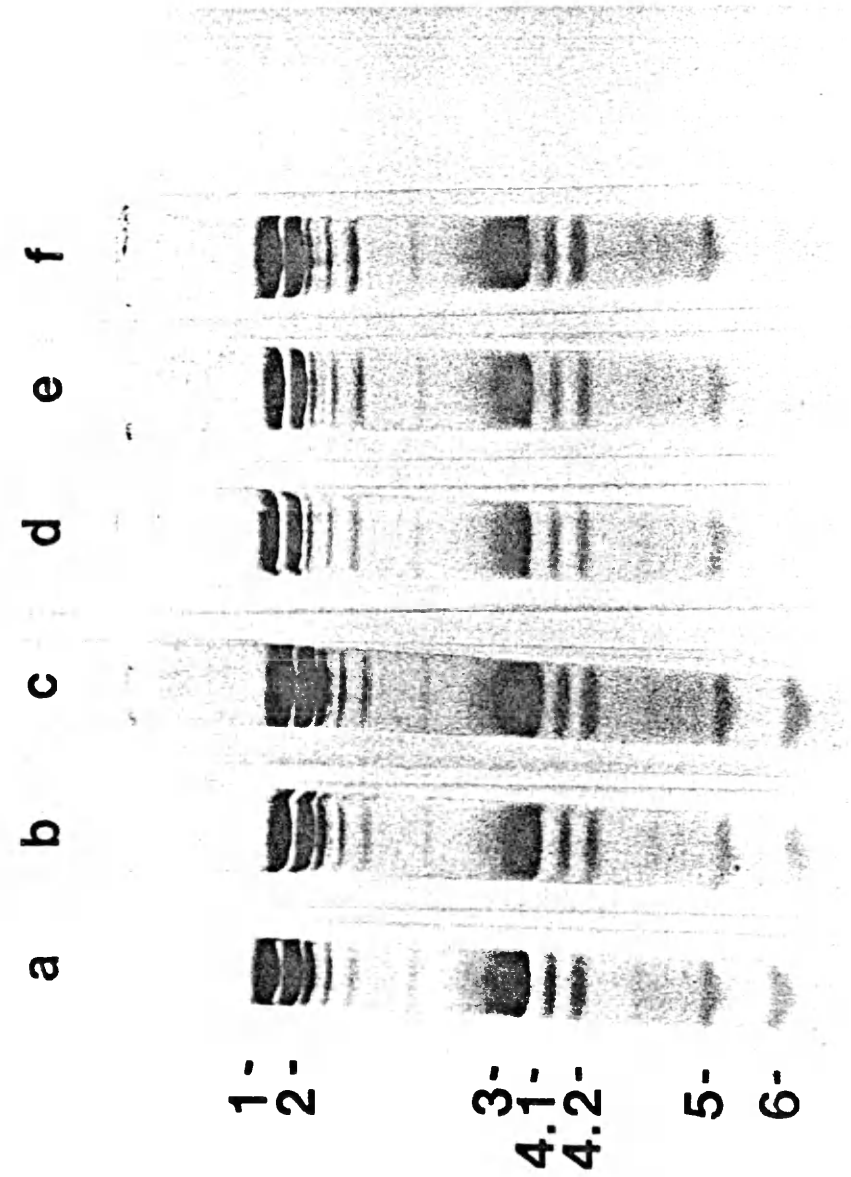
membrane's protein composition was analyzed by SDS electrophoresis. Gels in Figure 10 correspond to normal membrane proteins remaining in each sodium chloride treated preparation; gels in Figure 11 are the corresponding hereditary spherocytosis (patient AG) analogues of those in Figure 10. The gels in both Figures 10 and 11 indicate that band 6 concentration attached to the membrane decreases as a function of increasing sodium chloride concentration, with the most dramatic such decrease occurring between gels c and d. To determine whether band 6 elution from hereditary spherocytosis membranes was more sensitive to sodium chloride concentration than the elution from normal membranes, the gels were scanned at 570 nm. The area under band 6 for each gel was adjusted for membrane concentration by normalizing it with respect to the area under band 3. On a percent basis, membrane bound band 6 values differ significantly for patient AG and normals within the range $0.102\ M \leq [NaCl] \leq 0.150\ M$ (Figure 12). Values for patient AG are approximately half the normal value at sodium chloride concentrations of 0.136 and 0.150 M.

The isoelectric-focusing gel in Figure 13 contains staining profiles obtained from each of six sodium chloride treated, hereditary spherocytosis affected erythrocyte membranes (a - f). Profiles on both ends of this gel (B6) correspond to GAPDH isozymes isolated from normal membranes. Both normal and hereditary spherocytosis GAPDH isozymes appear to have the same isoelectric points.

As can be seen, GAPDH bands of hereditary spherocytosis membranes decrease in concentration as a function of increasing sodium chloride concentration (Figure 13). The most dramatic GAPDH decrease occurs

FIGURE 10

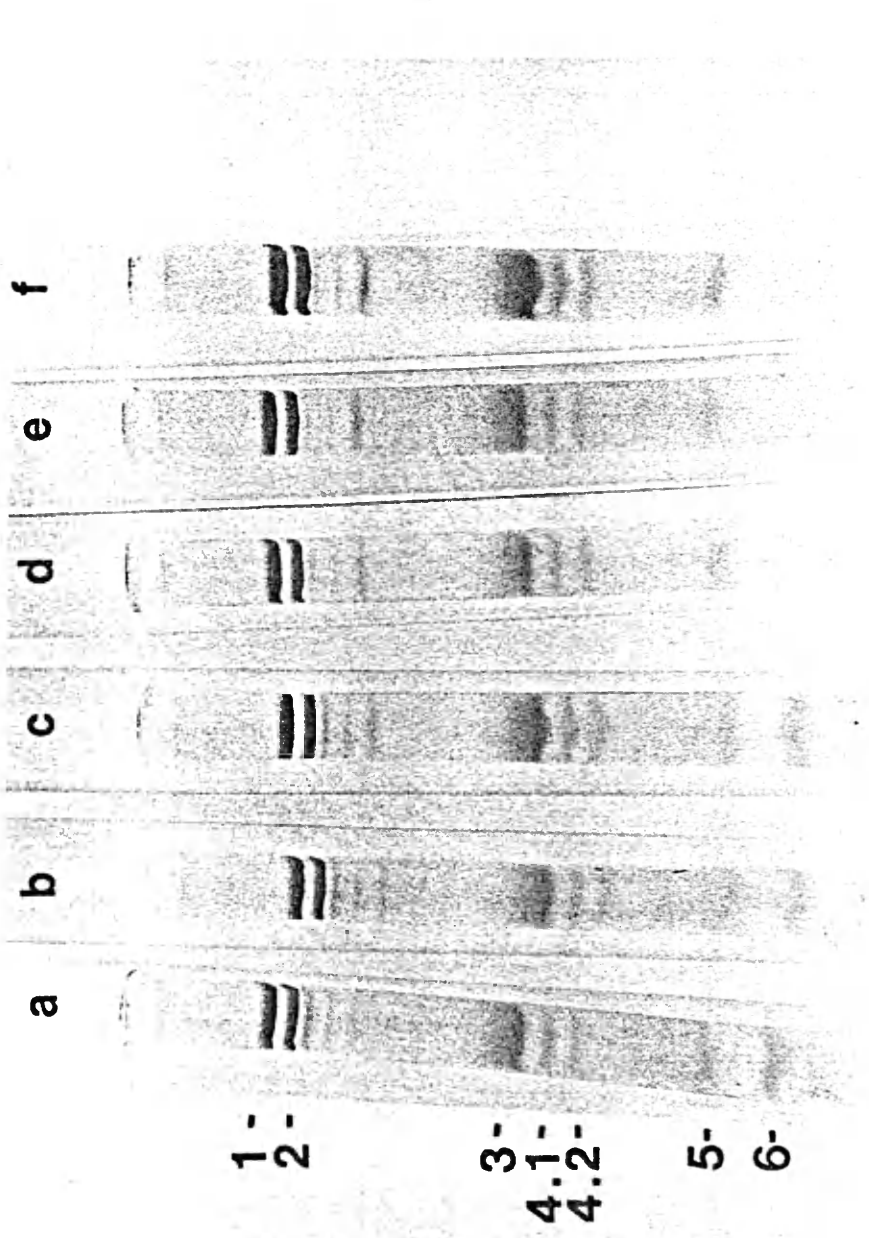
SDS ELECTROPHORESIS GELS SHOWING EFFECTS OF WASHING NORMAL ERYTHROCYTE MEMBRANES IN SOLUTIONS OF VARYING SODIUM CHLORIDE CONCENTRATION



Normal erythrocyte membranes were washed in 5P8 solutions of the following sodium chloride concentrations: (a) 0, (b) 0.034 M, (c) 0.068 M, (d) 0.102 M, (e) 0.136 M, and (f) 0.150 M. Samples were centrifuged and residual membranes were analyzed by SDS electrophoresis.

FIGURE 11

SDS ELECTROPHORESIS GELS
SHOWING EFFECTS OF WASHING HEREDITARY SPHEROCYTOSIS ERYTHROCYTE MEMBRANES
IN SOLUTIONS OF VARYING SODIUM CHLORIDE CONCENTRATION



Hereditary spherocytosis erythrocyte membranes of patient AG were washed in 5P8 solutions of the following sodium chloride concentrations: (a) 0, (b) 0.034 M, (c) 0.068 M, (d) 0.102 M, (e) 0.136 M, and (f) 0.150 M. Samples were centrifuged and residual membranes were analyzed by SDS electrophoresis.

Figure 12. Percent Erythrocyte Membrane Bound Band 6 as a Function of Sodium Chloride Concentration. Erythrocyte membranes were washed in 5P8 containing varying amounts of sodium chloride. Residual membranes were analyzed by SDS electrophoresis, and gels were stained and scanned as described. The area under the peak corresponding to band 6 was measured and corrected for membrane concentration. Individual affected with hereditary spherocytosis is patient AG.

FIGURE 12

PERCENT ERYTHROCYTE MEMBRANE BOUND BAND 6
AS A FUNCTION OF SODIUM CHLORIDE CONCENTRATION

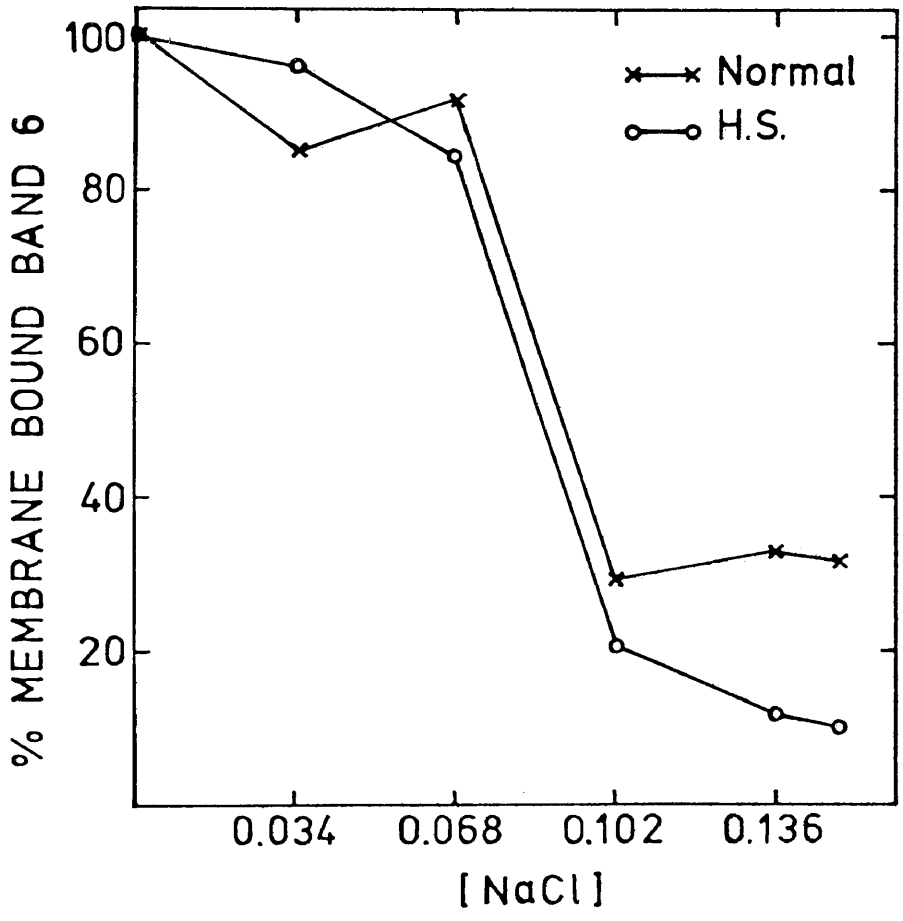
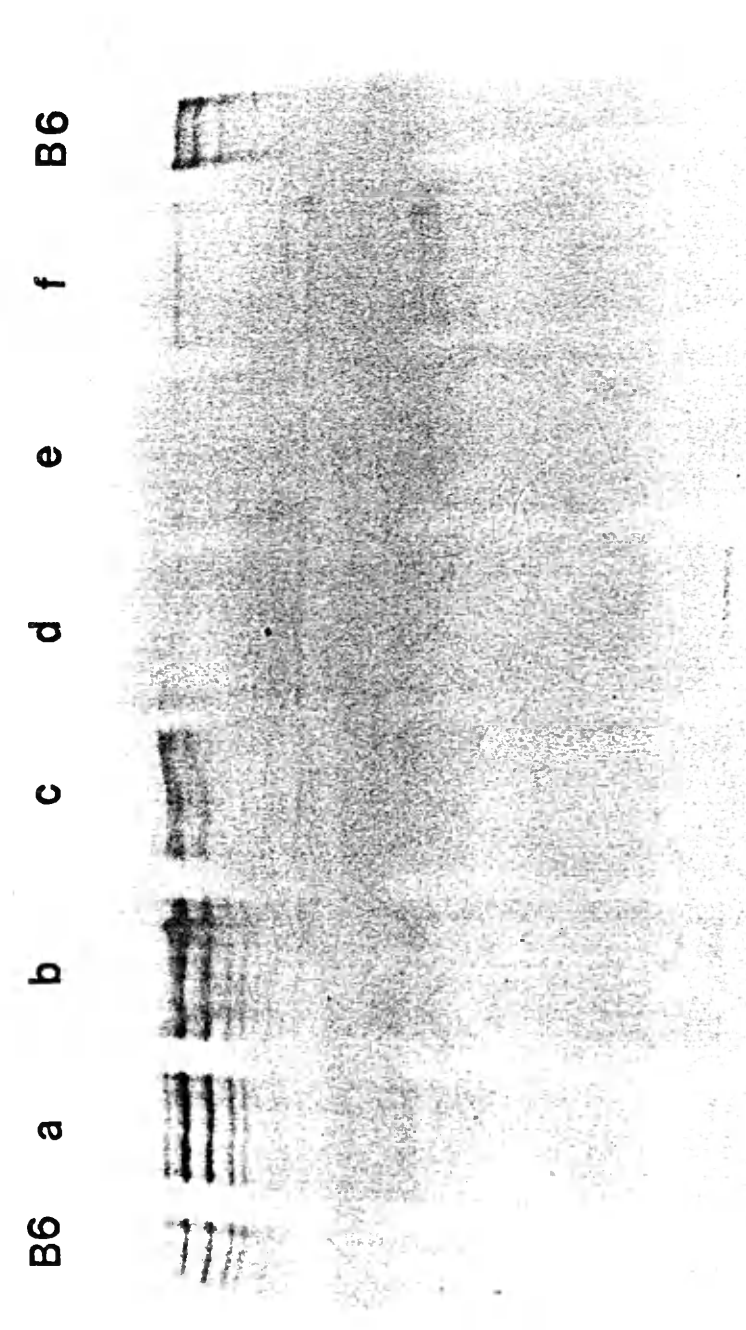


FIGURE 13

ISOELECTRIC-FOCUSING GEL
SHOWING EFFECTS OF WASHING HEREDITARY SPHEROCYTOSIS ERYTHROCYTE MEMBRANES
IN SOLUTIONS OF VARYING SODIUM CHLORIDE CONCENTRATION



Hereditary spherocytosis erythrocyte membranes of patient AG were washed in 5P8 solutions of the following sodium chloride concentrations: (a) 0, (b) 0.034 M, (c) 0.068 M, (d) 0.102 M, (e) 0.136 M, and (f) 0.150 M. Samples were centrifuged and analyzed by isoelectric-focusing. B6 (band 6) corresponds to GAPDH isolated from normal erythrocyte membranes.

between gels c and d, a finding which agrees with the SDS gels in Figure 11. Note that one GAPDH isozyme band in fraction f is observable (Figure 13). This observation was not reproduced in subsequent isoelectric-focusing runs of the same fraction. Similar GAPDH isozyme band decreases as a function of sodium chloride were obtained for normal membrane fractions (gel not shown). No isozyme bands appeared to be selectively insensitive to sodium chloride elution from these membranes.

DISCUSSION

Hereditary spherocytosis affected and unaffected erythrocyte membrane band 6 binding sensitivity to sodium chloride concentration has been examined in these studies by quantitative densitometry of SDS electrophoresis gels. Patient AG, who is an affected kindred member, manifests abnormal membrane affinity for band 6 when compared to normal controls, within a range of sodium chloride concentrations studied (see Figure 12). This finding provides a rationale for explaining why abnormal isoelectric-focusing staining profiles of patient AG were not consistently reproducible. It does not, however, distinguish between possible in vivo and in vitro ionic strength effects on this binding. On the one hand, the in vivo sodium concentration within spherocytes could possibly at times be high enough to cause dissociation of twice as much band 6 as occurs in normal erythrocytes. Subsequent isolation of membranes, involving lysis of cells, would then wash the previously dissociated enzyme out of the membranes, with the concentration of the remaining bound enzyme being below the detection limits of isoelectric-focusing. On the other hand,

in vitro lysis of spherocytes in the presence of elevated levels of sodium (i.e. above hypotonic 5P8) could lead to similar results as well.

Kant and Steck (31) have examined normal GAPDH membrane binding sensitivity as a function of sodium concentration by specific enzyme activity assays. They report approximately 12% soluble GAPDH activity (88% membrane bound) at 0.070 M NaCl and 77% (23% membrane bound) at 0.100 M NaCl for normal membranes at pH 8.0. Corresponding normal values obtained from the quantitative densitometry of SDS electrophoresis gels in the present study (see Figure 12) were 93% membrane bound band 6 at 0.068 M NaCl and 29% at 0.102 M NaCl. These two methods correlate well within this range of sodium chloride concentrations, despite the fact that the former measures soluble GAPDH activity and the latter measures membrane bound band 6.

Comparison of isoelectric-focusing staining profiles (B6) and (a) in Figure 13 indicates that membranes of patient AG are not deficient in GAPDH isozyme bands, as previously thought. Further, GAPDH isozyme bands of patient AG decrease in concentration with increasing sodium chloride concentration in the wash buffer at a rate similar to the rate decrease observed in band 6 on SDS gels (see Figure 11), since the greatest decrease in each occurs between fractions c and d.

Physiologic roles for erythrocyte membrane associated GAPDH are presently not clearly determined, although several suggestions have been offered. Bartlett (34) has suggested that GAPDH may catalytically control transport of phosphate into the erythrocyte. Parker and Hoffman (35) have proposed that GAPDH, coupled with phosphoglycerate

kinase, may generate energy for maintenance and control of the sodium pump of erythrocytes. A third major suggestion set forth by Schrier (36,37) is that GAPDH produces energy to control active transport into the erythrocyte. Each of these theories, based on varying degrees of scientific evidence, postulates a role for membrane bound GAPDH involving transport of some substance across erythrocyte membranes. An abnormality which affects binding of the enzyme to the membrane could therefore upset the proper intra- and extracellular balance of one or more specific substances. The fact that a hereditary spherocytosis affected kindred member manifests altered membrane affinity for band 6 is interesting in this regard since sodium levels have been shown to be elevated within spherocytes (4), even though the present studies are not conclusive of a relationship between the two.

CHAPTER IV

BINDING SENSITIVITY OF GAPDH TO ERYTHROCYTE MEMBRANES

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme found in carbohydrate metabolizing organisms. Comparative studies from mammals to yeast indicate that the enzyme is a tetramer of four probably identical polypeptide chains, each of which has a molecular weight of 36,000 daltons (38-41). Mammalian GAPDH contains four thiol groups per chain, one of which is located at the active site (42).

Human erythrocytes and erythrocyte membrane preparations contain GAPDH. As a membrane protein, it has been classified as extrinsic because it may easily be removed from the membrane (32). The fact that GAPDH adheres to erythrocyte membranes under membrane isolation conditions (i.e. hypotonic hemolysis in 5 mM sodium phosphate, pH 8.0) is not adequate criteria to conclude that it adheres in vivo. Its presence in isolated membrane preparations (5 - 7% of total membrane protein stainable on SDS electrophoresis gels, (31)) could represent electrostatic adsorption or hydrophobic interaction of the enzyme onto or with the membrane. Kant and Steck (31) have shown that, although random attachment does occur, GAPDH has specific binding sites for attachment to the inner surface of the erythrocyte membrane, probably on the protein designated as band 3. More recently Yu and Steck (43) have shown that band 6, the protomer of GAPDH, co-purifies

with band 3 and that the band 3 dimer binds two tetramers of the enzyme at the cytoplasmic surface of the erythrocyte.

Binding of GAPDH to erythrocyte membranes has been the subject of other studies as well. These studies have indicated that the binding may be affected by various metabolites (31), by bilirubin (44), and by salt solutions (31,45-49).

Isoelectric-focusing studies of erythrocyte membranes isolated from hereditary spherocytosis affected and unaffected members of a large kindred (see Chapters I and II) revealed that certain protein bands were frequently absent or greatly reduced in membrane preparations isolated from patients affected by hereditary spherocytosis compared to unaffected normals (28). These bands have been identified as the isozymes of GAPDH by separating erythrocyte membrane proteins using gel filtration chromatography followed by SDS electrophoresis and isoelectric-focusing analyses (see Chapter II). Subsequent GAPDH isolation from normal membranes confirmed the identification.

In chapter III band 6 binding sensitivity to sodium chloride concentration has been studied in normals and in one hereditary spherocytosis affected kindred member (patient AG). In these studies membrane preparations were washed in solutions of varying sodium chloride concentration and the quantity of band 6 remaining bound to the membrane was determined. Results indicated a more sensitive binding of band 6 to erythrocyte membranes in the hereditary spherocytosis affected patient examined compared to normals (see Chapter III).

It was of interest to confirm this increased binding sensitivity to sodium chloride concentration in hereditary spherocytosis affected

kindred members by measuring specific enzyme activity released into the surrounding solutions as a function of sodium chloride concentration. Enzyme activity release data for the kindred under examination as well as from hereditary spherocytosis affected patients from other unrelated kindreds are presented below.

EXPERIMENTAL

A. Materials

1. Chemicals. In addition to the chemicals in Chapter II, the following chemicals were obtained from:

Sigma Chemical Co.
Albumin from bovine serum
Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle)
DL-Glyceraldehyde-3-phosphoric acid, diethylacetal (monobarium salt)
β-Nicotinamide adenine dinucleotide
Dowex-50W hydrogen form

Fisher Scientific Co.
Cupric sulfate
Sodium carbonate
Sodium potassium tartrate
Sodium fluoride
Sodium arsenate
Sodium pyrophosphate

Eastman Kodak
L-(+)-Cysteine hydrochloride

Harleco
Phenol reagent Folin & Ciocalteu (2N)

2. Solutions.

a. Lowry Solution A
0.19 M Na_2CO_3
0.7 mM $NaKC_4H_4O_6 \cdot 4\ H_2O$
0.1 M NaOH

b. Lowry Solution B
0.5% (w/v) $CuSO_4 \cdot 5\ H_2O$

c. Lowry Solution C
0.186 M $NaCO_3$
0.69 mM $NaKC_4H_4O_6 \cdot 4\ H_2O$
0.1 M NaOH

d. Folin Reagent
50% (v/v) Stock phenol reagent Folin & Ciocalteu

B. Methods

1. Protein Determination. Protein concentration was determined by the method of Lowry (50). Protein in 20 µl aliquots was added to tubes containing 0.48 ml H_2O. The blank contained no protein and 0.5 ml H_2O. To each tube was added 2 ml of Lowry solution C. Tubes were then inverted. After 10 - 15 minutes, 0.25 ml of Folin reagent was added to each tube, followed by immediate inversion. Absorbance at 500 nm was measured 30 minutes after addition of Folin reagent (at room temperature). Standard curves using bovine serum albumin (BSA) were generated and unknown protein concentrations were determined directly from these curves.

2. Preparation of Glyceraldehyde-3-Phosphate. Glyceraldehyde-3-phosphate was prepared from the diethylacetal monobarium salt and standardized by the methods of Sigma Chemical Co.

3. GAPDH Enzyme Assay. The enzyme GAPDH was assayed by the method of Steck and Kant (51) according to the following reaction:

$$GAP + \beta\text{-}NAD^+ + H_2O \xrightarrow[\text{Arsenate}]{\text{GAPDH}} PGA + NADH + H^+$$

A few modifications were introduced. Aliquots of 2 - 100 µl of enzyme or membrane were mixed with one volume of 5P8. To this suspension 30 mM sodium pyrophosphate, pH 8.4, 4 mM cysteine-HCl (add cysteine-HCl just prior to use) was added to give a final volume

of 0.82 ml of solution. 30 µℓ of 0.4 M sodium arsenate was added followed by 50 µℓ of 20 mM β-NAD. Samples were then preincubated for 5 minutes at 37°C prior to addition of 100 µℓ of approximately 3 mM GAP, pH 7.0 (pH adjusted with NaOH just prior to use). At the concentrations of enzyme being assayed, GAP was found to be saturating. Absorbance at 340 nm was measured 1 minute and 30 minutes after addition of GAP as a measure of the NADH formed at 37°C.

4. GAPDH Elution From Erythrocyte Membranes. Erythrocyte membrane preparations were assayed for total protein by the method of Lowry (50) and the concentration was adjusted to 2.0 mg/ml using 5P8 for the necessary dilutions. 5 µℓ of these membranes were then added to 1 ml of 5P8 containing one of the following concentrations of NaCl: 0, 0.034, 0.068, 0.102, 0.136, or 0.150 M. Suspended membranes were incubated at 4°C for 90 minutes and then centrifuged at 20,000 x g for 10 minutes at 4°C. Supernatant samples of 100 µℓ were subsequently assayed for GAPDH activity released from the membranes.

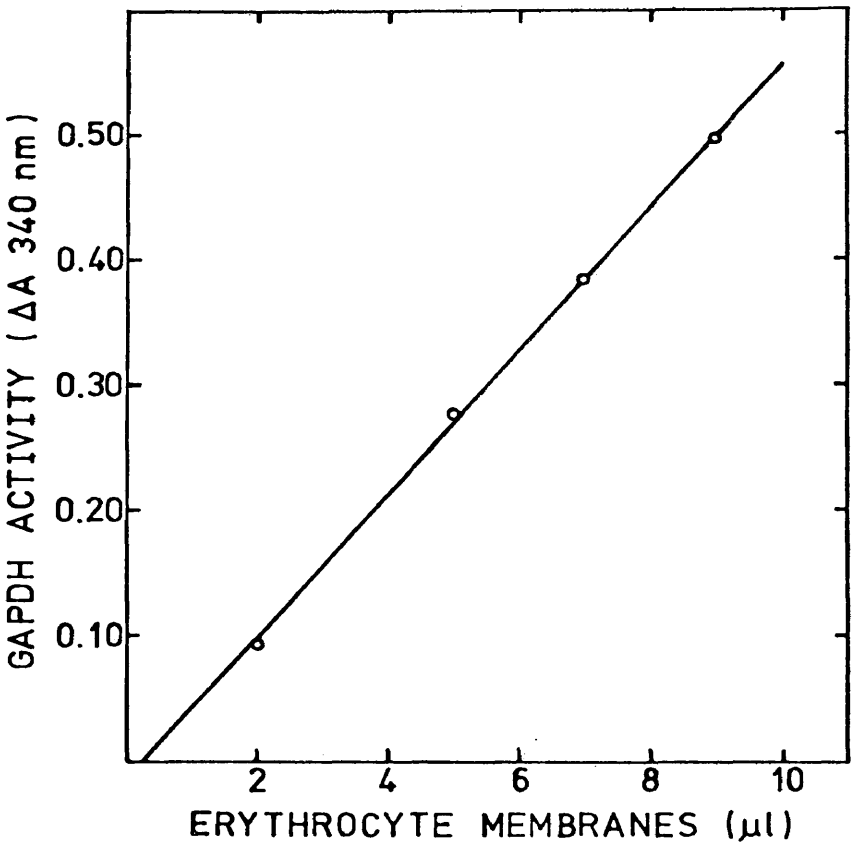
RESULTS

Relationship Between GAPDH Activity And Erythrocyte Membrane Concentration. Erythrocyte membranes prepared as described retain 60 - 85% of the erythrocyte's total GAPDH activity (31,49). As shown in Figure 14 the relationship between GAPDH activity and erythrocyte membranes was determined to be linear under our assay conditions up to 9 µℓ of membranes at protein concentrations of approximately 0.5 mg/ml. Steck and Kant (51) report that an accurate measure of the GAPDH activity in a sample is reflected in the

Figure 14. GAPDH Activity as a Function of Erythrocyte Membrane Concentration. Varying amounts of normal erythrocyte membranes of approximately 0.5 mg/ml were assayed for GAPDH activity using 3 mM GAP as substrate. Enzyme activity was determined by measuring absorbance change at 340 nm between 1 and 30 minutes after initiating reaction.

FIGURE 14

GAPDH ACTIVITY AS A FUNCTION OF ERYTHROCYTE MEMBRANE CONCENTRATION



absorbance change at 340 nm between 1 and 2 minutes after addition of substrate. GAP concentrations of approximately 3 mM were found to be saturating for thirty minutes after initiation of the reaction, as shown in Figure 14. This period of time was considered more desirable because it allowed for greater absorbance changes (less inherent sampling error) and more sensitivity in determining enzyme activity.

GAPDH Elution From Erythrocyte Membranes. Measuring the release of GAPDH activity from erythrocyte membranes was found to be another, more sensitive method for studying GAPDH binding affinity to membranes as a function of sodium chloride concentration. Typical elution curves were sigmoidal for normal and hereditary spherocytosis affected patients, with maximum GAPDH elution from membranes usually occurring at 0.136 M NaCl.

As shown in Figure 15 erythrocyte membranes from patient AG (curve a) were found to release approximately twice as much GAPDH activity per mg of membrane protein in the range 0.068 M $\leq$ [NaCl] $\leq$ 0.150 M when compared to unaffected, unrelated normals (curve d). Membranes from several unaffected normals were assayed for GAPDH release at 0.136 M NaCl in order to establish appropriate normal baseline activity values (Figure 16). The values for normals WY, RC, and RC´ (and others, not reported) were within an approximate range of 0.07 to 0.10 absorbance units/30 minutes, whereas values at the same sodium chloride concentration for patient AG fell within a range of 0.18 to 0.20 absorbance units/30 minutes.

Erythrocyte membranes from two children of patient AG, one hereditary spherocytosis affected (curve c, patient KG) and one normal

Figure 15. GAPDH Activity Released from Erythrocyte Membranes as a Function of Sodium Chloride Concentration. Erythrocyte membranes with protein concentrations of 2.0 mg/ml were incubated for 90 minutes at 4°C in 5P8 containing varying concentrations of sodium chloride. Suspensions were centrifuged at 20,000 x g at 4°C for 10 minutes. Soluble enzyme activity in 100 µl of supernatant solution was assayed at 340 nm as described. Results for patient AG (a) who manifests hereditary spherocytosis, for this patient's unaffected (b) and affected (c) children, and for an unaffected, unrelated normal individual (d) are given.

FIGURE 15

GAPDH ACTIVITY RELEASED FROM ERYTHROCYTE MEMBRANES
AS A FUNCTION OF SODIUM CHLORIDE CONCENTRATION

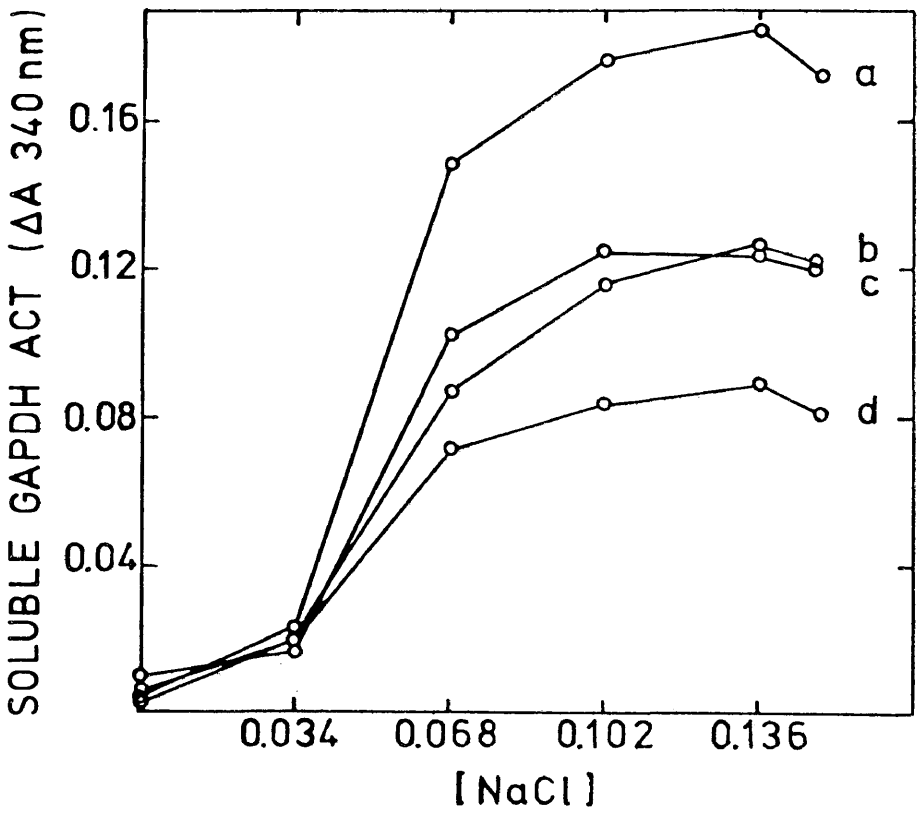
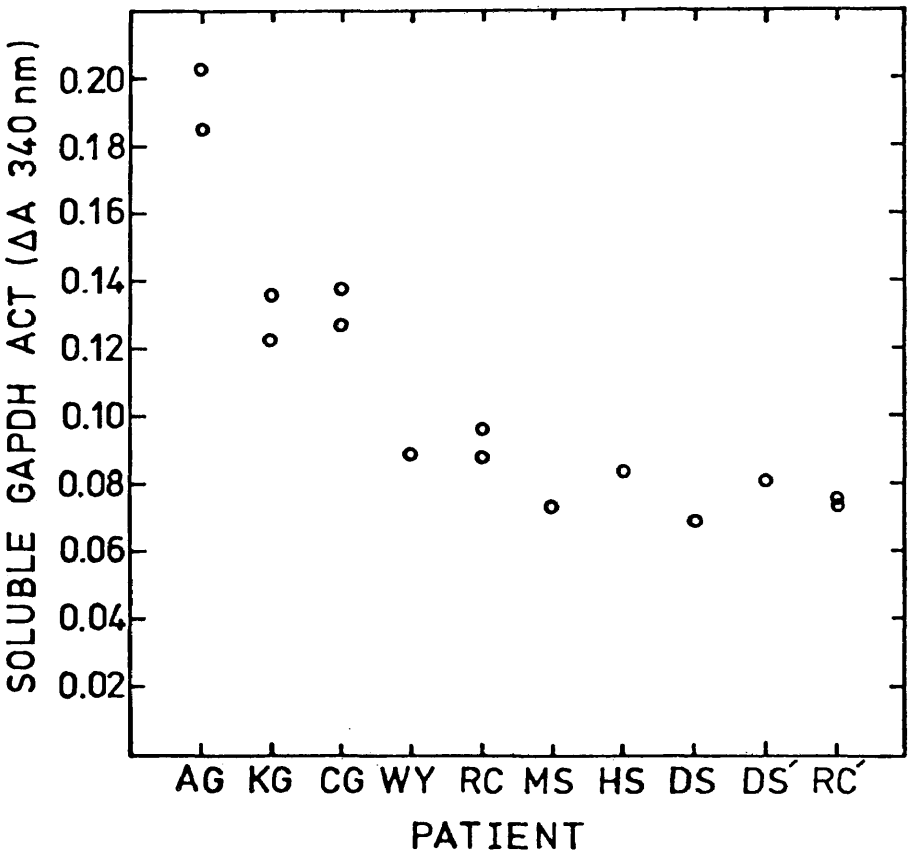


Figure 16. Soluble GAPDH Activity Eluted from Erythrocyte Membranes at 0.136 M NaCl. Erythrocyte membranes containing 2.0 mg/ml protein were incubated for 90 minutes at 4°C in 5P8 containing 0.136 M NaCl. After centrifuging, 100 µl of supernatant solution was assayed for GAPDH activity at 340 nm. Patients AG, KG, and CG are related. Patients MS, HS, DS, and DS´ are related and patients WY, RC, and RC´ are unrelated. Patients CG, WY, RC, MS, and RC´ are unaffected by hereditary spherocytosis and the others are affected by the disorder.

FIGURE 16

SOLUBLE GAPDH ACTIVITY ELUTED FROM ERYTHROCYTE MEMBRANES
AT 0.136 M NaCl



(curve b, patient CG), have also been analyzed by this method (Figures 15 and 16). As can be seen, values of GAPDH activity are quite similar for both offspring and lie intermediate between values for patient AG and normals in the 0.068 M $\leq$ [NaCl] $\leq$ 0.150 M range.

Another kindred manifesting hereditary spherocytosis was examined for GAPDH affinity to erythrocyte membranes (Figure 16). Enzyme activity released from the membranes for these patients (MS, HS, DS, and DS´) was found to lie entirely within the normal range, even though only patient MS was unaffected by hereditary spherocytosis.

DISCUSSION

In the studies which demonstrated the relationship between GAPDH activity and erythrocyte membrane concentration, 3 mM GAP was found to be saturating, as shown by the linear curve in Figure 14. Non-saturating levels of GAP substrate would have been indicated by a hyperbolic-shaped curve, with a flattened portion at high erythrocyte membrane concentrations. The maximum enzyme activity measured in Figure 14 was 0.496 absorbance units/30 minutes. Protein concentrations used in the enzyme elution experiments (see Figures 15 and 16) were chosen to insure that enzyme activities would always fall well within the linear portion of the curve in Figure 14; eluted enzyme would therefore be saturated with substrate as well.

Sigmoidal curves in Figure 15 indicate that more enzyme is released into the supernatant from erythrocyte membranes as the concentration of sodium chloride is increased, usually reaching a maximum value at 0.136 M NaCl. Coupled with this finding is the fact

that concomitant loss of band 6 on SDS electrophoresis gels occurs in a sigmoidal fashion with increasing sodium chloride concentration (see Chapter III). This loss of band 6 from the membranes, coupled with a gain in GAPDH activity in the supernatant solution adds a further measure of confidence in identifying the missing protein bands of isoelectric-focusing staining profiles of membranes isolated from hereditary spherocytosis affected kindred members (see Chapter II) as both band 6 and GAPDH.

GAPDH erythrocyte membrane binding sensitivity as a function of sodium chloride concentration has been examined in these studies by specific enzyme activity assays. This method correlates well with the method using quantitative densitometry of SDS electrophoresis gels (see Chapter III) within the range $0.102\ M \leq [NaCl] \leq 0.150\ M$, despite the fact that the former measures enzyme activity and the latter measures band 6 protein.

Since it has been shown that GAPDH can be displaced from erythrocyte membranes by various substances (31,44-49), it may be speculated that these substances either affect sites on the enzyme directly involved with membrane adherence or produce configurational changes in the membrane itself, leading to enzyme dissociation (52). This latter hypothesis may seem to be the one of greater probability in light of studies reported by Jenkins and Tanner (53). They have shown that the polypeptide corresponding to band 3 on SDS electrophoresis gels undergoes ionic-strength-dependent changes in structure. This finding is particularly interesting and relevant to results presented since the enzyme GAPDH binds to band 3. Findings reported in Chapter III

indicate that the GAPDH isozymes of a hereditary spherocytosis affected kindred member (patient AG) have the same isoelectric points as normal GAPDH isozymes (see Figure 12). A strong possibility, therefore, is that the band 3 binding site, not the enzyme, is altered in this patient. Ionic-strength-dependent changes in conformation of an abnormal band 3 might affect the membrane's affinity for GAPDH.

GAPDH binding to erythrocyte membranes of patient AG appears to be approximately twice as sensitive to ionic strength (when $0.068\ M \leq [NaCl] \leq 0.150\ M$) compared to normals. Corresponding values for the hereditary spherocytosis affected and unaffected children of patient AG are approximately 1.5 times more sensitive than normals (see Figure 15). These findings should be qualified by mentioning that residual levels of hemoglobin remaining attached to the membranes would cause inaccurate protein determinations and dilutions to 2.0 mg/ml. This did not seem to be much of a problem based on observation and on the narrow range of normal baseline GAPDH elution activities obtained (see Figure 16). Membrane aging, freezing and thawing of membranes, and concentrations of membranes while being stored were factors which affected the activity of the enzyme GAPDH in other unreported experiments. Presumably, these were not important problems in the reported results because membrane samples were examined as soon after isolation as possible.

It should be pointed out that the results reported in this thesis are not conclusive of a relationship between altered membrane affinity for GAPDH and hereditary spherocytosis. Data given should be interpreted as preliminary, since further studies are being planned.

The most serious of the unanswered questions is the finding that a second freshly isolated sample from patient AG resulted in normal range GAPDH elution from the membranes. Whether some effector other than sodium chloride concentration is involved, which could have changed in the condition of the patient between samples, is unknown. Membranes isolated from the spouse of patient AG gave normal range GAPDH elution values. A second sample from patients KG and CG was not obtained.

Supportive evidence which relates hereditary spherocytosis with increased GAPDH binding sensitivity may be found in the work of McCann et al (26). These investigators have studied another kindred in which GAPDH is reduced in the erythrocyte and which also exhibits hereditary spherocytosis, although they indicate that the two may not be related. Studies of GAPDH binding to erythrocyte membranes in another family with hereditary spherocytosis have been reported in Figure 16 (patients MS, HS, DS, and DS´). As can be seen, enzyme elution values for these patients fall within the normal range at 0.136 M NaCl. Further, of the two children of patient AG who show intermediate sensitivity of GAPDH binding (patients KG and CG), one clinically has hereditary spherocytosis and the other is normal. This finding may indicate that, if these are related occurrences, other factors relating to penetrance are involved in the clinical expression of hereditary spherocytosis. If hereditary spherocytosis and altered membrane affinity for GAPDH are not related occurrences, one possibility is that patient AG is homozygous for a GAPDH involved genetic abnormality, the children are heterozygous, and the other

parent is homozygous unaffected. This is a valid possibility particularly in light of work reported by Mueller and Morrison (54). These investigators have detected a variant of the protein corresponding to band 3, following proteolytic digestion of intact erythrocytes. One of the two possible structural alterations presented to represent the variant protein involves an additional segment incorporated into that portion of the molecule which extends inside the cell. This sort of variant could possibly affect proper binding of GAPDH to erythrocyte membranes, since band 3 has been shown to bind GAPDH (31,43). One way to test this theory would be to explore the prevalence of the GAPDH binding abnormality in the general population.

In a recent communication Karadsheh and Uyeda (55) have demonstrated the importance of binding of phosphofructokinase to erythrocyte membranes. They report that phosphofructokinase loses its sensitivity to the allosteric effectors ATP and 2,3-diphosphoglycerate when membrane bound. The fact that GAPDH binding may be altered in patient AG is interesting when viewed in relation to possible regulatory roles for membrane binding as indicated by the phosphofructokinase example.

Further examination of the erythrocyte membranes of the kindred described in this study may lead to a better understanding of the molecular abnormality of hereditary spherocytosis, to some important secondary effect of the disease, or to a new, unrelated abnormality as described above. Since hereditary spherocytosis is a good model for examining autosomal dominant disorders, the markers studied may further the understanding of the basis for these kinds of disorders,

since only a few are understood (56). The importance of certain membrane proteins, particularly involving the function of enzyme binding to membranes, and the roles they play in other genetic diseases may be expanded by the techniques described, particularly isoelectric-focusing in appropriate and effective detergents.

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