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PROPERTIES OF THE WATER DISSOLVED MEMBRANE PROTEINS OF HUMAN ERYTHROCYTES

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SUMMARY

Human erythrocyte membrane proteins were dissolved in water employing a modification of the method of Mazia and Ruby. About 40 % of ghost proteins were recovered in distilled water. More than 85 % of the water dissolved proteins remained in the supernatant on sucrose density gradient ultracentrifugation, forming one major peak with a sedimentation rate of approx. 3 S. All proteins in the supernatant penetrated a 5 % acrylamide gel on electrophoresis with a 5 mM Tris–38 mM glycine buffer system, suggesting that most of the water dissolved proteins are highly dissociated. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed about 15 protein components or polypeptide chains in the water dissolved protein fraction. The dissolution of ghost proteins into water is partially selective. Moderate amounts of virus receptor substance and complement inhibitor were detected in the water dissolved fraction while the membrane bound enzymes acetylcholinesterase and (Na^+-K^+)-dependent ATPase were almost absent. The water dissolved proteins aggregated readily in the presence of low concentrations of electrolytes. This tendency for aggregation prevented further fractionation of the water dissolved proteins by gel filtration or by preparative zone electrophoresis.

INTRODUCTION

The proteins of human erythrocyte membranes are completely solubilized by single or combined use of sodium dodecyl sulfate, formic acid, guanidine hydrochloride, and 2-mercaptoethanol[1–3]. Biological activities of many membrane proteins are destroyed during the treatment with these reagents. On the other hand, water soluble proteins with retained immunological or enzymatic activities have been extracted from human erythrocyte membranes by using organic solvents such as phenol, butanol, or pyridine[4–10]. The method for dissolution of beef erythrocyte membranes in water, described by MAZIA AND RUBY[11], is also likely to retain the biological activities of the membrane proteins. In this paper, physicochemical, immunological and enzymatic properties of water dissolved membrane proteins from human erythrocytes are described.

MATERIALS AND METHODS

Dissolution of human erythrocyte membranes

The preparation and dissolution of erythrocyte membranes was performed according to the method of MAZIA AND RUBY[11] with some minor modifications: 240 ml of human blood in acid–citrate–dextrose, no more than 5 days old, was used as starting material. Plasma and buffy coat were removed and the cells were washed with 0.3 M dextrose until the cells began to clump. The packed cells were poured into 480 ml of a 0.1 % Triton X-100 (Rohm and Haas, Philadelphia) solution containing 12 g of mixed bed resin (AG 501-X 8(D), Bio-Rad Lab., California). The suspension was decanted from the resin and centrifuged for 30 min at 35000 × *g*. The sediment was suspended in 20 mM ammonium acetate which was adjusted to pH 7.4 by 3 % ammonium hydroxide and centrifuged for 20 min at 35000 × *g*, 4–6 times, until the supernatant was colorless. The pellet showed a pale pink color. The pellet was suspended in distilled water to a protein concentration of 4–7 mg/ml, and dialyzed for 18 h against 4 l of distilled water, the pH of which was adjusted to 9.3–9.5 by 3 % ammonium hydroxide. After dialysis, the suspension was centrifuged twice for 60 min at 100000 × *g*. The supernatant was used as water dissolved membrane protein preparation. The entire procedure was performed at 4°.

Chemical analysis

Amino acid analysis was performed on a Beckman model 120-B automatic amino acid analyzer with the system of MOORE AND STEIN[12]. The protein was hydrolyzed in evacuated sealed tubes in 6 M HCl at 110° for 22 h. Protein concentrations were measured by the method of LOWRY *et al.*[13] by using bovine pancreas ribonuclease (Worthington Biochemical Corp., New Jersey) as standard. Phosphorus was assayed by the technique of BARTLETT[14], and cholesterol was measured by the method of ZLATKIS *et al.*[15]. Hemoglobin was assayed by the measurement of absorbance at 557 mμ after conversion into reduced pyridinehemochromogen, using the mmolar extinction coefficient of 34.7 (ref. 16).

Electrophoresis and isoelectric focusing

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in 0.5 % sodium dodecyl sulfate–0.1 M sodium phosphate buffer at pH 7.1, using a 7.5 % acrylamide gel, in discs of 5 mm diameter and 12 cm length. Samples were solubilized in 2 % sodium dodecyl sulfate, 1 % 2-mercaptoethanol, 10 % glycerol and 10 mM sodium phosphate buffer (pH 7.1) followed by incubation at 37° for 2 h. Electrophoresis was carried out for 8 h with 3 V/cm. The gels were fixed overnight in 20 % trichloroacetic acid and 20 % sulfosalicylic acid, stained in 0.25 % Coomassie Brilliant Blue R-250 for 10 h and washed with 7 % acetic acid to remove excess dye. Disc electrophoresis without sodium dodecyl sulfate was performed in 5 % acrylamide gels, using a 5 mM Tris–38 mM glycine buffer (pH 8.3) both as gel buffer and as electrode buffer. The gel was stained with 1 % Amido Black 10-B in 7 % acetic acid.

Isoelectric focusing in polyacrylamide gel was performed according to the method of DALE AND LATNER[17] with a minor modification: The 6.5 % acrylamide gel contained about 600 μg water dissolved membrane proteins and 1 % ampholine,

pH 3–10 (LKB-Produkter, Sweden). Isoelectric focusing was performed at room temperature with 110 V for 18 h. The gels were washed in 0.1 M sodium acetate buffer (pH 4.5) for 60 min, stained for 5–6 h with 0.05 % Naphthalene Black 12B in the acetate buffer, and destained in the same buffer. Immunoisoelectric focusing was carried out by placing one half of a longitudinally cut gel immediately following the separation by isoelectric focusing on a 1 % agar plate. Antisera channels were located at a 18 mm distance from the gel. Precipitates were stained by Amido Black 10-B after washing the plate with saline for several days.

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation was performed at 4° in 5 ml cellulose nitrate tubes in a swinging bucket SW 50 rotor with a Spinco Model L ultracentrifuge at 40000 rev./min for 8 h. Gradients were from 20 to 5 % sucrose or 40 to 20 % sucrose, respectively. The sedimentation rates of α_2-macroglobulin (19 S), transferrin (5.5 S), α_1-antitrypsin (3.4 S) and of β_2-glycoprotein I (2.9 S) were used as standards.

Immunological studies

Rabbit antisera were obtained by immunizing rabbits with ghosts prepared according to DODGE *et al.*[18] which were repeatedly frozen and thawed prior to injection or with water dissolved human erythrocyte membrane proteins. The viral hemagglutination inhibitory titer was determined by the method of KATHAN *et al.*[19], their definition of a viral hemagglutination inhibition unit (HAI) was adopted. RI/5+ strain of Influenza Virus A2 was kindly provided by Dr. Purnell W. Choppin, The Rockefeller University, New York. Complement inhibitory activity was assayed with a standard complement test[20]. Incubation at 37° was carried out for 40 min; the incubation mixture contained $5.0 \cdot 10^8$ sensitized sheep red cells, 2.5 50-% units of guinea pig complement, serial dilutions of samples, and isotonic veronal buffer[20] to a total volume of 7.5 ml. The quantity of samples required to obtain 50 % inhibition of hemolysis was determined from the plot of percentage inhibition *vs.* the quantity of inhibiting samples. One complement fixation inhibitory (CFI) unit of sample was defined as the amount which inhibits one 50-% unit of guinea pig complement. Sheep red cell, guinea pig complement, and amboceptor (anti-sheep red cell hemolysin) were purchased from Behring Diagnostics, Inc.

Enzyme assays

Acetylcholinesterase activity was determined with a Radiometer TTT automatic titrimeter. The reaction mixture of 10 ml contained 0.15 M NaCl, 10 mM acetylcholine chloride and 0.2–0.6 mg membrane protein. The temperature of the incubation mixture was 37° and pH was maintained at 7.6 by addition of 100 mM NaOH. The activity was calculated from the linear rate of NaOH addition in the first 8–20 min. (Na^+-K^+)-dependent ATPase activities were assayed by incubation for 60 min at 37° in media containing 1.5–2.5 mg membrane proteins, 2 mM ATP, 3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 1 mM cysteine, 1 mM EDTA, and 30 mM Tris with or without 10^{-4} M ouabain at pH 7.5. Inorganic phosphate was determined by the method of FISKE AND SUBBAROW[21].

Gel filtration and preparative electrophoresis

Gel filtration was performed on Sephadex G-200 and Sepharose 4B columns, 2.5 cm × 70 cm, equilibrated with 5 mM Tris–38 mM glycine buffer (pH 8.3). 7.5 ml of sample were applied to the column and eluted with the same buffer. Preparative Pevikon block electrophoresis was carried out in barbital buffer (I = 0.05, pH 8.6), at 8 V/cm for 20 h. 4 ml of sample were applied to the block of 15 cm × 46 cm. Human serum with bromphenol blue was run parallel to the sample as a marker.

RESULTS

Composition of water dissolved proteins

Table I shows the results of the chemical analysis of the water dissolved proteins. Approx. 40 % of the proteins present in the ghost preparations were recovered in the water dissolved fraction. Although the protein/lipid ratio changed substantially as indicated by the rise of the N/P ratio from 14.1 to 52.7, appreciable amounts of lipids were also recovered in the water dissolved fraction. When ghosts were washed with 20 mOsm sodium phosphate buffer (pH 7.4)[18], also about 40 % of the proteins were recovered from the ghosts. Sucrose density gradient ultracentrifugation of the water dissolved proteins of these preparations revealed consistently a slight increase of the sedimentation rate of the material present in the major peak (Fig. 4(C)).

TABLE I

ANALYSIS OF WATER DISSOLVED ERYTHROCYTE MEMBRANE PREPARATION

Figures are mean and range from 5 preparations of 5 different individuals.

	Water dissolved preparation		*Ghost*	
	Mean	*Range*	*Mean*	*Range*
Protein recovery (%)	42.8	38.1–52.2		
Phosphorus recovery (%)	11.6	7.7–17.8		
Cholesterol recovery (%)	8.9	5.0–16.7		
Hb contamination (% protein)	0.5	0.3– 0.7	n.t.	
N/P ratio (mole/mole)	52.7	34.5–67.5	14.1	9.1–19.1
Protein/cholesterol ratio (mg/mg)	18.8	14.0–27.4	3.2	2.8– 3.5

The amino acid composition of the water dissolved proteins is presented in Table II. The water dissolved human erythrocyte membrane proteins show an amino acid composition similar to that of water soluble membrane proteins from beef erythrocytes[11] and to that of lipid extracted membrane proteins from human erythrocytes[1]. Sodium dodecyl sulfate–polyacrylamide gel electrophoretic patterns of ghosts, water dissolved proteins and water insoluble ghost proteins are shown in Fig. 1. A fast migrating component of high carbohydrate and high lipid content was observed (Band A) by staining with Coomassie Brilliant Blue. Another fast migrating membrane protein component was consistently absent in ghosts prepared by the method of MAZIA AND RUBY in the modification described here (Band B; Fig. 1b). About 15 protein components are observed in the water dissolved protein fraction (Fig. 1c). The figure indicates that the dissolution of ghost proteins into water is partially selective. Some proteins are almost completely insoluble in water (Bands C and D)

TABLE II

AMINO ACID COMPOSITION OF ERYTHROCYTE MEMBRANE PROTEINS (IN MOLES PERCENT)

Amino acid	*Water dissolved membrane proteins of human erythrocytes*				*Water dissolved membrane proteins of beef erythrocytes* ***	*Lipid extracted membrane proteins of human erythrocytes* §
	*Total**		*Precipitable at pH 4.5* **	*Soluble at pH 4.5* **		
	Range	*Mean*				
Lys	5.7– 6.1	5.9	6.0	6.0	5.8	5.3
His	2.5– 2.8	2.7	2.7	2.7	2.1	2.5
Arg	5.2– 5.7	5.5	5.9	4.2	5.1	4.6
Asp	9.7–10.0	9.9	10.0	9.5	9.7	8.7
Thr	4.7– 5.1	4.9	4.8	6.4	5.7	6.0
Ser	5.4– 6.2	5.8	5.7	7.7	7.2	6.4
Glu	14.1–16.5	15.6	16.1	11.8	16.2	12.5
Pro	3.9– 4.7	4.2	3.9	6.1	4.5	4.4
Gly	5.7– 6.2	6.0	5.8	8.1	6.3	6.9
Ala	8.3– 8.7	8.4	8.5	8.1	7.3	8.4
Cys	0.3– 1.0	0.7	0.9	1.1	1.2	1.1
Val	5.4– 6.1	5.9	5.3	7.1	6.5	7.3
Met	1.3– 2.4	2.0	2.1	1.3	1.9	2.1
Ile	4.0– 4.5	4.3	4.2	3.7	4.1	5.4
Leu	11.7–12.1	11.9	12.1	9.3	9.8	11.6
Tyr	2.4– 2.7	2.6	2.6	2.8	2.7	2.5
Phe	3.5– 3.9	3.7	3.7	3.5	3.5	4.3

* Range observed in 4 preparations from 4 different individuals; each preparation was analyzed in duplicates.

** Mean values in 3 preparations from 3 different individuals; each preparation was analyzed in duplicates.

*** Ref. 11.

§ Calculated from ref. 1.

whereas other components were nearly completely dissolved (Band E, Figs. 1b–1d). The results of isoelectric focusing and immunoisoelectric focusing are shown in Fig. 2. Most of the water dissolved membrane proteins migrated to positions corresponding to an isoelectric point between pH 4 and 5; at least 9 different zones could be observed. Immunologic analysis revealed at least 5 different precipitate lines.

Solubility of the water dissolved membrane proteins

Fig. 3 shows the solubility of the water dissolved proteins as a function of pH. 10 mM sodium citrate–citric acid buffers were used for the pH gradient. Most of the water dissolved proteins precipitated between pH 3.9 and 5.4. The result is similar to the findings of MADDY on beef erythrocyte membrane proteins extracted with butanol[22]. About 8 % of proteins remained in the supernatant at pH 4.5 under these conditions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed the presence of one major protein and several minor protein components in the supernatant (Fig. 10c). Comparison of the total amino acid composition of the supernatant and precipitate proteins showed significant differences in some amino acids (Table II). In Table III the solubility of the water dissolved membrane proteins in the presence of various electrolytes is presented. The water dissolved membrane proteins are extremely sensitive to electrolytes, especially to divalent cations. The results of sucrose density gradient centrifugation experiments are given in Fig. 4. In the absence of

electrolytes or in the presence of only 0.03 mM NaOH, one major peak is observed with a sedimentation rate of approx. 3 S. The amount of protein sedimented to the bottom was 12 % of the protein applied. The proteins in the supernatant can penetrate

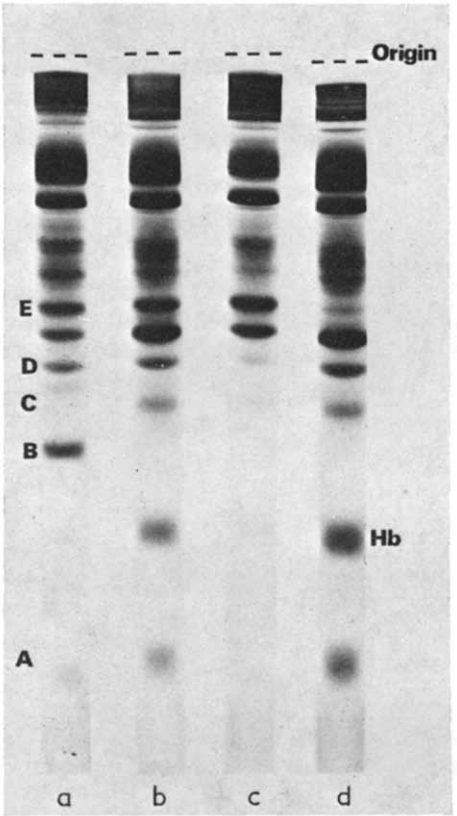

Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of ghost proteins and water dissolved erythrocyte membrane proteins. (a) Ghost prepared by the method of DODGE *et al.*[18]. (b) Ghost prepared by the modified method of MAZIA AND RUBY[11]. (c) Water dissolved membrane proteins. (d) Water extracted ghost. Hb: hemoglobin.

TABLE III

SOLUBILITY OF WATER DISSOLVED MEMBRANE PROTEINS

Electrolytes in solution	*pH*	*Fraction of protein in supernatant* (%)	
		After 20000 × g, 30 min	*After 100000 × g, 60 min*
20 mM NaCl	8.4*	86.3	42.5
20 mM KCl	8.4*	95.4	42.4
10 mM $CaCl_2$	8.4*	39.3	23.0
10 mM $MgCl_2$	8.4*	60.3	27.8
10 mM phosphate buffer	7.4	n.t.**	57.4
10 mM Tris buffer	8.4	n.t.	78.8
10 mM barbital buffer	8.7	n.t.	62.1

* pH was adjusted by ammonium hydroxide.
** n.t., not tested.

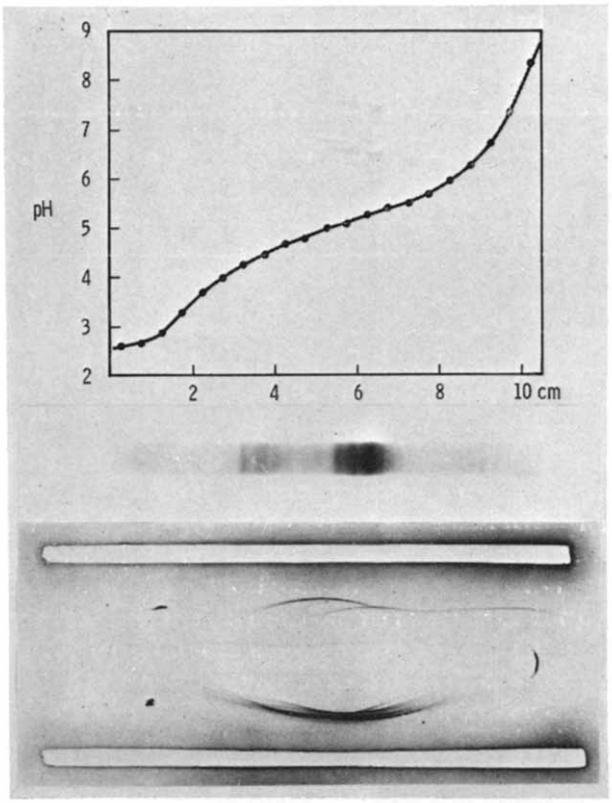

Fig. 2. Isoelectric focusing and immunoisoelectric focusing patterns of water dissolved proteins. pH gradient of the gel was determined by immersing 5-mm gel sections in 2 ml distilled water overnight and subsequent measurement of pH of the solution. Two different rabbit antisera were used for immunoisoelectric focusing.

completely a 5 % acrylamide gel on electrophoresis with a 5 mM Tris–38 mM glycine buffer (Fig. 5A, b). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed the presence of at least 12 protein components in the 3-S peak (Fig. 5B). Sodium or potassium phosphate at a concentration of as low as 1 mM at pH 7.4 resulted already in an increase of the sedimentation rate of the water dissolved membrane proteins.

Immunological properties

Water dissolved membrane proteins contain at least 5 different antigens (Fig. 6). One of these antigenic components is partially soluble at pH 4.5. Some of the antigenic components of the membrane are released from the membranes during the preparation of ghosts (Fig. 6B). Water dissolved membrane proteins contain moderate amounts of virus receptor substance and of complement inhibitor(s) (Table IV). A, B, and H blood group antigens were also present in the water dissolved fraction as determined by agglutination inhibition tests as well as variable amounts of M and N blood group antigens. Rh antigen was not detectable.

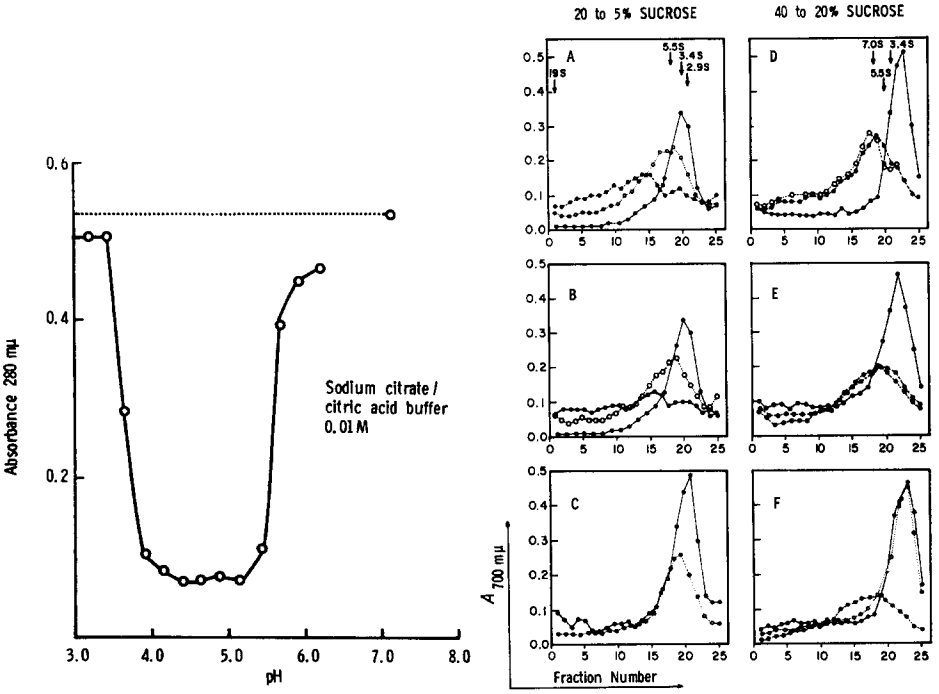

Fig. 3. Solubility of the water dissolved proteins in acidic pH. Absorbance of the supernatant at 280 mμ was measured after centrifugation for 20 min at 20000 × *g*.

Fig. 4. Sucrose density gradient ultracentrifugation patterns of the water dissolved membrane proteins in distilled water and in the presence of various electrolytes. Demonstrated are the influences of sodium phosphate (A), potassium phosphate (B and D), calcium chloride (E), $MgCl_2$ (E), and Tris buffer systems (F). (A) ●—●, in water; ○---○, in 1 mM sodium phosphate buffer (pH 7.4); ●---●, in 10 mM sodium phosphate buffer (pH 7.4). (B) ●—●, in water; ○---○, in 1 mM potassium phosphate buffer (pH 7.4); ●---●, in 10 mM potassium phosphate buffer (pH 7.4). (C) ●—●, water dissolved membrane protein prepared from acetate-washed ghosts; ○---○, water dissolved membrane proteins prepared from phosphate-washed ghosts. No electrolyte was added to the sucrose solution. (D) ●—●, in 0.03 mM NaOH (pH 7); ○---○, in 10 mM potassium phosphate buffer (pH 7.4); ●---●, in 100 mM potassium phosphate buffer (pH 7.4). (E) ●—●, in 0.03 mM NaOH (pH 7); ○---○, in 10 mM $CaCl_2$ and 0.03 mM NaOH (pH 6.9); ●---●, in 10 mM $MgCl_2$ and 0.03 mM NaOH (pH 6.9). (F) ●—●, in 0.03 mM NaOH (pH 7); ○---○, in 5 mM Tris–38 mM glycine buffer (pH 8.3); ●---●, in 100 mM Tris buffer (pH 7.4).

Enzymatic assays

The activities of acetylcholinesterase and the ouabain sensitive (Na^+-K^+)-dependent ATPase are very low in the water dissolved membrane protein fraction, the latter being in some preparations completely absent (Table IV). Acetylcholinesterase activity was also assayed in the hemolysate supernatant and wash solutions for preparations of ghosts. It was found that 40–80 % of the total acetylcholinesterase were released from the red cell membrane during hemolysis by 0.1 % Triton X-100 in distilled water and the subsequent washing procedure of ghosts with 0.02 M ammonium acetate at pH 7.4. This loss accounts for the relatively low specific activity of 1.06 in the ghost preparation. Also the specific activity of ghost ATPase is low (Table IV). It is not clear whether this low specific activity of ghost ATPase is due to inactivation of the enzyme or due to release of the enzyme from membranes during

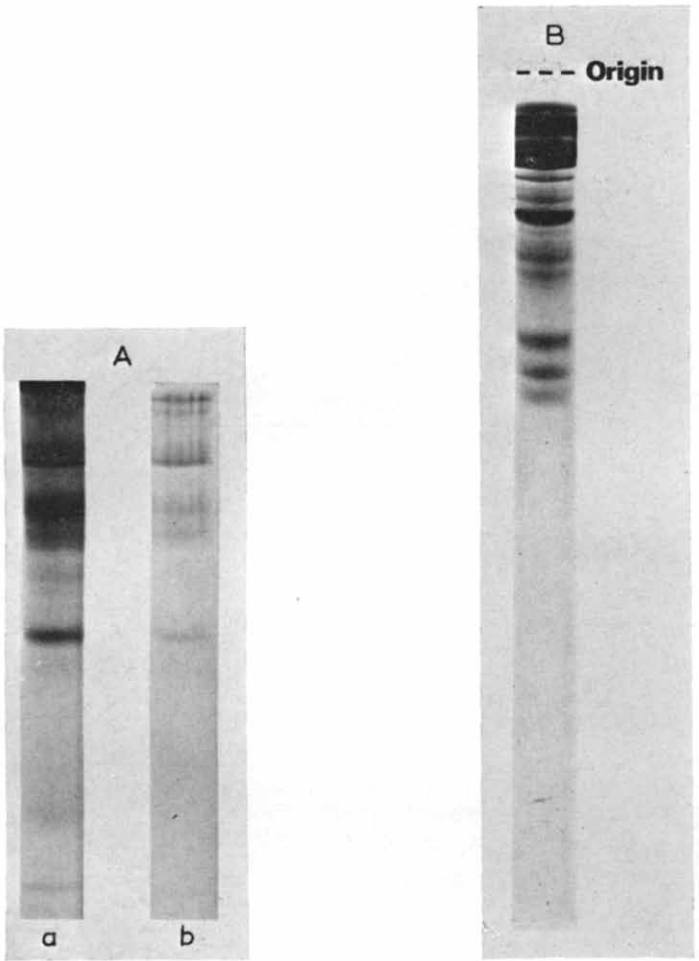

Fig. 5. (A) Disc electrophoresis patterns in 5% polyacrylamide gels with 5 mM Tris–38 mM glycine buffer system. a, water dissolved membrane proteins; b, proteins from the 3-S peak fraction in Fig. 4E. All of the proteins of other fractions in Fig. 4E also penetrated 5% acrylamide gel without sodium dodecyl sulfate. (B) Proteins from the 3-S peak fraction in Fig. 4E analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

the preparation. When ghosts were prepared according to the method of DODGE *et al.*[18], using a 6 mM Tris buffer (pH 7.5), the ouabain sensitive and ouabain insensitive (Na^+-K^+)-ATPase activities were 0.40 μmole P_i per mg protein per h and 0.29 μmole P_i per mg protein per h, respectively.

Gel filtration and preparative electrophoresis

In Fig. 7 the elution diagram of a Sepharose 4B column is given. 5 mM Tris–38 mM glycine buffer (pH 8.3) was chosen as eluent since on sucrose density gradient ultracentrifugations with this buffer system, protein aggregation was not apparent (Fig. 4F). The major portion of the membrane proteins was eluted in the void volume. The remainder was eluted in two small peaks. Sodium dodecyl sulfate–polyacrylamide

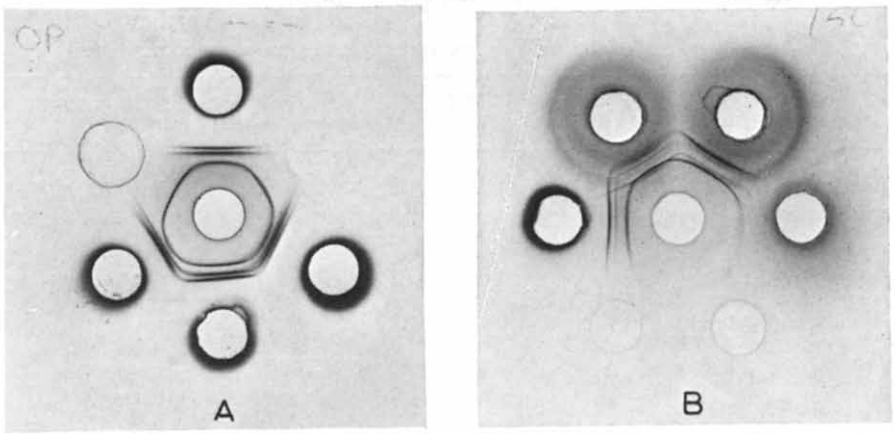

Fig. 6. (A) Ouchterlony test of the water dissolved membrane proteins, precipitates and supernatants after treatment at pH 4.5. From top clockwise: 1, water dissolved membrane proteins; 2, supernatant pH 4.5 after treatment with Ampholine pH 3–6; 3, precipitate pH 4.5 after treatment with Ampholine pH 3–6; 4, water dissolved membrane proteins; 5, precipitate pH 4.5 after treatment with sodium acetate; 6, supernatant pH 4.5 after treatment with sodium acetate. AS (center well): rabbit antiserum against phosphate-washed red cell ghosts. (B) Immunologic analysis of hemolysate and wash fluids for the preparation of ghosts (modified method of MAZIA AND RUBY). From upper left corner clockwise: 1, supernatant of hemolysate; 2, 1st wash; 3, 2nd wash; 4, 3rd wash; 5, 4th wash; 6, water dissolved membrane proteins. AS (center well): rabbit antiserum against phosphate-washed red cell ghosts.

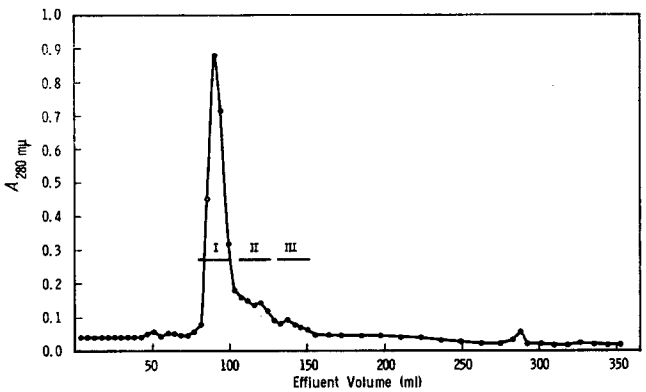

Fig. 7. Gel filtration of the water dissolved membrane proteins on Sepharose 4B in 5 mM Tris–38 mM glycine buffer.

gel electrophoresis showed that each fraction represented a complex mixture of several components and that distinct separation was not achieved (Fig. 8). On preparative zone electrophoresis, the water dissolved protein fraction migrated as a single component (Fig. 9). Rerun of this peak fraction on sodium dodecyl sulfate–polyacrylamide gels revealed the presence of almost all components of the water dissolved membrane protein fraction (Fig. 10d).

TABLE IV

IMMUNOLOGIC AND ENZYMATIC ACTIVITIES OF THE WATER DISSOLVED MEMBRANE PROTEINS

		Ghost	*Water dissolved preparation*	*Water insoluble fraction*
Viral hemagglutination inhibition* (HAI units/mg protein)				
	Mean	6.5	3.7	9.6
	Range	3.6 –10.1	0.8 –8.0	5.5 –14.2
Complement fixation inhibition* (CFI units/mg protein)				
	Mean	40.4	33.3	44.0
	Range	24.2 –55.2	13.0 –68.0	27.6 –67.5
Acetylcholinesterase** (μmoles acetate/mg protein per min)				
	Mean	1.06	0.15	2.14
	Range	0.77–1.21	0.07–0.20	2.00–2.30
$(Na^+ + K^+)$-ATPase*** (μmoles P_i/mg protein per h)				
Total	Mean	0.17	0.04	0.29
	Range	0.09–0.28	0.04–0.05	0.16–0.46
Ouabain sensitive	Mean	0.09	0.01	0.15
	Range	0.03–0.16	0.00–0.03	0.07–0.26
Ouabain insensitive	Mean	0.08	0.03	0.14
	Range	0.06–0.16	0.02–0.04	0.09–0.20

* Mean and range of 3 preparations from 3 individuals.
** Mean and range of 5 preparations from 5 individuals.
*** Mean and range of 4 preparations from 4 individuals.

DISCUSSION

About 40 % of the human erythrocyte membrane proteins are dissolved in water by the modified procedure of the method of MAZIA AND RUBY[11] described in this report. Their amino acid composition and N/P ratio are similar to those of water dissolved beef erythrocyte membrane proteins, but their recovery from human erythrocyte ghosts is only half of the reported recovery from beef erythrocytes ghosts. When ghosts were washed with 20 mM ammonium acetate (pH 6.5) according to the original method[11], the recovery of proteins from the ghost was less than 30 % of which one fifth was hemoglobin. These differences may be ascribed to differences in cohesive structures between human and beef erythrocyte membrane components as suggested by BURGER *et al.*[23].

It can be assumed that the water dissolved membrane proteins are obtained in highly dissociated states. This assumption is based on the observation that the proteins of this fraction can penetrate more or less completely polyacrylamide gels: (a) Most of the proteins of this fraction move towards their isoelectric points through a 6.5 % acrylamide gel when examined by isoelectric focusing. (b) All of the proteins in the supernatant of the sucrose density gradient penetrate 5 % acrylamide gels on electrophoresis with 5 mM Tris–38 mM glycine buffer.

The role of Triton X-100 in the method of MAZIA AND RUBY has not been clearly

defined[11]. In experiments (unpublished), in which Triton X-100 was not used, and hemolysis was achieved by distilled water only, the protein recovery from ghosts was less than 20 %, and the water dissolved protein fraction was strongly contaminated

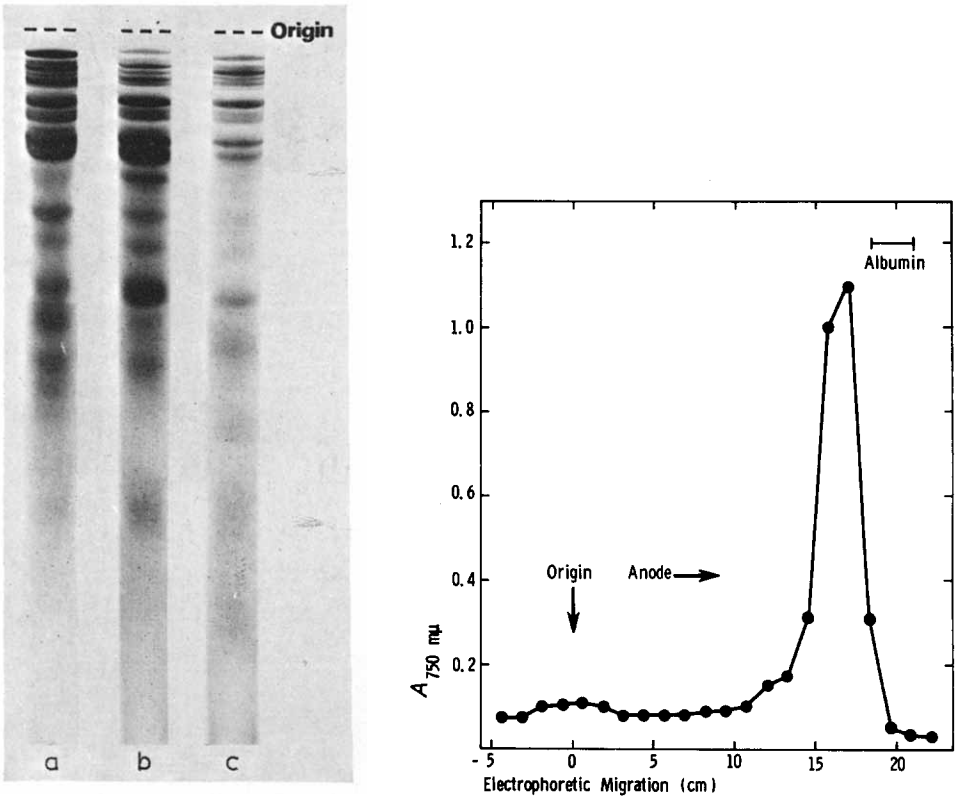

Fig. 8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of the fractions by Sepharose 4B gel filtration (Fig. 7). (a) Fraction I. (b) Fraction II. (c) Fraction III.

Fig. 9. Preparative Pevikon block electrophoresis of the water dissolved membrane proteins. Proteins were eluted from half inch sections of the block with barbital buffer (I = 0.05, pH 8.6) and measured by the method of LOWRY *et al.*[13]. The position of serum albumin is shown for comparison.

with hemoglobin. The N/P ratio of the preparation was approx. 200. The proteins penetrated completely a 5 % acrylamide gel in electrophoresis with 5 mM Tris–38 mM glycine buffer. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed apart from hemoglobin, at least 19 protein components. The pattern was clearly distinguishable from that of the water dissolved proteins prepared with the use of Triton X-100. In the absence of Triton X-100, acetylcholinesterase was not released from membranes during the preparation of ghosts. These data indicate that the use of Triton X-100 at the time of lysis not only increases the yield of dissolved proteins and lipids, but also results in the solubilization of proteins which are water insoluble in its absence.

The effects of electrolytes on the aggregation of water soluble erythrocyte mem-

brane proteins have been analyzed by previous investigators[10,11,22]. The data presented here show that while Ca^{2+} and Mg^{2+} have quite drastic effects on the aggregation of human erythrocyte membrane proteins, that also Na^{+} and K^{+} in rather low concentrations result in the formation of aggregates. This tendency towards aggregation prevents the effective fractionation of these proteins with conventional separation procedures.

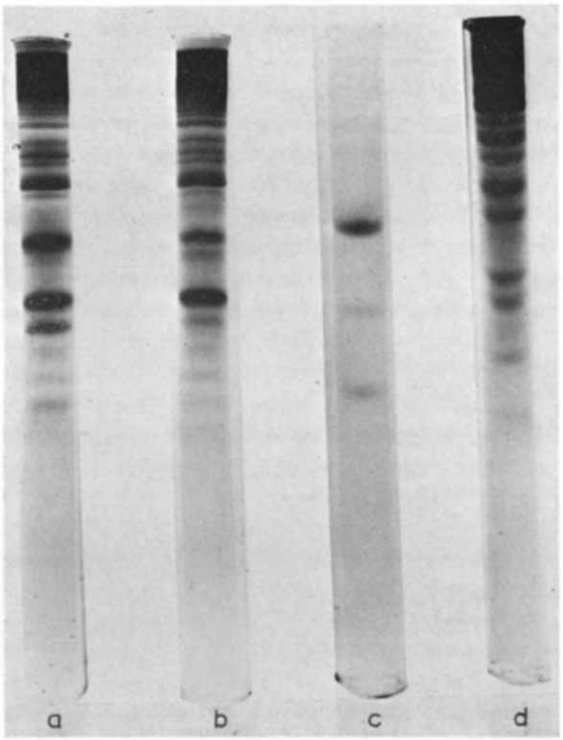

Fig. 10. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis patterns of fractions of water dissolved membrane proteins separated by precipitation at pH 4.5 and of major component obtained by preparative zone electrophoresis. (a) Water dissolved membrane proteins. (b) Precipitate after treatment at pH 4.5. (c) Supernatant after treatment at pH 4.5. (d) Major fast migrating component from preparative zone electrophoresis (Fig. 9).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis shows that the water dissolved membrane protein fraction is a complex mixture of at least 15 protein components. To relate these components to specific biological functions is a difficult task. Some of the components may be identified with the use of radioactively labelled chemicals[24,25]. Although the amino acid composition of the water dissolved proteins fraction from human erythrocyte membranes resembles those observed in other cell membranes from different species[11], we do not feel that the results of this study contribute to the controversial question of the presence of a structural protein in red cell membranes[26–28].

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