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Water-Soluble Proteins of the Human Red Cell Membrane

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Summary. Procedures were developed for preparation of red cell membranes almost free of hemoglobin but with minimal loss of membrane proteins. Two water-soluble protein fractions are described, each constituting about 25% of the ghost protein. The first is ionically bonded and can be solubilized in water rapidly at pH 7.0 and more slowly at higher ionic strength solutions, with a minimal rate at 20 mM. This fraction contains four major components with molecular weights ranging from 30,000 to 48,000. The second fraction can only be solubilized at an appreciable rate if Ca^{++} is absent and at higher pH (9.0). It is predominantly a single molecular weight component (150,000). It tends to aggregate at higher ionic strength and in the presence of Ca^{++}. Evidence is presented suggesting that the water-soluble proteins are present at the inner face of the membrane. The lipids remain in a water-insoluble residue that contains four major protein components ranging in molecular weight from 30,000 to 100,000. The latter is the predominant component. Only the residue contains the Na^{+}–K^{+}-activated ATPase, the cholinesterase, antigenic activity and most of the sialic acid and carbohydrate. The first water-soluble fraction contains a Mg^{++}-activated ATPase. The extraction of the water-soluble proteins is accompanied by anatomical changes resulting finally in the formation of small membranous vesicles.

Although approximately half of the mass of the red cell membrane is protein, its arrangement with respect to the other constituents, its role in determining structure, and its role in specific membrane functions are poorly understood. Recently a number of techniques have been applied in an attempt to learn more about membrane proteins. The most direct of these involves the dissociation of the membrane into lipids, proteins, and/or lipoproteins which can then be characterized chemically and in some cases functionally (for example, in terms of enzyme activity).

A variety of solubilization procedures have been used, most of them involving nonaqueous solvents [5, 7, 8, 23, 31, 34, 36, 39, 52], ionic deter-

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gents [5, 6, 8, 14, 40, 42], or nonionic detergents [5, 28, 45, 46, 50] usually used in combination with other agents or treatments including urea [3, 5, 19, 40, 42, 45], mercaptoethanol [3, 19, 24, 27, 40, 45, 48, 49], guanidine [5, 40], chelating agents [24, 27, 40, 43, 49], acid [3, 35, 37, 42, 46], alkali [5, 24, 38, 49], low [15, 16, 24, 28, 32, 43, 49] or high [30, 33, 40] ionic strength, chemical modification [36, 42], and sonication [2, 5, 31, 40, 41, 46]. Once solubilized, the components are separated by one or more of several techniques including centrifugation, density gradients, electrophoresis (usually gel), or chromatography. Because different agents, different sequences of manipulation, and different means of characterization are used, cross comparisons of the many reported procedures are most difficult.

Some membrane proteins can be dissolved without the use of solvents or detergents particularly by manipulation of ionic strength and pH, and usually in the presence of chelating agents and sometimes mercaptoethanol [5, 15, 16, 24, 27, 30, 32, 39, 40, 43]. Solubilization of a unique protein of relatively high molecular weight which is capable of forming large aggregates and fibers has been reported from three laboratories [15, 16, 24, 27, 43, 48]. The procedures for extraction are similar as are some of the properties, so it may be suspected that the same protein has been isolated in each case. The protein called "spectrin" by one laboratory has been chemically characterized in detail [24, 27, 48] and has been demonstrated in red blood cells from a number of species [49]. The same or a similar protein shows Ca^{++}-activated ATPase activity [43].

Most of the reported procedures are more or less empirical. They are aimed in most cases at solubilization of the membrane in whole or in part, fractionation of the components, and isolation and characterization of individual proteins in such terms as molecular weight, amino acid composition, end-group analysis, carbohydrate content, sialic acid content, antigenic behavior, enzymic activities, and tendency to aggregate in fibrils or other visible (with electron microscopy) structures. From the large variety of procedures that have already been attempted, it is abundantly clear that the erythrocyte membrane does not lend itself easily to solubilization, separation and characterization of components – in part because of the inherent properties of the proteins and in part because a fairly large number of proteins are present in a complex structural array with the membrane lipids.

The solubilization procedures are a necessary prerequisite to isolation and characterization. In addition, however, the nature of the procedures required for solubilization of particular components can potentially provide information concerning the nature of the bonding of those components

into the membrane. For example, proteins may in general be held in the membrane by forces related to simple ionic attractions, chelation, hydrogen bonding, hydrophobic attractions, or disulfide bonds. They will tend to dissolve when the attractive forces are reduced or eliminated, and when electrostatic repulsive forces are maximized. A systematic series of solubilization using different techniques may therefore provide considerable insight into the interactions of various membrane components, and perhaps their arrangements in the membrane. A further possibility relates to the location of components on the inner and outer sides of the membrane. Natural markers of the outer face of the membrane include the sialic acid [47] and antigenic groups. The outside is also characterized by specific anatomically visible structures, the plaques [17]. Fibrillar proteins have been localized on the inner boundary of the membrane [17, 25, 26, 43] as has the Na–K-activated ATPase (pump ATPase) [26, 51]. Ligands on the outer face can also be labeled by ligand-specific nonpenetrating chemical agents [6, 25].

Recently, in compiling a catalogue of membrane proteins, Rosenberg and Guiddoti [40] carried out a series of extractions starting with low ionic strength [with ethylenediaminetetraacetate (EDTA) and mercaptoethanol], followed by high ionic strength, solvent extraction, and finally treatment with the detergent sodium dodecylsulfate (SDS). In the present paper, a series of extractions ("serial solubilization") is also used with particular attention paid to a complete extraction and recovery of those proteins that are ionically bonded and that can be made soluble by simple manipulations of ionic strength, pH, and chelating agents. In preparing membranes relatively free of hemoglobin, it can be questioned if loosely bonded membrane proteins have been dissolved and lost in the lysing media. An answer to this was attempted by analysis of both hemoglobin and non-hemoglobin protein of membranes during a series of lyses. The nature and location of the solubilized proteins were determined by markers or properties such as enzyme activity, antigenic activity, sialic acid, carbohydrates, electron microscopy, molecular weight estimates, gel electrophoresis, and by use of nonpenetrating chemical markers. The solubilization characteristics of the various protein components were taken as indicators of the nature of their bonding to the membrane.

Materials and Methods

The Preparation of Red Cell Membranes (Ghosts)

Ghosts were prepared for most experiments from recently out-dated human blood [acid citrate dextrose (ACD) solution A, U.S.P. 65.25 ml plus 435 ml human blood].

For the preparation of white ghosts (containing less hemoglobin than 0.5% of the total protein of ghosts), a series of hemolyses was performed at 30 °C with NaCl-phosphate buffer solutions of pH 7.2 with osmolarities of 60, 30, and 20 mosm in the presence of EDTA. This is a modification of the method described earlier [39]. Polyethylene tubes were used to avoid hemolytic effects of glass.

For each preparation, one bag of ACD blood was divided equally into four centrifuge tubes (volume 250 ml). The cells were packed by centrifugation for 10 min at $3{,}000 \times g$, and the plasma and buffy coat were removed by aspiration. The remaining red cells were washed three times with three volumes of 1% NaCl. After the final washing, the supernatant was clear and colorless. The hemolysis was performed in three steps, each step repeated once. The appropriate dilutions of 60, 30, and 20 mosm were made from a stock buffer solution containing 0.200 M NaCl, 0.075 M Na_2HPO_4, and 0.025 M KH_2PO_4. After dilution, sodium EDTA was added to a level of 1 mM. The cells or ghosts were exposed in each case to eight times their original volume; the suspensions were shaken for 30 min at 30 °C, and then centrifuged at $10{,}000 \times g$ for 20 min. Each time the supernatants were aspirated very carefully. In the last steps, an occasional small red button of resistant red cells was also aspirated.

One series of experiments was carried out with fresh blood cells.

Polyacrylamide Gel Electrophoresis

Gel electrophoresis on solubilized fractions was carried out as a modification of the polyacrylamide system introduced by Ornstein and Davies [11] (sample and spacer gels were omitted). Polymerization of 5 ml of 7.5% gel solution took place in Pyrex tubes (inner diameter 8 mm; length 150 mm) with a total volume of 10 ml. After polymerization, the overlayered water was replaced with buffer solution. Up to 2-ml samples containing 10% sucrose were layered under the buffer solution in direct contact with the gel. Electrophoresis was performed in the cold room at a current of 1 mA per tube for 1 hr in order to concentrate the proteins on top of the gel by free electrophoresis in the sample solution. Thereafter the amperage was raised to 4 mA per tube for 4 hr. None of the proteins moved faster than the marker dye (bromphenol blue, 1 ml of a 1% solution) which was added to the buffer. The gels were stained overnight with a 1% Amido-Black solution in 7.5% acetic acid. Destaining was done electrophoretically in 7.5% acetic acid. In later work, the gels were fixed in 10% trichloracetic acid overnight and stained with Coomassie Blue (1 ml of 1% solution per tube) for 1 hr.

In order to estimate molecular weights, gel electrophoresis was carried out in the presence of sodium lauryl sulfate (SLS) by a modification of the methods of Ruckert and Dunkle [13]. The buffer used was 0.1 M Tris-Cl and the urea, mercaptoethanol and SLS concentrations of the sample were 6 M, 5%, and 1%, respectively.

Chemical and Assay Procedures

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall [20]. A solution of purified bovine serum albumin (Pentex) was used as an arbitrary standard. The amount of protein is usually expressed in percentages of the total protein present in the ghost preparation.

Hexose and hexosamine were measured by the methods of Winzler [52]. Sialic acid was measured by the method of Aminoff [1]. Hemoglobin was determined by the benzidine method [10] at low concentrations and by absorption at 550 nm at higher concentrations. Lipids were separated by silica paper chromatography [9] and detected by the tricomplex staining procedure [14].

ATPase was detected in the gels by the following method (G. Berg, *personal communication*). Immediately after electrophoresis, gels were quartered lengthwise and the quarter sections either stained or incubated in the ATPase medium at 37 °C. The ATPase medium consisted of 10 ml of 2 M KCl, 25 mM Tris-Cl, 1 mM $Pb(NO_3)_2$, and 3 mM ATP at pH 7.5. The activating effects of divalent cations were determined by adding the cation at a final concentration of 3 mM. ATPase was manifested as a whitish band of lead phosphate deposit.

In vitro assays of ATPase were conducted by incubating 0.1 ml of membranes in 0.5 ml of solution containing 80 mM Tris-histidine, 2 mM ATP, and cofactors at 37 °C for 45 min. The precipitate was spun down and the phosphate in the supernatant assayed by a modification of the Fiske-Subbarrow method [44]. The cofactors used were either 2 mM $MgCl_2$ or 2 mM $MgCl_2$ plus 80 mM NaCl plus 15 mM KCl.

Cholinesterase was estimated by the method of Michel [29]. Immunoassays were conducted by a hemagglutination inhibition test. The outer face of the membrane was labeled with 4-acetamido-4′-isothiocyanatestilbene-2,2′-disulfonic acid (SITS), a non-penetrating amino reagent [22]. One ml of 5 mM SITS was added to 5 ml of packed red cells suspended in 300 mosm phosphate buffer, pH 7.2. The cells were allowed to react for 5 min at room temperature, and the excess SITS was removed by washing several times in buffer. No hemolysis occurred during this procedure. Ghosts and membrane proteins were prepared from the SITS-labeled cells by the usual methods. The relative amount of SITS bound was estimated by scoring the fluorescence in equal weights of protein on a 0–4 basis, and by following the disappearance of fluorescence during serial twofold dilutions.

Phase and Electron Microscopy

The samples for phase microscopy were suspended in 20 mosM NaCl-phosphate buffer and examined with a Zeiss phase microscope. The ghosts and the ghost residues were studied by electron microscopy after negative staining with 2% phosphotungstic acid, or after fixation with osmium [26], sectioning and staining with various heavy metals.

Results

Because exposure of ghosts to water for even a short time results in solubilization of considerable protein, about 8% [39] methods of lysis were evolved that produced ghosts relatively free of hemoglobin, with a minimal loss of non-hemoglobin protein. The procedure as described in Methods involves serial lysis carried out in 60, 30, and 30 mosm salt. The resulting ghosts, virtually hemoglobin-free (less than 0.5% of the ghost protein), contained 3.47 mg protein per ml packed cells (range 3.24 to 3.72 in three determinations) or 9.07×10^{-10} mg per ghost. The amount of protein in ghosts prepared from fresh cells was not significantly different.

The ghosts, when washed with distilled water, released up to 47% of soluble protein that could be characterized by acrylamide gel electrophoresis. A number of bands of different staining intensity were found in a reproducible pattern as shown in Fig. 1. The band marked H represents residual traces of hemoglobin in the ghosts (as demonstrated by characteristic stain-

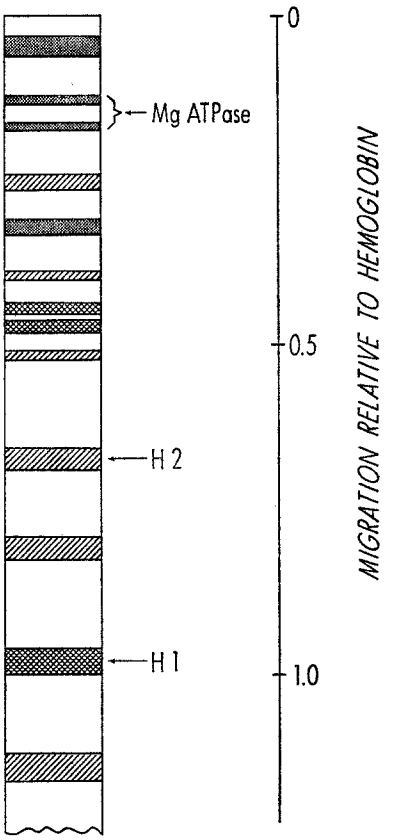

Fig. 1. Acrylamide gel patterns of water-soluble proteins. The traces of hemoglobin (marked *H* on pattern) are used as a reference protein

ing with benzidine peroxide). The hemoglobin band was used as a reference in calculating mobilities of other proteins in the gels. No traces of plasma proteins were detectable.

When the ghosts were washed with successive amounts of water, fractionation occurred. The residual hemoglobin and the more mobile proteins (in gel electrophoresis) predominated in the first wash, whereas slow-moving proteins, including some that barely entered the gels, predominated in the second wash (the dark band closest to the origin in Fig. 1). For convenience these will be called Proteins I and Proteins II.

The extraction of both fractions was influenced by pH, but not in a precisely parallel manner. Both were extracted minimally at pH 5.0 (Fig. 2). Although large amounts of protein were extractable at low pH, the band pattern on acrylamide gels was considerably altered. All subsequent extractions were therefore carried out at slightly alkaline pH. At pH 7.0, 8.0, and

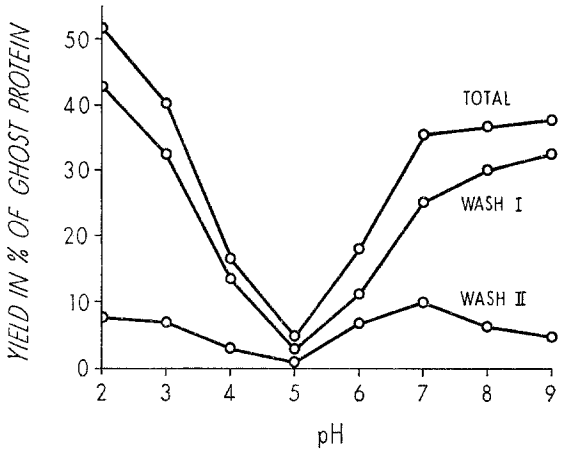

Fig. 2. The effect of pH on extraction of proteins in two consecutive water washes

9.0, the total amount of water-soluble protein extracted was about the same, but more of it came out in the first wash than in the second. This effect is almost entirely due to the increased extractability of the electrophoretically immobile protein (Protein II). Little of this material is extracted in one wash at pH 7.0, but it constitutes about one-half of the protein extracted in one wash at pH 9.0.

The difference in the rate of extraction of Proteins I and Protein II was also markedly influenced by ionic strength and by calcium. Protein I could be solubilized in the presence of salts, but the time required was greater than in water. Extraction in water was virtually complete in 30 to 60 min. In the presence of salt, a minimal rate of extraction was found at a concentration of 20 mM, but extraction was virtually complete at 4 °C after 16 hr at higher salt concentrations (Fig. 3). Results were similar with either KCl or K_2HPO_4 as the salt. The solubilization of Protein II, on the other hand, was particularly inhibited by salt. A good separation could therefore be made by exposing the ghosts overnight at 4 °C to 60 mM pH 7.0 buffer, in order to extract Proteins I, followed by a water extraction (at room temperature and pH 9.0 to remove Protein II). For optimal solubilization and separation, a somewhat more complicated procedure was adopted that is outlined in the flow sheet (Fig. 4).

Extraction of Protein II, but not of Proteins I, is influenced particularly by bivalent cations. Thus EDTA treatment does not enhance the extraction of Proteins I, but does enhance the extraction of Protein II. On the other hand, the presence of small concentrations of Ca^{++} or Mg^{++} (1 mM) completely inhibited the extraction of Protein II but not of Proteins I.

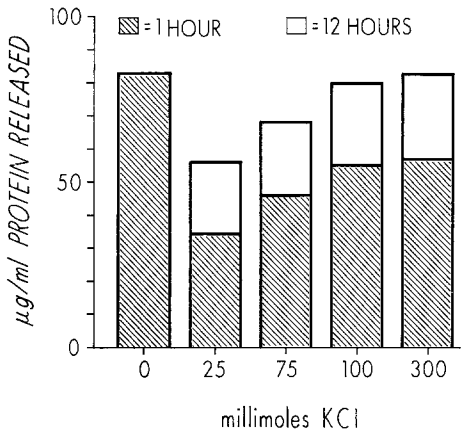

Fig. 3. The rate extraction of Proteins I as a function of ionic strength. The ordinate represents the amount of protein released per ml of ghost suspension in the stated time and ionic strength. Each ghost suspension was prepared from a red cell suspension containing 1 volume of cells to 8 volumes of salt solution

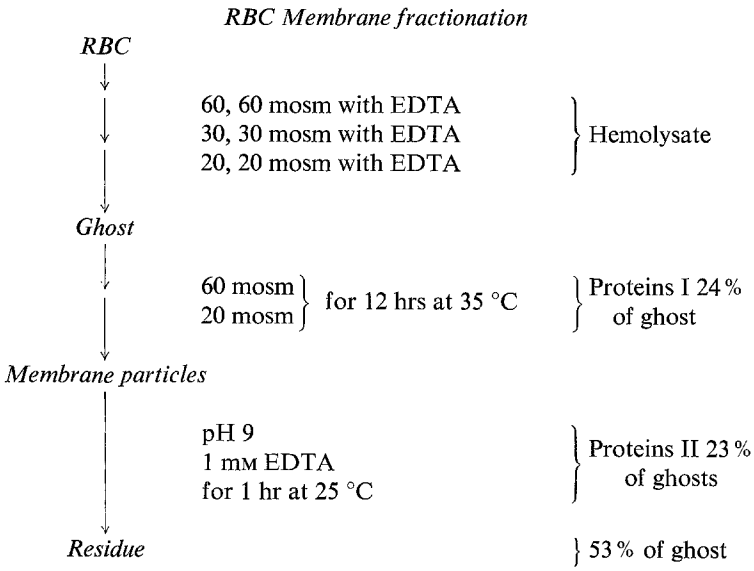

Fig. 4. Flow sheet for separation of Proteins I, Protein II, and residue

Proteins I and II constitute as much as 24% and 23% of total membrane protein, respectively, but no lipid could be detected in either fraction. Most of the lipid remains in a residue that can be removed by relatively low-speed centrifugation (1 to 3 hr at 40,000 to 50,000 $\times g$). This material is in the form of membranous vesicles (Fig. 5a) of about 0.5 μ and smaller that still retain surface plaques [17]. Vesicles have also been reported by others [24, 43].

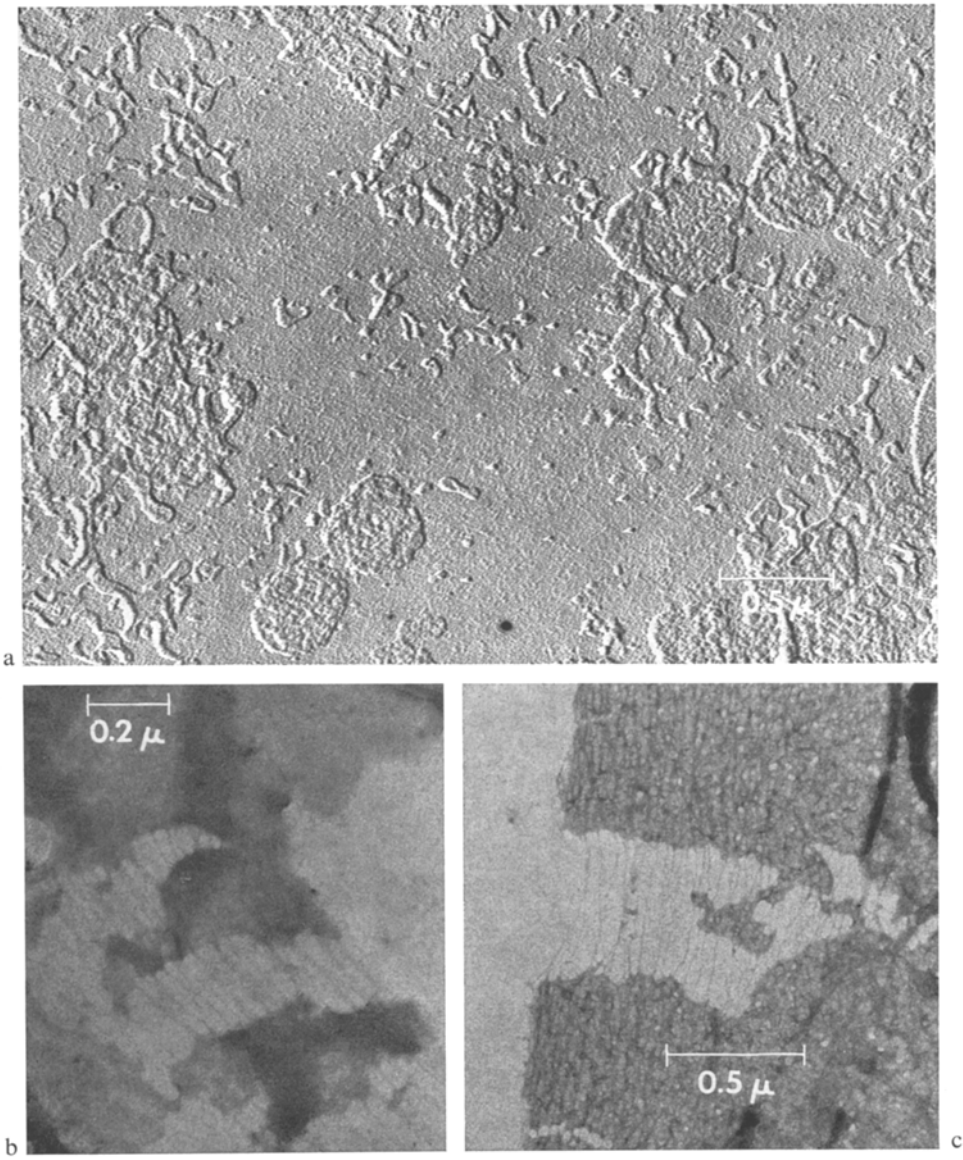

Fig. 5. a. pH 9 residue, after Proteins I and II were removed, air dried, and shadowed with chromium. The fragments are heterogeneous in size and shape and surface particles (about 150 A in diameter) are visible on most fragments. b, c. Whole ghosts present in negative stained (uranyl acetate) preparation. Fibers with diameters of 25 to 50 A and larger are seen extending from the material of the membrane

Complete separation of the lipid material from the Protein II fraction requires centrifugation at higher speeds (5 hr at 100,000 $\times g$). The additional lipid-containing material removed is presumably in the form of small mem-

Table 1. *Characteristics of membrane protein fractions*[a]

Characteristic	Fraction		
	Proteins I	Protein II	Insoluble residue
Solubilization in water	Rapid	Slow	No
Solubilization in water + Ca^{++}	Yes	No	No
Solubilization in salt solutions	Slow	No	No
Ghost protein	24	23	53
Lipid	<5%	<5%	>90%
Immunoassay (A & B antigen)	<5%	<10%	>80%
SITS-labeling of intact cells	<5%	<5%	>90%

[a] Numerical values are in % of total. The values for lipids, antigens, and SITS-fluorescence are estimates based on dilution series and on the least detectable amounts.

brane fragments that can be seen in the electron micrographs. Others have also reported fragmentation of the membrane in water [12, 16, 30, 34].

The transition from ghosts to smaller vesicles and fragments seems to involve a series of transitions associated with the reduction in ionic strength. Long tubular processes of approximately 0.2-μ diameter are formed which are similar to those reported by Baker [4]. By negative staining, a pebbled surface could be observed interspersed with a fibrous network. In some samples in which the membrane was stretched or broken in preparation, a network of long fibrils of diameter 15 A was observed (Fig. 5b and c). Attempts to display the unit membrane structure in the vesicles of the extracted residue were unsuccessful with osmium, permanganate, or uranyl stain.

The three membrane fractions (Proteins I, Protein II, and residue) show many other differences in addition to solubility and lipid distribution. The various assays used to characterize the fractions are summarized in Tables 1 and 2.

Proteins I contains little if any sialic acid or A or B antigen, and a small amount of hexose and hexosamine. Because sialic acid [47] and antigenic activity are considered to be located on the outer surface of the membrane, the data suggest but do not prove that Proteins I may be located at the inner face of the membrane. Most of the antigenic material, sialic acid, and carbohydrates remain with the residue but with small amounts in Protein II.

The question of location was further explored by the use of nonpenetrating amino-reactive agent SITS, first developed by Maddy [25]. This agent does not penetrate the membrane but reacts with superficial amino groups.

Table 2. *Assays of components in membrane fractions*[a]

Assay	Fraction				Total recovery (%)
	Ghost	Proteins I (%)	Protein II (%)	Residue (%)	
Sialic acid	30	2	13	87	102
Hexose	30	5	4	55	64
Hexosamine	21	11	12	82	105
Basal ATPase	0.70	36	31	92	158
Mg ATPase	1.12	0[b]	3	40	43
Na–K ATPase	0.87	−1	−2	35	32
Cholinesterase	1.00	0	0	34	34

[a] Units for sialic acid, hexose, and hexosamine are in μg/mg protein; for ATPases, moles $\times 10^{-8}$ Pi released per min per mg protein; for cholinesterase, the units are arbitrary. All values for fractions are expressed in percent recovery based on the amount in the ghost. The data represent the means of values from two batches of cells, each carried through the fraction. All analyses were carried out in duplicate or triplicate. The pattern shown by the means is similar to that of the individual values.

[b] Activity was found in bands of acrylamide gels (Fig. 1) by staining techniques.

SITS was allowed to react with intact cells, and the ghost fractions were then prepared. All of the SITS was located (by fluorescence) in the residue, with little if any detectable in Proteins I or II. In controls in which SITS was added to the isolated fractions and then the surplus dialyzed away, Proteins I and II gave a strong fluorescence. This experiment indicates that the free amino groups exposed to the environment are located in the residue, and that Proteins I and II are presumably located on the inner face of the membrane.

Several enzyme activities were assayed in the fractions (Table 2). Acetylcholinesterase was found only in the residue. Basal ATPase activity (no cations required) was found in all fractions, but almost all of the Mg^{++}-activated and the Na^{+}–K^{+}-activated ATPase was found in the residue. Some Mg^{++}-activated ATPase could be detected directly in the acrylamide gel in a pair of slow-moving bands (Fig. 1). In the fractionation, a considerable loss of cation-activated ATPase occurred, partly compensated by an increased recovery of basal ATPase. Cholinesterase activity was also lost on fractionation. No explanation for such changes in activity can be offered.

The molecular weights of each of the protein fractions were roughly estimated by use of acrylamide gel electrophoresis in the presence of the detergent SLS [11]. Each fraction had a unique pattern of molecular weight

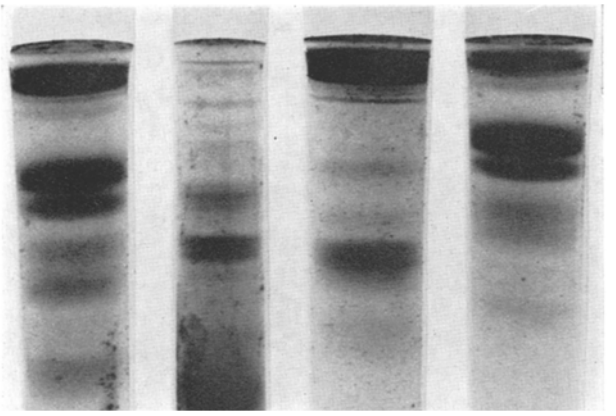

Fig. 6. SLS acrylamide gel patterns of proteins from ghosts, Proteins I, Protein II, and residue

distributions, indicating that unique proteins are present (Fig. 6). In the Proteins I fraction, at least four bands were observed, with molecular weights ranging from 30,000 to 48,000, with the 30,000 band predominant. Protein II gave primarily a single band of relatively high molecular weight (over 150,000), whereas the residue showed at least five bands of relatively low molecular weight from 30,000 to 100,000 with the latter predominant.

Discussion

About half of the protein of the red cell membrane can be solubilized by mild procedures involving reduced ionic strength and slightly alkaline solutions. The fraction called Proteins I is rapidly solubilized in water at pH 7.0, and can be dissolved, but considerably more slowly, at any ionic strength from 0 to 150 mM, with a distinct minimum in rate at 20 mM. It consists of a group of proteins containing no lipids, carbohydrates, sialic acid, or antigenic properties. These observations, taken together with the failure to bind the nonpenetrating amino reagent SITS, suggest that Proteins I are located on the inner surface of the membrane. These proteins are largely lost in many well-washed ghost preparations used to study membrane proteins.

Because Proteins I are washed out of the membrane with such mild procedures, one might question if they are really membrane proteins, and if so, how much is lost in preparation of the ghosts. These questions are difficult to answer in absolute terms but if hemoglobin is taken as a marker

for the behavior of soluble cellular proteins, then it is clear that Proteins I behave quite differently. For example, in six-step ghosts (as described in Methods), the hemoglobin content is reduced to less than 0.5% of the ghost protein, whereas Proteins I constitute 24% or a ratio of about 50/1; yet the ratio of non-hemoglobin to hemoglobin protein in the initial lysate is only 1/30 (hemoglobin is 300 mg/ml of packed cells and non-hemoglobin protein is 11.3 mg/ml) [21]. Thus the selective factor of the membrane for Proteins I over hemoglobin is as high as 1,500/1. These data certainly eliminate trapping of cytoplasmic non-hemoglobin proteins as an explanation. Nor does specific adsorption of the non-hemoglobin proteins during ghost preparation seem likely because prolonged exposure to the same salt concentrations as used in lysing will dissolve Proteins I. It would be difficult to explain how adsorption of proteins could occur under the same conditions that can result in their solubilization.

In another experiment, the lysates from each of the six steps were analyzed for hemoglobin and non-hemoglobin protein and were also run on acrylamide gels. The first two lysates were so rich in hemoglobin that all other bands in the gels were swamped out. In the later stages of lysis, however, the covering effects of hemoglobin were reduced and some of the same bands could be observed that are characteristic of Proteins I (as shown in Fig. 1). It is not entirely clear whether these bands represent Proteins I normally present in the cytoplasm or Proteins I that were associated with the membrane but were dissolved during the ghosting procedure. The fact that a continuous enrichment of non-hemoglobin protein to hemoglobin occurred from 1/30 in the first lysate to >10/1 in the last, and to 50/1 in the final ghosts, suggests the latter explanation to be at least partly true. On the other hand, Proteins I may be present in both the solution phase and the membrane, perhaps in some sort of equilibrium.

Proteins I are apparently held in the membrane by simple ionic bonds that can be broken rapidly at very low ionic strength at neutral pH, and more slowly at higher ionic strength (with a minimum at 20 mM). Presumably, at very low ionic strenth, the repulsive forces between like-charged groups of the proteins are maximized. The effects of ionic strength may also result from changes in surface pH due to the Donnan effect. The pH certainly has a large effect on the rate of solubilization (Fig. 2), presumably due to associations and dissociations of the ionic groups contributing to attractions and repulsions.

Protein II has entirely different properties than Proteins I. Ionic bonding is also presumably involved in its attachment to the membrane, but the bonding forces are greater. The protein dissolves much more slowly and

only in the virtual absence of salt and at higher pH. Chelation by Ca^{++} may also be important because small concentrations of the cation prevent solubilization. The fraction is predominantly homogeneous in molecular weight at about 150,000, but it tends to form higher molecular weight aggregates as the ionic strength is raised (unlike Proteins I which do not aggregate at high ionic strength). From its solubilization properties, its behavior, and its molecular weight, Protein II is undoubtedly identical to the protein spectrin which has already been characterized in some detail [24, 27, 48, 49] and is probably identical with the protein fractions identified as a Ca^{++}-activated ATPase [43], and a protein reported to form large aggregates [15, 16]. In fact, the only other water-soluble proteins (Proteins I) contain no protein with any of the prerequisite properties.

The solubilization of proteins is accompanied by major structural changes. Under conditions for extraction of Proteins I, long tubular processes about 0.2 μ in diameter, are extruded from the ghosts, as described by Baker [4]. These processes apparently are pinched off or fragmented because, as the extraction proceeds, the ghost residues become smaller in size. After extraction with water to remove Protein II, only membranous vesicles less than 0.5 μ and fragments are observed, as reported by others [24, 43]. It is of some interest that the surface plaques present in ghosts [17] are still visible in most of the fragments (Fig. 5a). Thus the plaques are part of the lipoprotein residue and do not contain Proteins I and II.

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