BBA 75770

STUDIES ON THE SUCCINYLATION OF ERYTHROCYTE MEMBRANES

CHARLES F. MOLDOW, DOROTHEA ZUCKER-FRANKLIN, ADRIENNE GORDON, VERNE HOSPELHORN AND ROBERT SILBER

Department of Medicine, New York University School of Medicine, New York, N.Y. 10016, Department of Biological Chemistry, Massachusetts General Hospital, Boston, Mass. 02114, and Will Rogers Hospital, Saranac Lake, N.Y. 12983 (U.S.A.)

(Received July 12th, 1971)

SUMMARY

A method is described for the solubilization of the erythrocyte membrane by means of succinylation. In this procedure lipids and proteins are solubilized into an aqueous phase. The physical properties of this preparation were studied by optical rotatory dispersion, circular dichroism and infrared spectroscopy. The results indicate that succinylation is associated with a decrease in ordered structure of the protein. After succinylation the soluble membrane proteins are still loosely associated with membrane lipids and retain the ABO blood group antigens and phytohemagglutinin binding activity. The membrane proteins and lipids can then be separated by centrifugation, gel filtration or polyacrylamide gel electrophoresis.

INTRODUCTION

Erythrocyte ghosts, which can be prepared with minimal cytoplasmic contamination[1,2], have been intensively studied with the ultimate goal of ascertaining the molecular organization of the component protein, lipid and carbohydrate moieties. In general, two approaches have been used to study this system. In the first, attempts are made to solubilize the entire membrane and then study the components of these preparations. Reports from several laboratories[2-7] show the erythrocyte membrane to consist of many groups of heterogeneous proteins. A second type of methodology differs in that a single membrane constituent is isolated and its characteristics defined. By the introduction of new solubilization techniques, MARCHESI *et al.*[8,9] have recently purified two major membrane proteins to apparent homogeneity.

Following the first approach cited above, the work reported here describes the solubilization of intact erythrocyte ghosts into an aqueous phase by succinylation. The proteins are partially characterized prior to and following dissociation from the lipids, and their heterogeneity confirmed. The data permit speculation on the effect of succinylation on the configuration of membrane proteins and the possible implications of this procedure on protein–lipid interaction in the erythrocyte membrane.

MATERIALS AND METHODS

All chemicals used were reagent grade. Succinic anhydride was obtained from Sigma, and urea was recrystallized from 70 % ethanol. Trypsin (bovine pancreatic)

and neuraminidase (*Clostridium perfringens*) were obtained from Sigma Chemical. Pronase (*Streptomyces griseus*) was purchased from Calbiochem and β-galactosidase (*Escherichia coli*) from Worthington Biochemicals. [4-^{14}C]Cholesterol (150 μC/mg) was obtained from New England Nuclear.

Protein was determined with bovine serum albumin as a standard[10]. Free amino groups were quantitated with ninhydrin[11]. Cholesterol was assayed by the method of ZLATKIS *et al.*[12]. Reducing sugars were quantitated by the method of DUBOIS *et al.*[13] with galactose as a standard. Sialic acid was determined according to WARREN[14]. ATPase was measured by the method of POST *et al.*[15]. Cholinesterase was determined according to ELLMAN *et al.*[16]. Following lipid extraction and acid hydrolysis[17], inorganic phosphorous was determined by the method of BARTLETT[18].

Solubilization procedure

Erythrocytes were obtained from fresh human blood anticoagulated with acid citrate dextrose. In some experiments, ghosts were prepared from the erythrocytes of rats that had been fed 20 μC ring labelled [^{14}C]cholesterol by stomach tube 18–24 h earlier. The cells were washed 3 times with 310 mosM Tris–HCl buffer, pH 7.4. The upper layer of red cells was discarded at each washing to insure removal of the buffy coat.

Ghosts were prepared by a minor modification of the method of DODGE *et al.*[1]. The washed, packed erythrocytes were suspended in 20 vol. of cold 15 mosM Tris–HCl buffer, pH 7.4, for 15 min and mixed with a magnetic stirrer. This hemolysate was centrifuged at 13000 × *g* in a Sorvall Centrifuge Model RC-2 for 40 min. The residue was resuspended in the same volume of buffer and washed at least three times or until the ghosts were creamy white. All procedures were performed at 4°.

The ghost suspension was then mixed with an equal volume of 8 M urea. Although succinylation proceeded in the absence of urea, the total amount of membrane protein that could be solubilized without urea was diminished by about 20 %. Solid succinic anhydride (2 mg per mg of ghost protein) was added with stirring at room temperature, as described by KLOTZ[19]. The pH was maintained between 8 and 9 by the gradual addition of 3 M NaOH. Succinylation of available amino groups was judged to be complete when ninhydrin reactive material was no longer demonstrable. The reaction mixture was then dialyzed for 48 h against several changes of water or buffer. After dialysis, the material was passed through a 0.45-μm Millipore filter; this filtrate is henceforth referred to as solubilized membrane. The solubilization and subsequent fractionation were monitored at each step with electron microscopy.

Electron microscopy

Precipitated ghosts (or pellets) were fixed in 3 % glutaraldehyde[20] and 2 % OsO_4, dehydrated in increasing concentrations of alcohol and propylene oxide, and embedded in Epon 812[21]. Thin sections were cut with an LKB ultratome and contrasted with uranyl acetate[22] and $Pb(OH)_2$[23]. Negative staining was accomplished by allowing a drop of the suspension to dry on a grid and subsequently contrasting it with 1 % phosphotungstic acid at pH 5.5, or by mixing the suspension with an equal volume of 2 % phosphotungstic acid and subsequent application to a carbon-coated Formvar covered grid. The specimens were viewed with a Siemens Elmiskop I electron micro-

scope with accelerating voltage of 60 kV at original magnifications ranging from 8000–40000.

Amino acid analysis

Material was hydrolyzed in evacuated sealed glass tubes with 6 M HCl at 110° for 18 h. The hydrolysate was analyzed in a Spinco model 120 C with a range expander or a Technicon amino acid analyzer[24].

Column chromatography

After dialysis against 0.15 M NaCl, adjusted to pH 7.0 with Tris–HCl buffer, the soluble membrane was chromatographed on Sephadex G-200, Sepharose 6B or 2B in 0.01 M Tris. The Sephadex G-200 was allowed to swell for three days and "fines" were removed by decanting before use. In some experiments, samples were incubated with 3 % sodium dodecylsulfate under sterile conditions for 24 h at 37° prior to chromatography in this detergent. Blue dextran (Pharmacia) and NaCl were used to measure the void volume (V_0) and total column volume (V_t), respectively.

Polyacrylamide gel electrophoresis

Gels prepared according to the method of ORNSTEIN[25] were run at pH 8.7 without urea; samples were applied directly in 25 % glycerol to 7.5 % gels. In the most frequently used system, acrylamide gels (7.5 %) in 0.1 % sodium dodecyl sulfate were prepared by the method of VINUELA *et al.*[26]. Samples after incubation in 1 % sodium dodecyl sulfate for 24 h at 37° were applied without stacking gels in 25 % glycerol. Electrophoresis was performed in sodium phosphate buffer (0.01 M, pH 7.2) with 0.1 % sodium dodecyl sulfate at room temperature for 2.5 h at 3 V/cm per gel with recirculation of the buffer. Following electrophoresis, the gels were fixed overnight in 20 % sulfosalicylic acid, stained with 0.2 % Coomassie blue for 2 h, and destained with 7 % acetic acid.

ORD and CD

ORD and CD spectra were determined with a Cary 6001 spectropolarimeter using 1-cm cells and protein concentrations of approx. 200 μg/ml[27]. Baseline buffer contained 0.15 M NaCl. Infrared spectroscopy was done on a Perkin–Elmer Model 221 infrared spectrophotometer on dried film samples[28].

Phytohemagglutinin binding

Purified phytohemagglutinin (lot No. 1553), generously donated by Burroughs Wellcome, was iodinated with carrier free $Na^{125}I$ according to MCCONAHEY AND DIXON[29] and further purified for erythroagglutinin activity by passage through Sephadex G-200. A specific activity of 1 μC/μg protein was achieved. Sucrose density gradient ultracentrifugation was performed at 4° with a swinging bucket rotor in a Spinco Model L-2 ultracentrifuge. Gradients were from 5–20 % sucrose in 0.15 M NaCl.

Hemagglutinin inhibition studies were done with Difco purified phytohemagglutinin prepared daily by a 1:20 dilution with 0.15 M saline. The hemagglutinin inhibition studies were performed as follows: Each assay tube contained 0.15 M NaCl, 0.1 ml purified phytohemagglutinin, and a dilution of the test sample in 0.1 ml. This

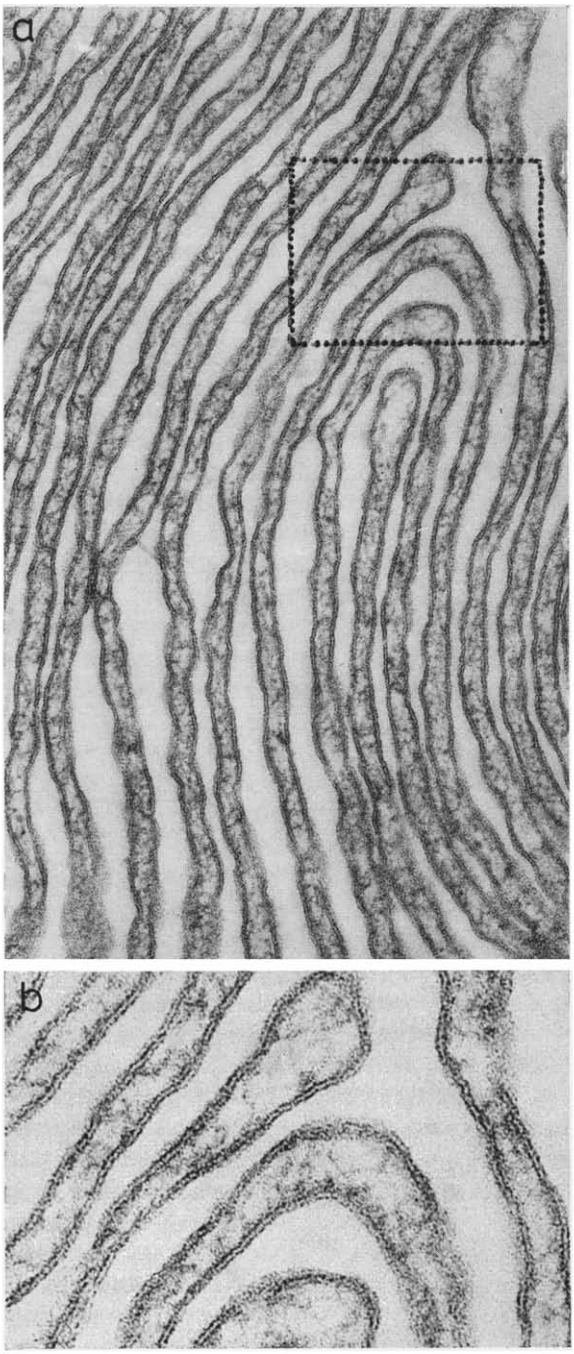

Fig. 1. Erythrocyte ghosts showing empty membrane sacs. Fibrillar material is attached to their inner surface. × 88000. The inset is illustrated at higher magnification on bottom (b) to show trilaminar structure to better advantage. × 180000.

was incubated for 10 min at room temperature and 0.1 ml of a 1 % solution of washed type O erythrocytes was added. This mixture was centrifuged at 100 × *g* for 30 sec and resuspended with gentle agitation. Units of activity expressed per mg of protein are defined as the reciprocal of the highest dilution of material which produced hemagglutination inhibition. A similar test system employing antisera and the appropriate erythrocyte suspension was used to ascertain the ABO and Rh activity of the succinylated membrane.

RESULTS

In thin section prior to succinylation the intact ghosts consisted essentially of empty sacs of unit membranes[30], except for some filamentous material attached to their inner surface which has been described in detail elsewhere[8]. Wherever these membranes were sectioned perpendicular to their surface, their trilamellar structure was readily apparent (Fig. 1a).

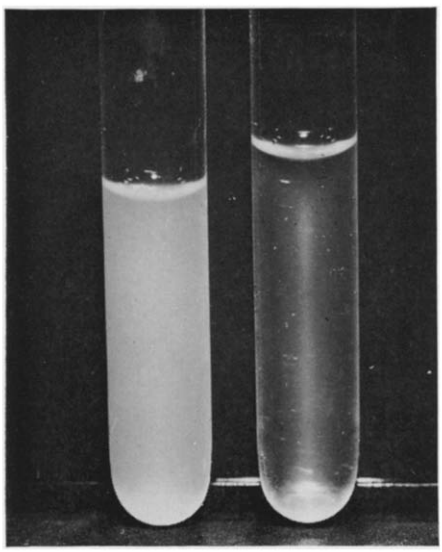

Fig. 2. Comparison of ghost suspension with succinylated membrane preparation. Ghosts shown on left; succinylated membrane shown on right.

TABLE I

COMPOSITION OF GHOSTS AND SOLUBILIZED MEMBRANE

	Ghost (mg)	*"Soluble" membrane (mg)*	*% of total in ghost solubilized (range)*	*% of solubilized membrane in 100000 × g supernatant (range)*
Protein	2.90	2.62	90 (75–105)	88 (67–95)
Cholesterol	0.72	0.62	86 (85–100)	4 (0–5)
Phospholipid	1.63	1.34	82 (70–95)	13 (0–15)
Neutral sugar	0.38	0.38	100 (75–105)	33 (30–55)
Sialic acid	0.05	0.05	100 (75–100)	50 (45–70)

Solubilization

During succinylation, the milky white ghost suspension became clear (Fig. 2). Representative data shown in Table I indicate that after dialysis and filtration through a 0.45-μm Millipore filter the relative concentrations of protein, lipid and carbohydrate in the succinylated membrane were similar to those in the original material. Usually 90 % of the ghost protein was present in this filtrate (range 75–100 %). The negatively stained solubilized membranes are illustrated in Fig. 3.

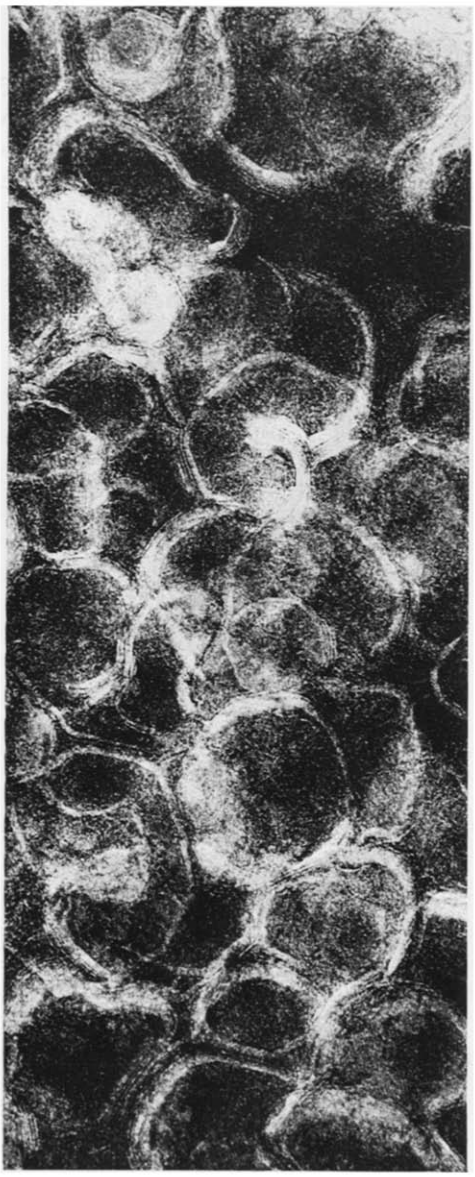

Fig. 3. Negative stained preparation of succinylated membranes also showing micellar structures. × 78000.

These preparations showed typical micellar structures. Data will be presented to support the concept that these microvesicles represent sedimentable lipoprotein aggregates. The myelin figures in this electron micrograph may have been produced either by the ultracentrifugation or during preparation of the sample for electron microscopy.

When the solubilized membrane was centrifuged for 12 h at 100000 × *g*, a pellet formed. The supernatant fluid contained from 67–95 % of the original protein. This fraction also contained 30–55 % of the total hexose and about 50 % of the sialic acid. Electron micrographs of negatively stained supernate preparations showed no discernible structure. There was complete loss of the microvesicles seen in the soluble membrane.

TABLE II

AMINO ACID COMPOSITION OF ERYTHROCYTE GHOSTS, SOLUBILIZED MEMBRANE AND FRACTIONS AFTER ULTRACENTRIFUGATION

Hydrolysis of similar samples for 18, 45 and 72 h gave 5% losses for serine and threonine. The values given are not corrected.

Amino acid	*Residues per 1000 residues**			
	Ghost	*Solubilized membrane*	*100000 × g supernatant*	*100000 × g pellet*
Aspartic acid	86	89	94	74
Threonine	57	58	59	57
Serine	81**	83**	73	124**
Glutamic acid	136	141	153	96
Proline	52	55	52	54
Glycine	71	69	66	80
Alanine	83	83	85	80
Half-cystine***	6.5	3.4	6.4	7.9
Valine	59	60	61	65
Methionine	20.1	20.5	18.3	15.7
Isoleucine	39.6	39.8	38.4	49
Leucine	114	113	110	121
Tyrosine	23.7	23.9	23.8	22
Phenylalanine	42	41	37.5	54
Lysine	55	52	60	49
Histidine	26.6	26.8	27.9	20.5
Arginine	50	55	52	47

* These compositions do not include tryptophan.
** Serine values are high because of presence of phosphatidyl serine in these fractions.
*** Half-cystine concentration is low since protein was not peroxidized with performic acid.

Characterization of the solubilized proteins

Amino acid analysis

The amino acid analyses of the ghost, solubilized membrane, and the 100000 × *g* supernatant were similar (Table II). The composition of the pellet will be discussed below.

ORD, CD and infrared spectra

ORD and CD spectra on the solubilized membrane (Fig. 4) revealed several differences from the pattern obtained with intact erythrocyte ghosts. The ORD trough shifts from 236 nm, the value for ghosts to 233 nm. The ORD crossover at

215 nm is considerably shifted from the value of 226.5 nm obtained with ghosts[27]. The CD spectrum reveals a prominent peak at 206 nm and a crossover at 199 nm, both of which are significantly different from the pattern in intact ghosts. The ORD and CD spectra of the 100 000 × *g* supernatant was similar to that obtained with solubilized membrane. It was not possible to resuspend the pellet in a fashion that would allow meaningful ORD, CD or infrared studies.

The presence or absence of urea during the succinylation process had no influence on the position of ORD and CD troughs. Infrared spectroscopy revealed a significant shoulder at 1635 cm^{-1}, characteristic of the β- or pleated sheet configuration[31], which is not found in spectra of whole ghosts.

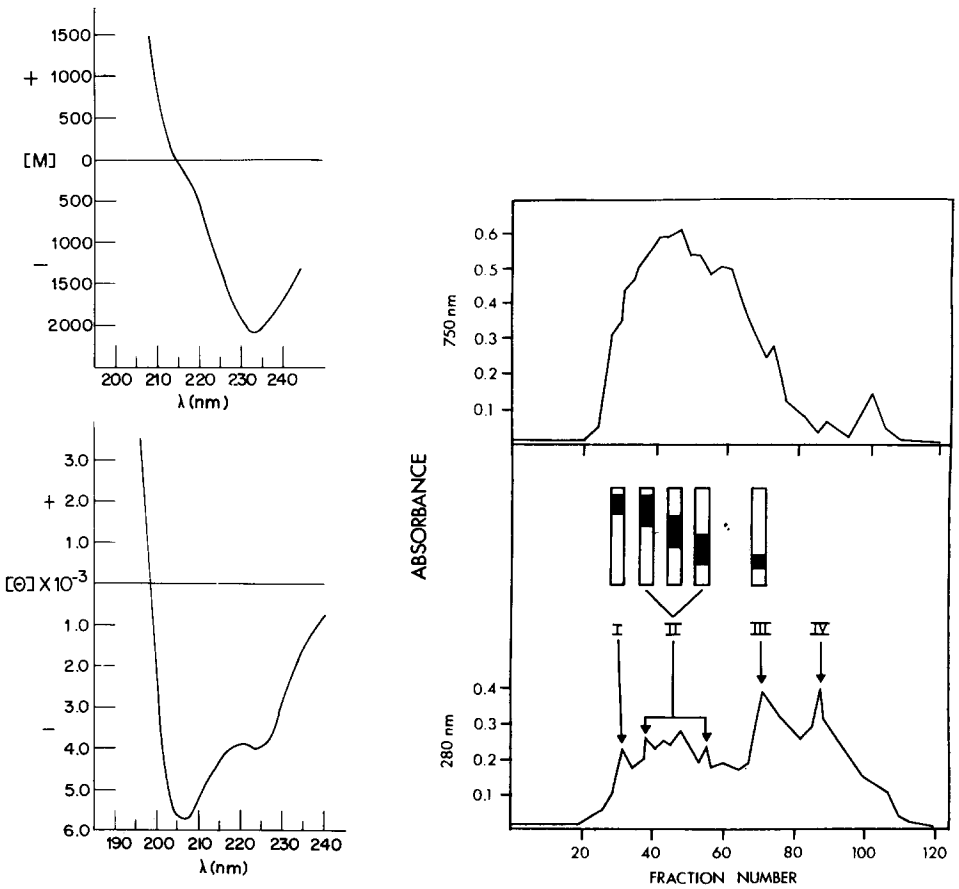

Fig. 4. Experimental ORD and CD spectra of solubilized membrane. Solvent 1 mM Tris–HCl, pH 7.4.

Fig. 5. Gel filtration of the solubilized membrane in the presence of sodium dodecyl sulfate. Solubilized membrane material was incubated in 3% sodium dodecyl sulfate for 24 h at 37°. Sepharose 6B column (70 cm × 2.5 cm) was eluted with unbuffered 3% sodium dodecyl sulfate at a flow rate of 15–20 ml/h, 4-ml fractions were collected. Upper panel: protein pattern. Lower panel depicts the gel electrophoresis patterns and absorbance at 280 nm.

Acrylamide gel electrophoresis

When the whole ghosts solubilized with 1 % sodium dodecyl sulfate were electrophoresed in the presence of 0.1 % sodium dodecyl sulfate, many bands, including an atypically staining band migrating in front of the tracking dye were observed. These findings were in confirmation of previous reports[7,32]. The results of one experiment using rat erythrocytes labeled with [^{14}C]cholesterol, indicate this leading band contains the label[33]. This band was not detectable in the 100000 × *g* supernatant. With Tris–glycine buffer (pH 8.7) in the absence of sodium dodecyl sulfate, succinylated ghosts migrated as a single band with a large amount of material remaining at the origin[34].

Gel filtration studies

Chromatography of the solubilized membrane on Sephadex G-200 and Sepharose 2B in the absence of sodium dodecyl sulfate revealed a sharp single peak emerging with the void volume. This same material equilibrated with 3 % sodium dodecyl sulfate and chromatographed on Sepharose 6B in the presence of this detergent, had markedly different elution pattern (Fig. 5). The first material (I) appeared with the void volume and was followed by a broad plateau (II) which was shown by gel electrophoresis to consist of a continuum of many bands presumably representing polypeptides of decreasing molecular weight as has been reported earlier[3]. Two sharper peaks (III and IV) which were eluted later had an absorbance at 280 nm

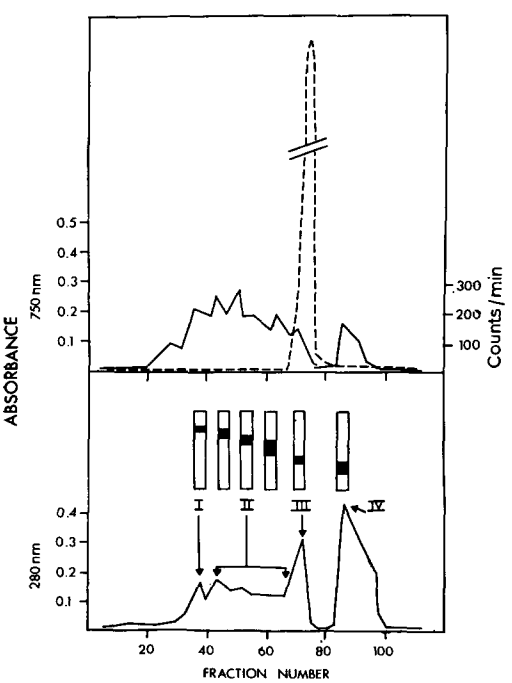

Fig. 6. Gel filtration of the rat erythrocyte solubilized membrane. Animals were fed [^{14}C]cholesterol 24 h before sacrifice. Erythrocytes were treated identically to the human material illustrated in Fig. 5. Upper panel: ———, protein; — — —, radioactivity. Lower panel depicts the gel electrophoresis patterns and absorbance at 280 nm.

greater than expected from their protein content; this may reflect light scattering due to the high lipid content of these peaks. Studies of succinylated rat erythrocyte membranes containing labeled cholesterol have a very similar elution profile (Fig. 6); Peak III contains all the radioactivity confirming the impression that this peak is lipid-rich.

Chromatography of the 100000 × *g* supernatant in the presence of 3 % sodium dodecyl sulfate on Sepharose 6B showed a pattern vaguely resembling that of the solubilized membrane (Fig. 7), however, there is better correlation between the absorbance at 280 nm and the protein content due to removal of the lipid during centrifugation.

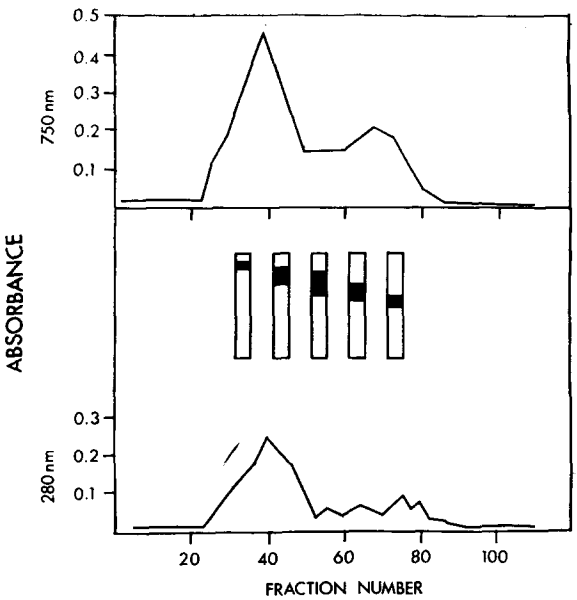

Fig. 7. Gel filtration of the 100000 × *g* supernatant chromatographed in the same manner as illustrated in Fig. 5.

Studies of the 100000 × g pellet

The pellet formed during ultracentrifugation contained practically all of the cholesterol and phospholipid (Table I). The protein, sialic acid and neutral sugar lost from the supernatant fluid was recovered in this pellet. When the pellet was washed with water, 50 % of the protein and all of the sialic acid were removed. The remainder of the protein was not removed from the pellet in the two additional washes. Succinylation of this protein fraction was complete as judged from the negative ninhydrin reaction. The pellet was resuspended in 3 % sodium dodecyl sulfate and chromatographed (Fig. 8). The elution pattern of the protein revealed a broad peak that did not correspond to the pattern obtained when absorbance at 280 nm was determined. Amino acid analysis of the proteins extracted from the washed pellet (Table II, column 4) clearly indicates decreased content of the acidic amino acids (glutamic and aspartic acid) while the concentration of some nonpolar amino acids such as glycine, phenylalanine and isoleucine was increased. A previous report[3] has noted variations

in amino acid composition of heterogeneous groups of membrane proteins. The lipid-associated pellet proteins may be related to Fraction IV of ROSENBERG AND GUIDOTTI[3].

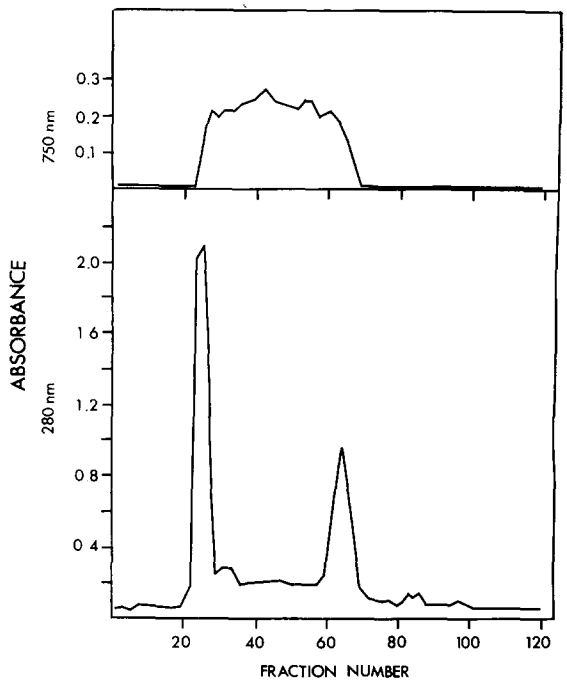

Fig. 8. Gel filtration of the 100000 × *g* pellet resuspended in 3% sodium dodecyl sulfate and incubated at 37° for 24 h. Column: Sepharose 6B (70 cm × 2.5 cm), elutant is unbuffered 3% sodium dodecyl sulfate, 4-ml fractions were collected. Upper panel: protein; lower panel represents absorbance at 280 nm.

On electron microscopic examination, intact membrane sacs were no longer found in this pellet (Fig. 9). However, fragments of variable length, devoid of any attached filamentous material still presented a trilaminar structure. Moreover, twisting of these short fragments resulted in frequent tangential sections of membrane sheets giving the impression that the bimolecular layer had been transected or "opened up" (arrow in Fig. 9).

Functional aspects of the solubilized membrane

The solubilized membrane lacked ATPase and cholinesterase activity and had no Rh blood group activity. The solubilized membrane and the 100000 × *g* supernatant fraction retained ABO specificity. Either preparation inhibited phytohemagglutinin-induced agglutination of erythrocytes. The binding of ^{125}I-labeled phytohemagglutinin to the succinylated membrane was demonstrated by two findings: (1) phytohemagglutinin was eluted sooner from a Sephadex G-200 column after preincubation of labeled phytohemagglutinin and solubilized membrane (Fig. 10); (2) the sedimentation velocity of ^{125}I-labeled phytohemagglutinin in a sucrose gradient

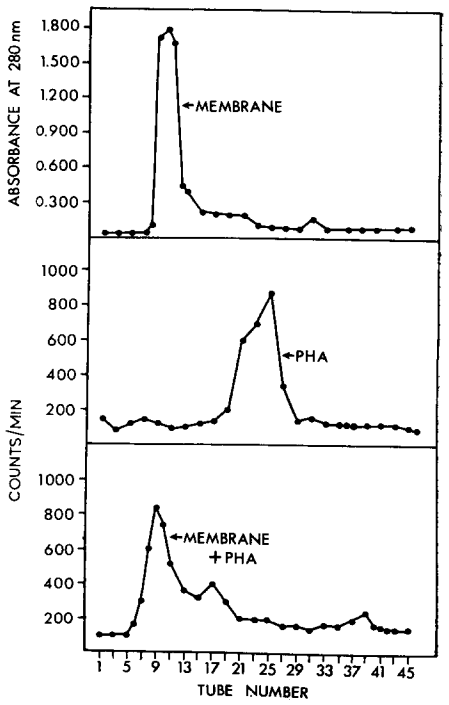

Fig. 9. Thin section obtained from pellet after succinylation of erythrocyte ghosts. Membranes are fragmented, devoid of fibrillar material and frequently twisted showing tangential sections at both ends (arrow). × 78000.

Fig. 10. Top: elution profile of solubilized membrane on a Sephadex G-200 (50 cm × 1 cm) column. Elutant was 0.01 M Tris–HCl, pH 7.4. The middle panel depicts the elution of [^{125}I]phytohemagglutinin under the same conditions, and the bottom panel shows the change in the migration of the phytohemagglutinin label after pre-incubation with solubilized membrane for 30 min at 37°. PHA = phytohemagglutinin.

TABLE III

EFFECT OF ENZYMATIC DEGRADATION ON PHYTOHEMAGGLUTININ BINDING SITE

All hemagglutinin inhibition tests performed at room temperature, incubations with pronase and trypsin were performed at pH 7.6, with incubation for 12 h at 37°, the activity of the enzymes after incubation was determined by their ability to release trichloroacetic acid soluble material from albumin. The sequential digestion with neuraminidase (pH 5.0) and β-galactosidase (pH 7.5) was done according to KORNFELD AND KORNFELD[35].

	Hemagglutinin inhibition units					
	No additive	*Pronase*	*Trypsin*	*Neuraminidase*	*β-Galactosidase*	*Neuraminidase followed by β-galactosidase*
Solubilized membrane	10	10	10	8.75	10	10
100000 × *g* supernatant (Erythrocyte membrane)	10	0	1.25	8.75	10	2.25
Trypsin digested ghosts	7.5	—	—	7.5	7.5	0
Succinylated bovine serum albumin	1.25	—	—	—	—	—

was increased in the presence of solubilized membrane when compared to control gradients containing phytohemagglutinin alone.

KORNFELD AND KORNFELD[35] have recently reported that sequential exposure to neuraminidase and β-galactosidase destroy a phytohemagglutinin-binding site in erythrocyte membranes. The effect of enzyme digestion on the phytohemagglutinin-inhibitory activity of solubilized membrane and 100000 × *g* supernatant is shown in Table III. Incubation of the 100000 × *g* supernatant with trypsin and pronase, destroyed the phytohemagglutinin binding activity. Sequential hydrolysis by neuraminidase and β-galactosidase also resulted in a marked diminution in the ability of the 100000 × *g* supernatant to inhibit phytohemagglutinin-induced hemagglutination. In contrast to this, the solubilized membrane, while a potent binder of phytohemagglutinin, was resistant to the action of trypsin, pronase, and the combined effect of neuraminidase and β-galactosidase. The possible significance of these differences is discussed below.

DISCUSSION

Succinylation of a lipid-free erythrocyte membrane preparation has been reported to be an effective method for the solubilization of membrane proteins[2]. In the experiments reported above, succinylation was found to be an effective means to solubilize whole erythrocyte ghosts. Succinic anhydride reacts with protein under mild conditions[19,36] introducing a negative charge by substituting the COO– group from the succinic anhydride for the ε-amino groups of lysine and other free amino groups. The resultant change in protein charge and/or configuration appears sufficient to cause breakdown of the membrane structure.

Electron microscopy of the soluble membrane revealed myelin forms. It is perhaps not surprising that this trilaminar ultrastructure was associated with the lipid. Similar trilaminar patterns have been demonstrated in lipid preparations which contained no protein at all and which were prepared by almost identical fixation and

embedding techniques[37]. Moreover, triple layered membranes have also been resolved in material from which all lipids had previously been removed with acetone[38]. These data and those of others[39] indicate that electron densities cannot yet be precisely correlated with chemical structures.

ORD, CD and infrared spectroscopy studies are compatible with a marked alteration in the configuration of the ghost proteins following succinylation. The positions of the ORD trough at 233 nm and the CD band at 223 nm of solubilized membrane are compatible with those of soluble proteins containing a measurable amount of α-helical confirmation. The CD band at 206 nm and crossover at 199 nm as well as the ORD crossover at 215 nm suggest that a significant amount of protein is in the beta conformation and that there is an increase in random coil. The shoulder observed at 1635 cm^{-1} with infrared spectroscopy further substantiates the presence of β-structure in the solubilized membrane. The increase in the β and random conformation are consistent with a diminution in α-helical structure as indicated by the decrease in magnitude of the mean residue ellipticity and mean residue rotation of solubilized membrane when compared with the values obtained with ghosts. Configurational changes similar to those observed have been produced in membrane exposed to extremes of pH[40]. However, in the present experiments pH was carefully controlled.

The observed changes could not be completely due to light scattering artifacts[41] since infrared spectrum of these solubilized membranes shows the presence of β conformation, a pattern not found in ghosts[31]. Moreover, there was no difference between the ORD and CD spectra of the solubilized membrane, which contains lipid and scatters light and that of the clear lipid-free 100000 $\times$ g supernatant.

It was anticipated that succinylated membrane proteins would have reduced affinity for the lipids[34]; ultracentrifugation resulted in this dissociation, but surprisingly the pellet formed during centrifugation proved to be rich in lipids and carbohydrates. The succinylated proteins in the supernate were virtually free of lipid.

In the procedure outlined above, the erythrocyte membrane proteins and lipids are solubilized in an aqueous environment. It seems probable that the lipids and proteins still remain associated in some fashion as suggested by their comigration during gel filtration in the absence of detergent. The observation that phytohemagglutinin binding activity of the solubilized membrane is unaffected by enzymatic digestion which destroys this site in the lipid-free 100000 $\times$ g supernatant, could be interpreted as indicating the association of lipid with the proteins making the glycoprotein site inaccessible to enzymatic inactivation. The loss of activity following proteolysis confirms the necessity of an intact protein backbone for full phytohemagglutinin binding as indicated by KORNFELD AND KORNFELD[42].

A limitation on the usefulness of solubilization of erythrocyte membranes by succinylation alone, is that this method leads to less dissociation of individual proteins than that achieved with sodium dodecyl sulfate, as judged by electrophoresis and gel filtration. Solubilization with sodium dodecyl sulfate, however, leads to a significant binding of the detergent to proteins, which cannot even be altered by extensive dialysis; this effect precludes many subsequent studies — antigen–antibody reactions would be affected and the hemolytic action of sodium dodecyl sulfate would prevent any of the many techniques involving hemagglutination. On the other hand, as illustrated above with the solubilization of the phytohemagglutinin-binding site and

ABO antigens, such studies can be performed after succinylation. The application of this technique to the solubilization of other antigens and virus receptor sites is currently under study.

The present work describes a method of solubilizing the erythrocyte membrane in an aqueous environment and investigatess the effect of this procedure on membrane conformation. Although the proteins remain associated with lipids after the initial solubilization, separation from lipids as well as further fractionation of proteins was achieved by ultracentrifugation.

ACKNOWLEDGMENT

We would like to acknowledge the expert technical assistance of Miss Rose Meyers. This investigation was supported in part by U.S. Public Health Service Research Grant No. CA 11655 and Hematology Training Grant No. AM 5282. Dr. Moldow is a postdoctoral fellow supported by U.S. Public Health Service Training Grant No. AM 5282. Dr. Zucker-Franklin is a U.S. Public Health Service Career Development Awardee. Dr. Silber is a Career Scientist of the Health Research Council of the City of New York.

REFERENCES

1 J. T. DODGE, C. MITCHELL AND D. J. HANAHAN, *Arch. Biochem. Biophys.*, 100 (1963) 119.
2 S. A. ROSENBERG AND G. GUIDOTTI, *J. Biol. Chem.*, 243 (1968) 1985.
3 S. A. ROSENBERG AND G. GUIDOTTI, *J. Biol. Chem.*, 244 (1969) 5118.
4 D. MAZZIA AND A. RUBY, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 1005.
5 A. M. MADDY, *Biochim. Biophys. Acta*, 117 (1966) 193.
6 R. F. A. ZWAAL AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 150 (1968) 323.
7 J. T. LENARD, *Biochemistry*, 9 (1970) 1129.
8 V. T. MARCHESI, E. STEERS, T. W. TILLACK AND S. L. MARCHESI, *The Red Cell Membrane, Structure and Function*, Lippincott, Philadelphia, Pa., 1969, p. 117.
9 V. T. MARCHESI, *Fed. Proc.*, 29 (1970) 600.
10 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
11 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
12 A. ZLATKIS, B. ZAK AND A. J. BOYLE, *J. Lab. Clin. Med.*, 41 (1953) 486.
13 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS AND F. SMITH, *Anal. Chem.*, 28 (1956) 350.
14 L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971.
15 R. L. POST, C. R. MERRITT, C. R. KINSDVING AND C. D. ALBRIGHT, *J. Biol. Chem.*, 235 (1960) 1796.
16 G. L. ELLMAN, K. D. COURTNEY, A. VALENTINO AND R. M. FEATHERSTONE, *Biochem. Pharmacol.*, 7 (1961) 88.
17 H. G. ROSE AND M. OKLANDER, *J. Lipid Res.*, 6 (1965) 428.
18 G. R. BARTLETT, *J. Biol. Chem.*, 234 (1959) 466.
19 I. M. KLOTZ, *Methods Enzymol.*, 11 (1967) 576.
20 D. D. SABATINI, K. BENSCH AND R. S. BARNETT, *J. Cell Biol.*, 17 (1963) 19.
21 J. H. LUFT, *J. Cell Biol.*, 9 (1961) 409.
22 M. L. WATSON, *J. Biophys. Biochem. Cytol.*, 4 (1958) 475.
23 G. MILLONIG, *J. Biophys. Biochem. Cytol.*, 11 (1954) 736.
24 S. MOORE AND W. H. STEIN, *Methods Enzymol.*, 6 (1963) 819.
25 L. ORNSTEIN, *Ann. N.Y. Acad. Sci.*, 121 (1964) 321.
26 E. VINUELA, I. D. ALGRANATI AND S. OCHOA, *Eur. J. Biochem.*, 1 (1967) 3.
27 A. S. GORDON, D. F. M. WALLACH AND J. H. STRAUS, *Biochim. Biophys. Acta*, 183 (1969) 405.
28 J. M. GRAHAM AND D. F. M. WALLACH, *Biochim. Biophys. Acta*, 193 (1969) 225.
29 P. J. MCCONAHEY AND F. I. DIXON, *Int. Arch. Allergy*, 29 (1966) 185.

30 J. D. ROBERTSON, *Biochem. Soc. Symp.*, 16 (1959) 3.
31 D. F. M. WALLACH AND P. H. ZAHLER, *Biochim. Biophys. Acta*, 150 (1968) 186.
32 M. T. LAICO, E. I. RUOSLAHTI, D. S. PAPERMASTER AND W. J. DREYER, *Proc. Natl. Acad. Sci. U.S.*, 67 (1970) 120.
33 K. L. GARAWAY AND D. KOBYLKA, *Arch. Biochem. Biophys.*, 219 (1970) 238.
34 P. E. BRAUN AND N. S. RADIN, *Biochemistry*, 8 (1969) 4310.
35 S. KORNFELD AND R. KORNFELD, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 1439.
36 D. M. MACLENNAN, A. TZAGOLOFF AND J. S. RISKE, *Arch. Biochem. Biophys.*, 109 (1965) 383.
37 F. A. HENN, G. L. DECKER, J. W. GREENAWALT AND T. E. THOMPSON, *J. Mol. Biol.*, 24 (1967) 51.
38 S. FLEISCHER, G. BRIERLY, M. KLOUWEN AND D. B. SLAUTTERBACK, *J. Biol. Chem.*, 237 (1962) 3264.
39 E. D. KORN, *Science*, 153 (1966) 1491.
40 D. F. M. WALLACH AND P. H. ZAHLER, *Biochim. Biophys. Acta*, 150 (1968) 186.
41 A. S. SCHNEIDER, M. J. SCHNEIDER AND K. ROSENHECK, *Proc. Natl. Acad. Sci. U.S.*, 66 (1970) 793.
42 S. KORNFELD AND R. KORNFELD, *J. Biol. Chem.*, 245 (1970) 2536.