BBA 75287

SULFANILIC ACID DIAZONIUM SALT: A LABEL FOR THE OUTSIDE OF THE HUMAN ERYTHROCYTE MEMBRANE

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(Received January 20th, 1969)

SUMMARY

1. The diazonium salt of [^{35}S]sulfanilic acid can be used as a label for outer components of the human erythrocyte membrane; the reagent does not penetrate intact cells.

2. Modified cells become permeable to Na^+ and K^+ but not to water-soluble nonelectrolytes. They eventually lyse in isotonic buffer.

3. About 20% of the label bound to intact cells can be recovered in an ethanol–ether membrane extract. Phospholipase D (cabbage) changes the way in which it partitions between ether and water.

4. If the residue left after ethanol–ether extraction is dissolved in 3% sodium dodecyl sulfate and sized by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate, a complex but reproducible pattern is observed on staining with Coomassie brilliant blue. The most intensely labeled material has a molecular weight of about 140000. Some peaks are free of label.

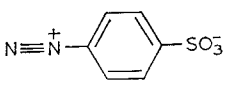
5. Similar patterns are obtained if intact membranes are dissolved in 3% sodium dodecyl sulfate; sodium dodecyl sulfate dissociates the protein and lipid as effectively as ethanol–ether.

6. If the residue left after ethanol–ether extraction is exposed first to 0.8 M NaCl, a medium in which it is largely insoluble, much of the protein shifts from high to low molecular weight. The most intensely labeled material does not. The membrane contains protein complexes which can be dissociated by sodium dodecyl sulfate after exposure to salt. The dissociation is less extensive when intact membranes are exposed to salt.

INTRODUCTION

The human erythrocyte membrane is readily permeable to lipid-soluble compounds, to very small water-soluble nonelectrolytes, and to inorganic anions; it is virtually impermeable to larger water-soluble molecules and to inorganic cations[1]. No systematic attempt has been made to determine the position within the membrane of the barrier or barriers responsible for this selectivity, or to determine which

structural components are located outside of the barrier(s), which are inside, and which go all the way through[2]. Our knowledge is limited mainly to the outermost surface of the cell, which has been exposed to antibodies and enzymes[3] and found to carry the blood group substances[4] and much of the sialic acid[5,6]. The permeability barriers in question may be inaccessible to most antibodies and enzymes and distant from the electrophoretic surface of shear. In this paper experiments are described in which human erythrocytes are treated with a small structural probe, the diazonium salt of sulfanilic acid:



This reagent forms azo, diazoamino, and *S*-azo or thio ether derivatives with proteins and lipids[7–10], but it does not go through the membrane; therefore, it can be used to identify components which are outside or partly outside the barrier which blocks its entry. The labeling reaction, its effects on permeability, and the fate of the label in different membrane fractions are described.

The emphasis of the work is on membrane protein as fractionated by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate. Sodium dodecyl sulfate is one of the few agents known to completely solubilize erythrocyte membranes[11,12] and their proteins[13]. Enough detergent is bound to a protein that its rate of migration through the gel depends on size rather than on native surface-charge[14], so modified and unmodified protein should move at the same rate (unless one is dissociated more than the other). This is not true for polyacrylamide- or starch-gel electrophoresis in the presence of urea, techniques which have been used extensively in studies of the erythrocyte[15–19].

The use of membrane-specific reagents has been discussed by MADDY[20], who treated ox erythrocytes with a fluorescent stilbene isothiocyanate. VAN STEVENINCK *et al.*[21] found that *p*-chloromercuribenzene sulfonic acid combined only with sulfhydryl groups on the outside of intact human erythrocytes. PRESSMAN *et al.*[22] prepared azo-erythrocytes, but they were not concerned with the permeability of the membrane. The present study is more in the spirit of that of PARDEE AND WATANABE[23], who have used the diazonium salt of 7-amino-1,3-naphthalene disulfonic acid to show that the sulfate-binding protein of *Salmonella typhimurium* is located outside the plasma membrane of that cell. It is not possible at this stage of the work to defend a particular structural model, but it is clear that the erythrocyte membrane is highly asymmetric.

METHODS

Reagents

Water was distilled, passed through a deionizer (Barnstead 8901) and dispersed from polyethylene. [^{32}S]Sulfanilic acid (Mallinckrodt reagent) was washed with dilute acetic acid and water and recryst. from boiling water. [^{35}S]Sulfanilic acid was obtained from the Radiochemical Centre, Amersham, England, at a specific activity of about 30 C/mole and diluted with the ^{32}S isotope as required. Sulfanilic acid diazonium salt was prepared immediately before use by the indirect method[9,10] and washed with a small amount of cold water. The diazonium salts of *p*-aminobenzoic

acid, *p*-nitroaniline, and *p*-iodoaniline (Eastman) were prepared by the direct method[9,10] with about a 10% molar excess of $NaNO_2$; *p*-(trimethylammonium)-benzenediazonium difluoroborate sodium fluoroborate[24] was the gift of Jean-Pierre Changeux, Institut Pasteur. Merck reagent urea was recryst. from 95% ethanol–0.1% ethylene diamine tetraacetic acid, erythritol was Calbiochem A grade, and sucrose was a Merck reagent. Sodium dodecyl sulfate (Fisher) was recryst. from 80% ethanol. ATP disodium salt was obtained from Sigma, and 2-mercaptoethanol was Sigma Type 1. Silicic acid was Fisher A-288; Hyflo Super Cel, Johns Manville. The phospholipid markers were L-α-cephalin dipalmitoyl (synthetic) CP (Mann), phosphatidyl-L-serine CP (Mann), sphingomyelin (beef brain) highly purified (Mann), and crude egg phospholipid (mostly phosphatidyl choline)[25]. Phospholipase D (EC 3.1.4.4, cabbage) was Calbiochem B grade. Acrylamide (Eastman X5521), *N*,*N*′-methylene-bisacrylamide (Eastman 8383), ethylene diacrylate (Borden 7529), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Eastman 8178), ammonium persulfate (Mallinckrodt), and Coomassie brilliant blue R-250 (Mann) were also used. The molecular-weight markers were fibrinogen Fraction 1 bovine (K and K 7211), bovine serum albumin Fraction V or cryst. (Mann 751 or 2506), ovalbumin 2 × cryst. (Mann 7338), and lysozyme cryst. (Armour 652). Liquifluor and hyamine hydroxide were obtained from Pilot Chemicals; the scintillators used in KINARD's[26] solution were from New England Nuclear.

Assays

Free amino groups were determined by the ninhydrin method of MOORE AND STEIN[27] and protein by the increment in free amino groups on hydrolysis in 2 M NaOH 2 h at 110°, assuming an amino acid molecular weight of 130 and ignoring the contribution from N-acetylated amino sugars. Total phosphorus was determined by the method of BARTLETT[28]. ^{35}S was assayed in a scintillation system or on planchettes in a gas-flow counter. Three scintillation media were used: toluene–Liquifluor (500:21, by vol.), 1,4-dioxane–anisole–1,2-dimethoxyethane–Liquifluor (18:3:3:1, by vol.) and KINARD's[26] solution.

Modification of intact cells

The reactions were run at room temperature on cells from human blood no more than 4 days old. Various blood types were used (A, B, AB and O). The buffy coat was removed by aspiration, and the cells were washed at 4° three times with 10 vol. of 0.9% NaCl and twice with 10 vol. of isotonic sodium phosphate ("phosphate buffer" (pH 7.5), containing in moles/l: Na^+, 185; K^+, 3.6; Cl^-, 97; HPO_4^{2-}, 42; and $H_2PO_4^-$, 8). The diazonium salts were added to the phosphate buffer at a concentration of 3 mM (assuming 100% yields in their preparation), and the pH was readjusted to 7.5. Some of the washed cells were set aside as a control ("control cells"). Those to be modified were warmed to room temperature and added to the reagent mixture. Suspensions of up to 50% (v/v) were used. In the usual case, the cells were agitated gently 20 min, then spun down and washed twice with 10 vol. of phosphate buffer at 4° ("modified cells").

Rate of evolution of acid

The reaction of sulfanilic acid diazonium salt was followed on a pH-stat in a buffer made by diluting 1 vol. of phosphate buffer with 49 vol. of 0.9% NaCl held at

pH 7.5 by the addition of 0.2 M NaOH. Reagent concentrations of 3 and 30 mM were used; the cell suspensions were 2% (v/v).

Effect of nitrite

Either $NaNO_2$ was added directly to a cell suspension or HONO was generated in a separate flask and bubbled through it in a stream of N_2. Concentrations of up to 50 mM were used.

Na^+ and K^+ permeability

Modified cells were suspended in phosphate buffer at room temperature. Aliquots were removed at different times and centrifuged 10 min at 1700 $\times$ *g*. The hemoglobin content of the supernatant fluid was determined from its absorbance at 540 mμ. The cells in the pellet were lysed in a known volume of water. The Na^+ and K^+ concentrations of the lysates were measured by flame photometry. Control cells were treated in the same way.

Permeability to water-soluble nonelectrolytes

Modified and control cells were used. Identical aliquots were transferred from stirred suspensions into tared tubes. The tubes were spun 5 min at 1700 $\times$ *g*, and the supernatant fluids were discarded. The pellets were resuspended in phosphate buffer, in phosphate buffer containing 0.4 M urea, in phosphate buffer containing 0.4 M erythritol, and in phosphate buffer containing 0.4 M sucrose, respectively. The tubes were spun 15 min at 1700 $\times$ *g*, the supernatant fluids were carefully removed, and the tubes were weighed.

Reaction with hemoglobin in broken and intact cells

A 20% cell suspension was divided into two parts. The cells in one part were broken ultrasonically. Enough [^{35}S]sulfanilic acid diazonium salt was added to each to make the extracellular reagent concentration 3 mM. After 20 min, the intact cells were spun down at 5000 $\times$ *g*, washed 3 times in 10 vol. of phosphate buffer, and lysed in 20 vol. of water. The sonicated suspension was diluted with water to the same volume, and both suspensions were centrifuged 40 min at 15000 $\times$ *g*. Samples from the top of the supernatant fluids were dialyzed exhaustively against phosphate buffer–water (1:20, by vol.) at 4°. Part of the dialyzed hemoglobin was purified by passage through a 45 cm $\times$ 2.5 cm Bio-Gel P-150 column. Heme was removed from all samples by the acid–acetone method of ANSON AND MIRSKY[29], and equal weights of globin were hydrolyzed in hot 6 M HCl, dried and counted on planchettes in a gas-flow counter.

Isolation of ghosts

Membranes were isolated at 4° from control and modified cells by the method of DODGE *et al.*[30] as applied by ROSENBERG AND GUIDOTTI[13]. The cells were washed first with isotonic Tris–HCl (pH 7.5). The centrifugation was done in polypropylene bottles. The final pellets contained intact ghosts of uniform size as judged by phase-contrast microscopy. Ghosts to be assayed without further extraction were frozen and stored at −23°.

Ethanol–ether extraction

Fresh erythrocyte ghosts were extracted with ethanol–ether and ether by the method of ROSENBERG AND GUIDOTTI[13]; the ether extractions were done at room temperature for only 24 h. The pooled ethanol–ether extracts were evaporated at room temperature in a flash evaporator and stored under N_2 at −23°. The protein (glycoprotein) residue was dried in air or in a vacuum desiccator and stored at −23°. The ether was evaporated, but the amount of residue was negligible (less than 1% of the protein by weight, free of label).

Chromatography of lipid

Neutral lipids and phospholipids were chromatographed on silicic acid and Hyflo Super Cel by the methods of WAYS AND HANAHAN[31]. Thin-layer chromatography was done on Eastman K301R2 Chromatogram sheet activated 35 min at 110° and eluted with chloroform–methanol–7 M aq. ammonia (46:18:3, by vol.)[32]. A number of phospholipid markers were used (see *Reagents*). The sheets were sprayed for amino groups and phosphorus[25], and the silica gel was scraped off and counted in toluene–Liquifluor.

Enzymatic hydrolysis of lipid

5-mg samples of the ethanol–ether extract were taken up in 5 ml of ether and shaken at room temperature 2.5 h with 5 ml of 0.1 M sodium acetate–0.01 M $CaCl_2$ (pH 5.6) containing either 10 mg of phospholipase D or 10 mg of phospholipase D inactivated by boiling. The reaction was stopped by the addition of 0.5 ml of 3 M trichloroacetic acid. The ether layer was removed, and the aqueous layer and a cloudy interfacial layer were extracted with 10 ml of ether three times. The NaCl concentration was raised to 0.5 M, and the solid material was centrifuged down and extracted with 2 ml of methanol. Samples of the ether, aqueous, and methanol fractions were counted on planchettes.

Exposure of the protein residue to salt

5-mg samples of the protein residue were powdered and tumbled slowly 27 h at room temperature with 0.5 ml of 0.8 M NaCl–15 mosM Tris–HCl (pH 7.5). The supernatant fluids were dialyzed 16 h at room temperature against 125 ml of water. The precipitates were dissolved in 3% sodium dodecyl sulfate and the contents of the dialysis sacs were made 3% sodium dodecyl sulfate by addition of solid detergent. Both were subjected to gel electrophoresis.

Exposure of intact ghosts to salt or to ATP–2-mercaptoethanol

Control-ghost suspensions were exposed to unbuffered 1 M NaCl by the method of MITCHELL AND HANAHAN[33] and to 0.3 mM ATP–50 mM 2-mercaptoethanol (pH 7.5) by the procedure of MARCHESI AND STEERS[19]. The pellets were ethanol–ether extracted, the supernatant fluids were dried and ethanol–ether extracted, and the residues were subjected to gel electrophoresis. The salt-supernatant residue was dialyzed against water and freeze-dried before being dissolved in sodium dodecyl sulfate; it was the only sample which did not dissolve completely.

Gel electrophoresis of ghosts and ghost protein

The method was adapted from VIÑUELA *et al.*[34]. The gels contained 5.0% (w/v) acrylamide and either 0.135% (w/v) *N,N'*-methylenebisacrylamide or 0.135 (v/v) ethylene diacrylate; the amount of glycerol in the sample buffer was reduced to 10%. Ghosts or ghost–protein samples were dissolved at room temperature in 3% (w/v) sodium dodecyl sulfate at a protein concentration of about 10 mg/ml; 4–50 μl of these solutions were mixed with 100 μl of the sample buffer and applied to the gels. The gels were run at 8 mA/tube 3–4 h, stained overnight in 0.25% (w/v) Coomassie brilliant blue in methanol–acetic acid–water (5:1:5, by vol.), and destained electrophoretically in methanol–acetic acid–water (2:3:35, by vol.). They were scanned at 515 or 550 mμ past a 1 mm $\times$ 0.1 mm slit mounted between the monochrometer and photomultiplier units of an Hitachi–Perkin–Elmer 139 Spectrophotometer at the rate of 0.085 mm/sec, and a signal proportional to absorbance was written out on a Bausch and Lomb VOM-5 recorder. Radioactive gels were scanned and frozen, sliced with a stack of razor blades, and counted by the method of VIÑUELA *et al.*[34].

A number of control experiments were run and scanned (not counted). In one, the 2-mercaptoethanol was omitted from the sample buffer. In another, samples were heated at 65° for 10 min in the sample buffer (with and without 2-mercaptoethanol) before being applied to the gels. In a third, the sodium dodecyl sulfate concentration was increased to 1%. In a fourth, *N,N'*-methylenebisacrylamide gels were polymerized, removed from their tubes, soaked 3 weeks in several changes of reservoir buffer and drawn back into the tubes before the protein was applied. In a fifth, a "run-true" experiment, two *N,N'*-methylenebisacrylamide gels were run, each with about 140 μg of protein; one was stained, destained and scanned; the other was frozen, cut into 7 pieces and thawed. The pieces were drawn into the ends of 7 gel-tubes. *N,N'*-Methylenebisacrylamide gels were polymerized in the empty sections, and the current was turned on so that the protein could continue to migrate in the direction it had before. The gels were stained, destained and compared with the copy of the original.

RESULTS AND DISCUSSION

None of the results depended on blood type, as far as I am aware. When a dilute suspension of cells was treated with 3 mM sulfanilic acid diazonium salt, no changes were apparent for more than an hour. Then, over a period of a few minutes, the suspension cleared and turned a deep reddish brown; the cells lysed, and the reagent combined with hemoglobin. In 50% suspensions this transition was much more gradual, because the cells were not able to swell initially to more than twice their original volume. The *p*-carboxy and *p*-trimethylammonium benzenediazonium salts behaved in the same way, but cells exposed to the *p*-nitro or *p*-iodo derivatives turned brown immediately. They did not lyse, even when added to water. These reagents penetrated the membranes and precipitated the hemoglobin; the precipitates could be seen inside the cells by ordinary light microscopy.

Rate of evolution of acid

Curves A and B in Fig. 1 are recordings from experiments in which cells were treated with 30 mM and 3 mM sulfanilic acid diazonium salt, respectively. The cells

were added to the reagent–buffer mixture at time 0. The reaction ran at a constant rate for about 23 min in A and 60 min in B, at which times it accelerated. These times coincided with the appearance of extracellular hemoglobin, as judged by the color of the supernatant fluids. In A, enough reagent combined with this hemoglobin to drive the pH-stat off-scale. In B, the reagent was exhausted, and the curve leveled off. Addition of more reagent in B caused the curve to move steeply upwards (not shown in the figure): hemoglobin was present in excess.

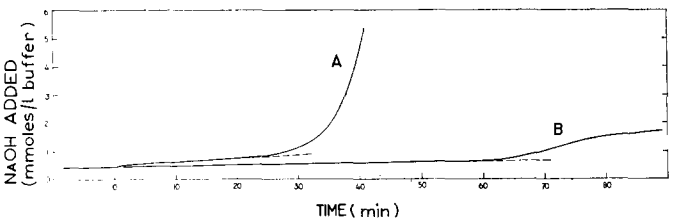


Fig. 1. The amount of base required to hold the pH of a 2% (v/v) suspension of cells at 7.5 when the supernatant fluid contained sulfanilic acid diazonium salt at a concentration of about 30 mM (A) or about 3 mM (B). The cells were added at time 0. The decomposition of the reagent at a concentration of 30 mM is shown between −9 and 0 min.

Effect of nitrite

Cells exposed to nitrite turned brown but did not lyse. They were as stable in isotonic buffer as control cells, and they lysed as rapidly in water. The effects observed with diazonium salts prepared by the direct method were not due to excess nitrite.

Na^+ and K^+ permeability

The cells lost K^+ and gained Na^+ before they lysed, Fig. 2. The change in salt composition occurred quite rapidly. The half-time for K^+ and Na^+ exchange in a normal cell or in one treated with a metabolic poison is about 36 h[1]. In the modified cells it was no more than a few minutes. Modified cells swell and lyse because they are unable to maintain the intracellular salt concentration required to offset the osmotic pressure due to hemoglobin. A similar situation was encountered in a study of 1-fluoro-2,4-dinitrobenzene, a reagent which goes through the membrane[35]. It was argued there[35] that the change in salt permeability might be due to a reduction in membrane charge. Exchange of K^+ and Na^+ prior to lysis also occurred with the trimethylammonium compound, a molecule which increases the total membrane charge; therefore, the reactivity of the diazonium group appears to be more important than the net charge on the molecule.

Permeability to water-soluble nonelectrolytes

The shrinkage of control and modified cells in hypertonic solutions of urea, erythritol, and sucrose is shown in Fig. 3. The osmotic responses of modified cells to urea, erythritol, and sucrose were not significantly different from those of control cells. Both kinds of cells were permeable to urea. They shrank in erythritol only slightly less than in sucrose. If the reagent makes holes in the membrane, the holes are too small to be used by erythritol.

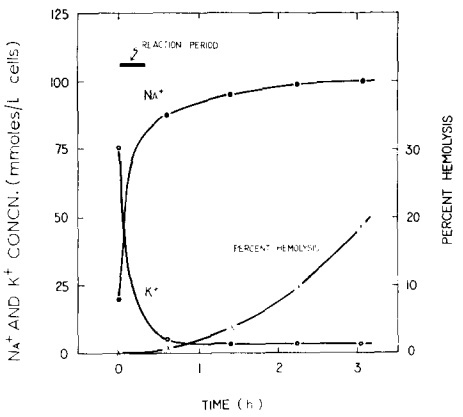


Fig. 2. The Na^+ and K^+ concentration and the percent hemolysis of cells treated 20 min with 3 mM sulfanilic acid diazonium salt.

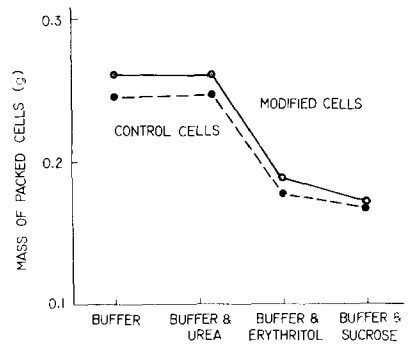


Fig. 3. The mass of equal numbers of packed cells after suspension in buffer or in buffer containing 0.4 M urea, erythritol or sucrose.

Reaction with hemoglobin in broken and intact cells

The amount of label associated with hemoglobin from intact cells was less than 0.2% of that from broken cells; the result was the same whether or not the samples were purified on Bio-Gel P-150 (background 9.3 $\pm$ 0.02 counts/min; intact-cell hemoglobin purified 11.1 $\pm$ 0.02 counts/min, unpurified 10.8 $\pm$ 0.02 counts/min; broken-cell hemoglobin 1072 counts/min). From the pH-stat experiments, Fig. 1, it is evident that there is more than enough hemoglobin in a 2% cell suspension to combine with all the reagent in a 6 mM solution in less than 20 min. Since the hemoglobin labeling experiment was done with a 20% cell suspension, it follows that the rate of reaction in intact cells was at most 10^{-5} of that in broken cells.

Composition of extracts

The ethanol–ether extracts were about 1% protein by weight, the protein (glycoprotein) residues about 0.15% phosphorus by weight (see also ref. 13). Table I gives the distribution of ^{35}S in the cell preparation on which most of the gel electrophoresis was done. Modified ghosts and the material derived from them were yellow.

Chromatography of lipid

The label was present in all but the neutral-lipid fractions when ethanol–ether extracts were chromatographed on silicic acid–Hyflo Super Cel[31]. On thin-layer chromatograms spots were observed at R_F 0.30 (phosphatidic acid?), 0.50 (phosphatidyl serine), 0.56 (sphingomyelin), 0.72 (phosphatidyl choline) and 0.88 (phosphatidyl ethanolamine). Some of the label remained at the origin; most of it was found between R_F 0.48–0.64 and 0.86–1.00. When suspensions of the extracts in 3% sodium dodecyl sulfate were run on polyacrylamide gels ($3.4 \cdot 10^4$ counts/min of the lipid of Table I per gel) no bands were found. These observations show that a number of lipid components or a number of derivatives of one component are involved. The strongest candidates are diazoamino derivatives of phosphatidyl ethanolamine[7].

Enzymatic hydrolysis of lipid

The enzyme reduced the amount of label soluble in ether, but it did not increase the amount soluble in water, Table II. The labeled product adsorbed to the pellet, from which it could be extracted with methanol. The results show that at least half of the label in the lipid fraction was bound to phospholipid.

TABLE I

LABEL BOUND TO LIPID AND PROTEIN

Type B, Rh-positive cells in a 50% (v/v) suspension were treated 20 min with [^{35}S]sulfanilic acid diazonium salt of specific activity $2.12 \cdot 10^{13}$ counts/min per mole. The initial concentration of reagent in the supernatant fluid was 2.5 mM. 3.5% of the reagent was bound to the ghosts. The table gives the distribution of these counts. The yield of protein was 4.35 mg/ml packed cells.

	Lipid	*Protein*
Number of counts (%)	20.3	79.7
Specific activity (counts/min per g)	$7 \cdot 10^7$	$3.4 \cdot 10^8$
g of material per mole of reagent bound	$3 \cdot 10^5$	$6.2 \cdot 10^4$

TABLE II

EXTRACTION OF LABEL FROM LIPID AFTER TREATMENT WITH PHOSPHOLIPASE D

The lipid was treated with phospholipase D (EC 3.1.4.4, cabbage) by the procedure given in the text (see METHODS). The table shows the counts recovered in the different fractions.

Fraction	*Counts recovered* (%)	
	Enzyme active	*Enzyme inactive*
Ether	14	60
Water	7	6
Methanol	53	6
Pellet	26	28

Gel electrophoresis of ghosts and ghost protein

These results are given in Figs. 4–7. The left and right margins of the figures correspond to the top and bottom edges of the gels, respectively. The solid curves are tracings of the recordings obtained with the scanner; spikes generated by diffraction of the beam at the top and bottom edges of the gels are not shown. The dots are the counting rates obtained from 1.3 mm slices of the gels (less background). No radioactive material remained in the sample buffers at the tops of the gels. In both the ethylene diacrylate and the *N,N'*-methylenebisacrylamide gels, fibrinogen, bovine serum albumin dimer, bovine serum albumin and ovalbumin could be fit with high precision to a log-molecular-weight *vs.* relative-displacement plot of the kind described by SHAPIRO *et al.*[14]. The molecular-weight scales shown in the figures were derived in this way from the displacements of fibrinogen, bovine serum albumin dimer and bovine serum albumin (molecular weights 330000, 134000 and 67000, respectively) in gels containing membrane protein but no 2-mercaptoethanol*.

* Fibrinogen is dissociated at pH 7.2 by sodium dodecyl sulfate and 2-mercaptoethanol but not by sodium dodecyl sulfate alone. Lysozyme was anomalous, appearing on the ethylene-diacrylate scale at molecular weight 24 000 and on the *N,N'*-methylenebisacrylamide scale at 18 000 (*cf.* ref. 14).

When 2-mercaptoethanol was omitted from the sample buffer, more membrane protein remained near the top of the gel, Fig. 7A. Nothing changed when the sample buffer–protein mixtures were heated: the patterns were identical for heated and unheated mixtures of similar composition. The band patterns did not change when the sodium dodecyl sulfate concentration was increased to 1%, but the staining was less intense. The patterns were the same when gels were run after they had been cleaned by soaking; therefore, ammonium persulfate oxidation artifacts[36,37] were not important. Finally, the "run-true" experiment ran true: the compound gels were blank except for regions corresponding to the sections cut from the original gel, and the same bands were present in each.

The patterns obtained from the protein and from intact ghosts were practically

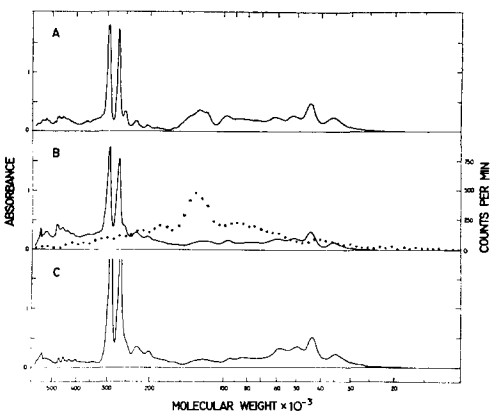


Fig. 4. Gel electrophoresis (ethylene diacrylate) of material from the cell preparation of Table I. (A) 12 μg of protein from control cells. (B) 37 μg of protein from modified cells. (C) Modified ghosts containing 59 μg of protein, as judged from ^{35}S content. A was scanned at 550 mμ, B and C at 515 mμ. The gel slices were each counted 6 min.

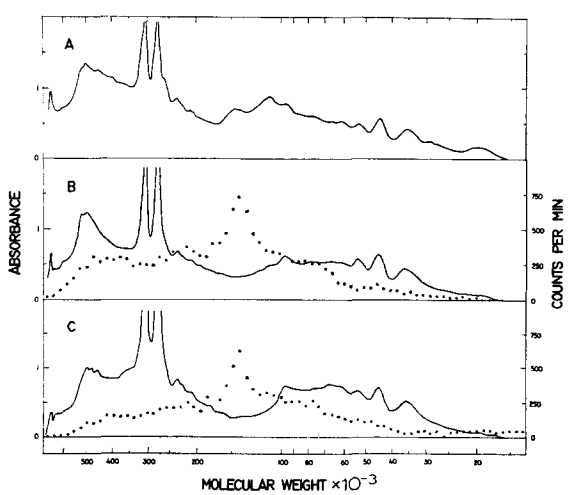


Fig. 5. Gel electrophoresis (ethylene diacrylate) of material from the cell preparation of Table I. (A) 130 μg of protein from control cells. (B) 145 μg of protein from modified cells. (C) Modified ghosts containing 190 μg of protein, as judged from ^{35}S content. The gels were scanned at 515 mμ, and the slices were each counted 12 min.

identical: compare Fig. 4B and C, Fig. 5B and C; the sodium dodecyl sulfate dissociated the lipid and protein as effectively as ethanol–ether. In the region containing most of the ^{35}S the modified protein was less intensely stained than the control protein: compare Fig. 4A and B, Fig. 5A and B. Coomassie brilliant blue binds to positive groups on proteins; the diazonium salt reduces their number; therefore, the modified protein should be less intensely stained. I tried to verify this supposition by scanning unstained gels at 280 mμ, but the amount of protein was too small to be detected. The most intensely labeled protein had a molecular weight of about 140000, Figs. 4B and 5B and C. The most prominent peaks, those around molecular weight 300000 and 270000, were label-free; no discontinuities in the amount of ^{35}S occurred at these positions. It is evident from the figures, especially Fig. 6, that the components were incompletely resolved. The peaks which were most distinct, *e.g.*, those in Fig. 4A at molecular weights 300000 and 43000, were as narrow as the peaks of protein standards of similar molecular weight, *e.g.*, fibrinogen and ovalbumin; the apparent low resolution was due to the complexity of the mixture.

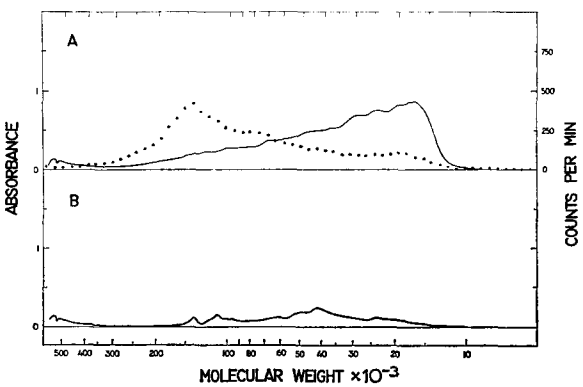


Fig. 6. Gel electrophoresis (ethylene diacrylate) of protein from the modified cells of Table I after exposure to salt. (A) Material which did not dissolve in 0.8 M NaCl–15 mosM Tris (pH 7.5). (B) Material which did dissolve. The amount of protein on each gel was proportional to the total amount in each fraction. The gels were scanned at 515 mμ and the slices were each counted 20 min. Gel B contained only 4% as much ^{35}S as A; it was not sectioned.

When the protein was exposed to salt, Fig. 6, the high-molecular-weight material shifted to low molecular weight; the most intensely labeled material did not. Only 17% of the protein dissolved, as judged from the areas of curves A and B; 10% of the ^{35}S went into solution, 4% with the protein, 6% as a small dialyzable component. The patterns for the control protein (not shown in the figure) were essentially identical to those of the modified protein. The salt solution did not dissolve the bulk of the protein; it converted it into a form which could be dissociated by sodium dodecyl sulfate.

Fig. 7 summarizes experiments in which intact ghosts were exposed to ATP–2-mercaptoethanol (curves B and C) or to 1 M NaCl (curves D and E). The presence of the 300000 and 270000 molecular-weight peaks in the ATP–2-mercaptoethanol supernatant Fraction, C, suggests that this material is related to the "Spectrin" of MARCHESI AND STEERS[19]. Except for an aggregate of very high molecular weight in C, the ATP–2-mercaptoethanol wash was conservative in the sense that the patterns for

the pellet protein, B, and supernatant protein, C, contained all of the prominent peaks of the pattern for the control protein, A. This was not true for the NaCl experiment, in which a large amount of material shifted to low molecular weight. The shift was less extensive than in the experiment of Fig. 6.

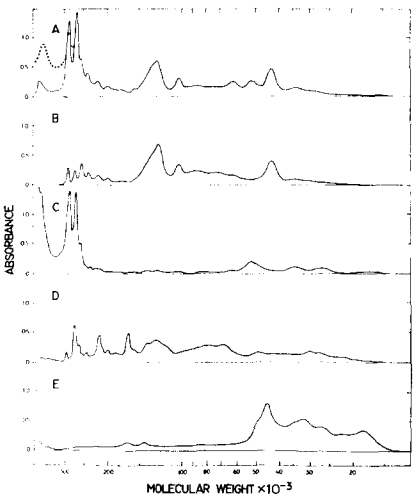


Fig. 7. Gel electrophoresis (*N,N'*-methylenebisacrylamide) of protein from type O, Rh-positive control cells. (A) Protein from unwashed ghosts; the dashed curve was obtained when 2-mercaptoethanol was omitted from the sample buffer (one of the control experiments). (B) Protein from the pellet following dialysis against ATP–2-mercaptoethanol. (C) Protein from the supernatant fluid following dialysis against ATP–2-mercaptoethanol. (D) Protein from the pellet following dialysis against 1 M NaCl. (E) Protein from the supernatant fluid following dialysis against 1 M NaCl. About 37 μg of protein from each fraction was used. The gels were scanned at 515 mμ.

In order to rule out the possibility that the components of molecular weight 300000 and 270000, Figs. 4 and 5, were label-free because they are unreactive, separations were made with a 150 cm × 2.5 cm descending Sepharose-4B column eluted with 1% sodium dodecyl sulfate[13]. In this system it was possible to separate a peak of low-specific activity which moved at about the rate of fibrinogen*, a peak containing a mixture of the components resolved in the 300000 molecular-weight region on the polyacrylamide gel. This material was precipitated with acetone and redissolved in 50 mM sodium phosphate (pH 7.5). When [^{35}S]sulfanilic acid diazonium salt was added, a nondialyzable yellow product formed which had a very high specific activity. Either the components of molecular weight 300000 and 270000 are on the inside of the membrane as such, or they are aggregates of material derived from the inside of the membrane.

CONCLUDING REMARKS

Membranes are difficult to study chemically because most techniques require that the molecules of interest be in solution or in crystals. Membranes in their natural state are neither soluble nor crystalline, and when they are solubilized (*e.g.*, with

* See ref. 13, Fig. 7, Fractions 80–100.

detergents) they lose most of the functional properties that make them interesting. It is necessary to identify these properties in advance. The properties of concern here are architectural: which components are outside of the permeability barrier(s), which are inside, and which go all the way through? A partial answer can be obtained by labeling the membrane from the outside, provided that the label does not alter the structural interrelationships in the membrane and that sufficiently powerful separation techniques are used. Components labeled from the outside may have copies on the inside, or they may extend all the way through. Components not labeled from the outside must be inside, provided that they are reactive. Since different reactivities and different permeability barriers may be involved, different sets of components may be distinguished by different reagents. Before trying to settle questions of reactivity, it seems to me more important that the labeling techniques be perfected to the point that different radioisotopes can be bound to either side of the membrane. One way to do this is to react the outside of the intact cell to completion with one reagent and then the entire cell (or ghost) with a second. Diazonium salts are poor candidates for this experiment, since lysis may occur before the outside reaction is complete.

ACKNOWLEDGMENTS

I am grateful to EUGENE P. KENNEDY for his comments on the manuscript, to STEVEN A. ROSENBERG and RICHARD R. BURGESS for advice, to Grant Bethel for help in obtaining cells, and to SUSAN KABAT and ROSALIND SHORENSTEIN for technical assistance.

This work was supported by Grant GM-14423 from the National Institutes of Health.

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