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ARTICLE *in* BIOCHEMISTRY · JULY 1971

Impact Factor: 3.02 · DOI: 10.1021/bi00789a030 · Source: PubMed

Electrophoretic Analysis of the Major Polypeptides of the Human Erythrocyte Membrane*

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ABSTRACT: The polypeptides of the human erythrocyte membrane were analyzed by polyacrylamide gel electrophoresis in 1% sodium dodecyl sulfate. Six major bands (I–VI) together make up over two-thirds of the protein staining profile. Component III (mol wt 89,000) predominates in the ghost membrane; it constitutes 30% of the protein and numbers over 10^6 chains/ghost. Components I and II form a slow-moving doublet (approximate mol wt 250,000) containing 25% of the protein. The molar amounts of I + II, IV (mol wt 77,500), V (mol wt 41,300), and VI (mol wt 36,200) are similar, falling in the range 3.4–4.6 $\times$ 10^5 chains/ghost. Four bands were recognized in gels stained by the periodic acid–Schiff procedure. A broad Schiff-positive zone just behind the tracking dye corresponds to membrane lipids. Three bands of lower mobility are sialoglycoproteins. The most prominent of these has an apparent molecular weight of 83,500 and contains at least 57% of the sialic acid of ghosts. The Schiff-positive bands were not colored by protein stains. Sialidase treatment of ghosts selectively increased the mobilities of the sialoglycoproteins without affecting the protein-staining profile. Attempts to produce subunits from the large polypeptides by treatment with various denaturing agents were unsuccessful. Normally, no polypeptides of size less than 15,000 were seen in ghost electrophorograms. However, heating ghosts with low levels of sodium dodecyl sulfate and high levels of salt produced diffuse bands of low average molecular weight. This highly variable effect is attributed to degradation by proteinases.

Components I, II, and V were solubilized by incubating ghosts at low ionic strength. Component VI was released by washing with buffered saline at concentrations above 0.1 M. Both elution procedures were rapid (15 min), complete, and selective; they were also conservative in that new bands were not created and the electrophorograms of released and retained material were complementary. The eluted material contained negligible sialic acid and no Schiff-positive lipids. Two classes of membrane protein were distinguished by their response to the elution procedures. Components I, II, V, and VI compose one class. They make up 30–35% of the protein and are tenuously related to the membrane, possibly by predominantly ionic bonds. The second class, which includes components III, IV, and the sialoglycoproteins, together with various minor components, constitutes 65–70% of the protein. These polypeptides are tightly bound; their properties may reflect participation in hydrophobic protein–protein and protein–lipid interactions.

The erythrocyte membrane has long served as a convenient model system for the testing of new concepts and methodology in membrane biochemistry. Hence, increasing interest in the role of membrane proteins in general has led to intensive efforts toward fractionating and characterizing the proteins of the erythrocyte membrane. Such studies have generally used erythrocyte "ghosts" freed of hemoglobin by hypotonic washing procedures similar to that of Dodge *et al.* (1963). These ghosts do not retain all of the functional and structural attributes of the intact cells, but are operationally well-defined and afford the advantages of reproducible composition and ready accessibility.

Up to 30% of the protein of such ghosts can be released by various simple ionic manipulations (Mitchell and Hanahan, 1966; Marchesi and Steers, 1968; Marchesi *et al.*, 1969a,b; Rosenberg and Guidotti, 1969a,b; Harris, 1969; Haggis, 1969) and over half is solubilized by disruption in 6 M guanidine hydrochloride (Gwynne and Tanford, 1970). In general, however, most of the protein remains membrane-associated or aggregated unless hydrophobic protein–protein and/or protein–lipid interactions are perturbed by effective organic solvents or detergents. Limited fractionations of the membrane, yielding partially purified preparations of M- and N-active sialoproteins, have been achieved after treatment with phenol–water mixtures (Kathan *et al.*, 1961; Morawiecki, 1964; Winzler, 1969a; additional references in Winzler, 1969b) or aqueous pyridine (Blumenfeld, 1968; Blumenfeld *et al.*, 1970; Furthmayr and Timpl, 1970). Large, but variable, proportions of membrane proteins have been extracted into butanol-saturated water (Maddy, 1964, 1966; Rega *et al.*, 1967), but this procedure cannot be readily controlled. Membrane proteins are dissolved by 2-chloroethanol (Zahler and Wallach, 1967; Zahler, 1968), but no high-resolution system for polypeptide fractionation in this solvent has been developed. The anionic detergent, SDS,[1] is also an effective solubilizing agent and molecular exclusion chromatography of ghost membranes dissolved in SDS has been used to resolve broad

* From the Biochemical Research Laboratory, Massachusetts General Hospital, Boston, Massachusetts 02114, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts. *Received December 17, 1970.* Supported by Contract No. NIH-69-2007 of the U. S. Public Health Service. G. F. was a Postdoctoral Fellow (No. 48078) of the National Science Foundation. T. L. S. was a Special Fellow (No. 5 F03 CA 28417-02) of the U. S. Public Health Service. D. F. H. W. was supported by the Andres Soriano Cancer Research Fund.

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; BIS, *N,N'*-methylenebisacrylamide; TEMED, *N,N,N',N'*-tetramethylethylenediamine; DTT, dithiothreitol (Cleland's reagent); PAS, periodic acid–Schiff reagent; 5P(8), 5 mM sodium phosphate (pH 8).

classes of polypeptides differing in average molecular weight (Rosenberg and Guidotti, 1968, 1969a,b).

All of the methods cited have failed to resolve the membrane polypeptides cleanly into discrete classes with distinct physicochemical characteristics—in some cases, because they did not completely disrupt aggregates; in others, because the effective solubilizing agent was not compatible with a process for separating the polypeptides with high resolution. To date, the most promising approach appears to be dissolution of the membrane in SDS followed by electrophoretic fractionation in SDS-containing polyacrylamide gels (Summers *et al.*, 1965; Maizel, 1966, 1969; Kiehn and Holland, 1968, 1970; Rosenberg and Guidotti, 1969b; Berg, 1969; Lenard, 1970a,b). We have applied this approach, using refinements of the method of Fairbanks (1969), and have achieved very reproducible, high resolution analytical fractionations of the ghost polypeptides. In this paper we describe the use of this method in characterizing the major components, including the glycopeptides. We also demonstrate complete, selective elution of certain components into aqueous solution without degradation or irreversible aggregation of the polypeptides. Finally, the high resolution of the technique, coupled with sensitive staining for protein and carbohydrate, revealed several methodological problems of general significance—we discuss the control of these, which was a prerequisite for our subsequent study of the disposition of the major polypeptides on the inner and outer surfaces of this membrane (Steck *et al.*, 1971).

TABLE I: Formulas for Stock Solutions, Buffers, and Gels.

A. Stock Solutions

Con AcBis	10× Buffer (pH 7.4)
Acrylamide (40 g)	1.0 M Tris (40 ml)
Bis (1.5 g)	2.0 M sodium acetate (10 ml)
H_2O to 100 ml	0.2 M EDTA (10 ml)
	acetic acid to pH 7.4
	H_2O to 100 ml

20% (w/w) SDS
1.5% (w/v) ammonium persulfate
0.5% (v/v) TEMED

B. Electrophoresis buffer (per liter)
10× Buffer (100 ml)
20% SDS (Matheson) (50 ml)
H_2O (850 ml)

C. Gels (per 10 ml of solution 5.6% in acrylamide)
Con AcBis (1.4 ml)
10× buffer (1.0 ml)
20% SDS (Mann) (0.5 ml)
H_2O (5.6 ml)
1.5% ammonium persulfate (1.0 ml)
0.5% TEMED (0.5 ml)

Materials and Methods

Materials. Sources of chemicals for electrophoresis were acrylamide, Eastman 5521; BIS, Eastman 8383; TEMED, Eastman 8178 and Canalco 204; SDS, Matheson Coleman & Bell DX2490 S7003 and Mann "Lauryl sodium sulfate, M.A." lyzed 12500-2696; Tris, Sigma" Trizma base"; coomassie brilliant blue R-250, Mann 9207; ponceau S, Millipore M 000002B (1.8% solution in 26.8% trichloroacetic acid plus 26.8% sulfosalicylic acid); Amido Black 10B (C. I. No. 20470), Merck (Darmstadt), 1167; pyronin Y (C.I. No. 45005), Fisher P-388; basic fuchsin (C.I. No. 42500), Allied (National Aniline) 434; DTT, Calbiochem 233155.

Molecular weight markers for SDS gel calibration were β-galactosidase (a preparation of K. Wallenfels given by D. Stathakos), phosphorylase *a* (Worthington PSA), bovine serum albumin (Mann 2506), human γ-globulin (Hyland 070-090, "plasma fraction II"), pepsin (Mann 2095), myoglobin (Mann 1155), and cytochrome *c* (Mann 1581).

The concentrated acrylamide–BIS stock solution (Table I) was deionized (to a conductivity below 5 μmhos/cm) using a column of mixed-bed resin (Rexyn 300, Fisher R-276).

The Schiff reagent was prepared by dissolving 2.5 g of basic fuchsin in 500 ml of water, then adding 5 g of sodium metabisulfite and 50 ml of 1 N HCl. The solution was stirred for several hours, and then decolorized with about 2 g of activated charcoal.

Preparation of Erythrocyte Ghosts. Ghosts were prepared by the method of Steck *et al.* (1970), which followed the principles of hypotonic lysis defined by Dodge *et al.* (1963). Phase-contract microscopy was used to monitor the procedure. Freshly drawn human blood (25 ml) was mixed with 1 ml of 0.2 M EDTA, then diluted with an equal volume of cold 5 mM sodium phosphate [pH 8 (5P(8))]–0.15 M NaCl. The suspension was centrifuged for $10^4 g_{max}$ min at 4°, using a swinging-bucket rotor, and the supernatant and "buffy coat" were aspirated. The packed cells were then washed three times by suspension in 5P(8)–0.15 M NaCl to 50 ml and centrifugation as above. In thoroughly aspirating the surface of the pellet after each spin, a total of about one-fourth of the original volume of erythrocytes was sacrificed. The yield of packed, washed cells was about 9 ml. The cells were lysed by mixing rapidly 1.0- to 1.2-ml portions into 40 ml of cold 5P(8). The suspensions were centrifuged for $3 \times 10^5 g_{max}$ min in an angle head. The resulting deep red supernatants were aspirated, leaving red, translucent pellets of packed ghosts over minute, opaque, cream-colored "buttons." Removal of the latter at this stage was essential in minimizing contamination of the ghosts with proteinases (see Discussion). Each tube was tilted and rotated to allow the loose ghost pellet to slide off the tightly packed button, which could then be aspirated with little loss of ghosts. After two additional washes under the same conditions, the pellets were homogeneous and white, and were composed of intact dimpled ghosts packed to a protein concentration of 3–4 mg/ml. A trace of residual hemoglobin was detectable; it could be removed, if desired, by an additional wash.

Assays. Protein was measured fluorometrically by the method of Resch *et al.* (1971). The membrane proteins dissolved in SDS (Mann 12500-2696) were diluted to 0.1–20 μg/ml in 0.1% SDS (Mann). An Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer recorded emission at 338 nm (bandwidth about 10 nm) with excitation at 285 nm (bandwidth 10 nm). Tryptophan was used as the standard; in 0.1% SDS the fluorescence of 1 μg of tryptophan was equal to that of 42 μg of membrane protein (based on fat-free membrane dry weight and protein determinations by the method of Lowry *et al.* (1951)).

The method of Warren (1959, 1963) was used to measure sialic acid. Determinations of total sialic acid were preceded by hydrolysis at 80° for 1 hr in 0.1 or 0.15 N H_2SO_4. The absorptions at 549 and 532 nm were measured and sialic acid

concentrations were calculated using eq 2 of Warren (1959), which corrects for the contribution of the minor chromophore at 532 nm. The validity of this procedure was checked with sialic acid (*N*-acetylneuramininic acid) and sialyllactose standards.

Sialidase Treatment. The sialidase of *Vibrio cholerae* ("Neuraminidase Behringwerke," Lloyd Brothers, Inc., Cincinnati, Ohio) was used. The reaction mixture contained ghosts at 2–3 mg of protein/ml, 20 mM sodium phosphate (pH 6.4), 1 mM $CaCl_2$, and 25 units (0.05 ml) of enzyme in a total volume of 0.99 ml. After incubation for 35 min at 37°, the suspension was chilled in an ice bath and 0.025 ml of 0.1 M EDTA, 0.125 ml of 0.5 M Na_2HPO_4, and 0.075 ml of 20% SDS (all at room temperature) were added in order. The clear solution was frozen immediately. An incubation control was prepared at the same time, with an equivalent volume of water substituted for the sialidase.

Polyacrylamide Gel Electrophoresis in SDS. The overall approach was that of Fairbanks (1969), with several modifications. Gel polymerization followed generally the "alternative procedure" of Davis (1964). Concentrated stock solutions were mixed in the order and proportions given in Table I and added to 10.8-cm glass (either flint or Pyrex) tubes of 8-mm o.d. that had been cleaned by soaking in chromic–sulfuric acid or by washing with hot SDS. Each column (height, 85 mm) was overlaid gently with a solution of 0.1% SDS, 0.15% ammonium persulfate, and 0.05% TEMED. (Gravity flow through a 2-μl Drummond microcap is convenient for applying the overlay.) When polymerization was complete (about 45 min at room temperature) the tops of the gels were rinsed and overlaid with about 0.5 ml of electrophoresis buffer (Table I). The gels were then left to stand for at least 12 hr.

Suspensions of erythrocyte ghost protein at 0.5–4.0 mg/ml were prepared for electrophoresis by adding the following (to the stated final concentrations): 1% SDS, 5–10% sucrose, 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8), 40 mM DTT, and 10 μg/ml of pyronin Y (tracking dye). Suspensions containing salts or organic solvents were first dialyzed overnight at 4° against 10 mM Tris-HCl–1 mM EDTA (pH 8) or overnight at 15° against the same buffer plus 1% SDS. The clear sample solutions were incubated at 37° for 15–30 min to promote reduction of disulfide bonds by DTT. Molecular weight markers were incubated in the same medium for 20 min at 45–50°. Reduction with DTT is particularly important when bovine serum albumin is used as a marker. When incompletely reduced, this protein exhibits an abnormally high mobility that can result in overestimates of molecular weight for polypeptides of size greater than about 60,000.

Electrophoresis was carried out in an apparatus equipped with convection-limiting baffles. About 400 ml of noncirculating buffer was used in each electrode compartment (six gels maximum). Each sample was taken up in a disposable micropipet and discharged gently beneath the upper buffer onto the top of the gel. Normally, electrophoresis was performed with the voltage gradient at about 7–8 V/cm and the current at 8 mA/tube (varying the voltage gradient in the range 5–12 V/cm did not affect the pattern). The running time under these conditions was about 2 hr.

Variations in absolute migration distances were minimized by removing the tubes from the electrophoresis apparatus one-by-one as the pyronin Y tracking dye band reached a mark 75 mm from the origin. The position of the tracking dye was marked in each gel by pricking it with a needle dipped in drafting ink.

The gels were normally stained for protein with coomassie blue. They were placed in slotted glass tubes suspended in a beaker to which was added the fixing, staining, and destaining solutions given below. The solutions were stirred vigorously at room temperature for the stated times; no less than 50 ml/gel was used at each stage: (1) 25% isopropyl alcohol, 10% acetic acid, 0.025–0.05% coomassie blue; overnight; (2) 10% isopropyl alcohol, 10% acetic acid, 0.0025–0.005% coomassie blue; 6–9 hr; (3) 10% acetic acid with 0.0025% or less coomassie blue; overnight; (4) 10% acetic acid; several hours, until the background was clear. Most of the pattern could be seen after the second step; *i.e.*, within 24 hr after electrophoresis. The third step intensified the pattern but was optional—if the dye concentration at step 2 was kept minimal, direct transfer of the gels to tubes containing about 10 ml of 10% acetic acid yielded an acceptably low background.

Isopropyl alcohol is apparently the primary fixing agent. Acetic acid could be omitted without effect, but fixation without alcohol resulted in gross broadening of bands and loss of protein from the gel. The effectiveness of the alcohol as fixative may be related to the fact that it increases the rate of SDS diffusion from the gel (T. L. Steck and G. Fairbanks, unpublished data). Fixation in 20% sulfosalicylic acid for 20 hr followed by staining and destaining in acetic acid (Maizel, 1966, 1969; Laico *et al.*, 1970) was tested for both 0.2 and 1.0% SDS gels. In comparison with equivalent gels subjected to the usual alcohol fixation, the sulfosalicylic acid fixed gels exhibited a lack of fine detail and apparent loss of low molecular weight material. In preliminary experiments, gels were fixed overnight at 4° in 15% trichloroacetic acid–25% isopropyl alcohol (Fairbanks, 1969). The use of the simpler protocol given above did not reduce the sharpness or intensity of any bands and yielded better recovery of the nonstaining lipid material just behind the tracking dye. Unreduced insulin (mol wt 6000) ran as a broad band of mobility about 0.95 relative to the tracking dye; 5 μg was easily detected with routine fixation and staining, indicating that loss of small proteins is unlikely with this procedure.

The presence of coomassie blue was not necessary for fixation; gels carried through the same process in the absence of dye showed equally high resolution. Amido Black or ponceau S was introduced into such fixed, unstained gels by overnight soaking with less than 0.01% dye in 10% acetic acid; unbound dye was removed by leaching into 10% acetic acid alone.

Gels were stained for carbohydrate using the PAS procedure. Because high concentrations of SDS produced an intense background, the SDS was removed before PAS staining by carrying out steps 1–4 above (omitting the protein stain). The fixed gels were left in the slotted staining tubes and treated as follows, using at least 80 ml of rinse solution per gel at each stage and stirring vigorously at room temperature: (1) 0.5% periodic acid (H_5IO_6); 2 hr; (2) 0.5% sodium arsenite, 5% acetic acid; 30–60 min; (3) 0.1% sodium arsenite, 5% acetic acid; 20 min—repeated twice; (4) acetic acid; 10–20 min. The gels were then (5) transferred to tubes containing 10 ml of Schiff reagent and left overnight; then (6) returned to the large bath in the slotted tubes for incubation in 0.1% sodium metabisulfite ($Na_2S_2O_5$), 0.01 N HCl, for several hours, repeated until the rinse solution failed to turn pink upon addition of formaldehyde. Rose-pink bands appeared after 5–10 min in the Schiff reagent (step 5) and slowly intensified as the reagent penetrated to the center of the gel. The subsequent soaking steps rid the gel of unreacted reagent, intensified the pattern, and retarded fading. A variable gradient of background absorption was seen. Its origin is uncertain, but might reflect the establishment of concentration gradients while the

gels stood overnight in the Schiff reagent without vigorous stirring.

Gels stained with coomassie blue were photographed through a deep yellow filter using Polaroid Type 55 P/N film. Gel densitometry was done using a Gilford spectrophotometer and Model 2410 linear transport accessory, scanning through a 0.1-mm slit at 550 nm for coomassie blue, 600 nm for Amido Black, 515 nm for ponceau S, and 560 nm for PAS.

Comparisons of the patterns of PAS and protein staining within single gels were made by dual scanning at 525 and 620 nm of PAS-stained gels that had been lightly superstained with Amido Black. For scanning at 620 nm, the recorder sensitivity was adjusted to set the peak heights in PAS-negative zones equal to the corresponding zones in the 525-nm profile. At 620 nm, absorption by PAS-positive bands was negligible, and the PAS pattern could be obtained by plotting the difference between the superimposed profiles. Refractile boundary artifacts at the top and bottom of the gel interfered with dual scanning of these regions; throughout most of the gel, however, the dual scanning result parallels that of PAS staining alone.

Critical Variables in SDS Gel Electrophoresis. Clean patterns with sharp, flat bands are obtained consistently only with careful control of critical aspects of sample preparation and electrophoresis. Thus, although DTT reduction does not change the mobility of any membrane polypeptide, it does prevent aggregation-induced streaking and entrapment of material at the origin. Particulate matter in the sample can also cause streaking; hence, the introduction of dust and fibers is to be avoided. Band curvature reduces effective resolution of the system, interferes with precise comparisons between gels, and complicates measurements of peak areas in densitometer scans. Curvature can result from dirty tubes and impure reagents, and is also critically dependent on the relative concentrations of SDS, ammonium persulfate, and TEMED. It becomes more pronounced as the SDS concentration is increased,[2] and to obtain flat bands in 1% SDS it was necessary to carry out the polymerization slowly using an unusually high ammonium persulfate:TEMED ratio. To ensure formation of a flat interface, the same amounts of catalyst and accelerator were included (with 0.1% SDS) in the overlay solution. Allowing the gels to stand overnight under electrophoresis buffer produced a further significant reduction in band curvature.

Results

Electrophoretic Fractionation of Ghost Membrane Proteins. Electrophoresis of ghost membrane proteins in 5.6% polyacrylamide gels containing 1% SDS yielded a pattern in which six well-resolved bands—arbitrarily labeled I–VI—predominate. This pattern was well reproduced when one protocol was adhered to, but the relative position, sharpness, and intensity of some minor bands were affected by changes in the conditions (pH, buffer composition, ammonium persulfate:TEMED ratio, concentrations of SDS and acrylamide) of electrophoresis. The intensity peaks between IV and V and just below VI appeared to represent overlapping minor components with differential responses to subtle changes in the fractionation process. In addition, the sharp major band, IV, appeared as a doublet when electrophoresis was performed with 0.1 or 0.2% SDS. No splitting of the other major components was seen under any of the conditions tested.

Quantitative densitometry of 1% SDS gels stained with coomassie blue, ponceau S, and Amido Black (Figure 1B) revealed that the six bands account for over two-thirds of the total integrated intensity in the staining profile (Table II). Importantly, also, the relative peak areas were the same with all three stains. Molecular weight estimates for components III–VI (Table II) were obtained by rigorous calibration of the gels with molecular weight markers. The calibration curve was extrapolated to yield a rough estimate of 250,000 for the molecular weight of I and II. From the data on size and relative abundance, the number of polypeptide chains per ghost and the molar ratios in relation to VI were calculated for each of the major species (Table II). There are two striking features of these results. One is the apparent molar predominance of component III; polypeptide chains of this type are two to three times more numerous in the ghost membrane than those of each of the other major components. It is also striking that the ratios (I + II):IV:V:VI approximate 1:1:1:1 within experimental error.

Electrophoretic comparisons of the proteins of blood plasma, erythrocyte cytoplasm, and ghost membranes (Figure 1A) showed no evidence of substantial contamination of the membrane preparation by soluble proteins from either aqueous compartment (except for trapped hemoglobin that was easily removed by an additional wash). We have examined electrophorograms of ghost proteins from healthy donors of both sexes and various blood types and have compared fresh to 21-day banked blood. No variations in the characteristic pattern have been detected. Normally, only 20–30 bands can be distinguished, though the system is capable of resolving at least 50. There may be additional minor components (possibly some specific to sex or blood type) that have not been seen because the amount of sample applied is kept low to prevent the distortion that accompanies overloading of the major bands I, II, and III.

Attempts to Reduce Large Polypeptides to Subunits. A large proportion of the polypeptides in this membrane system apparently are of unusually high molecular weight. This can be seen in Table II as well as in the results of Gwynne and Tanford (1970), Lenard (1970a), and Marchesi *et al.* (1969a). We have, accordingly, attempted to dissociate the ghost proteins using solvents known to reduce noncovalently bound polymers to subunits.

In one experiment, ghosts were first converted into vesicles by suspension in 1 mM Tris-HCl–1 mM EDTA (pH 8), overnight at 4°, then diluted fivefold with the following solutions: (1) 2:1 phenol–acetic acid, (2) 2-chloroethanol–0.01 N HCl, (3) dimethyl sulfoxide–0.01 N HCl, (4) methyl Cellosolve–0.01 N HCl, (5) dimethylformamide–0.01 N HCl, (6) 9:1 dimethylformamide–formic acid, (7) 6 M urea–10% formic acid, (8) 6 M urea–33% acetic acid, (9) 33% acetic acid, (10) 33% chloroacetic acid, (11) 33% 3-chloropropionic acid, and (12) 10% LiCl. The final protein concentration was approximately 1 mg/ml. After the mixtures had been incubated at 4° overnight, samples 1–3 and 8–9 were clear or slightly turbid, the others highly turbid. SDS was then added to a final concentra-

[2] Nevertheless, electrophoresis at high SDS concentrations was preferred for three reasons: (1) the patterns are independent of the buffer composition of the sample, so reproducible results and smooth calibration curves are obtained without dialyzing each sample to equilibrate it with the electrophoresis buffer; (2) the tracking dye, pyronin Y, runs as a sharp band in SDS above the critical micellar concentration, allowing precise mobility measurements and alignment of a series of gels for close comparison; and (3) high levels of SDS inhibit artifactual degradation by proteinases in the sample (see Discussion). None of these conditions prevails when electrophoresis is carried out at 0.1% SDS, below the critical micellar concentration.

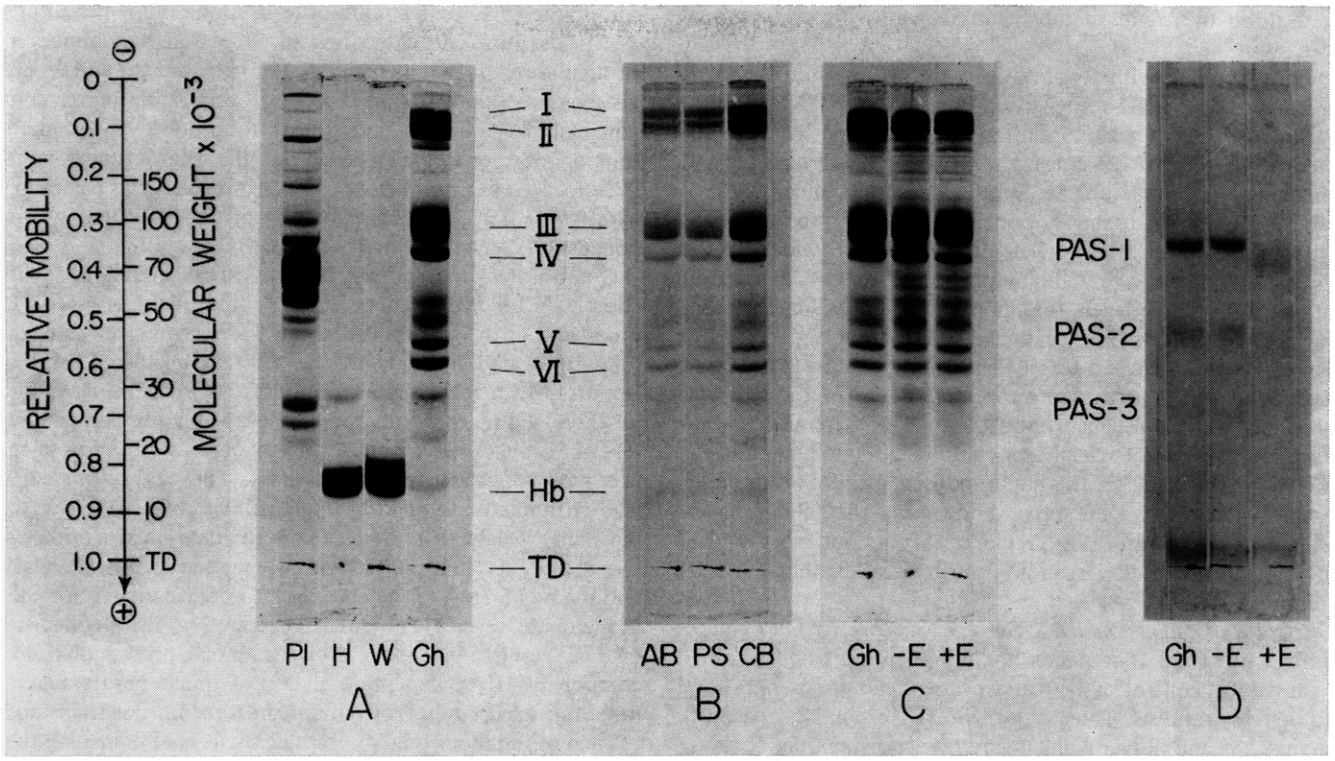

FIGURE 1: Electrophorograms of ghost membranes and other blood fractions. The relationship between relative mobility and polypeptide molecular weight is shown at the left. Details of sample preparation and staining are given in Materials and Methods. India ink marks inserted before fixation and staining show the final position of the pyronin Y tracking dye (TD) in each gel. (A) Electrophoretic analysis of proteins from plasma, red cell cytoplasm, and washed ghosts. Details of the preparation are given in Materials and Methods. Pl = plasma, 20 μl of a 4/50 dilution of the first supernatant. H = hemolysate supernatant, 25 μl of a 4/50 dilution. W = wash, 100 μl of a 4/10 dilution of the supernatant from the first ghost wash. Gh = ghosts, 25 μl of a 4/10 dilution of packed, washed ghosts (about 40 μg of protein). (B) Electrophorograms of ghost membrane proteins—comparison of Amido Black (AB), ponceau S (PS), and coomassie blue (CB) staining. The amount of protein applied to each gel was 28 μg. The gels were photographed through a deep blue-green filter. (C) The effect of sialidase on ghost membrane proteins—electrophorograms stained with coomassie blue. One sample of ghosts (Gh) was prepared for electrophoresis by the standard procedure; a second portion (−E) was an incubation control for the sialidase treatment; the third (+E) was treated with the *Vibrio cholerae* sialidase as described in Materials and Methods. The amount of protein applied to gel "Gh" was 34 μg; gels "−E" and "+E" each contained 23 μg. (D) Effect of sialidase on ghost membrane proteins—replicates of gels in (C) stained for carbohydrate by the PAS procedure and photographed through a blue-green filter.

tion of 1% and the samples were dialyzed for 24 hr at 15° against 10 mM Tris-HCl, 1 mM EDTA, 1% SDS, and 0.1 mM DTT (pH 8). After dialysis all samples were clear and were prepared for electrophoresis by adding concentrated sucrose and tracking dye. The electrophorograms showed no conversion of large polypeptides into smaller species of higher mobility in the gels. All major bands were unaltered, except that aggregation occurred in some cases, as evidenced by the appearance of stain at the origin with *selective* reduction in the intensity of band III.

In other experiments, ghosts were disrupted with 33% pyridine and dialyzed against water as described by Blumenfeld (1968). This did not alter the polypeptide size distribution on SDS gels. Samples could also be heated to 95° in 2% SDS (Maizel, 1969) without effect on the pattern.

Properties of the PAS-Positive Components. When stained for carbohydrate by the PAS procedure, electrophorograms of ghost components reproducibly gave the pattern illustrated in Figure 1D(Gh). An intense staining reaction was always seen just behind the tracking dye. We infer that this is a lipid zone. It does not bind any of the protein stains used, but can be detected as a bright zone of scattered light when viewed against a dark background. Its PAS-positive activity may be due in part to glycolipid (Lenard, 1970a), but must also be attributed to hydrolytic release of fatty aldehydes from plasmalogens (Rapport and Norton, 1962; Dodge and Phillips, 1967) and, possibly, to peroxidation of polyunsaturated fatty acids yielding malonaldehyde (Niehaus and Wold, 1970).

The three slower moving bands have been designated arbitrarily PAS-1, -2, and -3. PAS-1, the most intense, lies near bands III and IV. However, when the position of PAS-1 was marked with drafting ink and the gels were superstained with coomassie blue (after which the PAS bands normally cannot be seen because of their low intensity and rapid fading in 10% acetic acid), the mark fell on neither III nor IV, but in the lucent zone between them. The lesser PAS-positive bands likewise failed to coincide with any prominent bands stained with coomassie blue.

That the major PAS-positive components are distinct from bands I to VI was conclusively demonstrated by removing terminal sialic acid residues with the *Vibrio cholerae* sialidase (Figures 1C,D and 2). Comparison of the protein staining patterns in Figure 1C with the PAS patterns in 1D demonstrates that sialidase treatment altered the mobilities of the PAS-positive bands without affecting the distribution of coomassie blue in matching gels. The same effect is shown graphically in Figure 2. The PAS profiles in Figure 2A,B confirm the predominance of PAS-1 and also show a small shoulder trailing PAS-1, a continuous distribution of PAS-positive material between PAS-1 and -2, and the intense PAS-positive

TABLE II: Size and Abundance of Major Ghost Polypeptides.

Band	Mol Wt[a]	Relative Abundance[b]			Chains/Ghost[c]	Molar Ratios[d]
		Percentage of Stain				
		PS	AB	CB		
I + II	250,000	28.9	27.2	24.7 ± 1.0	340,000	0.7
III	89,000	26.1	29.7	30.0 ± 0.5	1,160,000	2.5
IV	77,500	7.8	7.6	7.8 ± 0.3	340,000	0.7
V	41,300	3.7	4.1	4.0 ± 0.1	340,000	0.7
VI	36,200	4.8	5.1	4.9 ± 0.2	460,000	1.0
		71.3	73.7	71.4		
PAS-1	83,500					
	(29,800)[e]		(2.3)[f] ?		(260,000)[f] ?	0.6 ?

[a] Measured by calibration of the gels with mixtures of molecular weight markers. From the range of mobilities in triplicate measurements on both markers and ghost polypeptides, the uncertainty in the values for bands III–VI was estimated as ±10%. A rough estimate of the size of I and II was obtained by extrapolation of the calibration curve. [b] Each stain—ponceau S (PS), Amido Black (AB), and coomassie blue (CB)—was applied to a series of four gels containing 14, 28, 41, and 55 μg of ghost protein. Staining and densitometry of each series were performed as described in Materials and Methods. Relative peak areas (percentage of stain) were obtained by cutting and weighing sections of the staining profiles. Each result is the average of four measurements. The standard deviation of the coomassie blue values is given. [c] Assuming 5.7×10^{-10} mg of protein/ghost, the average of three reported values (Dodge *et al.*, 1963: Weed *et al.*, 1963, 1969). The calculation used the proportions estimated by coomassie blue staining. [d] Relative to band VI. [e] Estimated size of the protein portion assuming PAS-1 is 35.7% protein–27.8% sialic acid (Winzler, 1969a,b). [f] Assuming that 60% of the total sialic acid is bound to PAS-1 (see text) and that ghosts contain 100 nM sialic acid/mg of protein (Table III).

response of the lipid zone. In Figure 2D–F the PAS profiles are superimposed on the protein-staining patterns. These gels were first stained with the Schiff reagent, then faintly superstained with Amido Black. The profiles of the two stains were revealed by dual scanning, taking advantage of negligible absorption at 620 nm by the PAS chromophore. The dual scans provide further evidence that PAS-1, -2, and -3 do not coincide with any major bands of protein stain and that they are uniquely responsive to sialidase treatment.

The sialic acid distribution in electrophorograms was also determined by applying the assay of Warren (1959, 1963) to transverse slices from large diameter gels. In unfixed gels, over 90% of the sialic acid applied was detected in the region of mobilities ranging from 0.25 to 0.75 relative to the tracking dye. Traces were detected in the lipid zone, but the amount is in doubt because of interference by other sugars, tracking dye, and polyunsaturated fatty acids (Niehaus and Wold, 1970; Saslaw *et al.*, 1966). The PAS-1 band contained 57% of the total sialic acid applied. In fixed gels, the same zone yielded a lower recovery, 46%, possibly due to slow hydrolytic release by acetic acid during fixation at room temperature or to destruction by excess acid when the slices were heated in 0.15 N H_2SO_4.

Although the sialic acid containing components, PAS-1, -2, and -3, fail to bind protein dyes, their low mobilities (corresponding to apparent molecular weights of 83,500, 45,600, and 25,500, respectively) suggest that they are glycoproteins. This supposition is strongly supported by the observation that the PAS pattern is altered by proteolytic treatment of intact erythrocytes, ghosts, and vesicles. These alterations are independent of the responses of bands I–VI to controlled proteolysis (Steck *et al.*, 1971). Winzler (1969a,b) reported that most of the erythrocyte membrane sialic acid is covalently bound to polypeptides. This finding is consistent with the observed sialic acid distribution in gels only if it is assumed that the major PAS-positive bands are sialoglycoproteins. If PAS-1 has the high sialic acid content of the M- and N-active blood group substance isolated by Winzler (1969a,b), its protein portion amounts to over 2% of the total membrane protein (Table II).

Figures 1D(+E), 2C, and 2F suggest that sialidase treatment converted the prominent PAS-positive components to forms with higher mobility, greater size dispersion, and lesser intensity. For PAS-1 and -2, the apparent molecular weight decrements were about 12,500 and 5000, respectively. The increased size dispersion may reflect the fact that the sialidase treatment released only 78% of the total sialic acid in the ghosts. The lower staining intensity is not surprising because galactose, the probable terminus after hydrolysis (Thomas and Winzler, 1969a,b), is less rapidly oxidized by periodate than sialic acid (Spiro, 1964).

A shoulder of PAS-positive material was consistently seen trailing PAS-1. It had an apparent molecular weight of about 100,000 and could represent either a minor glycoprotein component or a response of the trailing limb of band III. No sialic acid was detected in this region by analysis of transverse slices. Unlike the other PAS-positive bands, this component was unaffected by sialidase (Figure 2C,F).

Elution of Ghost Proteins at Low Ionic Strength. Several investigators have observed the release of substantial amounts of protein when erythrocyte ghosts are washed with or dialyzed against media of low ionic strength (Marchesi and Steers, 1968; Marchesi *et al.*, 1969a,b; Rosenberg and Guidotti, 1969a,b; Berg, 1969; Harris, 1969; Haggis, 1969; Furthmayr and Timpl, 1970; Hoogeveen *et al.*, 1970). The elution of these proteins parallels the generation of inside-out vesicles from

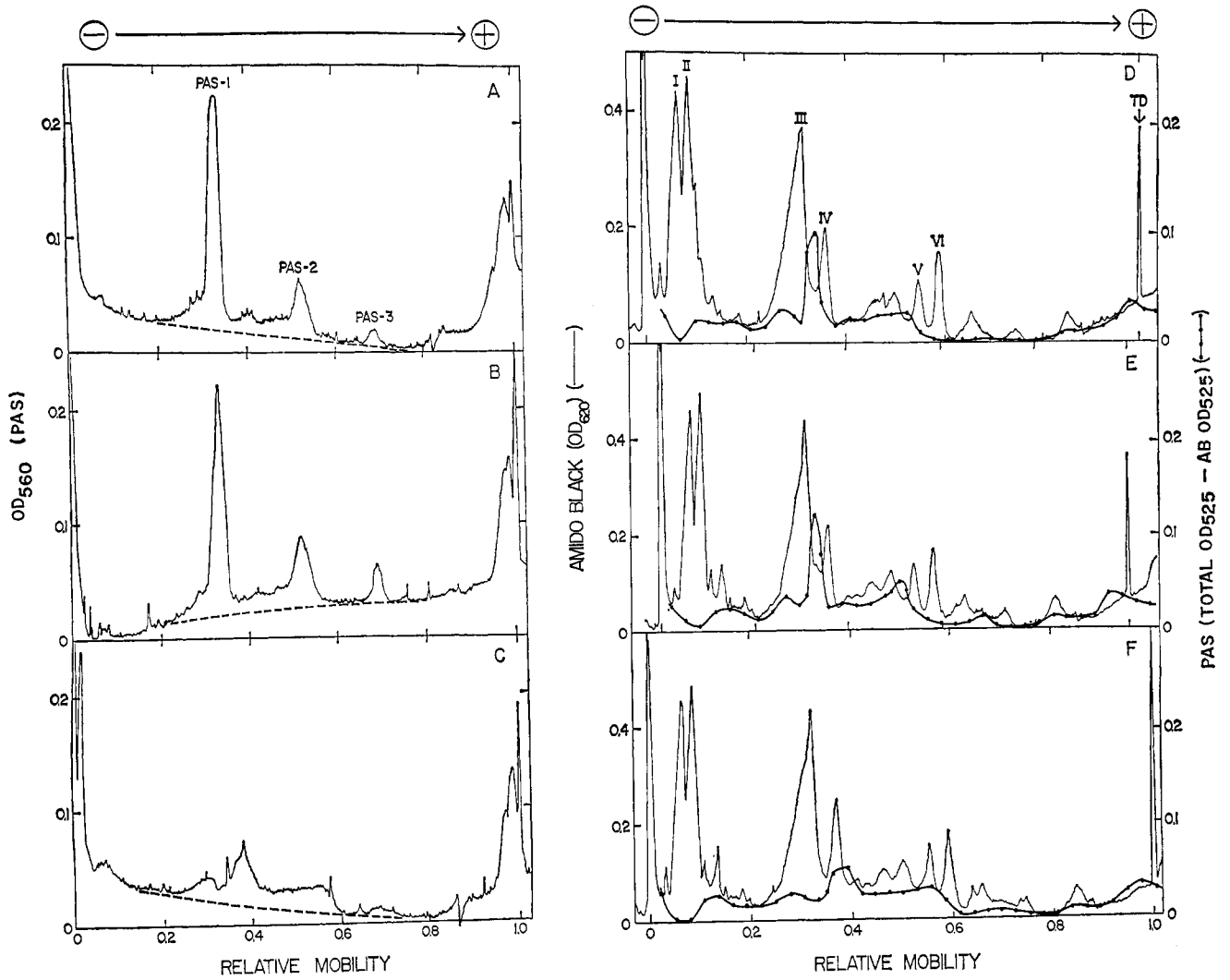

FIGURE 2: The PAS-positive components—their response to sialidase treatment and distribution relative to the Amido Black staining profile. (A–C) Replicates of gels in Figure 1C stained for carbohydrate with the Schiff reagent and scanned at 560 mμ. (D–F) Replicates of gels in Figure 1C stained with the Schiff reagent, lightly superstained with Amido Black, and analyzed by dual scanning at 525 and 620 mμ as described in Materials and Methods. The distribution of PAS-positive material (bold line) is superimposed on the profile of Amido Black absorbance at 620 mμ (fine line). Gels A and D contained, respectively, 34 and 28 μg of ghost protein dissolved in SDS by the standard procedure (*cf.* gels "Gh" in Figure 1C,D). Gels B and E contained 23 μg of protein from the sialidase incubation control (*cf.* gels "−E" in Figure 1C,D). Gels C and F contained 23 μg of protein from ghosts incubated with the *Vibrio cholerae* sialidase as described in Materials and Methods (*cf.* gels "+E" in Figure 1C,D).

ghosts, but the degree to which the two processes are related mechanistically remains to be established (Steck *et al.*, 1970, 1971).

As shown in Figure 3B, incubation of ghosts at low ionic strength can effect the complete and selective release of components I, II, and V. In the experiment illustrated, ghosts were diluted tenfold with warm 0.1 mM EDTA (pH 8) and incubated briefly at 37°. This rapid elution protocol is preferred to prolonged dialysis because it is convenient and minimizes the time during which aggregation and proteolysis artifacts can occur (see Discussion). The eluted material contained 24% of the ghost protein and no sialic acid or PAS-positive lipids (Table III). The release of protein is in satisfactory agreement with the amounts of the three components measured by scanning stained gels (Table II). Figure 3A,B demonstrates that the process is conservative; new bands were not produced, and the patterns of eluted (S) and retained (P) material are complementary.

Several aspects of the elution process were studied using both dialysis and dilution techniques. Spontaneous release of components I, II, and V was slow at 4°, but was completed within 15 min at 37°. Tris-HCl (pH 8) was inhibitory at a concentration of 10 mM, but not at 1 mM. Elution was not demonstrable at pH 5.8 and never occurred spontaneously when divalent cations were present.

Elution of Ghost Proteins at Elevated Ionic Strength. Brief incubation of ghosts with 0.5 M NaCl–5P(8) at 0° resulted in the complete elution of component VI (Figure 3C) together with minor components of higher mobility. The latter we judge to be hemoglobin monomers and other residual cytoplasmic components (see Figure 1A(H) and 1A(W)). Raising the incubation temperature to 37° had no effect on salt elution. The extract contained 4% of the protein, negligible sialic acid, and no PAS-positive lipids (Table III). The electrophorograms of the eluted (S) and retained (P) material are complementary, indicating that neither aggregation nor degradation occurred. Salt-treated ghosts were shrunken, spherical, and densely covered with spicules. They reverted to their normal

TABLE III: Protein and Sialic Acid Eluted from Ghosts.[a]

Fraction	Protein[b]		Sialic Acid[b]		Sialic Acid Protein (nM/mg)
	mg	%	nM	%	
Ghosts	2.20 ± 0.07	100	225[c]	100	102
Low-salt pellet	1.62 ± 0.05	74	240 ± 3	107	148
supernatant	0.53 ± 0.035	24	2.5 ± 4.8	1	5
Ghosts	1.22 ± 0.04	100	125[c]	100	102
High-salt pellet	1.22 ± 0.01	100	135 ± 3	108	111
supernatant	0.05 ± 0.004	4	0.3 ± 0.8	<0.5	6

[a] Low-salt treatment: ghosts in 0.9 ml of 5P(8) were diluted into 8.1 ml of 0.1 mM EDTA (pH 8) that had been warmed to 37°. The mixture was incubated at 37° for 20 min and then centrifuged for 30 min at 35,000 rpm in the Spinco 40 rotor at 4°. The pellet, consisting predominately of small vesicles, was resuspended in 0.8 ml of 5P(8) to a final volume of 1 ml. Samples of both supernatant and pellet were analyzed for protein and sialic acid and subjected to electrophoresis. High-salt treatment: ghosts in 0.5 ml of 5P(8) were diluted with 0.5 ml of 1 M NaCl in the same buffer. The mixture was allowed to stand for 20 min in an ice bath. The salt-treated ghosts were pelleted in 30 min at 35,000 rpm in the Spinco 40 rotor at 4°. The supernatant was decanted and the pellet resuspended in 0.95 ml of 5P(8). Both fractions were then dialyzed overnight against 10 mM Tris–1 mM EDTA (pH 8) at 4°. [b] Average of three determination plus and minus standard deviation. [c] Average of two determinations.

size and smooth biconcave contour when washed with 5P(8).

It is noteworthy that release of component VI was nearly complete when ghosts were treated with 0.15 M NaCl–5P(8). Intact erythrocytes retained this polypeptide through four washes in the same buffer.

Components III and IV. Like the sialoglycoproteins, components III and IV could not be solubilized under any of the conditions explored in elution experiments. Their release appeared to require substantial disruption of the membrane with certain effective solvents (*e.g.*, SDS, pyridine, phenol–acetic acid, and 2-chloroethanol).

Component III always appears as a sharp leading band with a diffuse trailing limb. We nevertheless consider this component to be either a single polypeptide or a family of closely related molecules. The most convincing evidence of this is the fact that controlled proteolysis of the inner membrane surface selectively degrades III (together with the sialoproteins), yielding a single, sharp major band of higher mobility plus minor low molecular weight products. This unique behavior involves both leading and trailing portions of the broad band (Steck *et al.*, 1971). We have referred above to the tendency of component III to aggregate selectively when ghosts are treated with organic solvents. Aggregation was also observed when ghosts were heated in the presence of low concentrations of SDS. This effect was particularly pronounced at high levels of salt. In all cases, such aggregation affected the entire band.

Component IV runs as a single sharp band in the presence of 1% SDS but splits into two equally sharp components when subjected to electrophoresis in 0.1 or 0.2% SDS. This and other recent observations (T. L. Steck, unpublished) suggest that IV is composed of roughly equal amounts of two distinct polypeptides whose mobilities are identical in 1% SDS.

Discussion

Our approach to the fractionation of erythrocyte membrane proteins evolved from earlier work of Fairbanks (1969) which demonstrated that polyacrylamide gel electrophoresis in the presence of high concentrations of SDS fractionates mixtures of polypeptides according to their molecular weight. The process of electrophoresis in SDS has been analyzed by many investigators. Control experiments confirm that the electrophoretic process does not normally introduce artifacts, but accurately reflects the state of the sample, whether prepared from soluble molecular weight standards (Shapiro *et al.*, 1967; Fairbanks, 1969; Weber and Osborn, 1969; Dunker and Rueckert, 1969) or dissolved membranes (Strauss *et al.*, 1968; Kiehn and Holland, 1968; Rosenberg and Guidotti, 1968; Fairbanks, 1969; Berg, 1969; Lenard, 1970a,b). There are minor reservations attached to this conclusion. Overloading the gel results in broadened bands that distort the pattern. Such overloaded bands increase the mobilities of minor components running just ahead of them and, in some cases, entrap material at their hypersharp leading edges (Fairbanks, 1969). Another reservation derives from the possibility that some artifacts due to intermolecular associations may occur; these have not been excluded in every case.

In the present study, the high sensitivity of coomassie blue staining has made possible the routine analysis of less than 50 μg of protein/gel, a level below that at which the resolution of bands I and II deteriorates and the pattern is distorted. It is unlikely that any of the major bands is an artifact generated by reversible intermolecular associations during electrophoresis, because various band combinations "run true" after isolation by elution. However, component III—if it is a single protein—migrates as if existing in more than one state. Its sharp leading edge and diffuse tail are suggestive of artifactual profiles seen when reversible association–dissociation processes occur during electrophoresis (Cann and Goad, 1968; Nichol *et al.*, 1964).

We wish to stress that although the electrophoretic process itself is relatively free from artifact, very dramatic artifacts may be produced in manipulating isolated membrane fractions and in preparing them for electrophoresis in SDS. Erythrocyte membrane proteins are prone to both (a) aggregation and (b) proteolytic degradation; under special circumstances, the two can be produced concurrently. Our routine protocol was designed to minimize these artifacts.

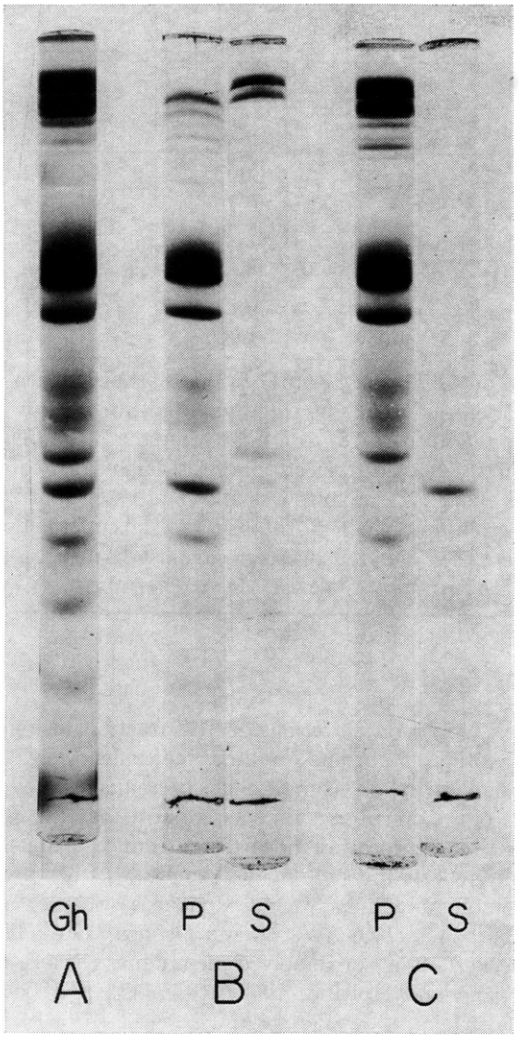

FIGURE 3: Electrophorograms of eluted polypeptides. Details of the elution procedures are given under Table III. (A) Intact ghosts washed with 5P(8)—24 μg applied. (B) Results of low-salt treatment: pellet (P—13 μg applied) and supernatant (S—2 μg applied) of $3 \times 10^6 g$ min spin after 1/10 dilution of ghosts into warm 0.1 mM EDTA (pH 8). (C) Results of high-salt treatment: pellet (P—24 μg applied) and supernatant (S—1 μg applied) of $3 \times 10^6 g$ min spin after incubation of ghosts at 0° with 0.5 M NaCl–5P(8).

We have observed irreversible aggregation of erythrocyte membrane proteins after exposure of the membranes to acid, organic solvents, and high temperatures. Component III aggregates most readily (the fate of the PAS-positive bands under these conditions was not studied) and, in this state, cannot be fractionated electrophoretically. There is no evidence that the observed aggregation—even that involving III selectively—is a reflection of specific interactions between polypeptide chains in the native membrane.

Proteolytic degradation is for two reasons a chronic problem in the analysis of samples by electrophoresis in SDS. First, the sample preparation involves protein denaturation by SDS, which can greatly increase the accessibility of peptide bonds to any relatively SDS-resistant proteinase in the mixture. Second, the electrophorograms are highly sensitive indicators of proteolysis because split products are dissociated and migrate faster than the intact polypeptides. We have observed—under circumstances that we normally avoid—the conversion of erythrocyte membrane polypeptides from the characteristic discrete set into a population with a lower average molecular weight and a diffuse electrophorogram. This phenomenon will be discussed in a future publication (G. Fairbanks and J. Avruch, in preparation). Here it suffices to summarize the observations that lead us to conclude that the apparent "depolymerization" results from proteolysis: at pH 7–8 this process is stimulated by salt and by low concentrations of SDS (SDS:protein ratios of about 1:2), but is prevented by high concentrations of SDS; furthermore it exhibits a temperature optimum.

The occurrence of proteinases in erythrocyte membranes has been suggested (Morrison and Neurath, 1953; Moore *et al.*, 1970), but we consider leukocytes a more likely source of proteinases. In preparing hemoglobin-free ghosts (Steck *et al.*, 1970; Dodge *et al.*, 1963), erythrocytes are washed four times in phosphate-buffered saline and, after each centrifugation, leukocytes are removed by aspiration of the "buffy coat" together with the top of the erythrocyte layer. Even when this is done most assiduously, however, white cells remain (aggregated platelets may also persist); after hypotonic lysis these sediment and aggregate to form an opaque "button" below the translucent ghost pellet. This "button" is very rich in proteolytic activity, and to prevent gross contamination of the ghosts, it is necessary to remove it efficiently (*e.g.*, by aspiration; see Materials and Methods) after the hemolyzed cells are first sedimented. Normally, some proteolytic activity remains after the usual three washes but is not manifested in electrophorograms because of the preventive measures taken: (a) rapid sample perparation; (b) high SDS concentrations during sample dissolution and electrophoresis; (c) low inorganic salt concentrations; and (d) inclusion of EDTA in sample and electrophoresis buffers. However, it can be detected when ghosts are incubated at moderate temperatures in the absence of SDS. (Compare, for example, the sialidase incubation control ("−E" in Figure 1C) with ghosts ("Gh" in Figure 1C) prepared for electrophoresis by the usual procedure.)

Laico *et al.* (1970) have suggested that the large erythrocyte membrane polypeptides are aggregates of small subunits ("miniproteins") that were missed in earlier investigations because the small polypeptides (a) were lost by leakage during dialysis in sample preparation, (b) migrated faster than the commonly used tracking dye, bromophenol blue, and (c) were not adequately fixed before staining. Our electrophoretic method circumvents all these potential difficulties. Dialysis is not required and the pyronin Y tracking dye moves ahead of lipids and small polypeptides. The fixation and staining procedure proved adequate to detect insulin and the small products of proteolytic degradation; it was, furthermore, found to yield sharper band patterns and better recoveries of low molecular weight material than 20-hr fixation in 20% sulfosalicylic acid (Laico *et al.*, 1970; Maizel, 1966, 1969). Clearly, if small polypeptides of 5000–10,000 daltons were present normally in samples of dissolved ghost membranes, they would have been detected by the electrophoretic method we have described. Yet no solubilization scheme—except those promoting peptide cleavage—yielded detectable amounts of "miniprotein" components. Even incubation of 95° with 2% SDS and DTT—conditions known to dissociate intransigent viral capsids (Maizel, 1969)—failed to alter the characteristic pattern of high molecular weight polypeptides. We therefore conclude that this represents the true size distribution of the erythrocyte membrane polypeptides and suggest that artifactual proteolysis is the most probable explanation for the apparent "depolymerization" of these large structures (Berg, 1969; Laico *et al.*, 1970; Kiehn and Holland, 1970; Furthmayr and Timpl, 1970).

The proteins of the erythrocyte membrane are unique in the predominance of high molecular weight components. In such a system proteolysis artifacts would be expected to be most dramatic. However, many other membrane preparations may contain proteinases and it is advisable to observe the precautions described above in dissolving all such samples.[3] Our experience with the red cell system also suggests a routine electrophoretic test for proteinases: their presence should be suspected if a shift toward lower average molecular weight is seen after incubation of the sample at a moderate temperature (30–50°) with a low level of SDS (*e.g.*, 0.1%) and a high concentration of salt (*e.g.*, 1 M).

The response of ghosts to manipulation of the ionic environment defined two distinct classes of membrane proteins. One set—comprising bands I, II, V, and VI—was readily eluted. The other—including components III and IV and the sialoglycoproteins—remained tightly bound to an extended framework with the membrane lipids. Densitometry of gels stained with three protein dyes indicated approximate molar equivalence for components (I + II), IV, V, and VI. Such measurements of relative abundance are accurate when applied to a group of similar proteins (*e.g.*, histones; Fambrough *et al.*, 1968) but are subject to large errors when the proteins vary greatly in composition and structure (Hansl, 1964; Fazekas de St. Groth, *et al.*, 1963). SDS appears to compensate for all but the most extreme variations by disrupting native conformation and neutralizing cationic groups. Thus, in SDS gel electrophorograms of *E. coli* envelope proteins, the profiles of coomassie blue staining and incorporated [^{14}C]-leucine are virtually identical (Fairbanks, 1969). Our densitometric measurements of the erythrocyte membrane polypeptides are supported by independent analyses of the protein in aqueous eluates. The molar ratio data encourage speculation that some of the major polypeptides may *in situ* be constituents of a larger multichain protein. It is evident, however, that because SDS solubilization disrupts quaternary structure, other methods must be used in studying the relationships between polypeptides.

The high molecular weight polypeptides, I and II, released by hypotonic treatment resemble the protein, "spectrin," first described by Marchesi and Steers (1968). Reported values for the size of the largest erythrocyte membrane polypeptides range from about 130,000 to 300,000 (Marchesi *et al.*, 1969a,b; Rosenberg and Guidotti, 1969a,b; Berg, 1969; Gwynne and Tanford, 1970; Furthmayr and Timpl, 1970; Lenard, 1970a; Hoogeveen *et al.*, 1970). In our electrophoretic system, these components exhibit molecular weights well above 200,000; by extrapolating the calibration curve above 130,000 (into a region where it is nonlinear on the semilog plot) we obtained a rough estimate of 250,000. This agrees most closely with the electrophoretic measurement by Lenard (1970a) and with sedimentation data of Gwynne and Tanford (1970) as corrected by them for an unusually high content of aliphatic amino acids.

The smaller component, V (mol wt 41,300), selectively eluted with I and II, was detected by Marchesi and Steers (1968) but has not been characterized, though our data (Table II) suggest a molar abundance near that of I plus II. Because I and II differ in size by about 40,000, it is conceivable that II and V are subunits of I. However, the calculated molar ratios (Table II) do not support this idea.

The selective elution of component VI (mol wt 36,200) with salt has not been described previously. Mitchell and Hanahan (1966) and Rosenberg and Guidotti (1969a,b) observed release of much larger amounts of protein during prolonged incubation at high salt levels. But our experience with this system leads us to suggest that much of this apparent solubilization resulted from proteolysis plus breakdown of the membranes to very small vesicles. We have observed that release of VI, together with degradation products from other polypeptides, occurs during limited proteolytic dissection of ghosts (Steck *et al.*, 1971).

Components III (mol wt 89,000) and IV (mol wt 77,500) appear to correspond to major bands recognized by Lenard (1970a,b), who assigned molecular weights of 108,000 and 86,000, respectively. The latter values may be slight overestimates reflecting the use of unreduced molecular weight markers including bovine serum albumin and its dimer (see Materials and Methods). The behavior of component III (mol wt 89,000) is of particular interest. It cannot be eluted from the ghost membrane by any of the simple means described; its molar frequency is more than twice that of the other major components studied; and it is distinguished by a tendency to aggregate under various conditions. Furthermore, component III, like the sialoglycoproteins, differs from the other major polypeptides in being cleaved by proteolytic attack at either surface of the membrane (Steck *et al.*, 1971). We suspect that these properties reflect hydrophobic associations and suggest that this predominant component may be a major structural element of the membrane. The data of Gwynne and Tanford (1970) are consistent with the idea that component III is tightly bound to lipids. Their gel filtration profiles in guanidine hydrochloride do not show a peak near 90,000, but otherwise resemble the molecular weight distribution in SDS gels. After the disruption of ghosts in guanidine hydrochloride, only about 60% of the protein was recovered in the clear lipid-free zone for gel filtration analysis (Gwynne and Tanford, 1970). The amount lost is consistent with our measurement of the abundance of III by densitometry of electrophorograms, and further experiments with guanidine hydrochloride have confirmed that, while the clear solution lacks component III, the lipid zone is enriched in this species (T. L. Steck, in preparation).

Our analysis of the PAS-positive components is consistent with earlier results of Winzler (1969a,b) and others showing that most of the erythrocyte membrane sialic acid is protein bound. However, we find that the major sialoglycoprotein (PAS-1) has an apparent molecular weight of 83,500 and a resistance to solubilization exceeded (among major components) only by III and IV. These results contrast with those of Kathan *et al.* (1961; Winzler, 1969a,b), who prepared the major sialoglycoprotein in a water-soluble form having an apparent molecular weight of 31,000. Using the compositional data of Winzler (Table II), it can be calculated that enzymatic hydrolysis of 78% of the bound sialic acid should have decreased the molecular weight of PAS-1 by 22%. The decrease observed was about two-thirds of this, suggesting that the contribution of sialic acid to the apparent molecular weight in SDS gels is less than that of protein or that the material analyzed by Winzler was partially degraded and thereby enriched in sialic acid.

The failure to detect PAS-1 with coomassie blue may be related to the unusually high proportion of acidic groups in the sialoglycoprotein. Pepsin, the only highly acidic protein

[3] This procedure is not, however, a panacea—no method of sample preparation tested protected the membrane proteins from extensive degradation when traces of pronase were added (T. L. Steck, unpublished data).

used as a molecular weight marker, also stains very poorly. In any case, the results caution us that major proteins of atypical composition may be missed in gels if only the common protein stains are used.

We have repeatedly observed striking differences between the properties of membrane proteins in intact erythrocytes and the properties of the same proteins in ghosts prepared by hypotonic lysis. No elution procedure released significant amounts of the major components from osmotically stabilized, intact erythrocytes. The behavior of component VI is an extreme example of the differential response to washing: after hemolysis, VI is substantially eluted by a single extraction with the same phosphate-buffered 0.15 M saline solution used in exhaustively washing the intact cells. We also find that, while the ghost membrane is susceptible to controlled proteolysis, the intact erythrocyte is extremely resistant to the same treatment (Steck *et al.*, 1971). Others have implied similar experience (Rosenberg and Guidotti, 1969b). The nature and degree of structural alteration that occurs in the membrane upon hemolysis are a question of general significance in membrane biochemistry, and are being actively investigated in our laboratories.

In summary, the results of properly controlled SDS gel electrophoresis demonstrate that the ghost membrane contains a collection of large polypeptides. By comparison to other membrane systems to which our analytical technique has been applied (J. Avruch, G. Fairbanks, B. R. Fernbach, A. E. Murray, and D. F. H. Wallach, unpublished observations) the red cell membrane is unique both in the large size of its predominant components, and in having a few components present in such large quantity that they together constitute over two-thirds of the protein. We have established systematic procedures for investigating erythrocyte membrane proteins that are distinguished by reproducibility, selectivity, high resolution, and freedom from artifact. The development of these methods has led to the recognition of three methodological problems that we stress because of their general significance. (1) The proteins are prone to degradation by proteolysis; (2) the major sialic acid bearing glycoprotein cannot be detected by the commonly used, highly sensitive protein stains; and (3) the composition of the washing medium dramatically influences the state of the membrane. The following paper (Steck *et al.*, 1971) demonstrates how, when these problems are carefully managed, the architecture of the isolated membrane can be analyzed by selective proteolysis of its inner and outer surfaces.

Acknowledgments

We are grateful to Drs. H. M. Kalckar and D. Stathakos for the valuable suggestion that proteolysis may be stimulated by SDS.

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Disposition of the Major Proteins in the Isolated Erythrocyte Membrane. Proteolytic Dissection*

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ABSTRACT: Vesicles derived from human erythrocyte membranes were separated into two fractions of normal and inside-out orientation. By treating each species with proteolytic enzymes, the two faces of the red cell membrane were selectively digested. The course of proteolysis was monitored by the consumption of alkali and by polyacrylamide gel electrophoresis of the membrane proteins dissolved in sodium dodecyl sulfate. By both criteria, whole ghosts and normally oriented vesicles were much more susceptible to proteolysis than inside-out vesicles. One protein was intrinsically resistant to proteolysis. All of the other major components that could be stained with coomassie blue were extensively digested at the outer surface. Only one of these, a predominant component of mol wt 89,000, was attacked by digestion of inside-out vesicles. The three sialoglycoproteins, which were detectable only by carbohydrate staining, were digested by proteolytic attack at either surface. The data are not consistent with a symmetrical membrane organization, but rather suggest a highly asymmetric arrangement of oriented proteins, at least some of which appear to span the thickness of the membrane.

The proteins of the human erythrocyte membrane have recently been analyzed and partially characterized in several investigations (see Fairbanks *et al.*, 1971). The molecular architecture of this plasma membrane has likewise been intensively studied, but remains more elusive. The present study sought to determine how the major component proteins were represented at the two faces of the isolated erythrocyte membrane. We asked: "Does each protein type equally and symmetrically populate each surface; do certain proteins exist at one surface and not the other; do any major proteins penetrate from the outer to the inner surface; and if there are penetrating proteins, are all molecules of one type oriented in the same direction or are they symmetrically or randomly disposed?"

Our study was made feasible by the development of methods for the preparation and purification of inside-out membrane vesicles, which bare the membrane's cytoplasmic face to selective attack, just as intact ghosts and right-side-out vesicles proffer the normal outer surface. It was assumed (see Discussion) that the proteins in these vesicles retained the orientation they held in the parent membrane. By means of gentle digestion with impermeable proteolytic enzymes, each of the membrane's two faces were probed separately; the consequences were assessed by polyacrylamide gel electrophoresis of the membrane proteins dissolved in SDS.[1]

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; IO, inside out; RO, right-side out; PAS, periodic acid–Schiff; TD, tracking dye; I–VI, numerals designating the six major protein components demonstrated by electrophoresis; PAS-1–3, the three major glycoprotein components demonstrated by electrophoresis (Fairbanks *et al.*, 1971).