Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 1733–1737, March 1984
Cell Biology

Detection of erythrocyte membrane proteins, sialoglycoproteins, and lipids in the same polyacrylamide gel using a double-staining technique

(silver stain/Coomassie blue/electrophoresis/sialic acid)

JAMES K. DZANDU, MERCY E. DEH, DENISE L. BARRATT, AND GARY E. WISE*

Texas College of Osteopathic Medicine/North Texas State University, Department of Anatomy, Camp Bowie at Montgomery, Fort Worth, TX 76107

Communicated by Keith R. Porter, November 30, 1983

ABSTRACT **A silver/Coomassie brilliant blue R-250 staining technique that permits a color-coded differentiation of erythrocyte membrane proteins, sialoglycoproteins, and lipids in a single one-dimensional NaDodSO$_4$/polyacrylamide gel has been described. Gels stained first with silver stain and then with Coomassie blue (CB) showed the characteristic blue staining of all conventional CB-sensitive membrane polypeptides, whereas periodic acid-Schiff reagent-sensitive sialoglycoproteins and lipids stained yellow. Several yellow Ag-stained bands corresponding to major and minor sialoglycoproteins were detected at $M_r \times 10^{-3}$ of 88, 72, 65, 41, 35, 31, 28, 24, and 20. Neuraminidase treatment of intact erythrocytes caused shifts in the electrophoretic mobilities of several yellow-stained bands without affecting the CB-stained polypeptide pattern. These observations afforded evidence that the yellow-staining bands were sialoglycoproteins and lipids. The double-staining technique was used in a topological analysis of the membrane surface of the erythrocyte using protease digestion and selective solubilization. Trypsin cleaved the yellow bands at M_r 88,000 and 41,000. Membrane-associated cleavage products were noted at M_r 58,000 and 38,000. Pronase treatment of intact cells gave membrane-associated cleavage products at M_r 38,000 (yellow) and two CB-stained bands at M_r 58,000 and 60,000. These results suggested that the double-staining technique may be applicable in compositional and topological analyses of other biological membranes.**

The successful application of NaDodSO$_4$/polyacrylamide gel electrophoresis as a technique for resolving complex mixtures of macromolecules has particularly enhanced the compositional and topological analysis of biological membranes. One consistent problem encountered in these studies, however, is the nonavailability of simple and sensitive methods for selective detection of the different classes of membrane constituents present in the same gel. Coomassie brilliant blue R-250 (CB) and periodic acid-Schiff (PAS) reagent staining of proteins and sialoglycoproteins (1), respectively, permit visualization of only one major class of membrane constituents in parallel gels. In addition, important compositional and topological information on trace membrane components are often lost because of the low sensitivity of these stains. Alternatively, covalent radioactive ligand labeling of membrane constituents has been used extensively to augment the sensitivity of macromolecule detection in gels (2–5).

We now report a silver/CB double-staining technique that permits a simple color-coded detection and direct visualization of human erythrocyte membrane proteins, sialoglycoproteins, and lipids in the same gel without recourse to radioisotopes. The technique to be described uses silver staining of gels first and then CB staining. It differs significantly from the recent double-staining protocol described by Irie *et al.* (6). In their description, Ag stain was used after CB to essentially increase the sensitivity of trace protein detection in gels without benefit of chemical selectivity. Conversely, our technique stains all the conventional CB-sensitive peripheral erythrocyte membrane polypeptides and the anion channel (band 3, Steck's nomenclature; see ref. 7) blue, whereas the sialoglycoproteins and lipids stain with Ag (yellow) in the same gel.

The effect of neuraminidase treatment of intact erythrocytes on the double-stained gel pattern of ghosts was used to decode the color differential and to provide experimental evidence for our assignment of the yellow color to sialoglycoproteins. We further used trypsin and Pronase digestion of intact erythrocytes to show specific applications of this technique in cell-membrane surface analysis.

MATERIALS AND METHODS

Materials. The following reagents were purchased from Sigma: *N*-acetylneuraminic acid (AcNeu), soybean trypsin inhibitor, neuraminidase (type VIII; lot 129C80851) purified from *Vibro cholerae*, and molecular weight protein standards. Trypsin L-(tosylamido 2-phenyl)ethyl chloromethyl ketone was obtained from Worthington. Pronase-CB was bought from Calbiochem and silver-stain kit was purchased from Bio-Rad. All other chemicals used were of the highest grade available, and glass distilled deionized water was used throughout. Heparinized blood samples were obtained from normal healthy volunteers. All gave informed consent.

Isolation of Erythrocytes. Heparinized whole human blood was routinely filtered through a bed of cotton wool to remove platelets and leukocytes (8). Erythrocytes were sedimented by centrifugation at 1000 × *g* for 5 min and then washed 3 times in 5 mM Tris·HCl/140 mM NaCl/1.0 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride (PhMeSO$_2$F), pH 7.4, at 0–4°C.

Trypsin and Pronase Treatment of Intact Erythrocytes. Isolated intact erythrocytes were washed 3 times in 5 mM Tris·HCl, pH 7.4/140 mM NaCl. Trypsin or Pronase was added to the cell suspension (20% hematocrit) to a final enzyme concentration of 100 μg/ml and then incubated at 25°C for 1 hr as described (5). Digested cells were washed 5 times in ice-cold 5 mM Tris·HCl/140 mM NaCl/1 mM EDTA/0.1 mM PhMeSO$_2$F, pH 7.4.

Neuraminidase Treatment of Intact Erythrocytes. Washed intact erythrocytes were incubated with neuraminidase as described by Aminoff *et al.* (9). Cells were suspended (50% hematocrit) in 5 mM sodium acetate/140 mM NaCl/10 mM CaCl$_2$/0.1 mM PhMeSO$_2$F, pH 5.1, and neuraminidase was added to a final concentration of 1.1 unit per ml of packed

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CB, Coomassie brilliant blue R-250; PAS, periodic acid-Schiff; PhMeSO$_2$F, phenylmethylsulfonyl fluoride; AcNeu, *N*-acetylneuraminic acid.

*To whom reprint requests should be addressed.

cells. Incubation at 37°C was accompanied by mechanical agitation. At various time intervals, 200-μl aliquots of cell suspension were removed and quenched in chilled tubes containing 100 μl of 5 mM Tris·HCl/140 mM NaCl/10 mM EDTA/0.1 mM $PhMeSO_2F$, pH 7.4. Cells were pelleted at 1000 × *g* for 5 min and duplicate 25-μl clear supernatant solutions were removed for the analysis of the liberated AcNeu. The corresponding cell pellets were washed and used for ghost preparation. A parallel incubation without neuraminidase was used both as control and for hematocrit determination.

Neuraminic Acid Determination. The thiobarbituric acid method of Warren (10) as modified by Skoza and Mohos (11) was used. Twenty microliters of packed ghosts (2.2×10^8 ghosts) and 25 μl of digest supernatant were used in the assays. All determinations were made in duplicate.

Preparation of Erythrocyte Membranes. Packed erythrocytes, control and enzyme treated, were hemolyzed in 30 vol of ice-cold 5 mM Tris·HCl/7 mM NaCl/1 mM EDTA/0.1 mM $PhMeSO_2F$, pH 8.0, and then centrifuged at 20,000 × *g* for 15 min in a Sorvall RC-2B centrifuge (0–4°C). Pelleted membranes were resuspended in the same buffer and washed repeatedly until creamy white.

Isolation of Integral Membrane Proteins. Ghosts were stripped of peripheral proteins by addition of 10 vol of ice-cold 0.1 M NaOH as described by Steck and Yu (12). Integral membrane proteins were sedimented by centrifugation at 20,000 × *g* for 15 min and then washed once without suspension in hemolysis buffer. The pellet was dissolved directly in $NaDodSO_4$ electrophoresis sample buffer (13).

Sialoglycoprotein Preparation. One volume of ghost suspension (50% hematocrit) in hemolysis buffer was extracted with 2.5 vol of chloroform/methanol/0.1 M HCl (vol/vol; 10:10:1) according to the procedure of Hamaguchi and Cleve (14). Sialoglycoproteins were recovered in the clarified aqueous phase.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out on isotropic 11% (wt/vol) acrylamide slab gels (16 × 18 × 0.15 cm) using the discontinuous buffer system of Laemmli (13). A constant current of 50 mA per slab was applied until the bromophenol blue tracking dye front was about 1.5 cm from the bottom of the gel. Molecular weight protein standards used were myosin heavy chain, galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase. Specific erythrocyte membrane polypeptide bands 1, 2, 4.1, 4.2, 5, 6, 7, and 8 were also used as internal molecular weight standards.

Double Staining. The silver-staining procedure was essentially a modification of the method of Merril *et al.* (15). Double deionized glass distilled water was used to prepare all solutions. Gentle mechanical agitation was used at all stages to facilitate reagent circulation. Handling of gels was minimized by using an aspirator to remove reagents and wash solutions. All staining procedures were carried out in Pyrex glass dishes (34 × 20 × 5.6 cm).

Step 1. Each gel slab was fixed in 500 ml of 40% (vol/vol) methanol/10% (vol/vol) acetic acid for 1 hr at room temperature. Residual $NaDodSO_4$ was removed by two consecutive 30-min washes in 500 ml of 10% (vol/vol) ethanol/5% (vol/vol) acetic acid.

Step 2. The gel was equilibrated with 200 ml of oxidizer reagent containing 3.4 mM potassium dichromate/3.2 mM nitric acid for 10 min followed by three washes with distilled water (500 ml per wash) until the background was clear.

Step 3. The gel was transferred into 200 ml of 20 mM silver nitrate for 30 min, followed by three 1-min rinses with 500 ml of distilled water to remove residual unbound silver. This was verified by the absence of cloudiness when one drop of 1 M HCl was added to 1 ml of the final rinse.

Step 4. Band development was initiated by the addition of 200 ml of warm (40°C) developer solution (0.28 M Na_2CO_3/0.008% paraformaldehyde), which was changed 2 or 3 times when it became light brown. Positively stained yellow bands and negative optical images of unstained bands appeared within 1 hr. The developer solution was decanted, the gel was rinsed with distilled water, and 200 ml of 10% (vol/vol) acetic acid was added to enhance the yellow color development. Gels were photographed on a fluorescent light box (Ladd Research Industries, Burlington, VT) using color film (Kodacolor CG 135) with orange filter.

Step 5. Each silver-stained gel was counter-stained with 200 ml of 0.1% (wt/vol) CB/25% (vol/vol) methanol/7.5% (vol/vol) acetic acid for 1 hr, destained with 25% (vol/vol) methanol/7.5% (vol/vol) acetic acid, and then photographed again in color.

One may use the silver-stain kit from Bio-Rad instead of making up the individual solutions. Similar Ag-stained patterns are observed with the Bio-Rad solutions.

Gels were loaded with samples ranging from 1.25 μl (6.5 μg of ghost protein) to 30 μl of packed ghost equivalents. No difference in the double-staining pattern was observed.

RESULTS

Comparison of Stains. Fig. 1 compares gel patterns of ghosts (Fig. 1, lanes A, D, and F), integral membrane fraction—i.e., ghost NaOH pellets—(lanes B, E, and G), and partially purified sialoglycoproteins (lanes C and H). These gels were stained with Ag only (lanes A–C), CB only (lanes D and E), and the Ag/CB double stain (lanes F–H).

Ag Stain Only. The results show yellow staining of specific bands in ghosts (lanes A and F), integral proteins (lanes B and G), and partially purified sialoglycoproteins (lanes C and H). Negative images are also observed at specific regions of the gel (lanes A–C). These clear negative images correspond to CB-stained membrane polypeptides. This is well illustrated by comparing lanes A–C with lanes F–H.

Overdevelopment of silver-stained gels (step 4 of Ag-staining protocol) using higher paraformaldehyde concentrations, longer development time, or higher temperatures results in indiscriminate staining of all polypeptides, including the negative images (not shown). Similarly, incomplete removal of residual unbound silver before initiation of band development caused increased background and indiscriminate staining.

Careful molecular weight measurements on 11% Laemmli gels showed that the yellow Ag-stained bands migrated with apparent $M_r \times 10^{-3}$ of 88, 72, 65, 41, 35, and 31. In addition, 3–5 yellow Ag-stained bands were detected in the 20,000–28,000 M_r range. A prominent yellow-staining band was consistently noted in the region just beyond the bromophenol blue tracking dye front. We inferred that this was lipid. Parallel gels stained with PAS reagent showed corresponding bands at $M_r \times 10^{-3}$ of 88, 65, 41, and 28 and in the lipid region (not shown). The PAS staining was less sensitive and did not detect the minor yellow Ag-stained bands. These results indicated that the yellow Ag-stained bands correspond to the membrane sialoglycoproteins and lipids.

CB Stain Only. Fig. 1 (lanes D and E) shows typical CB-staining patterns of unfractionated ghosts and NaOH pellet. All conventional CB-sensitive membrane polypeptides are clearly visible in the unfractionated ghost gel in accord with published results (1, 7, 16). Only bands 3 and 7 stain with CB in the ghost NaOH pellet (12). The partially purified major sialoglycoproteins at concentrations used were CB insensitive (not shown). The positions occupied by the CB-stained polypeptides (lanes D and E) correspond precisely with those of the negative images seen on gels stained with Ag only (lanes A–C).

Ag/CB Double Stain. The Ag/CB double-stained patterns

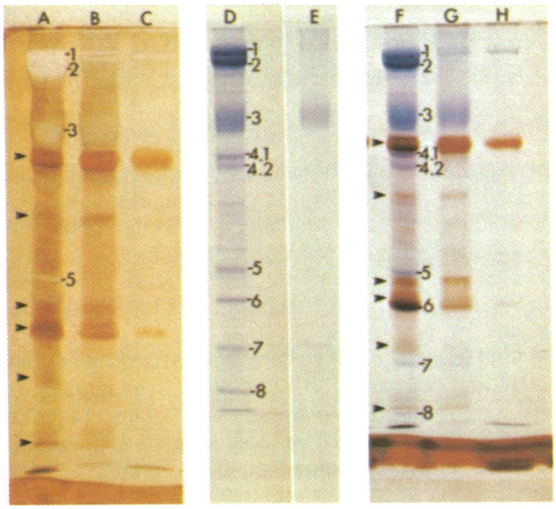

FIG. 1. Comparison of stains. Lanes: A–C, 11% Laemmli gels stained by Ag stain only; D and E, CB only; and F–H, Ag/CB. Gel patterns of unfractionated ghosts (lanes A, D, and F), insoluble ghost NaOH pellets (lanes B, E, and G), and sialoglycoproteins (lanes C and H) are shown. Arrowheads in lanes A and F denote yellow Ag-stained bands at $M_r \times 10^{-3}$ of 88, 65, 41, 35, 31, and 20. Identical yellow bands are seen in lanes B and G. Negative images or optically clear bands are also seen (lanes A–C), which correspond to several CB-sensitive polypeptides (lanes F–H). The partially purified sialoglycoproteins show distinct yellow bands. Fifteen microliters of packed ghosts or equivalents were applied to each gel slot.

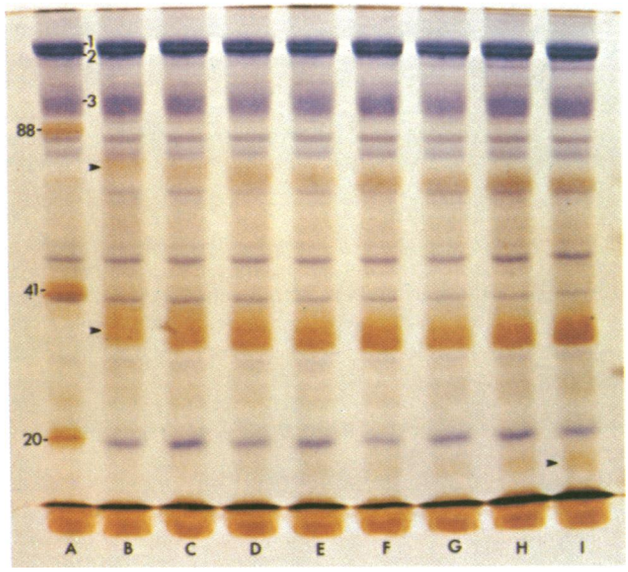

FIG. 2. Time course of desialylation of intact erythrocytes by neuraminidase treatment as assayed by Ag/CB double staining. Ghosts were isolated from cells taken at the time periods (min) indicated as follows. Lanes: A (0), B (5), C (10), D (15), E (30), F (40), G (60), H (75), and I (90). Electrophoresis was on 11% Laemmli gels; 10% of control (lane A) neuraminic acid remained in the 90-min time point sample (lane I). Note the shifts in the yellow bands at M_r 88,000, 41,000, and 20,000 to limiting M_r values of 66,500, 29,000, and 15,000, respectively.

of ghosts, integral proteins, and the major sialoglycoproteins are shown (Fig. 1, lanes F–H). All of the yellow Ag-stained sialoglycoproteins stained by Ag only (lane A) and all of the membrane proteins stained by CB only (lane D) are visible on the double-stained gel (lane F). The results show the selectivity of the double stain to differentiate CB-stained polypeptides and Ag-sensitive sialoglycoproteins with high fidelity. The anion channel (band 3), PAS-1, and band 4.1 can all be distinguished by their differential staining despite the fact that they almost overlap in this region of the gel (lane F). Monochromatic silver stain (lanes A–C), CB (lanes D and E), or PAS stain alone do not permit easy assessment of the relative positions occupied by these different membrane constituents in the same gel. This problem is overcome with the double stain (lanes F–H).

The double-stain pattern of the integral proteins (Fig. 1, lane G) shows that cold 0.1 M NaOH removes all CB-sensitive peripheral polypeptides and permits direct visualization of the transmembrane proteins. The intensity of the yellow bands relative to the CB-stained bands indicates the greater sensitivity of the Ag stain, given that the sialoglycoproteins comprise only 10% (wt/wt) total membrane proteins (17). The yellow Ag-stained bands seen as the major constituents of the Hamaguchi and Cleve (14) extract (lanes C and H) are further evidence that the sialoglycoproteins differentially stain yellow with Ag.

Fig. 2 shows the double-stained gel pattern of ghosts taken at various time intervals during neuraminidase treatment of intact cells. The zero time point sample showed specific yellow staining of all sialoglycoproteins and lipids. Correlative with the kinetics of AcNeu released from the cell surface (see Fig. 4), a systematic time-dependent shift and greater size dispersion of all yellow-staining sialoglycoproteins to

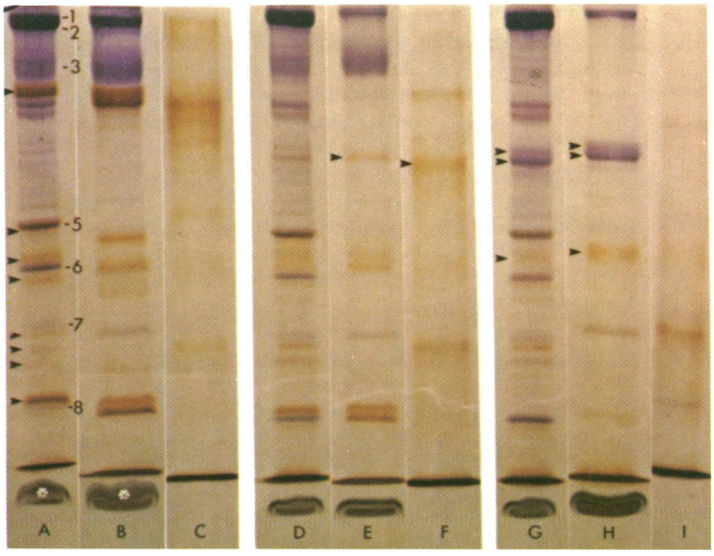

FIG. 3. Topological analysis. Isotropic 11% Laemmli gels of ghosts, insoluble ghost NaOH pellets, and sialoglycoproteins, respectively, from control untreated erythrocytes (lanes A–C), trypsinized cells (lanes D–F), and Pronase-digested cells (lanes G–I). All were double stained. The results of selective protease digestion is directly visualized as the disappearance of specific bands and concomitant appearance of lower M_r proteolytic membrane-associated cleavage products. Trypsin cleaved yellow Ag-stained bands at $M_r \times 10^{-3}$ values of 88 and 41 (lanes D–F). Cleavage products appeared at $M_r \times 10^{-3}$ of 58 and 38. Yellow bands at $M_r \times 10^{-3}$ of 28 and 20 were resistant to trypsinization. Pronase cleaved CB-stained band 3 and two Ag-stained bands at $M_r \times 10^{-3}$ of 88 and 41. Cleavage products can be seen at $M_r \times 10^{-3}$ of 60 and 58 (both CB-stained) and at M_r at 38,000 (yellow Ag-stained).

positions of higher electrophoretic mobilities ($M_r \times 10^{-3}$, 66.5, 29, and 15) was observed (Fig. 2). No change in the positions of the CB-stained polypeptides or the yellow Ag-stained lipid band was noted over the entire incubation period.

Proteolytic Analysis of the Erythrocyte Membrane Topology. The ability of the double-staining technique to simultaneously detect different membrane components in the same gel was exploited in the study of the organization of the membrane. Trypsin and Pronase were used to cleave specific externally located membrane polypeptides. Control ghosts and ghost derivatives prepared from cells digested with proteases were electrophoresed, and their double-staining patterns were analyzed for membrane-associated cleavage products.

Control Ghosts. Ghosts and derivatives prepared from unproteolyzed erythrocytes show typical double-staining patterns (Fig. 3, lanes A–C).

Trypsin-Treated Erythrocytes. Trypsin treatment caused the disappearance of the yellow Ag-stained bands at M_r 88,000 and 41,000 (Fig. 3, lanes D and E). Membrane-associated proteolytic products were noted at M_r 58,000 and 38,000. Trypsin did not cleave the yellow Ag-stained band at M_r 28,000 and 20,000. We observed a small diminution in the CB stain of band 3 as a result of trypsin treatment of intact cells.

Pronase-Treated Erythrocytes. Fig. 3 (lanes G–I) shows the effects of Pronase treatment on intact cells. Pronase cleaved band 3 (CB stained) and the yellow Ag-stained bands at M_r 88,000 and 41,000. Membrane-associated cleavage products were seen at M_r 60,000, 58,000 (both CB stained), and 38,000 (yellow Ag-stained).

Quantitation and Kinetics of Neuraminidase Treatment. AcNeu moieties present in covalent linkage on the external surface of the cell were subjected to enzymatic removal by neuraminidase. The time course of the AcNeu released into the incubation medium and residual covalently bound AcNeu remaining on the cell surface were determined. The rate and extent of AcNeu release is shown in Fig. 4. AcNeu content of control untreated erythrocytes from seven determinations was 399.3 ± 48.0 nmol per ml of packed erythrocytes. It can be seen (Fig. 4) that 90% of bound AcNeu was released as free AcNeu, leaving 10% still present in covalent linkage.

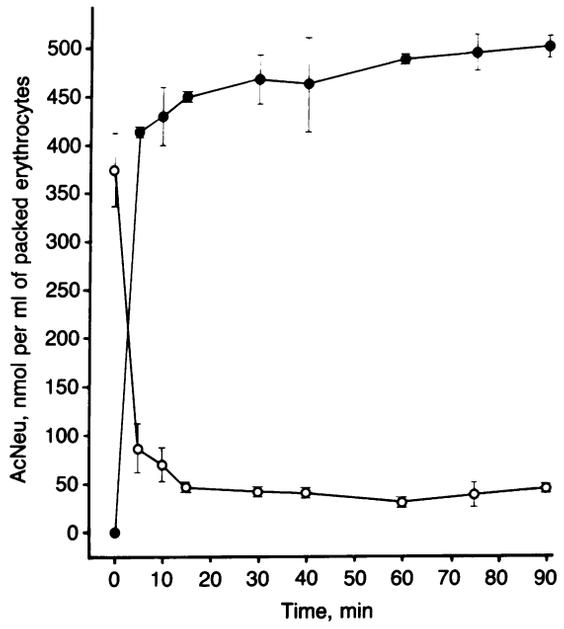

FIG. 4. Kinetics of AcNeu release from intact erythrocytes by neuraminidase treatment. Free AcNeu released into the incubation medium (●) and residual AcNeu present in covalent linkage in ghosts (○) were quantitated at the time periods indicated.

DISCUSSION

During Ag staining of normal human erythrocyte membranes on $NaDodSO_4$/polyacrylamide gels using a modification of the method described by Merril *et al.* (15), both positive and negative bands were observed (Fig. 1, lanes A–C). On counterstaining these gels with CB, the negatively stained bands stained blue (lanes F and G) without loss of the positive yellow bands.

The double-stained gel pattern of ghosts showed that the blue (CB) and yellow (Ag) bands stained in a mutually exclusive fashion. All the conventional CB-stained membrane polypeptides were present in stoichiometric amounts, suggesting that yellow Ag-stained bands represent a subclass of membrane constituents differentiated by their chemical reactivity toward the silver-stain reagents.

The yellow Ag-stained bands at $M_r \times (10^{-3})$ of 88, 72, 65, 41, 35, 28, 24, and 20 are consistent with the $M_r \times 10^{-3}$) values of 105, 68, 47, 38, 35, 27, and 24 reported for sialoglycoproteins by Mueller *et al.* (18) on 12.5% Laemmli gels. Thompson *et al.* (19) also reported the presence of several sialoglycoprotein bands on 11.5% Laemmli gels with the major components at $M_r \times 10^{-3}$ of 80, 38, and 24. The use of high acrylamide gel concentrations (≥11%; wt/vol) and the resolving power of the Laemmli buffer system clearly show the heterogeneity of this class of membrane components having in common terminal sialic acids.

Several lines of experimental evidence suggest that in the double-stain profile of ghosts, the yellow Ag-stained bands are sialoglycoproteins and lipids. First, partially purified authentic membrane sialoglycoproteins (Hamaguchi and Cleve ghost extracts) stained yellow in parallel gels (Fig. 1, lanes C and H) and migrated with identical electrophoretic mobilities under the same conditions. Second, parallel gels of ghosts stained with Ag only or with PAS reagent only showed identical bands at $M_r \times 10^{-3}$ of 88, 65, 41, 35, 28, and at the solvent front. However, because the Ag stain is more sensitive than PAS reagent, the former detected several minor bands not stained with PAS. In fact, the M_r 88,000 sialoglycoprotein was detected in as little as 1.25 μl of packed ghost. Third, we observed shifts in the M_r yellow Ag-stained bands toward lower M_r values on desialylation (Fig. 2). The kinetics of AcNeu released correlated with shifts in M_r (compare Figs. 2 and 4). The M_r shifts were accompanied by greater molecular size dispersion as inferred from increased bandwidths. It is unlikely that the shifts toward low M_r were due to proteolysis, because EDTA and $PhMeSO_2F$ were always included in buffers during ghost preparations. Furthermore, none of the CB-stained polypeptides present in the neuraminidase-treated samples showed any evidence of proteolysis.

Exhaustive neuraminidase treatment removed 90% of the total sialyl groups present on the cell surface, consistent with recent reports (20). Chemical hydrolysis in 0.05 M H_2SO_4 at 80°C for 1 hr, however, caused complete release of all sialyl groups from ghosts. We calculated that, based on the amount of desialylated ghosts electrophoresed (15 μl of packed ghosts), the residual sialoglycoprotein present was equivalent to 1.5 μl of control packed ghosts. This amount was high enough to stain yellow, which we also found (Fig. 2, lane I). Enzymatic release of 90% of total membrane-bound sialic acids caused yellow Ag-stained bands at $M_r \times 10^{-3}$ of 88, 41, and 20 to migrate at limiting $M_r \times 10^{-3}$ values of 66.5, 29, and 15, respectively. Based on the compositional data of Winzler (21, 22), the expected decrease in molecular weight of the M_r 88,000 sialoglycoprotein after release of

90% AcNeu would be 88,000 × 0.9 × 0.278 = 22,018, or 25%. We observed a decrease in M_r of 21,500, or 24.4%, in good agreement with theoretical predictions.

The presence of Ag staining beyond the bromophenol blue tracking dye front (best seen in Fig. 3, lanes A, B, D, E, G, and H) and its absence from the Hamaguchi-Cleve extract (lanes C, F, and I) suggests that Ag stains membrane lipids as well. Staining due to lipid derivatives such as fatty aldehydes (23) or malonaldehyde derived from peroxidation of polyunsaturated fatty acids (24) may also account for the yellow color.

With the Ag/CB double-staining format, polypeptides stained by CB and sialoglycoproteins stained by Ag can be differentially visualized in the same gel (e.g., Fig. 1, lane F). In previous studies (5, 12), band 3 and the sialoglycoproteins and lipids that constitute the integral membrane fractions were never directly observed simultaneously in the same gel, whereas they can be seen using double staining. Stripping the ghost of peripheral proteins also enhanced detection of various trace sialoglycoproteins that comigrated with CB-stained polypeptides. For example, removal of band 5 (M_r, 43,000) permits visualization of the M_r 41,000 sialoglycoprotein (Fig. 1, lane G). Moreover, the fidelity and sensitivity of the Ag stain in the double-stain format is as good as in monochromatic Ag-staining form. The minor sialoglycoproteins with $M_r \times 10^{-3}$ of 20, 22, 24, and 28 seen in gels stained with Ag only (Fig. 1, lanes A and C) are also visible in the double-stained gels (lane F).

The advantages of the double stain were exploited in a topological study of the membrane surface using trypsin or Pronase digestion and selective solubilization. In agreement with published results (5, 25), trypsin digestion of intact cells caused the disappearance of PAS-1 (yellow Ag-stained band; M_r, 88,000) and the appearance of the yellow Ag-stained cleavage product at M_r 38,000. Trypsin also cleaved the M_r 41,000 sialoglycoprotein. The M_r 38,000 cleavage product of PAS-1 trypsinization probably represents a dimer associated with the membrane as reported (5).

Pronase digestion caused complete hydrolysis of PAS-1 and band 3, as evidenced by the disappearance of both yellow (Ag) and blue (CB) bands in the band 3 region. The cleavage products were identified at M_r 60,000, 58,000, and 38,000. The cleavage products at M_r 60,000 and 58,000 were CB stained and derived from band 3. The M_r 38,000 yellow Ag-stained band was derived from PAS-1. Mueller and Morrison (25) identified genetic variants of band 3 (anion channel), which on Pronase treatment in intact erythrocytes yielded cleavage products at $M_r \times 10^{-3}$ of 60 and 63. Our results have shown that similar polypeptides (M_r, 58,000 and 60,000) are obtained when erythrocytes from some individuals are treated with Pronase.

It is suggested that the double-staining technique described represents a valuable tool for both compositional and topological analysis of cell membranes. In addition, this technique would facilitate the interpretation of two-dimensional gel patterns of membrane, because it permits differential detection of proteins, sialoglycoproteins, and lipids.

We thank Mr. Kelly J. Pace for photography, Ms. Mary H. Caffrey for the art work, and Ms. Patricia Webb for secretarial assistance. This work was supported in part by a Texas College of Osteopathic Medicine Faculty Research Grant to G.E.W.

1. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10,** 2606–2617.
2. Phillips, D. R. & Morrison, M. (1971) *Biochemistry* **10,** 1766–1771.
3. Staros, J. V. & Richards, F. M. (1974) *Biochemistry* **13,** 2720–2726.
4. Sears, D. A., Friedman, J. M. & George, J. N. (1977) *J. Biol. Chem.* **252,** 712–720.
5. Johnson, R. M., McGowan, M. W., Morse, P. D., II, & Dzandu, J. K. (1982) *Biochemistry* **21,** 3599–3604.
6. Irie, S., Sezaki, M. & Kato, Y. (1982) *Anal. Biochem.* **126,** 350–354.
7. Steck, T. L. (1974) *J. Cell Biol.* **62,** 1–19.
8. Beutler, E., West, C. & Blume, K. G. (1976) *J. Lab. Clin. Med.* **88,** 328–333.
9. Aminoff, D., Bell, W. C., Fulton, I. & Ingebrigtsen, N. (1976) *Am. J. Hematol.* **1,** 419–432.
10. Warren, L. (1959) *J. Biol. Chem.* **234,** 1971–1975.
11. Skoza, L. & Mohos, S. (1976) *Biochem. J.* **159,** 457–462.
12. Steck, T. L. & Yu, J. (1973) *J. Supramol. Struct.* **1,** 220–232.
13. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
14. Hamaguchi, H. & Cleve, H. (1972) *Biochim. Biophys. Acta* **278,** 271–280.
15. Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) *Science* **21,** 1437–1439.
16. Mueller, T. J. & Morrison, M. (1977) *J. Biol. Chem.* **252,** 6573–6576.
17. Winzler, R. J. (1970) *Int. Rev. Cytol.* **29,** 77–125.
18. Mueller, T. J., Dow, A. W. & Morrison, M. (1976) *Biochem. Biophys. Res. Commun.* **72,** 94–99.
19. Thompson, S., Rennie, C. M. & Maddy, A. H. (1980) *Biochim. Biophys. Acta* **600,** 756–768.
20. Stibler, H. & Borg, S. (1982) *Drug Alcohol Depend.* **10,** 85–98.
21. Winzler, R. J. (1969) in *Cellular Recognition*, eds. Smith, R. T. & Good, R. A. (Appleton-Century, New York), p. 11.
22. Winzler, R. J. (1969) in *Red Cell Membrane, Structure and Function*, eds. Jamieson, F. A. & Greenwalt, T. J. (Lippincott, Philadelphia), p. 157.
23. Dodge, J. T. & Phillips, G. B. (1967) *J. Lipid Res.* **8,** 667–675.
24. Niehaus, W. G., Jr., & Wold, F. (1970) *Biochim. Biophys. Acta* **196,** 170–175.
25. Mueller, T. J. & Morrison, M. (1977) *J. Biol. Chem.* **252,** 6573–6576.