Photochemical Labeling of the Surface Proteins of Human Erythrocytes†

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ABSTRACT: The reagent *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-taurine) has been synthesized and employed in a study of the human erythrocyte cell surface. Photolysis of NAP-taurine produces a highly reactive nitrene which acts as a general covalent labeling reagent. In leaky ghost preparations all detectable protein components are labeled as has been found with most other labeling procedures. In intact cells labeled from the exterior, sodium dodecyl sulfate gel bands 3, PAS-1, and PAS-2 are labeled as previously found by others (band nomenclature adapted from Fairbanks *et al.* (1971), *Biochemistry 10*, 2606). In addition bands 2.1, 2.2, 2.4, 2.5, and 2.6 are labeled with NAP-taurine. It is suggested that these components are present at the exterior surface, but that few if any nucleophilic groups are exposed for reaction with the small molecule reagents previously used and that they are inaccessible to the enzyme probes thus far employed.

Only recently have physical measurements such as differential scanning calorimetry (Steim *et al.*, 1969; Melchior *et al.*, 1970) and X-ray diffraction (Engelman, 1971; Wilkins *et al.*, 1971) confirmed the bilayer model of Gorter and Grendel (1925) as the principal structure of membrane lipids. Including the protein components which are normally about one-half of the mass, the present general model for a membrane is that of a lipid bilayer into which globular membrane proteins are inserted either partially or all the way through the membrane (see, for example, Singer, 1971; Vanderkooi and Green, 1970). This mosaic of lipid and protein is thought to resemble a two-dimensional liquid, with lipid–lipid, lipid–protein, and possibly protein–protein associations continually being made and broken. The lipid bilayer is seen as providing an inert barrier in which are positioned various functional sites (Stoeckenius, 1972).

With respect to the dimension normal to the membrane plane, the proteins in the above model can be placed in four topologically distinct classes (Figure 1). Several experimental approaches, including chemical labeling with hydrophilic, small molecule reagents (Berg, 1969; Bretscher, 1971a–c; Steck, 1972), chemical labeling *via* an enzymatically catalyzed reaction (Phillips and Morrison, 1971a,b, 1973), and proteolytic digestion (Bender *et al.*, 1971; Steck *et al.*, 1971; Steck, 1972; Triplett and Carraway, 1972), have been used in attempts to determine which of the above classes (or analogous groupings) are actually represented in a particular membrane, the plasma membrane of human erythrocytes. These studies have recently been reviewed by Bretscher (1973) and by Zwaal *et al.* (1973).

In these procedures positive identification of the position of a protein with respect to a given surface of the bilayer requires successful labeling with a reagent to which the membrane is demonstrably impermeable. Group specific, low molecular weight reagents normally must find a reactive nucleophile on the protein, for example, an amino group. In the enzyme mediated case, a lactoperoxidase-catalyzed iodination, the label is restricted to tyrosyl or histidyl residues of the membrane proteins which are accessible to the active site of the peroxidase (Phillips and Morrison, 1971a, 1973). Proteolysis also demands accessibility of a specific group, the susceptible peptide bond, to a macromolecular reagent, the proteolytic enzyme. In addition, proteolysis suffers the intrinsic problem of any degradative probing procedure, that early and unrecognized stages of degradation may lead to altered structures, and the latter, on continuation of the reaction, can be mistaken for native structures. Negative results in a labeling experiment by any of these techniques must be interpreted with extreme caution. Absence of reaction can be due to absence of an appropriate, accessible, functional group and not to absence of that protein component at the interface.

In this study we have concentrated on small molecule reagents to minimize accessibility problems, and, in the exact converse of affinity labeling, looked for high reactivity and low specificity in order to maximize the chances of labeling all accessible components. The recent successful introduction of aryl nitrenes as photoaffinity labels (Fleet *et al.*, 1969; Knowles, 1972) focused attention on this type of reactive group. Aryl nitrenes can have lifetimes in hydroxylic solvents in the 0.1–1-msec range (Reiser *et al.*, 1968) and are thus useful in principle as general modification reagents without relying on the proximity effects operating with affinity labels (Ruoho *et al.*, 1973). Hydrogen abstraction, the anticipated major side reaction for an aryl nitrene reagent (Smith, 1970), can be mitigated by judicious substitutions on the aryl ring (Smith and Hall, 1962). These considerations led us to the synthesis of a hydrophilic nitrene precursor reagent, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate (I) (NAP-taurine).[1] Once photoactivated this compound is capable of reacting with proteins, carbohydrates, and lipids. The predominant stable products are probably secondary amines pro-

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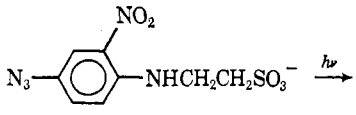
[1] Abbreviations used are: F-NAP, 4-fluoro-3-nitrophenyl azide; NAP-taurine, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate; Temed, *N,N,N',N'*-tetraethylenemethylenediamine; PAS, periodic acid–Schiff (stain).

duced by insertion of the nitrene into carbon–hydrogen bonds (Smith, 1970). Because aryl nitrenes can react with membrane components by insertion into carbon–hydrogen bonds, it is impossible to saturate potentially reactive sites, since each molecule of reagent adds such sites to any species with which it has reacted.

It must be emphasized that the labeling reagent employed in this study, *i.e.*, the reactive chemical species, is not I, the aryl azide precursor, but is *N*-(4-nitreno-2-nitrophenyl)-2-aminoethylsulfonate (II), the product of the photolytic loss of N_2 from I. The concentration of II is a function of the



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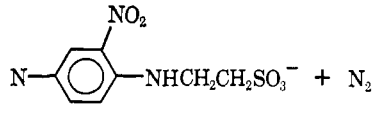

II

concentration of I and of the net light flux and is, therefore, in the course of these experiments, only a minute fraction of the original concentration of I. We will adopt the convention, however, of referring both to the aryl azide and to the photogenerated aryl nitrene as NAP-taurine, when it is clear to which species we are making reference.

Experimental Section

All chemical reagents were of ACS certified grade, or better. Water for these experiments was twice distilled and deionized with a Barnsted mixed bed resin.

Synthesis of [^{35}S]-*N*-(*4-Azido-2-nitrophenyl*)-*2-aminoethylsulfonate* (*NAP-Taurine*) (*I*). This reagent was synthesized and handled before photolysis in the dark, under very dim white light, or under a red safelight.

4-Fluoro-3-nitrophenyl azide (F-NAP) was synthesized according to the procedure outlined by Fleet *et al.* (1972).

[^{35}S]Taurine was procured from Amersham Searle at a specific activity of ~60 Ci/mol and was used as received. The radioactive taurine (50 μmol) was taken up in approximately 5 ml of water to which 100 μmol of sodium carbonate buffer (pH 9.8) was added from a concentrated solution. The pH was readjusted to 9.8 with dilute NaOH. This buffered solution was lyophilized, taken up in 0.2 ml of H_2O, and transferred into the tip of an acid-washed 2-ml conical centrifuge tube containing a micro Teflon-coated magnetic stirring bar. A four- to fivefold molar excess of F-NAP was added; the tube was sealed with attachment to a nitrogen supply and a mercury bubbler. The reaction chamber was flushed for 10–15 min with wet N_2, and then the tip of the chamber was immersed in a 65 ± 5° oil bath. The reaction was allowed to proceed for 35–40 hr at this temperature, with constant stirring. The reaction was terminated by cooling to room temperature, filtering the unreacted F-NAP onto a sintered glass funnel, washing the latter with 1–2 ml of H_2O, and adjusting the filtrate to pH 3 with dilute HCl. The product was separated from unreacted taurine by ion-exchange chromatography on an Aminex 50W X2 (Bio-Rad) column in the H^+ form, eluting with water. Approximately 15% of the eluted counts were in the NAP-taurine peak.

In order to confirm the structure of the [^{35}S]NAP-taurine, the nonradioactive reagent was synthesized by an analogous,

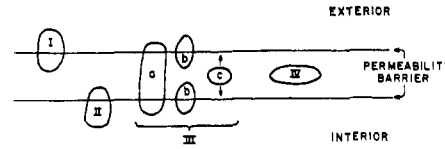

FIGURE 1: The four possible topological classes of membrane proteins: I, exterior surface; II, interior surface; III, transmembrane; IV, buried. This figure is entirely diagrammatic; sizes, shapes, and lateral distribution of the proteins are arbitrary. The possibilities of varying extent of exposure within any given class or of association within or between classes are not indicated. The classes are operationally distinguishable in principle by hydrophilic probes of the kind discussed in this paper. The subclasses of transmembrane proteins (III) illustrated are: (a) physically extended through the permeability barrier; (b) identical subunits on either surface, either in physical contact or not; (c) mobile. Subclass IIIb is transmembrane only in the sense that labeling from the exterior and the interior could not distinguish it from IIIa and IIIc. Although included for logical completeness, the authors consider unlikely the existence of IIIb or IIIc in functioning biological membranes.

large-scale method, and was isolated as the dihydrate of the sodium salt (Staros, 1974). Microanalysis of this compound (Spang Microanalytical Laboratories, Ann Arbor, Mich.) gave C, 27.78%; H, 3.34%; N, 20.24%; S, 9.37%; Na, 6.68%, as compared with C, 27.84%; H, 3.50%; N, 20.29%; S, 9.29%; Na, 6.66%, calculated for $C_8H_8N_5NaO_5S \cdot 2H_2O$. The compound is bright vermillion in aqueous solution with λ_{max} 471 nm and ϵ_{471} 4730. The proton nuclear magnetic resonance (nmr) spectrum of this compound in deuterated dimethyl sulfoxide consists of an aromatic quartet (2 protons) at 7.36 ppm (downfield of a tetramethylsilane internal standard), an aromatic doublet (1 proton) at 7.81 ppm, a methylene triplet (2 protons) at 2.90 ppm, a methylene multiplet (2 protons), partially obscured by the water peak, at 3.62 ppm, and a broad peak from the amino proton at 8.77 ppm. The position of the amino proton can be shifted by the addition of a source labile protons.

The ^{35}S reagent was identified with cold NAP-taurine in having the same elution position from the Aminex column, and by cochromatography on thin-layer silica gel plates (E. Merck) in methanol and in 1 : 1 chloroform–methanol.

Erythrocyte and Ghost Preparations. Fresh heparinized blood, or freshly drawn blood free of additives, was separated from plasma by centrifugation at 3° at 12,000g_{max} for ≤1 min (at ω_{max}) in an SS-34 rotor in a Sorvall RC-2B refrigerated centrifuge. The cells were maintained at 0–5° throughout the experimental procedure. The buffy coat was removed by aspiration and the erythrocytes were washed at least three times with phosphate buffered saline (0.15 M NaCl buffered with 14 mM sodium phosphate (pH 7.4)).

Ghosts were prepared by the method described by Heinz and Hoffman (1965), except that the volume of hemolysis solution used was 80 times the packed cell volume rather than 10 times. In those experiments in which the ghosts were to be reacted, they were washed in a phosphate wash buffer (15 mM NaCl–5 mM sodium phosphate (pH 7.4)) at least twice before reaction, and the ghosts were suspended in this buffer during the photolytic reaction.

After reaction with NAP-taurine the cells or ghosts were washed, and the cells were lysed. The membranes were further purified to remove radioactive side products from the photolysis on a discontinuous sucrose gradient in an SW41 rotor in a Beckman preparative ultracentrifuge: 5.0 ml of 25% (w/w) sucrose in 15 mM NaCl–5 mM Tris-HCl (pH 7.2) was layered onto 5.0 ml of 50% (w/w) sucrose in the same buffer. The suspension of ghosts in the NaCl–Tris wash buffer of Heinz

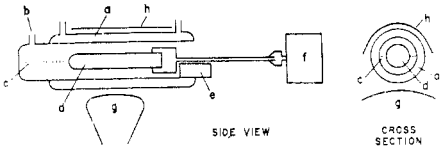

FIGURE 2: Apparatus for photolysis: (a) cooling jacket; (b) water inlet; (c) water level in reservoir; (d) sample chamber; (e) reservoir seal; (f) electric motor; (g) light source, Sylvania R-32 photoflood lamp; (h) reflector. The sample, sealed in chamber d, is mounted as illustrated. After the reservoir is sealed with e, the reservoir is filled with cold water to level c.

and Hoffman (1965) was layered on top of the 25% sucrose, and the rotor was spun at 20,000 rpm for 1 hr, giving a force at the 25%:50% sucrose interface of 52,000*g*. The purified ghosts were collected from the interface, washed once in the NaCl–Tris wash buffer, and resuspended in 0.3 ml of phosphate-buffered saline. A solubilizing solution containing 3% (w/v) sodium dodecyl sulfate, 25% (w/v) sucrose, 25 mM Tris-HCl (pH 8.0), 2.5 mM EDTA, 0.10 M dithiothreitol, and 25 μg/ml of pyronin Y (Fairbanks *et al.*, 1971) was added in an amount equal to two-thirds of the volume of the suspension. The dissolved membranes were incubated at 100° for 2 min in order to quench any proteolysis as well as to promote complete denaturation and reduction of the membrane polypeptides. Samples prepared by this procedure could be stored at −20° for 3 months without change in the sodium dodecyl sulfate polyacrylamide gel pattern obtained, as long as fresh reducing agent was added to the stored solution immediately before electrophoresis (usually 2% (v/v) 2-mercaptoethanol).

Reaction with NAP-Taurine. A 50% suspension of washed erythrocytes in phosphate-buffered saline, 0.5 ml, or of ghosts in phosphate wash buffer, containing 0.1–0.5 mM [^{35}S]NAP-taurine, was introduced into an acid washed 13 × 100 mm borosilicate glass test tube. The tube was closed by pushing it into a 12 mm annular slot cut to a depth of 12 mm in a No. 2 silicone rubber stopper. The outer surface of the stopper was wrapped with Teflon tape (Du Pont) to minimize friction, and the sealed tube was mounted in the apparatus shown in Figure 2.

The chamber external to the reaction vessel was filled with cold water to half-submerge the reaction vessel. A 2-propanol–water mixture at 0° was continuously pumped through the outer jacket of the chamber. The reaction vessel was rotated around its long axis during the 20-min photolysis time[2] used in these experiments. The average thickness of the reaction layer during photolysis was 0.15 mm.

After photolysis the labeled erythrocytes or ghosts were diluted to ~40 ml with the appropriate buffer, centrifuged out, and processed as described above. The supernatant was checked for hemoglobin, so as to monitor any lysis during the photolysis step.

Electrophoretic Analysis of Erythrocyte Membrane Polypeptides. Polyacrylamide gels, 10 cm, 4.5 and 9.0%, were cast in 16.5 cm × 0.6 cm precision bore glass tubes (Gilson Medical Electronics), which had been previously treated with a 1% aqueous solution of Siliclad (Clay Adams). The gel solution contained 4.5% (w/v) or 9.0% (w/v) electrophoresis grade acrylamide (Aldrich), 0.15% (w/v) or 0.30% (w/v) methylenebisacrylamide, 0.10 M sodium phosphate (pH 6.9), 0.5% (w/v) sodium dodecyl sulfate, 0.1% (w/v) $NH_4S_2O_8$, and 0.05% (v/v) Temed. The gel solutions were overlayered with a solution of 0.5% (w/v) sodium dodecyl sulfate, 0.1% (w/v) $NH_4S_2O_8$, and 0.05% (v/v) Temed. After allowing 1 hr for complete polymerization, the overlayer was removed and was replaced by at least 1 ml of electrophoresis buffer: 0.10 M sodium phosphate (pH 6.9)–0.5% (w/v) sodium dodecyl sulfate–0.1% (v/v) 3-mercaptopropionic acid. The gels were then left for at least 1 hr before use.

A sample of 0.1-ml solubilized membrane preparation (total protein ≅ 100 μg, by a modified Lowry assay (Bailey, 1967)) was applied to each gel. The polypeptides were loaded into the gel at low current (2 mA/gel for the 4.5% gels; 3 mA/gel for the 9% gels) for 0.5 hr. The gels were then run at 6–8 mA/gel for 6–10 hr until the pyronin Y tracking dye had migrated to within 1 cm of the ends of the gels. The gels were either fractionated directly for liquid scintillation counting or they were fixed and stained for protein or carbohydrate, sliced longitudinally, dried, and autoradiographed.

The electrophoresis buffer was removed from above those gels which were to be fractionated for liquid scintillation counting and was replaced with a fresh 10% (w/v) $NH_4S_2O_8$ solution. After 20–30 min, the ammonium persulfate solution was removed and a 1 cm layer of a new gel solution containing 10% (w/v) acrylamide, 0.33% methylenebisacrylamide, 0.5% sodium dodecyl sulfate, 6 mg/ml of myoglobin (as a high molecular weight dye), 0.13% $NH_4S_2O_8$, and 0.06% Temed was applied, which was, in turn, overlayered with H_2O. After polymerization, the gels were fractionated into 1-mm fractions on a Gilson aliquogel fractionator (Gilson Medical Electronics), eluting with 0.1 mg/ml of Pronase (Calbiochem, B grade), ~0.3 ml/fraction. Fractionation was continued until 1 or 2 ochre fractions from the myoglobin stain in the upper gel appeared, indicating that the electrophoretic gel had been entirely fractionated. The vials were capped, incubated at 37° overnight, and cooled. Scintillation fluid was added, 5 ml of toluene–Triton X-100–Liquifluor (New England Nuclear) (150:60:7), and the vials were sealed and counted.

Coomassie Blue staining for protein and PAS staining for carbohydrate were carried out essentially by the procedure of Fairbanks *et al.* (1971). Destaining of Coomassie Blue stained gels was carried out in 25% 2-propanol, 10% acetic acid, and terminated in 10% acetic acid, both without dye. Their PAS procedure was followed except that step 3 was eliminated and the volume of rinse solution per gel was 45 ml. Center quarter slices were dried and autoradiographed on Kodak RP/R54 film. The developed films were scanned for absorbance on a Joyce Loebl MkIII microdensitometer.

Results

In order for a chemical reagent to be useful as a topological probe, it must be shown to be reactive toward all components of the system under study, when they are made accessible. Previous studies utilizing a small molecule, membrane impermeant reagent (Bretscher, 1971a), enzyme mediated labeling (Phillips and Morrison, 1971) and proteolytic digestion (Bender *et al.*, 1971; Steck *et al.*, 1971; Triplett and Carraway, 1972) have shown that all of the detectable proteins of isolated ghosts are accessible to these probes (with the exception of band 6 to proteolysis).

When NAP-taurine labeled ghosts have been purified, dissolved, electrophoresed in 4.5% and in 9% sodium dodecyl

[2] Because the concentration of the aryl nitrene depends upon light flux (see introduction), one must determine optimal photolysis times for given experiments. We photolyzed suspensions of erythrocytes prepared as above for various times, in order to determine what time of photolysis resulted in maximizing the incorporation of NAP-taurine into membrane proteins while minimizing lysis. We used the 20-min photolysis time determined for intact erythrocytes for all of our experiments.

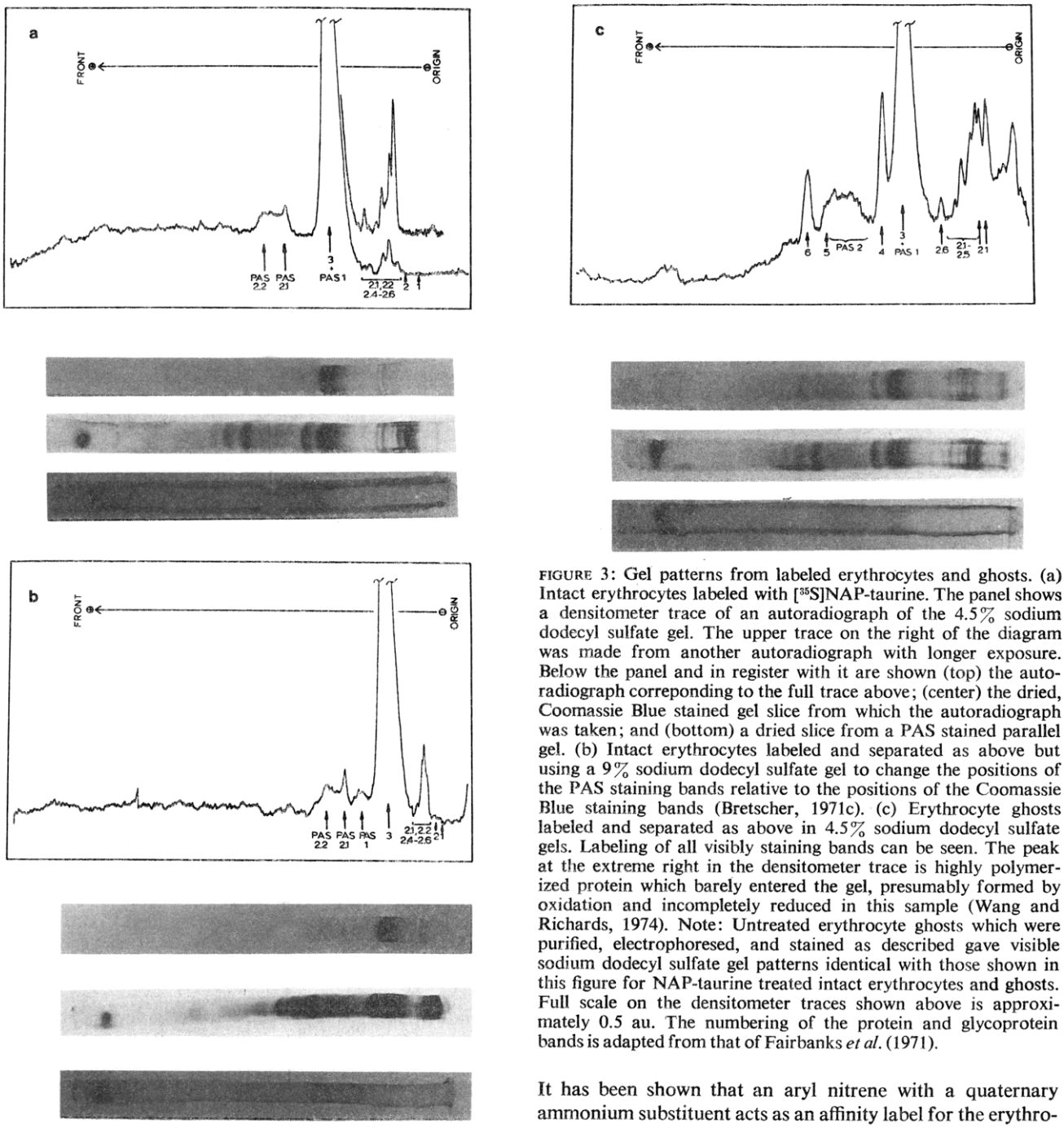

FIGURE 3: Gel patterns from labeled erythrocytes and ghosts. (a) Intact erythrocytes labeled with [^{35}S]NAP-taurine. The panel shows a densitometer trace of an autoradiograph of the 4.5% sodium dodecyl sulfate gel. The upper trace on the right of the diagram was made from another autoradiograph with longer exposure. Below the panel and in register with it are shown (top) the autoradiograph correponding to the full trace above; (center) the dried, Coomassie Blue stained gel slice from which the autoradiograph was taken; and (bottom) a dried slice from a PAS stained parallel gel. (b) Intact erythrocytes labeled and separated as above but using a 9% sodium dodecyl sulfate gel to change the positions of the PAS staining bands relative to the positions of the Coomassie Blue staining bands (Bretscher, 1971c). (c) Erythrocyte ghosts labeled and separated as above in 4.5% sodium dodecyl sulfate gels. Labeling of all visibly staining bands can be seen. The peak at the extreme right in the densitometer trace is highly polymerized protein which barely entered the gel, presumably formed by oxidation and incompletely reduced in this sample (Wang and Richards, 1974). Note: Untreated erythrocyte ghosts which were purified, electrophoresed, and stained as described gave visible sodium dodecyl sulfate gel patterns identical with those shown in this figure for NAP-taurine treated intact erythrocytes and ghosts. Full scale on the densitometer traces shown above is approximately 0.5 au. The numbering of the protein and glycoprotein bands is adapted from that of Fairbanks *et al.* (1971).

sulfate polyacrylamide gels, stained for protein with Coomassie Blue and for carbohydrate with PAS, and autoradiographed, all of the Coomassie Blue staining (protein) and PAS staining (glycoprotein) bands in the stained dried gels are superimposable onto bands of exposure on their corresponding autoradiographs (Figures 3c and 4A). If the proteolysis step is deleted, no covalent labeling is obtained, *i.e.*, the autoradiograph is blank.

It should be noted that although all of the stained bands from NAP-taurine reacted ghosts are observed to carry label, the NAP-taurine labeling is not proportional to the relative staining of the bands. In Figure 3c, it is shown that bands 1, 2, and 5 carry label at a relatively lower level than they stain. We cannot determine from our data whether these differences in relative labeling are due to differences in relative surface areas accessible to the reagent or to differential associations with the reagent, *e.g.*, by electrostatic attraction or repulsion.

It has been shown that an aryl nitrene with a quaternary ammonium substituent acts as an affinity label for the erythrocyte acetylcholinesterase (Kiefer *et al.*, 1970). Subsequent studies (Ruoho *et al.*, 1973) have shown, however, that while reaction does occur in the active site of the acetylcholinesterase, only ~0.1% of the reagent is incorporated into that site. The remainder reacts with other accessible protein sites. Likewise, we might expect our reagent to have some affinity for the anion channel, which has been associated with band 3 (Cabantchik and Rothstein, 1972), and to be repelled somewhat from PAS-1, to which is attached most of the sialic acid of the erythrocyte (Winzler, 1969) and which therefore is negatively charged. Any such differential association would not, however, affect the basic premise of this study, that those components of the intact erythrocyte which do react with NAP-taurine are accessible to the external environment of the cell.

In contrast to the pan-reactivity that NAP-taurine exhibits toward ghost proteins and glycoproteins, the labeling pattern obtained upon labeling intact erythrocytes with this reagent is quite selective (Figures 3a,b and 4B). Significantly, no label

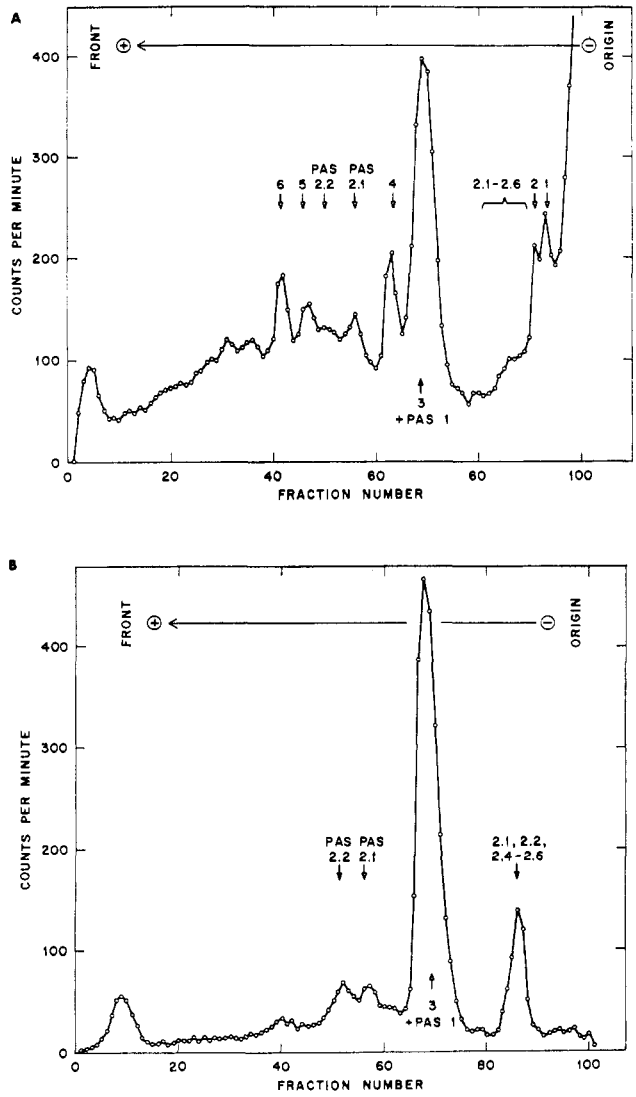

FIGURE 4: NAP-taurine labeled erythrocyte ghosts (A) and intact erythrocytes (B), electrophoresed in 4.5% gels and fractionated into 1-mm fractions, as described in the text. (For the material at the top of gel A and peak numbering system, see legend for Figure 3.) Integration of the three peak areas in (B) indicates that approximately 75% of the NAP-taurine bound to the proteins of those regions is bound to bands 3 plus PAS-1, 16% is bound to components of the 2.1–2.6 region, and 9% is bound to components of PAS-2. From the data shown here and the specific activity of the [^{35}S]NAP-taurine at the time of this experiment (~30 Ci/mol), we estimate that approximately 0.1 to 0.2 mol of NAP-taurine are bound per mole of 90,000 molecular weight protein in the major peak.

can be detected in bands 1 and 2 or in bands 4, 5, or 6. (Lysis during the photolysis was $\leq 2\%$ of the cells, as determined by measurement of OD_{541} of the supernatant.) Bands 3 and PAS-1 do carry label, in agreement with previous studies (see Discussion). In addition, at least five high molecular weight protein bands (2.1, 2.2, 2.4–2.6)[3] were labeled, as were at least two minor glycoproteins (PAS-2.1, PAS-2.2).

[3] Bands 2.1–2.6 have not been previously described *per se*, but bands corresponding to these have been shown in photographs and densitometer traces of stained gels prepared and run under a wide variety of conditions; *e.g.*, Lenard (1970), Figure 1; Steck *et al.* (1971), Figure 3, control gel; Bender *et al.* (1971), Figure 2a; Triplett and Carraway (1972), Figures 1–4, 6, control gels. All six of these bands were not necessarily seen in the above gels; our gel technique was developed with special attention to increasing the resolution in this region. Further, cross-linking studies in our laboratory (K. Wang and F. M. Richards, in preparation) indicate that the narrow bands in this region do correspond to distinct polypeptide chains.

When ~30 μg (measured by absorption at 541 nm) of hemoglobin isolated from cells labeled externally with NAP-taurine was precipitated with trichloroacetic acid, dissolved, electrophoresed in sodium dodecyl sulfate polyacrylamide (4.5%) gels, fractionated, and counted in a liquid scintillation counter, no radioactivity could be detected. This test would have detected protein labeled to a specific activity of approximately 1% of that observed for band 3.

Intact erythrocytes were preincubated with [^{35}S]NAP-taurine in phosphate buffered saline for 0, 30, or 60 min at 0° before photolysis. The isolated membranes were electrophoresed (by the method of Fairbanks *et al.* (1971) for this experiment only), sliced without staining, dried, and autoradiographed. There were no visible differences among the autoradiographs in the identity of the labeled bands or the relative extent of labeling.

While NAP-taurine does not measurably permeate into erythrocytes under the conditions described above, we have found that at 37° this reagent will slowly leak in. Preliminary studies of efflux from NAP-taurine loaded cells confirm this sharp difference in permeability of erythrocytes between 0 and 37°. Further, when NAP-taurine loaded cells are cooled to 0°, washed, and photolyzed, membrane proteins accessible to the cytoplasma, as well as hemoglobin, are clearly labeled. The negative controls reported above can thus be confidently interpreted as indicating negligible penetration of the reagent in the studies reported here. A detailed description of the efflux rates and interior labeling experiments will be presented elsewhere (J. V. Staros, B. E. Haley, and F. M. Richards, in preparation).

Discussion

The data presented above indicate that NAP-taurine, as a label for erythrocytes, satisfies the two basic requirements of a probe for investigating membrane topology: general reactivity and membrane impermeability. When NAP-taurine is reacted with ghosts, all of the sodium dodecyl sulfate polyacrylamide gel bands which stain with either PAS or Coomassie Blue carry label (Figures 3c and 4A). Since all detectable bands were labeled, our data do not contain evidence for the existence of any class IV proteins in ghosts.

The absence of label in the gel of hemoglobin isolated from labeled erythrocytes strongly suggests that the reagent does not penetrate into the cell under the conditions of these experiments. This contention is also supported by the finding that the labeling pattern of intact erythrocytes does not change with preincubation in NAP-taurine containing buffer prior to photolysis.

A third line of evidence that NAP-taurine is membrane impermeant is the absence of radioactivity in bands 1 and 2 (collectively called spectrin) from labeled intact erythrocytes (Figures 3a,b and 4B). These two large polypeptides are extracted together in equimolar proportions (Clarke, 1971) at low salt concentrations and in the presence of EDTA (Marchesi *et al.*, 1969), and they have been cross-linked in dilute solution, resulting in dimers (Clarke, 1971), and in ghosts, resulting in multimers (Wang and Richards, 1974), so that one may consider them together as one protein. This study, as well as previous work utilizing nucleophile specific small molecule probes (Berg, 1969; Bretscher, 1971a; Steck, 1972), lactoperoxidase catalyzed iodination, before and after surface proteolysis (Phillips and Morrison, 1971a,b, 1973), proteolysis (Bender *et al.*, 1971; Steck *et al.*, 1971; Triplett and Carraway, 1972), proteolysis followed by nucleophile specific small

molecule labeling (Bender *et al.*, 1971), and treatment with spectrin specific antibodies (Marchesi *et al.*, 1969; Furthmayr and Timpl, 1970), have all failed to detect any part of this protein on the outside of intact erythrocytes. Therefore, spectrin belongs to class II or IV (Figure 1) in the intact erythrocyte. It has been definitely localized on the cytoplasmic surface (class II) in ghosts using ferritin conjugated antispectrin antibodies (Nicolson *et al.*, 1971) and its solubility in water argues strongly against the possibility that it was ever totally buried in the membrane. Therefore, though we cannot argue that its precise conformation is identical in ghosts and intact erythrocytes, it is evident that it falls into class II in both structures; and thus the labeling of spectrin in intact erythrocytes can legitimately be used as an independent test of the permeability of a given probe through the erythrocyte membrane.

Using NAP-taurine, we have shown that at least nine distinct proteins and glycoproteins corresponding to bands 2.1, 2.2, 2.4–2.6, 3, PAS-1, 2.1, 2.2 are accessible to the exterior environment, and are therefore either class I or class III components. Of these nine, band 3 and the glycoproteins have been shown through previous studies to communicate with the external environment of the cell (Bretscher, 1971a, Bender *et al.*, 1971; Steck *et al.*, 1971; Steck, 1972; Phillips and Morrison, 1971b, 1973). The considerably wider spectrum of components of classes I and/or III identified in this study is apparently due to the very much higher reactivity of our probe combined with its accessibility characteristics.

The importance of a probe's accessibility to the surface is well illustrated by the lactoperoxidase catalyzed iodination experiments of Phillips and Morrison (1971a,b; 1973). Their earlier studies had shown that two membrane components, corresponding to bands 3 and PAS-1, were labeled when intact erythrocytes were iodintaed. They later calculated, however, that when all available sites had been iodinated, only about 2% of the total glycoprotein molecules had been labeled. Further, by partially trypsinizing the intact erythrocytes to the extent of removing about 20% of PAS-1 from the membrane surface, the efficiency of the iodination was increased by an order of magnitude for both band 3 and for the remaining undegraded glycoprotein. This increase in reaction efficiency is attributed to a partial removal of the "glycoprotein barrier," thereby allowing the lactoperoxidase a greater accessibility to the remaining proteins.

Bender *et al.* (1971) showed that the major peptide fragment remaining after degradation of the band 3 polypeptide with Pronase could be labeled when Pronase-treated cells were exposed to diazotized [^{35}S]sulfanilic acid, even though continued Pronase treatment would not further degrade this fragment. The reagent was shown not to penetrate either intact or Pronase-treated erythrocytes by the criterion of nonlabeling of spectrin (Figure 3 of Bender *et al.*, 1971). In addition, the authors showed that the facilitated diffusion of glucose is relatively unaffected by Pronase treatment but is strongly inhibited by treatment with diazotized sulfanilic acid.

The above examples clearly show that membrane proteins can be inaccessible to enzymes in the external environment, but are still exposed with respect to interaction with small molecules in that environment. We suggest that the proteins corresponding to bands 2.1, 2.2, 2.4–2.6 are such enzyme inaccessible proteins. Further, since they were not labeled by electrophilic reagents, diazotized sulfanilic acid (Berg, 1969; Bender *et al.*, 1971), formylmethionine sulfone methyl phosphate (Bretscher, 1971a), or trinitrobenzenesulfonate(Steck, 1972), these proteins do not have appropriate nucleophilic groups exposed to the external environment.

The PAS-2 region, in which we find at least two externally accessible glycoproteins (Figures 3a,b and 4B), PAS-2.1, 2.2, has been reported to be accessible to proteolytic digestion of intact erythrocytes (Steck *et al.*, 1971; Steck, 1972). The data presented by Bender *et al.* (1971) also suggest that diazotized sulfanilic acid is reactive toward one or more components of the PAS-2 region in intact erythrocytes.

The current picture of the nonlipid surface components of the human erythrocyte is one of great complexity. There are several glycoproteins in the membrane, all of which appear to be in contact with the external environment. The approximately 90,000-dalton protein, band 3, which recently has been reported to contain 5–10% carbohydrate (Guidotti, 1972a,b), but which does not stain as a glycoprotein, is, by all of the criteria listed above, externally accessible. This protein is by a considerable margin the dominant species of the membrane in terms of number of copies per cell (Fairbanks *et al.*, 1971). There are five high molecular weight proteins which are exposed to the external environment, as shown in this study, but which could not extend far into the extracellular aqueous phase, in that they are inaccessible to enzymes; and they exhibit few, if any, free nucleophiles to this phase, in that they have eluded detection by electrophilic reagents.

Anion permeability through the erythrocyte membrane can be inhibited by treatment with several stilbenedisulfonate derivatives, which appear to bind to band 3 (Cabantchik and Rothstein, 1972). Two other functions which can be inhibited by treatment at the exterior face (Bender *et al.*, 1971) are the acetylcholinesterase, by either Pronase or diazotized sulfanilic acid (as well as by reaction with quaternary ammonium aryl azides (Kiefer *et al.*, 1970)) and the facilitated transport system for glucose, which is blocked by the same reagent but is relatively immune to proteolysis. The acetylcholinesterase cannot be identified with any of the above bands because, with a molecular weight of 90,000 and a relative abundance of approximately 0.2% of the total membrane protein (Bellhorn *et al.*, 1970), it would be completely hidden by band 3, which has the same molecular weight and a relative abundance of 25–30% (Fairbanks *et al.*, 1971). The facilitated diffusion system for glucose has been estimated to be present at about 2×10^5 copies per cell (Kahlenberg *et al.*, 1971), which is of the same order as the estimates for the major visible bands, with the exception of band 3 (Fairbanks *et al.*, 1971). However, there is insufficient data at present to identify it with any of the known externally accessible proteins.

In summary, use of a nitrene surface probe has provided evidence for a new subclass of membrane proteins, one which is exposed to the extracellular aqueous environment but which cannot be appreciably extended into it. The discovery of several representatives of this subclass provides evidence for a greater degree of complexity in the exterior surface of the erythrocyte than had previously been recognized. The high reactivity of this probe to otherwise unreactive components makes it a good candidate for studies of subtle topological changes accompanying physiological changes. Such a study, involving the transition from intact cells to resealed ghosts, will be described elsewhere (J. V. Staros, B. E. Haley, and F. M. Richards, in preparation).

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