N-(4-Azido-2-Nitrophenyl)-2-Aminoethylsulfonate (NAP-Taurine) as a Photoaffinity Probe for Identifying Membrane Components Containing the Modifier Site of the Human Red Blood Cell Anion Exchange System

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ABSTRACT Exposure of cells to intense light with the photoactivatable reagent, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-taurine), present in the external medium results in irreversible inhibition of chloride or sulfate exchange. This irreversible inhibition seems to result from covalent reaction with the same sites to which NAP-taurine binds reversibly in the dark. As shown in the preceding paper, high chloride concentrations decrease the reversible inhibition by NAP-taurine in the dark, in a manner suggesting that NAP-taurine and chloride compete for the modifier site of the anion transport system. In a similar fashion, high chloride concentrations in the medium during exposure to light cause a decrease in both the irreversible binding of NAP-taurine to the membrane and the inhibition of chloride exchange. Most of the chloride-sensitive irreversibly bound NAP-taurine is found in the 95,000 dalton polypeptide known as band 3 and, after pronase treatment of intact cells, in the 65,000 dalton fragment of this protein produced by proteolytic cleavage. After chymotrypsin treatment of ghosts, the NAP-taurine is localized in the 17,000 dalton transmembrane portion of this fragment. Although the possible involvement of minor labeled proteins cannot be rigorously excluded, the modifier site labeled by external NAP-taurine appears, therefore, to be located in the same portion of the 95,000 dalton polypeptide as is the transport site.

INTRODUCTION

In the preceding paper (Knauf et al., 1978), it was found by kinetic analysis that in the dark external NAP-taurine appears to inhibit red cell anion exchange reversibly by acting at a nontransporting site, called the modifier site (Dalmark, 1976). Inasmuch as NAP-taurine contains an aryl azide, which can be activated by light to form a highly reactive nitrene, it should be possible to use this compound to label and thereby identify the component containing the modifier

site. This possibility can only be realized, however, if it can be shown that NAP-taurine acts as a true photoaffinity label; that is, if the reversibly bound reagent activated by light reacts covalently with the binding site before dissociating from it (Ruoho et al., 1973). If, on the other hand, NAP-taurine in solution simply reacts with the transport system by random collision when activated by light, the sites of covalent labeling might be entirely different from the sites of reversible inhibition.

The present communication describes experiments designed to determine whether or not NAP-taurine is a photoaffinity label with respect to membrane sites at which the agent inhibits anion exchange. Inasmuch as the reversible inhibition in the dark is decreased by high chloride concentrations, the effect of chloride on the covalent labeling of membrane proteins by NAP-taurine has also been examined. Proteolytic enzymes have been used to define more precisely the location of labeled sites in the proteins. Parts of this work have been previously presented in brief form (Knauf et al., 1976; Rothstein et al., 1977).

METHODS

Chloride Fluxes

Fresh blood from volunteer donors was collected by venipuncture into heparinized Vacutainers (Becton, Dickinson & Co., Rutherford, N. J.) The blood was cooled on ice and brought to the desired pH with CO_2. The blood was then washed three times in 165 mM NaCl and the buffy coat was removed.

Cells were treated with nystatin (Cass and Dalmark, 1973; Dalmark, 1975), chloride exchange was measured, and rate constants were determined as described in the preceding paper (Knauf et al., 1978). By use of nystatin, the chloride concentration was varied while keeping the inside and outside chloride concentrations nearly equal, thereby eliminating possible effects of changes in the chloride gradient or membrane potential. When cells were irradiated in the presence of NAP-taurine, they were loaded with ^{36}Cl after the irradiation and washing procedure, just before the efflux was measured.

Sulfate Fluxes

Sulfate and chloride appear to compete for the same transport system (Gunn, 1978; Passow and Wood, 1974; Cabantchik et al.[1]). Inasmuch as sulfate exchange occurs much more slowly than chloride exchange, in many experiments it was more convenient to use sulfate exchange as a measure of the activity of the red cell anion exchange system.

Bank blood (<3 wk after collection) was washed twice in 165 mM NaCl and the buffy coat was removed. The cells were then washed once in a Tris-sulfate buffer consisting of 5 mM Na_2SO_4, 20 mM NaCl, 200 mM sucrose, and 25 mM tris(hydroxymethyl)aminomethane (Tris) base, which had been titrated to pH 7.4 at room temperature with H_2SO_4, and were incubated in the same buffer at 5% hematocrit for 1 h at 37°C. The suspension was then centrifuged at 1800 *g* on a Sorvall GLC-2 centrifuge (DuPont Instruments-Sorvall, Wilmington, Del.) and the cells were resuspended at 10% hematocrit and incubated at 37°C for 1 h in Tris-sulfate containing a tracer quantity of $^{35}SO_4$ (Amersham/

[1] Cabantchik, Z. I., P. A. Knauf, and A. Rothstein. The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of "probes." *Biochim. Biophys. Acta.* In press.

Searle Corp., Arlington Heights, Ill.). The $^{35}SO_4$ loaded cells were washed at least three times in ice-cold Tris-sulfate buffer. For measurements of reversible inhibition, the washed, $^{35}SO_4^-$ loaded cells were resuspended at 5% hematocrit in Tris-sulfate medium containing various concentrations of NAP-taurine (Pierce Chemical Co., Rockford, Ill.). If irreversible inhibition was to be measured, the cells were irradiated at 27–28°C in the presence of NAP-taurine and washed as described in the next section on irradiation procedures. The $^{35}SO_4$ efflux was measured at 27–28°C in Tris-sulfate medium. Samples of the suspensions were taken at various times and centrifuged, the time for each sample taken as the time at which centrifugation was started. 1-ml samples of the supernates were added to 0.2 ml 30% trichloroacetic acid (TCA), and 0.8 ml of each TCA supernate was added to 8 ml of Triton-X-100-toluene scintillation fluid (New England Nuclear, Boston, Mass.). Radioactivity of the samples was determined in a Packard C2425 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). 1-ml samples of the total suspension were also taken and added directly to TCA (without centrifugation) for determination of the total $^{35}SO_4$ in the suspension ($P(\infty)$). The rate constant was determined by the method of least squares from the slope of the line obtained by plotting ln $(P(\infty)/(P(\infty) - P(t)))$ vs. time as described by Knauf et al., (1978). The square of the correlation coefficient (r^2) for the lines so obtained was almost always >0.99 and was always >0.97.

Irradiation Procedure

Unless otherwise indicated, 0.5 ml of cells to be irradiated were suspended in 1 ml of medium containing the indicated amount of NAP-taurine. For labeling experiments, ^{35}S-NAP-taurine, prepared from ^{35}S-taurine (Amersham-Searle Corp.) by the method of Staros and Richards (1974) and purified as described in the preceding paper (Knauf et al., 1978), was kindly supplied by Dr. Saul Ship. The cell suspension was placed in a 15 × 150-mm glass tube, which was inserted into a water-jacketed glass chamber. During the irradiation, the tube was held nearly horizontal and was rotated by a rotary evaporator (Buchler Instruments, Fort Lee, N. J.). The chamber contained sufficient water or ethanol-water mixture to wet the portion of the tube containing the cells, which was kept at the desired temperature (0°C or 27°C) by a Lauda K4R controlled-temperature circulator (Lauda Div., Brinkmann Instruments, Inc., Westbury, N. Y.). The suspension was irradiated for 20 min with light from a General Electric DX8 photospot (General Electric Co., Schenectady, N. Y.) positioned 10 cm from the tube containing the cells. Alternatively, cells were exposed for 10 min to light from a source constructed by Photochemical Research Associates, University of Western Ontario, consisting of a General Electric ELH projection lamp (operated at 92 V) positioned immediately adjacent to the water jacket. In this case, 0.1 ml of cells were irradiated. After irradiation, the cells were washed at least two times with medium containing 0.5% wt/vol bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and at least once more with buffer.

Pronase Treatment

Cells at 33% hematocrit were treated with freshly prepared 0.1 mg/ml pronase (Calbiochem, San Diego, Calif., lot no. 400899) for 10 min at 32°C. After pronase treatment, cells were washed three times with medium containing 0.5% albumin and then three times with medium to remove pronase.

Ghost Preparation and Polyacrylamide Gel Electrophoresis

Ghosts were prepared from labeled cells by lysing them in 60 mosM phosphate buffer and then washing once in 60 mosM phosphate buffer and four times in 20 mosM buffer

(Dodge et al., 1963). Protein was determined by the method of Lowry et al. (1951). For calculations of the number of molecules bound per ghost, it was assumed that each ghost contains 6×10^{-10} mg protein (Dodge et al., 1963). Aliquots of the ghost suspension were counted for radioactivity by adding 20 μl to 10 ml Protosol-Liquifluor-toluene (10% Protosol, 4.2% Liquifluor [New England Nuclear] in toluene). Efficiency of counting was determined by means of an external standard.

Ghosts were solubilized in sodium dodecyl sulfate (SDS) in the solution described by Fairbanks et al. (1971) by boiling for 3–5 mins and then incubating at 60°C for 30 min. Aliquots of the solubilizing solution were counted in Protosol-Liquifluor-toluene. Solubilized ghosts were electrophoresed in 5.6% or 7.5% polyacrylamide gels containing 1% SDS, fixed, and stained with Coomassie Brilliant Blue as described by Fairbanks et al. (1971). For determination of radioactivity, unstained gels were sliced with a scalpel blade and the slices were placed in scintillation vials to which 0.5 ml of Protosol-Toluene-Water (9:10:1) was added. After incubation overnight at 60°C, the vials were cooled and 8 ml of Protosol-Liquifluor-toluene was added. After incubation at 40°C for at least 8 h, and cooling in the dark for 3 h, the vials were counted. The results were corrected for the amount of protein added to the gels and for the gel length by dividing the number of counts in each slice by the number of micrograms of protein added to the gel and by the normalized length of the slice, l/l_o, where l is the length of the slice and l_o is the distance from the top of the gel to the marker dye, Pyronin Y.

Preparation of Alkali-Treated Ghosts

Freshly-drawn washed intact red cells at 10% hematocrit were exposed to light from the General Electric ELH projection lamp for 10 min in the presence of 300 μM ^{35}S-labeled NAP-taurine and washed as described above. Ghosts were prepared by lysis in 5 mM phosphate buffer, pH 8 (5P8) (Fairbanks et al., 1971). The ghosts were made up to a volume corresponding to twice the original volume of intact cells from which the ghosts were made, and a sample was saved for electrophoresis. The rest of the ghost suspension was incubated with ice-cold 10 mM NaOH, 0.1 mM EDTA for 5 min at 0°C to extract extrinsic proteins (Steck and Yu, 1973; Grinstein et al., 1978). After one wash in 5P8, the pellet was brought to the original volume and a portion was used for electrophoresis.

Chymotrypsin Treatment

To 1 vol of the ghost suspension was added 4 vol of 0.2 mg/ml chymotrypsin (Sigma Chemical Co.). The ghosts were frozen and thawed once to ensure that both surfaces of the membrane were exposed to chymotrypsin, and then incubated for 90 min at 37°C. The reaction was terminated by addition of 30 μg/ml phenylmethane sulfonyl fluoride (PMSF), and the chymotrypsin-treated ghosts were then washed once with 5P8, restored to their original volume and used for electrophoresis.

RESULTS AND DISCUSSION

Criteria for Photoaffinity Labeling

Ideal behavior of NAP-taurine as a photoaffinity probe is illustrated in Fig. 1. In the dark, the anionic probe (1) is assumed to equilibrate with modifier sites (2), which are probably positively charged, producing a reversible inhibition (Knauf et al., 1978). The reaction is presented as a simple one-to-one binding, with the degree of inhibition determined by the fraction of sites occupied. On exposure to light, the activated NAP-taurine in solution (3) is assumed to hydrolyze (5) without covalent interaction with the membrane sites, whereas the

NAP-taurine reversibly bound to the sites is assumed, on activation (4), to react covalently (6) before it escapes. The irreversible binding after exposure to light would therefore precisely reflect the reversible binding that existed in the dark. Thus, the binding sites identified by kinetic analysis in the dark as being the modifier sites (Knauf et al., 1978), could be labeled in the light and thereby localized in particular components.

One of the criteria which distinguish a photoaffinity reagent is that in the

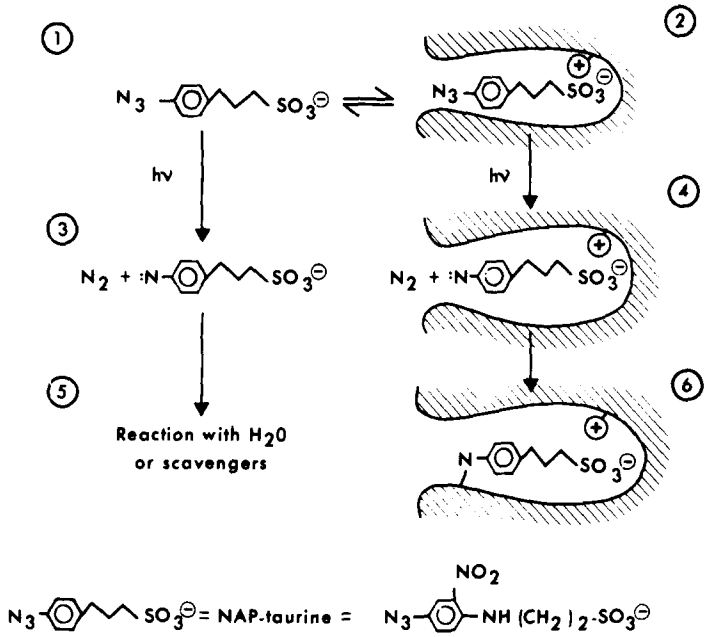

FIGURE 1. A proposed mechanism of photoaffinity labeling by NAP-taurine. In the dark, NAP-taurine in solution (1) can reversibly inhibit anion transport by binding to sites (2) which appear to be identical to the modifier sites of the transport system (Knauf et al., 1978), at which chloride and other halides cause a noncompetitive inhibition of transport. Upon exposure to light ($h\nu$) the aryl azide moiety of NAP-taurine loses nitrogen to form a highly reactive nitrene (3 and 4). In the case of an ideal photoaffinity reagent, the activated reagent at the site (4) forms a covalent bond with the site (6) before it can dissociate from the site. Activated reagent in solution (3) reacts with water or other compounds in solution (5) before it can interact with the membrane. The removal of activated reagent from solution can be accelerated by adding scavengers to the medium which react readily with the photo-generated nitrene.

dark its effect is reversible, but after exposure to light, irreversible. The reversibility in the dark is evident in Fig. 2 A, B, and C by comparing the low level of transport activity (measured by sulfate exchange) in the presence of NAP-taurine (the solid square point associated with the letter R on the abscissa), with the essentially normal level in other aliquots of the same cells in which the reversibly bound NAP-taurine was removed by extensive washing (O). On the

other hand, when cells were irradiated in the presence of NAP-taurine, irreversible inhibition became clearly evident in a time-dependent manner (△). Appropriate controls indicate that the effect is not due to the action of light alone (●). Furthermore, neither reversible nor irreversible effects are observed when thoroughly preirradiated NAP-taurine is added to cells (Rothstein et al., 1977). Thus, the irradiation of NAP-taurine must be carried out in the presence

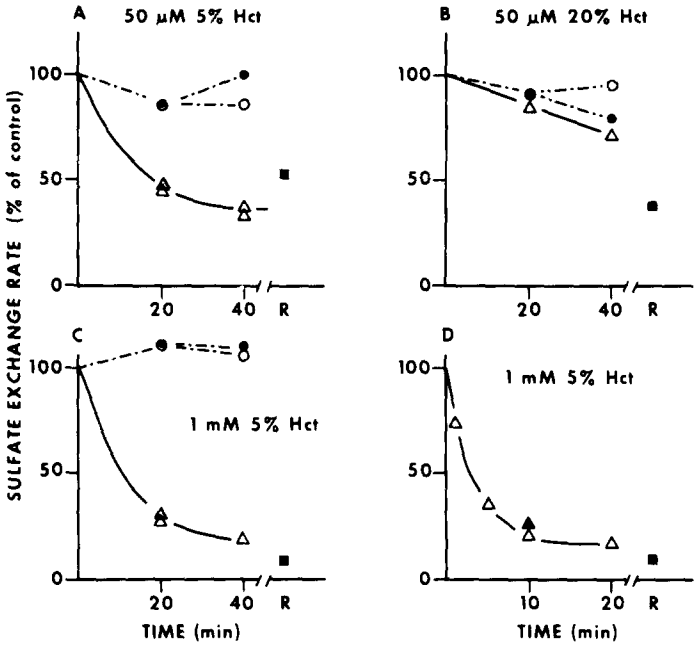

FIGURE 2. Effect of time of irradiation on the irreversible inhibition of sulfate fluxes by NAP-taurine. Cells were exposed to NAP-taurine or light or both for the times indicated on the abscissa, then washed with Tris-sulfate medium containing albumin. Sulfate exchange was measured as described in Methods. (○—○) NAP-taurine alone, no light; (●—●) light without NAP-taurine; (△——△) NAP-taurine present at the time of exposure to light. R (■) indicates the reversible inhibition obtained with NAP-taurine present in the flux medium in the dark. For (A) the value for reversible inhibition was taken from a series of nine similar experiments. The standard error was 1.2%. In (A–C) the light source was a General Electric photospot; in (D) it was a General Electric ELH projection lamp. (A) 50 μM NAP-taurine, 5% hematocrit (Hct); (B) 50 μM NAP-taurine 20% hematocrit; (C and D) 1 mM NAP-taurine, 5% hematocrit, except for (▲) which is for 10% hematocrit.

of cells if an irreversible effect is to be produced, another criterion of a photoaffinity probe. In quantitative terms, if certain assumptions regarding the rates of photoactivation of bound and free NAP-taurine hold (see Appendix), the probe should produce an irreversible inhibition after exposure to light that is of the same magnitude as the reversible inhibition in the dark, provided that the light-activated reaction is carried to completion. In Fig. 2 A, at a relatively

low concentration of NAP-taurine (50 μM) and low hematocrit (5%), the irreversible inhibition approaches a level which is slightly greater than the reversible inhibition (shown by the solid square labeled R on the abscissa) after 20–40 min of irradiation. If the NAP-taurine level is higher (1 mM), however, the conversion of reversible to irreversible inhibition (during the same time period) is somewhat less complete (Fig. 2 C), presumably because of the absorption of light by the larger amount of NAP-taurine present in the medium. The rate of production of irreversible inhibition can, however, be speeded up by using a more intense source of irradiation (ELH projection lamp) as illustrated in Fig. 2 D. Another important factor that determines the rate of the photo-sensitive reaction is the hematocrit. If the cell concentration is increased from 5 to 20% (as in Fig. 2 B) the rate is substantially decreased, presumably because the larger amount of hemoglobin in the cell suspension absorbs a larger portion of the available light.

Further comparisons of reversible and irreversible inhibition are presented in Fig. 3 in the form of a modified Dixon plot ($1/k$, where k is the rate constant for sulfate efflux, vs. the inhibitor (NAP-taurine) concentration). The solid symbols represent data obtained in the dark with NAP-taurine present during the flux measurements. As in the case of chloride (Knauf et al., 1978), the Dixon plot for sulfate was linear, suggesting that one molecule of NAP-taurine combines with each inhibitory site (Webb, 1963). The apparent K_i indicated by the x-intercept was 53 μM, somewhat larger than the values of K_i observed when chloride fluxes were measured at 0°C (Knauf et al., 1978). This is probably largely due to the higher temperature in these experiments, because at 37°C there is a further increase in K_i (data not shown).[2] The open symbols represent data for irreversible inhibition after exposure to light (20 min with photoflood) in the presence of NAP-taurine, followed by washing before flux measurements. For lower concentrations of the probe, the results are similar for reversible and irreversible inhibition. The small tendency toward higher inhibition in the light may simply reflect a slightly greater rate of photoactivation of the bound NAP-taurine, as discussed in the Appendix. At higher NAP-taurine concentrations, the irreversible inhibition is less than the reversible inhibition, probably because of the filtering of the incident light by the NAP-taurine in solution, as already illustrated in Fig. 2 (cf. panels A and C).

The data of Figs. 2 and 3 suggest that NAP-taurine is a good photoaffinity probe provided that the particular conditions allow completion of the light-sensitive reaction. The rate of the irreversible reaction is increased with higher light intensities and decreased with higher concentrations of NAP-taurine or of cells. It is essentially complete in 20–30 min with the photoflood system or 10 min with the projection lamp, at a hematocrit of 5% and with NAP-taurine concentrations <100 μM.

Another test for photo-affinity labeling is provided by the use of scavengers. Scavengers are compounds which react with the photoactivated form of the

[2] It is perhaps not surprising that the K_i for NAP-taurine increases with temperature if it is binding to the modifier site, since Brahm (1977) has found that the dissociation constant for chloride at the modifier site also increases substantially with temperature.

probe in the medium, thereby preventing it from reacting with the membrane. If the molecule is bound to a particular site and upon activation by light reacts at that site before dissociating from it, scavengers should have no effect. Ruoho et al. (1973) have shown that *p*-aminobenzoic acid (PABA) at 10 mM is an effective scavenger for aryl nitrenes, and can almost completely abolish the effect of a photoactivated compound resulting from random collision of activated molecules in the bulk solution with the membrane. When used with

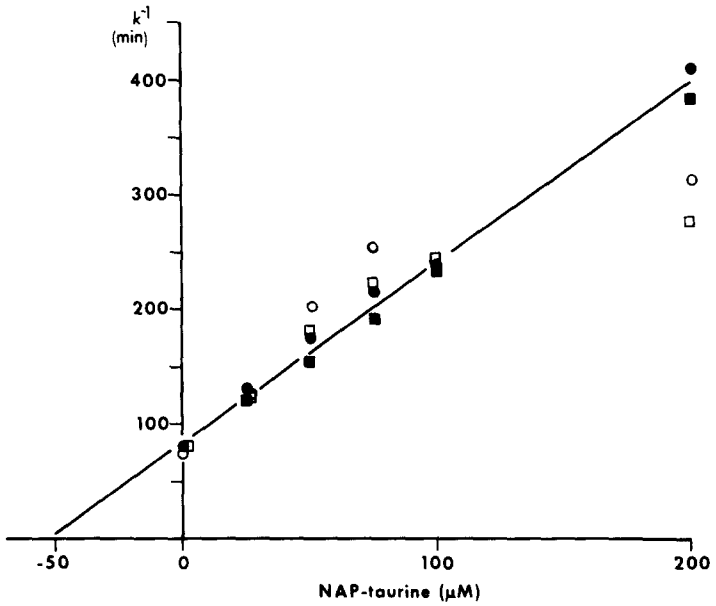

FIGURE 3. Modified Dixon plot of reversible and irreversible inhibition of sulfate exchange by NAP-taurine. The reciprocal of the rate constant for $^{35}SO_4$ efflux, k, is plotted against the NAP-taurine concentration in μM. Inasmuch as the sulfate concentration in these experiments was constant, the rate constant is proportional to the flux of sulfate, J, and the plot is analogous to the Dixon plot of $1/J$ vs. the inhibitor concentration. The circles and squares represent data from two separate experiments. The solid symbols represent the reversible inhibition with NAP-taurine present in the dark, whereas the open symbols represent the irreversible inhibition after exposure to light and extensive washing as described in Methods. The temperature for both fluxes and irradiation was 27–28°C. The line was calculated by the method of least squares for the reversible inhibition data. The x intercept corresponds to an apparent K_i of 53 μM.

the anion exchange system, PABA has the disadvantage that it is also a reversible inhibitor of the system, producing 68% inhibition at a concentration of 10 mM. It appears to compete with NAP-taurine for binding, because it reduces the reversible inhibition by NAP-taurine in the dark (Fig. 4), when its action as a scavenger is irrelevant. Despite this effect of PABA, it is still possible to compare the irreversible inhibition after exposure to light with the corresponding reversible inhibition in the presence of a constant amount of PABA. If the irreversible inhibition were caused by photoactivated NAP-taurine in the me-

dium, PABA should react with it and prevent its irreversible effect. From Fig. 4, it appears that, if anything, the irreversible inhibition is slightly larger than the reversible inhibition in the presence of PABA. It thus seems that reversibly bound NAP-taurine is activated by light and reacts at the site without contacting the PABA present in the bulk solution.

Effect of Chloride Concentration on Labeling of Membrane Components by NAP-Taurine

If, as seems likely from the preceding experiments, NAP-taurine acts as a photoaffinity label, the sites of reversible and irreversible binding should be

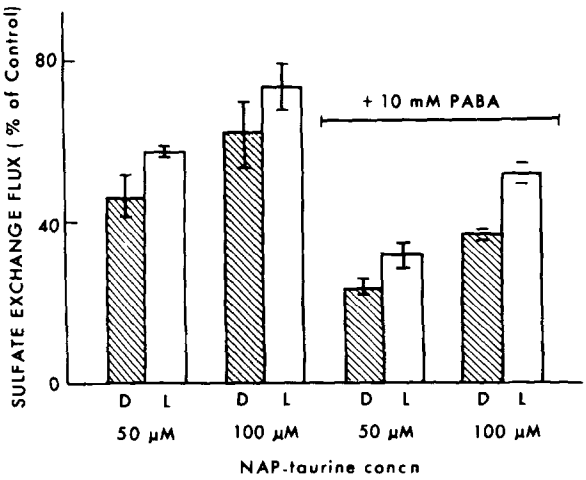

FIGURE 4. Comparison of reversible and irreversible effects of NAP-taurine on sulfate exchange in the presence and absence of a scavenger, *p*-aminobenzoic acid (PABA). Sulfate fluxes were measured as described in Methods. For the columns marked D, fluxes were measured in the presence of NAP-taurine in the dark. When PABA was present, it was added to the flux medium and the sulfate flux is expressed relative to a control with PABA but without NAP-taurine. For the columns marked L, cells were irradiated for 20 min by photoflood in the presence of NAP-taurine, with or without PABA, and then were washed as described in Methods to remove reversibly bound NAP-taurine and PABA. Bars represent standard deviations of at least three experiments except for the two rightmost columns, where the bars indicate the range of two determinations.

identical. In the accompanying paper (Knauf et al., 1978), it has been shown that relatively high chloride concentrations reduce the inhibitory effect of NAP-taurine, presumably by competing with NAP-taurine for binding to the modifier site of the transport system. To determine whether the covalent labeling of membrane components was similarly sensitive to chloride, cells were irradiated in the presence of ^{35}S-NAP-taurine in media of different chloride concentrations. The irreversible inhibition was measured as well as the binding to membrane proteins after separation by SDS-polyacrylamide gel electrophoresis. As is shown in Fig. 5, the extent of irreversible inhibition was reduced at a high chloride concentration (301 mM), as expected. The distribution of radioactivity

on polyacrylamide gels is shown in Fig. 6. As has been previously shown (Staros and Richards, 1974; Staros et al., 1974; Cabantchik et al., 1976; Rothstein et al., 1977), most of the ^{35}S-NAP-taurine (55–70%) is found in a peak which corresponds to band 3, the 95,000 dalton polypeptide which seems to be involved in anion exchange. From Fig. 6 it is also evident that the labeling of band 3 by NAP-taurine is decreased at the high chloride concentration roughly in parallel to the diminished inhibition. In this particular experiment, the inhibition was

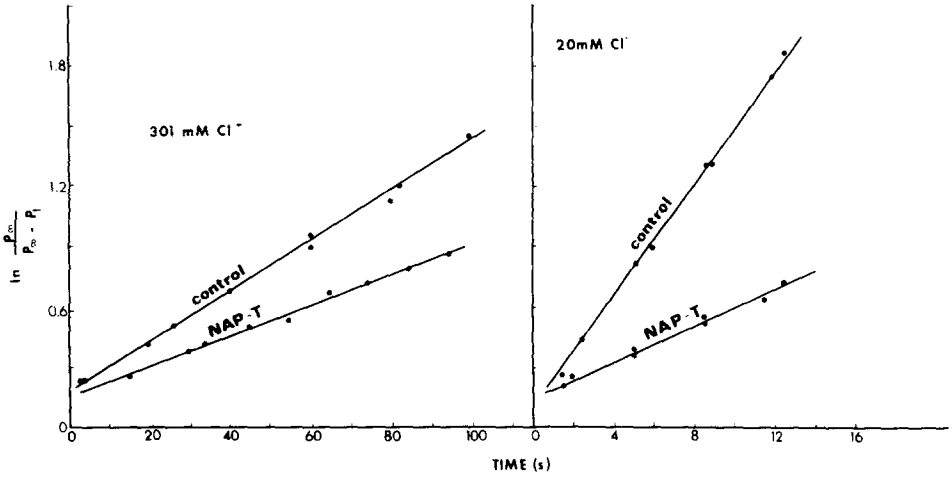

FIGURE 5. Irreversible inhibition of ^{36}Cl exchange by NAP-taurine at different chloride concentrations. Cells were equilibrated with either 20 or 301 mM chloride by the nystatin technique as described by Knauf et al. (1978), except that 14 mM Hepes (*N*-2-hydroxyethyl-piperazine-*N*'-2-ethane sulfonic acid) was substituted for 27 mM sucrose. They were then exposed to light from a photoflood for 20 min at 0–5°C in the presence of 53–55 μM ^{35}S-labeled NAP-taurine. After irradiation, the cells were washed three times with high or low chloride buffer containing 0.5% albumin, and then twice with buffer alone. They were then loaded with ^{36}Cl and the efflux of ^{36}Cl was measured at pH 7.8, 0°C as described by Knauf et al. (1978). The amount of ^{36}Cl appearing in the supernatant is plotted in a logarithmic fashion (as described in Methods) vs. time, so that the slope of the line is the rate-constant for ^{36}Cl exchange. Each line represents the least squares best fit to data points from two separate flux experiments. With 301 mM chloride present, the inhibition was only 40%, in comparison to 69% with 20 mM chloride. The Donnan ratio of internal to external chloride concentration was 0.95 with 301 mM chloride and 0.69 with 20 mM chloride. Thus, the increased inhibition with 20 mM chloride could not be ascribed to an increase in the Donnan ratio (see Discussion in Knauf et al., 1978).

decreased from 69% to 40%, a reversal of ~40%, whereas the counts in the band 3 peak were reduced by 30%. The smaller NAP-taurine peaks to the right and left of band 3 showed little apparent modulation by high chloride concentrations, but because of the small number of counts quantitation is difficult.

To more precisely define the site of chloride-sensitive NAP-taurine binding,

cells exposed to light in the presence of different concentrations of NAP-taurine and chloride were then treated with a low concentration of pronase. Under these conditions, pronase causes little or no inhibition of anion exchange (Cabantchik and Rothstein, 1974), but band 3 is cleaved to fragments of ~35,000

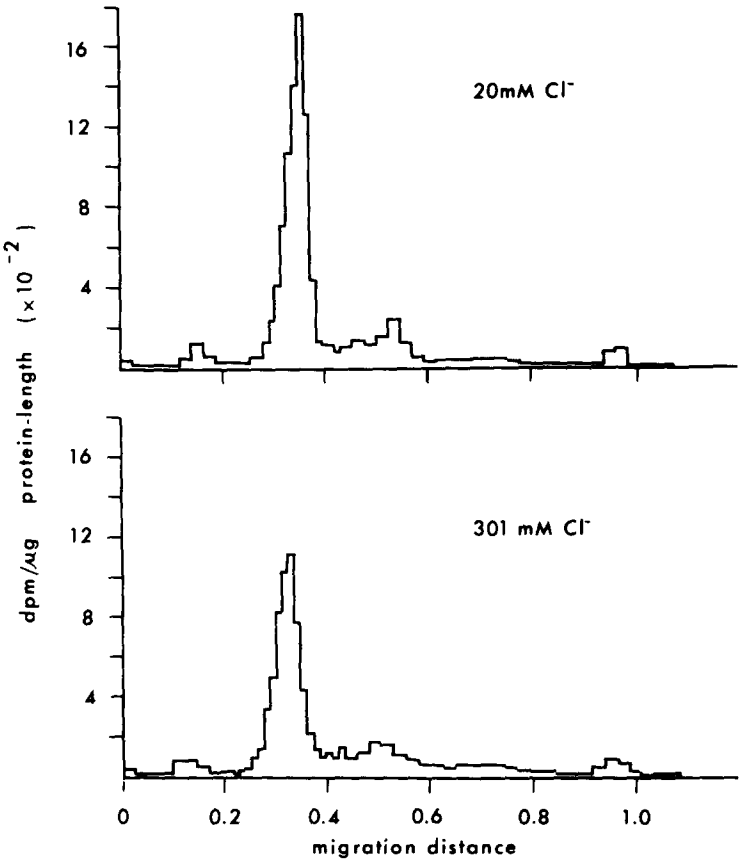

FIGURE 6. Labeling of membrane proteins by NAP-taurine in high and low chloride media. Ghosts were prepared from a portion of the cells whose chloride fluxes are shown in Fig. 5. The ghosts were solubilized and electrophoresed on 5.6% polyacrylamide gels, and the gels were sliced and counted as described in Methods. The radioactivity in each slice is presented on the ordinate as disintegrations per minute (dpm) per microgram of total ghost protein applied to the gel per length of the slice (see Methods). The abscissa represents the distance from the top of the gel (which is to the left in the figure), relative to the marker dye, Pyronin Y. The area under each peak is proportional to the number of NAP-taurine molecules bound per ghost. The major peak corresponds to the 95,000 dalton polypeptide known as band 3 (Fairbanks et al., 1971).

and 65,000 daltons molecular weight (Bender et al., 1971; Cabantchik and Rothstein, 1974). Approximately 80% of the NAP-taurine label in band 3 is recovered in the 65,000 dalton fragment (band 3'), but some of the minor NAP-taurine binding components are no longer evident (Cabantchik et al., 1976).

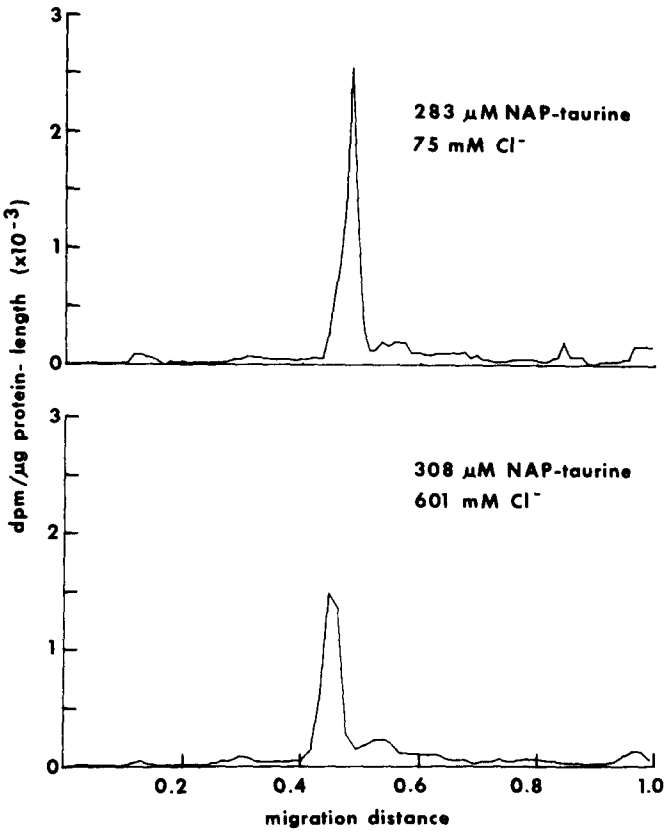

FIGURE 7. Labeling of membrane proteins by NAP-taurine in high and low chloride media after treatment of intact labeled cells with pronase. Cells loaded with either 75 mM or 601 mM chloride by the nystatin method (Knauf et al., 1978) were irradiated for 10 min at 0°C with light from an ELH projection lamp in the presence of the indicated concentrations of ^{35}S-labeled NAP-taurine. They were then washed three times in buffer containing 0.5% albumin, and then twice in buffer. Pronase treatment was carried out as described in Methods, and the cells were then washed three times in buffer with 0.5% albumin and then three times in buffer without albumin. Portions of the cells were loaded with ^{36}Cl, and chloride fluxes were measured as described in Methods. Ghosts were made from other portions of the cells and were electrophoresed on 5.6% polyacrylamide gels, sliced, and counted. The results are presented as described in Fig. 6. The areas under each peak correspond to the number of NAP-taurine molecules bound per ghost. The major peak corresponds to the position of the 65,000 dalton fragment of band 3 known as band 3'. Note that very little of the NAP-taurine remains in the region corresponding to intact band 3 (cf. Fig. 6). For the cells in 75 mM chloride, the inhibition of chloride flux was 67%; for the cells in 601 mM chloride it was reduced to 54%. The Donnan ratios were 1.05 and 0.96 for the cells in 75 and 601 mM Cl^-, respectively; the pH was 7.2 at 0°C.

The pronase-treated cells were extensively washed and ghosts from these cells were electrophoresed on SDS-polyacrylamide gels. The results for the highest NAP-taurine concentration used are shown in Fig. 7. The predominant peak of labeling, with 55% of the total, was the 65,000 dalton fragment, band 3′. The rest of the label was distributed throughout the gel. In the presence of high chloride (601 mM), the amount of NAP-taurine in band 3′ was substantially reduced.

Under the conditions of these experiments, in which internal and external chloride concentrations are nearly the same, the effects of chloride on NAP-

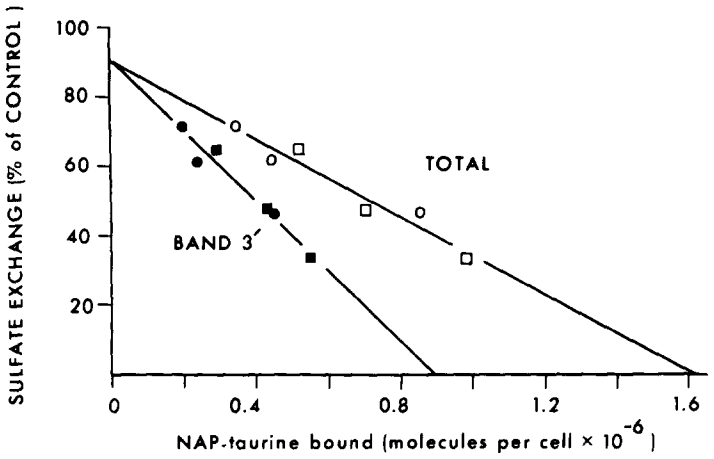

FIGURE 8. Relationship between chloride exchange rate and binding of NAP-taurine to pronase-treated ghosts and to band 3′. Cells were treated as described in Fig. 7. The number of NAP-taurine molecules per ghost (open symbols) was calculated from the radioactivity and the protein in samples of ghosts, assuming 6×10^{-10} mg protein per ghost. On this basis the number of sites might be slightly overestimated, because pronase treatment removes a small amount (<10%) of the membrane protein (Bender et al., 1971). The number of molecules bound to band 3′ (closed symbols) was calculated by multiplying the fraction of the total counts in the gel slices which are in band 3′ by the number of molecules of NAP-taurine per ghost. The squares refer to experiments with 75 mM chloride; the circles refer to experiments with 601 mM chloride. Lines were calculated by the method of least squares.

taurine labeling are probably due to competition for a common binding site. In previous experiments (Rothstein et al., 1976; 1977) in which only external chloride was varied, the inhibitory effect of NAP-taurine was changed to a greater degree than would be expected from such a direct competitive effect. As discussed in the preceding paper (Knauf et al., 1978), this observation may be attributed to effects of the chloride gradient on the accessibility of the modifier site. Under these circumstances the binding of NAP-taurine to band 3 (Rothstein et al., 1976) or to band 3′ (Rothstein et al., 1977) was also affected by the change in chloride concentration, in parallel with the effects on the

inhibitory potency of NAP-taurine. This provides further evidence that band 3′ is the site at which NAP-taurine binds and inhibits anion transport.

The binding of NAP-taurine to the ghosts and to band 3′, as modulated by Cl^- and NAP-taurine concentrations, both showed a reasonably linear relationship to the degree of inhibition (Fig. 8). The square of the correlation coefficient (r^2) for the total binding was 0.945 and the total number of sites associated with 100% inhibition was 1.6×10^6, similar to the value of 1.4×10^6 obtained earlier with cells which had not been exposed to pronase (Cabantchik et al., 1976). If only binding to band 3′ was considered, however, (the lower line in Fig. 8), the correlation was improved ($r^2 = 0.960$) and the number of sites was considerably less (9.0×10^5). This number is not very different from the number of DIDS binding sites in band 3′ per cell ($1–1.2 \times 10^6$, Lepke et al., 1976; Ship et al., 1977), and is similar to the number of band 3 monomers (1.16×10^6, Fairbanks et al., 1971), suggesting that most of the band 3 monomers are involved in transport and that one NAP-taurine molecule binds to each monomer.

Inasmuch as the total binding to the membrane and the binding to band 3′ both correlate well with inhibition of transport, it is clear that the total non-band 3′ binding must also be correlated to some extent with inhibition of anion exchange. Although this is true for the total, when the binding to each of the individual regions of the gel, particularly the minor peaks, was plotted against the fractional chloride exchange, the correlation was much poorer than in the case of band 3′. The values of r^2 ranged from 0.472 to 0.844. Thus, although these data do not rule out the possible participation of any of these minor peaks, it seems most probable that the chloride-sensitive NAP-taurine binding site associated with inhibition of anion exchange is located in band 3′.

Further evidence concerning the site of NAP-taurine binding is presented in Figs. 9 and 10. When intact cells were labeled with 300 μM ^{35}S-labeled NAP-taurine and ghosts from these cells were electrophoresed on 7.5% polyacrylamide gels, the distribution of radioactivity shown in Fig. 9 A was obtained. The pattern is essentially similar to that seen on 5.6% gels in Fig. 6. As shown in Fig. 10 A and A′, however, on 7.5% gels it is possible to more clearly distinguish the PAS-1 band (glycophorin), stained with the periodic acid-Schiff technique, from band 3. Most of the radioactivity is associated with band 3, and only a small shoulder (<7% of the total radioactivity) corresponds to the major glycoprotein, with a mobility from 0.25 to 0.28.

Treatment of the labeled ghosts with 10 mM NaOH and 0.1 mM EDTA removes many of the extrinsic ghost proteins (Steck and Yu, 1973), as illustrated in Fig. 10 B, but there is little change in the labeling pattern (Fig. 9 B), except that the labeling at high molecular weight in the mobility range near 0.1, which corresponds mainly to band 2.1 (Staros and Richards, 1974), is removed along with the corresponding Coomassie Blue stained band. This protein thus appears to be less tightly associated with the membrane than are the other components labeled by external NAP-taurine. In the case of the alkali-stripped ghosts, even less labeling (<4%) can be attributed to glycophorin.

As discussed above, band 3 can be cleaved by external pronase to yield band 3′. This can be further cleaved by protease treatment at the inside of the membrane to yield a 41,000 dalton soluble fragment and a 17,000 dalton

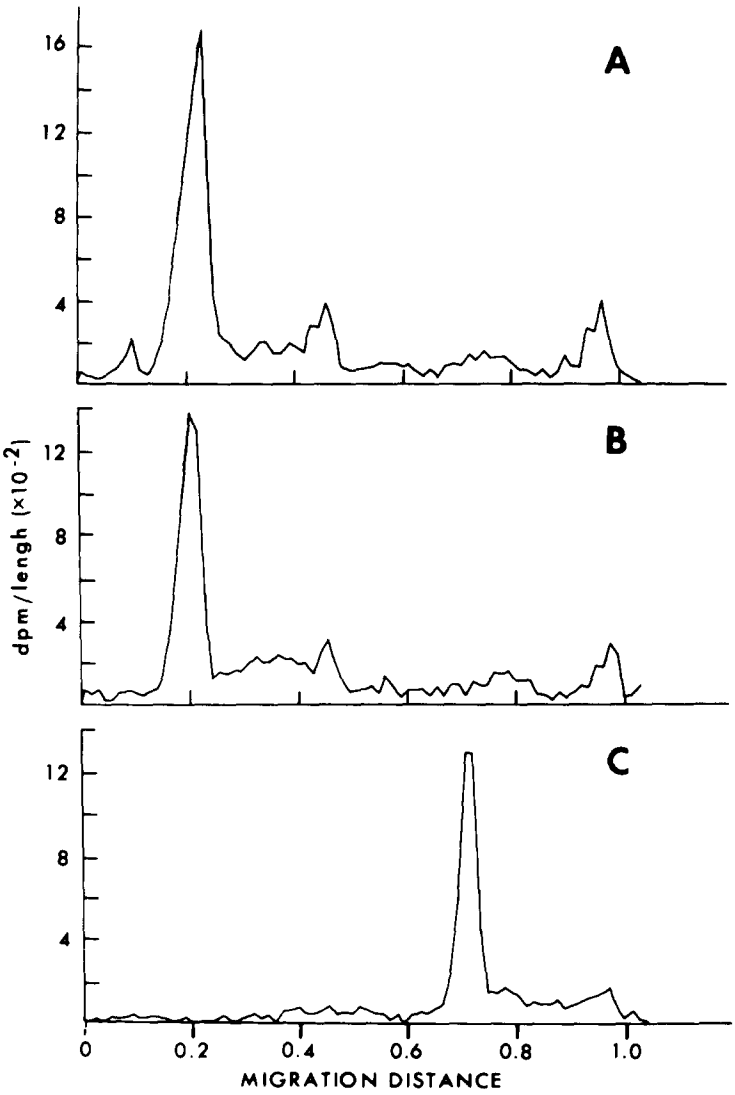

FIGURE 9. Separation of NAP-taurine labeled membrane proteins on 7.5% polyacrylamide gels: effects of alkali stripping and chymotrypsin treatment. Intact fresh red cells were labeled with 300 μM ^{35}S-labeled NAP-taurine as described in Methods. After washing with buffer containing 0.5% albumin, ghosts were prepared from the cells. Some of the ghosts were extracted with alkali and treated with chymotrypsin as described in Methods. After each treatment (NaOH extraction or chymotrypsin), the washed pellet was made up to the original weight of the ghost suspension with 5P8. Samples of these suspensions were solubilized in SDS and were applied to 7.5% polyacrylamide gels. The abscissa represents the distance from the top of the gel relative to the marker dye, Pyronin Y. The ordinate represents the number of disintegrations per minute (dpm) of ^{35}S per normalized length of the slice (see Methods). In this case, the disintegrations per minute were not normalized for the amount of ghost protein applied to the gel. Rather, to each gel was applied an amount of ghost suspension corresponding to 33 μl of packed intact cells ($\sim 3.7 \times 10^8$ cells), assuming complete recovery of the ghosts at all stages of treatment. The peak areas after treatment thus represent a minimum estimate of the recovery of label. (A) Ghosts from intact labeled cells; (B) alkali-stripped ghosts; (C) chymotrypsin treated, alkali-stripped ghosts.

transmembrane segment (Steck et al., 1976). The 41,000 dalton N-terminal segment seems to be entirely on the inside of the membrane based on its solubility behavior (Steck et al., 1976; 1978). Thus, the only part of band 3′ which should be labeled by external NAP-taurine is the 17,000 dalton fragment. This 17,000 dalton fragment can be generated directly by treatment of alkali-stripped ghosts with chymotrypsin. As shown in Fig. 10 C, this results in conversion of most of band 3 to a fragment of ~17,000 daltons molecular weight, with mobility of ~0.7. After chymotrypsin treatment, over 70% of the band 3 labeling in stripped ghosts (Fig. 9 B) is recovered in this fragment (Fig. 9 C). On this basis it seems that the modifier site labeled by external NAP-taurine is located in this 17,000 dalton transmembrane segment. Because this fragment has already been shown to contain the DIDS binding site (Grinstein et

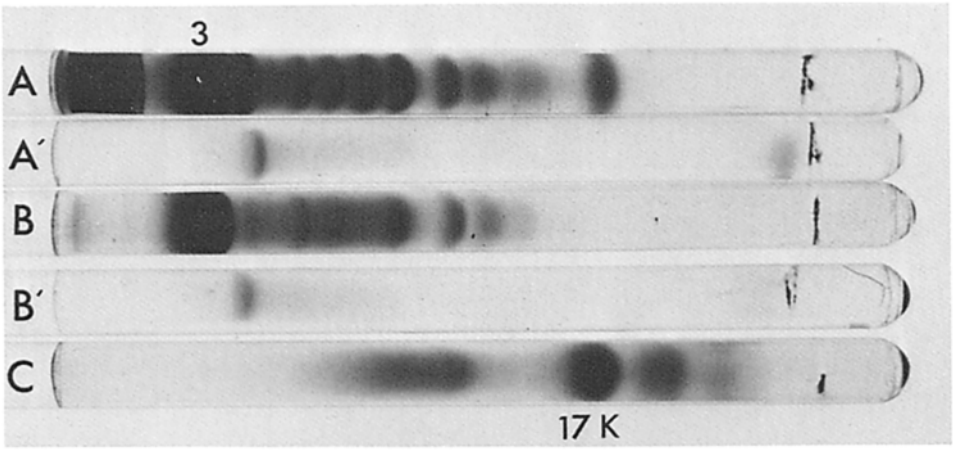

FIGURE 10. Coomassie Blue- and periodic acid-Schiff-stained proteins separated on 7.5% polyacrylamide gels, showing the effects of alkali stripping and chymotrypsin treatment. Coomassie Blue-stained proteins: (A) ghosts; (B) alkali-stripped ghosts; (C) chymotrypsin-treated, alkali-stripped ghosts. Periodic acid-Schiff staining: (A′) ghosts; (B′) alkali-stripped ghosts. The top of the gel is to the left in each case; the black line near the right hand end of the gel represents the migration distance of the marker dye, Pyronin Y. The same amounts of the same ghost suspensions were applied to the gels as in Fig. 9.

al., 1978), which appears to be the substrate site (Shami et al., 1978), and because the segment seems to be fully capable of supporting anion transport (Grinstein et al., 1978), the present findings would indicate that all of the known sites of the transport system are located in this fragment. As further analysis of the peptides comprising this segment becomes possible, it will be of interest to examine the relationship between the substrate site (labeled by DIDS) and the modifier site (labeled by external NAP-taurine). These two functionally related sites are not only present in the same segment of band 3, but may be closely associated in a geographic sense. Both sites are accessible to probes from the outside under conditions of minimal penetration, so both are presumably located in that portion of the 17,000 dalton segment that is exposed at the external face of the membrane. Furthermore, DIDS bound to the transport site

largely prevents the binding of NAP-taurine to band 3 and reciprocally NAP-taurine bound to the modifier site largely prevents the binding of DIDS to band 3 (Rothstein et al., 1976; Cabantchik et al., 1976). This finding can be readily explained if the two sites are adjacent, since the binding of a bulky anion to one site could prevent the binding of another large anion to the second site by steric hindrance. It must be kept in mind, however, that the mutual interference could also be explained by an allosteric phenomenon in which the binding of a probe to one site would decrease the binding affinity of a second site, at some distance.

APPENDIX

PHILIP A. KNAUF

The reversible interaction of NAP-taurine (N) with membrane transport sites (S) to form an NS complex can be described by the equation: $(N)\cdot(S)/(NS) = K_i$, where K_i is the apparent dissociation constant for NAP-taurine at the site. If the irreversible inhibition by NAP-taurine is supposed to occur by rapid and complete reaction of the photoactivated compound at the site, and if m is the rate of photoactivation, then:

$$\frac{d(NS_c)}{dt} = m(NS) = \frac{m(N)\cdot(S_t - NS_c)}{K_i + (N)}, \tag{1}$$

where S_t is the total number of sites, and NS_c represents sites which have reacted covalently with NAP-taurine. If the NAP-taurine in solution is photoactivated and reacts to form a compound which does not bind to the site, and k is the rate of photoactivation, then the concentration of unreacted NAP-taurine in solution, (N), as a function of time is given by:

$$(N) = N_0 \exp(-kt), \tag{2}$$

where N_0 is the NAP-taurine concentration at time zero. Substituting Eq. 2 into Eq. 1 and integrating, we obtain:

$$\frac{(NS_c)}{S_t} = \left(1 - \frac{(K_i + N_0 \exp(-kt))^{m/k}}{(K_i + N_0)^{m/k}}\right), \tag{3}$$

which, for long irradiation times (as t approaches infinity) is:

$$\frac{(NS_c)}{S_t} = \left\{1 - \left(\frac{K_i}{K_i + N_0}\right)^{\frac{m}{k}}\right\}. \tag{4}$$

For the simplest case, where the rates of photoactivation of the bound and free NAP-taurine are identical ($m = k$), Eq 4 reduces to:

$$\frac{(NS_c)}{S_t} = \frac{N_0}{K_i + N_0}. \tag{5}$$

If the fraction of occupied sites, $(NS_c)/S_t$, is equated with the fractional inhibition, it can be readily seen that the irreversible inhibition is exactly the same as the reversible inhibition under the same conditions, which would be given by:

$$\frac{(NS)}{S_t} = \frac{N_0}{K_i + N_0}. \tag{6}$$

If photoactivation at the binding site occurs more rapidly than in solution ($m > k$), then the irreversible inhibition will be greater than the reversible inhibition. This might happen, for example, if cells coating the walls of the reaction vessel received more exposure to light than did the bulk NAP-taurine in solution. The experimental data for 50 μM NAP-taurine suggest that m is slightly larger than k, inasmuch as the irreversible inhibition is slightly (9%) but significantly ($P < .02$) higher than the reversible inhibition.

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