

Asymmetry of the Red Cell Anion Exchange System

Different Mechanisms of Reversible Inhibition by N-(4-Azido-2-Nitrophenyl)-2-Aminoethylsulfonate (NAP-Taurine) at the Inside and Outside of the Membrane

P. A. KNAUF, S. SHIP, W. BREUER, L. McCULLOCH,
and A. ROTHSTEIN

From the Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8

ABSTRACT In the dark, the photoaffinity reagent, N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-aurine), acts as a reversible inhibitor of red cell anion exchange when it is present either within the cell or in the external solution. A detailed analysis of the inhibition kinetics, however, reveals substantial differences in the responses to the probe at the two sides of the membrane. On the inside of the cell, NAP-aurine is a relatively low affinity inhibitor of chloride exchange ($K_i = 370 \mu\text{M}$). Both the effects of chloride on NAP-aurine inhibition and the affinity of NAP-aurine for the system as a substrate are consistent with the concept that internal NAP-aurine competes with chloride for the substrate site of the anion exchange system. External NAP-aurine, on the other hand, is a far more potent inhibitor of chloride exchange ($K_i = 20 \mu\text{M}$). It acts at a site of considerably lower affinity for chloride than the substrate site, probably the modifier site, at which halide anions are reported to cause a noncompetitive inhibition of chloride transport. NAP-aurine therefore seems to interact preferentially with either the substrate or modifier site of the transport system, depending on the side of the membrane at which it is present. It is suggested that the modifier site is accessible to NAP-aurine only from the outside whereas the transport site may be accessible from either side.

INTRODUCTION

In recent years, our understanding of the anion exchange system of the human red cell has been greatly augmented by two sorts of information. On the one hand, kinetic studies have shown that the transport is almost exclusively a one-for-one exchange of anions and that the system displays properties consistent with a mobile carrier model (Wieth, 1972; Gunn et al., 1973; Hunter, 1971;

Sachs et al., 1975; Dalmark, 1975 *a*; Cabantchik et al.¹). On the other hand, the use of inhibitory chemical probes has led to the identification of the 95,000-dalton polypeptide, known as band 3 (Fairbanks et al., 1971), as the protein which apparently mediates the transport process (Cabantchik and Rothstein, 1974; Ho and Guidotti, 1975; Rothstein et al., 1976).

To integrate these two kinds of information, additional knowledge is required concerning the relationship between carrier sites postulated from kinetic analysis of transport and inhibitory sites in band 3 labeled by the covalent reactions of the chemical probes. The evaluation of the mechanism by which probes inhibit transport can be greatly facilitated by the use of "bimodal" inhibitors (Deuticke, 1977; Cabantchik et al.¹), that is, probes which are capable of covalent reaction for labeling purposes but which, under other circumstances, act as reversible inhibitors of the system, allowing the nature of their effects to be determined by a kinetic analysis. Photoaffinity reagents are particularly useful, because they can be used as reversible or irreversible probes under exactly the same conditions, the only difference being the presence or absence of light to activate the compound.

The photoaffinity reagent, *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate, or NAP-taurine (Fig. 1), inhibits sulfate and chloride fluxes in human red cells

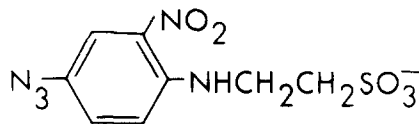


FIGURE 1. Structure of NAP-taurine.

(Cabantchik et al., 1976). This compound, synthesized by Staros and Richards (1974) as a surface label for membranes, is chemically unreactive in the dark. Upon exposure to light, it is converted to a very highly reactive nitrene, which is even capable of inserting into carbon-hydrogen bonds in its vicinity (Knowles, 1972). Its covalent reaction occurs largely at sites to which it was bound reversibly in the dark, so it can be used to label membrane components involved in its reversible inhibitory action (Knauf et al., 1978).

The present paper is primarily concerned with the reversible interactions of NAP-taurine with the anion exchange system in the dark. Previous work in this laboratory has shown that NAP-taurine appears to act as a substrate for the same anion exchange system which transports inorganic ions such as chloride and sulfate, in that its transport is sensitive to inhibitors of anion exchange, such as 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid (DIDS) and dipyridamole (Cabantchik et al., 1976). These observations might lead one to expect that NAP-taurine acts as a competitive inhibitor of anion transport by binding to the substrate site.

On the other hand, Dalmark (1976) has shown that substrate anions such as Cl^- and other halides can inhibit the chloride exchange system not only by

¹ Cabantchik, Z. I., P. A. Knauf, and A. Rothstein. 1978. 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.

competing at the substrate site, but also, at higher concentrations, by binding to a modifier site, at which they produce a noncompetitive inhibition of chloride exchange. Further data are therefore needed to determine the mechanism of NAP-aurine inhibition and thereby to identify the function of the site(s) in the transport system which can be labeled by NAP-aurine. To accomplish this, we have studied the effects of variations in the chloride concentration on the inhibitory potency of NAP-aurine. This has been done under conditions where internal and external chloride are nearly equal (Dalmark, 1976), to simplify the mathematical analysis of the system and to preclude possible effects of changes in membrane potential or ion gradients.

Chloride fluxes can be measured in short times at 0°C, conditions where little or no transmembrane flow of NAP-aurine occurs (Staros et al., 1975). Thus, we were able to compare the effects of internal and external NAP-aurine. The data indicate that NAP-aurine can inhibit by two different mechanisms, depending on the side of the membrane at which it is present, suggesting an asymmetry in the arrangement of the anion transport system in the membrane.

Some of the work described in this paper has been briefly presented elsewhere (Knauf et al., 1977).

METHODS

Cell Preparation

For most experiments, blood was obtained from volunteer donors by venipuncture and was collected in heparinized Vacutainer tubes (Becton, Dickinson & Co., Rutherford, N.J.). In some cases, where indicated, bank blood was used within 3 wks after collection. The cells were washed three times with 165 mM NaCl and the buffy coat was removed. For the chloride flux experiments, the cells were brought to the indicated pH with CO₂ before washing.

Nystatin Treatment

In order that cell chloride concentration could be varied in parallel with extracellular chloride concentration, the potassium content of the cells was adjusted using the nystatin method of Cass and Dalmark (1973), essentially as described by Dalmark (1975 *b*). Briefly, cells were washed with solutions containing from 19 to 300 mM KCl, 1 mM NaCl, and sufficient sucrose to make the solution approximately isotonic (or 27 mM sucrose for solutions which had 150 mM KCl or more). The cells were then resuspended at 5% hematocrit in these same solutions containing 75 µg/ml nystatin (Mycostatin, E. R. Squibb & Sons, Princeton, N. J.), added as 5-mg/ml solution in methanol. After incubation on ice for 10 min, the suspensions were centrifuged and were resuspended in solutions with the same KCl, NaCl, and nystatin concentrations, but with only 27 mM sucrose. (For cells to be loaded with 600 mM KCl, however, the first incubation was with 300 mM KCl, 1 mM NaCl, 27 mM sucrose and the second with 600 mM KCl, 1 mM NaCl, 27 mM sucrose). After a 10-min incubation on ice, the cells were washed three times at room temperature with the same solutions except without nystatin, to remove nystatin and restore the membrane permeability to normal (Cass and Dalmark, 1973). The cells were left overnight on ice in these solutions, and were washed again the following morning with solutions ("flux media") of the same KCl, NaCl, and sucrose concentrations, but containing in addition 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid), which had been brought to the desired pH with NaOH.

Chloride Fluxes

Cells were loaded with ^{36}Cl (Amersham/Searle Corp., Arlington Heights, Ill.) by incubation for at least 15 min on ice at 30% hematocrit in the appropriate flux medium to which 0.5–0.9 $\mu\text{Ci/ml}$ ^{36}Cl had been added, replacing an equivalent amount of non-radioactive chloride. Aliquots of suspension containing $\sim 50 \mu\text{l}$ of cells were transferred to small polyethylene tubes (Beckman) and centrifuged in a Beckman Microfuge for 1 min (Beckman Instruments, Inc., Fullerton, Calif.). The supernate was removed by aspiration and the end of the tube was cut off with a scalpel blade. To start the flux, a syringe containing $\sim 7 \text{ ml}$ of prechilled medium was attached to the top of the tube and the cells were flushed into a total of 30 ml of flux medium which contained the indicated concentration of NAP-aurine. At the low hematocrit used in these experiments, binding of NAP-aurine to the cells should cause the extracellular concentration to decrease by $< 0.1 \mu\text{M}$. The medium was maintained at 0°C in a water-jacketed chamber by a Lauda K4R controlled temperature circulator (Lauda Div., Brinkmann Instruments, Inc., Westbury, N.Y.). Samples of cell-free supernate were taken at appropriate intervals using the filtration technique described by Dalmark and Wieth (1972), with Millipore RAWP (1.2 μm) filters and AP prefilters (Millipore Corp., Bedford, Mass.). At the end of the flux experiment, duplicate 1-ml aliquots of the suspension were added to 0.2 ml of 30% trichloroacetic acid (TCA). Experiments with NAP-aurine were performed under dim white light.

A 0.8 ml portion of each cell-free supernate was added to 8 ml of Triton X-100-toluene scintillation fluid (New England Nuclear, Boston, Mass.), and radioactivity was determined in a Packard C2425 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). The supernates from the TCA-precipitated samples of the total suspension were counted in the same manner. Counts of standards with and without TCA demonstrated that TCA affected the counting efficiency of ^{36}Cl by less than 3%.

Determination of Cell Water and Cell Chloride Concentration

Nystatin-treated cells were incubated on ice at 20% hematocrit in the appropriate flux medium containing ^{36}Cl and [^3H]inulin (New England Nuclear), the latter as tracer for extracellular fluid (ECF). After at least 30 min incubation on ice, with occasional stirring, the suspension was centrifuged at 27,000 g for 10 min in a prechilled HB-4 rotor in a Sorvall RC2-B centrifuge (Dupont Instruments-Sorvall, Wilmington, Del.). By means of an Aliquanter (Hamilton Co., Reno, Nev.), 25- μl samples of supernate were transferred to tubes containing 1 ml of 5% TCA. After aspiration of the rest of the supernate, similar aliquots of the packed cells were added to TCA. These samples were mixed and centrifuged, and 0.8 ml of the supernate was transferred to scintillation vials and counted as described above. The rest of the packed cells were transferred to a tared aluminum pan and weighed. They were then dried at 80°C for at least 24 h, cooled in a dessicator and weighed again. Cell water was determined from the difference between wet and dry weight. The intracellular chloride concentration was calculated from the amount of ^{36}Cl in the cells (corrected for the trapped ECF, which was generally $< 5\%$), the specific activity of chloride-36 in the suspending medium, and the cell water. After nystatin treatment, the cells were slightly swollen, having a cell water volume/packed cell volume of 0.73–0.76, compared to about 0.7 in untreated cells. Such changes in cell volume, however, do not appear to affect chloride permeability significantly (Funder and Wieth, 1976).

Calculation of Rate Constants and Chloride Fluxes

Under the conditions of these experiments, chloride is at equilibrium; that is, there is no net chloride flux into or out of the cells. Provided that the isotope exchange can be described by a single rate constant, the time-course of the efflux of ^{36}Cl from ^{36}Cl loaded cells can be described by the equation:

$$\ln [P(\infty)/[P(\infty) - P(t)]] = kt,$$

where $P(\infty)$ represents the radioactivity per ml of total suspension, $P(t)$ is the radioactivity per milliliter of supernate at time t , and k is the rate constant for the isotope exchange process (Knauf and Rothstein, 1971). If $\ln [P(\infty)/[P(\infty) - P(t)]]$ is plotted against time, the rate constant can be determined directly from the slope of the line obtained by the method of least squares. In the experiments described in this paper, the square of the correlation coefficient (r^2) for such data was usually >0.99 , and was never <0.97 .

Based on the assumption that the properties of the ^{36}Cl tracer do not differ from those of the nonradioactive chloride, the unidirectional efflux of chloride, J , (which is equal to the influx) may be calculated from the rate constant for tracer exchange as follows (Gardos et al., 1969):

$$J = \frac{k S_i S_o}{S_i + S_o},$$

where S_i is the total amount of chloride in the cells, and S_o is the total amount of chloride in the medium. In these experiments, $S_i \ll S_o$, and therefore the flux $\cong k S_i$. If S_i is expressed as the amount of chloride per kilogram dry solids (equivalent to 3.1×10^{13} cells; Funder and Wieth, 1966), the flux may be obtained as millimoles per kilogram solids/minute. For fluxes at high chloride concentrations, the dry weight of the cells was corrected for the contribution of intracellular KCl, as suggested by Dalmark (1975 b).

NAP-Taurine Efflux

[^{35}S]NAP-*taurine* was synthesized from [^{35}S]taurine (Amersham-Searle; 77 mCi/mmol) as described by Staros and Richards (1974) and was purified by preparative thin-layer chromatography on silica gel plates with methanol-dichloroethane (1:1). Purity was checked by thin-layer chromatography with chloroform-methanol (3:1) and by comparing the spectrum of the compound formed with that of authentic NAP-*taurine*. In addition, NAP-*taurine* fluxes determined at different ratios of radioactive and nonradioactive NAP-*taurine* (Pierce Chemical Co., Rockford, Ill.) differed by $<10\%$, indicating that the labeled compound was very similar to the unlabeled NAP-*taurine* in its transport behavior.

For the efflux measurements, blood bank cells were washed in saline and suspended at 20% hematocrit in a buffer containing 137 mM NaCl, 5 mM Na_2SO_4 , 5 mM glucose, 20 mM Hepes (titrated to pH 7.2 at 37°C with NaOH), and 1 $\mu\text{g}/\text{ml}$ chloramphenicol. The desired concentration of ^{35}S -labeled NAP-*taurine* was added, and the suspension was incubated overnight. After incubation the suspension was cooled at 0°C, and the cells were washed three times with ice-cold buffer containing 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and then three times with ice-cold buffer. The cells were resuspended in buffer at 37°C, and samples were taken at various times and centrifuged. The radioactivity of the supernate was determined as described above, except that Aquasol (New England Nuclear) was used instead of Triton X-100-toluene. Because after 60 min there was little change in the radioactivity of the supernate, the $P(\infty)$ value was determined from the average radioactivity at 60 and 90 min. This

procedure was necessary inasmuch as ~10–15% of the total cellular NAP-aurine (as determined after TCA precipitation) exchanged very slowly, presumably because of binding to intracellular constituents. The amount of rapidly exchangeable NAP-aurine per dry weight of cells was determined from the amount of exchangeable NAP-aurine per milligram of hemoglobin (measured by the method of Crosby et al., 1954) and the amount of hemoglobin per dry weight of cells. The concentration of NAP-aurine in the cell water was determined in a similar manner. The intracellular to extracellular ratio of exchangeable NAP-aurine was somewhat higher (1.2–1.6) than the ratio for chloride (0.82), again suggesting some intracellular binding of NAP-aurine.

Chloride Fluxes in Resealed Ghosts Loaded with NAP-Taurine

Fresh washed red cells were brought to 50% hematocrit with 155 mM NaCl. To this suspension of cells at 0°C was added 10 vol of ice-cold hemolyzing solution (4 mM magnesium sulfate, 1.2 mM acetic acid, pH 3.2), and the resulting mixture was kept at 0°C for 5 min (Schwoch and Passow, 1973). Then 1 vol of ice-cold resealing solution (concentrated Hepes-buffered sodium chloride, pH 7.2) was added, to give the desired final buffer (10 mM) and chloride concentrations. The suspension was kept at 0°C for 10 min (Funder and Wieth, 1976), and then incubated at 37°C for 1 h. After cooling to 0°C, the resealed ghosts were centrifuged in a Sorvall SS-34 rotor at 20,000 *g* for 10 min, then washed three times with ice-cold 10 mM Hepes buffer, pH 7.2 at 0°C, containing the desired NaCl concentration. The ghosts were pink and stable in color.

To load the resealed ghosts with NAP-aurine, a 25% hematocrit suspension was incubated with a solution of NAP-aurine in buffer for 1 h at 37°C in the dark. After cooling to 0°C, the ghosts were centrifuged and then brought to 50% hematocrit with buffer. The rate constant for chloride self-exchange was determined at pH 7.2, 0°C in the usual way, as described above, except that the ³⁶Cl loaded ghosts were centrifuged at 10,000 *g* for 5 min in an Eppendorf model 3200 centrifuge before the start of the flux measurement (Eppendorf Div., Brinkmann Instruments, Westbury, N.Y.).

The concentration of NAP-aurine inside the resealed ghosts was determined by adding 10 vol of ice-cold acetone to 1 vol of 50% hematocrit ghosts, centrifuging, and reading the absorption of the supernate at 471 nm. This value was compared with that obtained for standard solutions of NAP-aurine.

RESULTS

Effects of External NAP-Taurine on Chloride Exchange

Fig. 2 depicts the time-course of the efflux of ³⁶Cl into the medium from ³⁶Cl-loaded cells. As has been observed in previous studies (Dalmark and Wieth, 1972; Gunn et al., 1973; Dalmark, 1975 *a*), >90% of the cellular chloride exchanges with a single rate constant, given by the slope of the line. Under the conditions shown in Fig. 2, 200 μM NAP-aurine inhibits chloride exchange by ~90%. In these experiments, cells were added at time zero to a medium containing NAP-aurine. The inhibitory effect of NAP-aurine appears to be complete within the time required to take the first sample, ~3 s. As NAP-aurine penetrates the membrane very slowly under these conditions, if at all (Staros et al., 1975), the site at which NAP-aurine acts must be readily accessible from the outside of the membrane.

Inasmuch as NAP-aurine appears to be a substrate for the anion exchange system (Cabantchik et al., 1976; Rothstein et al., 1977), its inhibitory effect might

reflect competition with chloride for the substrate site. To examine this possibility, the effects of NAP-taurine on chloride exchange were measured at different chloride concentrations, with the internal chloride concentration kept nearly equal to the external chloride concentration by means of the nystatin technique (Cass and Dalmark, 1973; Dalmark, 1975 *b*). In Fig. 3, the data at chloride concentrations between 20 and 150 μM are plotted by the method of Hanes (1932); that is, the substrate concentration divided by the flux is plotted against the substrate concentration. For a simple enzyme or carrier system, if there is competitive inhibition, the slopes of the lines for different inhibitor

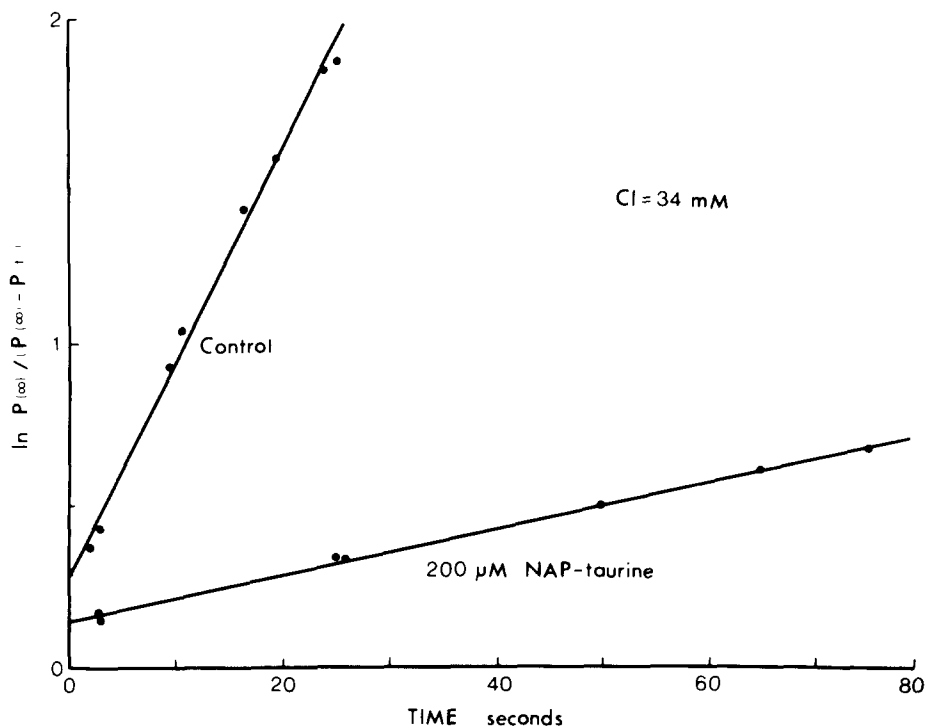


FIGURE 2. Effect of NAP-taurine on chloride exchange. Cells were treated with nystatin, and chloride flux was measured as described in Methods. The data are plotted against time in a logarithmic form, such that the slope is equal to the rate constant for chloride-36 exchange. Each line was determined from points for two separate flux experiments at 0°C, pH 7.2 by the method of least squares.

concentrations should be identical (Webb, 1963). Because of the presence of the modifier site, at which a low affinity binding of chloride or other halides causes a noncompetitive inhibition of exchange (Dalmark, 1976), the slopes of the lines should increase slightly with increasing inhibitor concentration (see Appendix). The expected result for competitive inhibition with 200 μM NAP-taurine is illustrated by the broken line in Fig. 3. It has been calculated using 65 mM (Brazy and Gunn, 1976) and 335 mM (Dalmark, 1976) as the dissociation constants for chloride at the substrate (K_s) and modifier (K_m) sites, respectively

(see Appendix). In contrast to the prediction based on competition for the transport site, the slopes of the experimental lines increase dramatically with increasing inhibitor concentration. The data are therefore inconsistent with competitive inhibition. On the other hand, the results are consistent with noncompetitive inhibition of transport by NAP-taurine. For this mode of inhibition, the slopes should increase substantially with NAP-taurine concentration, and the lines should intersect on the abscissa at -54 mM (see Appendix). In fact, the lines determined by the method of least squares for the data with 0, 50, 100, and 200 μ M NAP-taurine intersect the abscissa at -53.0 mM, -45.8 mM, -47.2 mM, and -48.2 mM, respectively, in good agreement with the expected result for noncompetitive inhibition.²

The data of Fig. 3 strongly indicate that external NAP-taurine does not inhibit

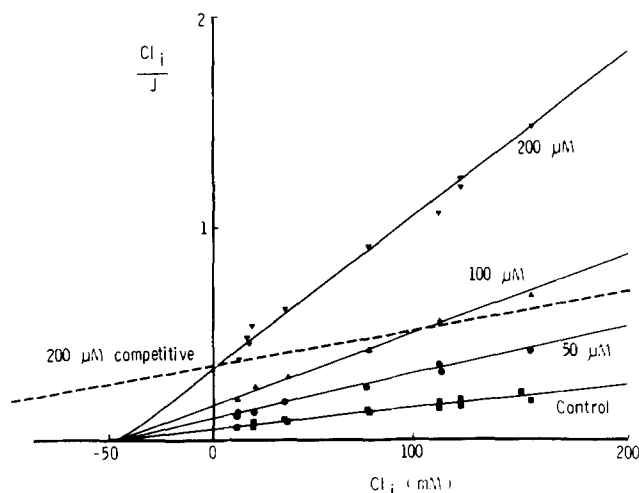


FIGURE 3. Hanes plot of the internal chloride concentration (Cl_i) in millimolar divided by the chloride equilibrium exchange flux (J) in millimoles/kilogram of cell solids per minute, vs. Cl_i . Rate constants and fluxes are from three separate experiments with nystatin-treated cells, performed as described in Methods and Fig. 2. Lines were calculated by the method of least squares. The pH was 7.2–7.5 and the temperature was 0°C .

chloride exchange by acting at the substrate site, but they do not rule out the possibility that NAP-taurine may act at a site with a lower affinity for chloride, such as the modifier site. The effects of NAP-taurine were therefore examined at higher chloride concentrations. The Hanes or Lineweaver-Burk plots are not useful under these circumstances because they become nonlinear, owing to the

² As can be seen from Eqs. 10, 13, and 16 of the Appendix, the plot of Cl_i/J curves upward at high Cl_i concentrations. This curvature was far less marked in the presence of NAP-taurine than in the control (data not shown). The model of completely noncompetitive inhibition predicts that the inhibitor will have no effect on the curvature (Eq. 10), in contrast to the experimental results. For the model in which NAP-taurine acts at the modifier site, however, the curvature should diminish at high inhibitor concentrations, since the term I/K_i in Eq. 13 will tend to dominate the Cl_i/K_s term which gives rise to the nonlinearity.

presence of a second chloride binding site (Webb, 1963). Straight lines can be obtained, however, by plotting the reciprocal of the fractional inhibition, i , against the reciprocal of the inhibitor concentration, I (Webb, 1963). The slope of the line is the apparent dissociation constant for the inhibitor, K_i^{app} . As can be seen from Fig. 4, the value of K_i^{app} does not increase substantially except at

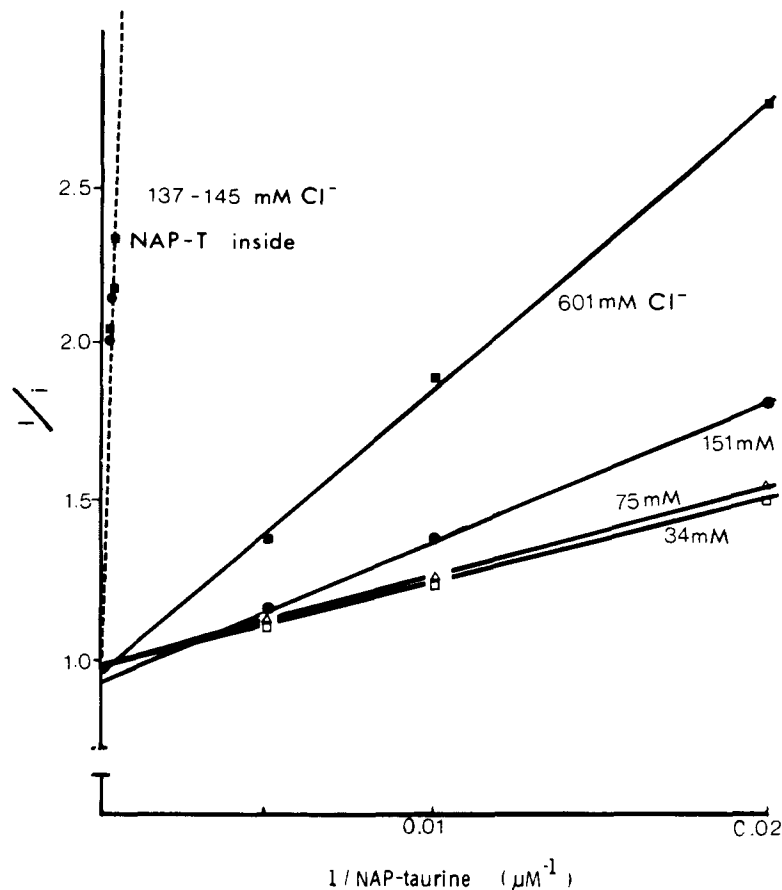


FIGURE 4. Plot of the reciprocal of the fractional inhibition of chloride exchange ($1/i$) vs. the reciprocal of the NAP-aurine concentration ($1/I$). The solid lines were calculated by the method of least squares from data with external NAP-aurine at the indicated chloride concentrations, pH 7.2, 0°C. The broken line depicts the effect of internal NAP-aurine on chloride efflux from NAP-aurine loaded cells. The slope of the lines gives the apparent dissociation constant for the inhibitor, K_i^{app} .

600 mM chloride, indicating that an apparent decrease in the affinity of NAP-aurine for its inhibitory site is readily noticed only at very high chloride levels. The y intercepts of the lines are not above 1, indicating that high concentrations of NAP-aurine are capable of completely inhibiting the system. The straight lines obtained both in this plot and in the Dixon plot ($1/\text{flux}$ vs. I , data not

shown) indicate that only one inhibitor molecule combines with each membrane site, at least over the concentration range from 25 to 100 μM .³

A more quantitative estimate of the effect of chloride on the apparent K_i for NAP-taurine can be obtained (Webb, 1963) from the Hunter-Downs plot in Fig. 5. In this case, $I(1 - i)/i$, a measure of the apparent K_i , is plotted against the chloride concentration. The y intercept gives the K_i at zero chloride concentra-

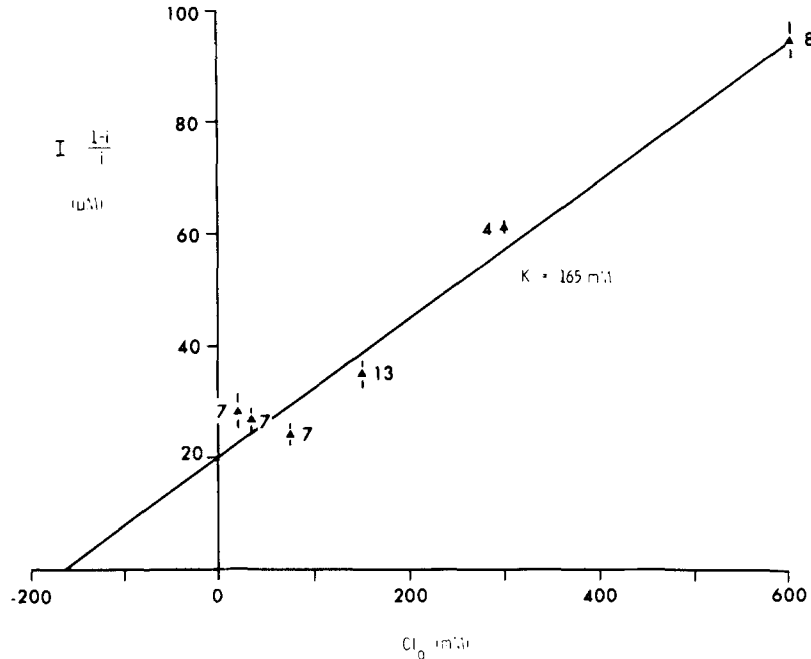


FIGURE 5. Hunter-Downs plot of the apparent K_i for inhibition by external NAP-taurine (calculated as $I(1 - i)/i$) vs. the external chloride concentration (Cl_0). Inhibition was measured in nystatin-treated cells at 0°C and pH 7.2 as described in Methods and illustrated in Fig. 2. The bars represent the standard error of the mean; the numbers adjacent to the points indicate the number of flux experiments. The line which best fit the data was determined by the method of least squares from 46 flux experiments on blood from three donors. The Donnan ratios varied from 0.91 to 1.04. The dissociation constant for chloride at the inhibitory site, determined from the x intercept, was 165 mM.

tion, while the x intercept indicates the dissociation constant for chloride at the site of NAP-taurine binding (see Appendix). The straight line, determined by the method of least squares, indicates a K_i of $20.2 \mu\text{M} \pm 1.7 \text{ SEM}$ and a dissociation constant for chloride of 165 mM (90% confidence interval from 132 to 202 mM). This value is significantly higher than the reported value of 65 mM

³ On Dixon plots ($1/j$ vs. I), the points for 200 μM NAP-taurine deviated upward from the line determined from the other points, suggesting additional inhibition due to interaction at a second site, probably the transport site. The presence of such a site is also consistent with the fact that the lines on the double reciprocal plots intersect the y axis slightly below 1.

(Brazy and Gunn, 1976) for the substrate site ($P < 0.001$), confirming the conclusion that NAP-aurine does not act by competing at the substrate site. The value is also significantly lower than the reported chloride dissociation constant (K_z) for the modifier site, 335 mM (Dalmark, 1976). It should be noted, however, that the determination of K_z is subject to considerable error (Dalmark, 1976; see Appendix). In addition, the data derived from experiments at 200 μM NAP-aurine may give values for the Cl^- dissociation constant at the inhibitory site that are too low, because some of the inhibition may result from interaction of the probe at the transport site, even though its affinity for that site is considerably lower (see later section). Such an interaction is suggested by the Dixon plots (1/flux vs. NAP-aurine concentration, not shown): for a series of chloride concentrations, the data points for lower concentrations (100 μM or less) of NAP-aurine fall along a straight line but for 200 μM are above the line, suggesting nonlinearity due to a second inhibitory site. If the data at 200 μM NAP-aurine are excluded from the Hunter-Downs plot, the K_i becomes 25.4 $\mu\text{M} \pm 3.8$ SEM, and the chloride dissociation constant is 250 mM, with a 90% confidence interval from 179 mM to 343 mM. This value is significantly different ($P < 0.001$) from that expected for action at the substrate site, but not significantly different ($P > 0.1$) from that expected if NAP-aurine acts at the modifier site.

The hypothesis that external NAP-aurine acts at the modifier site can be evaluated in a more direct way by testing the ability of this model to fit the actual measured values of chloride flux as a function of chloride concentration. Fig. 6 shows such data for red cells from a single donor (to avoid variations in the maximal flux rate in cells from different donors). The upper line was calculated using the values for K_s and K_z mentioned above, with the maximal flux (J_m) adjusted to fit the data with no inhibitor present. The lower line was calculated using exactly the same parameters, except that NAP-aurine was assumed to inhibit the flux by acting at the modifier site with a K_i of 25 μM (determined from the data at 150 mM Cl^-). As expected, the control flux reaches a maximum around 150 mM chloride, and thereafter declines due to inhibition by chloride acting at the modifier site. This self-inhibition is suppressed by NAP-aurine. The fit of the data to the model is fairly good, except at 600 mM chloride, where the inhibition is somewhat less than predicted. This discrepancy might reflect a small effect of ionic strength at this high chloride concentration, especially inasmuch as Dalmark has observed similar deviations at high chloride concentrations (e.g., Fig. 3; Dalmark, 1976). In general, therefore, the data are consistent with the hypothesis that external NAP-aurine acts primarily by binding to the modifier site.

Efflux of NAP-Taurine from Erythrocytes

In that NAP-aurine is reported to be transported by the chloride exchange system (Cabantchik et al., 1976), it was surprising to find that when added to the outside it appeared to inhibit chloride efflux by acting at the modifier site rather than the substrate site. Additional experiments were therefore carried out using NAP-aurine as a transport substrate, to estimate its affinity for the transport site. Cells were loaded with [^{35}S]NAP-aurine, and its efflux into a NAP-aurine-

free medium was measured. In Fig. 7, the reciprocal of the NAP-taurine efflux is plotted against the reciprocal of the intracellular NAP-taurine concentration. The maximal flux of NAP-taurine, calculated from the y intercept, is ~ 250 $\mu\text{mol/kg}$ cell solids per min, and the apparent affinity for the substrate site, K_s , is just over 2 mM. The latter value is far higher than 37 μM , the apparent K_i for external NAP-taurine as an inhibitor of Cl^- transport at the same Cl^- concentration (137 mM). These data indicate the NAP-taurine on the inside of the cell, acting as a substrate for the anion exchange system, interacts with a site which has a much lower affinity for NAP-taurine than does the inhibitory site at which external NAP-taurine acts.

The difference in affinities for NAP-taurine at the inside and outside is also reflected in the substantial inhibitory action of external NAP-taurine on its own efflux. For example, 350 μM external NAP-taurine inhibited efflux by 62%.⁴ In

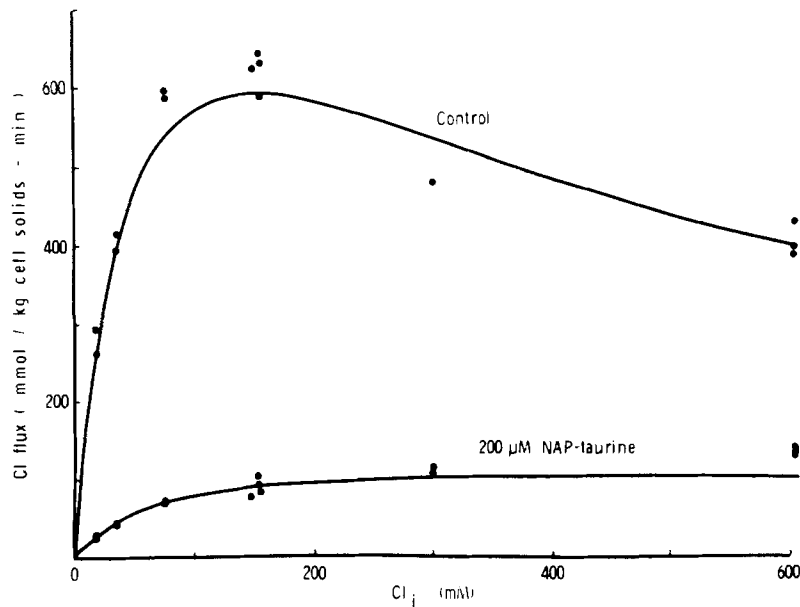


FIGURE 6. Chloride flux as a function of internal chloride concentration. The points represent flux measurements on nystatin-treated red cells from a single donor, performed as described in Methods. The upper curve was calculated with $K_s = 65$ mM, $K_z = 335$ mM, and $J_m = 1,250$ mmol/kg of dry solids per min. The lower curve was calculated using the same parameters, but assuming that NAP-taurine inhibits the chloride flux by interacting at the modifier site with a K_i of 25 μM .

⁴ The inhibition was somewhat less than would be produced by the same concentration of NAP-taurine on chloride fluxes at 0°C. Most of this disagreement can be explained by two factors. First, the apparent K_i for NAP-taurine increases with increasing temperature (as determined from the inhibition of sulfate fluxes), so only about an 80% inhibition would be expected at 37°C. Second, some of the NAP-taurine may cross the membrane by a mechanism other than the inorganic anion exchange system, consistent with the finding that dipyrindamole, another inhibitor of anion exchange, is also less effective in inhibiting NAP-taurine fluxes than sulfate fluxes (data not shown). Also, the inhibitor DIDS is only 90% effective against NAP-taurine fluxes (Cabantchik et al., 1976), but almost 100% effective against sulfate fluxes.

addition, the half-saturation concentration for NAP-aurine efflux was lowered by a factor of over 10 when the probe was present on the outside as well as the inside of the cell (at nearly equal concentrations). The maximal flux was lowered to a similar degree. This apparent increase in affinity and decrease in rate are exactly what would be expected if external NAP-aurine binds to the modifier site, because the response of the system to increasing NAP-aurine concentrations on both sides of the membrane will be dominated by its interaction with this high affinity inhibitory site.

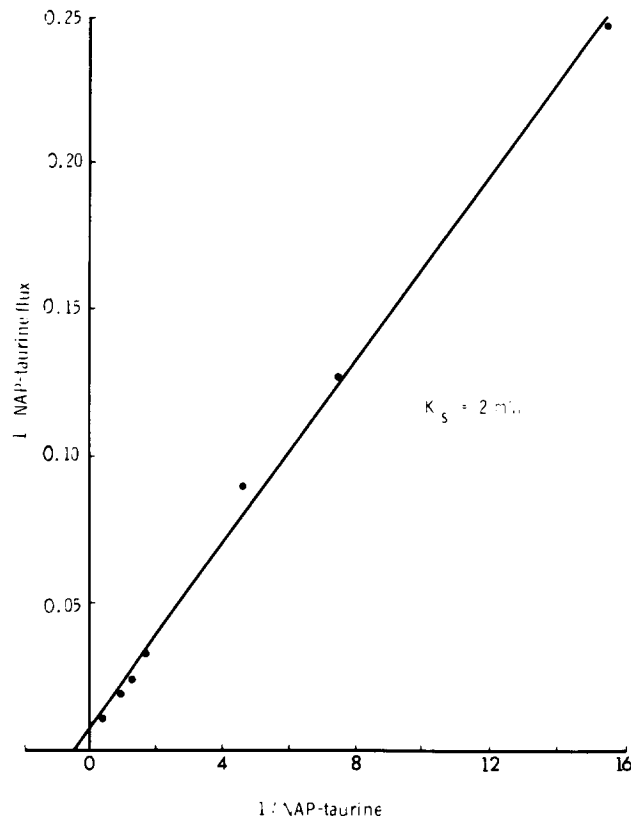


FIGURE 7. Lineweaver-Burk plot of the reciprocal of the NAP-aurine flux in micromole/liter of cell water per minute vs. the reciprocal of the internal concentration of exchangeable NAP-aurine in millimolar. Fluxes were measured at 37°C, pH 7.2 as described in Methods. The ratio of liters cell water to kilogram of cell solids was 1.90.

Effects of Internal NAP-Taurine on Chloride Exchange

The nature of the site with which NAP-aurine interacts at the inside of the membrane was further investigated by measuring the chloride efflux from resealed red cell ghosts loaded with various concentrations of NAP-aurine. Ghosts were used to minimize possible effects of intracellular binding on the determination of the intracellular NAP-aurine concentration. The data are displayed in

Fig. 8 on a Hunter-Downs plot of $I(1 - i)/i$ vs. the Cl^- concentration. The x intercept, which indicates the chloride dissociation constant of the inhibitory site, is 36.4 mM. This is not significantly different ($P > 0.2$) from the value of K_s , 65 mM, but is significantly lower ($P < 0.001$) than the corresponding value for the modifier site, 335 mM. The data are therefore compatible with the concept that inter-

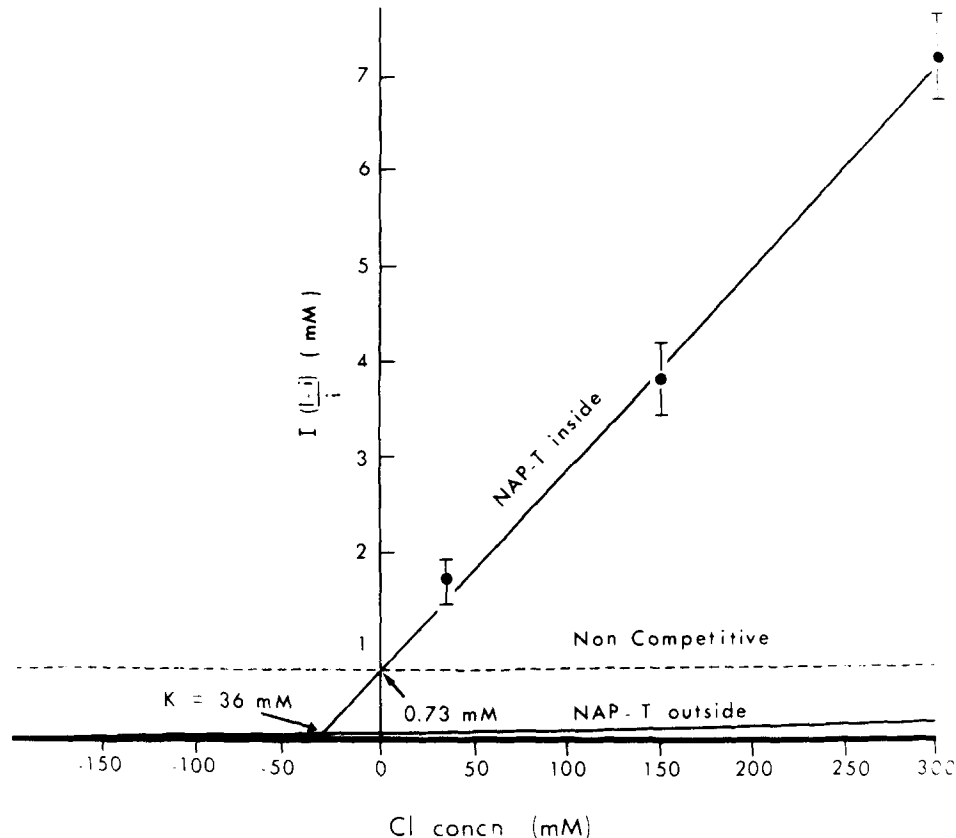


FIGURE 8. Hunter-Downs plot of the effect of internal NAP-taurine on chloride flux in red cell ghosts as a function of the external chloride concentration. The apparent K_i , determined as $I(1 - i)/i$, is plotted on the ordinate in millimolar. Fluxes were measured at 0°C, pH 7.2 as described in Methods. Each point represents at least four flux measurements and the bars indicate the standard error of the mean. The upper line was determined by the method of least squares to best fit the internal NAP-taurine data. The lower solid line depicts the data for external NAP-taurine taken from Fig. 5.

nal NAP-taurine inhibits by competing with chloride for the substrate site of the anion exchange system. The y intercept is $0.73 \text{ mM} \pm 0.58 \text{ SEM}$. Because under the conditions of these experiments ($\text{Cl}_i = \text{Cl}_o$) only half of the substrate sites are accessible to the inhibitor at any time (Dalmark, 1975 *a*; Cabantchik et al.¹), the K_i is actually half of this value, or 0.37 mM (see Appendix). Thus, NAP-

taurine is a less potent inhibitor at the inside of the membrane than at the outside by a factor of over 15. The substantial asymmetry between inside and outside is also evident by comparing in Fig. 8 the line for inside NAP-taurine with that from outside NAP-taurine (taken from Fig. 5). That this difference in potency is not due to a difference between intact cells and resealed ghosts is shown in Fig. 4, representing data on chloride fluxes in intact cells. From a comparison of the broken line (NAP-taurine-loaded cells) with the solid lines (NAP-taurine added externally), it is evident that the apparent K_i , given by the slope of the line, is much higher for internal NAP-taurine than for external NAP-taurine in cells, in agreement with the results obtained with resealed ghosts.

DISCUSSION

Side-Dependence of NAP-Taurine Effects

One of the most striking aspects of the data presented above is that the inhibitory potency of NAP-taurine is so strongly dependent on the side of the membrane to which it is added. At 0°C, the K_i for inhibition of chloride fluxes by external NAP-taurine is only 20.3 μM (Fig. 5), contrasting with an over 15-fold higher value of 370 μM for internal NAP-taurine (Fig. 8). The transport system thus exhibits considerable asymmetry in its response to this inhibitor.

From the effects of chloride concentration on the inhibitory potency (Fig. 8), it appears that internal NAP-taurine inhibits chloride fluxes by competing at a site with a chloride affinity similar to that of the substrate site. Furthermore, if NAP-taurine acts at the substrate site, its K_i as an inhibitor should be equal to its K_s as a substrate. In fact, the value of 2 mM obtained for K_s (Fig. 6) is not very different from the value of ~ 1.8 mM, which would be expected for the apparent K_i at the same chloride concentration (corrected for the fact that only one-half of the unoccupied substrate sites are accessible to the inhibitor). These values, although similar, must be compared with caution, because for technical reasons it was necessary to measure the chloride and NAP-taurine fluxes at different temperatures, 0°C and 37°C, respectively. The change in the apparent affinity of the exchange system for chloride (Brahm, 1977) with temperature would be expected to affect the competition between chloride and NAP-taurine for the substrate site. Also, the internal NAP-taurine concentration is difficult to define precisely in intact cells, because of binding to intracellular components. Nevertheless, the observed similarity in affinities of the system for internal NAP-taurine as substrate and inhibitor is consistent with its interaction at the substrate site.

In contrast, the much greater inhibitory potency of external NAP-taurine, together with the apparent absence of a competitive interaction with chloride at low concentrations (Fig. 3), leads to the conclusion that it acts at a different site. The simplest interpretation, consistent with the reduction of the inhibitory effect by high chloride concentrations (Figs. 4 and 5), is that NAP-taurine acts at the modifier site. The effect of chloride on NAP-taurine inhibition is somewhat larger than would be expected from Dalmark's (1976) value for the dissociation constant for chloride at the modifier site, but this may be partly due to some degree of interaction with the substrate site at concentrations of NAP-taurine

over 100 μM . If the dissociation constant for NAP-aurine at the substrate site is the same at the outside as at the inside of the membrane (370 μM), some binding to the substrate site would be expected at these concentrations. The data obtained at NAP-aurine concentrations of 100 μM or less are consistent with interaction at the modifier site, and even for the 200 μM data, the deviations of the results from this model are small (Fig. 6). Nevertheless, it should be emphasized that more complicated models for the action of NAP-aurine at the external surface of the membrane cannot be ruled out. For example, Brazy and Gunn (1976) postulated that another inhibitor, furosemide, acts at a site (F_1) where it both prevents transport and alters the affinity of chloride for the substrate site. Such a model does not appear to provide as good a fit to the NAP-aurine data as the modifier site model, but cannot be rigorously excluded. Perturbations of the system due to changes in ionic strength inherent in the procedure, while not obvious in previous studies of the anion exchange system (Gunn, 1978; Dalmark, 1976), may also play some role in modifying the effects of NAP-aurine.

If, as seems likely, external NAP-aurine acts at the modifier site, the data suggest that this site is only present at the external membrane surface. Less probably, the site could be exposed alternately to the inner and outer surface, but at the inner surface it would be accessible only to chloride and not to NAP-aurine. In either case, the results demonstrate asymmetric properties in a site which is closely related to transport.

It is of some interest that the halides and bicarbonate have a much higher affinity for the transport site than for the modifier site, whereas NAP-aurine has at least a 15-fold higher affinity for the modifier site. This difference may be related to a hydrophobic component in the binding of the organic anion.

Relation between Binding Sites for NAP-Taurine and DIDS

Recently in our laboratory it has been shown that the dihydro derivative of 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid (DIDS), known as H_2DIDS , appears to inhibit chloride exchange from the outside by acting at the substrate site (Shami et al., 1978). Inasmuch as external NAP-aurine appears to act at a different site, the displacement of NAP-aurine by DIDS or H_2DIDS and vice versa (Cabantchik et al., 1976) requires explanation. Although no data bear directly on the question, the apparent contradiction might be resolved in either of two ways. First, if the substrate and modifier sites are closely adjacent, a large molecule such as DIDS, binding to the chloride substrate site, may block access to the modifier site as well. Similarly, NAP-aurine bound to the modifier site would prevent the binding of DIDS to the substrate site. A second possibility is that NAP-aurine alters the affinity of the substrate site for chloride as well as for DIDS, either by steric hindrance or by some allosteric effect.

Effects of Chloride Gradient or Membrane Potential

Another apparent discrepancy deserves comment because it suggests an important property of the transport system. In previous studies, in which the external chloride concentration was varied by replacement with sucrose, small changes in external chloride concentration caused large changes in the inhibition of sulfate fluxes by external NAP-aurine (Cabantchik et al., 1976; Rothstein et al., 1977).

Because changes of this magnitude were not observed in the present study, when chloride concentrations were kept equal at both sides of the membrane, the reported effects may have been the result of alterations of membrane potential or chloride gradient which occur when only external chloride is varied (Sachs et al., 1975). Such effects could be explained if the accessibility of the modifier site to the external medium or else its affinity for NAP-taurine is

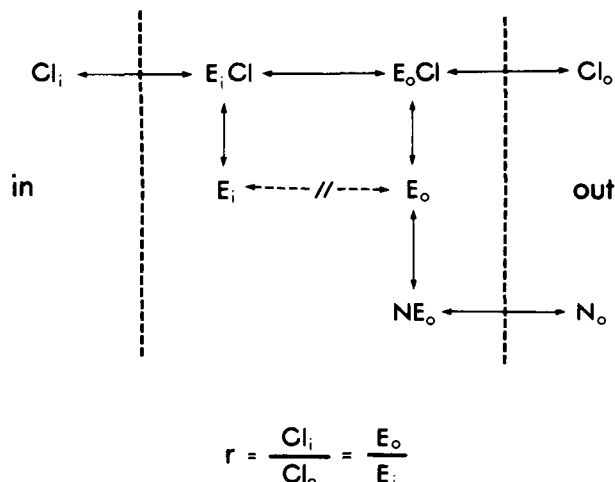


FIGURE 9. Sequential carrier model of the chloride exchange system. Chloride is assumed to cross the membrane by combining with a carrier, designated E . The carrier-chloride complex, ECl , can then move rapidly across the membrane. On the basis of the assumption that the affinity for chloride is the same at the two sides of the membrane, it can be calculated (Dalmark, 1975 *a*) that the unloaded forms of the carrier must be distributed according to the equation shown in order for the unidirectional influx and efflux of chloride to be equal (as they must be if the system is at equilibrium). Thus, the gradient of chloride across the membrane affects the distribution of unloaded carrier between the inside-facing and outside-facing forms, even though it is assumed in the model that the unloaded carrier cannot by itself cross the membrane (to explain the low electrical conductivity of the chloride exchange system). External NAP-taurine is shown interacting with a site (the modifier site) which is distinct from the substrate site. If NAP-taurine interacts with this site only when the transport site is in the outside-facing form, the distribution of chloride will affect the apparent affinity of the system for the probe, with higher values of the chloride ratio associated with larger inhibitory effects. For simplicity, the interaction of NAP-taurine with the enzyme-chloride complex is not shown. In fact, this reduces the effect of the chloride gradient, and at high chloride concentrations should completely abolish the effect.

affected either directly by the potential or indirectly by changes in the conformation of the substrate site. As Dalmark (1975 *a*) has shown, for a model in which the substrate site is alternately exposed to the inside or outside solutions (sequential carrier model), the ratio of inside to outside-facing carrier should depend on the chloride ratio (Fig. 9). The kinetics of anion exchange are consistent with a model in which the transport site shifts alternately from inside

to outside-facing conformations within the protein structure (Cabantchik et al.¹). If NAP-aurine interacts primarily with the form in which the substrate site faces outward (Fig. 9), then increases in the ratio of inside to outside chloride concentration (by reducing external chloride) will increase the amount of outside-facing carrier and therefore enhance the inhibitory potency of NAP-aurine. More detailed studies designed to examine such effects are currently in progress.

Labeling of Sites with NAP-Taurine

The results of the present study have shown that, depending on the side at which it is applied, NAP-aurine interacts with the substrate site or the modifier site. If the NAP-aurine is activated by exposure to light, it can therefore be used to label these sites. The use of external NAP-aurine as a label for the modifier site (Knauf et al., 1978) and of internal NAP-aurine as a label for the substrate site (Grinstein et al.⁵) are considered in related papers.

APPENDIX

PHILIP A. KNAUF

The presence of substrate inhibition, such as that which results from binding of chloride to the modifier site of the anion exchange system (Dalmark, 1976), makes the kinetic analysis of the action of inhibitors considerably more difficult. Although the kinetics of substrate inhibition (Webb, 1963; Cleland, 1970) and the general forms of the rate equations in the presence of an inhibitor (Webb, 1963) have been presented, the problems of graphical analysis of the mechanism of inhibition for a transport system have not been adequately treated. Such an analysis is essential to determine the site of action of inhibitory probes of the anion transport system. In the present study, two approaches have been used. In the first, the effect of chloride on the degree of inhibition is measured at low chloride concentrations, where interaction with the low affinity modifier site would not cause significant nonlinearity. The results are graphed on a Hanes plot of Cl/J (chloride concentration divided by flux) vs. chloride concentration (Cl), which has the advantage that the accuracy of Cl/J is not strongly affected by variations in Cl , so a statistical analysis of the data is meaningful (Wilkinson, 1961).

The expected results for various mechanisms of inhibition can be calculated from the model of Dalmark (1975 *a*), which can be used to describe a mobile carrier or any equivalent system with two conformational states, one of which has the substrate site facing inward and the other of which has it facing outward. The total amount of carrier, E_t , is given by the equation:

$$E_t = E_i + E_o + EC_i + EC_o + CE_i + CE_o + CEC_i + CEC_o, \quad (1)$$

where E represents the carrier (enzyme), C represents chloride, and the subscripts i and o refer to the forms of the carrier in which the substrate site faces inside and outside, respectively. When C stands to the right of E , it is bound to the substrate site; when C stands to the left of E , it is bound to the modifier site.

⁵ Grinstein, S., L. McCulloch, and A. Rothstein. 1978. Transmembrane effects of irreversible inhibitors of anion transport in red blood cells. Evidence for mobile transport sites. Manuscript submitted for publication.

The efflux, J , is given by:

$$J = k(EC_i), \quad (2)$$

where k is the rate constant for conversion of inside to outside-facing carrier, and vice versa. Inasmuch as no net flux occurs, the unidirectional influx and efflux must be equal, so

$$J = k(EC_i) = k(EC_o), \quad (3)$$

and

$$EC_i = EC_o. \quad (4)$$

Inasmuch as the maximum flux should occur when all of the enzyme is in the EC_i and EC_o forms, and inasmuch as half of the enzyme will be in each of these forms (Eq. 4), the maximal flux, J_m , is given by:

$$J_m = k(E_t/2). \quad (5)$$

The interactions of chloride with the substrate site are described by:

$$K_s = \frac{E_i \cdot C_i}{EC_i} = \frac{E_o \cdot C_o}{EC_o} = \frac{CE_i \cdot C_i}{CEC_i} = \frac{CE_o \cdot C_o}{CEC_o}, \quad (6)$$

where K_s is the dissociation constant for chloride at the substrate site. Note that internal chloride only interacts with the substrate site of those forms of the carrier (E_i and CE_i) in which the substrate site faces inward, and vice versa for external chloride. For simplicity, and in accordance with evidence presented in this paper, the modifier site is assumed to face the outside, regardless of the orientation of the substrate site. This may in fact be an oversimplification (see Discussion), but does not affect the kinetic predictions for cases where the internal and external chloride concentrations are equal. On this basis, the interactions of chloride with the modifier site are given by:

$$K_z = \frac{E_i \cdot C_o}{CE_i} = \frac{E_o \cdot C_o}{CE_o} = \frac{EC_i \cdot C_o}{CEC_i} = \frac{EC_o \cdot C_o}{CEC_o}, \quad (7)$$

where K_z is the dissociation constant for chloride at the modifier site.

To simplify the equations and to mimic the experimental situation, it has been assumed that the chloride concentrations inside and outside are equal, so $Cl = C_i = C_o$. Thus, from Eqs. 4, 6, and 7:

$$\frac{C_i}{C_o} = \frac{E_o}{E_i} = \frac{CE_o}{CE_i} = 1, \quad \text{and} \quad \frac{EC_o}{EC_i} = \frac{CEC_o}{CEC_i} = 1. \quad (8)$$

Significant variations of the Donnan ratio from 1 were only observed at the lowest chloride concentrations and, together with the more rapid isotope equilibration under these conditions ($t_{1/2} < 10$ s), may explain some deviations from the expected kinetic behavior under these circumstances (see, for example, Fig. 5). It has also been assumed that the inhibitor is present at the outside, but analogous equations are obtained with the inhibitor at the inside of the membrane. For quantitative calculations, the value of K_s used was that determined by Brazy and Gunn (1976) from flux measurements at low chloride concentrations, $65 \text{ mM} \pm 5 \text{ SEM}$, which agrees with Dalmark's (1976) value of 67 mM. The value for K_z cannot be determined directly, and is strongly influenced by the precise shape of the curve of chloride flux vs. chloride concentration (Dalmark, 1976).

Also, the possibility that binding to the modifier site causes only partial inhibition remains open. This would considerably lower the value of K_z . Therefore, at present no statistical confidence interval can be associated with this parameter, and the value of 335 mM (Dalmark, 1976) has been taken as the best available estimate. From this model, the Hanes plot lines can be calculated as follows: For completely noncompetitive inhibition, in which the inhibitor acts at a third site, which is distinct from both the transport and modifier sites, the interactions with inhibitor are described by:

$$K_i = \frac{E_i \cdot I}{E_i} = \frac{E_o \cdot I}{E_o} = \dots = \frac{CEC_o \cdot I}{CEC_o}, \quad (9)$$

plus similar terms for each form of the carrier shown in Eq. 1. The inhibitor is shown below E to indicate that it binds to a site different from both the substrate and modifier sites. By substituting Eqs. 2, 5, 6, 7, and 9 into Eq. 1 and rearranging,

$$\frac{Cl}{J} = \frac{1}{J_m} \left(1 + \frac{Cl}{K_z} \right) (Cl + K_s) \left(1 + \frac{I}{K_i} \right). \quad (10)$$

The plot of Cl/J vs. Cl is not a straight line, but instead curves upward at high chloride concentrations, as the Cl^2 term becomes larger. The tangent to the line at $Cl = 0$, which represents the linear approximation for low chloride concentrations, is given by:

$$\frac{Cl}{J} = \frac{1}{J_m} \left(K_s + Cl + \frac{K_s Cl}{K_z} \right) \left(1 + \frac{I}{K_i} \right). \quad (11)$$

The lines for different inhibitor concentrations intersect on the abscissa at $-K_s(1 + K_s/K_z)$ or -54 mM.

If the inhibitor acts at the modifier site, the equilibria for the inhibitor are described by:

$$K_i = \frac{E_i \cdot I}{IE_i} = \frac{E_o \cdot I}{IE_o} = \frac{EC_i \cdot I}{IEC_i} = \frac{EC_o \cdot I}{IEC_o}, \quad (12)$$

and the Hanes plot is given by:

$$\frac{Cl}{J} = \frac{1}{J_m} \left(1 + \frac{Cl}{K_z} + \frac{I}{K_i} \right) (Cl + K_s). \quad (13)$$

Note that in this case the curvature at high Cl is reduced at high inhibitor concentrations, since the I/K_i term dominates the Cl/K_z term which gives rise to the curvature. At low chloride concentrations the linear approximation is:

$$\frac{Cl}{J} = \frac{1}{J_m} \left\{ \left(1 + \frac{I}{K_i} \right) (Cl + K_s) + \frac{K_s Cl}{K_z} \right\}. \quad (14)$$

Lines for different inhibitor concentrations intersect at $Cl = -K_s$, that is, -65 mM and at $Cl/J = -K_s^2/K_z J_m$. Since the y intercept of the control line is K_s/J_m , the intersection point is located about 0.2 times this distance below the abscissa (because $K_s/K_z = 0.2$).

In the case of competitive inhibition at the substrate site, the interactions with inhibitor are described by:

$$K_i = \frac{E_o \cdot I}{EI_o} = \frac{CE_o \cdot I}{CEI_o}. \quad (15)$$

Interactions only occur with forms in which the transport site faces outward and is unoccupied by Cl . In this case,

$$\frac{Cl}{J} = \frac{1}{J_m} \left(1 + \frac{Cl}{K_z} \right) \left(Cl + K_s + \frac{K_s I}{2K_i} \right), \quad (16)$$

which, for low Cl concentrations, reduces to:

$$\frac{Cl}{J} = \frac{1}{J_m} \left\{ Cl + K_s \left(1 + \frac{I}{2K_i} \right) \left(1 + \frac{Cl}{K_z} \right) \right\}. \quad (17)$$

Note that in this case the inhibitor concentration which gives 50% inhibition at zero chloride concentration is $2K_i$, rather than K_i . This is because only half of the free transport sites (not complexed with inhibitor) are in the outside-facing form (Eq. 8), and therefore available to interact with the inhibitor.

The lines for different inhibitor concentrations intersect at $Cl = -K_z$, or -335 mM, and at $Cl/J = -K_z/J_m$, that is, about five times the y intercept value of the control curve below the abscissa. If there were no modifier site present, competitive inhibition would yield parallel straight lines for different inhibitor concentrations, whose slope is $1/J_m$ (Webb, 1963). Thus, because the affinities of substrate and modifier sites differ only by a factor of 5, the presence of substrate inhibition has some effect on the predicted kinetic behavior, even at chloride concentrations near zero.

The use of the Hanes plot at low chloride concentrations, while valuable for distinguishing competitive from noncompetitive inhibition, provides little quantitative information about interactions at the modifier site (but see text footnote 2). The predictions in the latter case are almost indistinguishable at low chloride concentrations from those for a completely noncompetitive inhibition. To observe competition of inhibitor and chloride at the modifier site, a second method has been employed, using the Hunter-Downs plot (Webb, 1963). In this case $I(1-i)/i$, a measure of the apparent K_i , is plotted against substrate concentration. Because inhibition is measured, rather than flux, the nonlinear terms cancel out, and one obtains straight lines for the three cases considered, as follows:

For noncompetitive inhibition,

$$I(1-i)/i = K_i. \quad (18)$$

Chloride has no effect on the apparent K_i , and so one obtains a straight line parallel to the abscissa, with y intercept at K_i .

For interaction of inhibitor with the modifier site, one obtains:

$$I(1-i)/i = K_i(1 + Cl/K_z). \quad (19)$$

The y intercept is again K_i , while the x intercept is $-K_z$, the negative of the dissociation constant for chloride at the modifier site, or -335 mM.

Competitive inhibition at the substrate site gives:

$$I(1-i)/i = 2K_i(1 + Cl/K_s). \quad (20)$$

The y intercept in this case is equal to $2K_i$, because only half of the substrate sites face the external solution in which the inhibitor is present. The x intercept is equal to the negative of the dissociation constant for chloride at the substrate site, -65 mM.

Similar equations are obtained for the case of an inhibitor acting at the inside surface of the membrane. To determine the values of the intercepts from the experimental data, lines were calculated by the method of least squares. Standard errors for the y intercept were calculated as described by Scheffler (1969). The confidence intervals for the x intercept were determined by calculating the points at which the confidence limits for the line intersect the x axis. The Hunter-Downs method has certain statistical disadvantages, because the errors in the apparent K_i 's are not necessarily equal at different

substrate concentrations. Nevertheless, this method provides a means for linearizing data obtained over the full range of substrate concentrations, and for determining, at least for some simple and plausible models, the chloride affinity of the site with which the inhibitor interacts.

This method can easily be extended to determine the affinity of the inhibitory sites for other ions added to the system, such as other halides or bicarbonate, thus providing a more precise description of the site in terms of its ion affinities. Experiments of this type are in progress.

Note Added in Proof In this paper, we postulate that two inhibitors with different modes of action, such as external NAP-taurine and DIDS, can mutually interfere with each other's binding to the membrane. Support for this concept comes from a recent report of Cousin and Motais⁶ that niflumic acid, a potent but strictly noncompetitive inhibitor of anion exchange, competes with SITS (4-acetamido-4'-isothiocyano-2,2'-stilbene-disulfonate), an analog of DIDS, for binding to inhibitory sites.

Assistance of Alex Gaarn, Patricia Harper, and Pamela Marchant is gratefully acknowledged, as well as the comments of Dr. Sergio Grinstein on the manuscript.

This study was supported by Medical Research Council (Canada) Grants MA5149 and MT4665.

Received for publication 10 February 1978.

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