Effects of the Transport Site Conformation on the Binding of External NAP-Taurine to the Human Erythrocyte Anion Exchange System

Evidence for Intrinsic Asymmetry

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ABSTRACT External *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate (NAP-taurine) inhibits human red cell chloride exchange by binding to a site that is distinct from the chloride transport site. Increases in the intracellular chloride concentration (at constant external chloride) cause an increase in the inhibitory potency of external NAP-taurine. This effect is not due to the changes in pH or membrane potential that usually accompany a chloride gradient, since even when these changes are reversed or eliminated the inhibitory potency remains high. According to the ping-pong model for anion exchange, such transmembrane effects of intracellular chloride on external NAP-taurine can be explained if NAP-taurine only binds to its site when the transport site is in the outward-facing (E_o or ECl_o) form. Since NAP-taurine prevents the conformational change from ECl_o to ECl_i, it must lock the system in the outward-facing form. NAP-taurine can therefore be used just like the competitive inhibitor H_2DIDS (4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid) to monitor the fraction of transport sites that face outward. A quantitative analysis of the effects of chloride gradients on the inhibitory potency of NAP-taurine and H_2DIDS reveals that the transport system is intrinsically asymmetric, such that when $Cl_i = Cl_o$, most of the unloaded transport sites face the cytoplasmic side of the membrane.

INTRODUCTION

The photoaffinity probe *N*-(4-azido-2-nitrophenyl)-2-aminoethyl sulfonate (NAP-taurine), when present in the outside medium, inhibits chloride or sulfate exchange by binding to a site that is distinct from the chloride transport site

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/84/05/0683/19 $1.00

Volume 83 May 1984 683–701

(Knauf et al., 1978*b*). Cytoplasmic (internal) NAP-taurine does not have access to this site. On the basis of its affinity for chloride (Knauf et al., 1978*b*), this modifier site seemed to be the same as the modifier site at which high concentrations of chloride inhibit anion exchange (Cass and Dalmark, 1973; Dalmark, 1976). Although some early data with red cell ghosts (Schnell et al., 1978) indicated that the chloride modifier site, like the NAP-taurine site, faces the external medium, a revised version of these data does not show this (Schnell, 1979). Recent experiments with intact cells in our laboratory (Knauf and Mann, 1984*a*) show that the inhibitory effect of chloride is primarily (and perhaps exclusively) the result of binding to a site at the cytoplasmic surface of the membrane. Thus, the site at which external NAP-taurine inhibits chloride exchange is both distinct from the cytoplasmic modifier site where chloride self-inhibition takes place and distinct from the chloride transport site.

Several pieces of evidence suggest that the binding sites for external NAP-taurine and for probes that act as competitive inhibitors (and thus probably bind to the transport site) are closely adjacent. NAP-taurine prevents the reaction of DIDS (4,4′-diisothiocyano-stilbene-2,2′-disulfonic acid) with the transport system and DIDS prevents the reaction of NAP-taurine with band 3 (Cabantchik et al., 1976). More recently, it has been demonstrated that covalently bound DIDS prevents the reversible binding of NAP-taurine to red cell ghosts (Macara and Cantley, 1981) and that the reversible binding of NAP-taurine is mutually exclusive with that of the competitive inhibitors BADS (4-benzamido-4′-amino-stilbene-2,2′-disulfonate) (Macara and Cantley, 1981) and DNDS (4,4′-dinitro-stilbene-2,2′-disulfonic acid) (Fröhlich and Gunn, 1982). Moreover, if NAP-taurine is activated by light to form a reactive nitrene, the site of covalent binding is located in the same 17,000-dalton segment of the band 3 protein that contains the primary H_2DIDS (4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid) labeling site (Knauf et al., 1978*a*).

According to the ping-pong model of anion exchange (Gunn and Fröhlich, 1979; Knauf, 1979), the band 3 protein can exist in either an E_i form, in which the transport site faces the cytoplasm, or an E_o form, in which it faces the outside medium. The data presented in the preceding paper (Furuya et al., 1984), as well as other evidence (Jennings, 1980, 1982), demonstrate that chloride gradients across the membrane affect the conformation of the transport site. In this paper, we make use of such methods to see whether or not the conformation of the transport site affects the ability of external NAP-taurine to inhibit chloride exchange.

It is clear that chloride gradients can skew the distribution of the conformations of band 3, but such experiments do not tell us directly whether or not the sites are symmetrically distributed when $Cl_i = Cl_o$. There would seem to be no a priori reason why the inward-facing and outward-facing forms of the transport site should have equal chloride affinities, nor is there any reason why the rate constant for outward translocation of chloride, k, should be the same as the rate constant for inward translocation, k' (see Fig. 1). If either the dissociation constants for chloride at the inside and outside, K_i and K_o, or the rate constants for inward and outward translocation are different, the system will exhibit an

asymmetric distribution of unloaded transport sites, E_o and E_i, even when $Cl_i = Cl_o$ (Knauf, 1979). As shown in the Appendix, Eq. A5, the ratio of E_o to E_i under these conditions can be defined as the asymmetry factor, A.

This factor can be determined by taking the quotient of the concentrations of chloride required to half-saturate the transport system at the outside and inside of the membrane, respectively (Appendix, Eq. A11). Such measurements by Schnell et al. (1978) indicated an asymmetry factor of 2.5, with more sites facing outward. More recent data of Gunn and Fröhlich (1979) suggest an asymmetry in the opposite direction, with more inward- than outward-facing sites. Experiments by Jennings (1980, 1982) on the sulfate-transporting form of the system also suggest that more of the sites face inward than outward. To help resolve this question, we have developed and applied a method for using the chemical

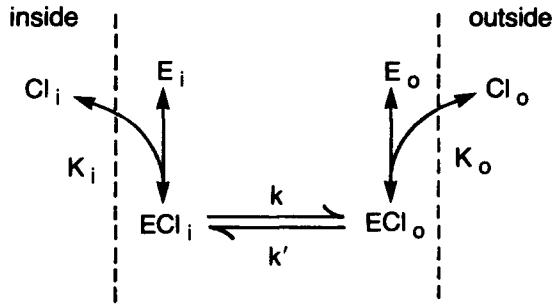

FIGURE 1. Ping-pong model of the anion transport system. The band 3 protein can exist in either an E_o form, with the transport site facing outward, or in an E_i form, with the transport site facing the cytoplasm. The chloride dissociation constants of the inward- and outward-facing forms are K_i and K_o, respectively. The rate constant for the conformational change from the inward-facing ECl_i form to the outward-facing ECl_o form is k, while the rate constant for the reverse conformational change is k'.

probes H_2DIDS and NAP-taurine to obtain an independent measure of the intrinsic asymmetry of the anion exchange system.

Preliminary reports of some of these data have been published (Knauf et al., 1980*a*, *b*; Knauf and Rothstein, 1980; Knauf, 1982) and have appeared in thesis form (Furuya, 1980).

METHODS

Preparation of Cells with High and Low Internal Chloride

Fresh human red blood cells were washed and treated with nystatin to alter the intracellular KCl concentration as described previously (Furuya et al., 1984). The inhibitory effects of NAP-taurine (Pierce Chemical Co., Rockford, IL) were measured at 0°C in a medium at pH 7.2 containing 10 mM KCl, 20 mM HEPES, and 27 mM sucrose for the low chloride cells or 245 mM sucrose for the high chloride cells. Chloride exchange fluxes were measured, rate constants were determined, and the data were analyzed statistically as described by Furuya et al. (1984). During the washing and isotope loading in 10 mM

Cl^- medium, the high Cl^- cells lost some Cl^- and decreased in volume, both because of the increase in intracellular pH and possibly because of an increased K^+ permeability due to nystatin. The volume decrease seemed to have no effect on the inhibitory potency of NAP-taurine, however, since when high Cl^- cells were suspended in medium with 150 mM chloride, 20 mM HEPES, addition of 245 mM sucrose had no effect on the ID_{50} for NAP-taurine.

pH and Membrane Potential Experiments

Fresh red cells were washed three times in 150 mM KCl, 33 mM sucrose, 5 mM HEPES, pH 7.2, at 0°C (150K-33 buffer). One group of cells (preincubated) was then incubated for 10 min at 0°C in the flux buffer containing 10 mM KCl, 280 mM sucrose, 5 mM HEPES, pH 7.2, at 0°C. Cells were washed once more in this buffer and then loaded with ^{36}Cl, and the chloride efflux was measured in this flux buffer at 0°C in the presence of various concentrations of NAP-taurine. A second group of cells (plunged) was washed and loaded with ^{36}Cl in high chloride 150K-33 buffer, and then was plunged directly into the low chloride flux buffer for measurement of the effects of NAP-taurine on ^{36}Cl exchange. A third group of cells (low pH) was treated like the plunged cells, except that the 150K-33 buffer was titrated to pH 6.9 rather than 7.2 at 0°C.

For some of the plunged cells, chloride fluxes were measured in the presence of 10 μM valinomycin and 1.33% ethanol. This treatment is sufficient to cause a reversal of the sign of the membrane potential (Furuya et al., 1984).

RESULTS

Effects of Chloride Gradients on NAP-Taurine Inhibitory Potency

As shown in the Appendix (Eq. A27), if NAP-taurine binds equally well to its site, regardless of the conformation of the transport site, changes in Cl_i at constant Cl_o will have no effect on the concentration of NAP-taurine required to inhibit chloride exchange by 50% (the ID_{50}). On the other hand, if NAP-taurine binds preferentially to its site when the transport site is in the E_o or ECl_o form, then if $Cl_i > Cl_o$, this will increase the ratio of E_o to E_i, thereby decreasing the concentration of NAP-taurine necessary to cause 50% inhibition (see Appendix, Eq. A30). On the other hand, if NAP-taurine binds preferentially to the E_i and ECl_i forms, imposition of a chloride gradient will have the opposite effect on the ID_{50} (i.e., will cause it to increase).

Cells with high or low internal chloride concentrations were prepared as described in the preceding paper (Furuya et al., 1984) and the inhibitory effect of external NAP-taurine on chloride exchange at 0°C was measured with 10 mM chloride present in the external medium. All of the experiments were done under subdued light, so photoactivation did not occur and the binding of NAP-taurine was reversible. The results are plotted on a modified form of the Dixon plot in Fig. 2. For the cells with $Cl_i > Cl_o$, the slope of this plot, which is equal to $1/ID_{50}$, was significantly greater than for the cells in which $Cl_i = Cl_o$. The x-intercept, which is the negative of the ID_{50} value, indicated that for the cells with a chloride gradient, the ID_{50} was less than half of that for the cells with no chloride gradient. Since external chloride and external ionic strength were constant in this experiment, the data strongly suggest that there is a transmem-

brane effect of internal chloride on the inhibitory potency of external NAP-taurine.

The data for four such experiments with NAP-taurine are shown in Table I. The difference between ID_{50} with and without (ID_{50}*) a chloride gradient was significant at the $P < 0.02$ level, and the 95% confidence interval (CI) for the ratio of ID_{50} to ID_{50}* was from 0.14 to 0.50. The confidence interval overlaps that seen with H_2DIDS (see Table I of Furuya et al., 1984).

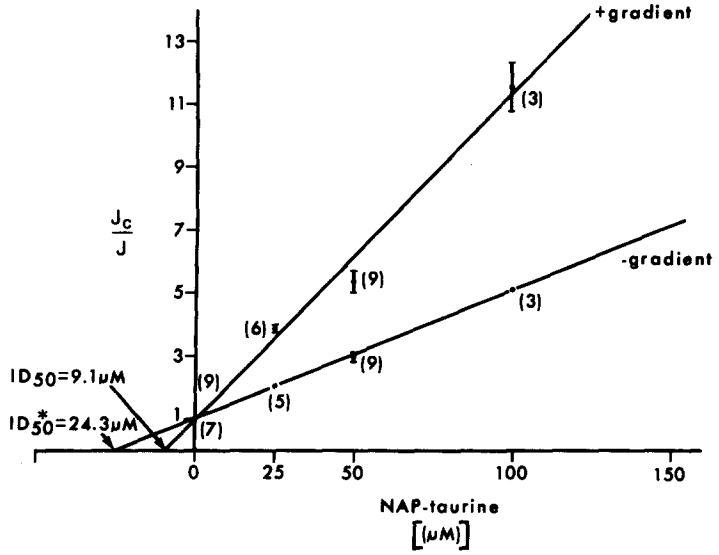

FIGURE 2. Modified Dixon plot of NAP-taurine inhibition of chloride exchange at 0°C. On the ordinate is plotted the control chloride flux, J_c, divided by the flux in the presence of various concentrations of NAP-taurine, J. The NAP-taurine concentration is plotted on the abscissa. Data from the four experiments in Table I are plotted together. For the cells plus gradient the Cl_i/Cl_o ratio ranged from 4.9 to 6.3; for the cells minus gradient it ranged from 0.8 to 0.93. Both groups of cells were pretreated with nystatin as described in Methods, and the extracellular chloride concentration was 10 mM. Bars indicate SEM; the numbers in parentheses indicate the number of flux measurements. Lines were determined by the method of least squares.

Possible Effects of pH

In the presence of a chloride gradient, Cl^-/OH^- and Cl^-/HCO_3^- exchange occur across the red cell membrane via the anion exchange system, so that at equilibrium Cl_i^-/Cl_o^- is equal to OH_i^-/OH_o^-. Since the pH of the external medium is buffered, when $Cl_i > Cl_o$ the cell interior should become more alkaline. To see whether or not such a change in intracellular pH might cause the increased inhibitory potency of NAP-taurine observed when $Cl_i > Cl_o$, we performed experiments in which fresh cells, instead of being preincubated with the low chloride medium, were loaded with ^{36}Cl in a high chloride medium and were

plunged into the low chloride medium at the beginning of the flux measurement. Under these conditions (Furuya et al., 1984), the pH change during the flux measurement is <0.1 pH unit.

The results are plotted on a modified Dixon plot in Fig. 3. If the change in pH were the cause of the increased inhibition seen when $Cl_i > Cl_o$, the slope for the "plunged" cells should have decreased to that characteristic of cells with $Cl_i = Cl_o$. If anything, the cells that were plunged into low chloride medium show a slight increase in slope, which would indicate an increase rather than a decrease in inhibitory potency.

Data are also shown for cells ("low pH") that were washed in high chloride buffer with a pH 0.3 unit more acidic than normal. The data for these cells, whose intracellular pH was never more alkaline than the usual control intracellular pH, fall in the usual range for cells with $Cl_i > Cl_o$. Thus, regardless of whether or not the intracellular pH becomes alkaline, the inhibitory potency of

TABLE I

Effect of a Chloride Gradient on NAP-Taurine Inhibition in Cells with $Cl_i > Cl_o$

Experiment	ID_{50}*	ID_{50}	ID_{50}/ID_{50}*	Cl_o/Cl_i
	μM	μM		
1	39.0	8.80	0.23	0.189
2	37.7	7.40	0.20	0.158
3	25.3	5.37	0.21	0.204
4	22.0	10.70	0.40	0.188
Mean	31.0	8.07	0.26	0.185
SEM	4.3	1.13	—	0.010
95% CI	17.3	4.49	0.14	0.154
	44.7	11.65	0.50	0.215

For the measurements of ID_{50}*, the mean intracellular Cl^- concentration was 8.5 mM; for the measurements of ID_{50} it was 54.7 mM. The extracellular Cl^- concentration was 10 mM and the temperature was 0°C.

NAP-taurine has the same high value, which demonstrates that the enhancement of inhibition when $Cl_i > Cl_o$ could not be due to the change in intracellular pH.

Possible Effects of Membrane Potential

Because of net chloride flow across the membrane, when $Cl_i > Cl_o$ the membrane potential will tend to become depolarized, that is, it will become positive inside with respect to outside. Since there are charged groups on the transport protein band 3, changes in membrane potential might affect the protein structure in such a way that the effectiveness of NAP-taurine as an inhibitor of chloride exchange is increased. To test this possibility, high chloride cells were plunged directly into medium with 10 mM chloride, either in the absence or the presence of 10 μM valinomycin, and the inhibition of ^{36}Cl exchange by NAP-taurine was measured. As shown in the preceding paper (Furuya et al., 1984), under these conditions valinomycin should raise the potassium permeability of the membrane to such an extent that the membrane potential will change from ~61 mV to

about −57 mV, that is, the membrane will become strongly hyperpolarized. If membrane potential were the critical variable affecting NAP-taurine inhibitory potency, this hyperpolarization would be expected to reverse the increase in inhibition produced under conditions of depolarization (when $Cl_i > Cl_o$). As Fig. 4 shows, there was only a small effect of valinomycin on the slope of the modified Dixon plot and almost no effect on the *x*-intercept. This minimal effect of membrane potential reversal is in sharp contrast to the very pronounced effects

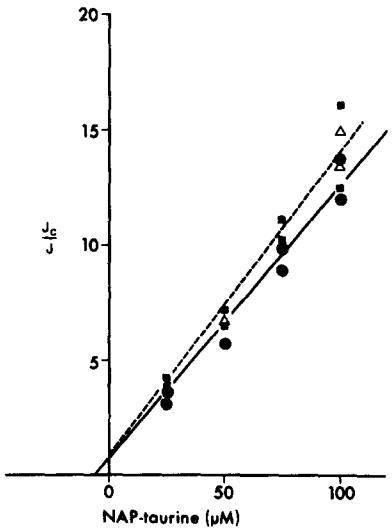

FIGURE 3. Inhibition of chloride exchange by NAP-taurine in low chloride medium. Fresh cells were washed and treated as described in Methods. The inhibition of chloride exchange at 0°C and pH 7.2 by external NAP-taurine in a 10-mM chloride medium is plotted on a modified Dixon plot as described in the legend to Fig. 2. The broken line is the least-squares best fit to the data (■) for the cells that were plunged into low chloride medium; the solid line is the corresponding best fit for cells preincubated with low chloride medium (○). Data for cells preincubated with pH 6.9 medium (low pH cells) are indicated by triangles (△). The mean intracellular Cl^- concentration and pH (calculated by assuming complete Cl^-/OH^- equilibration) for preincubated cells were 87.5 mM and pH 8.14; for plunged cells, 135 mM and pH 7.15; for low pH washed cells, 143.3 mM and pH 6.88. For the pre-equilibrated cells, the slope was 0.119 μM^{-1} and the *x*-intercept was −5.70 μM. For the plunged cells, the corresponding values were 0.132 μM^{-1} and −5.76 μM; for the low pH cells, 0.132 μM^{-1} and −6.32 μM (line not shown).

of chloride gradients seen in Fig. 2, and argues strongly that the change in inhibitory potency of NAP-taurine caused when $Cl_i > Cl_o$ is not due to the change in membrane potential.

Model for NAP-Taurine Inhibition

The data thus demonstrate that a chloride gradient across the membrane has the same effects (within the scatter of the data) on the NAP-taurine inhibitory

potency that it has on the potency of H_2DIDS (Furuya et al., 1984). As is shown in the Appendix, Eq. A30, such transmembrane effects of internal chloride on the binding of external NAP-taurine would be expected if NAP-taurine can only bind to its site when the transport site is in the outward-facing (E_o or ECl_o) conformation. This seems the simplest model that is compatible with the data.

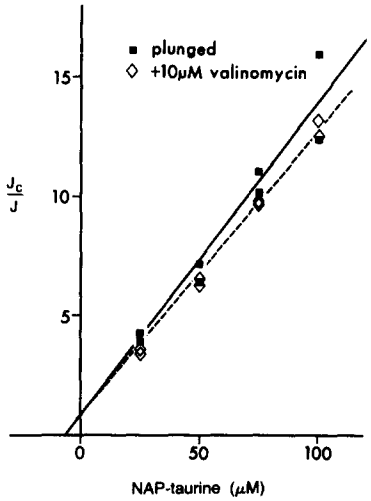

FIGURE 4. Effects of membrane potential changes on the inhibition of chloride exchange by NAP-taurine in low chloride medium. Fresh cells were washed and prepared as described in Methods. The inhibitory effects of NAP-taurine on chloride exchange are plotted on a modified Dixon plot as described in the legend of Fig. 2. The solid line is the least-squares best fit to the data for cells with a mean intracellular Cl^- concentration of 135 mM plunged into low (10 mM) chloride medium; the broken line is the corresponding best fit for cells similarly treated, except that the flux medium contained in addition 10 μM valinomycin and 1.33% ethanol. For the plunged cells, the slope was 0.132 μM^{-1} and the x-intercept was −5.76 μM; with valinomycin, the slope was 0.121 μM^{-1} and the x-intercept was −6.25 μM.

The experimental results, however, do not exclude the possibility that NAP-taurine also binds with a much lower affinity to band 3 when the transport site faces inward.

Intrinsic Asymmetry of the Transport System

From the data presented above, it is clear that the inhibitory potency of NAP-taurine, as well as the inhibitory potency of H_2DIDS (Furuya et al., 1984), can be used to monitor the conformation of the transport site of the anion exchange system. A quantitative analysis of the effects of chloride gradients on the inhibitory effects of these probes can also provide information concerning the intrinsic asymmetry of the transport system, in the following way: as shown in Fig. 5, for a system with more unloaded transport sites facing outward when $Cl_i = Cl_o$ ($A > 1$), most of the sites are already accessible to H_2DIDS or NAP-taurine. Thus, when a large chloride gradient ($Cl_i \gg Cl_o$) is applied across the membrane to

force most of the sites to the outside, there is only a small increment in the number of outward-facing sites and hence only a small increase in the inhibitory potency of H_2DIDS or NAP-taurine. Conversely, if the system has more inward-facing sites, that is, if $A < 1$, then few of the sites are accessible to H_2DIDS or NAP-taurine when $Cl_i = Cl_o$. Hence, when $Cl_i \gg Cl_o$, the number of outward-facing sites increases dramatically, and so should the inhibitory potency of H_2DIDS or NAP-taurine.

Quantitative predictions for a system in which half-saturation of the transport sites (with $Cl_i = Cl_o$) occurs at 65 mM Cl (Brazy and Gunn, 1976) and in which

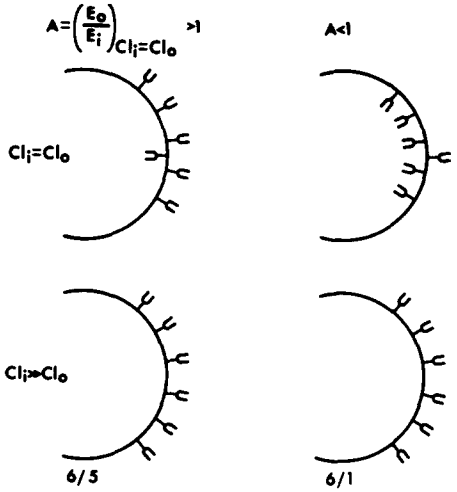

FIGURE 5. Effects of a chloride gradient on the relative number of outward-facing sites with varying degrees of asymmetry. For simplicity, only unloaded chloride transport sites are shown. If, as in the left-hand panel, there is a fivefold asymmetry in favor of outward-facing sites, then when a chloride gradient is imposed, with $Cl_i \gg Cl_o$, the number of outward-facing sites can only increase by a factor of 6/5. Thus, there will be only a small increase in the inhibitory effect of H_2DIDS or NAP-taurine, which bind to outward-facing sites. Conversely, if there are five times as many inward-facing sites with $Cl_i = Cl_o$ (right-hand panel), then when a chloride gradient is imposed there will be a sixfold increase in outward-facing sites and correspondingly a very large increase in the inhibitory potency of H_2DIDS or NAP-taurine.

Cl_o = 10 mM are shown in Fig. 6. Note that when A is 10, there is very little effect of the chloride gradient (Cl_o/Cl_i) on the ID_{50} ratio, in comparison with a symmetrical system (A = 1). When there are 10 times as many inward-facing as outward-facing sites (A = 0.1), the effects of the chloride gradient on the ID_{50} are much more dramatic, but a further increase in asymmetry (A = 0.05) has little additional effect. Thus, this method is very good for determining the direction of the asymmetry, but relatively poor for measuring the precise degree of asymmetry, particularly if the asymmetry is large.

Since the dispute in the literature concerns the direction of the asymmetry,

this method should provide useful information. For the data with H_2DIDS from the preceding paper (Furuya et al., 1984), when Cl_o/Cl_i is 0.184, the upper limit of the 95% CI for the ratio of ID_{50} to ID_{50}* is 0.69. This is just below the predicted value of 0.70 for a system with $A = 1.4$, which indicates that from the H_2DIDS data $A < 1.4$. The NAP-taurine data (Table I) give an upper 95% confidence limit of 0.50 when $Cl_o/Cl_i = 0.185$. This would correspond to an A value of <0.4, which indicates that there are at least 2.5 times as many inward-facing (E_i) as outward-facing (E_o) sites. Even if one were to consider the control ratio of Cl_i/Cl_o to be 0.8, the lowest value observed, rather than 1, the NAP-taurine data still provide evidence that there are 1.5 times as many inward-facing as outward-facing transport sites when $Cl_i = Cl_o$, which again demonstrates an

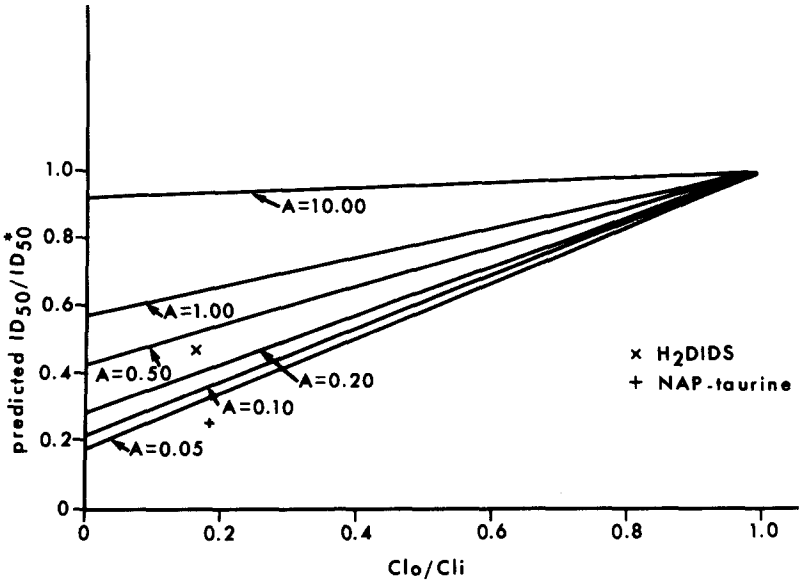

FIGURE 6. Predicted ID_{50}/ID_{50}* ratio as a function of Cl_o/Cl_i, at constant Cl_o. Lines are calculated for various values of the asymmetry ratio, A, assuming that K_c = 65 mM and the Cl_o = 10 mM, using Eq. A19 for H_2DIDS and Eq. A30 for NAP-taurine (these equations predict exactly the same effects for both probes).

asymmetry in favor of inward-facing sites. Comparison of these data with those of Schnell et al. (1978) and Gunn and Fröhlich (1979) in Table II shows that these results agree with Gunn and Fröhlich, but disagree with Schnell et al.

DISCUSSION

Mechanism of NAP-Taurine Inhibition

The data presented above (Fig. 2 and Table I) demonstrate that an increase in cytoplasmic chloride concentration causes an increase in the inhibitory potency of external NAP-taurine. This transmembrane effect is not accounted for by the changes in internal pH and membrane potential that normally accompany a

change in internal chloride, since even under conditions where the pH change does not occur (Fig. 3) or when the membrane potential is reversed (Fig. 4), the inhibitory effect of NAP-taurine remains high in the presence of a chloride gradient. These results demonstrate that NAP-taurine binds to band 3 preferentially and probably exclusively when the transport site is in the E_o or ECl_o form (Fig. 7). Since NAP-taurine does not interfere with chloride binding to the transport site (Knauf et al., 1978*b*), it must inhibit chloride exchange by preventing the conformational change from ECl_o to ECl_i or vice versa. Since NAP-taurine binds when the system is in the E_o or ECl_o form, and prevents the change to the ECl_i form, it must therefore lock the transport protein in the outward-facing E_o or ECl_o form.

The fact that the binding of NAP-taurine is strongly affected by the confor-

TABLE II

Comparison of Asymmetry Values Measured by Different Methods

Method	Reference	A	E_i/E_o
Effects of chloride gradient on H_2DIDS	Furuya et al., 1984	<1.4	>0.71
Effect of chloride gradient on NAP-taurine	This paper	<0.4	>2.5
Measurement of $K^o_{1/2}$(max)	Schnell et al., 1978	2.5	0.4
and $K^i_{1/2}$(max)	Gunn and Fröhlich, 1979	0.0638	15.6
	Schnell, 1979	0.476	2.1
Cl_o, which half-inhibits DNDS binding	Fröhlich, 1982	0.168	5.96
Extrapolation of $K^o_{1/2}$ to zero DNDS	Fröhlich, 1982	0.161	6.19
y-intercepts of Hunter-Downs plots with $Cl_i = Cl_o$ or with only Cl_o varied	Fröhlich, 1982	0.166	6.02
Same, but with different data for $Cl_o = Cl_i$	Barzilay and Cabantchik, 1979	0.230	4.36
Comparison of half-turnover chloride flux to number of band 3 molecules	Jennings, 1982	~0.11	~9

E_i/E_o given when $Cl_i = Cl_o$. The asymmetry is not calculated in the original references.

mation of the transport site provides further evidence that the transport and NAP-taurine sites may be closely adjacent. It also suggests that the conformational change of the transport site affects other regions of the transport protein, a point that is reinforced by other evidence that changes in transport site conformation affect the interactions of various inhibitors with the system (Knauf and Mann, 1984*b*; Passow et al., 1980*a*, *b*; Passow, 1982).

Intrinsic Asymmetry of the Anion Exchange System

The magnitude of the effects of chloride gradients on the inhibitory potency of both NAP-taurine and H_2DIDS (Fig. 6 and Table II) strongly supports the concept that with $Cl_i = Cl_o$ more of the unloaded transport sites face the inside than the outside ($E_i > E_o$). The only disagreement about the direction of the

asymmetry comes from the work of Schnell et al. (1978). After obtaining these results, we communicated with Dr. Schnell, and he informed us that a technical error in the computation of the fluxes caused an overestimate of $K^{\circ}_{1/2}$(max), leading in turn to an overestimate of A as calculated by Eq. A11. Dr. Schnell's revised data (Schnell, 1979) are in accordance with the idea that $E_i > E_o$ (Table II).

The true value of A can be determined by a variety of methods (see Appendix). In some cases recent experimental data permit calculation of A by these alternative techniques (Table II). From Fig. 6 of Fröhlich (1982), the concentration of external chloride that caused half-inhibition of DNDS binding to intact red cells was 6.2 mM when Cl_i was constant at 110 mM. From these data and Eq.

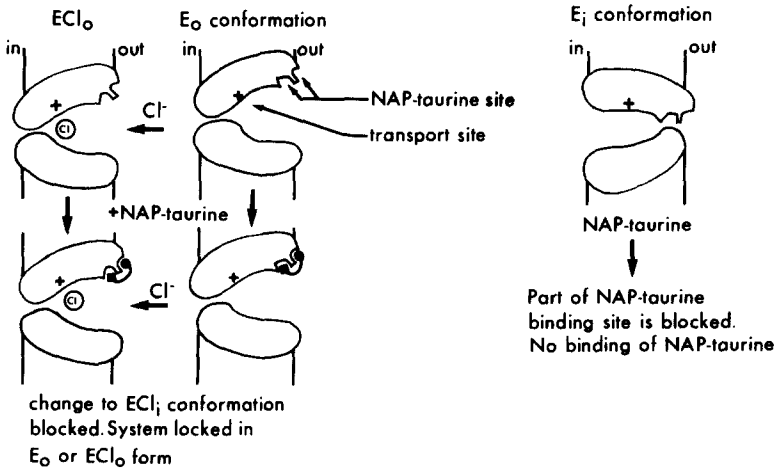

FIGURE 7. Model for the inhibitory action of NAP-taurine. When the transport site is in the outward-facing form, NAP-taurine binds to a separate site. This causes inhibition of the transport-related conformational change from $E\text{Cl}_o$ to $E\text{Cl}_i$. When the transport system is in the E_i or $E\text{Cl}_i$ form, part of the NAP-taurine binding site is inaccessible, so NAP-taurine binds very weakly or not at all when the transport site is in the inward-facing conformation. Note that the changes in protein conformation that change the orientation of the transport site are assumed to involve different domains of a single band 3 monomer.

A20, the value of A is 0.168, which corresponds to a sixfold excess of inward-facing sites. A similar calculation can be made from the $K^{\circ}_{1/2}$ data in Fig. 3 of Fröhlich (1982), which leads to an A of 0.161. From the y-intercepts of the Hunter-Downs plots obtained when external chloride (Fig. 6 of Fröhlich, 1982) or both internal and external chloride (Fig. 7 of Fröhlich, 1982) are varied, using Eq. A24, the value of A is calculated to be 0.166. If the data of Barzilay and Cabantchik (1979) are used for the Hunter-Downs y-intercept with $Cl_i = Cl_o$, an A value of 0.230 is obtained. The values of A calculated by different methods from the DNDS inhibition and binding data of Fröhlich (1982) are remarkably consistent, but the values of $K^{\circ}_{1/2}$ are higher than those originally reported by Gunn and Fröhlich (1979) and later confirmed by Milanick and Gunn (1981).

This leads to a higher estimate of A from the DNDS data. Since the direct measurement of $K^{\circ}_{1/2}$ is probably more accurate than the indirect methods involving the use of DNDS, the most precise measurement of the asymmetry is probably that of Gunn and Fröhlich, which would suggest that A is 0.0638 and thus that 15.6 times as many unloaded transport sites face the inside of the cell as face the outside. Jennings' (1982) measurements of the anion efflux associated with a half-turnover of band 3 are also compatible with the concept that most of the chloride transport sites face inward when $Cl_i = Cl_o$, although this method does not permit a very precise determination of A.

From a comparison of Cl/SO_4 and SO_4/SO_4 exchange fluxes, and from the effects of chloride gradients on DNDS inhibitory potency, Jennings (1980, 1982) concluded that there is a five- to eightfold asymmetry in favor of the inward-facing form of the protonated anion exchange carrier, which transports sulfate. Since these experiments were done in a pH range above or near the pK for the titratable group that converts the chloride carrier to a sulfate carrier (Milanick and Gunn, 1982), these results would imply that the pK of this group is similar at both sides of the membrane.

TABLE III

Comparison of Predicted and Actual ID_{50} Ratios When $Cl_o > Cl_i$

Condition	Cl_o/Cl_i	Experimental			Predicted
		ID_{50}/ID_{50}*	95% CI		ID_{50}/ID_{50}*
Ghosts	2.35	1.41	1.05	1.79	1.66
Cells	4.66	1.80	1.34	2.84	2.78

Experimental data are from Furuya et al. (1984). Predictions are based on Cl_o = 60 mM, K_c = 65 mM (Brazy and Gunn, 1976), A = 0.06383, and Eq. A19.

The value of A calculated from the data of Gunn and Fröhlich (1979) and from the experiments with chemical probes in the presence of outwardly directed chloride gradients should be consistent with the results obtained when the chloride gradient is reversed (Furuya et al., 1984). That this is so is shown in Table III, where the predicted ID_{50}/ID_{50}* ratios calculated with A = 0.0638 are compared with the data from Tables II and III of Furuya et al. (1984). In each case, the predicted values lie within the 95% confidence limits for the data. The mean values of the ID_{50} ratio, however, fall somewhat below the predicted values, possibly because of difficulties in maintaining and measuring the chloride gradients in such experiments.

In summary, all of the data are compatible with the concept that the red cell anion exchange system is highly asymmetric, with far more unloaded transport sites facing the cytoplasm than the external medium. There is no obvious reason why the system should require this property; it probably exists simply because the transport protein band 3 has two different conformations and these differ slightly in free energy or in chloride affinity. The relative contributions of these two possible sources of asymmetry are discussed in the following paper (Knauf and Mann, 1984*b*).

APPENDIX

Definition of Asymmetry Factor

If we assume that the anion exchange system can be in either of two forms—one (E_i) in which the transport site faces inward and the other (E_o) in which it faces outward—we can express the concentrations of the corresponding chloride-loaded forms of the system in terms of the dissociation constants for chloride at the inside (cytoplasmic) and outside surfaces of the membrane, K_i and K_o, as follows (where Cl to the right of E denotes binding of chloride to the transport site):

$$K_i = \frac{(E_i)(Cl_i)}{ECl_i} \qquad K_o = \frac{(E_o)(Cl_o)}{ECl_o}. \tag{A1}$$

The equations for the chloride influx and efflux, J_i and J_o, are:

$$J_i = k'ECl_o \qquad J_o = kECl_i \tag{A2}$$

and, since the net Cl^- flux is very small in comparison with the unidirectional fluxes:

$$J_i = J_o. \tag{A3}$$

Substituting Eqs. A1 and A2 into A3 and rearranging:

$$\frac{E_o}{E_i} = \frac{kK_oCl_i}{k'K_iCl_o}. \tag{A4}$$

If $k \neq k'$ and/or $K_i \neq K_o$, there will be an asymmetric distribution of E_i and E_o, even when $Cl_i = Cl_o$. This can be defined as the asymmetry factor, A:

$$A = \left(\frac{E_o}{E_i}\right)_{Cl_i=Cl_o} = \frac{kK_o}{k'K_i}. \tag{A5}$$

Determination of A from Chloride Half-Saturation Experiments

The maximum flux, ignoring modifier site interactions, is (see preceding paper, Furuya et al., 1984):

$$J_m = \frac{kE_t}{(1 + k/k')}, \tag{A6}$$

where E_t is the total amount of band 3 present in the membrane. E_t is given by the sum of all forms of band 3:

$$E_t = E_i + E_o + ECl_i + ECl_o. \tag{A7}$$

Substituting Eqs. A1–A3 and A6 into A7 and solving for the reciprocal of the efflux:

$$\frac{1}{J_o} = \frac{1}{J_m(1 + k/k')}\left[\frac{K_i}{Cl_i} + \frac{K_ok}{Cl_ok'} + 1 + \frac{k}{k'}\right]. \tag{A8}$$

Schnell et al. (1978) and Gunn and Fröhlich (1979) measured the chloride concentration on one side of the membrane required to half-saturate the transport system, when chloride was very high at the other side of the membrane. From these measurements, A can be determined as follows.

If Cl_i is kept constant and very high, so that $(K_i/Cl_i) \rightarrow 0$, and if Cl_o is varied, the Cl^- flux reaches half of its maximum value when the term containing Cl_o in the brackets of

Eq. A8 is equal to the other terms in the brackets. The concentration of Cl_o at which this occurs is $K^o_{1/2}(\max)$:

$$K^o_{1/2}(\max) = K_o/(1 + k'/k). \tag{A9}$$

Similarly, if Cl_o is kept high and constant, so that $(K_o/Cl_o) \rightarrow 0$, and if Cl_i is varied, the concentration of Cl_i at which the flux is half-maximal, $K^i_{1/2}(\max)$, is:

$$K^i_{1/2}(\max) = K_i/(1 + k/k'). \tag{A10}$$

Forming the quotient of A9 and A10:

$$\frac{K^o_{1/2}(\max)}{K^i_{1/2}(\max)} = \frac{K_o k}{K_i k'} = A. \tag{A11}$$

Thus, if $K^o_{1/2}(\max)$ and $K^i_{1/2}(\max)$ are known, A can be calculated. Moreover, K_c, the chloride concentration required to half-saturate the transport system when $Cl_i = Cl_o$, from Eq. A8, is:

$$K_c = \frac{K_i k' + K_o k}{k' + k} = K^i_{1/2}(\max) + K^o_{1/2}(\max). \tag{A12}$$

Thus, if K_c and either $K^o_{1/2}(\max)$ or $K^i_{1/2}(\max)$ are known, the other can be calculated, and therefore A can be determined. This is useful since $K^i_{1/2}(\max)$ is difficult to determine with precision (Gunn and Fröhlich, 1979). A similar derivation of A, using a slightly different model, has been presented by Fröhlich et al. (1983).

Dependence of the ID_{50} Ratio for H_2DIDS on A

If external H_2DIDS (D_o) acts as a reversible competitive inhibitor, it will bind only to E_o with dissociation constant K_d:

$$K_d = \frac{(E_o)(D_o)}{ED_o}. \tag{A13}$$

Cytoplasmic Cl^- (Knauf and Mann, 1984*a*; see Introduction) inhibits chloride exchange by binding to a modifier site with dissociation constant K_z, regardless of the orientation (E_o, E_i, ECl_o, ECl_i) of the transport site (Knauf and Mann, 1984*b*). (Even if the chloride modifier site is considered to be located at the outside surface (Schnell et al., 1978), this does not affect the outcome of any of the derivations in this Appendix.) Designating binding to the modifier site by placing Cl to the left of E, we obtain:

$$K_z = \frac{(E_o)(Cl_i)}{ClE_o} = \frac{(E_i)(Cl_i)}{ClE_i} = \frac{(ECl_o)(Cl_i)}{ClECl_o} = \frac{(ECl_i)(Cl_i)}{ClECl_i} = \frac{(ED_o)(Cl_i)}{ClED_o}. \tag{A14}$$

Including all of these forms, the equation for total band 3, E_t, becomes:

$$E_t = E_i + E_o + ECl_i + ECl_o + ED_o + ClE_i + ClE_o + ClECl_i + ClECl_o + ClED_o. \tag{A15}$$

Substituting Eqs. A1–A3, A6, A13, and A14 into A15, and solving for the reciprocal of the efflux:

$$\frac{1}{J_o} = \frac{1}{J_m(1 + k/k')}\left[\frac{K_i}{Cl_i} + \frac{K_o k}{Cl_o k'} + 1 + \frac{k}{k'} + \frac{D_o K_o k}{K_d Cl_o k'}\right]\left[1 + \frac{Cl_i}{K_z}\right]. \tag{A16}$$

When $D_o = ID_{50}$, the concentration that inhibits Cl^- exchange by 50%, $1/J_o$ is doubled

compared with its value when $D_o = 0$. Thus, the term in Eq. A16 containing D_o must equal all of the other terms in the same set of brackets:

$$\frac{ID_{50}K_o k}{K_d Cl_o k'} = \frac{K_i}{Cl_i} + \frac{K_o k}{Cl_o k'} + 1 + \frac{k}{k'}. \tag{A17}$$

Solving for ID_{50} and substituting for $k' + k$ from Eq. A12 and substituting A from Eq. A5:

$$ID_{50} = \frac{K_d}{A}\left[\frac{Cl_o}{Cl_i} + A + \frac{Cl_o}{K_c}(1 + A)\right]. \tag{A18}$$

This equation contains two unknowns, K_d and A. To eliminate K_d, we take the ratio of ID_{50} to the value (ID_{50}*) with $Cl_o = Cl_i$. If Cl_o is kept constant, we obtain:

$$\frac{ID_{50}}{ID_{50}^*} = \frac{(Cl_o/Cl_i) + A + (Cl_o/K_c)(1 + A)}{1 + A + (Cl_o/K_c)(1 + A)}. \tag{A19}$$

Determination of A from Hunter-Downs Plots

If Cl_o is varied at constant Cl_i and the ID_{50} for inhibition of chloride exchange by H_2DIDS (or another competitive inhibitor such as DNDS) is plotted against Cl_o, the x-intercept (defined as $-K_I$; Fröhlich, 1982) can be determined by setting ID_{50} equal to 0 in Eq. A18 and substituting $-K_I$ for Cl_o. Solving for A, we obtain:

$$A = \frac{K_I}{Cl_i}\left(\frac{K_c + Cl_i}{K_c - K_I}\right). \tag{A20}$$

If $Cl_o = Cl_i$, a Hunter-Downs plot of ID_{50} vs. Cl_o has an x-intercept at $Cl_o = -K_I'$ (Fröhlich, 1982). Setting ID_{50} at 0, Cl_o/Cl_i at 1, and substituting $-K_I'$ for Cl_o in Eq. A18, we obtain:

$$K_I'(1 + A) = K_c(1 + A). \tag{A21}$$

Thus, $K_I' = K_c$, regardless of the value of the asymmetry factor (Shami et al., 1978; Knauf et al., 1978*b*; Grinstein and Knauf, 1982). As Fröhlich (1982) has pointed out, Hunter-Downs plots with $Cl_i = Cl_o$ provide no information regarding A.

The y-intercept of such a plot, y_o, is given by setting $Cl_i = Cl_o = 0$ and $Cl_o/Cl_i = 1$ in Eq. A18:

$$y_o = (K_d/A)(1 + A). \tag{A22}$$

The y-intercept, z_o, of the Hunter-Downs plot when only Cl_o is varied, at constant Cl_i, is given by:

$$z_o = K_d. \tag{A23}$$

Thus, as Fröhlich (1982) has shown in a slightly different way:

$$A = z_o/(y_o - z_o). \tag{A24}$$

Dependence of the ID_{50} Ratio for NAP-Taurine on A

If external NAP-taurine binds reversibly to band 3, regardless of the orientation of the transport site, with dissociation constant K_t, the binding of NAP-taurine is described by:

$$K_t = \frac{(E_o)(T_o)}{TE_o} = \frac{(E_i)(T_o)}{TE_i} = \frac{(ECl_o)(T_o)}{TECl_o} = \frac{(ECl_i)(T_o)}{TECl_i}, \tag{A25}$$

where T_o is the concentration of NAP-taurine in the medium. External NAP-taurine competes with external Cl^- at a site different from both the transport site and the chloride self-inhibitory (modifier) site (Knauf and Mann, 1984*a*). Designating the chloride dissociation constant for this site as K_y, and deriving the equation for the reciprocal of the chloride efflux in a manner similar to that used to derive Eq. A16, we obtain:

$$\frac{1}{J_o} = \frac{1}{J_m(1 + k/k')}\left[\frac{K_i}{Cl_i} + \frac{K_o k}{Cl_o k'} + 1 + \frac{k}{k'}\right]\left[1 + \frac{Cl_i}{K_z}\right]\left[1 + \frac{Cl_o}{K_y} + \frac{T_o}{K_t}\right]. \quad \text{(A26)}$$

When $T_o = ID_{50}$, the term in T_o in the right-hand brackets is equal to the other terms, so

$$ID_{50} = K_t\left(1 + \frac{Cl_o}{K_y}\right). \quad \text{(A27)}$$

Thus, the ID_{50} does not depend on the chloride ratio.

If, on the other hand, NAP-taurine only binds to band 3 when it is in the E_o or ECl_o forms, then the equation for $1/J_o$ becomes:

$$\frac{1}{J_o} = \frac{1}{J_m(1 + k/k')}\left(\frac{K_i}{Cl_i} + 1 + \frac{K_o k}{Cl_o k'} + \frac{k}{k'}\right)\left(1 + \frac{Cl_o}{K_y}\right)\left(1 + \frac{Cl_i}{K_z}\right) + \left(\frac{K_o k}{Cl_o k'} + \frac{k}{k'}\right)\left(1 + \frac{Cl_i}{K_z}\right)\left(\frac{T_o}{K_t}\right). \quad \text{(A28)}$$

When $T_o = ID_{50}$, the term in T_o will equal the other terms so:

$$\frac{ID_{50}}{K_t}\left(\frac{K_o k}{Cl_o k'} + \frac{k}{k'}\right) = \left(\frac{K_i}{Cl_i} + 1 + \frac{K_o k}{Cl_o k'} + \frac{k}{k'}\right)\left(1 + \frac{Cl_o}{K_y}\right). \quad \text{(A29)}$$

Simplifying, forming the ratio ID_{50}/ID_{50}^*, substituting for $k + k'$ from Eq. A12, and inserting A from Eq. A5, if Cl_o is constant and Cl_i is varied, we obtain:

$$\frac{ID_{50}}{ID_{50}^*} = \frac{(Cl_o/Cl_i) + A + (Cl_o/K_c)(1 + A)}{1 + A + (Cl_o/K_c)(1 + A)}. \quad \text{(A30)}$$

This is exactly the same equation as Eq. A19, which was derived for the competitive inhibitor H_2DIDS. Thus, according to this model the chloride gradient should have exactly the same effects on the inhibitory potency of H_2DIDS and NAP-taurine, despite their different mechanisms of inhibition.

To derive Eq. A30, it was assumed that external Cl^- binds to the NAP-taurine site regardless of the conformation of the transport site. If, on the other hand, it is assumed that external Cl^- binds to the NAP-taurine site only when the transport site faces outward, a more complex expression is obtained. Under the conditions of these experiments, however, where $Cl_o \ll K_y$ (estimated to be ~165 mM; Knauf et al., 1978*b*), the terms in Cl_o/K_y are small and Eq. A30 provides a very good approximation for the ID_{50} ratio.

This research was supported by grants from the Medical Research Council, Canada (MT-5149) and from the U. S. Public Health Service (1R01-AM27495). It has been assigned Report No. UR-3490-2254.

Received for publication 14 April 1983.

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