ELSEVIER

Biochimica et Biophysica Acta 1193 (1994) 276–286

BBA Biochimica et Biophysica Acta

Inhibition of red cell urea flux by anion exchange inhibitors

Michael R. Toon, A.K. Solomon *

Biophysical Laboratory, Harvard Medical School, Boston, MA 02115, USA

Received 7 March 1994

Abstract

When they studied the chemical properties of red cell anion exchange inhibitors such as DIDS (4,4′-diisothiocyanate-2,2′-stilbene disulfonate), Barzilay et al. (1979) Membr. Biochem. 2, 227–254 also examined the benzene sulfonates. These molecules are structurally similar to half a DIDS molecule and are also specific anion exchange inhibitors with ID_{50} values measured in mM, rather than μM, as for the stilbene disulfonates. We have studied several inhibitors of the benzene sulfonate (BS) class and found that they also inhibit red cell urea flux by up to 92% and stimulate water flux by up to 58%. The values of $K_{inhib,app}$ for urea flux inhibition are the same as the ID_{50} values for anion flux inhibition; covalent DIDS completely suppresses the inhibition. These observations strongly suggest that the effect on urea flux is caused by BS binding at the stilbene site. Comparative studies on the short chain amides exclude lipid solubility and solute molar volume as factors that affect these BS actions. $K_{stim,app}$ for water flux stimulation is also related to the anion exchange ID_{50} values; covalent DIDS suppresses the water flux stimulation. These observations on urea and water fluxes are consistent with a common *driver*, located at the stilbene site, which is responsible for the BS actions on urea, water and anion fluxes. The subsequent steps are independent with separate *effectors* to modulate each of the individual fluxes. These *effectors* are presumably located in different regions of the protein or proteins and carry out their separate processes by allosteric means.

Key words: Red cell; Benzene sulfonate; Band 3; Anion exchange protein; Anion transport inhibitor; Water flux; Urea flux

1. Introduction

In their 1979 study of the chemical properties of anion transport inhibition sites in human red cells, Barzilay et al. [1] and Barzilay and Cabantchik [2] examined not only the disulfonic stilbene anion exchange inhibitors, similar to DIDS (4,4′-diisothiocyanate-2,2′-stilbene disulfonate), but also a related class of inhibitors, consisting of benzene sulfonate and its substituted derivatives. The benzene sulfonate class of compounds (denoted by BS) are structurally similar to half of a DIDS molecule and, like DIDS, are effective inhibitors of human red cell anion exchange, but the BS affinity is roughly three orders of magnitude lower. Millimolar concentrations of BS are required to effect the same degree of inhibition that can be attained with micromolar concentrations of the stilbene disulfonates. Brown et al. [3] had suggested that the anion transport protein, band 3, is also the locus of the water channel into the red cell; we [4] suggested that band 3 also serves as the urea channel and advanced a great deal of evidence supporting band 3 as the locus for the aqueous channel, though Mannuzzu et al. [5] believe the urea channel to be a separate protein. Now that the properties of CHIP28 as a water transport protein have been discovered by Preston et al. [6], it is important to reexamine the relationship of anion exchange to water and urea flux. Our finding that the BS class of inhibitors also affects red cell water and urea transport provides the opportunity to make this examination.

It is widely accepted that negative charges within the anion channel keep anions from diffusing freely across the red cell membrane and that a specialized mechanism is responsible for the one-for-one exchange of anions between the cytoplasm and the extracellular milieu. The inhibition of anion exchange by DIDS is directly proportional to the number of DIDS molecules bound to band 3, so that the anion flux goes essentially to zero when a single DIDS molecule has reacted with each band 3 [7,8]. Since the bulky DIDS molecule can

* Corresponding author. Fax: +1 (617) 4321144.

SSDI 0005-2736(94)00102-U

bind irreversibly to a lysine site within the anion exchange channel of band 3, it might be expected that DIDS would occlude the narrow aqueous pore and thus inhibit water flux. However this is not the case, since Brahm [9] has shown that 1 μM DIDS has no effect on diffusional water permeability. Subsequently, Toon and Solomon [10] found that incubation of NEM (*N*-ethylmaleimide) treated red cells with 10 μM DIDS leads to $11 \pm 3\%$ inhibition of osmotic water flux. If the mechanism by which the bulky DIDS molecule blocks anion exchange were steric hindrance within the channel, DIDS should also significantly inhibit water flux. However, it is more likely that DIDS inhibits anion exchange by some process related to movement of charged groups in the channel. If the DIDS binding site were in an antechamber to the narrowest region of the channel or in a pocket beside the channel, water flux would be affected minimally.

We initiated a study of the effects of the BS anion exchange inhibitors on urea and water fluxes. We found that these inhibitors cause both a profound inhibition of urea transport and simultaneously, a large increase in water flux. This increased water flux shows that BS inhibition of urea flux can not be ascribed to steric hindrance in a water filled channel; instead our results show that BS inhibitors exercise an important effect on the chemical and conformational properties of the channel or channels through which urea and water enter the red cell.

2. Materials and methods

2.1. Materials

Benzene sulfonic acid (sodium salt), 4-aminobenzene sulfonic acid, and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) were obtained from Sigma (St. Louis, MO). Acetamide, 4-chlorobenzene sulfonic acid, 3-nitrobenzene sulfonic acid (sodium salt) and 4-hydroxybenzene sulfonic acid (sodium salt) were supplied by Aldrich (Milwaukee, WI). DIDS was obtained from Molecular Probes (Eugene, OR); propionamide, butyramide and formamide from Eastman (Rochester, NY) and urea from Fisher (Fairlawn, NJ). Outdated bank blood was kindly supplied by the Children's Hospital (Boston, MA).

2.2. Methods

Outdated bank blood, after aspiration of buffy coat and plasma, was washed three times with a buffer (control buffer) of the following composition, in mM: NaCl, 140; KCl, 5; Hepes, 20; pH 7.4, 300 ± 5 mosM. Buffers which contained benzene sulfonates (BS) were prepared by decreasing the NaCl concentration by an appropriate amount to maintain constant osmolality. Measurements performed in the presence of BS were made with the indicated concentrations of BS present in both the suspensions and all mixing solutions. Osmolalities of all solutions were determined by a Fiske Model OS osmometer (Uxbridge, MA). Cells at 2% hematocrit were mixed with an equal volume of buffer made hyperosmolal by the addition of NaCl or permeant solute and the time course of red cell volume changes was measured by 90° scattered light using the stopped-flow apparatus of Terwilliger and Solomon [11]. The analog data were digitized and averaged by a Hewlett-Packard Model 217 computer which was also used for the data analysis. Experiments were carried out at 24–26°C. The hydraulic permeability coefficient, L_p, and the solute permeability coefficient, ω, were determined as described in detail by Toon and Solomon [12]. Briefly, the time course of 90° light scattering (which is proportional to cell volume) was recorded and digitized, and the results for a series of 25 (for L_p) or 35–45 (for ω) replicate runs were averaged over a period from 8 ms to 1.2 s. After subtraction of the average of an equivalent number of control runs (in which the cells are mixed with the buffer in which they are suspended, so there is no change in cell volume), the data were fitted to the relevant equations as described by Toon and Solomon [12] in a paper which includes a typical time course for our measurements (as their Fig. 2). The response of the cells to a 150 mosM gradient after mixing was the basis for all determinations of L_p. When higher osmotic gradients were used to determine L_p, the maximal fractional stimulation observed in the presence of benzene sulfonates was reduced. Solute permeability coefficients were determined by the minimum method of Sha'afi et al. [13] and were based on the response of the cells to 350–450 mosM solute gradients, after mixing.

Washed red cells were resuspended to 25% hematocrit in buffer with (or without) 10 μM DIDS and incubated at 37°C for 1 h. Cells were then centrifuged, washed with 25 volumes of buffer + 0.5% bovine serum albumin (BSA), followed by an additional wash with buffer without BSA. Cells were then resuspended for stopped-flow measurements.

3. Results and discussion

3.1. Effects of aromatic sulfonate inhibitors on urea fluxes

Interpretation of the action of sulfonate inhibitors rests upon the characterization of the DIDS anion transport inhibition site which has been studied in

detail, particularly by Ship et al. [7] and Lepke et al. [8]. These authors report that 90% of the DIDS binds to band 3 and that there is a 1:1 correlation between DIDS binding and anion exchange inhibition. There are two sites for anion exchange, a high affinity transport site and a low affinity modifier site, whose characteristics and inhibition have been reviewed by Cabantchik and Greger [14] and Passow [15]. The properties of the binding site for stilbene inhibitors of anion transport have been characterized by Barzilay et al. [1] and Barzilay and Cabantchik [2] and compared with those of a number of aromatic sulfonic acid inhibitors. Their careful study of the kinetics of anion exchange inhibition led them to conclude that both classes of inhibitor bound to the high affinity binding site. This view is accepted by Passow [15] who shows a schematic drawing of the binding with the sulfonates and the stilbenes occupying the same or closely adjacent sites.

We have chosen to study four representative BS anion exchange inhibitors: benzene sulfonate (BSate), 3-nitrobenzene sulfonate (3NBS), 4-chlorobenzene sulfonate (4CBS) and 4-hydroxybenzene sulfonate (4OHBS), whose anion exchange ID_{50} values range from 4.9 to 47 mM [1]. As these authors point out, ID_{50} for competitive inhibition in their experiments is equivalent to $K_{i,app}$. These BS anion exchange inhibitors are also very effective inhibitors of urea permeability, as shown in Fig. 1 for three experiments with 4CBS. The data have been fit to a single site binding curve with $K_{inhib,app} = 3.7 \pm 0.4$ mM and maximal inhibition of 92 $\pm$ 3%, as given in Table 1. The observation that 4CBS inhibition of urea flux is a saturable phenomenon means that the inhibition is driven by 4CBS reaction with a fixed number of sites and can not be attributed to dissolution in the membrane fabric which is not a saturable process. The fact that $K_{inhib,app}$ is similar to the ID_{50} of 5.5 $\pm$ 2 mM (Table 1) for inhibition of anion exchange suggests that a single inhibitor binding site is responsible for inhibition of both anion exchange and urea flux, though the ID_{50} values were obtained in a sulfate medium, rather than the chloride solution we have used.

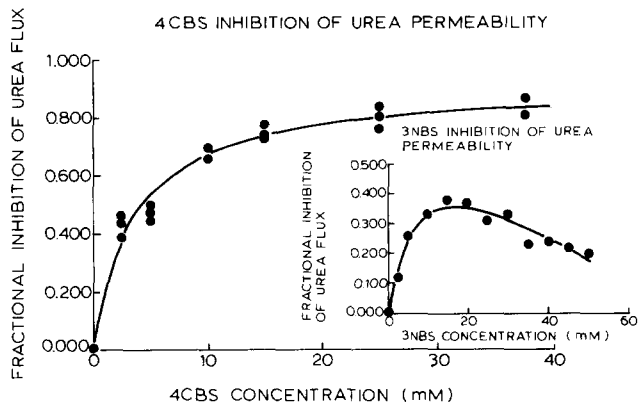

Fig. 1. Dependence of urea flux inhibition on 4CBS concentration in three experiments. Data fitted to a single site binding curve with $K_{inhib,app} = 3.7 \pm 0.4$ mM and maximal inhibition of 92 $\pm$ 3%. (Inset) Dependence of fractional inhibition of urea flux on 3NBS concentration. Data fitted empirically by non-linear least-squares fit to a single site binding curve plus a linear decrement.

Just as the ID_{50} is idiosyncratic to the nature of the substituent on the benzene molecule, so is the $K_{inhib,app}$; the shape of the inhibition curve also depends upon the substituent on the benzene ring. Thus, though the data for 4OHBS also fit a single site binding curve, the inhibition of urea flux produced by BSate and 3NBS decreases as the inhibitor concentration becomes greater than two to four times the $K_{inhib,app}$, similar to the effect of the modifier site on anion exchange. In the case of BSate ($K_{inhib,app}$ = 44 mM), urea flux inhibition decreases by about 10% at 150 mM and we have not included the 150 mM points in the fits. In the case

Table 1
Correlation of aromatic sulfonate effects on anion exchange with water and urea permeability

Sulfonic acid	Anion exchange inhibition [c] (ID_{50} mM)	Water flux stimulation		Urea flux inhibition	
		K_{stim} mM	fract stim	K_{inhib} mM	fract inhib
3-Nitrobenzene(8)	4.9 ± 2	14 ± 6	0.58 ± 0.12	4.8 ± 0.9	0.40 ± 0.03
4-Chlorobenzene(3)	5.5 ± 2	15 ± 10	0.52 ± 0.10 [a]	3.7 ± 0.4	0.92 ± 0.03
4-Hydroxybenzene(5)	14 ± 5	12 ± 7	0.13 ± 0.03	21 ± 15	0.37 ± 0.09
Benzene(5) [b]	47 ± 15	27 ± 13	0.18 ± 0.03	44 ± 14	0.63 ± 0.09

Three experiments with 4-aminobenzene sulfonate (4ABS, sulfanilic acid) were carried out and had no effect on urea and water permeability up to the maximum concentration we could use, 150 mM. Since the ID_{50} for 4ABS anion inhibition is 61 ± 20 mM, the highest of all those studied by Barzilay et al. [1] it is possible that water and urea effects might have been observable, had we been able to increase the 4ABS concentration in our system. The numbers of experiments are given in parentheses.

[a] In these 4CBS experiments, the maximum fractional stimulation was computed from a plot of (fractional stimulation)$^{-1}$ against (inhibitor concentration)$^{-1}$, analogous to a Lineweaver-Burk plot, since the asymptotes of the single site binding curves were poorly defined.

[b] K_{inhib} for BSate inhibition of acetamide permeability is 11 ± 7 mM and the maximum inhibition is 0.31 ± 0.04.

[c] Data from Barzilay et al. [1].

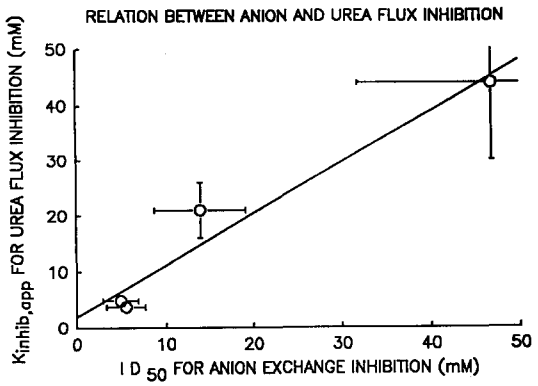

Fig. 2. Correlation of $K_{inhib,app}$ for urea flux inhibition (data from Table 1) with the ID_{50} for anion exchange inhibition [1]. $r = 0.99$, $P < 0.01$, t-test.

of 3NBS ($K_{inhib,app} = 4.8$ mM), the decrease begins at 15–20 mM as shown in the inset to Fig. 1. Since there is no clearly marked plateau with 3NBS, the curve is truncated as soon as the inhibition begins to fall, and $K_{inhib,app}$ is obtained from the rising phase of the inhibition curve. This procedure allows us to determine $K_{inhib,app}$ for 3NBS inhibition without making any assumptions as to the cause of the falling inhibition, which may be either a sequential or parallel process. The nature of the specific substituent on the benzene ring also determines the fraction of the flux inhibited, which ranges from 37 ± 9% for 4OHBS to 92 ± 3% for 4CBS, as shown in Table 1, together with the $K_{inhib,app}$ values.

The relationship of the $K_{inhib,app}$ and ID_{50} of 4CBS also extends to other members of the series as shown graphically in Fig. 2 in which $K_{inhib,app}$ is plotted as a function of ID_{50}. The two parameters are linearly related with a slope of 0.97 and an r of 0.99, which is significant at $P < 0.01$, t-test. Since the slope is indistinguishable from unity, Fig. 2 makes it likely that the same binding site governs anion exchange and urea flux inhibition.

This linkage between the site for anion transport inhibition and that for urea flux inhibition is supported by covalent DIDS binding experiments. As discussed above, Barzilay et al. [1,2] had shown that sulfate transport is competitively inhibited by either mono- or disulfonic inhibitors, thus showing that aromatic sulfonates and stilbene disulfonates bind to the same site, the high affinity anion binding site. Consequently, we used DIDS to show definitively that the anion transport inhibition site is directly implicated in urea flux inhibition. The anion exchange site was first blocked by covalent reaction with 10 μM DIDS, and the unreacted DIDS was washed away. Fig. 3 shows the results of one (of seven) 3NBS experiments in which DIDS not only abolishes the 3NBS inhibition of urea flux, but stimulates the flux slightly. The results of these DIDS experiments directly confirm the central role of the anion transport inhibition site in the regulation of red cell urea flux.

3.2. Inhibitor binding reaction

Since the binding site for the BS and stilbene disulfonate inhibitors is the same, and since the chemical structures are related, we expected that the binding reaction would be the same, though with very different rate constants. We had previously used fluorescence to measure the kinetics of DBDS (4,4′-dibenzamido-2,2′-stilbene disulfonate) binding to band 3 [16] and found that the first step is a bimolecular association too fast for us to measure, even by stopped flow. This first step is followed by a slow monomolecular conformation change which stabilizes the bound complex. Since the forward rate constant of the monomolecular step is 44 times faster than the backwards rate constant, the conformation change effectively locks the DBDS into position. In the limit, if the conformation change is irreversible, as for Michaelis-Menten kinetics, the kinetics (for an isolated binding reaction) conform to a single site binding curve; otherwise there is an additive constant which displaces the curve but does not alter the equilibrium constant.

The stoichiometry of BS binding to the anion exchange transport protein was established by Barzilay et al. [1] who showed that the slope of the Hill plot was essentially unity, indicating that a single inhibitor binds to each transport protein, like the Verkman et al. [16] reaction scheme in which a single DBDS binds to each band 3. A similar observation applies to the urea flux inhibition process, as shown in Fig. 4 in which the data from Fig. 1 are plotted using the Hill coordinates. The relation is linear with a Hill coefficient (slope) of 1.04 ± 0.08, and a correlation coefficient, $r = 0.96$ and $P < 0.01$.

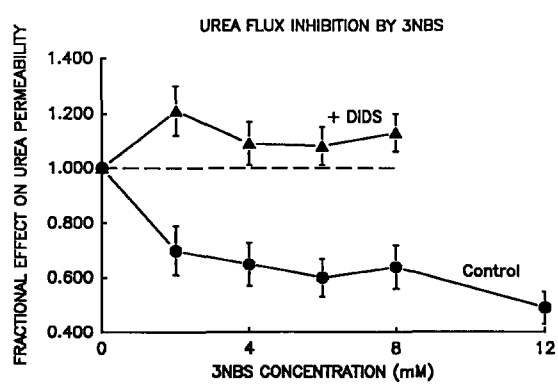

Fig. 3. Effect of covalent DIDS (10 μM) reaction on the inhibition of urea flux by 3NBS. One of seven experiments.

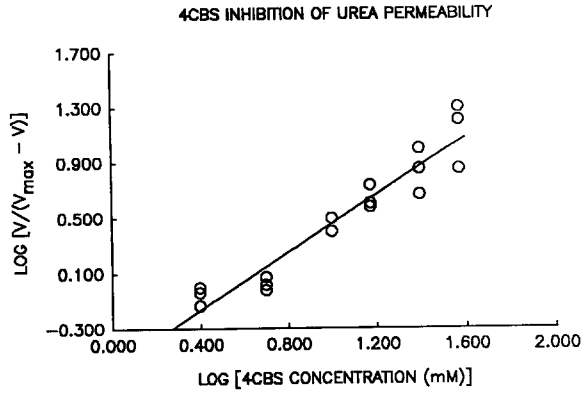

Fig. 4. Data from Fig. 1 plotted in the form of a Hill plot. The Hill coefficient (slope) is 1.04 ± 0.08 and $r = 0.96$, $P < 0.01$.

In the stopped-flow apparatus, the reaction between red cells (in one solution, called A) and the permeant solute (in the other solution, called B) begins when the two solutions meet in the reaction chamber. The dead-time of our apparatus is 10–15 ms and the first point is taken at 16 ms. To see whether the reaction with inhibitors of the BS class is complete in < 16 ms, the BS compound is added to solution B so that the binding reaction is only initiated when the red cells in solution A meet the BS in solution B in the mixing chamber, effectively setting a lower limit on the time constant we can resolve. Our results show that the binding is complete in this time (data not shown), so we conclude that the inhibition reaction is essentially instantaneous, with a time constant < 16 ms.

Barzilay and Cabantchik [2] have shown that the effect of 3NBS on anion exchange can be fully reversed by three washes with 10 volumes of medium. We find that the 3NBS effects on urea fluxes (and water fluxes as will be discussed below) can be reversed from 60 to 90% with three washes with 25 volumes of medium, in reasonable agreement with Barzilay and Cabantchik. In general, fewer washes, or smaller volumes of wash, decrease the fraction of 3NBS removed.

3.3. Kinetics of aromatic sulfonate effects on urea flux

Although the same binding site, which we call the *driver* site, is implicated in anion and urea flux inhibition, some differentiation in the subsequent events is to be expected, since urea and Cl^- are such different molecules. Evidence for this difference comes from the 3NBS experiments because the reversal of urea inhibition we have observed at high 3NBS concentrations is entirely absent for anion transport inhibition. Barzilay and Cabantchik have increased the 3NBS concentration up to 50 mM, twelve times the ID_{50}, with no signs of aberrant behavior in anion exchange inhibition. This means that there are different *effectors* for anion exchange and urea transport. Interaction with the *effector* site depends upon the substituent on the benzene sulfonate, since 4CBS and 4OHBS show no decrement in urea flux inhibition. If we assume that the 3NBS is effectively locked into the *driver* site, there must be an additional locus for the *effector* action, perhaps adjacent to the *driver* site, and responsible for control of urea transport, probably by an allosteric mechanism.

The kinetics of urea flux inhibition can be modeled using the mixed-type inhibition equations given by Segel [17]. A phenomenological adaptation of the relevant equation leads to:

$$\begin{array}{ccccc}
\text{site} + \text{urea}_{out} & \xleftarrow{K_m} & \text{site/urea} & \xrightarrow{k_{tr}} & \text{site} + \text{urea}_{in} \\
+ & & + & & \\
\text{BS} & & \text{BS} & & \\
\updownarrow & & \updownarrow & & \\
\text{site-BS} & & \text{site-BS/urea} & & \\
\downarrow & & \downarrow & & \\
\text{site-BS*} + \text{urea}_{out} & \xleftarrow{\alpha K_m} & \text{site-BS*/urea} & \xrightarrow{\beta k_{tr}} & \text{site-BS*} + \text{urea}_{in}
\end{array} \qquad (1)$$

According to the tripartite model we have proposed for urea permeation of the red cell membrane [12,18], a hydrated nonelectrolyte, like urea, has to exchange its waters of hydration for the H-bonds that line the aqueous channel, before it can enter the channel. The channel itself forms the sieve-selective region, whose diameter controls the flux of water. Urea makes the exchange at a site at the entrance to the channel, and exits the pore after a reverse exchange at an analogous site at the cytoplasmic face. The existence of these H-bond exchange sites is supported by our evidence, and that of others [19,20], not only that the transport of urea and other small hydrophilic amides is characterized by specific K_m values, but also that K_m values on different faces of the membrane are different [19]. In Eq. (1), the H-bond exchange site is represented by the reaction of urea_{out} with its external site, where it forms the transient complex called site/urea, whose apparent equilibrium constant is K_m, when no inhibitor is present. The rate constant k_{tr} is a phenomenological representation of all the steps in the ensuing rate of urea transport across the membrane with no implications about the mechanism. In a phenomenological sense, k_{tr} may be considered as the apparent rate constant for the *effector* reaction. The reaction of BS with the site, which is complete before any significant amount of urea has entered the cell, is shown as the vertical reaction at the left of Eq. (1) in which the site-BS complex, after its conformation change, is denoted as site-BS*. In the presence of inhibitor, the equilibrium constant for the reaction of urea_{out} with the site-inhibitor complex, site-BS*, be-

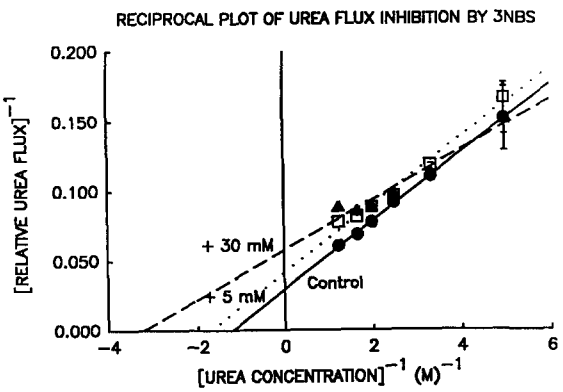

Fig. 5. Reciprocal plot of urea flux showing inhibition by 3NBS. One of four experiments. Control (●, solid line); +5 mM 3NBS (□, dotted line); +30 mM 3NBS (▲, dashed line).

comes αK_m, as shown in the bottom line of Eq. (1), and the rate constant for onward transport is denoted by βk_{tr}.

The class of inhibition consistent with our data can be determined by a reciprocal plot [1] of 3NBS inhibition, like Fig. 5 (one experiment, typical of four), which shows that the inhibitor increases the urea binding affinity (decreased K_m) and decreases V_{max}, characteristic of mixed inhibition [17]. At 5 mM 3NBS, which is the $K_{inhib,app}$ for urea transport inhibition, the equilibrium constant is decreased by multiplication by the factor $\alpha = 0.68$. This factor is almost the same as the fractional decrease in V_{max} with $\beta = 0.70$. In the other three experiments in this series, α ranges from a low of 0.40 to 0.68 for Fig. 5. Nonetheless, the α/β ratio remains very close to unity with a mean value of 0.95 ± 0.02. This correlation suggests that whatever change 3NBS induces in the site to increase its urea binding affinity, it also leads to a concomitant decrease in V_{max}, possibly because the urea, being more tightly bound, finds it difficult to escape from the site and continue its journey across the membrane. These observations about the effect of 3NBS on α and β are consistent with the tripartite model in which there are only two rate-determining steps for urea flux, the H-bond exchange site and the sieve-selective region. Since water transport is increased by 3NBS and urea flux is decreased, our model predicts that 3NBS should modify the H-bond exchange site; the effects on α and β show that such a change has occurred.

When the inhibitor concentration is increased by a factor of six to 30 mM, more than enough to saturate the site, the binding becomes even tighter (α ranging from 0.26 to 0.35), but the correlation with β disappears and the α/β ratio varies from 0.54 to 0.83, possibly because of increased deformation at these high inhibitor concentrations.

3.4. Inhibition of amide permeability by 3NBS

A set of experiments were carried out on the 3NBS inhibition of the permeability of the homologous amides from formamide through butyramide to study the effects of systematic increases in solute molar volume and lipid solubility. Sha'afi et al. [23], who characterized amide permeability in 1971, showed that there was a relationship among the permeability coefficient, ω_i, the lipid solubility, measured by the ether/water partition coefficient, k_{ether}, and the solute size, measured by the cylindrical radius determined from molecular models. When Sha'afi et al. plotted $\ln(\omega_i/k_{ether})$ as a function of solute radius, they found that all the amides from formamide through isovaleramide fell on a smooth curve which was characteristic of the members of the amide family. Our control studies in Fig. 6 show that the permeability decreases with the increase in cylindrical radius from formamide to propionamide (see Table 2) despite the increase in k_{ether}. However the increase in k_{ether} for butyramide outweighs the increased cylindrical radius and ω_i rises, in agreement with the results of Sha'afi et al. [23]. The striking observation, after inhibition with 15 mM 3NBS (approximately three times the $K_{inhib,app}$), is that the pro-

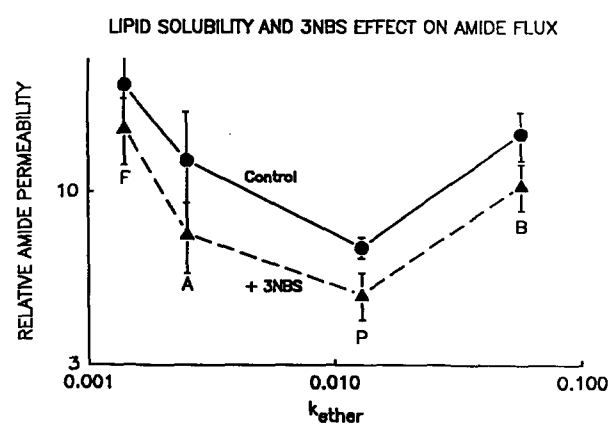

Fig. 6. Effect of 15 mM 3NBS on the permeability of formamide (F), acetamide (A), propionamide (P), and butyramide (B) plotted as a function of k_{ether}. Points shown are the average permeability coefficients ±S.D. for three experiments, in the presence or absence of 15 mM 3NBS. Values for k_{ether} are from Table 2.

[1] In the experiment shown in Fig. 5, the control K_m of 850 ± 110 mM is decreased to 580 ± 140 mM by 5 mM 3NBS and further to 310 ± 50 mM by 30 mM 3NBS. The control K_m is much greater than the mean K_m of 300 ± 70 mM determined previously by Toon and Solomon [12], though it is comparable to values of 680 up to 1 M given by Macey's group [21,22]. Though the Macey group remark upon the differences in K_m in different laboratories, they have no explanation, nor do we. Other values of our control K_m values in the experiments similar to that in Fig. 5 range from 780 ± 70 mM to 930 ± 90 mM, as determined from single site binding curves which do not depend upon the long extrapolations in Lineweaver-Burk plots.

Table 2
3NBS inhibition of amide permeability *

Solute	Permeability coeff (ω_i) mol dyne^{-1} s^{-1} ($\times 10^{-15}$)		k_{ether}	Cylindrical radius (A)
	control	+15 mM 3NBS		
Formamide [a]	21 ±5	16 ±4	0.0014	2.07
Acetamide	13 ±5	8 ±2	0.0025	2.38
Propionamide	6.8±0.5	4.9±0.8	0.013	2.61
Butyramide	15 ±3	11 ±2	0.058	2.68

* Data for k_{ether} and cylindrical radius taken from Sha'afi et al. [23].
[a] Three experiments were carried out for each amide.

portional decrease in permeability is essentially independent of the nature of the solute as can be seen in Fig. 6, notwithstanding the great variation in lipid solubility, with k_{ether} values that vary by a factor of forty from 0.0014 to 0.058. If lipid solubility were a factor in the inhibition, the relative effect of 3NBS treatment on butyramide, whose permeability through the lipids is an important component, would be very much different, and larger, than that of the other more hydrophilic solutes.

When the logarithm of ω_i/k_{ether} is plotted as a function of cylindrical radius (Fig. 7), the control data fall on a smooth curve in reasonable agreement with the data of Sha'afi et al. [23]. The effect of 3NBS causes a uniform downward shift in the four solutes in Fig. 7 which shows that the inhibition is essentially independent of cylindrical radius. If the inhibition of amide permeability could be attributed to steric hindrance, the effect would be larger for the larger molecules as they make a tighter and tighter fit to the pore radius.

If the urea/amide flux inhibition is not to be attributed to membrane solubility or steric hindrance, what is left? Kitagawa et al. [24] observed that the half-time for BS compound permeation into the rat red cell is of the order of 5–10 min which means that the urea/amide permeation process has come to equilibrium before the inhibitors have crossed the cell membrane. This places the rate controlling step on, or near, the outside face of the membrane. As we have discussed, Eq. (1) has two possible rate determining steps, urea binding to the H-bond exchange site with the equilibrium constant, K_m, and its onward transport with the rate constant, k_{tr}. Our kinetic experiments have shown that BS inhibitors reduce k_{tr} to βk_{tr} and that the factor β is 0.70 (5 mM 3NBS), consistent with control of the urea flux by this step in the reaction pathway. It is also possible that steric or other restrictions could affect the rate of the bimolecular association, and thus play a role in the inhibition pathway.

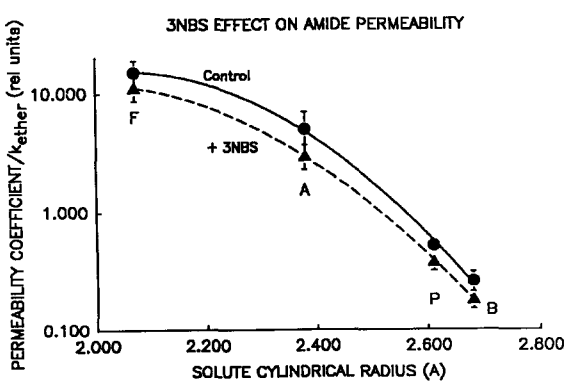

Fig. 7. The factor, ω_i/k_{ether}, plotted as a function of solute cylindrical radius (Table 2). The data have been fitted empirically to a second-order polynomial.

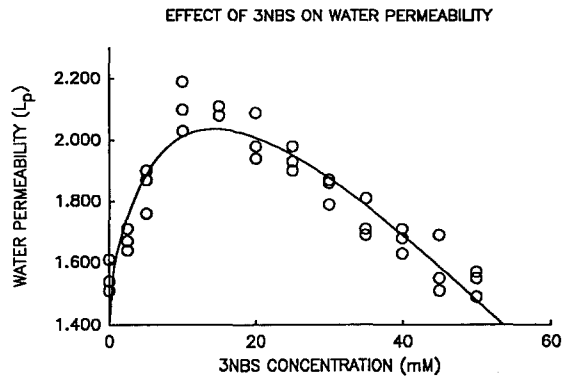

Fig. 8. Dependence of the osmotic water permeability coefficient (L_p, $\times 10^{-11}$ cm^3 dyne^{-1} s^{-1}) on 3NBS concentration. The curve has been drawn empirically as described in the text. Data from three experiments, each on a different blood donor.

3.5. Stimulation of water fluxes by BS anion exchange inhibitors

Though it is relatively easy to inhibit red cell water transport with mercurial sulfhydryl reagents, the variety of water transport inhibitors is limited and virtually no reagents are known which stimulate red cell water flux by more than 25%. Thus, our finding that the class of BS inhibitors which are effective urea transport inhibitors stimulate red cell water flux by up to 58% is unexpected, particularly because all the mercurial reagents which inhibit water flux are even more effective inhibitors of urea flux.

Fig. 8 shows that 3NBS causes a smooth increase in water flux up to concentrations of about 15 mM. At higher concentrations, there is a decremental phase, so that at 50 mM 3NBS, water permeability has returned to control values. The curve through the points in Fig. 8 has been drawn empirically as the sum of a single site binding curve (called phase one) plus a linear decrement (called phase two). A similar biphasic effect on water flux has been observed for the other BS inhibitors we have studied.

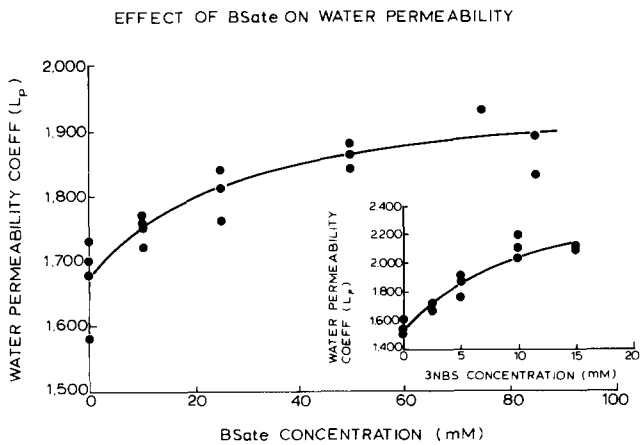

Fig. 9. Dependence of the phase one water flux stimulation on BSate concentration. Data from four experiments fit to a single site binding curve, with $K_{stim} = 27 \pm 13$ mM and maximal fractional stimulation of $18 \pm 3\%$. (Inset) Dependence of phase one water flux stimulation on 3NBS concentration. Data from three experiments, with $K_{stim} = 12 \pm 7$ mM and maximal fractional stimulation of $71 \pm 19\%$.

The use of a single site binding curve to fit phase one is based on our assumption that binding of a single inhibitor molecule to a membrane site leads to the conformational change responsible for the increased water permeability. Since there is no clearly marked plateau, the curve is truncated as soon as the permeability begins to fall, and phase one is defined in operational terms as the rising phase of the permeability curve. This procedure allows us to determine a $K_{stim,app}$ for water flux stimulation in phase one in the absence of any assumptions about phase two. Fig. 9 shows the phase one data for four experiments with BSate and, in the inset, three experiments with 3NBS. The five fold difference in the concentration scales shows that the binding affinity depends upon the nature of the substituents on the benzene ring. The relative magnitude of the stimulation for the experiments shown in Fig. 9 is also idiosyncratic to the

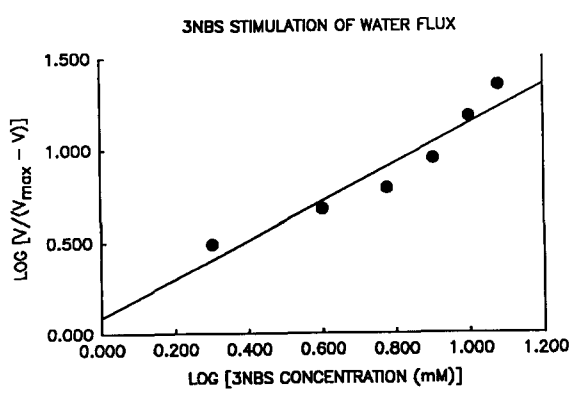

Fig. 10. Control curve data from Fig. 13 plotted in the form of a Hill plot. The Hill coefficient (slope) is 1.05 ± 0.17 and $r = 0.95$, $P < 0.01$.

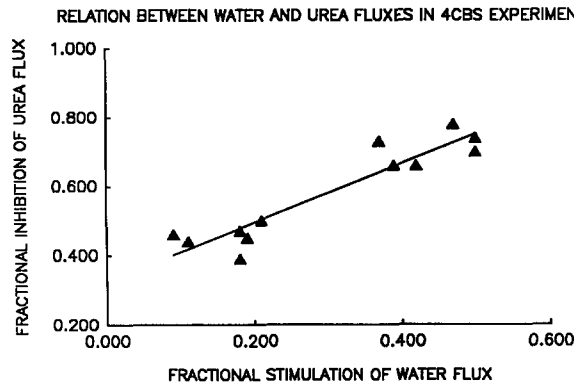

Fig. 11. Relation between the fractional stimulation of water flux (phase 1) with the fractional inhibition of urea flux for the three experiments with 4CBS. $r = 0.94$.

substituent, ranging from $18 \pm 3\%$ for BSate to $71 \pm 19\%$ for 3NBS in these specific experiments.

As we did for urea, we plotted the data from the control curve of Fig. 13 as a Hill curve in Fig. 10. The Hill coefficient (slope) is 1.05 ± 0.17 ($r = 0.95$ and $P < 0.01$), as required for binding of one 3NBS molecule for each binding site. Also, as for urea, the saturable nature of the single site binding curve is not compatible with dissolution of 3NBS in the membrane as the cause of the water flux stimulation.

Notwithstanding the difference in the direction of the effects, there is a close relationship between the action of BS on water, urea and anion fluxes. The average $K_{stim,app}$ values for water flux stimulation for all four BS compounds are given in Table 1 together with the urea flux inhibition and ID_{50} values for anion exchange [1]. The fractional stimulation of water flux is correlated linearly with the fractional inhibition of urea flux over the range shown in Fig. 11 for all three 4CBS experiments ($r = 0.94$, $P < 0.001$). A significant correlation is also observed with the other BS compounds

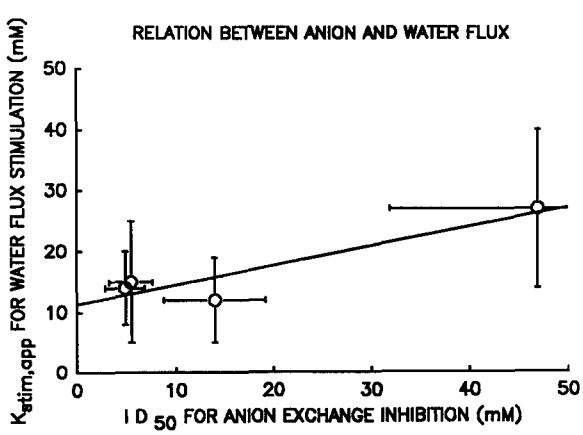

Fig. 12. Correlation of $K_{stim,app}$ for phase one water flux stimulation (data from Table 1) with ID_{50} for anion exchange inhibition [1]. $r = 0.93$.

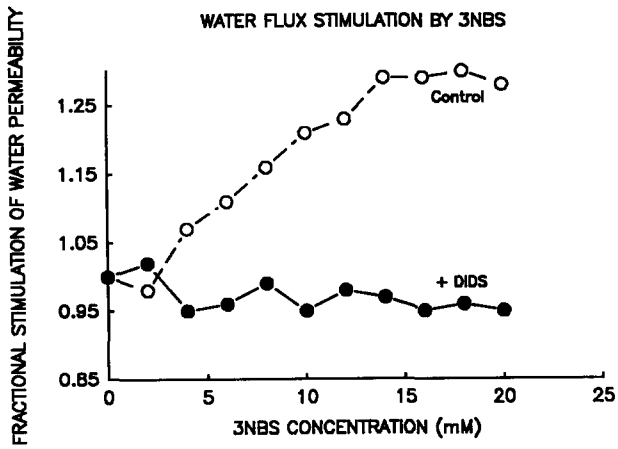

Fig. 13. Effect of covalent DIDS reaction on 3NBS phase one water flux stimulation. One of eight experiments.

as, for example, for the nine experiments with 3NBS. The correlation coefficient in six of these experiments is significant at $P < 0.01$, in two at $P < 0.05$ and in one at $P < 0.1$. These correlation coefficients show that there is a common rate-determining element responsible for both urea flux inhibition and water flux stimulation.

The relation between the BS binding site for water flux stimulation and that for anion flux inhibition can be seen in the graphical comparison between $K_{stim,app}$ for water flux and ID_{50} for anion flux inhibition in Fig. 12 ($r = 0.93$, $P < 0.1$). With 4 data pairs, a correlation coefficient of 0.95 is required to establish significance at the 5% level; consequently, these experiments only suggest that the conformational change responsible for anion exchange inhibition is also involved in water flux stimulation. We strengthened the inference about the linkage between sites for anion exchange inhibition and water flux stimulation by experiments with DIDS, as for urea. Fig. 13 shows that covalent DIDS abolishes the 3NBS phase one stimulation of water flux (one of eight DIDS/3NBS experiments) which shows that the anion inhibitor binding site plays a role in the water flux stimulation.

3.6. Structure-activity relationships of BS inhibitors

The primarily electrostatic nature of the binding of the BS compounds is supplemented by their electron acceptor capacity. This conclusion was put forward by Barzilay et al. [1] based on the importance of positive charges in attracting the sulfonate group. Passow [15] suggests that the BS compounds occupy a portion of the DIDS binding site close to a positive charge near one of the lysines implicated in anion transport. In order to relate the activity of the sulfonate inhibitors of anion transport to their specific molecular structure, Barzilay et al. [1] relied on the Hansch and the Hammett constants. The Hansch constant, π, is a measure of the octanol/water partition coefficient of each substituent on the benzene sulfonate molecule and can be used to compare, for example, the lipid solubility of the 3-nitro substituent with that of the 4-chloro substituent. The Hammett constant, σ, is a measure of the forward reaction velocity (or the equilibrium constant) of the substituted benzene sulfonic acid as compared to the parent compound. Barzilay et al. [1] measured the ID_{50} of 9 substituted benzene sulfonates and found the log of ID_{50}^{-1} to be linearly related to σ, so that the tightest binding to the anion transport inhibition site corresponded to the highest σ, or the greatest electronegativity. The correlation was somewhat better when a contribution from the Hansch π was included in the computation, but the major factor governing the tightness of binding of the BS compounds, as measured by anion transport inhibition, is the reactivity of the specific substituent on the benzene sulfonate. In phenomenological terms the ID_{50} may be considered as an index of the binding affinity of the *driver* reaction.

BS inhibitors cross the membrane by way of the anion exchange protein and the BS inhibitor flux is inversely correlated with σ in the rat red cell [24]. The log of the flux of BS inhibitors decreases linearly with increased σ, which means that the tighter the inhibitors are bound to their site, the slower they cross the membrane. This is exactly the same behavior that we observed with urea, as shown in Fig. 5, in which the increased affinity of urea binding caused by 3NBS is accompanied by a decrease in V_{max}. As discussed above, we took this observation to mean that the rate of debinding of urea from its site at the entrance to the pore is a rate determining step in urea transport. Similar to DBDS binding, the bimolecular association of the BS inhibitors with band 3 is rapid and not rate-determining, which leads to our suggestion that delay in the debinding process might account for the inverse relationship between BS inhibitor binding affinities and transmembrane BS inhibitor flux observed by Kitagawa et al. [24].

The four compounds we have characterized are too few to reveal any correlation of our $K_{inhib,app}$ values and $K_{stim,app}$ values for urea and water flux with the

Table 3
Values of Hansch and Hammett constants *

Sulfonic acid	Hammett constant (σ)	Hansch constant (π)
3-Nitrobenzene	0.71	−0.28
4-Chlorobenzene	0.23	0.71
Benzene	0	0
4-Hydroxybenzene	−0.37	−0.67
4-Aminobenzene	−0.66	−1.23

* Data from McDaniel and Brown [25] and Leo et al. [26].

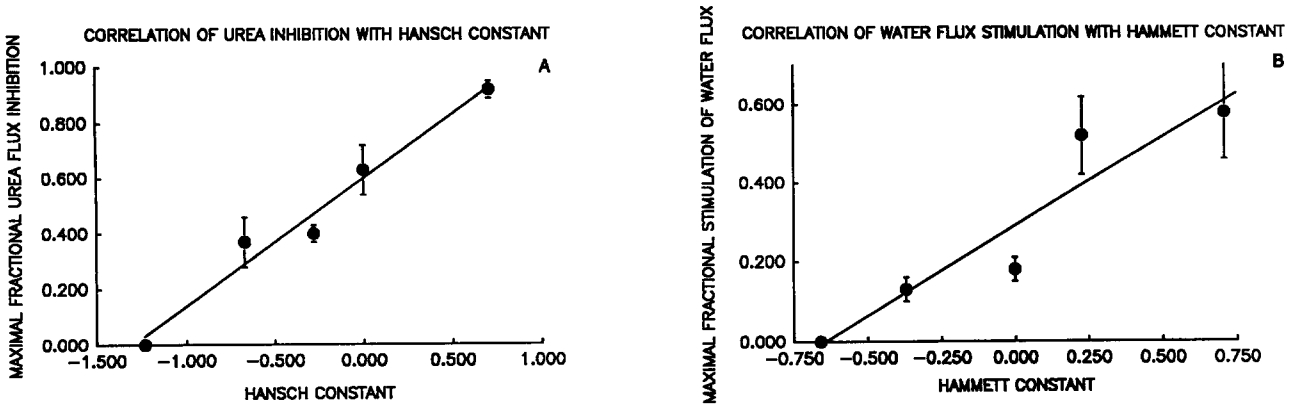

Fig. 14. (A) Correlation of the maximal fractional inhibition of urea flux (Table 1) with the Hansch constant of the substituent (Table 3). $r = 0.99$, $P < 0.01$. (B) Correlation of maximal stimulation (phase one) of water flux (Table 1) with the Hammett constant of the substituent (Table 3). $r = 0.94$, $P < 0.01$.

Hansch and Hammett constants given in Table 3. Correlations are evident, however, with measures of the amplitude of the effects, that is the maximal fractional inhibition (of urea flux) and stimulation (of water flux). Correlations with the amplitudes allow us to include our measurements with sulfanilic acid (4ABS), the member of the BS class with the smallest values of π and σ. Our observation that 4ABS has no detectable effect on either water or urea flux gives an additional point at zero amplitude, which makes it much easier to determine whether the correlations with π and σ are significant. As Fig. 14A shows, the amplitude of urea flux inhibition is significantly correlated with π ($r = 0.99$, $P < 0.01$), suggesting that the greater the lipid solubility of the BS substituent, the tighter urea is bound to its H-bonding site, and the more the flux is inhibited. In the case of water flux stimulation, the important factor is σ, which is significantly correlated with the amplitude of water flux stimulation ($r = 0.94$, $P < 0.01$) as can be seen in Fig. 14B. There appears to be no correlation in the other pairs, that is between σ and the amplitude of the urea flux inhibition, or between π and water flux stimulation.

The classical inhibitor of red cell urea transport is *p*-chloromercuribenzene sulfonate (pCMBS), whose binding to the urea flux inhibition site was characterized by Toon and Solomon [27]. They reported that the aromatic inhibitors bound two orders of magnitude more tightly than $HgCl_2$. Subsequently, Mannuzzu et al. [5] studied the mercurial spin label, pCMBN, whose octanol/water partition coefficient is more than four orders of magnitude greater than pCMBS. pCMBN inhibits red cell urea flux with a rate constant between three and four orders of magnitude greater than pCMBS. Thus, the results that we have obtained on the importance of hydrophobicity in BS inhibition of urea flux are similar to those found with mercurial inhibitors and suggest that the H-bond exchange site is either in, or adjacent to, a hydrophobic environment.

3.7. Site of action of BS compounds on water and urea flux

(i) The driver site

The common element which links urea flux, water flux and anion exchange is the *driver* site, the anion exchange inhibitor site on band 3. This conclusion is based on the experiments showing that covalently bound DIDS completely suppresses the 3NBS effects on urea and water fluxes (Figs. 3 and 13). In the case of urea, the identity of the *driver* site is further supported by the significant correlation between the $K_{inhib,app}$ values for urea flux with the ID_{50} for anion exchange inhibition shown in Fig. 2.

(ii) The effector processes: urea

The diverse actions that follow BS inhibitor binding to the *driver* site reflect very different molecular regulatory processes, possibly located in different regions, or even domains, of the protein and linked allosterically to the *driver* site. As we have discussed, anion transport is presumably regulated by the movement of charged groups in a band 3 channel.

According to our model, urea flux inhibition is effected by conformational changes in the urea H-bond exchange site. The reciprocal plot in Fig. 5 shows that 3NBS inhibition of urea flux causes V_{max} to decrease by the multiplication factor, β, which modifies, k_{tr}, to form the product, βk_{tr}, the rate constant for onward transmission of urea from the complex, site-BS*/urea. Since the rate constant changes, the enzyme conformation, that is, the conformation of the urea binding site, must also have changed. The affinity of this binding site is measured by the urea binding constant, K_m. As the reciprocal plot in Fig. 5 shows, 3NBS inhibition leads to multiplication of K_m by the factor α to form αK_m. The observed decrease in K_m shows that the requisite conformation change has taken place, in accordance with the requirements of our model.

How is urea transport related to the properties of CHIP28 which has been suggested to be the water transport protein in the red cell membrane [6], but is unable to transport urea [28]? If CHIP28 is the sole route of water transport, the relationship between urea flux inhibition and water flux stimulation that we have observed would require CHIP28 to be not only physically associated with, but also to have allosteric connections with, the protein, or proteins, associated with red cell urea flux. A urea transport protein has now been isolated from the rabbit kidney and cloned by You et al. [29] but these investigators were unable to find any related protein responsible for rabbit red cell urea transport.

(iii) The effector processes: water

There is a fundamental linkage between the effect of BS inhibitors on water flux and that on urea flux. As the urea flux goes down, the water flux increases proportionally, as illustrated in Fig. 11. This significant correlation is supported by other numerical correlations in individual experiments, as recounted in the text.

There is an additional step interposed between BS binding to the *driver* site and the water flux stimulation process, because there is a very great difference between the BS binding site affinities, $K_{stim,app}$ and $K_{inhib,app}$ (Table 1). It is possible that the affinities are related, since Fig. 12 shows a linear dependence of $K_{stim,app}$ on the ID_{50}, though the correlation coefficient of 0.93 ($P < 0.1$) is not significant. The simplest way to account for these altered affinities is to suppose that binding to the anion inhibitor site opens up a second, probably adjacent, BS binding site that is responsible for water flux stimulation. In light of Passow's [15] suggestion that BS binds to half of a DIDS site on band 3, a particularly attractive candidate is the other half of that site.

Not only are the effectors for urea flux inhibition and water flux stimulation distinguished by their difference in direction, they are probably located in different regions of the protein or proteins. This conclusion arises from a comparison of Figs. 14A,B: the maximal fractional inhibition of urea flux is correlated with the Hansch constant, π; the maximal fractional stimulation of water flux is correlated with the Hammett constant, σ. If these separate, though possibly adjacent, functional domains are both located on band 3, their diverse properties could be accomodated by the very great lability of band 3, so strongly emphasized by Passow [15].

Acknowledgements

Supported in part by The Council for Tobacco Research – U.S.A., Inc. and by a grant from Dr. Philemon T. Marvell. We should like to express our thanks to Dr. Frank Greco for his essential contributions to the maintenance of the stopped-flow apparatus.

References

[1] Barzilay, M., Ship, S. and Cabantchik, Z.I. (1979) Membr. Biochem. 2, 227–254.
[2] Barzilay, M. and Cabantchik, Z.I. (1979) Membr. Biochem. 2, 255–281.
[3] Brown, P.A., Feinstein, M.B. and Sha'afi, R.I. (1975) Nature 254, 523–525.
[4] Solomon, A.K., Chasan, B., Dix, J.A., Lukacovic, M.F., Toon, M.R. and Verkman, A.S. (1983) Ann. N.Y. Acad. Sci. 414, 97–124.
[5] Mannuzzu, L.M., Moronne, M.M. and Macey, R.F. (1993) J. Membr. Biol. 133, 85–97.
[6] Preston, G.M., Carroll, T.P., Guggino, W.B. and Agre, P. (1992) Science 256, 385–387.
[7] Ship, S., Shami, Y., Breuer, W. and Rothstein, A. (1977) J. Membr. Biol. 33, 311–323.
[8] Lepke, S., Fasold, H., Pring, M. and Passow, H. (1976) J. Membr. Biol. 29, 147–177.
[9] Brahm, J. (1982) J. Gen. Physiol. 79, 791–819.
[10] Toon, M.R. and Solomon, A.K. (1987) J. Membr. Biol. 99, 157–164.
[11] Terwilliger, T.C. and Solomon, A.K. (1981) J. Gen. Physiol. 77, 549–570.
[12] Toon, M.R. and Solomon, A.K. (1991) Biochim. Biophys. Acta 1063, 179–190.
[13] Sha'afi, R.I., Rich, G.T., Mikulecky, D.C. and Solomon, A.K. (1970) J. Gen. Physiol. 55, 427–450.
[14] Cabantchik, Z.I. and Greger, R. (1992) Am. J. Physiol. 262, C803-C827.
[15] Passow, H. (1986) Rev. Physiol. Biochem. Pharmacol. 103, 62–223.
[16] Verkman, A.S., Dix, J.A. and Solomon, A.K. (1983) J. Gen. Physiol. 81, 421–449.
[17] Segel, I.H. (1975) Enzyme Kinetics, pp. 178–192, Wiley-Interscience, New York.
[18] Toon, M.R. and Solomon, A.K. (1990) Biochim. Biophys. Acta 1022, 57–71.
[19] Mayrand, R.R. and Levitt, D.G. (1983) J. Gen. Physiol. 81, 221–237.
[20] Levitt, D.G. and Mlekoday, H.J. (1983) J. Gen. Physiol. 81, 239–253.
[21] Yousef, L.W. and Macey, R.I. (1989) Biochim. Biophys. Acta 984, 281–288.
[22] Karan, D.M. and Macey, R.I. (1990) Biochim. Biophys. Acta 1024, 271–277.
[23] Sha'afi, R.I., Gary-Bobo, C.M. and Solomon, A.K. (1971) J. Gen. Physiol. 58, 238–258.
[24] Kitagawa, S., Terada, H. and Kametani, F. (1982) J. Membr. Biol. 65, 49–54.
[25] McDaniel, D.H. and Brown, H.C. (1958) J. Org. Chem. 23, 420–427.
[26] Leo, A., Hansch, C. and Elkins, D. (1971) Chem. Rev. 71, 525–554.
[27] Toon, M.R. and Solomon, A.K. (1986) Biochim. Biophys. Acta 860, 361–375.
[28] Zeidel, M.L., Ambudkar, S.V., Smith, B.L. and Agre, P. (1992) Biochemistry 31, 7436–7440.
[29] You, G., Smith, C.P., Kanal, Y., Lee, W., Stelzner, M. and Hediger, M.A. (1993) Nature 365, 844–847.