Proton-Sulfate Co-Transport
Mechanism of H^+ and Sulfate Addition to the Chloride Transporter of Human Red Blood Cells

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ABSTRACT Proton and sulfate inhibition of the obligatory chloride-chloride exchange of human erythrocytes was measured at 0°C to determine their mechanism of reaction with the anion transporter. The proton and sulfate that are co-transported by this mechanism at higher temperatures behaved as nontransported inhibitors at 0°C. We analyzed the data in terms of four molecular mechanisms: (1) HSO_4^- addition to the transporter; (2) ordered addition with the proton first; (3) ordered addition with the sulfate first; (4) random addition to the transporter. The Dixon plots of $1/M_{Cl}$ vs. $[SO_4]$ at different proton concentrations were not parallel. Thus protons and sulfate ions were not mutually exclusive inhibitors. The slope of these Dixon plots was independent of pH above 7.0, which indicates that sulfate could bind to the unprotonated carrier and excludes the first two mechanisms. Protons were inhibitors of chloride flux in the absence of sulfate, which indicates that protons could bind to the unloaded carrier and excludes mechanism 3. The K_I for sulfate was 4.35 ± 0.36 mM. The pK for the protonatable group was 5.03 ± 0.02. The binding of either a proton or sulfate to the carrier decreased the K_I of the other by ninefold. The only simple mechanism consistent with the data is a random-ordered mechanism with more transporters loaded with a sulfate than loaded with a proton at the pH and sulfate concentrations of plasma.

INTRODUCTION AND BACKGROUND

Protons and sulfate can be co-transported across the red blood cell in exchange for chloride (44). Jennings found that during chloride/sulfate exchange in a bicarbonate-free system, the extracellular pH became more alkaline as sulfate influx accompanied chloride efflux. This alkalinization of the extracellular medium occurred even against an electrochemical gradient for protons. The ratio of chloride efflux to proton influx from a pH-6.8 medium was indistin-

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guishable from unity. The pH dependence of the exchange was more simply modeled by proton-sulfate co-transport than chloride and hydroxyl exchange for sulfate. Therefore, sulfate and protons appear to be co-transported with a 1:1 stoichiometry. However, from these data one cannot determine whether sulfate and proton ions react separately in a specific or random order or whether an ion pair, HSO_4^-, reacts with the transport site.

Chloride/sulfate exchange, including the proton flux, is consistent with the titratable model of anion transport. This model was originally proposed to explain three sets of observations (27); first, the interactions between the different inorganic anions and a common transporter (see below); second, the saturation of the chloride flux (36); and, third, the pH dependencies of the anion fluxes. As pH values increase from 6 to 8, the chloride flux increases (36). Above pH 8, the chloride flux across resealed red cell ghost membranes is essentially independent of pH (23). On the other hand, as pH values increase from 6.5 to 8, the sulfate flux decreases (55). At pH values below 6.5, the sulfate flux decreases as well (69). A revised titratable carrier model postulated three states for the carrier (29). The transporter that is unprotonated at a certain site can complex with small monovalent anions and rapidly transport them. After a single titration reaction at this certain site, the protonated transporter can complex and slowly transport divalent anions. A second titration reaction at another site inhibits all transport by the transporter. The model predicted that iodide (28) and bromide would have pH profiles similar to chloride and offered an explanation of why phosphate (16) behaved like sulfate. In this model the transporter has one more proton at a certain site when it is a divalent anion transporter than when it is a monovalent anion transporter. The early data on pH effects on sulfate fluxes only indicated that this proton *activated* the sulfate flux. Jennings' data now suggest that the proton that both alters the selectivity of the transporter and activates the sulfate flux may also be the transported proton.

An implicit assumption of the titratable carrier model was that the first proton-binding reaction took place before the divalent anion-binding step (26). However, three other simple mechanisms are also consistent with the published data.

The four possibilities for the order of divalent anion/proton binding to the transporter are: (1) the divalent anion, $X^=$, and the proton, H^+, first bind to each other and then the ion pair, HX^-, binds to the transporter. This possibility is more plausible for phosphate (pK_2 = 7.4, 25°C, zero ionic strength) than for sulfate (pK = 2); (2) an ordered reaction, as implied by the titratable model, with the proton binding before the divalent anion; (3) an ordered reaction with the divalent anion binding to the carrier before the proton. In contrast to the titratable carrier model, the divalent anion enables the transporter to bind a proton; (4) a random-ordered reaction for proton and divalent anion binding to the transporter. Thus either the divalent anion or the proton can bind first, followed by the other.

Most enzyme systems have two or more substrates and products (including H_2O). The order of addition of these substrates and the order of their release

as products are important characteristics used to classify enzyme mechanisms (reviewed in texts 7, 22, 73). In contrast, the order of addition for co-transport mechanisms has rarely been studied (10–12, 65). Even though co-transport systems are found in most cell membranes and are important in biology, they are difficult to quantitate. The usual observation that the transport of x requires the specific presence of y only proves that y activates the transport of x; it does not imply that y is also transported by the same system.

Two difficulties prevented us from determining the order of sulfate/proton binding in red cells from direct measurements of the sulfate and proton concentration dependence of sulfate flux. First, net sulfate transport is a significant portion (at least 20%) of the sulfate tracer exchange (49). This net transport has an unknown pH dependence and would change internal and, to a lesser extent, external sulfate concentrations during measurements. Second, a more fundamental difficulty is that both the inside and the outside order of sulfate binding will influence the proton and sulfate concentration dependence of the sulfate flux. Because there are no *a priori* constraints that require the sequence of binding to be the same on the inward- and outward-facing sites, there would be more possibilities than could be distinguished uniquely by these experiments.

Therefore, we have determined the order of sulfate/proton binding at the external surface by measuring the inhibition of chloride transport. We have thereby avoided the above difficulties and have exploited three fundamental characteristics of anion transport in red blood cells. First, all transported inorganic anions appear to share the same transporter (32). The evidence that sulfate and chloride share the same transporter is extensive and includes the following observations. Sulfate is a competitive inhibitor of chloride fluxes and chloride is a competitive inhibitor of sulfate fluxes (34, 69). When studied under identical conditions of temperature and pH, a given concentration of a reversible inhibitor produces the same percent inhibition of sulfate or chloride transport (51). The number of binding sites per erythrocyte that must be complexed with a stilbene to completely inhibit sulfate flux is about the same as that required to completely inhibit chloride flux, namely, 10^6 (21, 24, 63, 74).[1] A model with a shared transporter for sulfate and chloride and a single site with alternating access to the two solutions correctly predicts conformational recruitment assayed by sulfate transport in the presence of a chloride gradient (46). The second characteristic we have exploited is that even though sulfate and chloride share the same transporter, their transport rates are very different. Chloride exchange is 10^4 times faster than sulfate exchange (15, 51, 81). Thus at 0°C, chloride exchange has a half-time of ~10 s (36), whereas sulfate exchange has a half-time of 16 d (79). The third characteristic we have exploited is that chloride is primarily transported by a nonconducting pathway (30, 48, 66, 77). It is virtually an obligatory one-for-one exchange (42, 43, 49, 53). Less than 0.01% of the tracer chloride exchange

[1] The slight differences reported by different laboratories are probably due to the variation of sites per cell from different donors (21).

rate reflects potential net chloride ion permeability (43). Because of these characteristics we can evaluate proton and sulfate binding to the transport site by measuring the change in chloride flux.

The overall exchange of halides we believe is better described by a single-site, alternating-access mechanism with ping-pong kinetics (25, 33) than by a two-site simultaneous (sequential) exchange for halide concentrations less than those required to observe substrate inhibition (67, 68). The major conclusions of this paper do not require unequivocal resolution of which of these two models is correct. Our quantitative analysis, however, will be in terms of a ping-pong mechanism.

This paper presents data that rule out three of four possible mechanisms for sulfate-proton to the external transport site. We show that (*a*) there is no appreciable inhibition of chloride fluxes by HSO_4^-; (*b*) sulfate alone can inhibit chloride fluxes and thus sulfate may bind first; and (*c*) protons alone can inhibit chloride fluxes and thus protons may bind first. Therefore the mechanism of proton/sulfate addition to the carrier is a random-ordered reaction pathway. We also show that the binding of either ligand increases the apparent affinity of the transporter for the other ligand by ninefold.

A preliminary account of this work has been previously presented (59).

METHODS

Preparation of Cells

Fresh, heparinized whole blood from a normal adult human was centrifuged at 12,000 *g* for 10 min (Sorvall RC-5, Dupont Instruments-Sorvall Biomedical Div., Newtown, Conn.) After aspirating the plasma and buffy coat we washed the cells twice in a 165-mM KCl solution to remove most of the bicarbonate. Then we washed the cells three times in a 150-mM KCl, 27-mM glycylglycine medium titrated to pH 7.8 at 0°C. This wash medium as well as all the efflux media that had a pH >6 were bubbled for 1 h with nitrogen that had been passed through an alkaline trap to remove most of the residual bicarbonate and CO_2.

Homoexchange Flux of Chloride

The nonequilibrium exchange of intracellular radioactive chloride for extracellular chloride was measured using previously published techniques (33). ^{36}Cl (0.6 μCi/ml suspension) was added to the final washed 30% (vol/vol) suspension of cells and allowed to equilibrate across the cell membrane for 5 min at 0°C (>25 × $t_{1/2}$). These cells were then packed in nylon tubes in a centrifuge (Sorvall RC-5, SS-34) at 17,000 *g* for 12 min. These tubes were cut with a razor blade and the packed cells and supernate were saved separately on ice. Duplicate samples of packed cells were used to determine the ratio (wt/wt) of cell water to cell solids, d_{corr}, and the ratio of intracellular ^{36}Cl to extracellular ^{36}Cl concentration, r^*_{Cl}, including the correction for the 2.7% trapped extracellular space. These parameters were nearly constant in all experiments since the cells were washed and packed under identical conditions. The efflux coefficient was measured by injecting the cells into a well-stirred solution thermostated at 0°C. At known times, a sample $s(t)$ of the supernate was taken using the rapid filtration technique of Dalmark and Wieth (14). A sample of the suspension, $s(T)$, was set aside for at least 1 h at room temperature. A least-squares fit of the linear equation, $ln[1 - a(t)/a(T)] = bt + y_o$ was made to determine the initial rate

coefficient, b, where $a(t)$ is the average of the counts of duplicate samples of $s(t)$; and $a(T)$ is the average counts of the duplicate samples from the supernate of $s(T)$. The error caused by using $a(T)$ instead of $a(\infty)$, the equilibrium counts for pure exchange, is at most a few percent (33).

The fluxes were calculated by using the equation

$$M = b \times \mathrm{Cl}_i \times d_{\mathrm{corr}} \quad (1)$$

where Cl_i was determined from the product of r^*_{Cl} and $\mathrm{Cl}_{\mathrm{out}}$ when the cells were packed. Eq. 1 is a very good approximation of the formally correct equation

$$M = b' S_1 S_2/(S_1 + S_2)$$

where b' is the rate coefficient determined using $a(\infty)$ instead of $a(T)$, and S_1 and S_2 represent the respective intracellular and extracellular amounts of chloride.

Proton and Sulfate Inhibition of M_{Cl}

In most of the experiments described in this paper we measured ^{36}Cl efflux from human red blood cells. In each set of experiments the internal chloride concentration was 110 mmol/l cell water, the internal proton concentration was $r^{*-1}_{\mathrm{Cl}}\ 10^{-7.8}$ mol/l cell water, and the external chloride concentration was 6.6 mM. To assess the order of proton and sulfate binding, the external sulfate and proton concentrations were varied. Potassium sulfate was isosmotically substituted for potassium gluconate. Thus the media consisted of 6.6 mM KCl, 27 mM glycylglycine, y mM K_2SO_4 and $(144 - 1.13y)$ mM K^+-gluconate. The final osmolarity was 0.300. Because the highest sulfate concentration used was <20 mM, the ionic strength varied from 0.15 to 0.17 M. Fresh gluconic acid, made from passing a potassium gluconate solution through a Dowex cation exchange column, or KOH was used to titrate the solutions.

In preliminary experiments we added KOH to a "bicarbonate-free" cell suspension and measured the rate of movement of proton equivalents as the system returned to equilibrium. Even when the intracellular and extracellular concentrations differed by two and three orders of magnitude the proton flux was only 0.1–0.5 mmol/(kg cell solid·min), which is <0.1% of the chloride flux under these conditions. Both the linearity of our Dixon plots (Figs. 4 and 5) and the agreement of our proton flux values with those of Jennings (44, 45) are consistent with the idea that protons may be treated as nontransported inhibitors of chloride efflux under these conditions. These low proton flux values also indicate that the inhibition we observed at low values of $\mathrm{pH}_{\mathrm{out}}$ was not due to a decrease in internal pH. Decreasing the internal pH from 7.8 to 6.8 will only inhibit the chloride flux by 20% (34). Direct measurements of packed cell lysates demonstrated that the internal pH changed by no more than 0.2 pH units in 15 s at 0°C. Thus only 2–3% inhibition could be expected. We therefore feel justified in treating protons as nontransported inhibitors of chloride flux and in assuming that any effect on the chloride flux of changing the external pH was not due to subsequent changes in internal pH.

Theory

We will consider only the *outside* reaction of protons (H) and sulfate (S) with the anion transporter because protons and sulfate are transported only very slowly at 0°C ($t_{1/2} > 1$ d) (79; and Milanick and Gunn, unpublished observations).

Fig. 1 is a schematic representation of the four possible mechanisms by which external protons and sulfate could competitively inhibit the exchange of chloride on a transport system with ping-pong kinetics.

Table I provides a quantitative comparison of the four possible cases.[2] Each column represents a different case: column 1 is the case where the ion pair HSO_4^-, binds; column 2 is the case where the proton binds first; column 3 is the case where the sulfate binds first; and column 4 is the case where either sulfate or proton can bind to the carrier first, followed by the other.[3] The velocity equations of Cl efflux, M, for each case, derived using the King-Altman algorithm, are given in the first line of

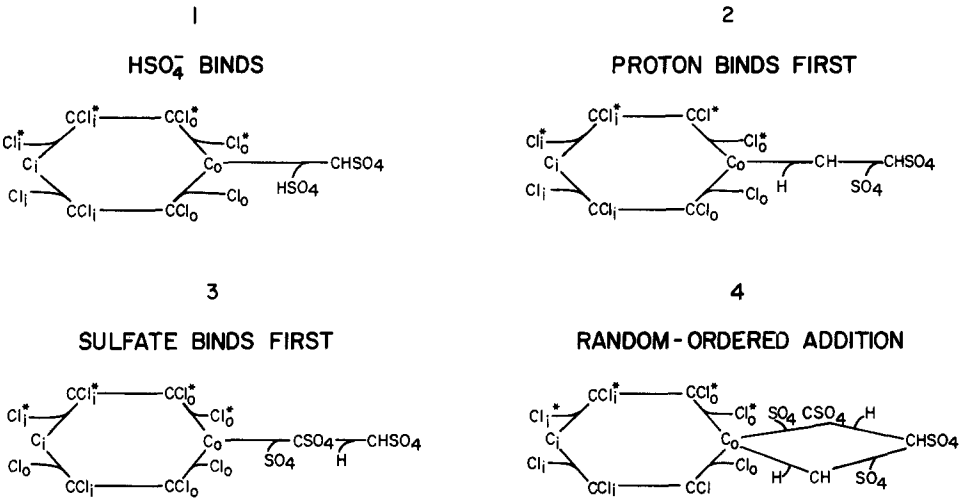

FIGURE 1. Four possible competitive inhibition schemes for proton and sulfate binding to the externally facing site of a transporter that transports chloride in a ping-pong manner. Cl* and Cl are tracer and nontracer chloride ions. Subscripts 0 and i refer to outside (extracellular) and inside (intracellular) concentrations. C is the molecular entity responsible for the rapid obligatory exchange of chloride (carrier). SO_4 is the sulfate anion and HSO_4^- is the ion pair formed by associated of sulfate and one proton ($pK = 2$). Case 1: only the performed ion pair, HSO_4^- binds to the empty transporter, C_o, with a dissociation/inhibition constant K_I. Case 2: an ordered reaction of a proton and then a sulfate anion to the empty transporter. K_H is the inhibition constant for the proton. Case 3: an ordered addition of sulfate anion and then a proton to the empty transporter C_o. K_S is the inhibition constant for sulfate. Case 4: either a sulfate anion or a proton can bind to the empty transporter. The other inhibitor can then bind with an altered inhibition constant, either αK_H or αK_S.

[2] The symbols used are: V_m, the maximum flux; Cl, the chloride concentration; $K_{1/2}$, the Michaelis constant for the concentration of substrate causing half-maximum flux in the absence of inhibitors and when all other substrates are at saturating concentrations, here for outside chloride denoted as K_o and for inside chloride as K_i (36); K_I, the inhibition constant for inhibitor, I; and, m, the ion flux.

[3] A comment on what we mean by the term "ordered reaction" is necessary here. (This comment is based on the insight of Hearon et al. [38].) When one considers a bisubstrate reaction with a catalyst, thermodynamic considerations prove that each pathway of the reaction has the same overall equilibrium constant; thus every such reaction has a random order, i.e., it proceeds along all of the pathways and a finite amount of each of the intermediates (enzyme-one-substrate complex) is formed. From a kinetic viewpoint, one of the pathways may make a negligible contribution to the rate of product formation so that the reaction appears to have a

Table I.[4] Because it is easiest to think in linear terms, the equations are written in reciprocal form. The rest of Table I can be considered in two analogous sections. In the first section are the parameters of the Dixon plots $1/M$ vs. $[SO_4]$ and in the second section are the parameters of the Dixon plots $1/M$ vs. [H]. Within each section, the particular parameters of the Dixon plot or replot are shown on separate lines.

TABLE I

	HSO_4 binds	H binds first	S binds first	Random order addition
1) Reciprocal velocity equations: $(1/M)$ $-\frac{1}{V_m} + \frac{K_i}{Cl_i V_m} + \frac{K_o}{V_m Cl_o} =$	$\frac{K_o(H)(S)}{V_m Cl_o K_I'}$	$\frac{K_o}{V_m Cl_o} \cdot \left[\frac{H}{K_H}\left(1 + \frac{S}{K_S}\right)\right]$	$\frac{K_o}{V_m Cl_o} \cdot \left[\frac{S}{K_S}\left(1 + \frac{H}{K_H}\right)\right]$	$\frac{K_o}{V_m Cl_o} \cdot \left[\frac{S}{K_S} + \frac{H}{K_H} +$
Dixon Plot $1/M$ vs. SO_4				
2) Slope $\times \frac{V_m Cl_o}{K_o}$	$\frac{H}{K_I'}$	$\frac{H}{K_H K_S}$	$\frac{1}{K_S}\left(1 + \frac{H}{K_H}\right)$	$\frac{1}{K_S}\left(1 + \frac{H}{\alpha K_H}\right.$
3) Replot of slope of $(1/M$ vs. S) vs. H has y-intercept	0	0	$\frac{K_o}{V_m Cl_o}\frac{1}{K_S}$	$\frac{K_o}{V_m Cl_o}\frac{1}{K_S}$
4) Replot of slope of $(1/M$ vs. S) vs. H has x-intercept	0	0	$-K_H$	$-\alpha K_H$
Dixon Plot $1/M$ vs. H				
5) Slope $\times \frac{V_m Cl_o}{K_o}$	$\frac{S}{K_I'}$	$\frac{1}{K_H}\left(1 + \frac{S}{K_S}\right)$	$\frac{S}{K_S K_H}$	$\left(1 + \frac{S}{\alpha K_S}\right)\frac{1}{K}$
6) Replot of slope of $(1/M$ vs. H) vs. S has y-intercept	0	$\frac{K_o}{V_m Cl_o}\frac{1}{K_H}$	0	$\frac{K_o}{V_m Cl_o}\frac{1}{K_H}$
7) Replot of slope of $(1/M$ vs. H) vs. S has x-intercept	0	$-K_S$	0	$-\alpha K_S$

The parameters of the Dixon plot and the replot of its slopes against the second inhibitor for the four competitive inhibition schemes as shown in Fig. 1. Note that the y-intercept of the replot shown in line 3 differentiates between two pairs of models (1, 2 vs. 3, 4), depending upon whether the value is zero or nonzero. Similarly, the replot in line 6 differentiates between two other pairs of models (1, 3 vs. 2, 4). See the Theory section for more details. $K_I' = K_{eq}^{HSO_4} \times K_I$.

particular or preferred order. Also, the amounts of some intermediates may be negligible. Nevertheless, some small portion of the products comes from these other pathways because of finite fluctuations in the conformational and energy states of the molecules consistent with the thermodynamic viewpoint. If both pathways make a substantial contribution to the rate of product formation, then the reaction appears to have no preferred order, i.e., it appears to be random. As an alternative, one can say that the reaction has a random order if each of the intermediates is present in significant concentrations. One can alter the contributions of each pathway and the concentrations of the intermediates by changing the substrate concentrations. To differentiate ordered from random, one must consider the contributions from each pathway at saturating substrate concentrations.

[4] The velocity equations for the first three cases in Table I are derived from the more general assumption of steady state for each intermediary complex. The random-order velocity equation was derived assuming that the proton-binding steps and the sulfate-binding steps equilibrate

The possibilities are distinguished by whether the value of the ordinate intercept of the replot is zero or nonzero. Thus the replot of the slope of ($1/M$ vs. [S]) vs. [H] eliminates two mechanisms (line 3 of Table I) and the replot of the slope of ($1/M$ vs. [H]) vs. [S] (line 6 of Table I) will eliminate one of the remaining two, leaving only one mechanism consistent with the data.

Calculation of Kinetic Parameters

We have graphed the chloride fluxes that are inhibited by sulfate or protons as Dixon plots and replots from the Dixon plots. These plots are easier to inspect visually than the nonreciprocal, nonlinear plots. As has been pointed out by others (see especially Wilkinson [83] and Cleland [5]) the linear Dixon plots and replots from the Dixon plots, if evaluated by a linear least-squares fit, incorrectly weight the data points. Consequently, we have fitted the Dixon plot data to the nonlinear equation. Initial estimates of the parameters were obtained by a weighted ($1/M^4$) least-squares fit to the linear plot, $1/M$ vs. I. An iterative procedure was then used to refine the parameters by fitting the Gauss-Newton approximation of the nonlinear equation. Student's t test was used to determine confidence limits. We used a linear least-squares program to fit the replots with each value weighted by the reciprocal of its variance. We have also fit all the data to the original nonlinear equation (Table I, line 1, column 4) by use of the Gauss-Newton iterative procedure. The nonlinear replot procedure and the direct nonlinear procedure gave results that were not significantly different.

Determination of Kinetic Parameters for the Random-Ordered Case

The random-ordered mechanism is characterized by three parameters, the inhibition constants K_S and K_H for each of the inhibitors, and the interaction factor α. αK_S is the inhibition constant for sulfate binding to the protonated carrier; αK_H is similarly defined as the inhibition constant for proton binding to the sulfate-loaded carrier. Values for V_{max} (1,236 mmol Cl/[kg cell solids·min]), K_o (6.3 mM), and K_i (60 mM) were obtained in separate experiments and were similar to values reported previously (33). Chloride fluxes have substrate inhibition at high external chloride concentrations; the above values were obtained from experiments with $[Cl]_{out}$ <30 mM. K_S was determined from the ordinate intercept of the replot slope vs. [H] (line 3, column 4, Table I). Similarly, K_H was determined from the ordinate intercept value of the replot, slope vs. [S] (line 6, column 4, Table I).

The equation for the random-ordered mechanism (see line 5, column 4, Table I) is:

$$V_m Cl_o\ (\text{slope}_H)/K_o = (1 + S/\alpha K_S)/K_H \qquad (1)$$

where slope_H is the slope of the Dixon plot $1/M$ vs. [H]. By plotting log ($V_m Cl_o$ (slope_H)/K_o) vs. log ($1 + S/\alpha K_S$) one can read the pK (= $-\log K_H$) of the titratable group directly from the ordinate intercept of the graph.

If K_S is known, it is possible to calculate α from the abscissa-intercept of (slope_H)

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rapidly. The more general steady-state analysis of this case would introduce terms quadratic in H and S. Though all the data values are fitted by linear Dixon plots, i.e., they are fit by first-power terms in H and S, that does not imply that the rapid equilibrium assumption is correct (8). Nevertheless, as far as determining the order of binding is concerned, introducing terms in H^2 and S^2 into the random-order equation, column 4, Table I, would have no significant effect on the analysis. That is, the analysis is only dependent upon whether the replot of the slopes from the Dixon plots has a zero or nonzero intercept; terms in H^2 or S^2 will not change that distinction.

vs. [S] (line 7, column 4, Table I). Similarly, of course, one can calculate α from the abscissa intercept of (slope$_S$) vs. [H] when one knows K_H (line 4, column 4, Table I).

The Symbols H and S for Protons and Sulfate

The reactions involving protons that are described in this paper probably involved the hydrated proton H_3O^+. Despite this, we have used the less precise term "proton" to mean this species and have always written it as H^+ or H, although this is not strictly correct. A similar simplification has been often unknowingly used by biologists with regard to sulfate. Since sulfate readily forms complexes with monovalent cations in water, there is always a significant concentration of HSO_4^-, KSO_4^-, and $NaSO_4^-$ in solutions of K^+ or Na^+, depending on the pH and ionic strength and other cation concentrations. We have used the terms sulfate and sulfate anion, and the symbols S or SO_4 to denote the total $SO_4^=$, KSO_4^-, and $NaSO_4^-$ concentration. Our experiments do not attempt to distinguish between them. Therefore the simply sketched reaction of the transporter, *C*, with a proton and a sulfate moiety may be more complex than indicated in Fig. 1.

RESULTS

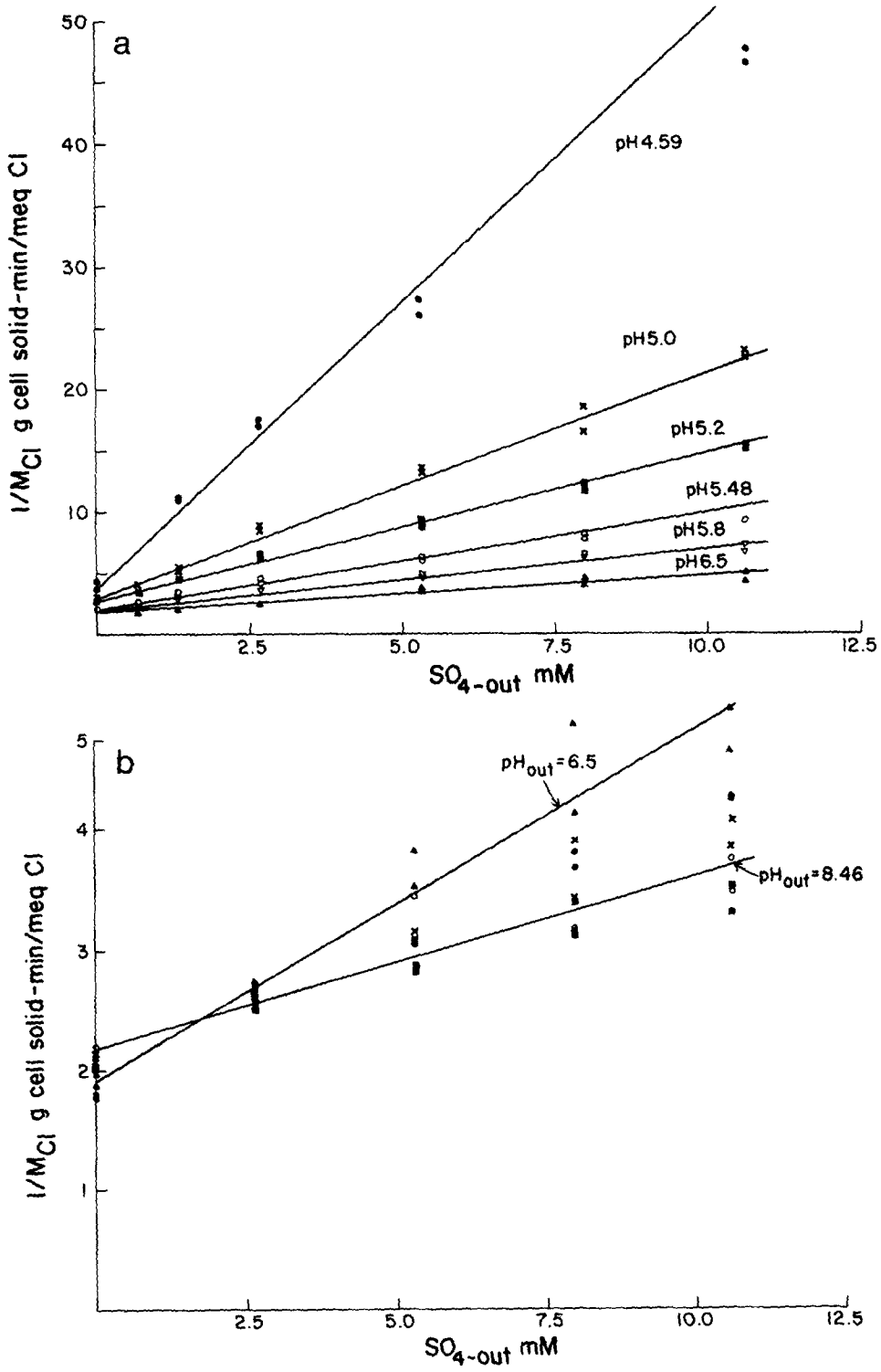
Spectator Anion and Buffer

We have designed the inhibition experiments such that the changes in the chloride efflux are due solely to the interactions of chloride, proton, sulfate, and the anion transporter on the external face of the cell. Most of our experiments were performed at physiological ionic strength (150 mM) and so required a spectator anion to substitute for sulfate when its concentration was changed. Our experience has been that at pH 7.8 citrate is the best choice in the sense that it does not inhibit chloride fluxes, but at lower pH values citrate increasingly inhibits anion transport (33). This presumably occurs because of the decreased net charge on citrate as it is protonated at low pH (pK = 6.2). All simple inorganic anions that we have tested inhibit chloride transport, e.g., Br^-, I^-, $H_2PO_4^-$, $SO_4^=$, $SO_3^=$, and $CrO_4^=$. When we tested several organic anions, acetate, para-aminohippurate, and pyromellitate, they also inhibited chloride fluxes. Wieth (80) and ourselves have found that gluconate may be a weak inhibitor above pH 8.5 (K_I > 150 mM). However, below pH 7.5, chloride efflux into a medium with 3 mM chloride has the same value whether sucrose or gluconate is the major osmotic component. In both solutions the flux is pH independent between 6.0 and 7.5. This suggests that any slight gluconate inhibition is constant over this pH range. Thus gluconate should not interfere with the measurement of the pH dependence of sulfate inhibition of chloride efflux.

At pH 8.5 near the pK of glycylglycine, 210 mM glycylglycine has no effect on chloride efflux into a chloride medium as compared with an isotonic isoionic citrate/sucrose medium. Therefore our use of 27 mM glycylglycine as a buffer and osmotic equivalent of intracellular hemoglobin does not interfere with the flux measurements or the interpretation of them.

Sulfate Inhibition

We examined the pH dependence of sulfate inhibition of chloride efflux. Fig. 2 is a Dixon plot, $1/M_{Cl}$ vs. [SO_4], of a typical series of experiments that


a
pH4.59
pH5.0
pH5.2
pH5.48
pH5.8
pH6.5
1/MCl g cell solid-min/meq Cl
SO4-out mM
b
pHout=6.5
pHout=8.46
1/MCl g cell solid-min/meq Cl
SO4-out mM

illustrates two important points. First, in Fig. 2a at low pH values (high proton concentrations), protons assisted sulfate inhibition of chloride flux and increased $1/M_{Cl}$. This was not due solely to a separate and additional inhibition by protons because the lines are not parallel (73). If H and S were mutually exclusive inhibitors, there would be no term in the flux equation that included both [H] and [S] as factors. In that case, the slope of $1/M$ vs. S could not be dependent upon H and would be the same for all values of [H]. Since the experimentally observed slope does depend upon [H], protons and sulfate are not mutually exclusive (73). Second, at pH values above 7.0 (Fig. 2b) the sulfate inhibition was independent of proton concentration. This shows that sulfate did not require a proton bound either to itself (as HSO_4^-, case 1) or to the carrier (as CH^+, case 2) in order to inhibit chloride flux.

A replot of the slopes of the lines in Fig. 2 as a function of sulfate concentration is shown in Fig. 3. From the intercept with the ordinate we determined the value for K_S: 4.35 ± 0.36 mM.

Proton Inhibition

We measured the sulfate concentration dependence of proton inhibition of chloride flux in order to distinguish the remaining two possibilities. These are the sulfate-binds-first mechanism and the random-ordered mechanism. The independent variable in Fig. 4 is the proton concentration. The reciprocal of the flux is a linear function of the proton concentration at each fixed sulfate concentration (see also Fig. 5). Any appreciable proton-chloride co-transport could make these Dixon plots nonlinear, because proton-chloride co-transport would be described by an equation that includes proton concentration terms in both numerator and denominator, e.g.,

$$M = [V_m(Cl) + W_m(H)(Cl)]/[K_{1/2} + Cl + A(H) + B(H)(Cl)].$$

The linearity is therefore additional support for the assumption that protons may be treated as nontransported inhibitors under these experimental condi-

FIGURE 2. Typical Dixon plots of sulfate inhibition of chloride flux at different external pH values. It can be seen that the slopes decrease with the proton concentration and approach a limiting value at pH values >7.0. For each type of efflux medium, duplicate flux measurements were made from the same set of packed cells that had an internal chloride content of 110 meq/(kg intracellular water) and an internal proton concentration of $(r^*_{Cl})^{-1}\ 10^{-7.8} = 10^{-7.6}$. The external chloride concentration was 6.6 mM. The best nonlinear fit to the data generates the following Dixon lines: $1/M_{Cl} = a[SO_4] + b$.

Symbol	pH_{out}	a	b
●	4.59	4.90±0.02	4.00±0.04
×	5.0	1.79±0.02	2.94±0.04
■	5.2	1.22±0.02	2.72±0.04
○	5.48	0.823±0.011	1.96±0.03
▽	5.8	0.550±0.012	1.86±0.05
▲	6.5	0.292±0.007	1.90±0.05
●	6.96	0.238±0.004	1.88±0.04
×	7.51	0.183±0.003	2.12±0.05
■	7.98	0.140±0.003	2.07±0.06
○	8.46	0.145±0.006	2.18±0.11

tions; that is, protons are not partial (hyperbolic) inhibitors of chloride fluxes under these conditions (73). It can be seen again in Fig. 4, as in Fig. 2, that the lines are not parallel and therefore H^+ and sulfate were not mutually exclusive inhibitors.

Fig. 5 is a Dixon plot, $1/M_{Cl}$ vs. [H], of a separate experiment in the complete absence of sulfate. It is evident that protons alone were linear inhibitors of chloride fluxes ($P < 0.001$).

Fig. 6 is a graph of the slopes of the Dixon plots $1/M_{Cl}$ vs. [H], from Fig. 4, replotted vs. [SO_4]. It can be seen that the value of the ordinate intercept of the line extrapolated from the nonzero sulfate concentration points agrees with the value measured at [SO_4] = 0. We conclude that sulfate assists the inhibition of M_{Cl} in conjunction with the same proton that alone inhibits

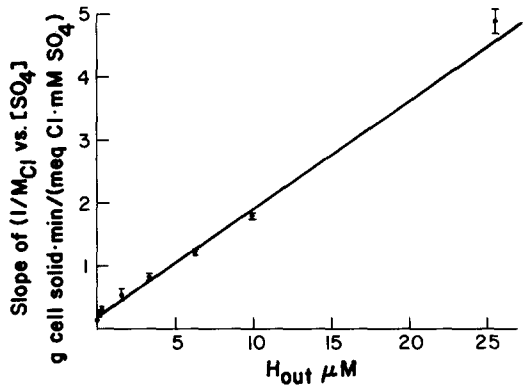


FIGURE 3. The slopes of $1/M_{Cl}$ vs. [SO_4] from Fig. 2 are replotted vs. [H].The y-intercept of this plot is clearly different from zero ($P < 0.005$). This shows that sulfate would inhibit in the absence of protons. This excludes the mechanisms in which HSO_4^- binds and in which the proton binds first. The best nonlinear fit is: slope = 0.172 ± 0.011 [H] + 0.178 ± 0.002. The inhibition constant for sulfate is K_S = 4.35 ± 0.36 mM.

chloride flux. The sulfate-binds-first mechanism is not consistent with the data presented in Figs. 4–6 because protons inhibited when sulfate was absent. In conclusion, the only mechanism consistent with the data is the random-ordered mechanism.

Fig. 7 is a log-log plot of normalized slopes ($slope_H$) versus a function of sulfate concentration. The apparent pK for the reaction of protons with the inhibitory group is given by the ordinate intercept (see Theory). The pK was 5.03 ± 0.02.

The values for the inhibition constants and the abscissa intercepts of the replots (Figs. 3 and 6) were used to calculate the interaction factor, α: α = (abscissa intercept of Fig. 6)/K_S = 0.111 ± 0.011 and α = (abscissa intercept of Fig. 3)/K_H = 0.108 ± 0.005. When all data were directly fit to the random-ordered nonlinear equation, we obtained the following parameters: K_H = 9.4 ± 0.9 × 10^{-6} M, K_S = 3.88 ± 0.22 mM, and α = 0.116 ± 0.01. These values

are not significantly different from the values taken from the separate graphs. In addition, each value is significantly different from zero. Again this implies a random-ordered model and excludes the other three models shown in Fig. 1.

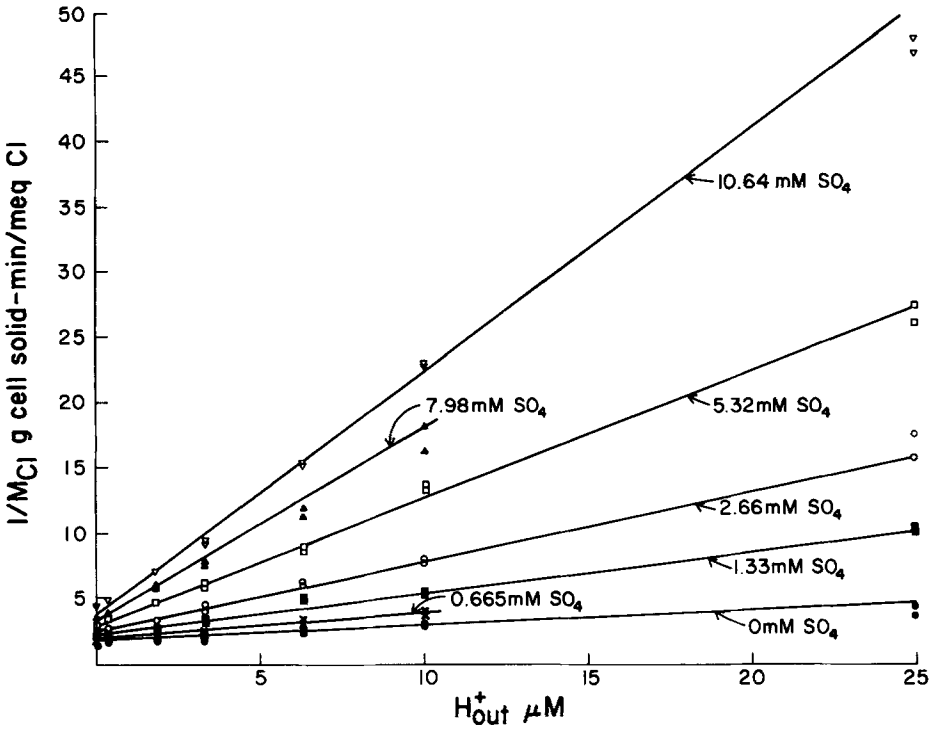


FIGURE 4. Dixon plot of proton inhibition of chloride flux at different external sulfate concentrations. This experiment is the same as the one plotted in Fig. 2. The best nonlinear fits to the data generate the following Dixon lines: $1/M_{Cl} = c[H] + d$:

SO_4	c	d
	mM	
0.0	0.082±0.004	1.94±0.09
0.665	0.20±0.001	1.98±0.07
1.33	0.33±0.001	2.16±0.056
2.66	0.50±0.001	2.55±0.02
5.32	0.99±0.02	3.08±0.06
7.98	1.51±0.04	3.38±0.09
10.64	1.93±0.05	3.75±0.10

DISCUSSION

This paper reports the first demonstration of which intermediates exist on the pathway by which two proven co-transported molecules, H^+ and $SO_4^=$, can react with their transporter in animal cell membranes. It also is the first report to quantitate the change in apparent affinity of one transported ion as a result

of the binding of the other co-transported ion. The proton-sulfate co-transport in red cells is probably not a unique coupling of ion flows in these membranes, since the anion exchange system can also carry Na^+ and $CO_3^=$, Li^+ and $CO_3^=$, and probably other combinations of monovalent cations and divalent anions (18, 24, 79). Co-transport mechanisms are generally the primary way in which the gradients of actively transported molecules are used to effect secondary active transport of molecules without a direct energy source. Although this importance of co-transport systems is well recognized, the detailed kinetics of how such a system operates are still unknown.

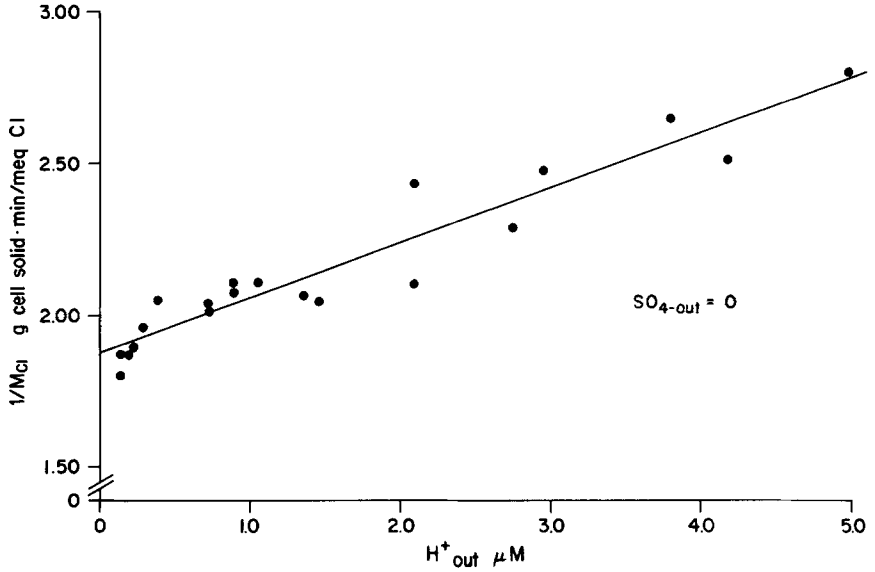


FIGURE 5. Dixon plot of proton inhibition of chloride fluxes with no sulfate present in the external medium. Because the slope of this line is nonzero ($P < 0.001$), protons are inhibitors of chloride flux in the absence of sulfate. These intact cells had an internal chloride content of 110 meq/(kg intracellular water) and internal proton concentrations of $(r^*_{Cl})^{-1}$ $10^{-7.8}$. The external [Cl] = 6.6 mM. The best nonlinear fit generates the Dixon line: $1/M_{Cl} = (1.9 \pm 0.4) + (0.16 \pm 0.02)$ [H].

Other Co-Transport Systems

Some recent reviews of the work establishing co-transport and measuring stoichiometries in different systems include: Crane (9), Schultz (70), Gunn (31), and Lever (57). Some potassium co-transport systems of human red cells (66) appear to require chloride (17, 54), at least as an activator. Epithelial tissues have been shown to have several Na co-transport mechanisms: Na/Cl (20), Na/amino acid (13, 58), Na/sugar (8, 71), Na/phosphate (41), and Na/sulfate (76). Na/amino acid and Na/sugar co-transport have also been studied in some mammalian tumor cell lines (6, 39, 56, 57, 62, 65). Even in some of the best studied of these systems there is controversy over the exact stoichiometry (47). Proton gradients, rather than sodium gradients, seem to supply

much of the driving force for nutrient transport in lower organisms (19, 40, 50, 72, 75, 78).

Serious attempts have been made in two putative co-transport systems in microorganisms to determine the order of substrate binding. Cuppoletti and Segal (10–12) have studied sulfate transport in *Penicillium notatum.* They have

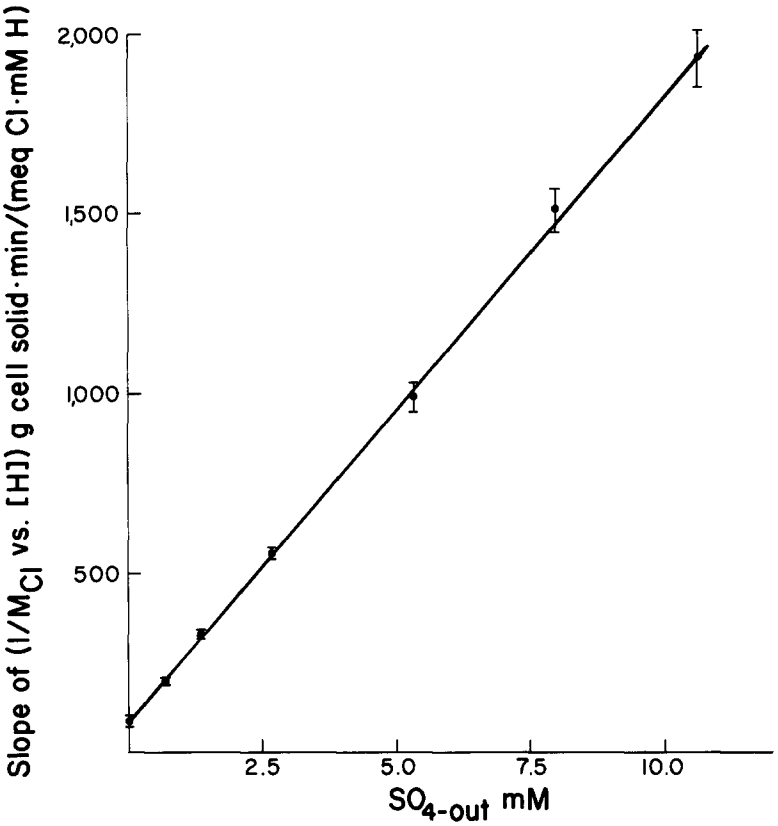


FIGURE 6. The slopes of $1/M_{Cl}$ vs. [H] from Fig. 4 are replotted vs. [SO_4]. The ordinate intercept of the line through the values for experiments with nonzero sulfate concentrations has the same value as the value obtained at zero sulfate concentration. Thus the proton that inhibits chloride fluxes in the absence of sulfate is the proton whose inhibition of M_{Cl} is assisted by sulfate. The parameters of the weighted nonlinear best fit generate the equation: $\text{slope}_H = (175 \pm 20)$ [SO_4] + (83.4 ± 4.0).

determined the order of binding of the three activators/substrates of this system, H, Ca, and SO_4. Unfortunately, their data do not rule out the possibility that calcium ions and protons were merely activators and not transported by the system. Roomans et al. (65) have studied proton/sulfate uptake by the yeast *Saccharomyces cerevisiae.* Their kinetic data are consistent with a kinetic model in which three protons bind before sulfate ions but they have not yet ruled out alternative models of binding order.

Ion Pair Formation and Transport: HSO_4^-

The co-transport of protons and sulfate may be due to the separate addition of the two substrates to the carrier. Alternatively, it may be due to the addition of the preformed ion pair HSO_4^-, which results from the reaction $H^+ + SO_4^= \leftrightarrows HSO_4^-$ ($pK = 2$ at 25°C). Both addition pathways are consistent with the pH dependence of sulfate fluxes between pH 8 and 6.5. Below pH 6.5, both require a separate inactivating titration reaction to explain the decreased flux. Although two previous observations suggested that ion pairs were un-

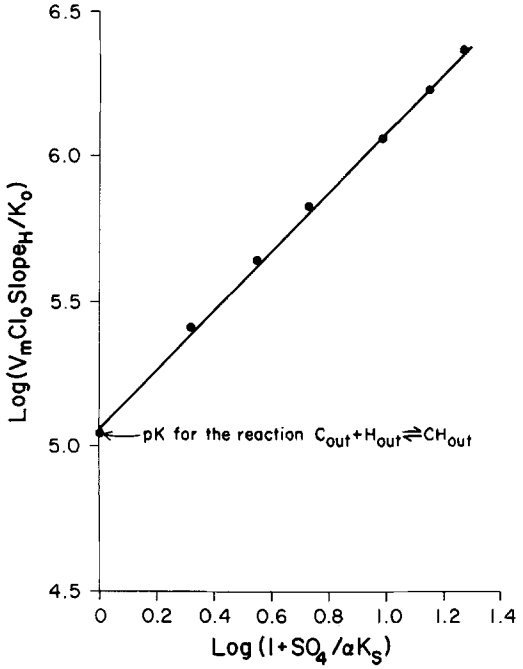


FIGURE 7. The log of the normalized slopes of $1/M_{Cl}$ vs. [H] from Fig. 4 are plotted vs. log $(1 + [SO_4]/\alpha K_S)$ $(=x)$ corresponding to the equation: log $(V_m Cl_o$ $slope_H/K_o) = -\log(K_H) + \log (1 + [SO_4]/\alpha K_S)$. The value of the y-intercept is the pK for the reaction of the inhibitory proton with the unloaded carrier. This proton can bind to the sulfate-loaded or unloaded carrier, thus the only mechanism consistent with all the data is the random-ordered mechanism. The best linear fit is $y = (5.03 \pm 0.02) + (1.0 \pm 0.05)x$.

important, these reports were not conclusive. First, sulfate or HSO_4^- self-exchange fluxes saturated at millimolar sulfate concentrations pH 7.0 (29, 69) or at <1 μM HSO_4^-. This half-saturation concentration for the ion pair is much lower than for all other transported anions but is the same magnitude as the K_I for disulfonic stilbenes (48). Second, the sulfate (HSO_4^-) flux at saturation was increased by increasing the proton concentration (69). Although this is unexpected for a simple saturation model if HSO_4^- is the substrate, Schnell et al. (69) found that the sulfate dependence of the flux was

not a simple hyperbolic dependence. They reported substrate inhibition at a modifier site that was especially prominent at lower pH values. To retain the suggestion that the ion pair HSO_4^- is the substrate for the transporter, one need only postulate that OH ions interact at the modifier site (even when the transport site is saturated with HSO_4^-). At low pH the absence of OH^- at the modifier site would both allow HSO_4^- to bind at that site at high concentrations, thus making the substrate inhibition more evident, and would increase the peak flux, thus mimicking proton activation of the maximum flux. These actions of OH ions would be like those observed for acetate and furosemide anions at the modifier site (4, 29). The present data, however, make such suggestions unnecessary because they effectively rule out a major role for HSO_4^- binding to the transporter at pH values above 4.6.

Parameters of the Random-Order Scheme

The value of K_S (4.35 ± 0.36 mM) can be compared with the outside $K_{1/2}$ values for the other anions. When the surface complexation reactions equilibrate rapidly compared to the translocation reactions, then K_S, $K_{1/2}$, and K_D approach the same value, k_{off}/k_{on}.

The value of $K_{S\text{-out}}$ is not too different from $K_{1/2\text{-out}}^{Cl}$ (3.9 ± 0.1 mM) (33), $K_{1/2\text{-out}}^{HCO_3}$ (0.55 mM) (52), $K_{1/2\text{-out}}^{iodide}$ (2.6 ± 0.5 mM) (60), and $K_{1/2\text{-out}}^{Br}$ (1–3 mM) (33). The value of $K_{S\text{-out}}$ is also similar to $K_{I\text{-out}}^{iodide}$ (3 ± 1 mM) (60), but is much smaller than the K_I values for monovalent phosphate (40 mM) and divalent phosphate (>150 mM) (37).

The similarity of these values (except for the phosphate values) suggests that the surface reactions for both oxyanions such as sulfate and bicarbonate and the halides such as chloride, bromide, and iodide, are probably not too different. If a divalent sulfate anion, $SO_4^=$ has the same affinity as a monovalent anion, I^-, then the electrostatic component in the binding reaction cannot be great. Nor can the field strength at the external site be as great as has been suggested (64), unless coincidentally other factors fully compensate. If KSO_4^- (see below) is the reactive sulfate moiety, then $K_I^{KSO_4^-}$ is 0.4 mM and a high field strength at the binding site is consistent with our results.

Fig. 8 is a summary of our findings. It is simplest to assume that the proton reactions we measure are related to the co-transported proton reaction since we find no evidence for another external proton that interacts with sulfate. Furthermore, preliminary experiments in our laboratory show that sulfate influx is activated by an external proton with a similar pK value at 0°C. The pK for proton binding to the unloaded carrier is 5.03 ± 0.02, which is lower than for the titratable group which inhibits chloride flux on the inside of the cell (p$K \cong 6.2$) (34, 35). After the first substrate is bound, the affinity for the other co-transported substrate is increased ninefold as measured by the interaction factor, α (= 0.11 at 0°C). Thus the pK for the reaction of the sulfate-loaded carrier with the proton is 5.99. Sulfate has a ninefold higher affinity for the protonated carrier than for the empty carrier. Its K_I at saturating proton concentrations is reduced from 4.35 to 0.48 mM. If the proton binding site is located near the sulfate binding site, then some of this

increased affinity may be due to the change in local electrostatic field strength upon binding of the other ion. Of course, conformational changes may also alter the affinity. The contributions of each of these factors to this observed change in apparent affinity must await further experiments.

Most previous sulfate flux measurements have been at temperatures above 0°C, where our present experiments were performed. We may apply our values to higher temperatures with some confidence since the $K_{1/2}^{\text{self-exchange}}$ value of chloride changes little between 0 and 37°C (3) and the $K_{1/2}^{\text{self-exchange}}$ of sulfate is constant between 25 and 37°C (29). In this case, if the pH value is above 6.0 and sulfate concentration is above 1 mM, most of the transporters that are not loaded with chloride on the outside are loaded with sulfate and

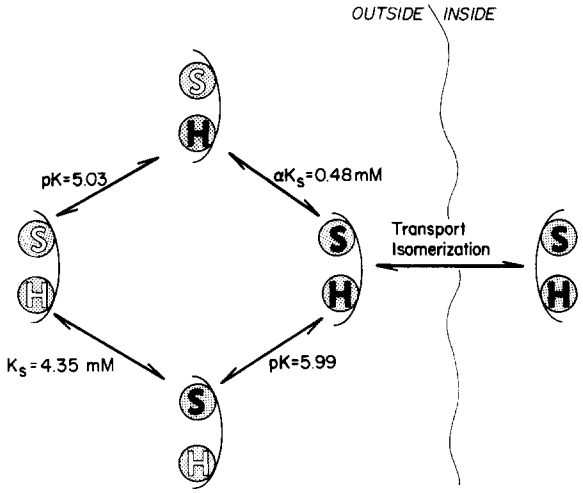


FIGURE 8. Schematic diagram of random-ordered addition of sulfate and protons to the co-transport mechanism. The binding at the external face, as depicted on the left, may proceed along either the upper or the lower path. After the first ion has been bound, the affinity for the other ion is increased ninefold as compared with its affinity for the unloaded carrier. After both ions have been bound to the transporter, they can be co-transported to the inside of the cell. Our experiments did not determine the mechanism of unloading at the internal face so these steps are not included.

only a few are loaded with a proton or loaded with both a proton and a sulfate. Normal plasma has a pH = 7.38; so $[H^+] = 0.0045\ K_H$. The normal concentration of all plasma sulfate complexes is 0.6 mM (1); so $[S] = 0.14\ K_S$. If the K_I values are the same at 38 as at 0°C, then in normal plasma, about 10 times more transporters are loaded with only a sulfate than with only a proton, but >95% of the transport sites that have either a chloride or bicarbonate bound face externally.

Alternative Models

In the Theory section we showed that one can distinguish between mechanisms with different orders of addition in this system if one replots the slopes of the

Dixon plots ($1/M$ vs. first inhibitor concentration) vs. the second inhibitor concentration. The fact that the replot vs. proton concentration in Fig. 3 has a nonzero y-intercept ($P < 0.005$) allowed us to conclude that our data were inconsistent with the proton-binds-first mechanism. Similarly, the fact that the replot vs. sulfate concentration in Fig. 6 has a nonzero y-intercept ($P < 0.001$) allows us to conclude that our data were inconsistent with the sulfate-binds-first mechanism. Thus the only simple mechanism consistent with our data is that proton and sulfate can bind in a random order to the external site of the anion carrier. Alternative—but more complex—models are now considered.

The analysis detailed in the Theory section included five assumptions: 1) sulfate may be treated as a nontransported inhibitor of chloride fluxes at 0°C; 2) protons are also nontransported inhibitors of chloride fluxes at 0°C; 3) proton inhibition is competitive; 4) only one proton is involved in the inhibition; and 5) HSO_4^- and $SO_4^=$ are the only sulfate complexes present in our solutions.

It is clear from the relative rates of chloride and sulfate transport that assumption 1 is reasonable. Jennings (44, 45) has shown that pH equilibration at 25°C across red cells in a bicarbonate-free media is mediated by the anion transporter. When sulfate is available, Cl-(H + SO_4) exchange moves protons and the rate is governed by the sulfate transport rate. In an all-chloride medium, protons can still be transported. Their rate is slow and must be about an order of magnitude smaller than sulfate/proton co-transport (45).[5] As these proton movements show the same temperature dependence as anion exchange ($Q_{10} = 8$; $E_A = 110$ kJ/mol), proton movements will be at least as slow as sulfate movements at 0°C (45). Furthermore, the experiments described here are most consistent with the treatment of protons as nontransported inhibitors at 0°C. We now consider the last three assumptions in some detail.

I. Other Types of Proton Inhibitions

In the Theory section of Methods we presented the four simplest models for proton and sulfate inhibition of chloride flux. The four models all assumed that the proton was a competitive inhibitor of chloride binding to the outward-facing carrier. We now wish to show that our conclusion that protons and sulfate bind in a random order only depends upon the assumption that the protons can bind to the unloaded transporter, C, i.e., they bind randomly as long as the proton is a competitive, noncompetitive, or mixed inhibitor. If one of the two latter cases is true, the reciprocal velocity equation would be:

$$\frac{1}{V} = \frac{1}{V_m} + \frac{K_o}{V_m Cl_o} + \frac{K_i}{V_m Cl_i} + \frac{K_o}{V_m Cl_o}\left[\frac{H}{K_H} + \frac{S}{K_S} + \frac{HS}{\alpha K_H K_S}\right] + \frac{1}{V_m}\left[\frac{H}{K'_H}\right] \quad (2)$$

[5] This proton/chloride co-transport may account for the small difference between the measured proton/sulfate stoichiometry of 0.8 to 0.9 and the theoretical value of 1.0 (49).

where K_H' is the inhibition constant of the proton when it binds to the chloride-loaded carrier, CCl. For noncompetitive proton inhibition, $K_H = K_H'$. Even though Eq. 2 contains an extra term, $(1/V_m) \times (H/K_H')$, compared with the competitive random-order case (line 1, column 4, Table I), this term does not enter into the sulfate analysis. The replot of $(\text{slope})_S$ vs. [H] will still have a nonzero y-intercept if S can bind without a proton. Both equations also give the same K_S value. The value of K_H is no longer given simply by the y-intercept of the replot of $(\text{slope})_H$ vs. [S], since in Eq. 2 the y-intercept is

$$\frac{1}{V_m Cl_o}\left[\frac{K_o}{K_H} + \frac{Cl_o}{K'_H}\right],$$

whereas previously the expression used to calculate K_H from the y-intercept in a competitive mechanism was $\frac{1}{V_m Cl_o}\left(\frac{K_o}{K_H}\right)$. Although by using these different models we will calculate different pK values, the proton can add first in each model if and only if the y-intercept of the replot $(\text{slope})_H$ vs. [S] is nonzero (Fig. 7).

If the proton were an uncompetitive inhibitor, that is, if it could only bind to an anion-loaded carrier, CCl or CSO_4, the reciprocal equation would be:

$$\frac{1}{V} = \frac{1}{V_m} + \frac{K_o}{V_m Cl_o} + \frac{K_i}{V_m Cl_i} + \frac{K_o}{V_m Cl_o}\left[\frac{S}{K_S} + \frac{HS}{K_H K_S}\right] + \frac{1}{V_m}\left[\frac{H}{K'_H}\right].$$

This mechanism has a nonzero y-intercept for the replot $(\text{slope})_H$ vs. [S] and is therefore consistent with the results of Figs. 4–6. However, this is not a random-ordered mechanism, as the proton does not bind to the unloaded transporter, C. We have preliminary data that rule out uncompetitive inhibition by protons but are not yet sufficient to distinguish between competitive and mixed (noncompetitive being a special case of mixed) inhibition (M. A. Milanick and R. B. Gunn, unpublished observations). Thus, we feel that protons can bind to C and the reaction is random ordered.

II. Different Protons

We cannot rule out the possibility that there are two different, mutually exclusive protons that inhibit chloride transport on the outside. It is possible that the proton that inhibits the chloride flux observed in Fig. 5 is different from the proton that assists sulfate inhibition of chloride fluxes. However, our data are completely consistent with the reaction of only one proton.

The only mechanisms consistent with our data with two different external protons affecting anion transport and with $5 < pK < 8$ are: (*a*) an ordered mechanism with first sulfate binding and then one proton (H_1) binding to a site with $pK = 5.99$, and another proton (H_2) binding to a separate site with $pK = 5.03$. This second proton would independently inhibit chloride flux and be mutually exclusive with the sulfate and the first proton H_1. (*b*) A random-ordered addition of sulfate and proton, H_1, as well as an additional proton, H_2, that competes with sulfate, H_1, and chloride. Although these models are

consistent with our data, we will not consider them further since our simpler explanation works as well.

III. HSO_4^-

It may seem possible to argue that only S binds to the transporter at high pH values (where $[HSO_4^-]$ would be much less than its K_I value) and that as the pH is lowered, HSO_4^- concentration and inhibition becomes appreciable. This seems to us to be a very unlikely hypothesis. First, HSO_4^- and S must be mutually exclusive as our Dixon plots $1/M$ vs. S are linear. ($1/M$ would show a parabolic response as a function of S if both HSO_4^- and S could bind to the same transporter). Secondly, HSO_4^- and H would be mutually exclusive (since otherwise one would expect supralinear Dixon plots at high sulfate and proton concentrations).[6] These two points mean that if both HSO_4^- and S bind, the sulfate flux measurements represent a very complex set of reactions. This is probably not the case because recent studies show that at the external surface, sulfate uptake is a simple function of the external proton and sulfate concentrations (61; and M. Jennings, personal communication). Thus we feel that above pH 4.6, there is negligible HSO_4^- interaction with the external face of the transporter, although at lower pH values, as the HSO_4^- concentration increases, HSO_4^- may make a non-negligible contribution to the kinetics.

IV. KSO_4^-

The pK for potassium binding to sulfate is 0.8 (corrected to zero ionic strength) (2). In 150 mM potassium solutions, the Debye-Huckel approximation for activity coefficients, γ, shows that about one-tenth of the sulfate is complexed as the monovalent anion KSO_4^-.

$$[KSO_4^-] = \frac{[K][SO_4]}{K_{eq} \cdot D}$$

where D is $\gamma_{KSO_4}/(\gamma_K \cdot \gamma_{SO_4})$.

It is therefore possible that the ion pair KSO_4^-, and not divalent sulfate, is the reactive moiety of sulfate. Because these experiments were performed at a fixed potassium concentration, the ratio of divalent sulfate to complexed sulfate is fixed and the experiments described above cannot distinguish KSO_4^- inhibition from $SO_4^=$ inhibition. Whichever form of sulfate binds $SO_4^=$ or KSO_4^-, there is a proton that binds in random order with it. Because the pK for the binding of all alkali metals to sulfate is 0.8, substitution experiments using Na^+ probably would not clarify this point.

RELATIONS TO PREVIOUS WORK

We have examined here the proton inhibition of chloride fluxes on the external surface of the membrane at much greater concentrations than previous reports. Both Wieth's laboratory (82) and our laboratory (34) reported that there was

[6] At pH 4.6 and 10.6 mM sulfate and 6.6 mM chloride, proton/sulfate co-transport influx could be 1–10% of the chloride influx under these conditions; this could easily account for the possible 5% discrepancy between the fitted lines and that data point on Fig. 2.

little or no external proton inhibition of chloride fluxes in the range of pH 5.5 to pH 8 when the internal pH was 7.8. We both concluded that the inhibition of chloride flux reported in this pH range when internal and external pH values were equal must be due to a protonation on the inside surface. Although this conclusion is correct if Cl_o <50 mM, our present experiments show that there is also an inhibitory protonation reaction for chloride flux on the outside surface. This external reaction is probably identical to the protonation which activates sulfate influx.

Knauf (48) in a review article argued from self-exchange measurements that sulfate and chloride can bind to both protonated and unprotonated forms of the transporter. From the arguments above concerning HSO_4^- ion pairs and the range of pH values in the experiments he considered, we believe those arguments were premature. Whether or not chloride can bind to both protonated and unprotonated forms on the outside is unknown at present. And whether or not sulfate and chloride can bind to the protonated and unprotonated transporter at the internal facing conformation deserves further study. Knauf came to his prescient conclusion, which may apply to outside sulfate, because the $K_{1/2}^{\text{self-exchange}}$ for sulfate increased only threefold between pH 7.3 and pH 8.3 and because the maximum sulfate self-exchange flux increased only two- to threefold between pH 7.5 and pH 6.5. But these results alone could be explained by several alternative models without assuming that external sulfate binds to both protonated and unprotonated transporters. We have reported elsewhere (61) that (*a*) the pK for the external protonation reaction that activates sulfate influx into chloride-loaded cells is less than pH 6 at 23°C, although sulfate self-exchange fluxes peak at pH 6.5; and (*b*) there is no inhibition of sulfate influx by high external proton concentration. From these observations it is not difficult to construct other models that have the characteristics of sulfate self-exchange. For example, if one chooses appropriate pK values for activation that are smaller than the pK for the inhibition of sulfate fluxes, one can model the changes in $K_{1/2}$ and V_{max} values indicated above. Consequently, we feel that the previous data and arguments in the literature merely suggested what we have directly shown here; namely, that external protons and sulfate can add separately to the chloride transporter.

CONCLUSION

We have shown that both sulfate anions and protons can bind separately to the transporter from the outside solution and inhibit chloride fluxes. Our data over a wide range of proton and sulfate concentrations are consistent with the simple mechanism of random-order binding of protons and sulfate anions, resulting in inhibition of chloride fluxes. This reaction pathway is the most likely mechanism by which the 1:1 proton-sulfate co-transport sites react with their two external substrates. The pK values for the external proton reactive group with and without a bound sulfate are 5.99 and 5.03, respectively. The K_I values for sulfate with and without a proton bound to the carrier are 0.48 and 4.35 mM, respectively. Consequently, the binding of one substrate enhances the apparent affinity of the transporter for the second substrate by ninefold.

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