Rapid Electrogenic Sulfate-Chloride Exchange Mediated by Chemically Modified Band 3 in Human Erythrocytes

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ABSTRACT One of the modes of action of the red blood cell anion transport protein is the electrically silent net exchange of 1 Cl\textsuperscript{−} for 1 SO\textsubscript{4}\textsuperscript{2−} and 1 H\textsuperscript{+}. Net SO\textsubscript{4}\textsuperscript{2−}-Cl\textsuperscript{−} exchange is accelerated by low pH or by conversion of the side chain of glutamate 681 into an alcohol by treatment of intact cells with Woodward's reagent K (WRK) and BH\textsubscript{4}. The studies described here were performed to characterize the electrical properties of net SO\textsubscript{4}\textsuperscript{2−}-Cl\textsuperscript{−} exchange in cells modified with WRK/BH\textsubscript{4}. The SO\textsubscript{4}\textsuperscript{2−} conductance measured in 100 mM SO\textsubscript{4}\textsuperscript{2−} medium is smaller in modified cells than in control cells. However, the efflux of [\textsuperscript{35}S] SO\textsubscript{4}\textsuperscript{2−} into a 150-mM KCl medium is 80-fold larger in modified cells than in control cells and is inhibited 99\% by 10 μM H\textsubscript{2}DIDS. No detectable H\textsuperscript{+} flux is associated with SO\textsubscript{4}\textsuperscript{2−}-Cl\textsuperscript{−} exchange in modified cells. In the presence of gramicidin to increase the cation permeability, the stoichiometry of SO\textsubscript{4}\textsuperscript{2−}-Cl\textsuperscript{−} exchange is not distinguishable from 1:1. In modified cells loaded with SO\textsubscript{4}\textsuperscript{2−}, the valinomycin-mediated efflux of \textsuperscript{86}Rb\textsuperscript{+} into an Na-glucuronate medium is immediately stimulated by the addition of 5 mM extracellular Cl\textsuperscript{−}. Therefore, SO\textsubscript{4}\textsuperscript{2−}-Cl\textsuperscript{−} exchange in modified cells causes an outward movement of negative charge, as expected for an obligatory 1:1 SO\textsubscript{4}\textsuperscript{2−}-Cl\textsuperscript{−} exchange. This is the first example of an obligatory, electrogenic exchange process in band 3 and demonstrates that the coupling between influx and efflux does not require that the overall exchange be electrically neutral. The effects of membrane potential on SO\textsubscript{4}\textsuperscript{2−}-SO\textsubscript{4}\textsuperscript{2−} exchange and SO\textsubscript{4}\textsuperscript{2−}-Cl\textsuperscript{−} exchange in modified cells are consistent with a model in which nearly a full net positive charge moves inward through the transmembrane field during the inward Cl\textsuperscript{−} translocation event, and a small net negative charge moves with SO\textsubscript{4}\textsuperscript{2−} during the SO\textsubscript{4}\textsuperscript{2−} translocation event. This result suggests that, in normal cells, the negative charge on Glu 681 traverses most of the transmembrane electric field, accompanied by Cl\textsuperscript{−} and the equivalent of two protein-bound positive charges.

INTRODUCTION

The rapid exchange of Cl\textsuperscript{−} for HCO\textsubscript{3}\textsuperscript{−} across the red blood cell membrane is mediated by the transmembrane protein known as band 3, capnophorin, or AE1 (see Knauf, 1979; Passow, 1986; Jennings, 1989b). Band 3 is well suited for the detailed study of coupled transport, in part because anion fluxes can be measured more precisely in red cells than in most other natural cells and expression systems. In
addition, the determination of the structure of band 3 is progressing relatively rapidly (Reithmeier, 1993). For these reasons band 3 has been used widely as a model system for the study of the molecular mechanism of coupled transport.

Although the physiological substrates are Cl\(^-\) and HCO\(_3^-\), band 3 also mediates the transport of the divalent anion SO\(_4^{2-}\) (Ku, Jennings, and Passow, 1979). The characteristics of SO\(_4^{2-}\) transport are quite different from those of Cl\(^-\). For example, SO\(_4^{2-}\) is transported much more slowly than Cl\(^-\); at pH 7.4 and 37°C, the rate constant for Cl\(^-\)-Cl\(^-\) exchange (Brahm, 1977) is over 10,000 times that for SO\(_4^{2-}\)-SO\(_4^{2-}\) exchange (Lepke and Passow, 1971). Another notable difference is pH dependence. Lowering the extracellular pH at neutral intracellular pH inhibits Cl\(^-\)-Cl\(^-\) exchange (Wieth and Brahm, 1985; Milanick and Gunn, 1982), and accelerates the influx of SO\(_4^{2-}\) into Cl\(^-\)-loaded cells (Jennings, 1980; Milanick and Gunn, 1984). The opposite effects of pH on Cl\(^-\) and SO\(_4^{2-}\) exchange can be explained by the titratable carrier model of Gunn (1978), who postulated that protonation of a titratable group converts the transporter from the normal monovalent (Cl\(^-\)/HCO\(_3^-\)) anion exchanger to one that transports SO\(_4^{2-}\) at an appreciable rate. As predicted by the titratable carrier model, the net exchange of Cl\(^-\) for SO\(_4^{2-}\) is accompanied by an obligatory, stoichiometric

![Diagram of catalytic cycles for the net exchange of Cl\(^-\) for SO\(_4^{2-}\) by way of a ping-pong mechanism.](image)

**Figure 1.** Catalytic cycles for the net exchange of Cl\(^-\) for SO\(_4^{2-}\) by way of a ping-pong mechanism. (A) Electroneutral exchange of 1 Cl\(^-\) for 1 SO\(_4^{2-}\) and 1 H\(^+\) by a ping-pong mechanism, as is believed to take place in normal band 3. The other three cycles are possible mechanisms of Cl\(^-\)-SO\(_4^{2-}\) exchange in band 3 modified to remove the H\(^+\)-titratable group (Glu 681) associated with H\(^+\)-SO\(_4^{2-}\) cotransport. (B) Electroneutral exchange of 2 Cl\(^-\) for 1 SO\(_4^{2-}\). (C) Electrogenic exchange of 1 SO\(_4^{2-}\) for 1 Cl\(^-\), with a net negative charge moving with SO\(_4^{2-}\). (D) Electrogenic exchange of 1 SO\(_4^{2-}\) for 1 Cl\(^-\), with a net positive charge moving with Cl\(^-\).
cotransport of H\(^{+}\) with SO\(_{4}^{2-}\) (Jennings, 1976; Milanick and Gunn, 1984), depicted schematically in Fig. 1 A.

The pK of the protonation event that inhibits Cl\(^{-}\) exchange and accelerates SO\(_{4}^{2-}\) influx is 5.0–5.5 (Milanick and Gunn, 1982, 1984), consistent with a carboxyl group. Chemical modification of intact red cells with Woodward’s reagent K and BH\(_{4}^{-}\) converts a particular glutamate side chain, Glu 681, into the uncharged alcohol (Jennings and Anderson, 1987; Jennings and Smith, 1992) and alters band 3 function in several ways, including inhibition of monovalent (Cl\(^{-}\)-Br\(^{-}\)) anion exchange, activation of Cl\(^{-}\)-SO\(_{4}^{2-}\) exchange, and elimination of the H\(^{+}\) flux that normally accompanies Cl\(^{-}\)-SO\(_{4}^{2-}\) exchange (Jennings and Al-Rhaiyel, 1988). The simplest interpretation of these results is that Glu 681 is the titratable residue which, when protonated, activates SO\(_{4}^{2-}\) exchange and inhibits Cl\(^{-}\) exchange. This residue is conserved in band 3 (AE1) and in the related proteins AE2 and AE3 of all species for which sequences are known (Kopito, 1990).

The above interpretation of the effects of Woodward’s reagent K and BH\(_{4}^{-}\) implies that the modification causes permanent conversion of the transporter from the normal Cl\(^{-}\)-transporting form to one that is missing one negative charge at a critical site in the transport pathway. According to this interpretation, net Cl\(^{-}\)-SO\(_{4}^{2-}\) exchange in modified cells could take place as an electroneutral exchange of 2 Cl\(^{-}\) for 1 SO\(_{4}^{2-}\) or as an electrogenic 1:1 exchange (Fig. 1). The experimental results described here show that the exchange in modified cells is electrogenic. These results not only support the idea that Glu 681 is the titratable residue associated with SO\(_{4}^{2-}\)-H\(^{+}\) cotransport but also provide the first example of an electrogenic anion exchange mediated by band 3. The effects of membrane potential on the net exchange are consistent with a ping-pong mechanism in which, in modified cells, a small amount of negative charge is translocated in the SO\(_{4}^{2-}\) limb of the cycle, and nearly a full net positive charge is translocated with Cl\(^{-}\). Therefore, the equivalent of nearly two protein-bound positive charges move through the transmembrane electric field during the anion translocation event.

**MATERIALS AND METHODS**

**Materials**

Human blood was drawn from lab personnel into EDTA and used after at most 10 d of storage as whole blood at 4°C. Most of the experiments were performed on red cells stored no more than 4 d; no effect of storage on anion exchange was detected. Woodward’s reagent K (N-ethyl-5-phenylisoxazolium 3’sulfonate) and NaBH\(_{4}\) were purchased from Sigma Chemical Co. (St. Louis, MO). The N-methyl d-glucammonium (NMG) salts of glutamic acid, HCl, or H\(_{2}\)SO\(_{4}\) were prepared by titrating the acid with N-methyl d-glucamine (Sigma Chemical Co.). N-methyl d-glucammonium d-gluconate was prepared by the same method, using d-gluconic acid (Fluka Chemical Co., Ronkonkoma, NY) and decolorizing the solution with activated charcoal. Gramicidin (87% gramicidin A) and valinomycin were from Calbiochem Corp. (San Diego, CA). H\(_{2}\)DIDS (4,4’-diisothiocyanatodihydrostilbene-2,2’-disulfonate) was prepared as described previously (Jennings, Adams-Lackey, and Denney, 1984). All other salts, buffers, and reagents were from either Sigma Chemical Co. or Fisher Scientific (Pittsburgh, PA). Radionuclides (Na\(^{35}\)SO\(_{4}\), H\(^{36}\)Cl, and \(^{86}\)RbCl) were from Dupont NEN (Boston, MA).
Cell Preparation

Intact cells were treated with 2 mM Woodward's reagent K and BH₄⁻ as described previously (Jennings and Al-Rhaiyel, 1988; Jennings and Smith, 1992). Briefly, cells were washed in 150 mM KCl, 10 mM MOPS, pH 7, chilled on ice, and then exposed to 2 mM WRK for 10 min followed by two successive additions of 2 mM NaBH₄ at 5-min intervals. This procedure modifies ~80% of the copies of band 3 at Glu 681 (Jennings and Al-Rhaiyel, 1988), leaving ~20% of the protein unmodified. Cells were then washed once in 150 mM KCl, 10 mM MOPS. Before ³⁶Cl⁻ efflux experiments, cells were washed further with 150 mM KCl buffered with 10 mM MOPS, pH 7, or 10 mM HEPES, pH 7.4, and then loaded with ³⁶Cl⁻ (2 μCi/ml cells). For loading with SO₄²⁻ after treatment with WRK/BH₄⁻, cells were washed three times in at least 20 vol of 100 mM K₂SO₄, 10 mM Na-HEPES, pH 7.4. Before each centrifugation in the K₂SO₄ medium, the suspension was incubated 10 min at 37°C to allow SO₄²⁻ influx. For loading with [³⁵S] SO₄²⁻, cells were then incubated at 30% hematocrit for 1 h at 37°C in 80–100 mM K₂SO₄, 10 mM Na-HEPES, pH 7.4 plus 10 μCi/ml [³⁵S] SO₄²⁻. For loading with ⁸⁶Rb⁺, the same procedure was followed except that the last incubation was 90 min, 37°C in 100 mM Na₂SO₄, 10 mM HEPES, pH 7.4, 10 mM glucose, and 10 μCi ⁸⁶Rb⁺/ml. In some experiments, cells were preloaded with ⁸⁶Rb⁺ by incubation for 90 min in HEPES-buffered physiological saline (10 mM glucose) before chemical modification and SO₄²⁻ loading. Cells equilibrated with 100 mM K₂SO₄, pH 7.4, were found to have an intracellular SO₄²⁻ concentration of 50 mM (measured with ³⁵S), consistent with the expected Donnan ratio of ~0.7 at pH 7.4 and 20°C (Sachs, Knauf, and Dunham, 1975; Gunn, Dalmark, Tosteson, and Wieth, 1973). The ³⁵S SO₄²⁻ flux experiments were performed on cells loaded in either 80 mM or 100 mM K₂SO₄. In 80 mM K₂SO₄, 10 mM Na-HEPES, pH 7.4, the cells have normal water content (0.71 g H₂O/ml cells). In 100 mM K₂SO₄/HEPES, cell volume is ~15% less than normal, but cell volume itself does not have a significant effect on band 3-mediated anion transport (Funder and Wieth, 1976). Fluxes measured in 80 mM K₂SO₄, 10 mM Na-HEPES were indistinguishable from those measured in 100 mM K₂SO₄, 10 mM Na-HEPES.

Transport Measurements

The efflux of ³⁶Cl⁻ in cells modified with WRK/BH₄⁻ was measured in a 150 mM KCl medium by an inhibitor stop method (counting pellets to allow measurement of rate constants up to 10/min) described previously (Jennings, Allen, and Schulz, 1990). For measurement of efflux of ³⁵S SO₄²⁻ or ⁸⁶Rb⁺, cells were washed twice in cold 100 mM K₂SO₄, 10 mM HEPES, pH 7.4, once in cold 250 mM sucrose, 10 mM HEPES, pH 7.4, and resuspended at a 1–2% hematocrit in media described in the figure legends. The reason for the sucrose wash was to minimize the extracellular SO₄²⁻ concentration. The time course of tracer efflux was determined as described previously (Jennings and Al-Rhaiyel, 1988). Under the net exchange conditions used here, the time course is not necessarily a simple exponential, because the flux is, in general, a function of the ion concentrations, which change continuously during net exchange. However, in nearly all of these experiments (exceptions noted below), a plot of ln [1 – CPMₜ/t/CPMₜ(t)] was indistinguishable from a straight line over the times that were used in the data analysis (linear regression coefficient r was at least 0.99 and usually more than 0.995 for 3–4 time points). The flux was calculated from the rate constant (min⁻¹) derived from the semilog plot, multiplied by the initial cellular SO₄²⁻ contents (μmol/ml cells).

In two classes of experiments, the time course was systematically nonexponential. The time course for the last 25% of the tracer SO₄²⁻ efflux in the presence of ionophore and outward K⁺ gradient is nonexponential because the intracellular compartment (total number of anions per cell) becomes progressively smaller during the efflux; under these circumstances the data were
analyzed only over the first 50–75% of the efflux, during which the time course was not distinguishable from an exponential. The time course also deviated from an exponential for \( \text{SO}_4^- \) efflux into a \( \text{Cl}^- \) medium in unmodified cells, because the incoming \( \text{Cl}^- \) causes progressive inhibition of \( \text{SO}_4^- \) efflux. For this experiment the initial flux was estimated from the slope of the efflux over the first 3–6% of the efflux. In modified cells, the \( \text{SO}_4^- \) efflux into a \( \text{Cl}^- \) medium is not distinguishable from a single exponential. We believe that the reason for the exponential efflux is that the affinities of \( \text{SO}_4^- \) and \( \text{Cl}^- \) for inward-facing transport sites are similar in modified cells, so that the incoming \( \text{Cl}^- \) does not strongly inhibit the \( \text{SO}_4^- \) efflux. A systematic study of the \( \text{SO}_4^- \) and \( \text{Cl}^- \) concentration dependences of \( \text{SO}_4^- \) transport in modified cells is beyond the scope of this paper. For the present purposes, the important point is that the time course of \( \text{SO}_4^- \) efflux into a \( \text{Cl}^- \) medium is a single exponential and the efflux may therefore be characterized by a single rate constant.

The influx of \( ^{36}\text{Cl}^- \) was measured in the same media used for \( ^{35}\text{SO}_4^- \) efflux, with \( ^{36}\text{Cl}^- \) at a specific activity of 10⁴ CPM/μmol in the extracellular medium. Aliquots (1 ml) of the suspension were removed at various times and mixed with 9 ml of ice-cold stop solution consisting of 100 mM \( \text{K}_2\text{SO}_4 \), 20 μM \( \text{H}_2\text{DIDS} \). Cells were centrifuged within 3 min of addition to the stop solution and were then washed once in 9 ml of the cold stop solution. Radioactivity in the pellet was determined as described previously (Jennings, Allen, and Schulz, 1990). The flux was calculated from the extracellular specific activity, intracellular CPM, and the amount of hemoglobin in the suspension, measured with Drabkin’s reagent (absorbance at 540 nm), assuming a mM extinction coefficient of 44 (Sigma technical bulletin). All fluxes are presented as μmol/ml cells-min, with 1 ml of standard cells defined as containing \( 1.1 \times 10^{10} \) cells and 5.0 μmol hemoglobin.

**Modification of Membrane Potential**

To examine the electrical properties of \( \text{SO}_4^- \) transport in modified cells, the membrane potential was altered with valinomycin (2 μM; 0.2% ethanol) for \( \text{SO}_4^-\text{SO}_4^- \) exchange or gramicidin (5–10 μg/ml cells; 0.2% ethanol) for \( \text{SO}_4^-\text{Cl}^- \) exchange. To estimate the cation permeability induced by gramicidin, \( ^{86}\text{Rb}^+ \) efflux was measured at the same temperature (20°C) and hematocrit (2%) as were used in the anion transport experiments. The rate constant for \( ^{86}\text{Rb}^+ \) efflux in 150 mM KCl medium was 3.5/min at 2.5 μg/ml cells and was too fast to estimate at higher gramicidin concentrations. At the gramicidin doses used in these experiments (5–10 μg/ml cells, as specified in the figure and table legends), the rate constant for \( ^{86}\text{Rb}^+ \) efflux should be at least 7/min. This is 10 times higher than the rate constant for \( \text{SO}_4^- \) efflux into a \( \text{Cl}^- \) medium in modified cells.

For most conditions, the membrane potential in the presence of gramicidin can be approximated by the Nernst potential for total Na⁺ + K⁺. However, for \( \text{SO}_4^- \) efflux into a high \( \text{Cl}^- \) medium, a correction is necessary to account for the fact that the \( \text{SO}_4^-\text{Cl}^- \) exchange flux is not completely negligible compared with the conductive cation fluxes. For this kind of experiment the membrane potential was calculated from the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949), with the following assumptions. (a) The total intracellular Na⁺ and K⁺ concentration is 150 mM. (b) Within 0.5 min after the addition of gramicidin, Na⁺ and K⁺ will exchange until transmembrane gradients of Na⁺ and K⁺ are equal. (c) The conductive cation flux has a permeability coefficient equivalent to an efflux rate constant of 10/min. (d) The \( \text{SO}_4^-\text{Cl}^- \) exchange flux contributes to the membrane potential similarly to a net monovalent anion efflux with rate constant 0.7/min. (e) The \( \text{Cl}^- \) conductance
(rate constant 0.15/min) makes a negligible contribution to the membrane potential because it appears as an additive term in the numerator with the $P_{K^+}[K^+]$ term, which is quite large.

For example, in a medium containing 15 mM K$^+$ + Na$^+$, 120 mM Cl$^-$, and cells with 40 mM intracellular SO$_4^{2-}$, the membrane potential is given by:

$$V_m = -25.3 \ln \left( \frac{[10 \times 150]}{[(10 \times 15) + (0.7 \times 40)]} \right) = -54 \text{ mV.}$$

For SO$_4^{2-}$--Cl$^-$ exchange at low (3 mM) extracellular Cl$^-$ concentration, the expected contribution of SO$_4^{2-}$--Cl$^-$ exchange to the membrane potential is very small (~1 mV) and has been ignored.

Estimates of Anion Conductance

The Cl$^-$ and SO$_4^{2-}$ conductances of normal and WRK/BH$_4$-treated cells were estimated under conditions of symmetric anion distributions (Donnan ratio 0.9-1.0). Cells were loaded with $^{86}$Rb$^+$ as described above, and the valinomycin-mediated efflux of $^{86}$Rb$^+$ was measured in the following media (each buffered with 10 mM MOPS, pH 7.0): 150 mM KC1, 150 mM NaCl, 100 mM Na$_2$SO$_4$, or 100 mM K$_2$SO$_4$. In each case the rate constant (min$^{-1}$) for $^{86}$Rb$^+$ efflux was measured from the exponential time course of efflux. The data were analyzed assuming that the conductive fluxes of all permeant ions obey the constant field equation (Goldman, 1943; Hodgkin and Katz, 1949). With this assumption, an expression for the rate constant $k_{Rb}$ for $^{86}$Rb$^+$ efflux can be written as follows:

$$k_{Rb} = k_{Rb}^0[FV/RT]/(1 - \exp[-FV/RT]),$$

where $V$ is the membrane potential, $k_{Rb}^0$ is the rate constant for efflux at zero membrane potential, and $F$, $R$, and $T$ are Faraday’s constant, the gas constant, and the absolute temperature. In a Cl$^-$ medium the membrane potential is given by the following (using the notation of Knauf et al., 1977):

$$FV/RT = -\ln B,$$

where

$$B = (P_{K^+}K_i + P_{Cl}Cl_i)/(P_{K^+}K_o + P_{Cl}Cl_o),$$

and $K_i$, $Cl_i$, et cetera are the intracellular and extracellular K$^+$ and Cl$^-$ activities. In a 150 mM KCl medium, the rate constant for $^{86}$Rb$^+$ efflux is an estimate of $k_{Rb}^0$ because the membrane potential is very small and $B$ is close to unity. From the rate of $^{86}$Rb$^+$ efflux into a NaCl medium, $P_{Cl}$ was estimated from Eqs. 2–4.

In a SO$_4^{2-}$-medium, the expression for $B$ is much more complex (Knauf et al., 1977). In a K$^+$-free medium,

$$B = \frac{P_{K^+}K_o + (P_{K^+}K_i^2 + 16P_{K^+}S_i [P_{K^+}K_i + 4P_{K^+}S_o])^{1/2}}{8P_{SO_4}S_i},$$

where $P_5$ is the conductive SO$_4^{2-}$ permeability coefficient and $S_o$ and $S_i$ are respectively the extracellular and intracellular SO$_4^{2-}$ activities. From the rate constants for $^{86}$Rb$^+$ efflux into K$_2$SO$_4$ and Na$_2$SO$_4$ media, Eq. 5 was used to estimate the ratio of $P_5$ to $P_5$ in control and
modified cells in the presence of 2 μM valinomycin. The numerical value of $P_s$ (min$^{-1}$) was converted to a permeability coefficient (cm/s) by multiplying by the ratio of solvent volume to surface area of the cell (see Knauf et al., 1977).

**RESULTS**

**Effects of WRK/Borohydride on Chloride and Sulfate Conductance**

Before attempting to determine the electrical properties of net SO$_4^-$/Cl$^-$ exchange in WRK/BH$_4^-$-treated cells, it is necessary to estimate the effects of the treatment on Cl$^-$ and SO$_4^-$ conductances. Treatment of cells with WRK/BH$_4^-$ causes a severalfold increase in the rate of valinomycin-mediated $^{86}$Rb$^+$ efflux into NaCl medium, indicating an increase in Cl$^-$ conductance, in agreement with previously published studies in which gramicidin rather than valinomycin was used as the ionophore (Jennings and Smith, 1992). The data in Fig. 2 show that the same WRK/BH$_4^-$ treatment has no stimulatory effect (slight inhibition) on SO$_4^-$ conductance measured under analogous conditions: outward K$^+$ gradient with cells initially at Donnan equilibrium in 100 mM SO$_4^-$, pH 7. The permeability coefficient for conductive SO$_4^-$ flux in untreated cells at 20°C is $\sim 1.7 \times 10^{-9}$ cm/s, which is sevenfold smaller than the Cl$^-$ conductance measured at the same temperature and pH. This selectivity of the conductive pathway for Cl$^-$ over SO$_4^-$ is in agreement with the data of Knauf and co-workers (Knauf, Fuhrmann, Rothstein, and Rothstein, 1977), obtained at higher temperature. In cells treated with WRK and BH$_4^-$, the conductive Cl$^-$ permeability is nearly 100-fold larger than the conductive SO$_4^-$ permeability.

It should be noted that the magnitude of $P_{Cl}$ is difficult to measure by using valinomycin in treated cells because the $^{86}$Rb$^+$ efflux into an NaCl medium is only slightly smaller than that into a KCl medium (Fig. 2), and some of the $^{86}$Rb$^+$ into a KCl medium represents exchange diffusion (Bennekou and Christofersen, 1986). Valinomycin-mediated exchange diffusion may also cause minor errors in the estimate of $P_s$, but the exact values of $P_s$ and $P_{Cl}$ are not important for this
manuscript. The main point of Fig. 2 for the present purpose is that WRK/BH$_4^-$ treatment does not increase the conductive SO$_4^{2-}$ permeability.

**Acceleration of SO$_4^{2-}$-SO$_4^{2-}$ and SO$_4^{2-}$-Cl$^-$ Exchange by WRK/BH$_4^-$**

Previous work showed that WRK/BH$_4^-$ treatment causes an acceleration of SO$_4^{2-}$-SO$_4^{2-}$ exchange and of SO$_4^{2-}$ influx into Cl$^-$-containing cells (Jennings and Al-Rhaiyel, 1988). The size of the effect depends on the pH of the flux measurement; at pH 7.4, the acceleration of SO$_4^{2-}$-SO$_4^{2-}$ exchange and Cl$^-$-SO$_4^{2-}$ exchange (SO$_4^{2-}$ influx) is 5–10-fold. We found recently that the acceleration of Cl$^-$-SO$_4^{2-}$ exchange is far larger when measured as $^3$H$^3$SO$_4^-$-Cl$^-$ exchange (SO$_4^{2-}$ efflux). Fig. 3 depicts the initial efflux of $^3$H$^3$SO$_4^-$ from control and treated cells suspended in sucrose, SO$_4^-$, or Cl$^-$ media. The $^3$H$^3$SO$_4^-$ efflux into a SO$_4^-$ medium is severalfold higher in treated cells than in control cells, in agreement with previous results at slightly lower extracellular pH (Jennings and Al-Rhaiyel, 1988). The initial $^3$H$^3$SO$_4^-$ efflux into a Cl$^-$ medium is much larger (80-fold) in treated cells than in control cells; the flux is inhibited 99% by 10 $\mu$M H$_2$DIDS in the flux medium. The net SO$_4^-$ efflux into a Cl$^-$ medium is over 100 times larger than would be predicted from the SO$_4^-$ conductance of these cells (Fig. 2), indicating that the SO$_4^-$-Cl$^-$ exchange flux in treated cells represents an obligatory exchange rather than a conductive SO$_4^-$ flux. (The possibility that external Cl$^-$ somehow induces a large SO$_4^-$ conductance is considered below.)

**Lack of H$^+$-SO$_4^{2-}$ Cotransport**

In normal red cells the exchange of SO$_4^{2-}$ for Cl$^-$ is accompanied by an obligatory cotransport of H$^+$ with SO$_4^{2-}$ (Jennings, 1976). This H$^+$ flux is most readily detectable...
during SO$_4^{2-}$ influx into Cl$^-$-containing cells at low extracellular pH (Jennings, 1976; Milanick and Gunn, 1984). A net H$^+$ cotransport with SO$_4^{2-}$ has also been demonstrated during initial SO$_4^{2-}$ efflux into a Cl$^-$ medium (Berghout, Raida, Legrum, and Passow, 1988).

In WRK/BH$_4$-modified band 3, no H$^+$ flux associated with net SO$_4^{2-}$-Cl$^-$ exchange (SO$_4^{2-}$ influx) was detectable (Jennings and Al-Rhaiyel, 1988). To determine whether an H$^+$ flux accompanies SO$_4^{2-}$ efflux, cells were treated with WRK/BH$_4$, loaded with SO$_4^{2-}$ at pH 7.4, and suspended at 20°C in a 150 mM KCl medium buffered very weakly with 0.1 mM HEPES. The CO$_2$/HCO$_3^-$ content of the medium was minimized by bubbling with nitrogen. The extracellular pH during net SO$_4^{2-}$-Cl$^-$ exchange in modified cells is remarkably stable (data not shown). Any H$^+$ flux associated with SO$_4^{2-}$-Cl$^-$ exchange in these cells, if present at all, has a magnitude <1% of the SO$_4^{2-}$ efflux. To a first approximation, then, the SO$_4^{2-}$ efflux in modified cells is not accompanied by H$^+$-SO$_4^{2-}$ cotransport, in agreement with earlier results for SO$_4^{2-}$ influx (Jennings and Al-Rhaiyel, 1988).

**FIGURE 4.** Effect of increasing extracellular Cl$^-$ concentrations on the rate constant (min$^{-1}$) for $^{35}$SO$_4^{2-}$ efflux from cells pretreated with WRK/BH$_4$. Cells were initially at Donnan equilibrium in 100 mM K$_2$SO$_4$, 10 mM HEPES, pH 7.4, and $^{35}$SO$_4^{2-}$ efflux was measured in mixtures of 250 mM sucrose and 150 mM KCl, 150 mM HEPES pH 7.4 (open symbols; no gramicidin). To measure efflux with the membrane potential clamped, the extracellular medium contained 60 mM K-gluconate, 0–90 mM NMG-Cl, and 150–0 mM sucrose, and 5 µg gramicidin/ml cells (filled symbols). Symbols represent the mean and standard deviation of 2–3 flux determinations. If not shown, the error bar is smaller than the symbol.

**Dependence of Sulfate Efflux on Extracellular Chloride**

Fig. 4 shows the effect of various extracellular Cl$^-$ concentrations on the $^{35}$SO$_4^{2-}$ efflux from cells pretreated with WRK and BH$_4$. The half-maximal activation of the efflux is at ~25 mM extracellular Cl$^-$, and the Cl$^-$ concentration dependence cannot be fit well as a simple hyperbolic function of extracellular Cl$^-$. One explanation of the complex extracellular Cl$^-$ dependence of SO$_4^{2-}$ efflux in treated cells is that SO$_4^{2-}$-Cl$^-$ exchange requires two extracellular Cl$^-$ ions. However, this interpretation is not supported by experiments in which the exchange was measured under conditions of constant membrane potential (clamped with gramicidin in a 60-mM K-gluconate medium). With the membrane potential clamped, the SO$_4^{2-}$ efflux is a simple, saturable function of extracellular Cl$^-$, with half-saturation at ~10 mM (Fig. 4). At low extracellular Cl$^-$ concentrations, gramicidin accelerates SO$_4^{2-}$ efflux by a factor of 3–4. The relatively slow exchange flux at low extracellular Cl$^-$ in the absence of gramicidin suggests that SO$_4^{2-}$-Cl$^-$ exchange is electrogenic and generates
a positive (inside) membrane potential, which slows down the exchange. At high extracellular Cl\textsuperscript{-}, there is little effect of gramicidin, because an inward conductive Cl\textsuperscript{-} flux balances the outward flux of negative charge associated with SO\textsubscript{4}\textsuperscript{2-}-Cl\textsuperscript{-} exchange.

**Stoichiometry of SO\textsubscript{4}\textsuperscript{2-}-Cl\textsuperscript{-} Exchange**

An attempt was made to determine the stoichiometry of SO\textsubscript{4}\textsuperscript{2-}-Cl\textsuperscript{-} exchange by measuring the SO\textsubscript{4}\textsuperscript{2-} efflux and Cl\textsuperscript{-} influx in the same cell preparations. The Cl\textsuperscript{-} influx was measured using an inhibitor stop method with H\textsubscript{2}DIDS. In the absence of ionophore, the global stoichiometry of the exchange is expected to be 2 Cl\textsuperscript{-}: 1 SO\textsubscript{4}\textsuperscript{2-} because of the lack of H\textsuperscript{+} flux and the fact that Na\textsuperscript{+} and K\textsuperscript{+} fluxes are much smaller than the SO\textsubscript{4}\textsuperscript{2-} and Cl\textsuperscript{-} fluxes. That is, electroneutrality requires that the overall stoichiometry be 2 Cl\textsuperscript{-}: 1 SO\textsubscript{4}\textsuperscript{2-}, which could result from an obligatory 1 Cl\textsuperscript{-}: 1 SO\textsubscript{4}\textsuperscript{2-} electrogenic exchange in parallel with a Cl\textsuperscript{-} conductance, both catalyzed by band 3. The measured stoichiometry is slightly <2 Cl\textsuperscript{-}: 1 SO\textsubscript{4}\textsuperscript{2-}, probably because of a systematic underestimate of the Cl\textsuperscript{-} influx (Table I) caused by losses of Cl\textsuperscript{-} during washing in H\textsubscript{2}DIDS stop solution. Gluconate influx is not likely to account for the observed stoichiometry of <2:1, because the SO\textsubscript{4}\textsuperscript{2-} efflux into Cl\textsuperscript{-}-free gluconate medium is quite small (Fig. 4). In any case, given the difficulties of measuring Cl\textsuperscript{-} influx in red cells, the stoichiometry of SO\textsubscript{4}\textsuperscript{2-}-Cl\textsuperscript{-} exchange is reasonably close to the expected value of 2:1 in the absence of ionophore.

When the membrane permeabilities to K\textsuperscript{+} and Na\textsuperscript{+} are increased by adding gramicidin, the stoichiometry of the exchange is close to 1 Cl\textsuperscript{-}:1 SO\textsubscript{4}\textsuperscript{2-} (Table I). The 1:1 stoichiometry (and lack of proton flux) implies that a net charge is transported during the anion exchange; the charge is presumably balanced by an outward K\textsuperscript{+} flux mediated by gramicidin. In the absence of ionophore, the charge is balanced by an inward conductive Cl\textsuperscript{-} flux (also through band 3), resulting in a global stoichiometry of 2:1.

| Gramicidin | Extracellular SO\textsubscript{4}\textsuperscript{2-} Efflux | Cl\textsuperscript{-} Influx | J\textsuperscript{-}/J\textsuperscript{+} |  |
|------------|---------------------------------|----------------|----------------|--------|
|            | Cl\textsuperscript{-} (mM) | \mu mol/ml-min | \mu mol/ml-min |   |
| -          | 6                              | 2.26           | 3.41           | 1.51   |
| -          | 12                             | 4.37           | 6.87           | 1.57   |
| -          | 24                             | 7.64           | 12.64          | 1.65   |
| +          | 6                              | 4.80           | 3.49           | 0.73   |
| +          | 12                             | 7.65           | 6.19           | 0.81   |
| +          | 24                             | 10.63          | 10.44          | 0.98   |

*Ratio of Cl\textsuperscript{-} influx to SO\textsubscript{4}\textsuperscript{2-} efflux.

Values are from a single determination of initial \[^{35}\text{SO}_4^2-\] efflux and \[^{36}\text{Cl}^-\] influx (3-4 time points each) in parallel preparations of cells on the same day. Cells were treated with 2 mM WRK/BH\textsubscript{4} and loaded with SO\textsubscript{4}\textsuperscript{2-} in 100 mM K\textsubscript{2}SO\textsubscript{4}, 10 mM HEPES, pH 7.4. The flux medium contained 90 mM K-Gluconate, 6-24 mM NMG-Cl, sucrose to 300 mosm, 10 mM Na-HEPES, pH 7.4, 20°C. Gramicidin, when present, was at a concentration of 0.1 \mu g/ml suspension (5 \mu g/ml cells). Ethanol (0.1%) was present in both control and gramicidin-containing suspensions.
Outward Movement of Negative Charge During SO\textsuperscript{4–}–Cl\textsuperscript{–} Exchange

We attempted to estimate membrane potential in the presence of opposing SO\textsuperscript{4–} and Cl\textsuperscript{–} gradients in treated cells, using the fluorescent dye Di-S-C\textsubscript{3}(5) (Hoffman and Laris, 1974) and also the method of Macey and co-workers (Macey, Adorante, and Orme, 1978), which uses extracellular pH measurements in the presence of the protonophore CCCP. Neither method is satisfactory in modified cells at the temperature (20°C) used here, because the opposing SO\textsuperscript{4–} and Cl\textsuperscript{–} gradients dissipate before a reliable potential can be measured.

As an alternate approach to testing the idea that net SO\textsuperscript{4–}–Cl\textsuperscript{–} exchange in modified cells is electrogenic, WRK/BH\textsubscript{4}–treated cells were loaded with \textsuperscript{86}Rb\textsuperscript{+} and nonradioactive SO\textsuperscript{4–}. Efflux of \textsuperscript{86}Rb\textsuperscript{+} was measured into an Na-gluconate/sucrose medium in the presence of valinomycin (Fig. 5, top). Efflux is initially slow because the SO\textsuperscript{4–} conductance is low, and the outward K\textsuperscript{+} gradient generates a large negative membrane potential. In control cells the addition of 5 mM NaCl has no effect on the efflux of \textsuperscript{86}Rb\textsuperscript{+}. In treated cells, however, the addition of 5 mM NaCl causes an
immediate increase in the efflux of \(^{86}\text{Rb}^+\), indicating that CI\(^-\) influx into SO\(_4^+\)-loaded cells causes net efflux of negative charge.

It is significant that, after addition of extracellular CI\(^-\), the rate of \(^{86}\text{Rb}^+\) efflux increases without any detectable delay and then does not increase further as the SO\(_4^+-\text{Cl}^-\) exchange proceeds (Fig. 5, top). The accelerating effect of CI\(^-\) on \(^{86}\text{Rb}^+\) efflux therefore, is not a consequence of an outward conductive CI\(^-\) after accumulation of intracellular CI\(^-\). To estimate the magnitude of the outward CI\(^-\) conductive flux under these conditions, cells were equilibrated with media consisting of 60 mM gluconate and mixtures of 60 mM NaCl and 40 mM Na\(_2\text{SO}_4\). The valinomycin-mediated \(^{86}\text{Rb}^+\) efflux was measured under conditions of symmetric distributions of Cl\(^-\) and SO\(_4^+\). The results (Fig. 5, bottom) show that, in treated cells, there is large increase in the \(^{86}\text{Rb}^+\) efflux as the CI\(^-\) concentration increases at the expense of SO\(_4^+\), as expected from Fig. 2. The effect of CI\(^-\)/SO\(_4^+\) replacement is rather nonlinear in treated cells. That is, in the absence of net anion exchange, the valinomycin-mediated \(^{86}\text{Rb}^+\) efflux is far slower at CI\(^-\) concentrations of 0–30 mM than that observed during net SO\(_4^+\) efflux into a Cl\(^-\) containing medium. The rapid \(^{86}\text{Rb}^+\) efflux in Fig. 5 (top) is therefore a consequence of net negative charge efflux during SO\(_4^+\)–Cl\(^-\) exchange (SO\(_4^+\) efflux) rather than the accumulation of Cl\(^-\).

**Effect of Membrane Potential on Self Exchange and Net Exchange**

The above data provide evidence that SO\(_4^+\)–Cl\(^-\) exchange in modified cells is an electrogenic 1:1 exchange with no H\(^+\) flux. If this exchange takes place by way of a ping-pong mechanism, the net charge could be translocated during the SO\(_4^+\) efflux, the Cl\(^-\) influx, or both limbs of the catalytic cycle (Fig. 1). To attempt to determine the current-carrying step in the cycle, \(^{35}\text{SO}_4^+\) efflux was measured with the membrane potential clamped at either ~0 mV or ~−60 mV with gramicidin. The efflux was measured into media containing either 80 mM SO\(_4^+\), 120 mM Cl\(^-\), or 3 mM Cl\(^-\) (and 117 mM glutamate). A negative membrane potential slightly inhibits the \(^{35}\text{SO}_4^+\) efflux into the 80 mM SO\(_4^+\) medium (Fig. 6). A negative potential accelerates \(^{35}\text{SO}_4^+\) efflux from the same cells into either a 120 mM Cl\(^-\) or 3 mM Cl\(^-\) medium (Fig. 6). In the
120 mM Cl⁻ medium the acceleration is only ~25%, which is far smaller than would be expected if external Cl⁻ induces a conductive SO₄⁻ pathway (see below). In a 3 mM Cl⁻ medium, the acceleration of ³⁵SO₄ efflux by a negative potential is considerably greater, ~90%. The effects of potential in all three cases are consistent with the idea that most (0.8–0.85) of the net charge transported during SO₄⁻–Cl⁻ exchange is net positive charge moving inward during the Cl⁻ translocation event, with a much smaller (0.15–0.2) net negative charge moving outward during the SO₄⁺ translocation event, as discussed below.

Asymmetry of SO₄⁺–Cl⁻ Exchange in Control and Modified Cells

To analyze the effect of membrane potential on SO₄⁺–SO₄⁺ exchange in modified cells, it is necessary to have an estimate of the proportions of inward-facing and outward-facing states (see Grygorczyk, Schwarz, and Passow, 1987). In unmodified cells at neutral pH and the membrane potential near 0 mV, SO₄⁺ transport is fairly symmetric: the initial SO₄⁺ efflux into a Cl⁻ medium is similar to the initial SO₄⁺ influx into Cl⁻-loaded cells (Fig. 7). The initial SO₄⁺–Cl⁻ exchange flux in each direction is ~2.5–3.5 times the SO₄⁺–SO₄⁺ exchange flux at Donnan equilibrium in the same medium. In modified cells, the initial SO₄⁺ influx into Cl⁻-loaded cells is about twice the SO₄⁺–SO₄⁺ exchange flux, but the initial SO₄⁺ efflux into a Cl⁻ medium is ~20 times the SO₄⁺–SO₄⁺ exchange flux. The asymmetry in the SO₄⁺–Cl⁻ exchange in modified cells indicates that the SO₄⁺ efflux translocation rate constant is ~10 times faster than that for influx. This 10-fold asymmetry in SO₄⁺ translocation in modified cells is used below to estimate the net charge movement associated with the translocation event.

Estimate of Cl⁻ Translocation Rates in Modified Cells

To analyze the effect of membrane potential on SO₄⁺–Cl⁻ exchange in modified cells, it is necessary to estimate the relative magnitudes of the SO₄⁺ and Cl⁻ translocation...
events. In normal cells, Cl\textsuperscript{−}−Cl\textsuperscript{−} exchange is over 10,000-fold faster than that of SO\textsubscript{4}\textsuperscript{2−} at pH 7.4 (Brahm, 1977; Lepke and Passow, 1971), but modification by WRK/BH\textsubscript{4} accelerates SO\textsubscript{4}\textsuperscript{2−} transport and inhibits Cl\textsuperscript{−} exchange. The rate of Cl\textsuperscript{−}−Cl\textsuperscript{−} exchange mediated by modified band 3 is therefore difficult to estimate, because unmodified copies of band 3 (20% of the total under the usual conditions used here) make a large contribution to the Cl\textsuperscript{−}−Cl\textsuperscript{−} exchange flux. To try to estimate the Cl\textsuperscript{−}−Cl\textsuperscript{−} exchange flux through modified band 3, the WRK/BH\textsubscript{4} treatment must be performed under conditions in which well over 95% of the copies of the protein are modified.

There is no evidence for negative or positive cooperativity in the inhibition of Cl\textsuperscript{−}−Cl\textsuperscript{−} exchange by WRK/BH\textsubscript{4}. For example, a single treatment with 2 mM WRK/BH\textsubscript{4} inhibits Cl\textsuperscript{−}−Cl\textsuperscript{−} exchange (measured at 0°C) by ~79% (data not shown); a very similar inhibition (76−78%) of Cl\textsuperscript{−}−Br\textsuperscript{−} exchange was found previously (Jennings and Al-Rhaiyel, 1988). The residual Cl\textsuperscript{−}−Cl\textsuperscript{−} exchange flux is inhibited 76% by a second treatment at the same WRK/BH\textsubscript{4} concentration. Accordingly, unmodified copies of band 3 appear to be equally reactive with WRK, independent of how many modified copies are present.

Although repeat treatments with WRK/BH\textsubscript{4} make it possible to modify essentially all copies of band 3, the repeat treatments modify sites other than Glu 681, as indicated by inhibition rather than acceleration of SO\textsubscript{4}\textsuperscript{2−} transport (data not shown). We found, however, that repeat treatments with WRK alone, with BH\textsubscript{4} added only after the final WRK incubation, cause modification of over 95% of the copies of band 3 without major reaction at sites that cause inhibition of SO\textsubscript{4}\textsuperscript{2−} transport (Table II). In cells treated with two additions of 2 mM WRK, followed after two washes by BH\textsubscript{4}, the

| Treatment of cells | Cl\textsuperscript{−}−Cl\textsuperscript{−} flux | SO\textsubscript{4}\textsuperscript{2−}−Cl\textsuperscript{−} flux |
|-------------------|-----------------|-----------------|
| None | 8445 | 0.26 ± 0.1 (5) |
| WRK/WRK/wash/BH\textsubscript{4} | 376 ± 28 (6) | 17 ± 4 (6) |
| WRK/WRK/wash/WRK/WRK/wash/BH\textsubscript{4} | 150 ± 20 (4) | 12 ± 0.5 (4) |

*"WRK/WRK" indicates the addition of 2 mM WRK (solid added directly to suspension in 150 mM KCl, 10 mM MOPS, pH 7), 10-min incubation at 0°C, addition of 4 mM HEPES hemisodium to return the pH to near 7, addition of another 2 mM WRK, and a further 10 min at 0°C. "Wash" indicates two centrifugations from KCl/MOPS, pH 7, at 0°C. BH\textsubscript{4} was added to a final concentration of 2 mM to the cold suspension in KCl/MOPS followed by 5 min at 0°C.

\textsuperscript{1}Cl\textsuperscript{−}−Cl\textsuperscript{−} exchange flux (μmol/ml cells/min) was measured at 20°C in 150 mM KCl, 10 mM MOPS, pH 7. The flux at pH 7 is indistinguishable from that at pH 7.4, but the rate constant is slightly lower (and easier to measure) because of the larger internal Cl\textsuperscript{−} compartment. Data represent mean ± SD (n).

\textsuperscript{2}Cells were loaded with \textsuperscript{35}SO\textsubscript{4}\textsuperscript{2−} in 80 or 100 mM K\textsubscript{2}SO\textsubscript{4}, 10 mM HEPES, pH 7.4, and the efflux (μmol/ml cells/min) was measured at 20°C in 120 or 150 mM KCl, 10 mM HEPES, pH 7.4. Data represent mean ± SD (n).

\textsuperscript{3}Cl\textsuperscript{−}−Cl\textsuperscript{−} exchange flux in control cells at 20°C is from Brahm (1977), Table Ia (23.8 mol/3.1 × 10\textsuperscript{13} cells/min).

\textsuperscript{4}Flux in these cells was inhibited a further 97% by 10 μM H\textsubscript{2}DIDS in the flux medium.
rate constant for Cl\(^--\)Cl\(^--\) exchange is 4.5/min, or ~5% of that expected for control cells at 20°C and pH 7 (Brahm, 1977). Therefore, this treatment modifies at least 95% of the copies of band 3. Treatment with \(4 \times 2\) mM WRK (washes after the second and fourth incubations, followed by BH\(_4^--\)) causes a further inhibition by ~2.5-fold. The residual flux in these cells is still inhibited 97% by 10 \(\mu\)M H\(_2\)DIDS in the flux medium.

The residual flux (rate constant 2.0/min at 20°C) after four exposures to WRK followed by BH\(_4^--\) contains contributions from band 3 modified at Glu 681 as well as residual unmodified band 3. The latter can be estimated if it is assumed that prior modification of 95% of the copies of band 3 with WRK/BH\(_4^--\) does not affect the subsequent reaction of WRK/BH\(_4^--\) with the remaining 5%. This assumption is unproven, but, as mentioned above, there is no evidence that modification of one copy can affect adjacent copies.

Accordingly, we assume that if a given treatment modifies 95% of the copies of the protein, then a subsequent treatment will modify 95% of the remainder, leaving 0.25% of the copies unmodified. Therefore, the contribution of unmodified band 3 to the residual flux after treatment with \(4 \times 2\) mM WRK is ~0.25/min (i.e., 5 × 5% of the control rate constant of 90/min), compared with an observed rate constant of 2/min. From these data, we estimate that the Cl\(^--\)Cl\(^--\) exchange flux through band 3 modified at Glu 681 is 150 \(\mu\)mol/ml cells/min, or 1.5% of the flux in unmodified cells. This flux, though small compared with the control flux, is 10 times larger than the SO\(_4^{2--}\)Cl\(^--\) exchange flux in modified band 3. The relative magnitudes of SO\(_4^{2--}\)Cl\(^--\) and Cl\(^--\)Cl\(^--\) exchange are used below in the analysis of the effect of membrane potential on SO\(_4^{2--}\)Cl\(^--\) exchange.

**DISCUSSION**

The results presented here define some of the characteristics of band 3-mediated net SO\(_4^{2--}\)Cl\(^--\) exchange in human red blood cells modified with Woodward's reagent K and BH\(_4^--\), which removes the negative charge on the side chain of Glu 681 (Jennings and Smith, 1992). In control cells, SO\(_4^{2--}\)Cl\(^--\) exchange is an electroneutral exchange of 1 Cl\(^--\) for 1 SO\(_4^{2--}\) + 1 H\(^+\) (Jennings, 1976). In modified cells the stoichiometry is also 1:1 (Table I), but there is no H\(^+\) cotransported with SO\(_4^{2--}\), implying that the exchange is electrogenic. Further evidence for electrogenercity of SO\(_4^{2--}\)Cl\(^--\) exchange is that the addition of extracellular Cl\(^--\) accelerates the valinomycin-mediated \(^{86}\)Rb\(^+\) efflux from SO\(_4^{2--}\)-loaded cells (Fig. 5).

A possible alternate explanation of these data is that, in modified cells, extracellular Cl\(^--\) induces a H\(_2\)DIDS-sensitive conductive pathway for SO\(_4^{2--}\) rather than a SO\(_4^{2--}\) efflux tightly coupled to Cl\(^--\) influx. However, the effect of membrane potential on the SO\(_4^{2--}\) efflux into a 120 mM Cl\(^--\) medium (Fig. 6) is much smaller than would be expected for a simple (constant field) conductive pathway for divalent SO\(_4^{2--}\). A change in potential from 0 to ~50 mV should increase the unidirectional SO\(_4^{2--}\) efflux through a constant field channel by a factor of 4, in contrast with the observed 1.25-fold acceleration of the efflux into a 120 mM Cl\(^--\) medium. Therefore, SO\(_4^{2--}\)Cl\(^--\) exchange is electrogenic but nonetheless is an obligatory exchange. This result demonstrates that coupled exchange through band 3 does not absolutely require that the exchange be electrically neutral. Accordingly, the nature of the coupling of electroneutral
exchange in band 3 may not be fundamentally different from that of ion exchangers (e.g., mitochondrial ATP-ADP; 3 Na⁺-Ca²⁺) in which the catalytic cycle transports a net charge (Klingenberg, 1989; Philipson and Nicoll, 1993).

**Most of the Charge is Moved During the Cl⁻ Limb of the Cycle**

For the present purposes, the effects of membrane potential on tracer SO₄²⁻ flux in modified cells are discussed in the context of a simple ping-pong model (Fröhlich and Gunn, 1986; Knauf, 1979), with translocation (rather than binding or release) the rate-limiting step. In normal cells, very little charge moves through the transmembrane field with the translocation step for either Cl⁻ or SO₄²⁻ (+H⁺) (Milanick and Gunn, 1984; Jennings, Allen, and Schulz, 1990; Jennings, 1989a). In modified cells, the two simplest mechanisms for electrogenic SO₄²⁻-Cl⁻ exchange are the following: net negative charge is transported outward with SO₄²⁻, or net positive charge is transported inward with Cl⁻ (Fig. 1). The experimental data are in agreement with a mechanism in which the main electrogenic step is Cl⁻ translocation.

The data in Fig. 6 represent the effect of a change in membrane potential of ~ −55 mV on ³⁵SO₄²⁻ efflux into media containing 80 mM SO₄²⁻ (15% inhibition), 120 mM Cl⁻ (25% acceleration), or 3 mM Cl⁻ (90% acceleration). The Appendix contains algebraic expressions (from the ping-pong model) for the voltage dependence of ³⁵SO₄²⁻ efflux into each of these media. The predicted effect of a ~ −55-mV potential change on each of these fluxes is plotted in Fig. 8 as a function of the net charge that moves through the transmembrane field in the Cl⁻ translocation limb of the catalytic cycle. Under all three conditions, the data are consistent with the idea that nearly a full net positive charge (0.80–0.85) is translocated inward with Cl⁻.

![Figure 8. Comparison of the SO₄²⁻ efflux data (solid symbols) from Fig. 6 with the predictions of a ping-pong mechanism, with various amounts of net negative charge translocated through the transmembrane potential with SO₄²⁻. The data are plotted as the ratio of the SO₄²⁻ efflux at ~ −55 mV to that at ~ −5 mV. The calculated pairs of membrane potentials (in millivolts) for the three kinds of experiments are the following: 80 mM K₂SO₄, +2 and −58; 120 mM KCl, −4 and −54; 3 mM KCl, −5 and −58. The model in the Appendix assumes that if 0 charge is transported with SO₄²⁻, then 1.0 positive charge is transported inward through the entire transmembrane field during the Cl⁻ influx step. If 0.2 negative charge is transported with SO₄²⁻, then 0.8 positive charge is transported with Cl⁻, etcetera. The experimental data for all three kinds of efflux experiment are consistent with the transport of 0.75–0.85 net positive charges with Cl⁻ and 0.15–0.25 net negative charges with SO₄²⁻.](image-url)
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Electrogenic Anion Exchange through Modified Band 3

Qualitatively, the effects of potential can be explained as follows. For \(SO_4^-\)–\(SO_4^-\) exchange, outward translocation is faster than inward translocation in modified cells (judging from the asymmetry of \(SO_4^-\)–\(Cl^-\) exchange; Fig. 7). Therefore, most of the transporters should be in the outward-facing configuration in 80 mM \(K_2SO_4\). A negative potential of \(-60\ \text{mV}\) has a very slight (but consistent) inhibitory effect on \(SO_4^-\)–\(SO_4^-\) exchange under these conditions because a small net negative charge is translocated with \(SO_4^-\), and the catalytic cycle is limited mainly by inward translocation.

In a medium containing 120 mM \(Cl^-\), a change in potential of \(-50\ \text{mV}\) has a slight (25%) accelerating effect on \(3^\text{35}SO_4^-\) efflux, because under these conditions the influx limb of the catalytic cycle is faster than the efflux limb. The effect of potential is modest because most of the charge is moved with \(Cl^-\) rather than \(SO_4^-\). An analogous situation is the \(Na^+, K^+-\text{ATPase}\), which has an electrogenic catalytic cycle, but in which the voltage-dependent step is in general not rate limiting (see De Weer, Gadsby, and Rakowski, 1988).

The effect of potential on \(SO_4^-\)–\(Cl^-\) exchange is larger at low extracellular \(Cl^-\) concentration (3 mM). The \(Cl^-\) translocation event is still more rapid than that of \(SO_4^-\), but the total number of outward-facing states is large because of the low extracellular \(Cl^-\) concentration. The outward-facing states that are occupied by \(Cl^-\) will tend to be driven into the inward-facing state by the negative membrane potential, thus providing more inward-facing states for \(SO_4^-\) efflux. Accordingly, the effect of membrane potential is expected to be larger at low extracellular \(Cl^-\). Quantitatively, if 0.8 net positive charges move inward during \(Cl^-\) translocation, then a potential change from \(-5\) to \(-58\ \text{mV}\) should cause a 1.9-fold increase in the \(SO_4^-\) efflux in a medium containing 3 mM \(Cl^-\), very close to the observed value.

We interpret these data, therefore, as evidence that the charge translocation during \(SO_4^-\)–\(Cl^-\) exchange in modified cells takes place mainly as net positive charge moved with \(Cl^-\) during the \(Cl^-\) translocation event. This interpretation implies that, during \(SO_4^-\) translocation, the equivalent of two protein-bound positive charges participate in the \(SO_4^-\) translocation event to approximately cancel the two negative charges on \(SO_4^-\). Moreover, during normal \(Cl^-\) translocation (unmodified cells), the negative charge on Glu 681 must traverse most of the transmembrane electric field, because, when this charge is absent, a net positive charge is translocated with \(Cl^-\). Therefore, during the normal, physiological translocation event, one monovalent anion, two positive charges, and the negative charge on Glu 681 all participate in the electro-neutral translocation event. This conclusion agrees with earlier evidence, based on the intracellular and extracellular pH dependences of \(SO_4^-\) transport, that Glu 681 can cross the permeability barrier (Jennings and Al-Rhaiyel, 1988).

**Asymmetry of Anion Exchange in Modified Cells**

In addition to the effects on the electrical properties of \(Cl^-\) and \(SO_4^-\) transport, the modification by WRK/BH\(_4^-\) also has major effects on the kinetics of \(SO_4^-\)–\(Cl^-\) and \(SO_4^-\)–\(SO_4^-\) exchange. In control cells at Donnan equilibrium in an all-\(SO_4^-\) medium at neutral pH, there appear to be roughly equal numbers of inward-facing and outward-facing transporters (Jennings, 1980). In cells modified with WRK/BH\(_4^-\), the asymmetry of \(Cl^-\)–\(SO_4^-\) exchange is altered. The initial net \(SO_4^-\) efflux into a \(Cl^-\)
medium is over 10 times larger than the initial net \( \text{SO}_4^{2-} \) influx into Cl\(^{-}\)-containing cells. If the catalytic cycle is ping pong, this result indicates that, in modified cells, the unimolecular rate constant for outward translocation of \( \text{SO}_4^{2-} \) is considerably larger than that for inward translocation. In modified cells, therefore, unlike normal cells, most of the transporters are apparently in the outward-facing configuration at Donnan equilibrium in an all-SO\(_4^{2-}\) medium.

It is of interest to compare the apparent asymmetry of \( \text{SO}_4^{2-} \) transport at pH 7.4 in modified cells with that in normal cells at pH 5.8, which is near the lowest pH at which \( \text{SO}_4^{2-} \) transport can be studied at Donnan equilibrium in red cells without interference from an acid-induced leak (Gunn, Wieth, and Tosteson, 1975). In both situations (modified cells at pH 7.4 and unmodified cells at pH 5.8), the charge on Glu 681 is at least partially neutralized. In unmodified cells at pH 5.8, net \( \text{SO}_4^{2-} \) influx into Cl\(^{-}\)-containing cells is \( \sim 10 \) times larger than the initial net \( \text{SO}_4^{2-} \) efflux into a Cl\(^{-}\) medium (Jennings, 1980). This asymmetry is in the opposite direction from that observed in WRK/BH\(_4\)-modified cells at neutral pH (see above). This result suggests that there is another titratable group with acid pK\(_a\) which, when protonated, inhibits outward \( \text{SO}_4^{2-} \) translocation. This group is at least partially protonated in normal cells at pH 5.8 but not in modified cells at pH 7.4. The identity of this group is not known, but it could be a second carboxyl group that reacts with WRK more slowly than Glu 681. The present results agree with previous evidence for a second inhibitory titration in the acid pH range in band 3 (Berghout, Raida, Legrum, and Passow, 1988). The acid-titratable group could be the carboxyl group modified by water-soluble carbodi-imides (Bjerrum, Andersen, Borders, and Wieth, 1989).

**Ping Pong Versus Simultaneous**

The experimental data presented here have been analyzed in terms of a ping-pong mechanism, in which each anion crosses the membrane in a binary complex consisting of one transported anion with the transport protein (Gunn and Fröhlich, 1979; Knauf, 1979; Jennings, 1980, 1982). The simplest alternative to a ping-pong model is a simultaneous mechanism, in which the rate limiting translocation event involves a ternary complex among the protein and the inward-going and outward-going anions (Knauf, 1979; Restrepo, Kozody, Spinelli, and Knauf, 1989; Restrepo, Cronise, Snyder, Spinelli, and Knauf, 1991). Most of the present results are in agreement with a ping-pong mechanism. However, the magnitude of the trans acceleration of \( \text{SO}_4^{2-} \) flux by Cl\(^{-}\) in modified cells (Fig. 7) is larger than can be explained by a ping-pong/recruitment mechanism. A simple simultaneous mechanism for electrogenic \( \text{SO}_4^{2-}-\text{Cl}^{-} \) exchange can explain the trans acceleration of \( \text{SO}_4^{2-} \) flux by Cl\(^{-}\), because the rate constants for the translocation event should depend on both anions but may nonetheless be asymmetric, and there are enough adjustable parameters in the model to account for the asymmetry.

However, a simultaneous mechanism cannot account for the electrical properties of the exchange. A change in membrane potential from \(-5\) to \(-55\) mV might cause the observed 25% acceleration of the translocation event (at 120 mM extracellular Cl\(^{-}\); Fig. 6) if the net charge moved during the simultaneous exchange event crosses only a small portion of the transmembrane field. However, at low extracellular Cl\(^{-}\) the
translocation event should have the same dependence on membrane potential. There should be fewer ternary complexes at low extracellular Cl\(^-\), but the translocation event should still be accelerated 25% by a potential change of \(-55\) mV. The observed acceleration is significantly higher (90%). In two further experiments (not shown) at a more negative potential (\(~ -80\) mV), the effect of potential at 3 mM Cl\(^-\) was also far larger than at 120 mM Cl\(^-\). Although it may be possible to modify a simultaneous mechanism to account for these data, a mechanism with a single rate-limiting simultaneous translocation event cannot explain the effects of membrane potential on SO\(_4^{2-}\)-Cl\(^-\) exchange in modified cells. A simple simultaneous mechanism also cannot account for the small (but very consistent) inhibition of SO\(_4^{2-}\)-SO\(_4^{2-}\) exchange by a negative potential in modified cells. A ping-pong mechanism, with no freely adjustable parameters, can account for the potential dependence of the exchange.

Although the ping-pong mechanism can explain the electrical properties of SO\(_4^{2-}\)-Cl\(^-\) exchange quite readily, the fact remains that a simple ping-pong mechanism cannot account for the magnitudes of the SO\(_4^{2-}\)-Cl\(^-\) exchange fluxes relative to the SO\(_4^{2-}\)-SO\(_4^{2-}\) exchange flux (Fig. 7) in modified cells. Replacement of SO\(_4^{2-}\) with Cl\(^-\) must cause acceleration of the unidirectional SO\(_4^{2-}\) flux by some mechanism beyond the recruitment of transporters into the inward-facing or outward-facing configuration. The presence of self-inhibitory modifier sites on band 3 is well established (Dalmark, 1976; Knauf and Mann, 1986; Passow, 1986). It is possible that WRK/BH\(_4\) treatment changes the characteristics of an existing modifier site such that occupancy with SO\(_4^{2-}\) has a larger effect than occupancy with Cl\(^-\). Replacement of SO\(_4^{2-}\) with Cl\(^-\) would relieve some of the inhibition. It is of interest in this context that addition of bilateral Cl\(^-\) (10-20 mM) accelerates SO\(_4^{2-}\) equilibrium exchange by a factor of nearly 2 in modified cells but not in control cells (data not shown), again indicating that Cl\(^-\) can accelerate SO\(_4^{2-}\) transport by a mechanism other than recruitment. Bilateral acetate has a similar effect. An anion-activated SO\(_4^{2-}\)-SO\(_4^{2-}\) exchange flux has been identified in mouse red cells by Passow and co-workers (Karbach et al., 1992). The relationship between that transporter and the modified human band 3 studied here is not clear; an important difference between the two systems is that the SO\(_4^{2-}\) flux in modified human band 3 is still highly sensitive to stilbenedisulfonate derivatives.

A complete characterization of the kinetics of SO\(_4^{2-}\)-Cl\(^-\) and SO\(_4^{2-}\)-SO\(_4^{2-}\) exchange in modified band 3 is beyond the scope of this paper, but clearly a simple ping-pong mechanism cannot account quantitatively for the trans acceleration of SO\(_4^{2-}\) flux by Cl\(^-\). On the other hand, a ping-pong model accounts extremely well for the effects of membrane potential on SO\(_4^{2-}\)-SO\(_4^{2-}\) exchange and on SO\(_4^{2-}\)-Cl\(^-\) exchange in both high and low Cl\(^-\) media. A simple simultaneous model does not account for the potential dependences. Our data are consistent with the idea that the potential-dependent steps of the net anion exchange cycle involve only one anion (SO\(_4^{2-}\) or Cl\(^-\)) at a time, in keeping with the ping-pong mechanism. The potential-dependent step is presumably translocation, since it is at least partially rate limiting and involves substantial charge movement through the transmembrane field. A complete explanation of the rates of SO\(_4^{2-}\)-Cl\(^-\) exchange in modified cells, however, requires further sites and interactions beyond those of a simple ping-pong mechanism.
**Conductance Mechanism**

Although the emphasis of this paper is on the mechanism of net SO\textsubscript{4}\textsuperscript{2-}-Cl\textsuperscript{-} exchange in WRK/BH\textsubscript{4}-treated cells, the data in Figs. 2 and 5 should be discussed in reference to the SO\textsubscript{4}\textsuperscript{2-} conductance mechanism in normal and treated cells. Treatment with WRK/BH\textsubscript{4} inhibits SO\textsubscript{4}\textsuperscript{2-} conductance by ~50\% when it is measured in 100 mM SO\textsubscript{4}\textsuperscript{2-}, but there is no detectable inhibition of conductive SO\textsubscript{4}\textsuperscript{2-} efflux into a gluconate medium (Fig. 5, top, first three data points). One possible explanation for the inhibition of conductance in a SO\textsubscript{4} medium is that extracellular SO\textsubscript{4}\textsuperscript{2-} inhibits outward SO\textsubscript{4}\textsuperscript{2-} conductance in treated cells. A "slippage" mechanism (return of empty transporter from outward-facing to inward-facing configuration) is not likely to be the major mode of Cl\textsuperscript{-} conductance through normal band 3 (see Fröhlich, 1984), but slippage could contribute to SO\textsubscript{4}\textsuperscript{2-} conductance in modified cells. Inhibition of SO\textsubscript{4}\textsuperscript{2-} conductance by extracellular SO\textsubscript{4}\textsuperscript{2-} in modified cells is consistent with a slippage mechanism, but many other explanations are possible and we do not interpret these data as evidence either for or against any particular conductance mechanism.

**Structure-Function Relations**

The above conclusion that a net positive charge moves inward with Cl\textsuperscript{-} in modified cells implies that the Cl\textsuperscript{-} translocation event is associated with the movement of the equivalent of two membrane-bound positive charges through most of the transmembrane electric field. This in turn implies that, in normal cells, the negative charge on Glu 681, the negative charge on Cl\textsuperscript{-}, and the equivalent of two protein-bound positive charges all move through the transmembrane field, resulting in no net charge movement. The physical distance through which this movement takes place is very likely limited to a few Angströms. The transmembrane potential profile is unknown, but, in order for side chains to actually move through most of the field, the potential gradient must be rather nonlinear, and potential change must be concentrated in the vicinity of groups that participate in the rate-limiting translocation event. The details of this event are of course unknown in the absence of knowledge of structure.

Under the conditions used here, the only carboxyl group that is detectably converted to an alcohol in band 3 is Glu 681 (Jennings and Smith, 1992). The location of this residue in the sequence is of interest in the context of the idea that it can cross the permeability barrier. The glycosylation site, Asn 642, is undoubtedly extracellular; the carbohydrate can be cleaved in intact cells with endo β-galactosidase (Mueller, Li, and Morrison, 1979). Between Asn 642 and Glu 681 the sequence is reasonably hydrophilic except for a very hydrophobic stretch of 22 residues immediately preceding Glu 681. In the absence of other information, Glu 681 would be expected to be on the cytoplasmic side of a transmembrane helix. However, this residue can be labeled by short (10 min) exposures at 0°C to the hydrophilic reagents WRK and BH\textsubscript{4}, indicating that it is accessible from the extracellular side of the permeability barrier. The functional effects of WRK/BH\textsubscript{4} suggest that Glu 681 can straddle the permeability barrier; its location in the sequence is consistent with this idea. The hydrophobic sequence immediately preceding Glu 681 may be a pitched
helix that positions Glu 681 within the membrane but accessible to WRK/BH₄⁻ through a channel leading to the extracellular medium.

APPENDIX

Effect of Membrane Potential on SO₄²⁻–SO₄²⁻ and SO₄²⁻–Cl⁻ Exchange

The following expression for the initial efflux of ³⁵SO₄²⁻ from WRK/BH₄⁻-modified cells was derived assuming a ping-pong mechanism (see Fröhlich and Gunn, 1986; Knauf, 1979) in which binding is much more rapid than translocation. Initially, SO₄²⁻ is the only permeant or competing anion inside the cells, and A is the only permeant or competing extracellular anion. In the experiments described here, [A] is one of the following: 80 mM SO₄²⁻, 120 mM Cl⁻, or 3 mM Cl⁻. The initial efflux of ³⁵SO₄²⁻ is:

\[
J_s = \frac{k_s[S]k_i[A]_o}{k_s[S]+(K_s+[A]_o)+k_i[A]_o(K_s+|S|)},
\]

where \(J_s\) is the SO₄²⁻ efflux (ions per transporter per minute); \(k_s\) is the unimolecular rate constant (min⁻¹) for outward SO₄²⁻ translocation; \(k_i\) is the unimolecular rate constant (min⁻¹) for the inward translocation of A; \([S]_i\) is the intracellular SO₄²⁻ concentration (activity); \([A]_o\) is the extracellular concentration of A; and \(K_s\) and \(K_i\) are respectively the dissociation constants for SO₄²⁻ binding to the inward-facing and A binding to outward-facing transport sites.

Effect of Potential on SO₄²⁻–SO₄²⁻ Exchange

The SO₄²⁻ concentration is far larger than the \(K_{1/2}\) for equilibrium exchange in modified cells (1–2 mM, estimated from ³⁵SO₄²⁻–SO₄²⁻ exchange at equilibrium in SO₄²⁻/acetate mixtures; data not shown). Therefore, although there is a detectable effect of membrane potential on the apparent affinity for extracellular SO₄²⁻ in normal cells (Jennings, Allen, and Schulz, 1990), it is very unlikely that a potential-induced change in affinity has a measurable effect on the SO₄²⁻–SO₄²⁻ exchange flux at this SO₄²⁻ concentration. Accordingly, it is assumed that the only effect of potential is on the rate-limiting translocation event. Let \(\epsilon\) = the net charge translocated outward through the transmembrane electric field during the outward SO₄²⁻ translocation event. Assuming a symmetric barrier, the potential dependence of the unimolecular rate constant for SO₄²⁻ efflux is:

\[
k_s = k_{so}\exp(eFV/2RT),
\]

where \(k_{so}\) is the rate constant for outward SO₄²⁻ translocation at zero membrane potential, \(V\) is the membrane potential, \(F\) is the Faraday constant, \(R\) is the gas constant, and \(T\) is the absolute temperature.

The expression for the initial efflux of ³⁵SO₄²⁻ (Eq. A1) applies for any exchange partner A. When A is SO₄²⁻, the dependence of the inward translocation rate constant on potential is the following:

\[
k_i = k_{io}\exp(-eFV/2RT),
\]
where \( k_{so} \) is the unimolecular rate constant for \( \text{SO}_4^- \) influx at zero membrane potential.

Eqs. A2 and A3 can be substituted into Eq. A1 to give an expression for the \( ^{35}\text{SO}_4^- \) efflux into an 80 mM \( \text{SO}_4^- \) medium as a function of membrane potential. The precise values for the influx and efflux translocation rate constants at zero potential are not known, but it is known that the initial \( \text{SO}_4^- \) efflux into a \( \text{Cl}^- \) medium is 10 times the initial \( \text{SO}_4^- \) influx into \( \text{Cl}^- \)-containing cells (Fig. 7). This asymmetry suggests that the efflux rate constant \( k_{so} \) is \( \sim 10 \) times as large as the influx rate constant \( k_{so} \). With Eqs. A2 and A3 substituted into A1, and the estimate that \( k_{so} \) is 10 times \( k_{so} \), the expression for the flux becomes:

\[
J_x = \frac{k_{so}}{10 \exp(\varepsilon FV/2RT) + \exp(-\varepsilon FV/2RT)}.
\]

The observed 14% inhibition of \( \text{SO}_4^- \) efflux by a potential change of \(-60\) mV (from \( +2 \) to \(-58\) mV) is consistent with a value of \( \varepsilon \) of \(-0.15 \) (Fig. 8). That is, a net negative charge of 0.15 traverses the transmembrane field during the \( \text{SO}_4^- \) translocation event in WRK/BH\(_4^-\)-modified cells. This estimate is not strongly dependent on the exact numerical value of the ratio of influx to efflux translocation rates, as long as the ratio is greater than \( \sim 3 \). For example, if the ratio is 5 instead of 10, the estimated charge \( \varepsilon \) is \(-0.18 \) instead of \(-0.15 \).

**Effect of Potential on \( \text{SO}_4^- \) Efflux into a \( \text{Cl}^- \) Medium**

If the exchange partner for intracellular \( ^{35}\text{SO}_4^- \) is \( \text{Cl}^- \) rather than \( \text{SO}_4^- \), a net positive charge enters the cells with each complete catalytic cycle. Therefore, if a charge of \( \varepsilon \) leaves the cells during \( \text{SO}_4^- \) efflux, then a charge of \( 1 + \varepsilon \) must enter the cells during \( \text{Cl}^- \) influx, giving an overall net inward movement of one positive charge. Therefore, the potential dependence of inward \( \text{Cl}^- \) translocation (\( k_c \)) is given by:

\[
k_c = k_{co} \exp(-[1 + \varepsilon]FV/2RT).
\]

The right side of Eq. (A5) can be substituted for \( k_c \) in Eq. A1 to give an expression for the potential dependence of the initial \( \text{SO}_4^- \) efflux into a medium in which \( \text{Cl}^- \) is the only permeant anion. In contrast to the situation for \( \text{SO}_4^-\)–\( \text{SO}_4^- \) exchange, the numerator now contains a term \( \exp(-FV/2RT) \), because one negative charge is transported outward per catalytic cycle. Substituting Eqs. A2 and A5 into A1 gives the following:

\[
J_x = \frac{k_{so} \exp(\varepsilon FV/2RT)}{[S]_i [K_c + [C]_o] + k_{co} \exp(-[1 + \varepsilon]FV/2RT)} \frac{[C]_o}{[S]_i}.
\]

where \( [C]_o \) is the extracellular \( \text{Cl}^- \) concentration and \( K_c \) is the dissociation constant for \( \text{Cl}^- \) binding to the outward-facing substrate site, and the other parameters have the same meaning as previously.

The efflux of \( ^{35}\text{SO}_4^- \) into 120 mM \( \text{Cl}^- \) was measured at two membrane potentials: \(-4\) mV (120 mM \( \text{K}^+ \) and 5 mM \( \text{Na}^+ \)) and \(-54\) mV (10 mM \( \text{K}^+ \) and 5 mM \( \text{Na}^+ \)). The \( ^{35}\text{SO}_4^- \) efflux at \(-4\) mV is (from Eq. A6):

\[
J_x = \frac{k_{so} \exp(-.080 \varepsilon)}{[S]_i [K_c + [C]_o] + k_{co} \exp(.080 \varepsilon)} [C]_o [K_c + [S]_i].
\]
The $^{35}\text{SO}_4^{2-}$ efflux from the same cells at a membrane potential of $-54$ mV is:

$$J_s = \frac{k_{so}\exp(-1.066\epsilon)[S]k_{co}\exp(1.066[1 + \epsilon])[C]_o}{k_{so}\exp(-1.066\epsilon)[S](K_c + [C]_o) + k_{co}\exp(1.066[1 + \epsilon])(C)_o(K_s + [S]_i)}.$$ (A8)

The ratio of the flux at $V_m = -54$ to that at $V_m = -4$ is given by dividing the right side of Eq. A8 by that of Eq. A7:

$$\frac{\exp(0.986)[k_{so}\exp(-0.080\epsilon)[S]_i(K_c + [C]_o) + k_{co}\exp(0.080[1 + \epsilon])(C)_o(K_s + [S]_i)]}{k_{so}\exp(-1.066\epsilon)[S](K_c + [C]_o) + k_{co}\exp(1.066[1 + \epsilon])(C)_o(K_s + [S]_i)}.$$ (A9)

This expression can be rewritten in the following form:

$$\frac{J_s(-54)}{J_s(-4)} = \frac{\exp(0.986)[b \exp(-0.080\epsilon) + \exp(0.080[1 + \epsilon])]}{b \exp(-1.066\epsilon) + \exp(1.066[1 + \epsilon])},$$ (A10)

where $b$ is a lumped variable that is a function of the anion concentrations, dissociation constants, and translocation rate constants:

$$b = \frac{k_{so}[S]_i(K_c + [C]_o)}{k_{co}[C]_o(K_s + [S]_i)}.$$ (A11)

The actual numerical values of $k_{so}$, $k_{co}$, $K_c$, and $K_s$ are not known precisely, but estimates of the relative values of these parameters may be made from existing flux data. At 120 mM extracellular $\text{Cl}^-$ and 40 mM intracellular $\text{SO}_4^{2-}$, the factors $(K_c + [C]_o)/[C]_o$ and $[S]_i/(K_s + [S]_i)$ should be close to unity, because the $\text{Cl}^-$ and $\text{SO}_4^{2-}$ concentrations are high. We do not know the actual numerical values of either $K_c$ or $K_s$, but we have estimated the concentration dependence of $\text{SO}_4^{2-}$ equilibrium exchange (acetate substitution) under these conditions and have found that the $K_{1/2}$ is 1–2 mM (data not shown). It is therefore likely that at $[S]_i$ of 40 mM, $[S]_i$ is much higher than $K_s$.

From independent estimates of $\text{SO}_4^{2-}$--$\text{Cl}^-$ vs $\text{Cl}^-$--$\text{Cl}^-$ exchange rates in modified cells at the same temperature, we estimate that the parameter $b$ is roughly 0.1, because the $\text{Cl}^-$--$\text{Cl}^-$ exchange rate is $\sim 10$ times the $\text{SO}_4^{2-}$--$\text{Cl}^-$ exchange rate (Table II). The basis of this estimate for $b$ is that the influx limb of the catalytic cycle for $\text{Cl}^-$--$\text{Cl}^-$ exchange must be no slower than the overall turnover number. Since the turnover number for $\text{Cl}^-$--$\text{Cl}^-$ exchange is $\sim 10$ times that for $\text{SO}_4^{2-}$--$\text{Cl}^-$ exchange (Table II), the influx translocation rate constant for $\text{Cl}^-$ must be at least 10 times the efflux translocation rate constant for $\text{SO}_4^{2-}$. The observed ratio of the flux at $V_m = -54$ mV to that at $V_m = -4$ mV is 1.25. The value of $\epsilon$ that gives this ratio in Eq. A8 is $-0.2$. That is, if there is a net efflux of $-0.2$ charges during outward $\text{SO}_4^{2-}$ translocation and a net influx of $+0.8$ charges during inward $\text{Cl}^-$ translocation, then a membrane potential change of $-50$ mV would accelerate the efflux by a factor of 1.25. This estimate of the amount of charge translocated is in good agreement with that obtained from the potential dependence of $\text{SO}_4^{2-}$--$\text{SO}_4^{2-}$ exchange.

It should be emphasized that the actual magnitude of the lumped parameter $b$ is not known because the overall $\text{Cl}^-$--$\text{Cl}^-$ exchange flux does not allow an estimate of the individual rate constant for $\text{Cl}^-$ influx. However, the estimate of the charge $\epsilon$ is not strongly dependent on the numerical value of $b$. For example, if $b$ were 0.5...
instead of 0.1, the calculated value of $\varepsilon$ would be $-0.14$; if $b$ were 0.01 instead of 0.1, $\varepsilon$ would be $-0.21$. The $SO_4^-$–$Cl^-$ exchange data at high extracellular $Cl^-$, then, are consistent with a mechanism in which most of the charge is transported with $Cl^-$ rather than with $SO_4^-$.

**Potential Dependence of $SO_4^-$ Efflux into 3 mM $Cl^-$**

At an extracellular $Cl^-$ concentration of 3 mM, a membrane potential of $-58$ mV has a more substantial (1.9-fold) accelerating effect on $^{35}SO_4^-$ efflux. At the low $Cl^-$ concentration, the numerical value of $b$ will be much higher than 0.1, because $[C]_o$ appears in the denominator. The rate constant for the initial $^{35}SO_4^-$ efflux into a medium containing 3 mM $Cl^-$, 117 mM K-glutamate, with the membrane potential clamped at near zero with gramicidin, is 0.14–0.17/min (range, four determinations on a total of two preparations of cells). This is 0.25 times the $SO_4^-$ efflux at zero membrane potential from the same cells into a 120 mM $Cl^-$ medium. Therefore, an extracellular $Cl^-$ concentration of 3 mM corresponds to $\sim 0.3$ times the $K_{1/2}$ for extracellular $Cl^-$, similar to the result found in Fig. 4 under slightly different conditions (gluconate rather than glutamate and slightly negative membrane potential). According to the ping-pong mechanism, the $K_{1/2}$ for extracellular $Cl^-$ is related to the real affinity and the translocation rates as follows:

$$K_{1/2} = \frac{K_c k_o[S][S]}{(k_i[S] + k_c(K_s + [S]))}.$$  \hspace{1cm} \text{(A12)}

Because $[C]_o = 0.3 K_{1/2}$, then Eq. A12 can be rewritten with 3.33 $[C]_o$ substituted for $K_{1/2}$. This expression for $[C]_o$ can in turn be substituted into Eq. A11 to give an expression for $b$ at an extracellular $Cl^-$ concentration of $0.3 \times K_{1/2}$:

$$b = \frac{(1.3 k_{in}(S_i) + k_{co}(K_s + [S]))}{0.3 k_{co}(K_s + [S])}. \hspace{1cm} \text{(A13)}$$

As discussed above in the case of high extracellular $Cl^-$, the numerical value of $b$ depends on the ratio of the $Cl^-$ influx translocation rate constant to that for $SO_4^-$ efflux. However, inspection of Eq. A13 shows that, at low extracellular $Cl^-$, the parameter $b$ is not a steeply varying function of the values of the translocation rate constants. For example, if the $Cl^-$ influx rate constant is 10 times as fast as that for $SO_4^-$ efflux, and if $[S]$ is five times $K_s$, the value of $b$ calculated from Eq. A13 is 3.69. If the $Cl^-$ influx rate constant is 20 times that for $SO_4^-$ efflux, then $b = 3.51$. (The dependence on $[S]$ is very weak; it would make very little difference if $[S]$ were, say, twice $K_s$ rather than five times $K_s$.)

Fig. 8 shows the predicted effect of a change in membrane potential from $-5$ mV to $-58$ mV on the $SO_4^-$ efflux into a 3 mM $Cl^-$ medium as a function of the net charge $\varepsilon$ translocated outward with $SO_4^-$, assuming $b = 3.69$. The dependence on $\varepsilon$ is not steep, but the predicted range for $\varepsilon$ between $-0.1$ and $-0.25$ is remarkably close to the experimentally observed ratio of 1.9. The data at low $Cl^-$, then, are again consistent with the idea that a small negative charge traverses the membrane field with $SO_4^-$, and most of the net charge transfer ($\sim 0.8$ charges) is as net positive charge moving inward with $Cl^-$.
ratio of the Cl\(^{-}\) influx to SO\(_{4}\)\(^{2-}\) efflux rate constants was estimated independently (Table II), and the ping-pong model (with most of the charge moving with Cl\(^{-}\)) predicts a 1.8 to 2-fold effect of a \(\sim -55\) mV potential change for all ratios of \(k_{in}/k_{out}\) between 0.001 and 0.2.

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