# 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 $Cl^-$ for 1 $SO_4^=$ and 1 $H^+$. Net $SO_4^=$–$Cl^-$ 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 $BH_4^-$. The studies described here were performed to characterize the electrical properties of net $SO_4^=$–$Cl^-$ exchange in cells modified with WRK/$BH_4^-$. The $SO_4^=$ conductance measured in 100 mM $SO_4^=$ medium is smaller in modified cells than in control cells. However, the efflux of [$^{35}$S] $SO_4^=$ into a 150-mM KCl medium is 80-fold larger in modified cells than in control cells and is inhibited 99% by 10 μM $H_2$DIDS. No detectable $H^+$ flux is associated with $SO_4^=$–$Cl^-$ exchange in modified cells. In the presence of gramicidin to increase the cation permeability, the stoichiometry of $SO_4^=$–$Cl^-$ exchange is not distinguishable from 1:1. In modified cells loaded with $SO_4^=$, the valinomycin-mediated efflux of $^{86}Rb^+$ into an Na-gluconate medium is immediately stimulated by the addition of 5 mM extracellular $Cl^-$. Therefore, $SO_4^=$–$Cl^-$ exchange in modified cells causes an outward movement of negative charge, as expected for an obligatory 1:1 $SO_4^=$–$Cl^-$ 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 $SO_4^=$–$SO_4^=$ exchange and $SO_4^=$–$Cl^-$ 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 $Cl^-$ translocation event, and a small net negative charge moves with $SO_4^=$ during the $SO_4^=$ translocation event. This result suggests that, in normal cells, the negative charge on Glu 681 traverses most of the transmembrane electric field, accompanied by $Cl^-$ and the equivalent of two protein-bound positive charges.

## INTRODUCTION

The rapid exchange of $Cl^-$ for $HCO_3^-$ 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, 1989*b*). 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

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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 $SO_4^=$ (Ku, Jennings, and Passow, 1979). The characteristics of $SO_4^=$ transport are quite different from those of $Cl^-$. For example, $SO_4^=$ 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 $SO_4^=$–$SO_4^=$ exchange (Lepke and Passow, 1971). Another notable difference is pH dependence. Lowering the extracellular pH at neutral intracellular pH inhibits $Cl^-$–$Cl^-$ exchange (Wieth

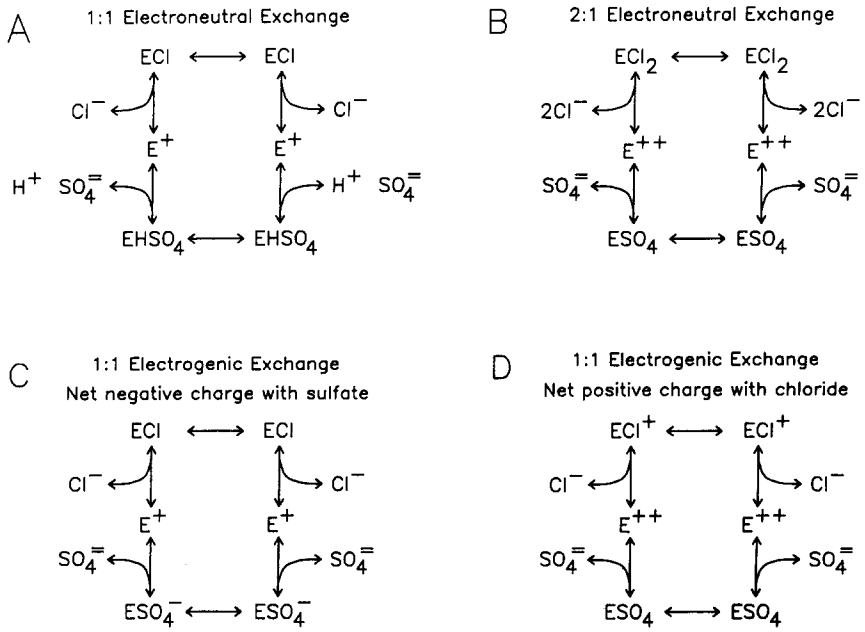


FIGURE 1. Catalytic cycles for the net exchange of $Cl^-$ for $SO_4^=$ by way of a ping-pong mechanism. (*A*) Electroneutral exchange of 1 $Cl^-$ for 1 $SO_4^=$ and 1 $H^+$ by a ping-pong mechanism, as is believed to take place in normal band 3. The other three cycles are possible mechanisms of $Cl^-$–$SO_4^=$ exchange in band 3 modified to remove the $H^+$-titratable group (Glu 681) associated with $H^+$–$SO_4^=$ cotransport. (*B*) Electroneutral exchange of 2 $Cl^-$ for 1 $SO_4^=$. (*C*) Electrogenic exchange of 1 $SO_4^=$ for 1 $Cl^-$, with a net negative charge moving with $SO_4^=$. (*D*) Electrogenic exchange of 1 $SO_4^=$ for 1 $Cl^-$, with a net positive charge moving with $Cl^-$.

and Brahm, 1985; Milanick and Gunn, 1982), and accelerates the influx of $SO_4^=$ into $Cl^-$-loaded cells (Jennings, 1980; Milanick and Gunn, 1984). The opposite effects of pH on $Cl^-$ and $SO_4^=$ 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 $SO_4^=$ at an appreciable rate. As predicted by the titratable carrier model, the net exchange of $Cl^-$ for $SO_4^=$ is accompanied by an obligatory, stoichiometric

cotransport of $H^+$ with $SO_4^=$ (Jennings, 1976; Milanick and Gunn, 1984), depicted schematically in Fig. 1 *A*.

The pK of the protonation event that inhibits $Cl^-$ exchange and accelerates $SO_4^=$ 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 $Cl^-$–$SO_4^=$ exchange, and elimination of the $H^+$ flux that normally accompanies $Cl^-$–$SO_4^=$ exchange (Jennings and Al-Rhaiyel, 1988). The simplest interpretation of these results is that Glu 681 is the titratable residue which, when protonated, activates $SO_4^=$ 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 $Cl^-$–$SO_4^=$ exchange in modified cells could take place as an electroneutral exchange of 2 $Cl^-$ for 1 $SO_4^=$ 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 $SO_4^=$-$H^+$ 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 $SO_4^=$ 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 $H_2SO_4$ 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 ($Na_2{}^{35}SO_4$, $H^{36}Cl$, and $^{86}RbCl$) were from Dupont NEN (Boston, MA).

*Cell Preparation*

Intact cells were treated with 2 mM Woodward's reagent K and $BH_4^-$ 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 $NaBH_4$ 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 $^{36}Cl^-$ 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 $^{36}Cl^-$ (2 μCi/ml cells). For loading with $SO_4^=$ after treatment with WRK/$BH_4^-$, cells were washed three times in at least 20 vol of 100 mM $K_2SO_4$, 10 mM Na-HEPES, pH 7.4. Before each centrifugation in the $K_2SO_4$ medium, the suspension was incubated 10 min at 37°C to allow $SO_4^=$ influx. For loading with [$^{35}S$] $SO_4^=$, cells were then incubated at 30% hematocrit for 1 h at 37°C in 80–100 mM $K_2SO_4$, 10 mM Na-HEPES, pH 7.4 plus 10 μCi/ml [$^{35}S$] $SO_4^=$. For loading with $^{86}Rb^+$, the same procedure was followed except that the last incubation was 90 min, 37°C in 100 mM $Na_2SO_4$, 10 mM HEPES, pH 7.4, 10 mM glucose, and 10 μCi $^{86}Rb^+$/ml. In some experiments, cells were preloaded with $^{86}Rb^+$ by incubation for 90 min in HEPES-buffered physiological saline (10 mM glucose) before chemical modification and $SO_4^=$ loading. Cells equilibrated with 100 mM $K_2SO_4$, pH 7.4, were found to have an intracellular $SO_4^=$ concentration of 50 mM (measured with $^{35}S$), 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 $^{35}SO_4^=$ flux experiments were performed on cells loaded in either 80 mM or 100 mM $K_2SO_4$. In 80 mM $K_2SO_4$, 10 mM Na-HEPES, pH 7.4, the cells have normal water content (0.71 g $H_2O$/ml cells). In 100 mM $K_2SO_4$/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 $K_2SO_4$, 10 mM Na-HEPES were indistinguishable from those measured in 100 mM $K_2SO_4$, 10 mM Na-HEPES.

*Transport Measurements*

The efflux of $^{36}Cl^-$ in cells modified with WRK/$BH_4^-$ 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 $^{35}SO_4^=$ or $^{86}Rb^+$, cells were washed twice in cold 100 mM $K_2SO_4$, 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 $SO_4^=$ 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 $[1 - CPM_o(t)/CPM_o(\infty)]$ was indistinguishable from a straight line over the times that were used in the data analysis (linear regression coefficient r was at least .99 and usually more than .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 $SO_4^=$ 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 $SO_4^=$ 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 $SO_4^=$ efflux into a $Cl^-$ medium in unmodified cells, because the incoming $Cl^-$ causes progressive inhibition of $SO_4^=$ 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 $SO_4^=$ efflux into a $Cl^-$ medium is not distinguishable from a single exponential. We believe that the reason for the exponential efflux is that the affinities of $SO_4^=$ and $Cl^-$ for inward-facing transport sites are similar in modified cells, so that the incoming $Cl^-$ does not strongly inhibit the $SO_4^=$ efflux. A systematic study of the $SO_4^=$ and $Cl^-$ concentration dependences of $SO_4^=$ transport in modified cells is beyond the scope of this paper. For the present purposes, the important point is that the time course of $SO_4^=$ efflux into a $Cl^-$ medium is a single exponential and the efflux may therefore be characterized by a single rate constant.

The influx of $^{36}Cl^-$ was measured in the same media used for $^{35}SO_4^=$ efflux, with $^{36}Cl^-$ at a specific activity of $10^4$ 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 $K_2SO_4$, 20 μM $H_2$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 $SO_4^=$ transport in modified cells, the membrane potential was altered with valinomycin (2 μM; 0.2% ethanol) for $SO_4^=$–$SO_4^=$ exchange or gramicidin (5–10 μg/ml cells; 0.2% ethanol) for $SO_4^=$–$Cl^-$ exchange. To estimate the cation permeability induced by gramicidin, $^{86}Rb^+$ efflux was measured at the same temperature (20°C) and hematocrit (2%) as were used in the anion transport experiments. The rate constant for $^{86}Rb^+$ 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 $^{86}Rb^+$ efflux should be at least 7/min. This is 10 times higher than the rate constant for $SO_4^=$ efflux into a $Cl^-$ 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 $SO_4^=$ efflux into a high $Cl^-$ medium, a correction is necessary to account for the fact that the $SO_4^=$–$Cl^-$ 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 gramidicin, $Na^+$ 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 $SO_4^=$–$Cl^-$ exchange flux contributes to the membrane potential similarly to a net monovalent anion efflux with rate constant 0.7/min. (*e*) The $Cl^-$ 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 $P_K[K^+]_i$ term, which is quite large.

For example, in a medium containing 15 mM $K^+$ + $Na^+$, 120 mM $Cl^-$, and cells with 40 mM intracellular $SO_4^=$, the membrane potential is given by:

$$V_m = -25.3 \ln \frac{[10 \times 150]}{[(10 \times 15) + (0.7 \times 40)]} = -54 \text{ mV}. \quad (1)$$

For $SO_4^=$–$Cl^-$ exchange at low (3 mM) extracellular $Cl^-$ concentration, the expected contribution of $SO_4^=$–$Cl^-$ exchange to the membrane potential is very small (~1 mV) and has been ignored.

### *Estimates of Anion Conductance*

The $Cl^-$ and $SO_4^=$ 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 KCl, 150 mM NaCl, 100 mM $Na_2SO_4$, or 100 mM $K_2SO_4$. 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:

$$k_{Rb} = k^o_{Rb}[FV/FT]/(1 - \exp[-FV/RT]), \quad (2)$$

where $V$ is the membrane potential, $k^o_{Rb}$ 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):

$$FV/RT = -\ln B, \quad (3)$$

where

$$B = (P_K K_i + P_{Cl}Cl_o)/(P_K K_o + P_{Cl}Cl_i), \quad (4)$$

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 $k^o_{Rb}$ 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 $SO_4^=$ medium, the expression for $B$ is much more complex (Knauf et al., 1977). In a $K^+$-free medium,

$$B = \frac{P_K K_i + (P_K^2 K_i^2 + 16 P_S S_i [P_K K_i + 4 P_S S_o])^{1/2}}{8 P_S S_i}, \quad (5)$$

where $P_S$ is the conductive $SO_4^=$ permeability coefficient and $S_o$ and $S_i$ are respectively the extracellular and intracellular $SO_4^=$ activities. From the rate constants for $^{86}Rb^+$ efflux into $K_2SO_4$ and $Na_2SO_4$ media, Eq. 5 was used to estimate the ratio of $P_K$ to $P_S$ 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

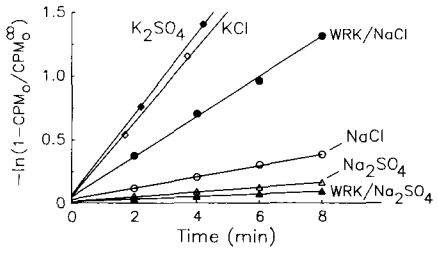


FIGURE 2. Time course of $^{86}Rb^+$ efflux into KCl, NaCl, or $Na_2SO_4$ media at 20°C in the presence of 2 μM valinomycin (0.2% ethanol). Cells were loaded with $^{86}Rb^+$ by incubation in physiological saline, then washed and treated at 0°C with or without 2 mM WRK/$BH_4^-$ in 150 mM KCl/10 mM MOPS, pH 7. Half of each suspension (control and treated) was then loaded with $SO_4^=$ by washing in 100 mM $Na_2SO_4$, 10 mM MOPS, pH 7. Finally, $Cl^-$-loaded cells were suspended in 150 mM KCl or NaCl, 10 mM MOPS, pH 7, and $SO_4^=$-loaded cells were suspended in 100 mM $K_2SO_4$ or $Na_2SO_4$, 10 mM MOPS, pH 7. Valinomycin was added at $t = 0$. The efflux of $^{86}Rb^+$ into KCl and $K_2SO_4$ media are shown for control cells, but the efflux is unaffected by pretreatment with WRK/$BH_4^-$.

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 $SO_4^=$ permeability.

*Acceleration of $SO_4^=$–$SO_4^=$ and $SO_4^=$–$Cl^-$ Exchange by WRK/$BH_4^-$*

Previous work showed that WRK/$BH_4^-$ treatment causes an acceleration of $SO_4^=$–$SO_4^=$ exchange and of $SO_4^=$ 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 $SO_4^=$–$SO_4^=$ exchange and $Cl^-$–$SO_4^=$ exchange ($SO_4^=$ influx) is 5–10-fold. We found recently that the acceleration of $Cl^-$–$SO_4^=$ exchange is far larger when measured as $^{35}SO_4^=$–$Cl^-$ exchange ($SO_4^=$ efflux). Fig. 3 depicts the initial efflux of $^{35}SO_4^=$ from control and treated cells suspended in sucrose, $SO_4^=$, or $Cl^-$ media. The $^{35}SO_4^=$ efflux into a $SO_4^=$ medium is severalfold higher in treated cells than in

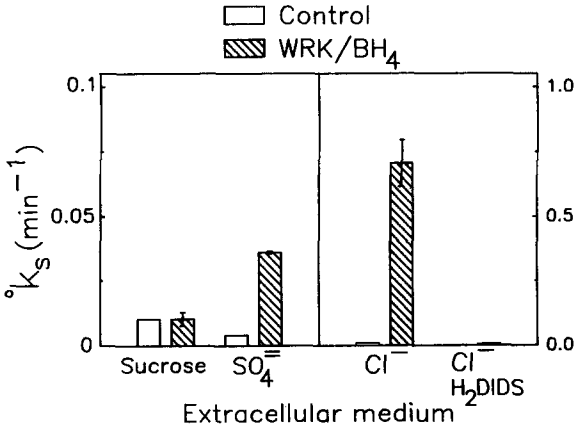


FIGURE 3. Effect of treatment with WRK/$BH_4^-$ on $^{35}SO_4^=$ efflux into $SO_4^=$ or $Cl^-$ media. Cells were loaded with $^{35}SO_4^=$ at Donnan equilibrium in 80 mM $K_2SO_4$, 10 mM HEPES pH 7.4, and the efflux was measured in the same medium or in 120 mM KCl, 10 mM HEPES pH 7.4, 20°C. Each bar represents two flux determinations in a single preparation of cells. The vertical scale for $SO_4^=$–$Cl^-$ exchange (right) is a factor of 10 larger than that for $SO_4^=$–$SO_4^=$ exchange. The large acceleration of $SO_4^=$–$Cl^-$ exchange by pretreatment with WRK/$BH_4^-$ was observed in each of at least 10 separate preparations.

control cells, in agreement with previous results at slightly lower extracellular pH (Jennings and Al-Rhaiyel, 1988). The initial $^{35}SO_4^=$ efflux into a $Cl^-$ medium is *much* larger (80-fold) in treated cells than in control cells; the flux is inhibited 99% by 10 μM $H_2$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 $H^+$–$SO_4^=$ Cotransport*

In normal red cells the exchange of $SO_4^=$ for $Cl^-$ is accompanied by an obligatory cotransport of $H^+$ with $SO_4^=$ (Jennings, 1976). This $H^+$ flux is most readily detectable

during $SO_4^=$ influx into $Cl^-$-containing cells at low extracellular pH (Jennings, 1976; Milanick and Gunn, 1984). A net $H^+$ cotransport with $SO_4^=$ has also been demonstrated during initial $SO_4^=$ efflux into a $Cl^-$ medium (Berghout, Raida, Legrum, and Passow, 1988).

In WRK/$BH_4^-$-modified band 3, no $H^+$ flux associated with net $SO_4^=$–$Cl^-$ exchange ($SO_4^=$ influx) was detectable (Jennings and Al-Rhaiyel, 1988). To determine whether an $H^+$ flux accompanies $SO_4^=$ efflux, cells were treated with WRK/$BH_4^-$, loaded with $SO_4^=$ 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 $SO_4^=$–$Cl^-$ exchange in modified cells is remarkably stable (data not shown). Any $H^+$ flux associated with $SO_4^=$–$Cl^-$ exchange in these cells, if present at all, has a magnitude $<1\%$ of the $SO_4^=$ efflux. To a first approximation, then, the $SO_4^=$ efflux in modified cells is not accompanied by $H^+$–$SO_4^=$ cotransport, in agreement with earlier results for $SO_4^=$ influx (Jennings and Al-Rhaiyel, 1988).

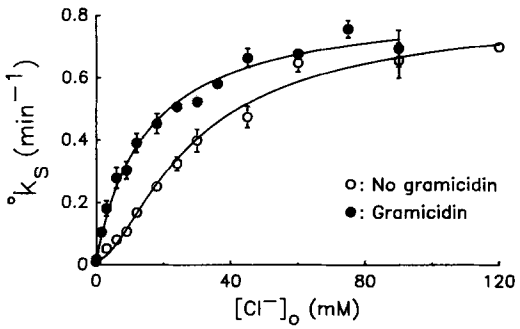


FIGURE 4. Effect of increasing extracellular $Cl^-$ concentrations on the rate constant ($min^{-1}$) for $^{35}SO_4^=$ efflux from cells pretreated with WRK/$BH_4^-$. Cells were initially at Donnan equilibrium in 100 mM $K_2SO_4$, 10 mM HEPES, pH 7.4, and $^{35}SO_4^=$ efflux was measured in mixtures of 250 mM sucrose and 150 mM KCl, 10 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 $^{35}SO_4^=$ 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 $SO_4^=$ efflux in treated cells is that $SO_4^=$–$Cl^-$ 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 $SO_4^=$ efflux is a simple, saturable function of extracellular $Cl^-$, with half-saturation at ~10 mM (Fig. 4). At low extracellular $Cl^-$ concentrations, gramicidin accelerates $SO_4^=$ efflux by a factor of 3–4. The relatively slow exchange flux at low extracellular $Cl^-$ in the absence of gramicidin suggests that $SO_4^=$–$Cl^-$ exchange is electrogenic and generates

a positive (inside) membrane potential, which slows down the exchange. At high extracellular $Cl^-$, there is little effect of gramicidin, because an inward conductive $Cl^-$ flux balances the outward flux of negative charge associated with $SO_4^=$–$Cl^-$ exchange.

*Stoichiometry of $SO_4^=$–$Cl^-$ Exchange*

An attempt was made to determine the stoichiometry of $SO_4^=$–$Cl^-$ exchange by measuring the $SO_4^=$ efflux and $Cl^-$ influx in the same cell preparations. The $Cl^-$ influx was measured using an inhibitor stop method with $H_2$DIDS. In the absence of ionophore, the global stoichiometry of the exchange is expected to be 2 $Cl^-$: 1 $SO_4^=$ because of the lack of $H^+$ flux and the fact that $Na^+$ and $K^+$ fluxes are much smaller than the $SO_4^=$ and $Cl^-$ fluxes. That is, electroneutrality requires that the overall stoichiometry be 2 $Cl^-$: 1 $SO_4^=$, which could result from an obligatory 1 $Cl^-$: 1 $SO_4^=$ electrogenic exchange in parallel with a $Cl^-$ conductance, both catalyzed by band 3. The measured stoichiometry is slightly $<2$ $Cl^-$: 1 $SO_4^=$, probably because of a systematic underestimate of the $Cl^-$ influx (Table I) caused by losses of $Cl^-$ during

TABLE I

*Stoichiometry of $SO_4^=$–$Cl^-$ Exchange in Cells Pretreated with WRK/$BH_4^-$*

| Gramicidin | Extracellular | $SO_4^=$ Efflux | $Cl^-$ Influx | $J_c/J_{SO_4}^*$ |
|---|---|---|---|---|
| | $Cl^-$ *mM* | *μmol/ml-min* | *μmol/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 |

Values are from a single determination of initial $^{35}SO_4^=$ efflux and $^{36}Cl^-$ influx (3–4 time points each) in parallel preparations of cells on the same day. Cells were treated with 2 mM WRK/$BH_4^-$ and loaded with $SO_4^=$ in 100 mM $K_2SO_4$, 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 μg/ml suspension (5 μg/ml cells). Ethanol (0.1%) was present in both control and gramicidin-containing suspensions.

*Ratio of $Cl^-$ influx to $SO_4^=$ efflux.

washing in $H_2$DIDS stop solution. Gluconate influx is not likely to account for the observed stoichiometry of $<2$:1, because the $SO_4^=$ efflux into $Cl^-$-free gluconate medium is quite small (Fig. 4). In any case, given the difficulties of measuring $Cl^-$ influx in red cells, the stoichiometry of $SO_4^=$–$Cl^-$ exchange is reasonably close to the expected value of 2:1 in the absence of ionophore.

When the membrane permeabilities to $K^+$ and $Na^+$ are increased by adding gramicidin, the stoichiometry of the exchange is close to 1 $Cl^-$:1 $SO_4^=$ (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 $K^+$ flux mediated by gramicidin. In the absence of ionophore, the charge is balanced by an inward conductive $Cl^-$ flux (also through band 3), resulting in a global stoichiometry of 2:1.

### *Outward Movement of Negative Charge During $SO_4^=$–$Cl^-$ Exchange*

We attempted to estimate membrane potential in the presence of opposing $SO_4^=$ and $Cl^-$ gradients in treated cells, using the fluorescent dye Di-S-$C_3$(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 protonphore CCCP. Neither method is satisfactory in modified cells at the temperature (20°C) used here, because the opposing $SO_4^=$ and $Cl^-$ gradients dissipate before a reliable potential can be measured.

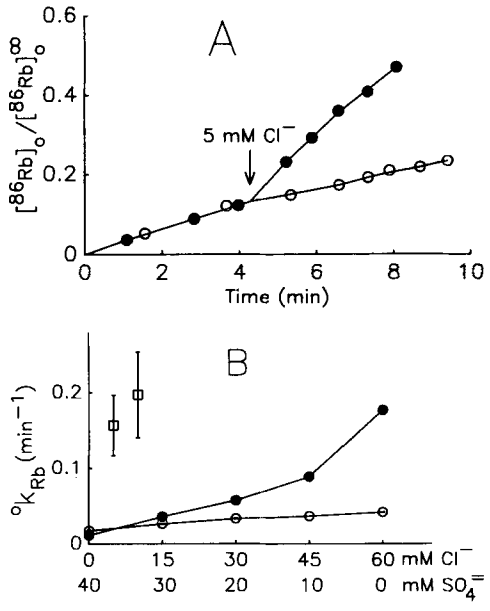


FIGURE 5. Demonstration of net charge transport during $SO_4^=$–$Cl^-$ exchange in cells pretreated with WRK/$BH_4^-$. Cells were treated with (*filled symbols*) or without (*open symbols*) WRK/$BH_4^-$ and loaded with $^{86}Rb^+$. (*A*) Cells were equilibrated with a medium consisting of 80 mM $K_2SO_4$, 10 mM HEPES, pH 7.4 and then resuspended at 20°C in 150 mM Na-gluconate, 10 mM HEPES, pH 7.4; valinomycin was added at $t = 0$. At the indicated time, 5 mM NaCl was added to initiate exchange of internal $SO_4^=$ for external $Cl^-$. The data are from a single experiment, representative of a total of five pairs of fluxes.

(*B*) Demonstration of low total anion conductance in cells containing $SO_4^=$ and 0–15 mM $Cl^-$. Cells were treated with (*filled symbols*) or without (*open symbols*) WRK/$BH_4^-$, loaded with $^{86}Rb^+$, and equilibrated with media consisting of 60 mM Na-gluconate, 10 mM HEPES, pH 7.4, and mixtures of 60 mM NaCl and 40 mM $Na_2SO_4$. Valinomycin was added and the rate constant for $^{86}Rb^+$ efflux was measured. Data represent the mean and range of two determinations on a single preparation of cells. For comparison, the initial rates of $^{86}Rb^+$ efflux after adding 5 mM or 10 mM $Cl^-$ to $SO_4^=$-loaded cells in a gluconate medium (conditions of Fig. 5 *A*) are plotted (*squares;* mean and range of two determinations at each $Cl^-$ concentration). The intracellular $Cl^-$ concentration during net $SO_4^=$–$Cl^-$ exchange increased with time, but an initial flux could be measured before the intracellular $Cl^-$ was larger than 15 mM.

As an alternate approach to testing the idea that net $SO_4^=$–$Cl^-$ exchange in modified cells is electrogenic, WRK/$BH_4^-$-treated cells were loaded with $^{86}Rb^+$ and nonradioactive $SO_4^=$. Efflux of $^{86}Rb^+$ was measured into an Na-gluconate/sucrose medium in the presence of valinomycin (Fig. 5, *top*). Efflux is initially slow because the $SO_4^=$ conductance is low, and the outward $K^+$ gradient generates a large negative membrane potential. In control cells the addition of 5 mM NaCl has no effect on the efflux of $^{86}Rb^+$. In treated cells, however, the addition of 5 mM NaCl causes an

immediate increase in the efflux of $^{86}Rb^+$, indicating that $Cl^-$ influx into $SO_4^=$-loaded cells causes net efflux of negative charge.

It is significant that, after addition of extracellular $Cl^-$, the rate of $^{86}Rb^+$ efflux increases without any detectable delay and then does not increase further as the $SO_4^=$–$Cl^-$ exchange proceeds (Fig. 5, *top*). The accelerating effect of $Cl^-$ on $^{86}Rb^+$ efflux therefore, is not a consequence of an outward conductive $Cl^-$ after accumulation of intracellular $Cl^-$. To estimate the magnitude of the outward $Cl^-$ conductive flux under these conditions, cells were equilibrated with media consisting of 60 mM gluconate and mixtures of 60 mM NaCl and 40 mM $Na_2SO_4^=$. The valinomycin-mediated $^{86}Rb^+$ 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 $^{86}Rb^+$ efflux as the $Cl^-$ concentration increases at the expense of $SO_4^=$, as expected from Fig. 2. The effect of $Cl^-/SO_4^=$ replacement is rather nonlinear in treated cells. That is, in the absence of net anion exchange, the valinomycin-mediated $^{86}Rb^+$ efflux is far slower at $Cl^-$ concentrations of 0–30 mM than that

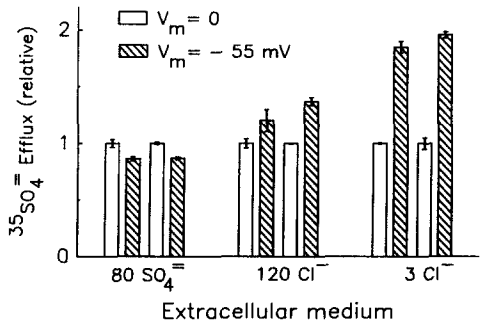


FIGURE 6. Effect of gramicidin-mediated changes in membrane potential on $^{35}SO_4^=$ efflux into $SO_4^=$, low $Cl^-$, or high $Cl^-$ media. In all cases, cells were pretreated with WRK/$BH_4^-$ and loaded with $^{35}SO_4^=$ at Donnan equilibrium in 80 mM $K_2SO_4$, 10 mM HEPES, pH 7.4. Efflux of $^{35}SO_4^=$ was measured in media containing 80 mM $SO_4^=$, 120 mM $Cl^-$, or 3 mM $Cl^-$ (117 mM glutamate). For each, the membrane potential was varied by replacing all but 10 mM $K^+$ with $NMG^+$. All extracellular media contained 5 mM $Na^+$, added as the hemisodium salt of 10 mM HEPES. (*Open bars*) $V_m = 0 \pm 5$ mV. (*Hatched bars*) $V_m = -55 \pm 5$ mV. Each bar represents 2–4 fluxes (±SD) on a single preparation of cells.

observed during net $SO_4^=$ efflux into a $Cl^-$ containing medium. The rapid $^{86}Rb^+$ 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, $^{35}SO_4^=$ 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 $^{35}SO_4^=$ efflux into the 80 mM $SO_4^=$ medium (Fig. 6). A negative potential accelerates $^{35}SO_4^=$ 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 $SO_4^=$ pathway (see below). In a 3 mM $Cl^-$ medium, the acceleration of $^{35}SO_4^=$ 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 $SO_4^=$–$Cl^-$ 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 $SO_4^=$ translocation event, as discussed below.

*Asymmetry of $SO_4^=$–$Cl^-$ Exchange in Control and Modified Cells*

To analyze the effect of membrane potential on $SO_4^=$–$SO_4^=$ 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, $SO_4^=$ transport is fairly

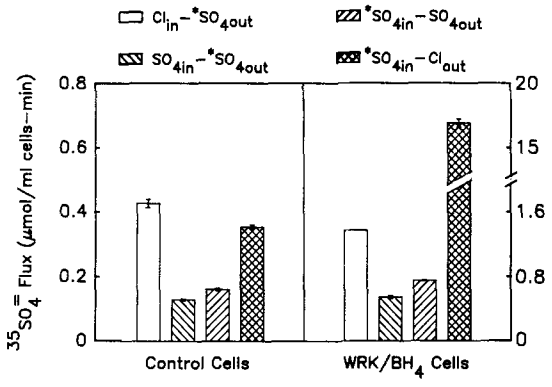


FIGURE 7. Asymmetry of $SO_4^=$–$Cl^-$ exchange in control cells and those pretreated with WRK/$BH_4^-$. Cells were pretreated in KCl medium as usual and then incubated in either 60 mM KCl, 120 mM sucrose, or 40 mM $K_2SO_4$, 120 mM sucrose, each buffered with 10 mM HEPES, pH 7.4. In the $SO_4^=$ medium the Donnan ratio is very close to unity (intracellular $SO_4^=$ concentration is 40 mM). The equilibrium exchange flux of $SO_4^=$ was measured in the 40 mM $K_2SO_4$ medium as either $^{35}SO_4^=$ influx or efflux. The $^{35}SO_4^=$ influx was also measured into $Cl^-$-loaded cells, and the $^{35}SO_4^=$ efflux was measured in the 60 mM KCl/sucrose medium.

symmetric: the initial $SO_4^=$ efflux into a $Cl^-$ medium is similar to the initial $SO_4^=$ influx into $Cl^-$-loaded cells (Fig. 7). The initial $SO_4^=$–$Cl^-$ exchange flux in each direction is ~2.5–3.5 times the $SO_4^=$–$SO_4^=$ exchange flux at Donnan equilibrium in the same medium. In modified cells, the initial $SO_4^=$ influx into $Cl^-$-loaded cells is about twice the $SO_4^=$–$SO_4^=$ exchange flux, but the initial $SO_4^=$ efflux into a $Cl^-$ medium is ~20 times the $SO_4^=$–$SO_4^=$ exchange flux. The asymmetry in the $SO_4^=$–$Cl^-$ exchange in modified cells indicates that the $SO_4^=$ efflux translocation rate constant is ~10 times faster than that for influx. This 10-fold asymmetry in $SO_4^=$ 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 $SO_4^=$–$Cl^-$ exchange in modified cells, it is necessary to estimate the relative magnitudes of the $SO_4^=$ and $Cl^-$ translocation

events. In normal cells, $Cl^-$–$Cl^-$ exchange is over 10,000-fold faster than that of $SO_4^=$ at pH 7.4 (Brahm, 1977; Lepke and Passow, 1971), but modification by WRK/$BH_4^-$ accelerates $SO_4^=$ transport and inhibits $Cl^-$ exchange. The rate of $Cl^-$–$Cl^-$ 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 $Cl^-$–$Cl^-$ exchange flux. To try to estimate the $Cl^-$–$Cl^-$ exchange flux through modified band 3, the WRK/$BH_4^-$ 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 $Cl^-$–$Cl^-$ exchange by WRK/$BH_4^-$. For example, a single treatment with 2 mM WRK/$BH_4^-$ inhibits $Cl^-$–$Cl^-$ exchange (measured at 0°C) by ~79% (data not shown); a very

TABLE II

*Effect of Multiple Treatments with Woodward's Reagent K on $Cl^-$–$Cl^-$ and $SO_4^=$–$Cl^-$ Exchange Fluxes at 20°C*

| Treatment of cells* | $Cl^-$–$Cl^-$ flux‡ | $SO_4^=$–$Cl^-$ flux§ |
|---|---|---|
| None | 8445‖ | 0.26 ± 0.1 (3) |
| WRK/WRK/wash/$BH_4^-$ | 376 ± 28 (6) | 17 ± 4 (6) |
| WRK/WRK/wash/WRK/WRK/wash/$BH_4^-$ | 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°, 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°. "Wash" indicates two centrifugations from KCl/MOPS, pH 7, at 0°C. $BH_4^-$ was added to a final concentration of 2 mM to the cold suspension in KCl/MOPS followed by 5 min at 0°C.

‡$Cl^-$–$Cl^-$ 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 $Cl^-$ compartment. Data represent mean ± SD (*n*).

§Cells were loaded with $^{35}SO_4^=$ in 80 or 100 mM $K_2SO_4$, 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*).

‖$Cl^-$–$Cl^-$ exchange flux in control cells at 20°C is from Brahm (1977), Table II*a* (23.8 mol/3.1 × $10^{13}$ cells/min).

¶Flux in these cells was inhibited a further 97% by 10 μM $H_2$DIDS in the flux medium.

similar inhibition (76–78%) of $Cl^-$-$Br^-$ exchange was found previously (Jennings and Al-Rhaiyel, 1988). The residual $Cl^-$–$Cl^-$ exchange flux is inhibited 76% by a second treatment at the same WRK/$BH_4^-$ 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/$BH_4^-$ 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 $SO_4^=$ transport (data not shown). We found, however, that repeat treatments with WRK alone, with $BH_4^-$ 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 $SO_4^=$ transport (Table II). In cells treated with two additions of 2 mM WRK, followed after two washes by $BH_4^-$, the

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 × 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 μM $H_2$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 × 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 μmol/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 $SO_4^=$–$Cl^-$ exchange flux in modified band 3. The relative magnitudes of $SO_4^=$–$Cl^-$ and $Cl^-$–$Cl^-$ exchange are used below in the analysis of the effect of membrane potential on $SO_4^=$–$Cl^-$ exchange.

## DISCUSSION

The results presented here define some of the characteristics of band 3-mediated net $SO_4^=$–$Cl^-$ 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, $SO_4^=$–$Cl^-$ exchange is an electroneutral exchange of 1 $Cl^-$ for 1 $SO_4^=$ + 1 $H^+$ (Jennings, 1976). In modified cells the stoichiometry is also 1:1 (Table I), but there is no $H^+$ cotransported with $SO_4^=$, implying that the exchange is electrogenic. Further evidence for electrogenicity of $SO_4^=$–$Cl^-$ exchange is that the addition of extracellular $Cl^-$ accelerates the valinomycin-mediated $^{86}Rb^+$ efflux from $SO_4^=$-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 $SO_4^=$ rather than a $SO_4^=$ efflux tightly coupled to $Cl^-$ influx. However, the effect of membrane potential on the $SO_4^=$ 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 $SO_4^=$. A change in potential from 0 to −50 mV should increase the unidirectional $SO_4^=$ 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, $SO_4^=$–$Cl^-$ 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 $Na^+$-$Ca^{++}$) 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 $SO_4^=$ 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 $SO_4^=$ ($+H^+$) (Milanick and Gunn, 1984; Jennings, Allen, and Schulz, 1990; Jennings, 1989*a*). In modified cells, the two simplest mechanisms for electrogenic $SO_4^=$–$Cl^-$ exchange are the following: net negative charge is transported outward with $SO_4^=$, or net positive

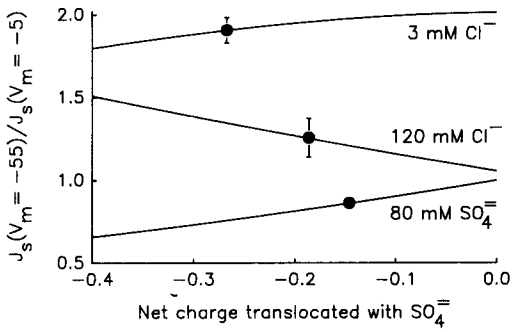


FIGURE 8. Comparison of the $SO_4^=$ efflux data (*solid symbols*) from Fig. 6 with the predictions of a ping-pong mechanism, with various amounts of net negative charge translocated through the transmembrane potential with $SO_4^=$. The data are plotted as the ratio of the $SO_4^=$ efflux at ~ −55 mV to that at ~ −5 mV. The calculated pairs of membrane potentials (in millivolts) for the three kinds of experiments are the following: 80 mM $K_2SO_4$, +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 $SO_4^=$, 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 $SO_4^=$, then 0.8 positive charge is transported with $Cl^-$, et cetera. 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 $SO_4^=$.

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 $^{35}SO_4^=$ efflux into media containing 80 mM $SO_4^=$ (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 $^{35}SO_4^=$ 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 $Cl^-$.

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 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 mV has a slight (25%) accelerating effect on $^{35}SO_4^=$ 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 $Na^+$, $K^+$-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 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 electroneutral 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 $SO_4^=$ 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 $SO_4^=$ 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-$SO_4^=$ medium.

It is of interest to compare the apparent asymmetry of $SO_4^=$ 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 $SO_4^=$ 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 $SO_4^=$ influx into $Cl^-$-containing cells is $\sim 10$ times larger than the initial net $SO_4^=$ 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 $SO_4^=$ 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 carbodiimides (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 $SO_4^=$ 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 $SO_4^=$–$Cl^-$ exchange can explain the *trans* acceleration of $SO_4^=$ 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 $SO_4^=$–$Cl^-$ exchange in modified cells. A simple simultaneous mechanism also cannot account for the small (but very consistent) inhibition of $SO_4^=$–$SO_4^=$ 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 $SO_4^=$–$Cl^-$ exchange quite readily, the fact remains that a simple ping-pong mechanism cannot account for the magnitudes of the $SO_4^=$–$Cl^-$ exchange fluxes relative to the $SO_4^=$–$SO_4^=$ exchange flux (Fig. 7) in modified cells. Replacement of $SO_4^=$ with $Cl^-$ must cause acceleration of the unidirectional $SO_4^=$ 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 $SO_4^=$ has a larger effect than occupancy with $Cl^-$. Replacement of $SO_4^=$ with $Cl^-$ would relieve some of the inhibition. It is of interest in this context that addition of bilateral $Cl^-$ (10–20 mM) accelerates $SO_4^=$ 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 $SO_4^=$ transport by a mechanism other than recruitment. Bilateral acetate has a similar effect. An anion-activated $SO_4^=$–$SO_4^=$ 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 $^{35}SO_4^=$ flux in modified human band 3 is still highly sensitive to stilbenedisulfonate derivatives.

A complete characterization of the kinetics of $SO_4^=$–$Cl^-$ and $SO_4^=$–$SO_4^=$ 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 $^{35}SO_4^=$ flux by $Cl^-$. On the other hand, a ping-pong model accounts extremely well for the effects of membrane potential on $^{35}SO_4^=$–$SO_4^=$ exchange and on $^{35}SO_4^=$–$Cl^-$ 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 ($SO_4^=$ 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 $SO_4^=$–$Cl^-$ 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 $SO_4^=$–$Cl^-$ exchange in WRK/$BH_4^-$-treated cells, the data in Figs. 2 and 5 should be discussed in reference to the $SO_4^=$ conductance mechanism in normal and treated cells. Treatment with WRK/$BH_4^-$ inhibits $SO_4^=$ conductance by ~50% when it is measured in 100 mM $SO_4^=$, but there is no detectable inhibition of conductive $SO_4^=$ efflux into a gluconate medium (Fig. 5, *top, first three data points*). One possible explanation for the inhibition of conductance in a $SO_4^=$ medium is that extracellular $SO_4^=$ inhibits outward $SO_4^=$ 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 $Cl^-$ conductance through normal band 3 (see Fröhlich, 1984), but slippage could contribute to $SO_4^=$ conductance in modified cells. Inhibition of $SO_4^=$ conductance by extracellular $SO_4^=$ 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 $Cl^-$ in modified cells implies that the $Cl^-$ 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 $Cl^-$, 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 Angstroms. 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 $BH_4^-$, indicating that it is accessible from the extracellular side of the permeability barrier. The functional effects of WRK/$BH_4^-$ 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/$BH_4^-$ through a channel leading to the extracellular medium.

## APPENDIX

### *Effect of Membrane Potential on* $SO_4^=$–$SO_4^=$ *and* $SO_4^=$–$Cl^-$ *Exchange*

The following expression for the initial efflux of $^{35}SO_4^=$ from WRK/$BH_4^-$-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, $SO_4^=$ 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 $SO_4^=$, 120 mM $Cl^-$, or 3 mM $Cl^-$. The initial efflux of $^{35}SO_4^=$ is:

$$J_s = \frac{k_s[S]_i k_a[A]_o}{k_s[S]_i(K_a + [A]_o) + k_a[A]_o(K_s + [S]_i)}, \tag{A1}$$

where $J_s$ is the $SO_4^=$ efflux (ions per transporter per minute); $k_s$ is the unimolecular rate constant ($min^{-1}$) for outward $SO_4^=$ translocation; $k_a$ is the unimolecular rate constant ($min^{-1}$) for the inward translocation of $A$; $[S]_i$ is the intracellular $SO_4^=$ concentration (activity); $[A]_o$ is the extracellular concentration of $A$; and $K_s$ and $K_a$ are respectively the dissociation constants for $SO_4^=$ binding to the inward-facing and $A$ binding to outward-facing transport sites.

### *Effect of Potential on* $SO_4^=$–$SO_4^=$ *Exchange*

The $SO_4^=$ concentration is far larger than the $K_{1/2}$ for equilibrium exchange in modified cells (1–2 mM, estimated from $^{35}SO_4^=$–$SO_4^=$ exchange at equilibrium in $SO_4^=$/acetate mixtures; data not shown). Therefore, although there is a detectable effect of membrane potential on the apparent affinity for extracellular $SO_4^=$ 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 $SO_4^=$–$SO_4^=$ exchange flux at this $SO_4^=$ 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 $SO_4^=$ translocation event. Assuming a symmetric barrier, the potential dependence of the unimolecular rate constant for $SO_4^=$ efflux is:

$$k_s = k_{so}\exp(\epsilon FV/2RT), \tag{A2}$$

where $k_{so}$ is the rate constant for outward $SO_4^=$ 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 $^{35}SO_4^=$ (Eq. A1) applies for any exchange partner $A$. When $A$ is $SO_4^=$, the dependence of the inward translocation rate constant on potential is the following:

$$k_a = k_{ao}\exp(-\epsilon FV/2RT), \tag{A3}$$

where $k_{ao}$ is the unimolecular rate constant for $SO_4^=$ influx at zero membrane potential.

Eqs. A2 and A3 can be substituted into Eq. A1 to give an expression for the $^{35}SO_4^=$ efflux into an 80 mM $SO_4^=$ 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 $SO_4^=$ efflux into a $Cl^-$ medium is 10 times the initial $SO_4^=$ influx into $Cl^-$-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 $k_{ao}$. With Eqs. A2 and A3 substituted into A1, and the estimate that $k_{so}$ is 10 times $k_{ao}$, the expression for the flux becomes:

$$J_s = k_{so}/[10\exp(\epsilon FV/2RT) + \exp(-\epsilon FV/2RT)]. \quad \text{(A4)}$$

The observed 14% inhibition of $SO_4^=$ efflux by a potential change of $-60$ mV (from $+2$ to $-58$ mV) is consistent with a value of $\epsilon$ of $-0.15$ (Fig. 8). That is, a net negative charge of 0.15 traverses the transmembrane field during the $SO_4^=$ 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 $\epsilon$ is $-0.18$ instead of $-0.15$.

*Effect of Potential on $SO_4^=$ Efflux into a $Cl^-$ Medium*

If the exchange partner for intracellular $^{35}SO_4^=$ is $Cl^-$ rather than $SO_4^=$, a net positive charge enters the cells with each complete catalytic cycle. Therefore, if a charge of $\epsilon$ leaves the cells during $SO_4^=$ efflux, then a charge of $1 + \epsilon$ must enter the cells during $Cl^-$ influx, giving an overall net inward movement of one positive charge. Therefore, the potential dependence of inward $Cl^-$ translocation ($k_c$) is given by:

$$k_c = k_{co}\exp(-[1 + \epsilon]FV/2RT). \quad \text{(A5)}$$

The right side of Eq. (A5) can be substituted for $k_a$ in Eq. A1 to give an expression for the potential dependence of the initial $SO_4^=$ efflux into a medium in which $Cl^-$ is the only permeant anion. In contrast to the situation for $SO_4^=$–$SO_4^=$ 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:

$$J_s = \frac{k_{so}\exp(\epsilon FV/2RT)[S]_i k_{co}\exp(-[1 + \epsilon]FV/2RT)[C]_o}{k_{so}\exp(\epsilon FV/2RT)[S]_i(K_c + [C]_o) + k_{co}\exp(-[1 + \epsilon]FV/2RT)[C]_o(K_s + [S]_i)}, \quad \text{(A6)}$$

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

The efflux of $^{35}SO_4^=$ into 120 mM $Cl^-$ was measured at two membrane potentials: $-4$ mV (120 mM $K^+$ and 5 mM $Na^+$) and $-54$ mV (10 mM $K^+$ and 5 mM $Na^+$). The $^{35}SO_4^=$ efflux at $-4$ mV is (from Eq. A6):

$$J_s = \frac{k_{so}\exp(-.080\,\epsilon)[S]_i k_{co}\exp(.080\,[1 + \epsilon])[C]_o}{k_{so}\exp(-.080\,\epsilon)[S]_i(K_c + [C]_o) + k_{co}\exp(.080[1 + \epsilon])[C]_o(K_s + [S]_i)}. \quad \text{(A7)}$$

The $^{35}SO_4^=$ efflux from the same cells at a membrane potential of −54 mV is:

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

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:

$$\frac{\exp(0.986)[k_{so}\exp(-.080\,\epsilon)[S]_i(K_c+[C]_o)+k_{co}\exp(.080[1+\epsilon])[C]_o(K_s+[S]_i)]}{k_{so}\exp(-1.066\,\epsilon)[S]_i(K_c+[C]_o)+k_{co}\exp(1.066[1+\epsilon])[C]_o(K_s+[S]_i)}. \quad \text{(A9)}$$

This expression can be rewritten in the following form:

$$J_s(-54)/J_s(-4) = \frac{\exp(0.986)[b\exp(-.080\,\epsilon)+\exp(.080[1+\epsilon])]}{b\exp(-1.066\,\epsilon)+\exp(1.066[1+\epsilon])}, \quad \text{(A10)}$$

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

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

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 $Cl^-$ and 40 mM intracellular $SO_4^=$, the factors $(K_c + [C]_o)/[C]_o$ and $[S]_i/(K_s + [S]_i)$ should be close to unity, because the $Cl^-$ and $SO_4^=$ 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 $SO_4^=$ 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 $SO_4^=$–$Cl^-$ vs $Cl^-$–$Cl^-$ exchange rates in modified cells at the same temperature, we estimate that the parameter $b$ is roughly 0.1, because the $Cl^-$–$Cl^-$ exchange rate is ~10 times the $SO_4^=$–$Cl^-$ exchange rate (Table II). The basis of this estimate for $b$ is that the influx limb of the catalytic cycle for $Cl^-$–$Cl^-$ exchange must be no slower than the overall turnover number. Since the turnover number for $Cl^-$–$Cl^-$ exchange is ~10 times that for $SO_4^=$–$Cl^-$ exchange (Table II), the influx translocation rate constant for $Cl^-$ must be at least 10 times the efflux translocation rate constant for $SO_4^=$. 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 $SO_4^=$ translocation and a net influx of +0.8 charges during inward $Cl^-$ 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 $SO_4^=$–$SO_4^=$ exchange.

It should be emphasized that the actual magnitude of the lumped parameter $b$ is not known because the overall $Cl^-$–$Cl^-$ exchange flux does not allow an estimate of the individual rate constant for $Cl^-$ 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 $\epsilon$ would be $-0.14$; if b were 0.01 instead of 0.1, $\epsilon$ 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–.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:

$$K_{1/2} = \frac{K_c k_s [S]_i}{(k_s [S]_i + k_c (K_s + [S]_i))}. \tag{A12}$$

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 $0.3 \times K_{1/2}$:

$$b = \frac{(1.3\ k_{so}(S]_i + k_{co}(K_s + [S]_i)}{0.3\ k_{co}(K_s + [S]_i)}. \tag{A13}$$

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 $[S]_i$ 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 $[S]_i$ is very weak; it would make very little difference if $[S]_i$ 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 $\epsilon$ translocated outward with $SO_4^=$, assuming $b = 3.69$. The dependence on $\epsilon$ is not steep, but the predicted range for $\epsilon$ 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 $Cl^-$. It is noteworthy that the predicted flux ratio of $\sim 1.9$ (at $-58$ vs $-5$ mV) at 3 mM $Cl^-$ was derived with no freely adjustable parameters. The

ratio of the $Cl^-$ influx to $SO_4^=$ 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 $k_{so}/k_{co}$ between 0.001 and 0.2.

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