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Proton Fluxes Associated with Erythrocyte Membrane Anion Exchange

Michael L. Jennings

Biophysical Laboratory, Harvard Medical School, Boston, Massachusetts 02115

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Summary. Transient extracellular pH changes accompany the exchange of chloride for sulfate across the erythrocyte membrane. The direction of the extracellular pH change during chloride efflux and sulfate influx depends on experimental conditions. When bicarbonate is present, the extracellular pH drops sharply at the outset of the anion exchange and tends to follow the partial ionic equilibrium described by Wilbrandt (W. Wilbrandt, 1942. *Pfluegers Arch.* 246:291). When bicarbonate is absent, however, the anion exchange causes the pH to rise, indicating that protons are cotransported with sulfate during chloride-sulfate exchange. The pH rise can be reversed by the addition of HCO_3^- (4 μM) or 2,4-dinitrophenol (90 μM). This demonstrates that the proton-sulfate cotransport can drive proton transport uphill. The stoichiometry of the transport is that one chloride exchanges for one sulfate plus one proton. These results support the titratable carrier model proposed by Gunn (Gunn, R.B. 1972. *In:* Oxygen Affinity of Hemoglobin and Red Cell Acid-Base Status. M. Rorth and P. Astrup, editors. p. 823. Munksgaard, Copenhagen) for erythrocyte membrane anion exchange.

The mechanism of the transport of inorganic anions across the human red cell membrane has been the subject of considerable investigation, which recently has been reviewed by Passow and Wood (1973). Anion transport across the red cell membrane is passive and consists mainly of anion exchanges. The experiments of Hunter (1971) on human red cells and of Lassen (1972) on amphiuma red cells have shown that over 99% of the chloride flux across the membrane occurs by way of an electrically neutral exchange mechanism. Passow and Wood (1973) report that at pH 6.6, 98% of the sulfate permeation at Donnan equilibrium is through an exchange pathway, the percent being lower at higher pH values. Equilibrium tracer fluxes of monovalent and divalent anions have an apparent activation energy of about 30 kcal/mole (Passow, 1964; Dalmark&Wieth, 1972). Various inhibitors have the same effect on both monovalent and divalent anion transport (*see* Passow&Wood, 1973). These similarities between tracer fluxes of monovalent and divalent

anions at Donnan equilibrium indicate that both classes of anions share the same transport system, which has been shown by Cabantchik and Rothstein (1974) to be associated with a membrane protein which migrates on SDS polyacrylamide gels as a single polypeptide.

Gunn (1972) has proposed a titratable carrier model for monovalent and divalent anion exchange across the red cell membrane. According to this model, transport occurs by way of electrically neutral complexes between anions and membrane-associated carrier molecules. Monovalent anions such as chloride are transported on a carrier molecule which possesses a single positive charge (C_1^+). Divalent anions such as sulfate are transported on a doubly charged form of the carrier (C_2^{++}). The two forms of the carrier are interconvertible by a gain or loss of a proton:

$$C_1^+ + H^+ \leftrightharpoons C_2^{++}.$$

Among the phenomena explained by this model are the opposite pH dependences of monovalent (Wieth, Dalmark, Gunn & Tosteson, 1973) and divalent (Lepke & Passow, 1971) anion fluxes, the saturation behavior of chloride transport (Wieth *et al.*, 1973), and the kinetics of inhibition of divalent anion fluxes by monovalent anions (Lepke & Passow, 1971), all of the fluxes being tracer fluxes measured in the presence of no net anion transport. The applicability of the Gunn model to *net* anion exchange, however, has not been explored. This paper examines the implications of the titratable carrier model for net chloride-sulfate exchange and the transient extracellular pH changes which accompany the anion exchange.

Materials and Methods

Measurement of Qualitative Change in External pH in the presence of a Chloride-sulfate Counterflux

Human blood from healthy donors was drawn by venipuncture into heparin and was used immediately or after one day of storage at 4 °C. Identical results were obtained with fresh or one day old blood. Cells were washed four times at room temperature in unbuffered 154 mM NaCl. After each wash the top layer of cells was removed to ensure no contamination by white cells. Cells were then titrated with 1 N HCl to an external pH of 7.37 ± 0.03 or 6.85 ± 0.04. In the bicarbonate-free experiments, CO_2 was removed from the suspension by bubbling N_2 through the suspension for at least 30 min. The extent of CO_2 removed by this method was estimated from pH changes in an unbuffered 154 mM NaCl solution. As a result of the N_2 bubbling, the pH changed from the initial value of about 5.7 to a value equal to or greater than 6.8. This indicates that the CO_2 concentration has been reduced by a factor of greater than 100.

The N_2 bubbling was performed on suspensions of about 10% hematocrit to reduce foaming. After the N_2 treatment, the cells were spun down in a closed centrifuge tube, most of the supernatant aspirated under N_2, and the cells suspended in the remaining supernatant to make a suspension of hematocrit of about 0.6. Cells were then added from a gas-tight syringe (Hamilton Co., Reno, Nevada) to 100 mM Na_2SO_4, unbuffered, pH 7.37 or pH 6.85, 23 °±1 °C to a final hematocrit of 0.3 to 0.5%, with or without CO_2 removal as specified. The pH of the extracellular medium was followed continuously with a glass electrode attached to a pH meter and strip chart recorder.

In the experiments shown in Fig. 5, the additions of $NaHCO_3$ or 2,4–dinitrophenol (DNP) were made to the cell suspension from concentrated stock solutions: 150 mM $NaHCO_3$, 90 mM DNP. Immediately prior to the additions, the stock solutions were titrated to the same pH as was the external pH of the cell suspension at the time of the additions. This ensures that pH changes subsequent to the additions result from proton or hydroxyl transport across the cell membranes rather than from protons directly added to the medium. The DNP stock solution was made CO_2 free by N_2 bubbling. DNP was obtained from Sigma Chemical Co. (St. Louis, Mo.) and recrystallized twice from water before use. All salts were analytical reagent grade.

Determination of the Stoichiometry of Proton Flux Associated with Chloride-Sulfate Exchange

Fresh blood drawn as above was washed three times at room temperature in unbuffered 100 mM Na_2SO_4, and titrated with 1 N H_2SO_4 to an external pH of 6.22–6.25. Sufficient time was allowed between washes for equilibration of the sulfate across the membrane. These preliminary washes reduced the internal chloride concentration to about 5 mM, sulfate having replaced most of the chloride initially in the cells. The cells were then washed three more times in a final washing medium consisting of either (in mM) 93.5 Na_2SO_4, 10 NaCl; 97 Na_2SO_4, 5.0 NaCl; or 99 Na_2SO_4, 2.0 NaCl. After the final wash, CO_2 was removed with N_2 as previously, and a final suspension of 0.50–0.75 hematocrit was prepared. One half ml of this suspension was added to 75 ml of CO_2-free 100 mM Na_2SO_4, buffered with either hemoglobin or citrate (chosen because they are nonpenetrating buffers) at pH 6.25, or 6.22, 26 °C. The buffering capacity of the medium was determined by standard additions of acid or base. The external pH as a function of time was followed as above.

The hemoglobin was added in the form of lysates prepared from red cells thoroughly washed in 100 mM Na_2SO_4. Lysates were used rather than pure hemoglobin because foaming during the N_2 bubbling appeared to be less for lysates. The small amounts of organic phosphates included (final concentration $< 20\,\mu M$) in the lysates are believed not to influence the anion transport. To ensure that the H^+ flows did not depend on the nature of the extracellular buffer, citrate (133 or 267 μM) was used as the buffer in one set of experiments. The results obtained in the citrate buffer do not differ significantly from those in the lysate buffer.

Before each set of experiments an aliquot of cells was added to the final washing medium, unbuffered, pH 6.24±0.01, 26 °C, in order to establish the precise equilibrium extracellular pH, which was 6.25 for two of the sets of experiments, and 6.22 for the third. To establish that the washing procedure did in fact replace the chloride with sulfate, controls were performed in which chloride was excluded from the final washing medium. Results of these controls are included in the legend of Table 1.

In these experiments the only net transport occurring was the efflux of the small amount of chloride initially inside the cells in exchange for external sulfate, and the associated proton flux. To determine the stoichiometry of the proton transport accompanying this exchange, the total number of moles of chloride initially inside the cells was compared

with the total number of moles of protons transported into the cells as a result of the chloride-sulfate counterflux. The total number of moles of chloride in the cells was determined from the volume of cell suspension added to the sulfate medium, the suspension hematocrit, cell water, external chloride concentration in the suspension, and the Donnan ratio r_{Cl}, the ratio of chloride concentrations in intra- and extracellular water. The hematocrit of the suspension was determined by centrifugation at $2000 \times g$ for 30 min; trapped volume was taken into account. Trapped volume under these conditions was 2.8%, as measured by the distribution of ^{14}C sucrose in supernatant and pellet. The Donnan ratio r_{Cl} was determined from the distribution of ^{36}Cl between cell water and medium. In 97 mM Na_2SO_4, 5 mM NaCl, pH 6.25, 25 °C, and N_2 atmosphere, r_{Cl} is 1.05 ± 0.05. Cell water content was determined by comparing wet and dry weight (dried to constant weight at 80 °C) of 0.2 or 0.5 ml of the suspension.

Determination of the Time Course of Chloride Efflux

The time course of the chloride efflux into chloride-free sulfate media was followed as $^{36}Cl^-$ efflux, using the Millipore filtration technique employed by Dalmark and Wieth (1972) to measure the time course of tracer anion equilibration in the presence of no net flux of anions. In our experiments, the time course of the appearance of $^{36}Cl^-$ in the extracellular medium is the same as the time course of the total net chloride efflux, since the specific activity of the chloride is constant throughout the experiment. The filtration assemblies were constructed as described by Dalmark and Wieth (1972), with the omission of the ring of ice in the lower half of the filter holder.

Cells and media were prepared as described for the experiments in which the pH was followed, except that ^{36}Cl was added to the cells as $H^{36}Cl$ (New England Nuclear, Boston, Mass.) after the final washes. The amount of added acid changed the equilibrium pH of the cells by about 0.02 units. The ^{36}Cl-containing cells were added to a stirred chloride-free Na_2SO_4 medium to a final hematocrit of about 0.4%, and samples of the extracellular medium were withdrawn by the filtration method at various time intervals and counted. About 30% of the total counts added were originally in the extracellular space of the stock red cell suspension. This nontransported radioactivity was subtracted from the radioactivity in the extracellular medium at each time point in order to obtain the time course of the chloride efflux, which is plotted in Figs. 3, 5, and 6 as $[Cl_t - Cl_0]/[Cl_\infty - Cl_0]$ *vs.* time.

Proton Flux Predicted by Titratable Carrier Model

Chloride-sulfate exchange across the red cell membrane occurs with half-times ranging from several seconds to twenty minutes, depending on species and on experimental conditions such as temperature, pH, and anion concentrations (Parpart, 1940; Jacobs & Stewart, 1942; Wilbrandt, 1942; Schwietzer & Passow, 1953). Although the rates of self-exchange of chloride and of sulfate across the human red cell membrane are known (Wieth *et al.*, 1973; Lepke & Passow, 1971) it is difficult to

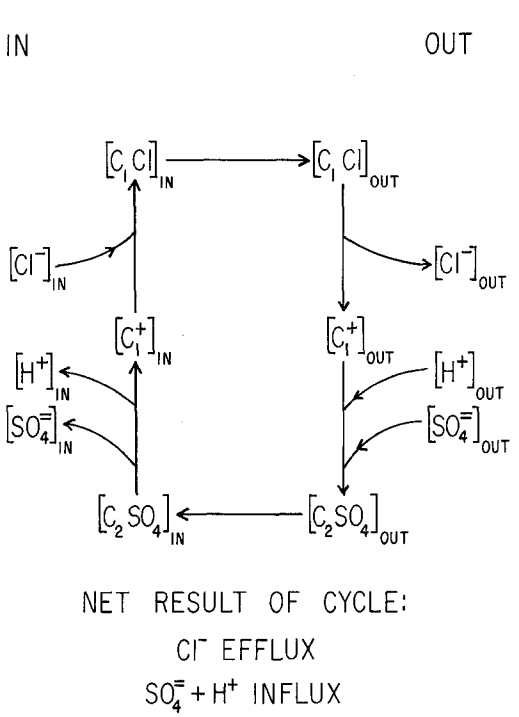

Fig. 1. Titratable carrier mechanism for the net exchange of intracellular chloride for extracellular sulfate. *See text*

compute the rate of net exchange of these ions predicted by the titratable carrier model; no attempt at such a prediction is made here. However, the model does make a specific prediction about proton transport associated with net chloride-sulfate exchange. Suppose that cells containing chloride as the only penetrating anion are added to a medium in which sulfate is the only anion. According to the titratable carrier model, the ensuing chloride-sulfate exchange should occur by way of the following sequence of events (Fig. 1). An internal chloride combines with a singly charged carrier (C_1^+), is transported outward and dissociates from the carrier, leaving a singly positive carrier C_1^+ facing outward. Sulfate can be transported inward only on the doubly charged form of the carrier ($C_2^{+\,+}$). Therefore, the C_1^+ carrier which has transported a chloride outward must gain a proton in order for the carrier to transport sulfate inward. Once a proton and a sulfate have combined with C_1^+, the resultant electrically neutral complex (C_2SO_4) is transported inward. Sulfate then dissociates from the carrier, leaving an inward facing $C_2^{+\,+}$ molecule. This $C_2^{+\,+}$ must lose a proton in order to transport another chloride outward. The net result of the cycle in Fig. 1 is that one internal chloride

ion has exchanged for one external proton plus one external sulfate. The experiments described below test this prediction of Gunn's model of anion transport, i.e., that a chloride efflux against a sulfate influx should produce a pH rise in the extracellular medium.

In discussing proton movements associated with anion transport, it is first necessary to establish that any pH changes in the extracellular medium which occur during anion counterflux are not a result of sodium or potassium transport. In all of the experiments presented here, sodium is the only extracellular cation (other than protons), and potassium is the major intracellular cation. Therefore, the cells and medium are not at equilibrium with respect to cations, and, although cation movements across the red cell membrane are very slow compared with anion transport, cation transport could conceivably contribute to proton fluxes across the membrane. This possibility was ruled out by washing cells in media consisting of mixtures of 154 mM NaCl and 100 mM Na_2SO_4, in various proportions (from 0% to 100% chloride), and titrating the cells to various equilibrium pH values between 6 and 7.4 in an air or nitrogen atmosphere. When cells so prepared are resuspended in the same medium (and same pH) as the washing medium, no significant proton movements are observed; the pH of the unbuffered extracellular medium changes by at most 0.02 units after resuspension of cells, even though sodium and potassium gradients across the membrane are present. The pH is quite stable; it changes by at most 0.04 units in three hours of incubation. The external pH does change, however, when there are anion gradients across the membrane, as discussed below. Therefore, the proton movements we have observed are related to anion transport and do not result from cation gradients across the membrane.

Another consideration in these experiments is the possibility that the pH changes observed result not from the anion fluxes themselves but rather from (*A*) the shrinkage of the cells which occurs as a result of the chloride-sulfate exchange, or (*B*) a direct effect of chloride or sulfate on the hydrogen ion binding of hemoglobin, which is the major buffer present. These possibilities have been excluded by the following experiment. A suspension of NaCl-washed red cells is titrated to some equilibrium extracellular pH, e.g., 6.9, in either air or nitrogen atmosphere. These cells are suspended in 100 mM Na_2SO_4 (in air or N_2), and incubated until the chloride-sulfate exchange is complete. The extracellular pH at equilibrium in the sulfate medium is within 0.05 pH units of the initial value in the chloride medium, even though the cells have shrunk and sulfate has replaced virtually all the chloride initially

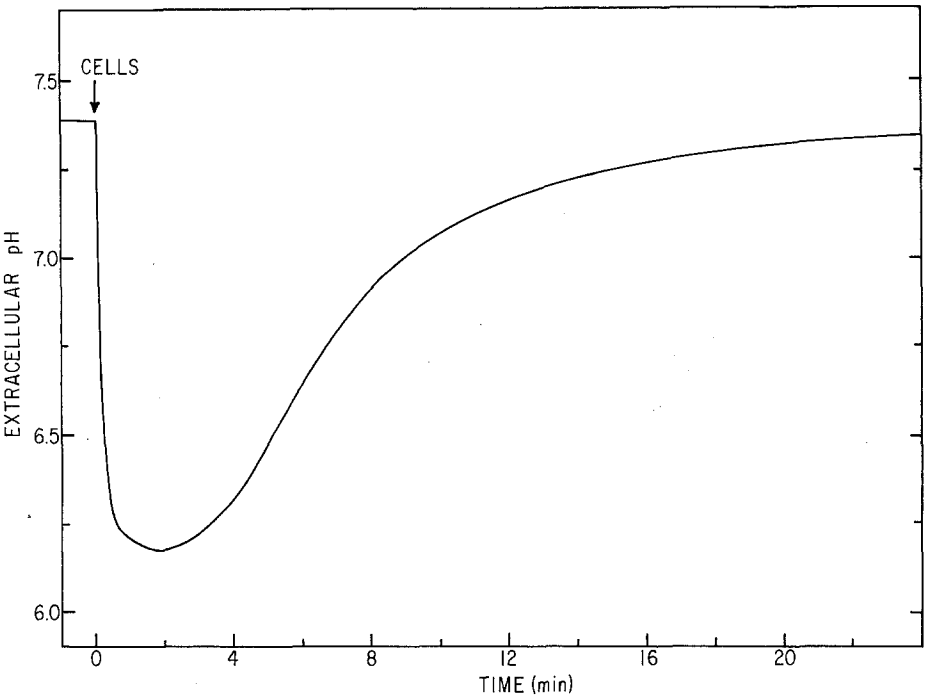

Fig. 2. Time course of extracellular pH during chloride-sulfate counterflux. Cells equilibrated with 154 mM NaCl, pH 7.37, are added to 100 mM Na_2SO_4, unbuffered pH 7.39. Final hematocrit approximately 0.4%. Temperature 23±1 °C

inside the cells. The large pH changes discussed below are therefore transient and do not represent a change in the equilibrium pH value.

Qualitative Change in External pH During Anion Counterflux

Changes in the external pH during chloride-sulfate counterflux across the red cell membrane have been studied several times in the past (Wilbrandt, 1942; Schwietzer & Passow, 1953). We have repeated these experiments and obtained results similar to the earlier studies. When cells washed in isotonic NaCl and titrated to an external pH of 7.4 are suspended in unbuffered 100 mM sodium sulfate (initially at pH 7.4), the pH rapidly (half–time of less than one min) drops more than 1.0 unit, and slowly rises (half–time of about 3 min) to the equilibrium pH, as shown in Fig. 2. The final result of the transport is that one sulfate ion has replaced each two chloride ions inside the cells with very little change in the equilibrium pH. The Gunn model of anion

transport (Fig. 1) predicts that the external pH should rise when the chloride-containing cells are suspended in the sulfate medium. The observed pH drop at the outset of the anion exchange is contrary to this prediction.

There are two reasons for the pH drop after addition of the cells. First, although the bulk of chloride transport across the red cell membrane is electrically silent, the electrical potential difference across the membrane still follows the Nernst potential for chloride (Lassen, 1972). Suspending chloride-containing cells in a chloride-free medium will therefore result in a positive membrane potential (inside with respect to outside). This membrane potential will tend to drive hydroxyl ions into and hydrogen ions out of the cells, thereby causing a drop in the external pH.

The second reason results from the fact that exchanges of monovalent anions across the red cell membrane are much faster than those of divalent anions (*see* Passow & Wood, 1973). When the chloride-containing cells are suspended in the sulfate medium, the most rapid anion exchanges across the membrane are those between monovalent anions. Since hydroxyl and bicarbonate (present as a result of CO_2 in the air) are the only external monovalent anions, these anions rapidly exchange for the intracellular chloride, driving the external pH down.

For these reasons, the outward chloride gradient tends to drive hydroxyl ions into the cells toward the following situation, which Wilbrandt (1942) termed "partial ionic equilibrium": $[Cl^-]_{in}/[Cl^-]_{out} = [OH^-]_{in}/[OH^-]_{out}$. Since, initially, $[Cl^-]_{in}/[Cl^-]_{out}$ is very large, hydroxyl will be transported inward in exchange for chloride, and the external pH rapidly drops. As the chloride exchange for external sulfate proceeds, $[Cl^-]_{in}/[Cl^-]_{out}$ becomes progressively lower in magnitude, and the external pH rises toward the final equilibrium value. If the pH is allowed to follow the dictates of partial ionic equilibrium, then, the extracellular pH will drop when chloride-containing cells are suspended in the sulfate medium, i.e., the pH will move in a direction opposite that predicted by the Gunn model. Equilibration of pH across the membrane must therefore be prevented before a chloride-sulfate counterflux experiment can be used to test the titratable carrier model of anion exchange.

The mechanism of pH equilibration across the red cell membrane involves anion transport rather than the transport of protons themselves (Jacobs & Papart, 1932; Scarpa, Cecchetto & Azzone, 1970). Equilibration of pH occurs primarily by way of the chloride-bicarbonate exchange cycle (utilizing carbonic anhydrase) described by Jacobs and Stewart

(1942) and more recently by Deuticke (1972). When cells and medium are not at Donnan equilibrium with respect to pH, chloride-bicarbonate exchange, along with the hydration-dehydration reactions between CO_2 and H_2O, result in an apparent chloride-hydroxyl exchange, which leads to pH equilibration. Hydroxyl ion itself is responsible for only a small fraction of the apparent chloride-hydroxyl exchange because it is usually present at much smaller concentrations than bicarbonate. Data on the solubility of CO_2 (Van Slyke, Sendroy, Hastings & Neill, 1928; Severinghaus, Stupfel & Bradley, 1956) indicate that in an aqueous solution at equilibrium with atmospheric CO_2, the bicarbonate concentration will be over 100 times larger than the hydroxyl concentration. If CO_2 (and therefore bicarbonate) is removed by bubbling nitrogen through all media, the rate at which pH equilibrates across the membrane will be diminished. In the absence of bicarbonate, then, any proton movements which may accompany the chloride-sulfate exchange should be easier to detect.

Fig. 3 illustrates the effect of bicarbonate removal on the behavior of the external pH in the presence of a chloride-sulfate counterflux. In both experiments human red cells were washed 4 times in isotonic sodium chloride, titrated to an external pH of 6.85 and suspended in unbuffered 100 mM Na_2SO_4, initially at pH 6.85. (This pH was chosen because bicarbonate removal by nitrogen is more complete at pH 6.85 than at pH 7.4.) In Fig. 3*A*, bicarbonate is present as a result of CO_2 in the air. The external pH, after addition of the cells, drops to about 6.05 in 1 min and subsequently rises to the equilibrium pH of 6.85, i.e., the external pH tends to follow partial ionic equilibrium as in Fig. 2. The lower part of Fig. 3*A* shows that the half-time for the chloride efflux under these conditions is about 1.5 min. Since in this experiment the chloride-containing cells are added to a chloride-free medium, the ratio Cl^-_{in}/Cl^-_{out} is very large throughout most of the chloride efflux, implying that the pH should drop well below 6 if partial ionic equilibrium is actually attained. As the pH approaches the minimum of 6.05, however, the influx of hydroxyl and bicarbonate occurs more slowly because the concentration of both of these ions is quite low at this pH. For this reason, the pH does not strictly follow partial ionic equilibrium until after the chloride efflux is more than 90% complete (5 min).

If there were no proton movements associated with chloride-sulfate exchange, the expected result of bicarbonate removal on the time course of the pH during the anion exchange would be that the pH drop toward partial ionic equilibrium would occur more slowly. Fig. 3*B* shows, howev-

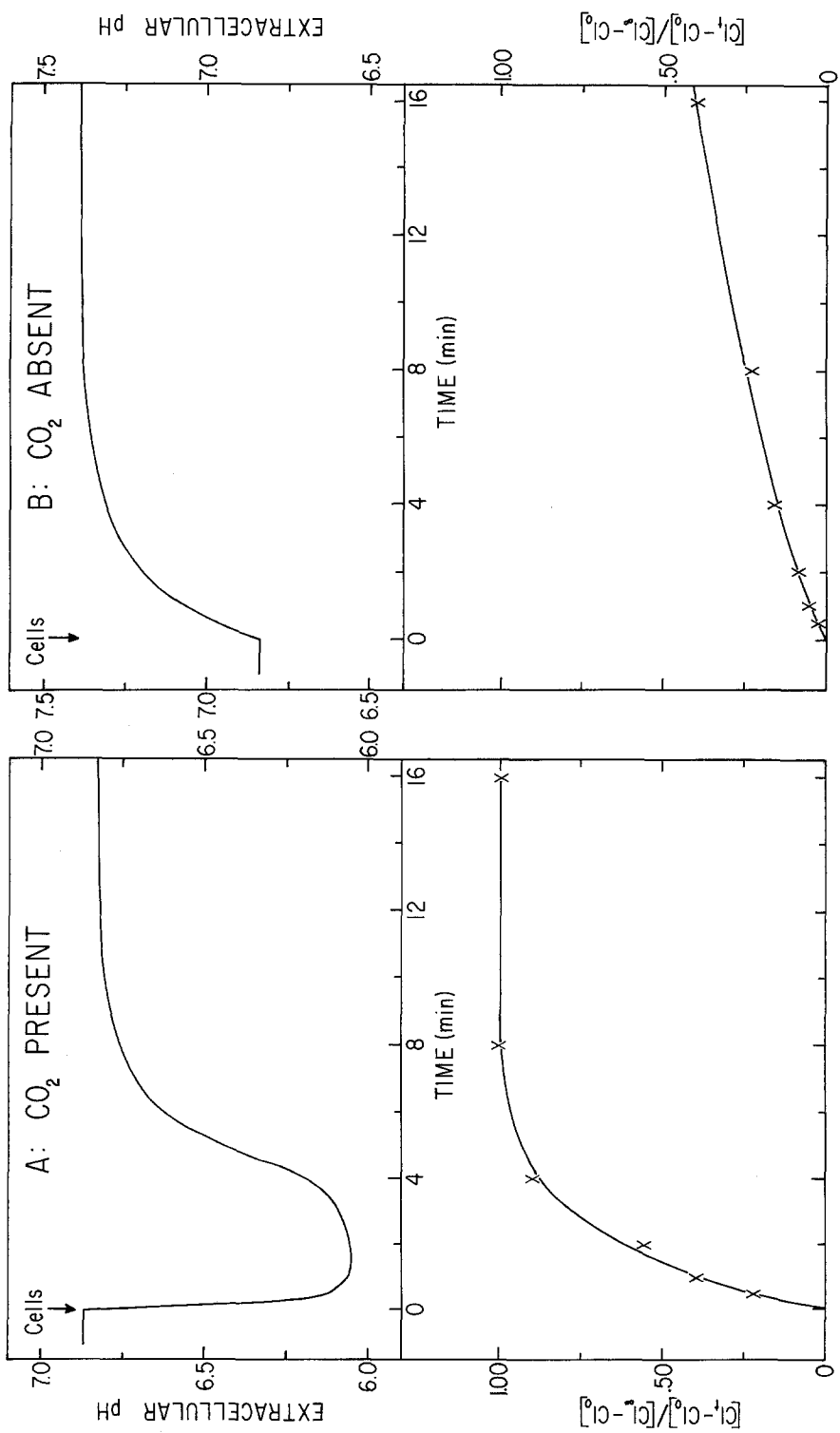

Fig. 3. *Upper*: Effect of presence (*A*) and absence (*B*) of CO_2 on the time course of the extracellular pH during chloride-sulfate counterflux. Equilibrium pH 6.85. Final hematocrit 0.4%. Temperature 23 ± 1 °C. *Lower*: Time course of chloride efflux under the same conditions in the presence (*A*) and absence (*B*) of CO_2. "CO_2 Present" signifies that media are at equilibrium with atmospheric CO_2. "CO_2 Absent" signifies that CO_2 has been removed by bubbling N_2 through media

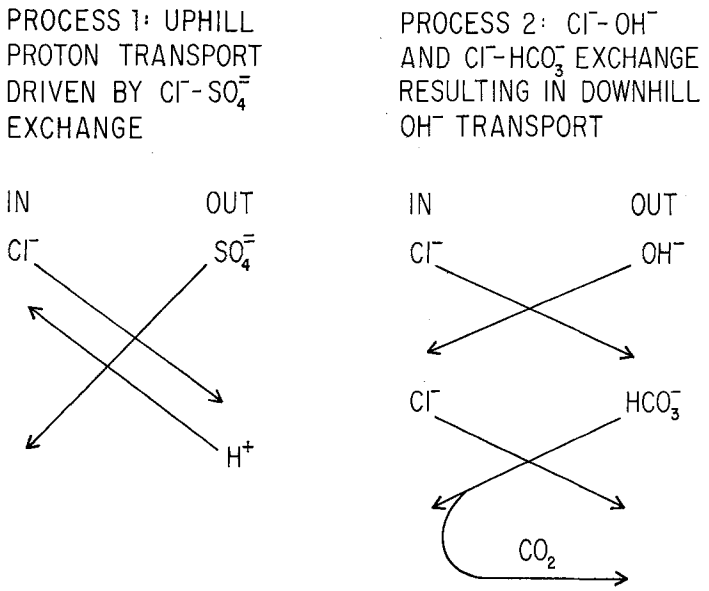

Fig. 4. Summary of the two types of anion exchanges occurring during chloride-sulfate counterflux

er, that in the absence of bicarbonate an entirely different behavior is observed. After the cells are added, the external pH *rises* to about 7.4 and very slowly drops toward the equilibrium pH (0.01 units in 20 min). That is, the external pH under these conditions moves in a direction *opposite* that dictated by partial ionic equilibrium. Fig. 3*B* (*lower*) shows that the chloride efflux in the absence of bicarbonate is much slower; after 20 min only 45% of the chloride has left the cells. This is consistent with the earlier observation by Jacobs and Stewart (1942) that addition of bicarbonate drastically accelerates chloride-sulfate exchange across the red cell membrane.

The results in Fig. 3 are explained by the Gunn model as follows. During the chloride-sulfate counterflux there are two types of anion exchange events occurring, designated here for future reference as *Process* 1 and *Process* 2 (Fig. 4). Both processes occur by way of the same transport system, but one produces inward proton transport and the other results in inward hydroxyl transport. In *Process* 1, one internal chloride ion is exchanged for one external sulfate plus one external proton, according to the titratable carrier mechanism in Fig. 1. This tends to drive the external pH upward, *away* from partial ionic equilibrium:

$$\text{Process 1} \quad Cl^-_{in} \leftrightarrow H^+_{out} + SO^=_{4out}.$$

The anion exchanges of *Process* 2 consist of exchanges of external bicarbonate (and hydroxyl) for internal chloride. These anion exchanges result in an inward hydroxyl flux and the associated pH drop *toward* partial ionic equilibrium:

$$\textit{Process } 2 \quad Cl^-_{in} \leftrightarrow OH^-_{out} \text{ or } HCO^-_{3\,out}.$$

The anion exchanges of *Processes* 1 and 2 eventually result in a new equilibrium state, in which one sulfate ion has replaced each two chloride ions inside the cells, with no change in the equilibrium pH. The transient changes in the external pH during the chloride-sulfate exchange depend entirely on the relative magnitudes of proton and hydroxyl transport by *Process* 1 and *Process* 2. In Fig. 3*A* above bicarbonate is present, so *Process* 2 dictates the changes in external pH, i.e., there is an initial hydroxyl influx toward partial ionic equilibrium, as discussed above. In Fig. 3*B*, however, apparent hydroxyl influx by *Process* 2 has been slowed by bicarbonate removal. This allows the proton influx by *Process* 1 to drive the external pH upward, creating a pH disequilibrium across the membrane. The external pH continues to rise until hydroxyl influx by *Process* 2 becomes large enough to balance the proton influx by *Process* 1, causing the external pH to remain nearly constant, even though the chloride efflux is not complete.

If this balance of proton influx by *Process* 1 and hydroxyl influx by *Process* 2 is upset, the external pH will rapidly drop towards its equilibrium value. This can be demonstrated in two ways, as shown in Fig. 5. In both experiments, cells prepared as in Fig. 3 were added to 100 mM Na_2SO_4, unbuffered, pH 6.85, in an N_2 atmosphere. As in Fig. 3*B*, the external pH rises after addition of the cells and remains nearly constant for 30 min. In Fig. 5*A*, $NaHCO_3$ was added to a final concentration of 4 μM. The bicarbonate addition allows the pH to equilibrate by the Jacobs-Stewart cycle mentioned above; the bicarbonate increases apparent hydroxyl transport by *Process* 2, and the pH drops toward equilibrium. In Fig. 5*B*, 2,4-dinitrophenol was added to a final concentration of 90 μM. Dinitrophenol, an uncoupler of oxidative phosphorylation, facilitates proton transport across membranes (Lea & Croghan, 1969; McLaughlin, 1972) and allows the pH to drop toward equilibrium. In this case, the uphill proton transport driven by the chloride-sulfate exchange is overcome by downhill proton transport catalyzed by dinitrophenol. In both Fig. 5*A* and 5*B* the pH subsequent to the additions of HCO_3^- or DNP drops slightly below and then rises to the final equilibrium pH of 6.85. This occurs because, at the time of

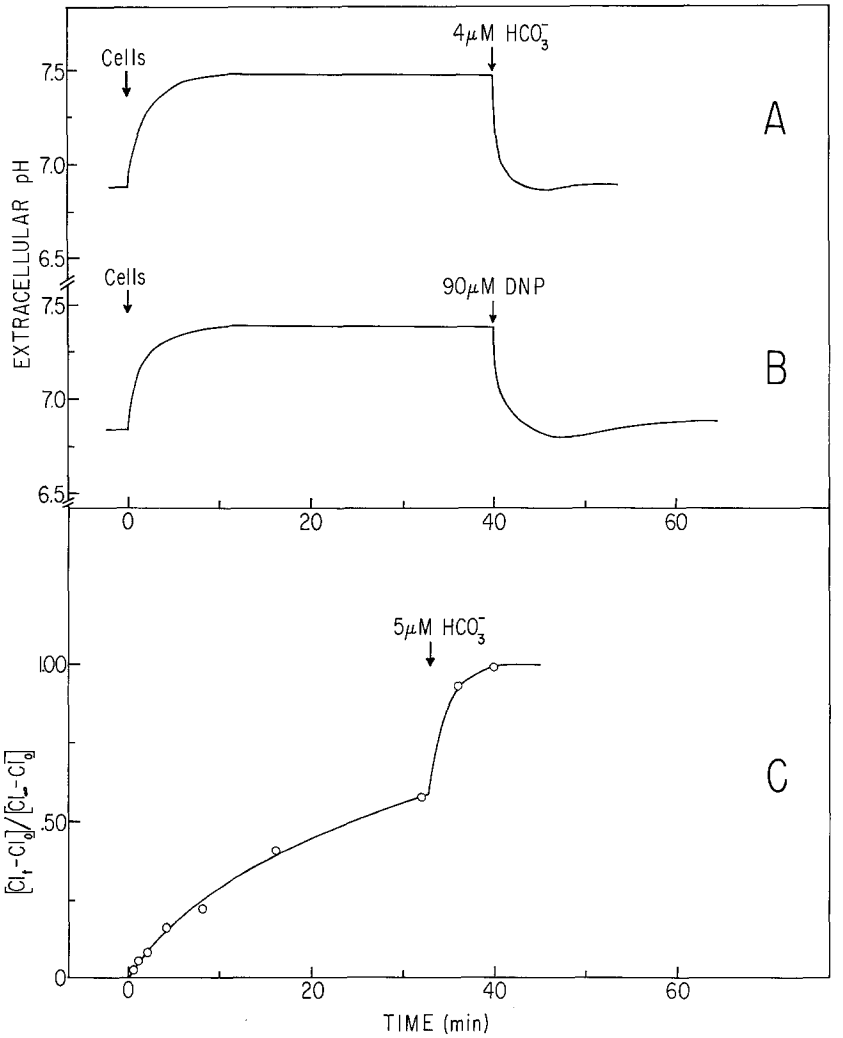

Fig. 5. Reversal of anion exchange-induced pH rise by 4 μM bicarbonate (*A*) and 90 μM 2,4-dinitrophenol (*B*). Lower portion of figure (*C*) shows time course of the chloride efflux under the same conditions. Final hematocrit 0.4%, equilibrium pH 6.85, temperature 23 °C, N_2 atmosphere

the additions, there is still a chloride gradient directed outward from the cells, so that the pH specified by partial ionic equilibrium is below pH 6.85 until the chloride gradient is dissipated. Note that the pH remains slightly below 6.85 for somewhat longer after the DNP addition (Fig. 5*B*) than after HCO_3 is added (Fig. 5*A*). This results from the fact that DNP is an inhibitor of red cell anion transport (Omachi, 1964). The final equilibration of Cl and SO_4 (and pH) therefore takes longer in

the presence of DNP than it does after the HCO_3 addition. Direct measurement of the chloride efflux under these conditions show that the efflux is only about 60% complete after 30 min (Fig. 5*C*). Chloride equilibration is complete within 5 min after bicarbonate addition.

The above results show that protons are cotransported with sulfate during chloride-sulfate exchange across the erythrocyte membrane, and that the anion exchange can drive proton transport uphill, away from electrochemical equilibrium. The uphill proton transport can only be observed when the normal means of pH equilibration across the membrane (i.e., bicarbonate) is removed. This proton-sulfate cotransport is in accordance with the titratable carrier model for anion transport.

Stoichiometry of the Anion Transport-linked Proton Flux

In addition to accounting for the qualitative change in the external pH in the presence of a sulfate influx and chloride efflux, the Gunn model predicts that virtually *all* of the sulfate entering the cell in exchange for chloride should carry a proton with it, in the absence of hydroxyl movements (assuming that the flux resulting from two chlorides crossing on C_2^{++} is negligible). That is, if hydroxyl transport is not allowed to occur, the model predicts that if cells containing a total of N moles of chloride are added (to a very low hematocrit) to a chloride-free sulfate medium, then N moles of protons should be transported into the cells.

The difficulty of experimentally establishing the stoichiometry of the sulfate-proton cotransport results from the fact that any hydroxyl movements which occur by way of *Process* 2 will obscure the actual stoichiometry of the transport. The experiments in Figs. 3*B* and 5 are inadequate for the purpose of measuring stoichiometry because the initial large chloride gradient is only partially dissipated by the time hydroxyl influx (*Process* 2) becomes large enough to balance the proton influx (*Process* 1). Experimental conditions must be chosen, therefore, such that the chloride efflux by *Process* 1 is complete before significant influx of hydroxyl into the cells by *Process* 2 has occurred. In order for *Process* 1 to be much faster than *Process* 2, the equilibrium pH must be low (facilitating CO_2 removal by nitrogen), and the proton influx resulting from the chloride-sulfate exchange must generate only a relatively small pH gradient across the membrane, because a large pH gradient will produce a large hydroxyl flux by *Process* 2. To do this, the medium must be buffered, so that a given removal of protons from the medium produces a smaller pH change, and the initial concentration of chloride

Table 1. Stoichiometry of the exchange of chloride for sulfate and protons[a]

Initial Internal chloride concentration (mM)	Total chloride initially inside cells (μmoles)	Total protons entering cells during chloride efflux (μmoles)	Stoichiometry of exchange: [Proton Influx]/ [Chloride Efflux]
2.1	0.565	0.50	0.88
2.1	0.565	0.49	0.87
2.1	0.565	0.50	0.88
5.25	1.23	1.00	0.81
5.25	1.23	1.06	0.86
5.25	1.23	1.05	0.85
5.25	1.23	1.06	0.86
10.5	1.84	1.50	0.81
10.5	1.84	1.46	0.79
10.5	1.84	1.57	0.85

[a] Cells containing approximately 100 mM sulfate and the indicated amount of chloride were suspended in chloride-free 100 mM Na_2SO_4, buffered with hemoglobin (0.2–0.4 mg/ml, experiments 1–7) or citrate (133–267 μM, experiments 8–10). Final hematocrit: 0.33–0.5%. Temperature: 26 °C. Equilibrium extracellular pH = 6.22–6.25. Uncertainties in both chloride content of cells and proton influx are about 5%. Control experiments in which chloride was excluded from the final washing medium resulted in a proton influx of <0.01 to 0.03 μmoles (5 experiments). Addition of 10 μmoles of NaCl directly to the buffer medium (Hb or citrate) resulted in no pH change (<0.01 units).

inside the cells must be small (again so that only a small pH gradient is developed). The following experimental conditions fulfill these requirements. Cells which have been washed with a predominantly Na_2SO_4 medium which contains either 2, 5, or 10 mM chloride, pH 6.25, CO_2-free, are added to 100 mM Na_2SO_4, CO_2-free, buffered with hemoglobin or citrate at pH 6.25. Ten such experiments were performed, the results of which are summarized in Table 1. The recorder trace of a representative experiment is shown in the upper portion of Fig. 6. The time course of the chloride efflux under the same conditions is shown in the lower portion of Fig. 6. Addition of the cells to the chloride-free medium produces an external pH rise resulting from the small amount of internal chloride exchanging for external sulfate and protons. After 1 min, the pH reaches a maximum and the chloride efflux is complete. In contrast to the experiments in Fig. 5, the pH remains at this maximum value for only 10 sec; pH equilibration by *Process* 2 is evident immediately after the maximum is reached. This results from the fact that the chloride efflux by *Process* 1 is complete at the time the pH maximum occurs, so that pH equilibration proceeds without any opposing proton influx.

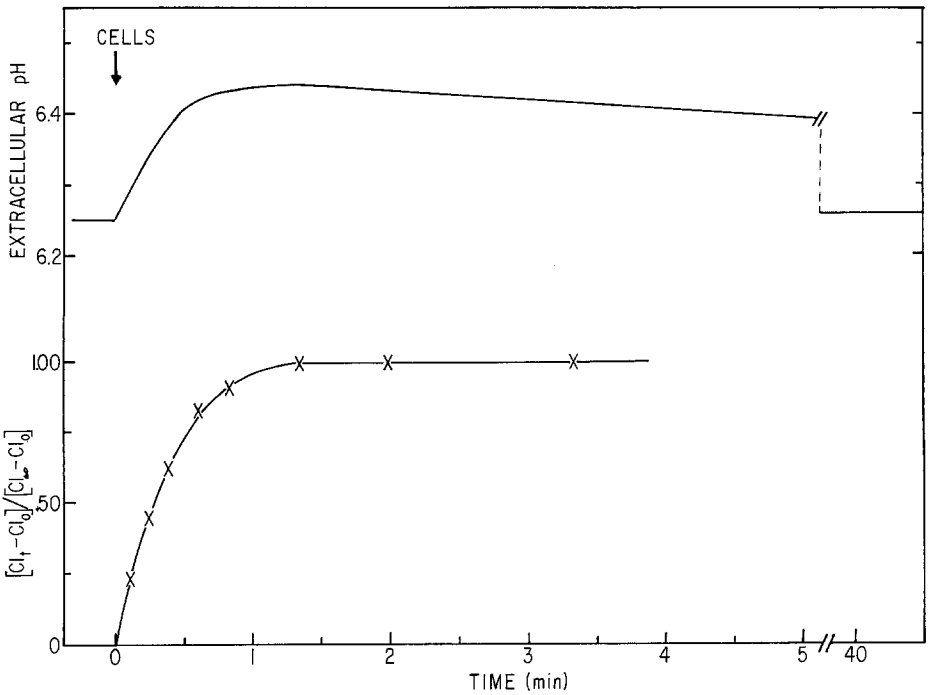

Fig. 6. *Upper*: Time course of extracellular pH after cells equilibrated with 97 mM Na_2SO_4, 5 mM NaCl, pH 6.25, are added to chloride-free 100 mM Na_2SO_4, buffered with hemoglobin at pH 6.25. A pH change of 0.1 corresponds to 0.55 μmoles H^+. Final hematocrit 0.5%, temperature 26 °C, N_2 atmosphere. *Lower*: Time course of the chloride efflux under the same conditions

In this case the pH equilibration consists mainly of exchanges of external hydroxyl for internal sulfate, since the internal chloride concentration is quite low. The initial pH rise corresponds (from the measured buffering capacity of the medium) to a proton influx equal to about 85% of the total amount of chloride initially inside the cells (Table 1). That is, the pH rise is 85% of that predicted if one proton plus one sulfate ion exchanged for one chloride ion, and if *Process* 2 were infinitely slow. Fig. 5 shows, however, that pH equilibration by *Process* 2 occurs at an appreciable rate (half-time about 10 min under these conditions), thereby lowering the pH rise produced by the anion exchange. If correction is made for this rate of pH equilibration, the pH rise is calculated to be $95 \pm 10\%$ of the prediction of the Gunn model, i.e., that the stoichiometry of the exchange is one chloride exchanging for one sulfate plus one proton.

According to the titratable carrier model of anion exchange, the stoichiometry of the exchange of chloride for sulfate and protons will

be one proton entering the cell for each chloride leaving the cell *only* if the efflux of chloride occurs solely by the singly charged carrier (Fig. 1). If an appreciable fraction of the exchange occurs by way of two chlorides being transported on the doubly charged carrier, then the stoichiometry of the exchange will be such that the number of protons entering the cell with the sulfate will be less than the number of chlorides leaving the cell. The results in Table 1 and the calculation from Fig. 6, however, show that at pH 6.25 the stoichiometry of the exchange is very close to one proton plus one sulfate exchanging for one chloride. This means that only a very small fraction of the chloride efflux occurs by way of the doubly charged carrier, even though the doubly charged carrier is believed to be the predominant form at this pH (Gunn, 1972).

Possible Role of Bisulfate Transport

An alternative explanation of the observed proton-sulfate cotransport is that the membrane permeability to bisulfate (HSO_4^-, $pK_A = 1.9$) is many orders of magnitude higher than its permeability to sulfate. A bisulfate influx against a chloride efflux would also result in a pH rise in the external medium. Our experimental results could then be interpreted to mean that the vast majority of the sulfate flux across the red cell membrane is actually bisulfate flux, and that the protons crossing the membrane are not the result of a titratable transport system. The following facts argue against this explanation. Phosphate tracer exchange fluxes are similar to sulfate exchange fluxes under similar conditions, according to the data of Gruber and Deuticke (1973) for phosphate and of Lepke and Passow (1971) for sulfate transport. At a given concentration of sulfate or phosphate, the singly negative form of phosphate ($H_2PO_4^-$, $pK \cong 7$) is present at about 10^5 times as high a concentration as the singly charged bisulfate. The sulfate and phosphate fluxes, however, are similar, so that if bisulfate comprises the bulk of the sulfate flux, the membrane permeability to HSO_4^- must be about 10^5 times the permeability to $H_2PO_4^-$. Such an enormous selectivity between monovalent inorganic anions of similar size is unlikely. It is, therefore, improbable that bisulfate transport can account for the proton-sulfate cotransport we have observed.

Explanation of Transport Rates by Gunn Model

The arguments in this paper are based on the direction and extent of proton movements in the presence of chloride-sulfate exchange across

the red cell membrane, and not on rates of transport. The times required to complete the chloride-sulfate exchanges in our experiments vary considerably, depending on the experimental conditions. The variations in the rates of the transport, however, are consistent with the titratable carrier model. For example, much more time is required for the chloride and sulfate to equilibrate across the membrane in Fig. 3*B* than in Fig. 3*A*. The titratable carrier model can account for this difference in transport rate on the basis of the availability of outward facing C_2^{++} molecules, which are necessary for sulfate influx. In Fig. 3*A* the sulfate influx occurs more rapidly because the external pH during most of the experiment is low, favoring formation of C_2^{++}. In Fig. 3*B*, however, the sulfate influx is slower because the pH rise to 7.4 results in a smaller number of outward facing C_2^{++} molecules. In Fig. 6, the chloride efflux is complete in about 60 sec (although the pH takes several more minutes to equilibrate). The short time for completion of the chloride efflux in this experiment results from the large number of C_2^{++} carriers for the sulfate influx at low pH, and also from the higher temperature ($E_a \cong 30$ kcal/mole; Jennings, 1976).

The experiments presented here show that chloride-sulfate exchange across the red cell membrane is accompanied by a stoichiometrically exact proton flux. This proton-sulfate cotransport can be detected only when bicarbonate transport is eliminated, i.e., when chloride-sulfate exchange is the only anion exchange occurring. The proton flux drives the external pH away from electrochemical equilibrium, as illustrated by the fact that either bicarbonate or 2,4–dinitrophenol is able to reverse the pH rise associated with the anion exchange. The direction and stoichiometry of the proton flux are in quantitative agreement with the titratable carrier model of anion transport proposed by Gunn. In addition, the Gunn model accounts qualitatively for the variations in transport rates under different experimental conditions. Our anion exchange studies, therefore, provide new experimental support for the titratable carrier model of erythrocyte membrane anion transport.

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