Sulfate Transport in Human Neutrophils

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ABSTRACT The mechanism by which \( \text{SO}_4^{2-} \) is transported across the plasma membrane of isolated human neutrophils was investigated. Unlike the situation in erythrocytes, \( \text{SO}_4^{2-} \) and other divalent anions are not substrates for the principal \( \text{Cl}^-/\text{HCO}_3^- \) exchange system in these cells. At an extracellular concentration of 2 mM, total one-way \( \text{SO}_4^{2-} \) influx and efflux in steady-state cells amounted to \( \sim 17 \mu\text{mol/liter of cell water per min} \). The intracellular \( \text{SO}_4^{2-} \) content was \( \sim 1 \text{mM} \), 25-fold higher than the passive distribution level. Internal \( \text{Cl}^- \) stimulated \( \text{SO}_4^{2-} \) influx. Conversely, \( \text{SO}_4^{2-} \) efflux was stimulated by external \( \text{Cl}^- \) (\( K_m \sim 25 \text{mM} \)) and by external \( \text{SO}_4^{2-} \) (\( K_m \sim 14 \text{mM} \)), implying the presence of a \( \text{SO}_4^{2-}/\text{Cl}^- \) countertransport mechanism. The exchange is noncompetitively inhibited by 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS) (\( K_i \sim 50 \mu\text{M} \)) and competitively blocked by \( \alpha \)-cyano-4-hydroxycinnamate (\( K_i \sim 230 \mu\text{M} \)) and by ethacrynate (\( K_i \sim 7 \mu\text{M} \)); furosemide and probenecid also suppressed activity. The carrier exhibits broad specificity for a variety of monovalent (\( \text{NO}_3^- > \text{Cl}^- > \text{Br}^- > \text{formate}^- > \text{I}^- > \text{p-aminophenylurate}^- \)) and divalent \( \text{WO}_4^{2-} > \text{oxalate}^2^- > \text{SO}_4^{2-} > \text{MoO}_4^{2-} > \text{SeO}_4^{2-} > \text{AsO}_4^{2-} \) anions. There was little, if any, affinity for \( \text{HCO}_3^- \), phosphate, or glucuronate. The influx of \( \text{SO}_4^{2-} \) is accompanied by an equivalent cotransport of \( \text{H}^+ \), the ion pair \( \text{H}^+ + \text{SO}_4^{2-} \) being transported together in exchange for \( \text{Cl}^- \), thereby preserving electroneutrality. These findings indicate the existence of a separate \( \text{SO}_4^{2-}/\text{Cl}^- \) exchange carrier that is distinct from the neutrophil's \( \text{Cl}^-/\text{HCO}_3^- \) exchanger. The \( \text{SO}_4^{2-} \) carrier shares several properties in common with the classical inorganic anion exchange mechanism of erythrocytes and with other \( \text{SO}_4^{2-} \) transport systems in renal and intestinal epithelia, Ehrlich ascites tumor cells, and astroglia.

INTRODUCTION

In our previous studies on chloride fluxes across isolated human peripheral blood neutrophils (Simchowitz and De Weer, 1986; Simchowitz et al., 1986), we reported on three distinct pathways to account for all of the steady-state \( ^{36}\text{Cl}^- \) movements. These included anion exchange, active transport, and electrodiffusion. The bulk (~70%) of the total one-way \( ^{36}\text{Cl}^- \) influx and efflux occur through an anion exchange mechanism that is insensitive to disulfonic stilbenes. However, the
exchange carrier can be competitively inhibited by α-cyano-4-hydroxycinnamate (CHC), which is known to block monocarboxylate transport and anion exchange in red blood cells and in mitochondria (Halestrap and Denton, 1975; Halestrap, 1976; Deuticke, 1982). We also pointed out that, although 36Cl⁻ fluxes in neutrophils were resistant to 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS) (Simchowitz and De Weer, 1986), Korchak et al. (1980, 1982) have reported that this compound effectively blocks 35SO₄²⁻ fluxes in these cells. Therefore, it follows that the vast proportion of 36Cl⁻ and 35SO₄²⁻ fluxes must cross the cell membrane via different routes. Further work on the substrate selectivity of the principal anion (i.e., Cl⁻/Cl⁻) exchange carrier (Simchowitz, 1988a), which functions physiologically as a Cl⁻/HCO₃⁻ exchanger in pH recovery from alkaline loads (Simchowitz and Roos, 1985), supports the impression that the Cl⁻/Cl⁻ exchange mechanism is essentially devoid of affinity for SO₄²⁻ while Cl⁻ binds with high affinity (Kₘ ~5 mM [Simchowitz et al., 1986]). This feature is distinctive since Cl⁻ and SO₄²⁻ display comparable kinetic constants for binding to the inorganic anion exchange system (band 3 protein) of human erythrocytes (Schnell et al., 1977; Gunn, 1978; Barzilay and Cabantchick, 1979; Milanick and Gunn, 1984). On this basis alone, we felt it important to our understanding of membrane transport in neutrophils to elucidate the pathway(s) for SO₄²⁻ movements since the mechanism(s) is apt to be different from that in red cells.

Our interest in the subject of SO₄²⁻ transport was given further impetus by a number of recent reports indicating that inhibitors of 35SO₄²⁻ fluxes, namely the disulfonic stilbenes, effectively block certain stimulated functional responses in neutrophils (Korchak et al., 1980, 1982; Smolen, 1984; Smith et al., 1984). These include lysosomal enzyme release, but not superoxide radical generation, induced by chemotactic factors, ionophores, lectins, and immune complexes. These studies then provided a further justification for an in-depth analysis of 35SO₄²⁻ fluxes in human neutrophils since the pathway(s) for SO₄²⁻ movements might play an important role in cell activation. Thus, this knowledge could contribute to our basic understanding of stimulus–response coupling and eventually lead to new insights into the functional behavior of these and other phagocytic cells.

In the studies presented herein, we find that electrodiffusive 35SO₄²⁻ fluxes are negligibly small and that SO₄²⁻ can be considered to be essentially impermeant for all practical purposes. All of the steady-state 35SO₄²⁻ movements may be accounted for through a carrier that mediates an exchange of SO₄²⁻ for Cl⁻. This countertransport is voltage insensitive, independent of Na⁺, and is inhibited by SITS, CHC, ethacrylate, probenecid, and furosemide. As in red blood cells (Jennings, 1976; Milanick and Gunn, 1982; 1984), the movement of the divalent SO₄²⁻ anion is probably linked to the cotransport of H⁺, thereby preserving electroneutrality as the ion pair is transported together in exchange for Cl⁻.

MATERIALS AND METHODS

Incubation Media

The standard medium used in this study had the following composition: 137 mM NaCl, 5 mM KCl, 2 mM Na₂SO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 5 mM N-2-hydroxyethyl-
Piperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.40, and 1 mg/ml of crystalline bovine serum albumin. An all-SO₄²⁻ medium was prepared by substituting SO₄²⁻ salts for those with Cl⁻. This medium contained 100 mM SO₄²⁻, 194 mM Na⁺, and 5 mM K⁺ in addition to the normal amounts of Ca²⁺ (1 mM), Mg²⁺ (0.5 mM), and HEPES (5 mM). To test the effects of varying external Cl⁻ or SO₄²⁻, glucuronate was employed as the replacement anion. The cation composition of the media was manipulated by substituting equimolar amounts of either K⁺ or N-methyl-D-glucamine for Na⁺. In order to preserve isotonicity, all of the media were adjusted to a total osmolality of 305 ± 2 mosmol/liter, as monitored by a vapor pressure osmometer (model 5500; Wescor Inc., Logan, UT). For experiments in which the pHₐ of the media was varied between 5.6 and 8.6, the solutions were buffered with 2-(N-morpholino)ethanesulfonic acid (MES, pK' 6.0), HEPES (pK' 7.3), N-tris(hydroxymethyl)methylglycine (Tricine, pK' 7.8), or 2-(N-cyclohexylamino)ethanesulfonic acid (CHES, pK' 9.3) as appropriate. (In preliminary experiments comparable to those of Fig. 7 which were conducted in an otherwise inert Tris-buffered glucuronate medium [see below], we had ascertained that these buffers did not compete with SO₄²⁻.)

When the effects of varying concentrations of external SO₄²⁻ and all monovalent anions were evaluated, the amounts of Ca²⁺ and Mg²⁺ in the medium were kept constant at 1 and 0.5 mM, respectively. However, when foreign divalent anions such as tungstate, oxalate, and molybdate were tested, the experiments were conducted under divalent cation-free conditions to prevent precipitation of these salts. The presence or absence of extracellular Ca²⁺ and/or Mg²⁺ had no measurable consequences on any of the fluxes reported in this article.

Bicarbonate-containing media were obtained in the following manner. A 25 mM HCO₃⁻ stock solution was prepared by substituting 25 mM NaHCO₃ for NaCl in the standard medium described above. This medium was then equilibrated with a 5% CO₂/95% air mixture at 37°C in an incubator and the pH₀ readjusted to 7.40 if necessary. This stock was later diluted with other CO₂/HCO₃⁻-free medium (that had been exhaustively bubbled with N₂ to eliminate the CO₂), also at pH₀ 7.40, in order to obtain a set of solutions of known HCO₃⁻ concentration. The tubes containing these solutions were overlaid with mineral oil and capped during the cell incubations so as to retard diffusion of CO₂.

Neutrophils

Human peripheral neutrophils were isolated by sequential dextran sedimentation at 37°C followed by Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) gradient centrifugation at room temperature (Boyum, 1968). Contaminating erythrocytes were removed by hypotonic lysis in distilled water for 30 s. The neutrophils were washed three times and then counted. The purity of the neutrophil suspensions averaged 98% using Wright's staining. Viability, as assessed by eosin Y exclusion, was ≥99% and was not affected by any of the agents or incubation conditions tested. The cells were routinely isolated using unrefrigerated centrifuges and medium that was warmed to 37°C. In addition, the cells were kept in the standard (2 mM SO₄²⁻, 145 mM Cl⁻) medium for 1 h at 37°C before experimentation.

Reagents and Chemicals

All inorganic salts were obtained from Fisher Scientific Co., St. Louis, MO. The following reagents were purchased from Sigma Chemical Co., St. Louis, MO: crystalline bovine serum albumin, N-methyl-D-glucamine, HEPES, MES, CHES, Tricine, sodium glucuronate, glucuronic acid, p-aminophenyl acid (PAH), sodium PAH, D-glucose, ethacrynic acid, probenecid, and 5,5-dimethyl-2,4-dione (DMO). α-Cyano-4-hydroxycinnamate (CHC) was bought from Aldrich Chemicals, Milwaukee, WI; sodium 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) and SITS from Pierce Chemical Co., Rockford, IL; and nigericin from Cal-
Biochem-Behring Corp., La Jolla, CA. The 5-N,N-hexamethylene analogue of amiloride was a generous gift of Dr. Edward J. Cragoe, Jr., of the Merck, Sharp & Dohme Research Laboratories, West Point, PA and furosemide was kindly supplied by Hoechst-Roussel Laboratories, Somerville, NJ. Isotopes (Na$_2$SO$_4^-$, BaCl$_2$, [14C]DMO, and [3H]H$_2$O were purchased from New England Nuclear, Boston, MA: the $^{35}$SO$_4^{2-}$ was carrier-free; the specific activity of the $^{183}$Ba$^{2+}$ was 6.6 mCi/mg Ba.

**Unidirectional Sulfate Fluxes**

The technique described by Naccache et al. (1977) was used. The incubations were performed at 37°C in capped, plastic tubes (Falcon Labware, Oxnard, CA) under various experimental conditions (neutrophils $8-12 \times 10^6$/ml). Influx experiments were performed in the presence of $^{35}$SO$_4^{2-}$ (8.0 mCi/ml). At stated intervals, triplicate aliquots of the cell suspensions were layered on 0.5 ml of silicone oil (Versilube F-50; Harwick Chemical Corp., Akron, OH) contained in 1.5-ml plastic tubes and centrifuged for 1 min at 8,000 g in a microcentrifuge (Beckman Instruments, Inc., Fullerton, CA). Cell separation occurred in <5 s. The aqueous and oil phases were aspirated and discarded. The neutrophil pellets were excised and counted in a liquid scintillation counter (LS 7000, Beckman Instruments, Inc.).

For the efflux studies, neutrophils were first suspended at 2–3 $\times$ 10$^6$/ml in 2 mM SO$_4^{2-}$, 145 mM Cl$^-$ medium and incubated with $^{35}$SO$_4^{2-}$ (6.0 mCi/ml) for ~2 h at 37°C after which the cells were washed twice in unlabeled medium. Triplicate samples were then taken at stated intervals and handled as described above.

**Chemical Determinations of [SO$_4^{2-}$]**

The internal free SO$_4^{2-}$ content of the cells was determined chemically by precipitation with $^{183}$Ba$^{2+}$. Neutrophils were incubated in either SO$_4^{2-}$-free or in 2 mM SO$_4^{2-}$-containing standard medium for 4 h at 37°C. The neutrophil pellets (25.0 $\times$ 10$^6$ cells) were isolated in quadruplicate as described above and the cells were then lysed in 1% Triton X-100 in water. The cellular debris was removed by centrifugation and the cell lysates mixed with an equal volume of BaCl$_2$ solution labeled with $^{183}$Ba$^{2+}$ (final concentration 10 mM Ba$^{2+}$, 5 mCi/ml $^{183}$Ba$^{2+}$). The insoluble $^{183}$BaSO$_4$ precipitate was pelleted through a mixture of silicone oils (7 parts Versilube F-50 oil + 3 parts SF-9650 oil to give a specific gravity of 1.010) and counted in a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). In order to correct for the small amount of apparently non-SO$_4^{2-}$ precipitable $^{183}$Ba$^{2+}$ counts, the values for the controls (i.e., those neutrophils kept in SO$_4^{2-}$-free medium for several hours and presumably reflecting little or no free internal SO$_4^{2-}$) was subtracted in calculating the intracellular SO$_4^{2-}$ concentration of steady-state cells bathed in 2 mM SO$_4^{2-}$ medium. The internal SO$_4^{2-}$ contents were calculated as millimoles per liter of cell water using an intracellular water volume of 0.274 pl per cell (Simchowitz et al., 1982; Simchowitz and De Weer, 1986).

**Intracellular pH Measurements**

We derived pH$_i$ from the distribution of the $^{14}$C-labeled weak acid DMO ($pK'$ 6.13 at 37°C). Our use of the DMO method (see Roos and Boron, 1981 for review) has been described in a previous report (Simchowitz and Roos, 1985).

**Data Analysis**

In most cases, the influx of $^{35}$SO$_4^{2-}$, corrected for zero-time "uptake," which represents label trapped in the extracellular space, followed equations of the form:

$$C_t = C_0[1 - \exp(-kt)]$$  \hspace{1cm} (1)
where $C_t$ is the cell label at time $t$, $C_\infty$ is the cell label at steady-state, and $k$ is the rate coefficient. Eq. 1 was fit to the data by a nonlinear least-squares program, and the initial influx rate computed from the product $kC_\infty$. As indicated in the figure legends, the change in some of the measured variables often appeared to be linear over the period of study; in those cases, the influx rate was computed from the slope of the linear regression line.

The efflux of $^{35}\text{SO}_4^{2-}$ from the cells followed single exponentials of the form:

$$C_t = C_0 \exp (-kt)$$

where $C_0$ is the cell label at zero time. Curves representing the equation were fit to the data by the least-squares method.

**RESULTS**

**Steady-State $^{35}\text{SO}_4^{2-}$ Fluxes: Effects of Inhibitors**

Measurements of one-way $^{35}\text{SO}_4^{2-}$ influx and efflux in steady-state human neutrophils are presented in Figs. 1 and 2. The time course of $^{35}\text{SO}_4^{2-}$ influx into cells from a 2 mM $\text{SO}_4^{2-}$, 145 mM $\text{Cl}^{-}$ medium is shown in Fig. 1. The entry of $^{35}\text{SO}_4^{2-}$ followed single exponential kinetics with an initial rate of $17.2 \pm 3.1 \, \mu\text{mol/liter-min}$: the final, extrapolated uptake level was $915 \pm 202 \, \mu\text{mol/liter of cell water}$. Chemical determinations of the total free $\text{SO}_4^{2-}$ content of these presumably steady-state cells by means of precipitation with $^{138}\text{Ba}^{2+}$ yielded a value of $1,129 \pm 184 \, \mu\text{mol/liter of cell water}$, not statistically different from that determined above. This similarity indi-
icates that for practical purposes, all cellular SO₄²⁻ is freely exchangeable with extracellular SO₄²⁻.

The steady [SO₄²⁻] of ~1 mM is considerably greater (~25-fold) than the expected passive distribution level (~0.04 mM) for cells with a resting membrane voltage (V_m) of ~-53 mV (Seligmann and Gallin, 1980; Simchowitz et al., 1982) that are bathed in 2 mM SO₄²⁻ medium. It seems evident then that SO₄²⁻ is not passively distributed across the plasma membrane of steady-state neutrophils. Some active metabolic process or indirect coupling such as via secondary active transport must therefore be invoked to explain the relatively large intracellular accumulation of SO₄²⁻ to an extent ~25-fold greater than that expected on thermodynamic grounds.

Data on the efflux of ⁵⁸SO₄²⁻ into a standard 2 mM SO₄²⁻, 145 mM Cl⁻ medium are given in Fig. 2. Efflux from control cells was first-order with a rate coefficient of 0.0168 ± 0.0008 min⁻¹. Assuming the internal SO₄²⁻ concentration to be 1000 μmol/liter of cell water, this signifies an efflux rate of 16.8 ± 0.8 μmol/liter·min, a value comparable to that for one-way ⁵⁸SO₄²⁻ influx (17.2 ± 3.1 μmol/liter·min) measured under identical conditions. This similarity indicates that the cells were in a true steady-state with respect to SO₄²⁻. By way of comparison, these one-way ⁵⁸SO₄²⁻ fluxes are substantially lower than those for ⁴²K⁺ and ²²Na⁺ (each ~0.9 meq/liter·min [Simchowitz et al., 1982]) and for ⁵¹Cl⁻ (~1.4 meq/liter·min [Simchowitz and De Weer, 1986]). In an attempt to elucidate the nature of the pathways for ⁵⁸SO₄²⁻ movements across the neutrophil, we studied the effect of manipulating the ionic environment of the bathing medium and also of a number of commonly used inhibitors of membrane transport.
We first considered the possibility that $^{35}\text{SO}_4^{2-}$ fluxes might occur by simple electrodiffusion through a low-conductance pathway. If this were indeed the case, one-way $^{35}\text{SO}_4^{2-}$ influx and efflux should be strongly voltage dependent. For example, if constant field assumptions apply and $\text{SO}_4^{2-}$ ions behave independently, an $\sim 60$-mV change in membrane potential should cause a corresponding $\sim 10$-fold change in the magnitude of one-way $^{35}\text{SO}_4^{2-}$ fluxes, influx and efflux being affected oppositely. For a $\sim 60$-mV depolarization, the predicted direction would be an increase in influx and a decrease in efflux. However, it is clear from the data of Fig. 1 that depolarizing the cells from their normal resting membrane voltage of $\sim -53$ to $\sim 0$ mV by raising external K$^+$ from 5 to 120 mM (Seligmann and Gallin, 1980; Simchowitz et al., 1982; Simchowitz and De Weer, 1986) had a rather modest effect on influx. Increasing [K$^+$]$_o$ from 5 to 120 mM led to only a relatively slight enhancement of influx rate from 17.2 to 23.1 $\pm$ 1.6 $\mu$mol/liter$\cdot$min, an increase of 34%. Moreover, $^{35}\text{SO}_4^{2-}$ efflux was little affected if at all by depolarizing the cells in 120 mM K$^+$ (16.8 $\pm$ 0.8 vs. 15.3 $\pm$ 1.0 $\mu$mol/liter$\cdot$min, Fig. 2). These data leave little doubt that the predominant mechanism for $\text{SO}_4^{2-}$ movement into or out of the cell is other than by current-carrying pathways and most likely occurs through electroneutral transport via carriers and/or pumps.

In renal as well as in intestinal epithelia, $\text{SO}_4^{2-}$ is also transported into the cell by voltage-insensitive means: i.e., by either a 2 Na$^+$ + $\text{SO}_4^{2-}$ cotransport system (Lucke et al., 1979; Murer and Burckhardt, 1983; Schneider et al., 1984) or by anion exchange (Grinstein et al., 1980; Ulrich et al., 1980; Brazy and Dennis, 1981; Langridge-Smith and Field, 1981). The former mechanism, however, cannot explain the bulk of $^{35}\text{SO}_4^{2-}$ influx into human neutrophils since uptake was found to be independent of extracellular Na$^+$. As shown in Fig. 1, complete replacement of external Na$^+$ by N-methyl-d-glucamine (140 mM) had no effect on either the initial rate or the final extent of $^{35}\text{SO}_4^{2-}$ influx (initial rates for the Na$^+$ and Na$^+$-free data were 17.2 $\pm$ 3.1 and 17.7 $\pm$ 1.3 $\mu$mol/liter$\cdot$min, respectively). Concomitant depletion of all external K$^+$ likewise did not alter these results (data not shown).

In an effort to gain insight into the relevant mechanism(s) of $\text{SO}_4^{2-}$ entry and exit in neutrophils, the effect of a number of commonly used transport inhibitors was tested. Two inhibitors of anion exchange in a wide variety of cells, SITS and CHC (for reviews, see Sachs et al., 1975; Knauf, 1979; Gunn, 1979; Deuticke, 1982; Hoffmann, 1986), caused marked inhibition of the rate of $^{35}\text{SO}_4^{2-}$ influx. In the presence of 1 mM SITS or 20 mM CHC, influx was reduced from 17.2 to 2.9 $\pm$ 0.3 and 3.4 $\pm$ 0.4 $\mu$mol/liter$\cdot$min, respectively; inhibitions of 83% and 80%. In erythrocytes and other cell types, SITS and other disulfonic stilbenes have been shown to block anion (i.e., Cl$^-$/Cl$^-$ and Cl$^-$/HCO$_3^-$) exchange, whereas these compounds have little effect against the anion exchange carrier of human neutrophils (Simchowitz and De Weer, 1986). In neutrophils, however, anion exchange is competitively inhibited by CHC (apparent $K_i$ $\sim$ 9 mM), a drug which also blocks monocarboxylate transport as well as anion exchange in mitochondria and erythrocytes (Halestrap and Denton, 1975; Halestrap, 1976; Deuticke, 1982). (For comparison, apparent $K_i$ values of 0.006, 0.06, and 0.13 mM have been reported for inhibition of rat liver mitochondrial monocarboxylate, human red cell monocarboxylate, and human red cell inorganic anion transport, respectively.) Probenecid, which has also been shown
to suppress carrier-mediated anion fluxes in different biological systems (Blomstedt and Aronson, 1980) also had a significant inhibitory effect on $^{35}\text{SO}_4^{2-}$ influx into neutrophils although less so than for SITS or CHC. A drug concentration of 1 mM caused 59% inhibition of the initial rate of influx. Likewise, $^{35}\text{SO}_4^{2-}$ influx was substantially reduced by treating the cells with the "loop" diuretics ethacrynic acid and furosemide. In the presence of 1 mM of either of these agents, inhibitions of 81% and 48%, respectively, were observed.

Fig. 2 shows that, at the same concentrations as stated above, each of these drugs had a comparable inhibitory effect on $^{35}\text{SO}_4^{2-}$ efflux from the cells into a 2 mM SO$_4^{2-}$, 145 mM Cl$^-$ medium. Relative to a control efflux rate of 16.8 ± 0.8 μmol/liter·min, that into 20 mM CHC or 1 mM of either SITS, ethacrynic acid, probenecid, or furosemide was 2.4 ± 0.8, 2.5 ± 0.2, 3.3 ± 0.8, 8.3 ± 0.5, and 9.8 ± 0.7 μmol/liter·min, respectively. These values represent drug-dependent inhibitions of 86%, 85%, 80%, 51%, and 42%.

The marked inhibition of $^{35}\text{SO}_4^{2-}$ fluxes by SITS, in contrast to the lack of effect of the compound on Cl$^-$/Cl$^-$ self-exchange under the same conditions (Simchowitz and De Weer, 1986), strongly implies that SO$_4^{2-}$ does not cross the cell membrane of human neutrophils via the principal anion exchange carrier. Another line of evidence from this laboratory also supports the contention that the major anion exchange mechanism of neutrophils, which functions physiologically as a Cl$^-$/HCO$_3^-$ exchanger in pH recovery from alkaline loads (Simchowitz and Roos, 1985), is essentially devoid of affinity for SO$_4^{2-}$: external SO$_4^{2-}$ (0–95 mM, replacing glucuronate) has no effect whatsoever on the influx of $^{35}\text{Cl}^-$ from a 5 mM Cl$^-$ medium (Simchowitz, 1988a).

The inhibition of one-way $^{35}\text{SO}_4^{2-}$ influx and efflux by SITS, CHC, and probenecid, all of which have been shown to alter anion transport in other cell types, suggested the possibility that $^{35}\text{SO}_4^{2-}$ transport might reflect some form of carrier-mediated anion exchange. One obvious possibility is that Cl$^-$ represents the internal counter anion in the exchange process since the normal resting internal CI$^-$ content is high (~80 meq/liter of cell water [Simchowitz and De Weer, 1986]). To test this hypothesis, we prepared a batch of internally Cl$^-$-depleted cells ([Cl$^-$]$_i$ ~5 mM) by prolonged (~4 h) incubation in Cl$^-$-free, 145 mM PAH medium. After this pretreatment period, the cells were resuspended in a 2 mM SO$_4^{2-}$, 145 mM glucuronate medium. Glucuronate, rather than Cl$^-$, was selected as the predominant extracellular anion in order to prevent the reaccumulation of intracellular Cl$^-$ since glucuronate is inert with respect to the Cl$^-$/Cl$^-$ exchange carrier and also, as will be shown shortly, to SO$_4^{2-}$ transport. As shown in Fig. 3, $^{35}\text{SO}_4^{2-}$ influx into normal Cl$^-$ cells from a 2 mM SO$_4^{2-}$, 145 mM glucuronate medium proceeded at an initial rate of 77.0 ± 10.2 μmol/liter·min, approximately fivefold faster than from Cl$^-$ medium (17.2 μmol/liter·min, Fig. 1). Influx of $^{35}\text{SO}_4^{2-}$ is much more rapid from glucuronate as compared to Cl$^-$ medium because glucuronate is devoid of affinity whereas Cl$^-$ behaves as an alternate substrate which competes with SO$_4^{2-}$ for binding to the carrier (see below). As compared to normal Cl$^-$ cells, $^{35}\text{SO}_4^{2-}$ influx from a 2 mM SO$_4^{2-}$, 145 mM glucuronate medium into Cl$^-$-depleted, PAH-loaded cells was markedly slowed. The initial influx rate was only 8.9 ± 0.8 μmol/liter·min, roughly 12%
that into normal Cl⁻ cells (77.0 ± 10.2 μmol/liter-min). The addition of 1 mM SITS had only a slight further inhibitory effect, lowering the initial influx rate to 6.0 ± 0.6 μmol/liter-min, a value similar to that observed with normal Cl⁻ cells (5.6 ± 0.5 μmol/liter-min) when measured under identical conditions. This is as expected if Cl⁻ were serving as the intracellular exchange partner for external ³⁵SO₄²⁻. These data do not rule out the possibility that another physiologically relevant anion in addition to Cl⁻ could have been simultaneously depleted along with Cl⁻ during the prolonged incubation in PAH medium.

If the mechanism were truly that of an exchange of SO₄²⁻ for Cl⁻ (we will address the issue of stoichiometry later on), then one would expect to find certain proper-

![Figure 3](image.png)

**FIGURE 3.** Effect of intracellular Cl⁻ depletion on ³⁵SO₄²⁻ influx from a 2 mM SO₄²⁻, 145 mM glucuronate medium. A batch of internally Cl⁻-depleted ([Cl⁻]ᵢ ~ 5 mM) neutrophils was prepared by incubating cells in a Cl⁻-free, 2 mM SO₄²⁻, 145 mM PAH medium for ~4 h at 37°C. The normal Cl⁻ cells were kept in 2 mM SO₄²⁻, 145 mM Cl⁻ medium at 37°C throughout the entire pretreatment period. At zero time, the neutrophil pellets were resuspended in a 2 mM SO₄²⁻, 145 mM glucuronate medium in the presence or absence of 1 mM SITS. The bathing solutions also contained 85 mM K⁺ and 2 μM nigericin (for rationale, see text). At stated times, samples of the neutrophil suspensions were removed and the cell pellets counted for radioactivity. The top curve labeled Control (normal Cl⁻ cells) was fit to a single exponential equation (Eq. 1) which yielded an initial ³⁵SO₄²⁻ influx rate of 77.0 ± 10.2 μmol/liter-min. The middle and lower two curves were also fit to Eq. 1. The middle curve (Cl⁻-depleted cells) had an initial influx rate of 8.91 ± 0.75 μmol/liter-min. The lower curve (combined data for normal Cl⁻ and Cl⁻-depleted cells each in the presence of 1 mM SITS) exhibited an initial influx rate of 5.82 ± 0.39 μmol/liter-min. Results are from three experiments.

**Trans Effects: Substrate Saturation**

The data alluded to above, that ³⁵SO₄²⁻ influx is greater into normal Cl⁻ as compared to internally Cl⁻-depleted cells, in essence show trans stimulation of ³⁵SO₄²⁻ influx by intracellular Cl⁻. In addition, trans stimulation of ³⁵SO₄²⁻ efflux by external
Cl\(^{-}\) and by external \(\text{SO}_4^{2-}\) are shown in Fig. 4. For these experiments, the rate of \(\text{SO}_4^{2-}\) efflux was measured into media in which the concentration of either \(\text{Cl}^{-}\) (0–140 mM) or \(\text{SO}_4^{2-}\) (0–80 mM) was varied by replacement of glucuronate (the isotonicity of all of the media was maintained at \(\sim 305\) mosmol/liter). The media also contained 85 mM K\(^{+}\) and 2 \(\mu M\) nigericin, a K\(^{+}\)/H\(^{+}\) exchanging ionophore (Pressman, 1969), in order to pH-clamp the neutrophils at their normal resting pHi of \(\sim 7.25\) (Simchowitz and Roos, 1985). Otherwise, a reduction in [\(\text{Cl}^{-}\)]\(_{o}\) would lead (via the principal anion exchange carrier) to an exchange of internal \(\text{Cl}^{-}\) for the trace amounts of external HCO\(_3^{-}\) present in the otherwise inert glucuronate medium. The resulting intracellular alkalinization would then cause undesirable complications that would interfere with the interpretation of our results. When steps were taken to avoid these potential pitfalls using the high [K\(^{+}\)]\(_{o}\)/nigericin technique, pH\(_{i}\) remained essentially constant at \(\sim 7.25\). Under these conditions, as displayed in Fig. 4, raising [\(\text{Cl}^{-}\)]\(_{o}\) between 0 and 140 mM caused a progressive increase in the rate of \(\text{SO}_4^{2-}\) efflux from the cells. External \(\text{Cl}^{-}\) stimulated \(\text{SO}_4^{2-}\) efflux along a Michaelis-Menten activation curve with apparent \(K_m\) (\(\text{Cl}^{-}\)) \(= 22.4 \pm 8.1\) mM and \(V_{\text{max}} = 21.7 \pm 2.6\) \(\mu\)mol/liter-min; for \(\text{SO}_4^{2-}\), apparent \(K_m = 15.5 \pm 7.8\) mM and \(V_{\text{max}} = 7.24 \pm 1.32\) \(\mu\)mol/liter-min. Results have been taken from three to four separate experiments.

\[
\text{FIGURE 4. Substrate saturation: stimulation by external Cl}^{-} \text{ or SO}_4^{2-} \text{ of the rate of SO}_4^{2-} \text{ efflux from the cells. Neutrophils were labeled with SO}_4^{2-} \text{ as described in Fig. 2. There-}
\]

\[
\text{after, the cells were resuspended in media in which either the extracellular Cl}^{-} (0–140 mM) \text{ or SO}_4^{2-} (0–80 mM) \text{ concentrations were varied by}
\]

\[
\text{replacement of glucuronate. The media also contained 85 mM K}^{+} \text{ and 2 \(\mu M\) nigericin to pH-clamp the cells}
\]

\[
\text{at their normal resting pHi of \(\sim 7.25\) (for rationale, see text). The efflux of}
\]

\[
\text{SO}_4^{2-} \text{ was measured at 10 and 20}
\]

\[
\text{min and the efflux rates were calculated as in Fig. 2 on the assumption}
\]

\[
[\text{SO}_4^{2-}]_{i} = 1,000 \text{ \(\mu\)mol/liter of}
\]

\[
\text{cell water (see text and Figs. 1 and 3). After subtraction of the minor SITS-}
\]

\[
\text{insensitive background (2.4 \(\mu\)mol/liter-min), presumably representing some form of leak}
\]

\[
\text{flux, the data points were fit to Michaelis-Menten activation equations. The equation yielded}
\]

\[
\text{the following kinetic parameters: for Cl}^{-}, \text{ apparent } K_m = 22.4 \pm 8.1\text{ mM and } V_{\text{max}} = 21.7 \pm 2.6\text{ \(\mu\)mol/liter-min; for SO}_4^{2-}, \text{ apparent } K_m = 15.5 \pm 7.8\text{ mM and } V_{\text{max}} = 7.24 \pm 1.32\text{ \(\mu\)mol/liter-min. Results have been taken from three to four separate experiments.}
\]
SO$_4^{2-}$ exchange was much less than that for external Cl$^-$. At present, the data of Fig. 4 are compatible with the notion that Cl$^-$ binds to the external translocation site of the carrier with apparent K$_m$ ~22 mM and that extracellular Cl$^-$ and SO$_4^{2-}$ both stimulate the exchange of internal SO$_4^{2-}$, but that the rate of Cl$^-$/SO$_4^{2-}$ exchange is much faster than that of SO$_4^{2-}$/SO$_4^{2-}$ exchange.

As noted above, the data of Fig. 4 demonstrate substrate saturation of the carrier by external Cl$^-$ and SO$_4^{2-}$. Substrate saturation of $^{35}$SO$_4^{2-}$ influx is further shown in Fig. 5. For these experiments, the rate of $^{35}$SO$_4^{2-}$ influx into normal Cl$^-$ cells was measured from media in which the concentration of extracellular SO$_4^{2-}$ was gradually varied between 0.3 and 80 mM, replacing either Cl$^-$, a moderately high affinity substrate, or glucuronate, an inert anion. (The fact that no SITS-sensitive $^{35}$SO$_4^{2-}$ flux could be detected into an all-glucuronate [148 mM] medium supports the contention that this ion is truly inert with respect to the SO$_4^{2-}$ carrier.) When [SO$_4^{2-}$]$_o$ was varied reciprocally with [Cl$^-$]$_o$ such that isotonicity was maintained (osmolality ~305 mosmol/liter), the rate of $^{35}$SO$_4^{2-}$ influx rose linearly with [SO$_4^{2-}$]$_o$, with no evidence of saturation (i.e., the $^{35}$SO$_4^{2-}$ influx rate was a direct function of [SO$_4^{2-}$]$_o$). However, when the experiment was repeated, but this time with [SO$_4^{2-}$]$_o$ varying reciprocally with [glucuronate]$_o$, substrate saturation was clearly evident. External SO$_4^{2-}$ stimulated its own influx along a Michaelis-Menten activation curve with apparent K$_m$ (SO$_4^{2-}$) = 12.5 ± 3.0 mM and V$_{max}$ = 667 ± 63 μmol/liter·min. The value of ~14 mM for the apparent binding constant of external SO$_4^{2-}$ (Figs. 4 and 5) helps

![Figure 5](https://via.placeholder.com/150)
to explain the nonsaturability shown in Fig. 5 where SO_{4}^{2-} and Cl\textsuperscript{-} were varied reciprocally. This is as expected for two competing substrates when the apparent K_m of the replacement anion (i.e., Cl\textsuperscript{-}) is roughly equal to the apparent K_m of the measured anion (i.e., SO_{4}^{2-}). At this point, it is evident that the carrier possesses roughly similar affinities (within a factor of 2) for external Cl\textsuperscript{-} and SO_{4}^{2-}, but that the affinity for glucuronate is much less than that for either Cl\textsuperscript{-} or SO_{4}^{2-}. In fact, it appears that the SO_{4}^{2-} carrier is essentially devoid of affinity for glucuronate and that for all practical purposes, glucuronate may be considered to be inert.

**Inhibition by Drugs**

Specific inhibition of \textsuperscript{35}SO_{4}^{2-} influx is shown in Fig. 6. As mentioned above in connection with the results of Figs. 1 and 2, SITS, CHC, and ethacrynic acid all caused marked inhibition of steady-state \textsuperscript{35}SO_{4}^{2-} fluxes in 2 mM SO_{4}^{2-}, 145 mM Cl\textsuperscript{-} medium. In Fig. 6, A–C, the dose-dependence of each of these drugs on \textsuperscript{35}SO_{4}^{2-} influx from 2 mM (replacing glucuronate) and from 100 mM SO_{4}^{2-} media was tested. The studies were performed at two different external SO_{4}^{2-} concentrations, 2 and 100 mM SO_{4}^{2-} (or ~0.1 and ~7 times apparent K_m[SO_{4}^{2-}]) in order to evaluate whether the type of inhibition was competitive, noncompetitive, or mixed. In all instances, the dose–response curves could be fit by a simple Michaelis-Menten inhibition equation and the data displayed in the form of Dixon plots. For SITS (Fig. 6 A), the apparent K_i values at 100 and 2 mM SO_{4}^{2-} were essentially identical (49.4 ± 10.3 and 56.0 ± 8.5 μM, respectively) indicating a noncompetitive type of inhibition. In contrast, for CHC and ethacrynic (Fig. 6, B and C, respectively), there was a decrease in apparent drug K_i as [SO_{4}^{2-}]_o was lowered from 100 to 2 mM, thereby signifying an increase in apparent affinity. For example, the apparent K_i value for CHC fell from 2.08 ± 0.39 to 0.25 ± 0.06 mM, while that for ethacrynic fell from 59.0 ± 19.4 to 7.1 ± 1.8 μM at [SO_{4}^{2-}]_o = 100 and 2 mM, respectively. In these two cases, the substrate–inhibitor interactions were found to be strictly competitive in nature. The inhibitory effects of CHC and ethacrynic were readily reversible upon washing the cells and then resuspending them in drug-free medium. On the other hand, the inhibition by SITS persisted, suggesting a large irreversible component.

**Anion Selectivity**

Substrate competition is documented in Figs. 7 and 8 where the ability of other external anions to compete with external SO_{4}^{2-} for binding to the exchange carrier (and thus to inhibit \textsuperscript{35}SO_{4}^{2-} influx from a 2 mM SO_{4}^{2-} medium [balance glucuronate]) is shown. As displayed in Fig. 7 for monovalent anions, increasing the extracellular concentrations of either Cl\textsuperscript{-}, Br\textsuperscript{-}, I\textsuperscript{-}, NO\textsubscript{3}\textsuperscript{-}, formate\textsuperscript{-}, or PAH\textsuperscript{-} between 0 and 140 mM led to progressive reductions in the rate of \textsuperscript{35}SO_{4}^{2-} influx. The relationship was well fit by Michaelis-Menten kinetics with apparent K_i values of 21.1 ± 4.8, 31.9 ± 7.2, 43.6 ± 8.5, 81.8 ± 15.6, 125 ± 29, and 141 ± 31 mM, respectively, for NO\textsubscript{3}\textsuperscript{-}, Cl\textsuperscript{-}, Br\textsuperscript{-}, formate\textsuperscript{-}, I\textsuperscript{-}, and PAH\textsuperscript{-}, respectively. In contrast, HCO\textsubscript{3}\textsuperscript{-} (0–20 mM) had no inhibitory effect at all. It should be pointed out that for a system characterized by competition kinetics (and assuming a one-to-one stoichiometry), the
in the form of Dixon plots ($1/v$ vs. $I$). For each drug, the "true" $K_i$ values were calculated from Eq. 3 on the assumption that true $K_m$ ($SO_4^{2-}$) = 13.7 mM (Table I). Results are from three to four experiments for each condition. (A) Data for SITS: at 100 mM $SO_4^{2-}$, apparent $K_i = 49.4 \pm 10.3 \mu M$; at 2 mM $SO_4^{2-}$, apparent $K_i = 56.0 \pm 8.5 \mu M$; true $K_i = 52.7 \pm 6.6 \mu M$. (B) Data for CHC: at 100 mM $SO_4^{2-}$, apparent $K_i = 2.08 \pm 0.39 \mu M$; at 2 mM $SO_4^{2-}$, apparent $K_i = 0.252 \pm 0.057 \mu M$; true $K_i = 0.234 \pm 0.034 \mu M$. (C) Data for ethacrynate: at 100 mM $SO_4^{2-}$, apparent $K_i = 59.0 \pm 19.4 \mu M$; at 2 mM $SO_4^{2-}$, apparent $K_i = 7.08 \pm 1.78 \mu M$; true $K_i = 6.64 \pm 1.37 \mu M$. 

**FIGURE 6.** Dose dependence of inhibition of $^{35}SO_4^{2-}$ influx by various drugs. The uptake of $^{35}SO_4^{2-}$ into neutrophils from a 2 mM $SO_4^{2-}$ (balance glucuronate) and from a 100 mM $SO_4^{2-}$ medium was measured in the presence of varying concentrations of either SITS (0–800 μM), CHC (0–20 mM), or ethacrynic acid (0–1,000 μM). All media contained 85 mM $K^+$ and 2 μM nigericin. The initial $^{35}SO_4^{2-}$ influx rates were calculated as in Fig. 1 by fitting the internal $^{35}SO_4^{2-}$ contents at different time intervals to single exponentials (Eq. 1). A background subtraction of a SITS- or CHC-resistant influx of ~3 or ~150 μmol/liter ⋅ min at 2 and 100 mM $SO_4^{2-}$ was then applied. The individual sets of data are displayed
FIGURE 7. Substrate competition: inhibition of $^{35}$SO$_4^{2-}$ influx from a 2 mM SO$_4^{2-}$ medium by various monovalent anions. The bathing solutions contained 85 mM K$^+$ and 2 $\mu$M nigericin in which the concentrations of the different anions (Cl$^-$, Br$^-$, I$^-$, NO$_3^-$, HCO$_3^-$, formate, and PAH$^-$) were varied between 0 and 140 mM by replacement of glucuronate. The $^{35}$SO$_4^{2-}$ influx rates were calculated as in Fig. 5 (after correction for the SITS-insensitive leak influx ~3 $\mu$mol/liter-min) and plotted against the external concentrations of the competing anions. The curves are least-squares fits of the data to Michaelis-Menten inhibition equations which yielded apparent $K_i$ values for NO$_3^-$, Cl$^-$, Br$^-$, formate$^-$, I$^-$, and PAH$^-$ of 21.2 ± 4.8, 31.9 ± 7.2, 43.6 ± 8.5, 81.8 ± 15.6, 125 ± 29, and 141 ± 31 mM, respectively. These parameters signify the apparent $K_m$ values for each of the various anions in the presence of 2 mM SO$_4^{2-}$. From Eq. 3, taking $K_m$(SO$_4^{2-}$) as 13.7 mM (Table I), the “true” $K_m$ values are 0.87 times those listed above. The data for HCO$_3^-$ were fit to a straight line with a slope of 0.022 ± 0.297. Results represent three to four experiments for each monovalent anion.

FIGURE 8. Substrate competition by various divalent anions. See legend to Fig. 7. The external concentrations of the different anions (oxalate, tungstate, molybdate, arsenate, selenate, and phosphate) were varied between 0 and 90 mM by replacement of glucuronate; [SO$_4^{2-}$]$_o$ was 2 mM. The curves are fits to Michaelis-Menten inhibition equations which yielded apparent $K_i$ values for tungstate, oxalate, molybdate, selenate, and arsenate of 1.19 ± 0.18, 7.38 ± 1.86, 52.7 ± 15.2, 70.9 ± 18.6, and 116 ± 26 mM, respectively. On the assumption that true $K_m$(SO$_4^{2-}$) = 13.7 mM (Table I), the above parameters correspond to true $K_m$ values that are 0.87 times those listed above (see Eq. 3). The data for phosphate were fit to a straight line with slope = −0.0405 ± 0.0588 which could not be distinguished from zero. Results represent three to four experiments for each divalent anion.
relationship between the true and apparent binding constants for a substrate $S_1$ in the presence of a competing substrate $S_2$ is given by:

$$\text{apparent } K_m(S_1) = \text{true } K_m(S_1) \left[ 1 + \frac{[S_2]}{K_m(S_2)} \right]$$

(3)

Entering the appropriate values for $SO_4^{2-}$ (i.e., $S_2$, where $[SO_4^{2-}]_o = 2$ mM and $K_m(SO_4^{2-}) = 13.7$ mM [see Table I]), it follows from Eq. 3 that the true $K_m$ value for each of the anions tested is 0.87 times its apparent $K_i$ measured under the conditions outlined above.

### Table I

<table>
<thead>
<tr>
<th>Anion</th>
<th>$K_m$ (mM)</th>
<th>$K_i$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Divalent</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfate</td>
<td>13.7 ± 2.0</td>
<td>(-)</td>
</tr>
<tr>
<td>Tungstate</td>
<td>1.0 ± 0.2</td>
<td>(--)</td>
</tr>
<tr>
<td>Oxalate</td>
<td>6.4 ± 1.6</td>
<td>(--)</td>
</tr>
<tr>
<td>Molybdate</td>
<td>45.8 ± 13.2</td>
<td>(--)</td>
</tr>
<tr>
<td>Selenate</td>
<td>61.7 ± 16.2</td>
<td>(--)</td>
</tr>
<tr>
<td>Arsenate</td>
<td>101 ± 23</td>
<td>(--)</td>
</tr>
<tr>
<td>Phosphate</td>
<td>---</td>
<td>(--)</td>
</tr>
<tr>
<td><strong>Monovalent</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>25.1 ± 5.1</td>
<td>(5.0 ± 0.8)</td>
</tr>
<tr>
<td>Nitrate</td>
<td>18.4 ± 4.2</td>
<td>(8.4 ± 1.3)</td>
</tr>
<tr>
<td>Bromide</td>
<td>37.9 ± 7.4</td>
<td>(9.4 ± 1.0)</td>
</tr>
<tr>
<td>Formate</td>
<td>71.2 ± 13.6</td>
<td>(13.0 ± 2.5)</td>
</tr>
<tr>
<td>Iodide</td>
<td>109 ± 25</td>
<td>(44.2 ± 7.5)</td>
</tr>
<tr>
<td>$p$-Aminohippurate</td>
<td>123 ± 27</td>
<td>(50.3 ± 14.9)</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>---</td>
<td>(4.1 ± 0.9)</td>
</tr>
<tr>
<td>Glucuronate</td>
<td>---</td>
<td>(--)</td>
</tr>
</tbody>
</table>

The true Michaelis constants for the various mono- and divalent anions were calculated from Eq. 3 using the data of Figs. 7 and 8. The kinetic constant for $SO_4^{2-}$ was obtained from the weighted averages of the combined data of Figs. 4, 5, and 10; that for $Cl^-$, from Figs. 4 and 7. The calculations are based on the finding that the exchange carrier has no affinity for glucuronate. Analogous data for the principal anion ($Cl^-$/$HCO_3^-$) exchanger of neutrophils (enclosed in parentheses in the right-hand column and taken from Simchowitz et al., 1986 and Simchowitz, 1988a, b) are also shown for comparison.

*No apparent affinity.

Other divalent anions, such as oxalate, molybdate, arsenate, tungstate, selenate, and phosphate (each at concentrations ranging from 0 to 90 mM) were also evaluated and the results given in Fig. 8. Phosphate had no appreciable effect on $^{35}SO_4^{2-}$ influx at concentrations as high as 90 mM. However, raising the concentrations of tungstate, oxalate, molybdate, arsenate, or selenate between 0 and 60 mM caused a gradual decline in the rate of $^{35}SO_4^{2-}$ influx from a 2 mM $SO_4^{2-}$ medium. Fits of the data to Michaelis-Menten inhibition equations yielded apparent $K_i$ values of 1.2 ± 0.2, 7.4 ± 1.9, 52.7 ± 15.2, 70.9 ± 18.6, and 116 ± 26 mM for tungstate, oxalate,
molybdate, selenate, and arsenate, respectively. Over the concentration range tested, none of these polyvalent anions altered cell viability. Citrate and succinate (tri- and dicarboxylates) inhibited $^{35}$SO$_4^{2-}$ influx with apparent $K_i$ values of ~80 mM (data not shown). Among the various mono- and divalent anions tested, the carrier appeared to exhibit the highest affinity for tungstate. In fact, its apparent $K_m$ was $\leq$0.1 that of Cl$^-$/SO$_4^{2-}$, presumably the natural substrates.

That the interaction between these test anions and SO$_4^{2-}$ at the external transport site of the carrier was indeed competitive could be shown in the next series of studies. When the experiments of Figs. 7 and 8 were repeated, but this time in the presence of 50 mM SO$_4^{2-}$ in the bathing solutions, there was a substantial shift towards lower affinities. Comparing 2 and 50 mM SO$_4^{2-}$, the apparent $K_i$ values for tungstate, oxalate, and NO$_3^-$ rose from 1.2, 7.4, and 21.1 mM to reach 4.4 ± 1.0, 33.1 ±
5.9, and 94.7 ± 21.6 mM, respectively. The approximately fourfold increase in apparent inhibition constants in going from 2 to 50 mM SO$_4^{2-}$ is entirely compatible with simple competition kinetics (Eq. 3), given a true $K_m$ for SO$_4^{2-}$ of ~14 mM.

**Effect of pH: Stoichiometry**

At this point, a question arises as to the stoichiometry of the SO$_4^{2-}$ exchange carrier. The data of Figs. 1 and 2 indicate that one-way $^{35}$SO$_4^{2-}$ influx and efflux occur via electroneutral mechanisms. Since the relevant pathway seems to be that of an electrically silent exchange of SO$_4^{2-}$ for Cl$^-$, a simple hypothesis to account for all of these observations would be a one-for-one exchange of SO$_4^{2-}$ for Cl$^-$ where one of the two negative charges of SO$_4^{2-}$ is offset by the equivalent cotransport of H$^+$ together with SO$_4^{2-}$. Thus, the carrier effectively mediates the countertransport of Cl$^-$ for H$^+$ + SO$_4^{2-}$ (i.e., Cl$^-$/[H$^+$ + SO$_4^{2-}$] exchange). Such a mechanism has been described and examined in great detail in human erythrocytes where SO$_4^{2-}$ transport takes place through the classical inorganic anion exchanger (band 3 protein) (Jennings, 1976; Knauf, 1979; Milanick and Gunn, 1982, 1984).

Several lines of evidence directly support the existence of an analogous mechanism in human neutrophils that is distinct from the principal anion (i.e., Cl$^-$/Cl$^-$, Cl$^-$/HCO$_3^-$) exchanger. The first piece of data is shown in Fig. 9 where the pH dependence of $^{35}$SO$_4^{2-}$ influx from a 2 mM SO$_4^{2-}$, 145 mM Cl$^-$ medium is presented. For these experiments, the $^{35}$SO$_4^{2-}$ flux was measured at 2.5 and 5 min in order to minimize any secondary pH$_i$ changes. As previously reported (Simchowitz and Roos, 1985), while the pH$_i$ tended to slowly drift in the same direction with extracellular acidification or alkalinization, over the pH$_o$ range 5.6-8.6, the pH$_i$ remained within 0.15 pH U of the control value (7.31). The rate of $^{35}$SO$_4^{2-}$ uptake was markedly sensitive to changes in pH$_o$. Relative to the influx rate at pH$_o$ 7.40, extracellular acidification enhanced the uptake of $^{35}$SO$_4^{2-}$, while extracellular alkalinization inhibited it. Between pH$_o$ 5.6 and 8.6, the relationship between pH$_o$ and $^{35}$SO$_4^{2-}$ influx was sigmoidal with an apparent pK of 7.15 ± 0.14. The fact that the relationship can be adequately represented by a simple titration curve is consistent with the proposal that a single H$^+$ ion is needed to activate the exchange (Hill coefficient = 1.0). Thus, the data of Fig. 9 reveal cis activation of $^{35}$SO$_4^{2-}$ influx by H$^+$. This is as expected if H$^+$ were a substrate for the exchange.

As shown in Fig. 10, the striking sensitivity of $^{35}$SO$_4^{2-}$ influx to pH$_o$ did not result from a shift in the apparent $K_m$ for SO$_4^{2-}$. As pH$_o$ was lowered from 7.4 to 6.7 to 6.0 (and influx rates rose by factors of ~2 and ~3, respectively), the apparent $K_m$ for SO$_4^{2-}$ remained essentially the same: 12.5 ± 3.0, 17.6 ± 4.8, and 10.1 ± 2.2 mM, respectively. The rather low $^{35}$SO$_4^{2-}$ influx rates at pH$_o$ ≥ 8.0 made determinations of $K_m$(SO$_4^{2-}$) in the alkaline range unfeasible. The lack of effect of H$^+$ on the $K_m$ for extracellular SO$_4^{2-}$ is similar to the situation in red cells where Milanick and Gunn (1984) showed that the half-saturation constants for SO$_4^{2-}$ transport remained constant (4–5 mM) between pH$_o$ 5.5 and 9.0. These data do not rule out the possibility that the main effect of pH$_o$ is on the maximal transport rate ($V_{max}$) of the exchange carrier. However, this alternative was excluded in the next series of experiments (Fig. 9). These studies were based on the rationale that if the effect of pH were solely on $V_{max}$, then pH$_o$ should alter cis and trans SO$_4^{2-}$ fluxes to the same extent.
However, if the effect of pHo were chiefly related to the H⁺ ion's being a reactant in the exchange, then only cis $^{35}$SO₄⁻ fluxes should be materially affected. As can be seen in Fig. 9, in contrast to the dramatic consequences of changes in pHo on $^{35}$SO₄⁻ influx, varying pHo between 5.6 and 8.6 had little effect on $^{35}$SO₄⁻ efflux under the same conditions. These data imply that the effect of pH is not on the $V_{max}$ of the exchange carrier. Rather, it is a direct result of cis activation by H⁺ acting as a substrate for the carrier that stimulates the counter-transport mechanism.

The cis activation of $^{35}$SO₄⁻ efflux by internal H⁺ is shown in the inset to Fig. 9. For these experiments, neutrophils were acidified by varying [K⁺]o between 2 and 120 mM in the presence of 2 μM nigericin, a K⁺/H⁺ exchanging ionophore (Pressman, 1969). Since, under these conditions, [K⁺]o/[K⁺]i = [H⁺]o/[H⁺]i and resting [K⁺]i ~ 120 mM and pHi = 7.40, the pHi could be estimated on the assumption that equilibrium had been reached. Thus, according to the above equation, at

\[
\frac{[K^+]_o}{[K^+]_i} = 1.9, 3.8, 7.5, 15, 30, 60, \text{ and } 120 \text{ mM, the pHi should be } \sim 5.60, 5.90, 6.20, 6.50, 6.80, 7.10, \text{ and } 7.40, \text{ respectively. These expectations were validated by measuring the pHi under the conditions outlined above (data not shown). The data indicate that lowering pHi below 7.0 stimulated $^{35}$SO₄⁻ efflux from the cells. A comparison of the efflux data of the inset to Fig. 9 and the influx data of the main figure indicates that changes in the cis H⁺ concentration had a similar enhancing effect on $^{35}$SO₄⁻ efflux as on influx.}

A direct demonstration of the action of H⁺ as a substrate that is transported along with SO₄⁻ in exchange for Cl⁻ is presented in Fig. 11. In these studies, we reasoned that the net influx of H⁺ into the cell coupled with that of SO₄⁻ should lead to intracellular acidification. Moreover, the pH changes in the intracellular compartment should be sensitive to SITS, which inhibits $^{35}$SO₄⁻ fluxes in these cells (Figs. 1 and 2). These expectations were verified by the data of Fig. 11. For these
experiments, the neutrophils were suspended in a bathing medium containing 100 mM SO$_4^{2-}$ at pH$_o$ 6.70 in order to enhance the net H$^+$ influx across the plasma membrane. As displayed in Fig. 11, the influx of SO$_4^{2-}$ was associated with a gradual decline in pH$_i$ from 7.27 $\pm$ 0.03 to 7.04 $\pm$ 0.04 over the course of 30 min. The fall in pH$_i$ appeared to be linear over this observation period. (When the studies were extended to 45 and 60 min [not shown], a definite trend towards a gradual leveling off of pH$_i$ at $\sim$6.9 could be appreciated. Whether this "plateau" is related to the fact that the SO$_4^{2-}$ gradient is changing or that other regulatory mechanisms are now controlling pH$_i$, we do not know.) This pH$_i$ transient was abolished by treatment with 1 mM SITS. In 100 mM SO$_4^{2-}$ medium containing SITS, the pH$_i$ was similar to that under control conditions: in the presence of either 148 mM Cl$^-$ or glucuronate. The small decline in pH$_i$ over time in the controls is related to the fact that the experiments took place under an extracellular acidification (pHo 6.70) as compared to normal (pHo 7.40). Together, these data provide strong confirmatory evidence in support of the notion that H$^+$ and SO$_4^{2-}$ are transported together in exchange for Cl$^-$. The intracellular acidification (0.23 pH U/30 min) appeared to be linear at a rate of 0.0077 pH/min. Taking intrinsic intracellular buffering power as 50 mM/pH (Simchowitz and Roos, 1985), this rate of pH$_i$ change signifies an H$^+$ influx of 0.39 mmol/liter-min, a value that is substantially lower than that measured for SO$_4^{2-}$ influx (1.3 mmol/liter-min, Fig. 10) under similar conditions. This disparity could be due to the antagonizing effect of a pH$_i$ recovery process that is stimulated by intracellular acidification. Though an obvious candidate, Na$^+$/H$^+$ exchange cannot
explain this since the degree of internal acidification was not altered by the addition of 10 μM of the 5-N,N-hexamethylene analogue of amiloride, which blocks Na+/H+ countertransport (Simchowitz and Cragoe, 1987). These results are, however, compatible with the existence of some other as yet unidentified pH-i-regulatory mechanism(s) in these cells.

Steady-State Intracellular Sulfate Concentration

What then are the factors that determine the steady-state [SO4²⁻]? As mentioned above, for human neutrophils bathed in a 2 mM SO₄²⁻, 145 mM Cl⁻ medium, this value is ~1 mM as measured by isotopic uptake at equilibrium and by chemical means. As pointed out at the beginning of the Results section, this value is ~25-fold greater than the expected passive distribution level (~0.04 mM) for cells with a resting potential of ~−53 mV. Some form of active transport (i.e., primary, secondary, or tertiary) therefore seems quite likely. A similar ratio of 0.5 for the internal/external SO₄²⁻ concentration ratio has been reported by Korchak et al. (1980) although their studies were conducted at [SO₄²⁻]₀ = 1 mM.

It should also be noted that Cl⁻ is accumulated intracellularly to a concentration of ~80 meq/liter of cell water, which is approximately fourfold greater than expected on thermodynamic grounds for passive distribution (Simchowitz and De Weer, 1986). Presumably, this relatively high resting [Cl⁻], is maintained by the active Cl⁻ transport system which seems to be dependent, directly or indirectly, on metabolic energy to serve as the driving force for net Cl⁻ uptake. We would like to propose that it is the chemical energy invested in the Cl⁻ gradient that ultimately provides the driving force for SO₄²⁻ accumulation. From the standpoint of SO₄²⁻, therefore, this scheme represents a form of secondary (or tertiary) active transport where the net uptake of SO₄²⁻ is energetically linked to the downhill movement of Cl⁻. We shall devote the remainder of this section to proving that the steady-state [SO₄²⁻], follows from thermodynamic considerations of the SO₄²⁻/Cl⁻ countertransport system.

For an electroneutral system that exchanges one Cl⁻ for one SO₄²⁻ plus one H⁺, the relationship between the SO₄²⁻ and Cl⁻ distributions across the cell membrane at equilibrium is given by:

\[
\frac{[H^+]_i}{[H^+]_o} \cdot \frac{[SO_4^{2-}]_i}{[SO_4^{2-}]_o} = \frac{[Cl^-]_i}{[Cl^-]_o}.
\]

Substituting the appropriate values (pHᵢ = 7.27, pH₀ = 7.40, [SO₄²⁻]₀ = 0.92 ± 0.20 mM, [SO₄²⁻]₀ = 2 mM, [Cl⁻]ᵢ = ~80 mM, and [Cl⁻]₀ = 145 mM), we find that the left-hand side of the equation comes to 0.62 ± 0.13, while the right-hand side equals 0.55. Considering the uncertainties in our estimates of pHᵢ, [SO₄²⁻]₀, and [Cl⁻]ᵢ, the foregoing calculations are compatible with the idea that the steady-state distribution of SO₄²⁻ across the neutrophil plasma membrane obeys Eq. 4. This relationship (Eq. 4) is independent of the relative or absolute Kᵢ values for SO₄²⁻ and Cl⁻ and holds true for asymmetrical, as well as symmetrical carriers.

The implications of this scheme are that varying any of the other five parameters of Eq. 4, should lead to a predictable change in the steady-state [SO₄²⁻], level. These expectations are verified by the data of Fig. 12. In the first set of experiments (Fig.
12A), [Cl\(^-\)]\(_o\) was kept constant at 120 mM, while [SO\(_4^{2-}\)]\(_o\) was varied between 0.3 and 20 mM, replacing glucuronate. The pH\(_o\) was 6.0 and measurements were made at 90 min. A pH\(_o\) of 6.0 was chosen for two reasons: (a) to completely inhibit the principal anion (Cl\(^-\)/Cl\(^-\)) exchange carrier (whose activity is abolished by extracellular acidification to pH\(_o\) 6.0 [Simchowitz, 1988b]), thereby preventing further redistribution of Cl\(^-\) via this route, and (b) to accelerate the rate of SO\(_4^{2-}\)/Cl\(^-\) exchange and

![Figure 12](image)

**Figure 12.** Relationship between steady-state internal SO\(_4^{2-}\) concentration and either [SO\(_4^{2-}\)]\(_o\) or [Cl\(^-\)]. (A) Steady-state [SO\(_4^{2-}\)]\(_i\) as a function of [SO\(_4^{2-}\)]\(_o\). Neutrophils (normal Cl\(^-\) cells, [Cl\(^-\)] \sim 80 meq/liter of cell water) were suspended in 120 mM Cl\(^-\) medium, pH\(_o\) 6.0, in which the concentration of external SO\(_4^{2-}\) (labeled with \(^{35}\)SO\(_4^{2-}\)) was varied between 0.3 and 20 mM by replacement of glucuronate. The influx of \(^{35}\)SO\(_4^{2-}\) was measured at 90 min, by which time the intracellular uptake of \(^{35}\)SO\(_4^{2-}\) had reached a steady level. The internal SO\(_4^{2-}\) contents were calculated from the specific activity of \(^{35}\)SO\(_4^{2-}\) in the reaction mixtures (after correction for the small SITS-resistant leak influxes as in Fig. 5) and plotted against the prevailing [SO\(_4^{2-}\)]\(_o\) of the medium. A fit of the data points to a straight line gave a least-squares slope of 0.455 ± 0.028. (B) Steady-state [SO\(_4^{2-}\)]\(_i\) as a function of [Cl\(^-\)]. Neutrophils were depleted of their internal Cl\(^-\) to varying degrees by incubating them in Cl\(^-\)-free, 2 mM SO\(_4^{2-}\), 145 mM PAH medium for 0–3 h at 37°C. The cells were then resuspended in 2 mM SO\(_4^{2-}\), 145 mM Cl\(^-\) medium at pH\(_o\) 6.0. The influx of \(^{35}\)SO\(_4^{2-}\) was measured after 90 min. The internal \(^{35}\)SO\(_4^{2-}\) concentrations have been graphed against the corresponding internal Cl\(^-\) contents, measured by coulometry. The relationship was linear and a least-squares fit of the data to a straight line yielded a slope of 0.0149 ± 0.0009.

thus shorten the time necessary for isotopic equilibrium to be reached. The internal Cl\(^-\) content of neutrophils placed in 120 mM Cl\(^-\) medium at pH\(_o\) 6.0 gradually fell with time from an initial value of 78.3 ± 5.6 meq/liter of cell water to reach 56.3 ± 8.4 meq/liter of cell water at 90 min. The reduction in intracellular Cl\(^-\) most likely occurs via the following mechanism. In steady-state cells bathed in 148 mM Cl\(^-\) medium at pH\(_o\) 7.40 (where \(V_m\) \sim 60 mV), net passive Cl\(^-\) efflux amounts to \sim 0.3 meq/liter-min (Simchowitz and De Weer, 1986). The relatively high internal Cl\(^-\)
level of ~80 meq/liter of cell water (fourfold higher than the passive distribution level) is maintained by an active inward Cl\(^-\) transport system that is dependent on metabolic energy (i.e., is sensitive to inhibition by 2-DOG which leads to intracellular ATP depletion). The extracellular acidification to pH\(_o\) 6.0 apparently results in marked suppression of active Cl\(^-\) uptake as no 2-deoxyglucose-sensitive \(^36\)Cl\(^-\) influx (which we have operationally defined as the active transport component) could be detected under these conditions. Since the resting membrane potential remains the same, the net passive Cl\(^-\) efflux of ~0.3 meq/liter-min is left unchecked and [Cl\(^-\)]\(_i\) gradually falls over time. At this rate, the level of intracellular Cl\(^-\) might be expected to have fallen by ~25 meq/liter of cell water during the 90 min incubation, which in fact it did.

Moreover, as previously reported (Simchowitz and Roos, 1985), marked extracellular acidification leads to a gradual reduction in pH\(_o\). When resting cells, previously bathed in 145 mM Cl\(^-\) medium at pH\(_o\) 7.40 were transferred to 120 mM Cl\(^-\) at pH\(_o\) 6.0, the pH\(_o\) fell from a control value of 7.30 ± 0.04 to reach values of 6.71 ± 0.05, 6.32 ± 0.08, and 6.07 ± 0.11 at 30, 60, and 90 min, respectively.

As shown in Fig. 12 A, the steady-state [SO\(_4^{2-}\)]\(_i\), measured at 90 min at pH\(_o\) 6.0, was markedly dependent on [SO\(_4^{2-}\)]\(_o\). Over the range 0.31–20 mM (i.e., 64-fold concentration range), steady-state [SO\(_4^{2-}\)] varied in direct proportion to the increase in [SO\(_4^{2-}\)]\(_o\). Whether this relationship conforms to the chemical equilibrium state as dictated by Eq. 4 was examined in the following manner. By rearranging Eq. 4, a plot of [SO\(_4^{2-}\)]\(_i\) vs. [SO\(_4^{2-}\)]\(_o\) as in Fig. 12 A should give a straight line with a slope equal to [Cl\(^-\)]\(_o\)·[H\(^+\)]\(_o\)/[Cl\(^-\)]\(_i\)·[H\(^+\)]\(_i\). Substituting 56 and 120 mM for [Cl\(^-\)] and [Cl\(^-\)]\(_o\) and 6.07 and 6.00 for pH\(_o\) and pH\(_o\), respectively, we calculate a slope of 0.55, not very far removed from the observed value of 0.46 ± 0.03 (Fig. 12 A).

Similarly, altering [Cl\(^-\)]\(_i\), had predictable consequences for the steady-state SO\(_4^{2-}\) distribution (Fig. 12 B). In these studies, neutrophils were first depleted of internal Cl\(^-\) to different extents by incubating them for varying periods of time (0–3 h) in Cl\(^-\)-free, 2 mM SO\(_4^{2-}\), 145 mM PAH medium. After the pretreatment period in Cl\(^-\)-free medium, the cells were then resuspended in 2 mM SO\(_4^{2-}\), 145 mM Cl\(^-\) medium at pH\(_o\) 6.0 and the steady-state [SO\(_4^{2-}\)]\(_i\), measured at 90 min by isotopic incorporation. The internal Cl\(^-\) contents of the cells at the end of the 90-min incubation at pH\(_o\) 6.0 were determined by chemical means (chloridometry). As shown in Fig. 12 B, progressive reductions in [Cl\(^-\)] from ~60 to ~10 mM led to a concomitant decline in steady-state [SO\(_4^{2-}\)]\(_i\), levels. The fall in [SO\(_4^{2-}\)]\(_o\), was linearly proportional to that in [Cl\(^-\)]. Similar to the analysis of results of Fig. 12 A, if the SO\(_4^{2-}\) and Cl\(^-\) distributions across the neutrophil plasma membrane were to follow those for chemical equilibrium as given by Eq. 4, a plot of [SO\(_4^{2-}\)]\(_i\) vs. [Cl\(^-\)]\(_i\) should be linear with a slope equal to [SO\(_4^{2-}\)]\(_o\)·[H\(^+\)]\(_o\)/[Cl\(^-\)]\(_i\)·[H\(^+\)]\(_i\). Substituting the values discussed above, the calculation yielded a value of 0.016, in good agreement with the measured value of 0.015 ± 0.001 (Fig. 12 B). In summary, the data of Fig. 12 A and B indicate that Eq. 4 adequately describes the factors governing steady-state SO\(_4^{2-}\) distribution in human neutrophils.

**SITS-Resistant Fluxes**

In our previous studies on Cl\(^-\) movements in human neutrophils (Simchowitz and De Weer, 1986; Simchowitz et al., 1986), we noted that CHC competitively inhibi-
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ited Cl⁻/Cl⁻ exchange and that CHC-insensitive fluxes represented passive electrodiffusive Cl⁻ fluxes through ion channels. Initially, we reasoned that, by analogy, the small SITS- and CHC-resistant ³⁵SO₄⁻ fluxes observed here might also represent passive diffusion of SO₄⁻ through permeability channels. From the data of Figs. 1 and 2, it is apparent that if indeed the SITS-resistant ³⁵SO₄⁻ fluxes are due to permeation via current-carrying pathways, they cannot account for >~10% of total one-way ³⁵SO₄⁻ influx or efflux. Measurement of such minor components requires maximal suppression of the ³⁵SO₄⁻ fluxes by the exchange carrier. This was accomplished by conducting experiments in the presence of 1 mM SITS. When the resting potential was depolarized from ~−60 to ~0 mV by raising external K⁺ from 5 to 120 mM, only a modest approximately twofold increase in the rate of ³⁵SO₄⁻ influx was observed, whereas a ~10-fold enhancement is predicted for electrodiffusion based on constant field behavior. Moreover, little if any change in the rate of ³⁵SO₄⁻ efflux could be detected. Taken together, these findings imply that in all likelihood, the majority of the SITS-insensitive fluxes do not represent passive fluxes through ion permeability channels. These fluxes were for the most part independent of [SO₄²⁻]₀ (contrary to what is expected for electrodiffusive ³⁵SO₄⁻ influx) and probably reflect leakage of the tracer into or from the small number of injured or nonviable cells. In fact, corrections for these presumed leaks were applied to the measurements of total one-way ³⁵SO₄⁻ influx and efflux in Figs. 4–10 and 12 in order to analyze the effects of different inhibitors and substrates on the true magnitude of the carrier-mediated exchange flux. It would appear then that there are little if any channel-mediated fluxes of SO₄⁻ across the cell membrane of human neutrophils.

**DISCUSSION**

Steady-state human peripheral blood neutrophils bathed in a 2 mM SO₄²⁻, 145 mM Cl⁻ medium transport ³⁵SO₄⁻ across the plasma membrane at the rate of ~17 μmol/liter-min. For all practical purposes, passive electrodiffusive ³⁵SO₄⁻ influx and efflux through ion permeability channels can be discounted as negligibly small. The sum total of all of the one-way ³⁵SO₄⁻ influx and efflux represents a carrier-mediated exchange of SO₄⁻ for Cl⁻. This anion countertransport system is voltage insensitive and independent of Na⁺. The exchange is activated by SO₄²⁻ and by Cl⁻ which bind to the carrier's external translocation site with closely similar Kₘ values of ~14 and ~25 mM, respectively. The mechanism of transport seems to be that of a carrier-mediated exchange of Cl⁻ for the ion pair H⁺ + SO₄²⁻, thereby preserving electroneutrality.

The exchange carrier is noncompetitively blocked by SITS with a Kᵢ of ~50 μM and is competitively inhibited by CHC and by ethacrynate with Kᵢ values of 250 and 7 μM, respectively. One-way ³⁵SO₄⁻ fluxes were also sensitive to furosemide and to probenecid although the nature of the inhibition (i.e., competitive or otherwise) was not investigated since the degree of inhibition at a concentration of 1 mM was only ~50%. These compounds are all weak acids by virtue of their sulfonic or carboxyl groups which are almost completely ionized at physiological pH. Thus, the negatively charged (anionic) forms of these drugs presumably bind to the anion-recognition sites on the carrier even more avidly than either SO₄²⁻ or Cl⁻ and thus block transport of its natural substrates. Presently, we have no direct evidence as to
whether these inhibitors are themselves actually transported. However, since each of these agents blocks $^{35}$SO$_4^{2-}$ influx and efflux to roughly the same extent, it would appear that these drugs exert their effects by immobilizing the carrier. These findings confirm some of the original observations of Korchak et al. (1980, 1982) that $^{35}$SO$_4^{2-}$ influx and efflux are sensitive to inhibition by these agents. However, Korchak et al. (1980, 1982) reported that $^{35}$SO$_4^{2-}$ influx and efflux were reduced by ~90% in the presence of 0.1 mM DIDS. We, on the other hand, could detect no effect at this concentration (data not shown). The reason for this discrepancy is not known at present.

**Comparison with the Principal Anion (Cl$^-$/HCO$_3^-$) Exchanger of Human Neutrophils**

Table I lists the true kinetic constants of the SO$_4^{2-}$ exchange carrier for a number of the different anions tested. For convenience, values for the major anion (Cl$^-$/Cl$^-$, Cl$^-$/HCO$_3^-$) exchanger of human neutrophils (Simchowitz et al., 1986; Simchowitz, 1988a, b) are shown for comparison. Besides the dramatic differences in their drug sensitivities already alluded to, it is evident that the two anion countertransport mechanisms display rather marked differences in their substrate selectivity towards various anions. This contrast relates both to the absolute values of the kinetic constants as well as to the relative sequence of binding affinities. The most striking disparities involve the handling of SO$_4^{2-}$ and HCO$_3^-$.

The principal anion exchange carrier binds Cl$^-$ and HCO$_3^-$ with relatively high affinity (true $K_m$ values of 5.0 and 4.1 mM, respectively), but is essentially devoid of affinity for SO$_4^{2-}$ (Simchowitz, 1988a). By way of contrast, the SO$_4^{2-}$ exchanger binds Cl$^-$ and SO$_4^{2-}$ with roughly comparable affinities (true $K_m$ values of ~14 and ~25 mM, respectively), but has little affinity for HCO$_3^-$. In addition, the SO$_4^{2-}$ exchange carrier is sensitive to SITS, furosemide, and ethacrynic acid, whereas these compounds are inactive against Cl$^-$/Cl$^-$ self-exchange (Simchowitz and De Weer, 1986). The two carriers, however, share a common feature in that both are inhibited by CHC with true $K_i$ values of 230 and 290 $\mu$M, respectively. Taken together, the combined weight of all of these findings leaves little doubt that SO$_4^{2-}$/Cl$^-$ and Cl$^-$/HCO$_3^-$ exchanges are mediated by two carriers with distinctly different properties.

A related issue concerns the biologic significance of these observations for SO$_4^{2-}$ transport in human neutrophils. It might appear somewhat unusual that if in fact SO$_4^{2-}$ were a natural substrate for the SO$_4^{2-}$/Cl$^-$ exchange carrier described here, that its affinity should be so low ($K_m$ ~ 14 mM), especially when one considers that concentrations in this range are unlikely to exist in vivo, where the prevailing extracellular SO$_4^{2-}$ concentration has been estimated at 0.6–1.9 mM ([Cl$^-$]$_o$ ~ 90–110 mM [Van Harreveld et al., 1966]). Thus, under these conditions, the carrier would always be highly unsaturated with respect to SO$_4^{2-}$. The fact that no other significant pathway for SO$_4^{2-}$ transport could be identified in these cells suggests that SO$_4^{2-}$/Cl$^-$ exchange is indeed the relevant mechanism for the uptake of this physiologically important ion.

Such a system would seem to be especially pertinent to neutrophils and other phagocytic cells which contain sulfated macromolecules essential to their normal function (for details, see Parmley et al., 1983, 1986). For some time, sulfate-contain-
ing glycoconjugates have been known to be localized cytochemically within cytoplasmic granules and transport vesicles of human, rabbit, and feline neutrophils. The staining is restricted to immature primary (lysosomal) and tertiary granules which have been shown to contain chondroitin sulfate and other N-sulfated glycosaminoglycans such as heparan and dermatan sulfate. The absence of staining in mature lysosomes results from a masking of SO₄²⁻ groups rather than from their loss. Biochemical studies that glycosaminoglycans inhibit a number of hydrolytic enzymes support the hypothesis that they function by complexing with certain granule components, thereby rendering them inactive and facilitating their storage within lysosomes. Thus, complex formation provides a mechanism for maintaining lysosomal enzymes in latent form. Moreover, these complexes have been shown to dissociate during phagocytosis as intragranular substances become mobilized during the degranulation phase that accompanies phagolysosome formation. These sulfated macromolecules also redistribute to the cell surface after exocytosis where they are thought to influence cell adhesion and enhance surface attachment of particles. Clearly, there is a dynamic interaction between these sulfated glycosaminoglycans and other intragranular components during lysosomal packaging, storage, and release.

**Comparison with Sulfate Transport in Erythrocytes**

The SITS-sensitivity as well as the inhibition of SO₄²⁻/Cl⁻ exchange in neutrophils by CHC, furosemide, and ethacrynic acid demonstrated herein is reminiscent of the constellation of properties generally associated with the inorganic anion exchanger (band 3 protein) of human red blood cells (Sachs et al., 1975; Gunn, 1979; Knauf, 1979). This commonality of features is further enforced by the fact that both exchange carriers display very similar kinetic constants for the binding of SO₄²⁻ and Cl⁻ to their external translocation sites. Again, this is in marked contrast to the situation obtained for the principal anion (Cl⁻/HCO₃⁻) exchanger of human neutrophils which binds Cl⁻ with high affinity, but appears to be devoid of affinity for SO₄²⁻ (Simchowitz, 1988a).

In human red blood cells, ³⁵SO₄²⁻ fluxes are very pH-dependent: inward transport is accelerated by extracellular acidification and reduced by alkalinization (Jennings, 1976; Gunn, 1978). The nature of the sensitivity to pH has been extensively studied by Milanick and Gunn (1982, 1984). From their investigations, it is clear that the mechanism of SO₄²⁻ transport in red cells is that of an exchange of Cl⁻ for the ion pair H⁺ + SO₄²⁻. The co-transport of H⁺ and SO₄²⁻ helps to explain the cis activation of ³⁵SO₄²⁻ fluxes by H⁺ and also accounts for the electroneutrality of the exchange which otherwise appears to be a one-for-one SO₄²⁻/Cl⁻ exchange. In addition, the transport rate for SO₄²⁻ in both cell types is slower than for Cl⁻: threefold less in neutrophils, but 10⁴-fold less in erythrocytes. It seems evident then that qualitatively, the SO₄²⁻/Cl⁻ exchange carrier of human neutrophils shares a number of characteristics in common with the inorganic anion exchange system of red blood cells. This may help to explain the existence of determinants on the neutrophil plasma membrane that cross-react with antibodies to band 3 protein of human erythrocytes (Kay et al., 1983).
Comparison with Other Modes of $SO_4^{2-}$ Transport in Epithelia and Isolated Cell Preparations

It has been recognized for some time that $SO_4^{2-}$ may be transported by two basic mechanisms: (a) coupled Na$^+$ + $SO_4^{2-}$ cotransport which is energized by the Na$^+$ gradient (Lucke et al., 1979; Schneider et al., 1984) and (b) Na$^+$-independent anion exchange (Grinstein et al., 1980; Ullrich et al., 1980; Brazy and Dennis, 1981; Langridge-Smith and Field, 1981). The two systems may coexist in the same cell type as for intestinal and renal epithelia, whereas only the anion exchange carrier seems to be present in erythrocytes, Ehrlich ascites tumor cells, hepatocytes, astroglia, and neutrophils (for reviews, see Gunn, 1979; Knauf, 1979; Murer and Burckhardt, 1983; Hoffmann, 1986). As will be briefly summarized below, the $SO_4^{2-}$/Cl$^-$ exchange carrier described here for human neutrophils shares many general properties in common with $SO_4^{2-}$-transporting exchangers in other cell types.

In vesicles prepared from the brush border of rabbit ileum, pH-gradient stimulated $SO_4^{2-}$ uptake has been ascribed to an electroneutral $SO_4^{2-}$/OH$^-$ exchange that is sensitive to SITS, DIDS, and furosemide (Schron et al., 1985). The carrier does not transport HCO$_3^-$, whereas in the basolateral membrane of the rat intestine (Weinberg et al., 1986), a nonselective exchanger has been characterized that accepts a variety of inorganic and organic anions (e.g., taurocholate, pyruvate, PAH, Cl$^-$, HCO$_3^-$, and $SO_4^{2-}$). Similar electroneutral SITS- and DIDS-sensitive $SO_4^{2-}$/HCO$_3^-$ exchange carriers have been reported in brush border vesicles from the kidneys of mammals (Pritchard and Renfro, 1983; Low et al., 1984; Hagenbuch et al., 1985; Pritchard, 1987) and marine teleosts (Renfro and Dickman, 1980; Renfro and Pritchard, 1983) and from human placentas (Cole, 1984; Boyd and Shennan, 1986). In these systems, the $K_m$ for $SO_4^{2-}$ is ~1 mM, considerably lower than that for neutrophils (~14 mM). In all cases, however, there seems to be a rather broad acceptance of inorganic mono- and divalent anions as well as a variety of monocarboxylates such as formate, acetate, lactate, and PAH.

A $SO_4^{2-}$/anion exchanger has also been characterized in hepatocytes (Bracht et al., 1981; von Dippe and Levy, 1982; Hugentobler and Meier, 1986) where the affinity for $SO_4^{2-}$ ($K_m$ 7–16 mM) is much closer to that in neutrophils. The activity of the carrier is insensitive to membrane voltage and may be blocked by DIDS, probenecid, and N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate-taurine. $Ci$s inhibition and trans stimulation of $SO_4^{2-}$ fluxes were observed with thiosulfate, oxalate, succinate, and cholate, but not with Cl$^-$, HCO$_3^-$, lactate, or PAH.

Ehrlich ascites tumor cells (Levinson and Villereal, 1975; Villereal and Levinson, 1977; Hoffmann, 1986) possess a $SO_4^{2-}$/Cl$^-$ exchange mechanism that displays a high affinity for $SO_4^{2-}$ ($K_m$ ~ 2 mM). This carrier is inhibitable by SITS, furosemide, and phloretin. $SO_4^{2-}$ uptake is probably mediated by the same transport system that is responsible for Cl$^-$/HCO$_3^-$ exchange (Levinson, 1978), as in red blood cells where $SO_4^{2-}$ and Cl$^-$ share a common mechanism (Gunn, 1979; Knauf, 1979). However, in Ehrlich cells, the maximal transport rate for Cl$^-$ is ~10-fold greater than for $SO_4^{2-}$, in contrast to a factor of ~10$^4$ for human erythrocytes.

In surveying the literature, perhaps the strongest resemblance to neutrophils is that of $SO_4^{2-}$ transport in astroglia. In studying a glioma cell line, Wolpaw and Martin (1986) observed that $SO_4^{2-}$ influx is mediated by a countertransport system that
is distinct from the principal Cl⁻/HCO₃⁻ exchanger. The SO₄²⁻ carrier is electroneutral, is blocked by SITS, DIDS, and furosemide, and is activated by SO₄²⁻ and by Cl⁻ with $K_m$ values of 0.2 and 15 mM, respectively. As in neutrophils (Simchowitz, 1988a), SO₄²⁻ does not appear to be a substrate for the Cl⁻/HCO₃⁻ exchange system, which accounts for the vast majority of all Cl⁻ movements, the Cl⁻ flux via the SO₄²⁻ pathway amounting to only a minor fraction of the total Cl⁻ flux.

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REFERENCES


Smolen, J. E. 1984. Lag period for superoxide anion generation and lysosomal enzyme release


