Characterization of Oxalate Transport by the Human Erythrocyte Band 3 Protein

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ABSTRACT This paper describes characteristics of the transport of oxalate across the human erythrocyte membrane. Treatment of cells with low concentrations of H_2DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonate) inhibits Cl^-–Cl^- and oxalate–oxalate exchange to the same extent, suggesting that band 3 is the major transport pathway for oxalate. The kinetics of oxalate and Cl^- self-exchange fluxes indicate that the two ions compete for a common transport site; the apparent Cl^- affinity is two to three times higher than that of oxalate. The net exchange of oxalate for Cl^-, in either direction, is accompanied by a flux of H^+ with oxalate, as is also true of net Cl^-–SO_4^{2-} exchange. The transport of oxalate, however, is much faster than that of SO_4^{2-} or other divalent anions. Oxalate influx into Cl^--containing cells has an extracellular pH optimum of ~5.5 at 0°C. At extracellular pH below 5.5 (neutral intracellular pH), net Cl^--oxalate exchange is nearly as fast as Cl^-–Cl^- exchange. The rapid Cl^--oxalate exchange at acid extracellular pH is not likely to be a consequence of Cl^- exchange for monovalent oxalate ($HOOC\text{-}COO^-$; pK_a = 4.2) because monocarboxylates of similar structure exchange for Cl^- much more slowly than does oxalate. The activation energy of Cl^-–oxalate exchange is about 35 kCal/mol at temperatures between 0 and 15°C; the rapid oxalate influx is therefore not a consequence of a low activation energy. The protein phosphatase inhibitor okadaic acid has no detectable effect on oxalate self-exchange, in contrast to a recent finding in another laboratory (Baggio, B., L. Bordin, G. Clari, G. Gambaro, and V. Moret. 1993. *Biochim. Biophys. Acta.* 1148:157–160.); our data provide no evidence for physiological regulation of anion exchange in red cells.

INTRODUCTION

The rates of exchange of Cl^- and HCO_3^- across the red blood cell membrane are very high compared with those of most other cells (reviewed in Jennings, 1992). The high Cl^-/HCO_3^- flux in red cells is a consequence of the presence of a large number of copies of a protein (known as band 3, capnophorin, or AE1) that catalyzes an obligatory exchange of these anions (Knauf, 1979; Passow, 1986). The function of Cl^-/HCO_3^- exchange is to increase the CO_2 carrying capacity of blood (Wieth et al., 1982). In addition to Cl^- and HCO_3^-, many other anions are also transported by band 3; the transport of these anions takes place at widely varying rates and with characteristics that may be quite different from those of Cl^- and HCO_3^- (see Passow, 1986).

This paper describes some of the properties of oxalate transport in human red blood cells. There were several reasons for doing these experiments. First, there is evidence that red cell oxalate transport is abnormal in humans who tend to form kidney stones (Baggio et al., 1984, 1986; see, however, Motola et al., 1992). Characterization of oxalate transport in normal red cells could provide background information for mechanistic studies of oxalate transport in relation to nephrolithiasis.

Cousin and Motais (1976, 1979) have shown that various reversibly acting inhibitors have parallel effects on Cl^-–Cl^- and oxalate–oxalate exchange, indicating that red cell oxalate transport is mediated by band 3. It is possible, however, that there is a separate transporter for divalent organic anions, just as there is a separate transporter for monocarboxylates (Halestrap, 1976; Deuticke et al., 1978; Deuticke, 1982). In the kidney there are transport pathways for oxalate that are distinct from the Cl^-–HCO_3^- exchanger (Aronson, 1989); one or more of these transporters could be present in red cells. It is therefore worthwhile to test the assumption that oxalate transport is primarily through band 3 in red cells.

If oxalate is in fact transported mainly by band 3, a comparison of oxalate transport with that of other anions may provide information about substrate specificity of the band 3 protein. Another reason for character-

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izing band 3-mediated oxalate transport is to determine the potential for using oxalate as a band 3 substrate in studies of functional expression of band 3 (AE1) in *Xenopus* oocytes or other expression systems. Finally, oxalate transport in red cells has been reported to be stimulated by the protein phosphatase inhibitor okadaic acid (Baggio et al., 1993); one of the goals of this study was to characterize further the effect of okadaic acid on oxalate transport.

MATERIALS AND METHODS

Materials

Venous blood was obtained from each of the authors and one other colleague; none of the donors has a personal or family history of kidney stones. Blood was drawn into EDTA and stored as whole blood for up to 7 d at 4°C before use. Radionuclides ($H^{36}Cl$, [^{14}C] oxalic acid, $^{86}RbCl$) were purchased from Dupont NEN (Boston, MA). Okadaic acid (1 mg/ml in dimethylformamide) was purchased from Moana Bioproducts (Honolulu, HI). H_2DIDS was prepared as described previously (Jennings et al., 1984). Phloretin was purchased from Sigma Chem. Co. (St. Louis, MO). All other buffers and salts were from Sigma or Fisher Chemicals (Houston, TX).

Cell Preparation

Cells were washed three times in the media described in the figure legends. For loading with oxalate, cells and medium were incubated 10 min at 22°C before each centrifugation. The half-time for Cl^-–oxalate exchange is less than 10 s at 22°C and the pH range used here; three incubations for 10 min are therefore sufficient to equilibrate cells with the Cl^-/oxalate media. Cells were then resuspended at a 50–75% hematocrit in the same medium, and $^{36}Cl^-$ or [^{14}C]oxalate was added (1 μCi/ml of suspension) and incubated for at least five half-times of tracer self-exchange. The cellular content (μmol/g cell solids) of Cl^- or oxalate was calculated from the total radioactivity present, the extracellular radioactivity after loading, and the dry weight of cells in the loading suspension. In the experiments in Fig. 2, Cl^- and oxalate contents were not determined directly; instead, the fluxes were calculated assuming that the intracellular Cl^- (oxalate) contents were proportional to the extracellular Cl^- (oxalate) concentration.

Preincubation with H_2DIDS

Cells were washed three times and suspended at a 5% hematocrit in Hepes-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na-phosphate, 10 mM Hepes, pH 7.4). Then 0, 0.25, 0.5, or 1 μM H_2DIDS was added and the suspensions were incubated 1 h at 37°C. Cells were washed twice in 150 mM KCl, 10 mM Hepes, pH 7.4, plus 0.2% bovine serum albumin to remove any unreacted H_2DIDS (Cabantchik and Rothstein, 1974). Each suspension was then split in half for measurement of Cl^- and oxalate self-exchange.

Transport Measurements

The tracer efflux was determined using a variation of the inhibitor stop method described previously (Ku et al., 1979). The stop solution consisted of 100 mM K_2SO_4, 10 mM Hepes, pH 7.4, 200 μM phloretin, or 20 μM H_2DIDS. In most experiments pellets rather than supernatants were counted (Jennings et al., 1990) to obtain more accurate data for faster effluxes. The rate constant (min^{-1}) for Cl^-–Cl^- or oxalate–oxalate exchange was determined from a nonlinear least squares fit (NFIT software; Island Products, Galveston, TX) to a single exponential approach to equilibrium. The intracellular radioactivity at equilibrium ($[^*Cl^-]_i^\infty$ in Figs. 4–5) was estimated by allowing the suspension to incubate for at least eight half-times for tracer efflux and then mixing a sample with stop solution as for the other time points. The flux (μmol/g solids-min) is equal to the rate constant times the intracellular Cl^- or oxalate content (μmol/g solids).

Net anion exchange does not necessarily have an exponential time course, because the anion concentrations are not constant. Experimentally, the time course of the net Cl^- efflux into an oxalate medium is linear for longer than would be expected for an exponential (see Results). For these experiments the efflux was calculated from the decrease in Cl^- content per minute.

The pH of the flux solutions was measured at 20–22°C to avoid equilibrating the pH electrode at 0°C. Because of the temperature dependence of the pK_a of MOPS (Sankar and Bates, 1978), a solution buffered at pH 7.0 at 20°C in 10 mM MOPS, 10 mM citrate, will have a pH of ~7.17 at 0°C. The correction is much less at lower pH because the pK_a of citrate is much less temperature dependent (Dean, 1979). The pH values plotted in Figs. 6 and 7 were not corrected for temperature.

Effect of Okadaic Acid on Oxalate and Rubidium Transport

Fresh red cells were washed three times in Hepes-buffered physiological saline and incubated at 10% hematocrit in the same medium plus 10 mM glucose and 1% dimethylformamide ± 1 μM okadaic acid. After 90 min at 37°C, each suspension was divided in half for measurement of oxalate–oxalate exchange (as described above) and swelling-stimulated Rb^+ transport, which is known to be inhibited by okadaic acid (Kaji and Tsukitani, 1991; Jennings and Schulz, 1991). In preparation for $^{86}Rb^+$ influx measurements, cells were washed twice in 160 mM NaCl, 10 mM Hepes, pH 7.4. Cells were then suspended at 37°C in either 2.5 ml 160 mM NaCl, 10 mM Hepes, pH 7.5, or 1.5 ml 160 mM NaCl, 10 mM Hepes, pH 7.5, plus 1 ml water to swell the cells. All media contained 10^{-4} M ouabain. After 10 min at 37°C in the isotonic or hypotonic medium, 5 mM KCl and 1 μCi $^{86}Rb^+$/ml were added. The influx of $^{86}Rb^+$ was determined as described previously (Jennings and Schulz, 1991).

RESULTS

Irreversible Inhibition of Oxalate and Cl^- Transport

To test the idea that oxalate is transported by band 3, red cells were reacted with varying (0–1 μM) concentrations of H_2DIDS. Except at the highest concentration, the total number of moles of H_2DIDS was less than the total number of moles of band 3. Under these conditions, the free concentration of H_2DIDS was extremely low, because band 3 binds H_2DIDS reversibly with high affinity before the covalent reaction (see Knauf, 1979; Passow, 1986). Therefore, the covalent reaction of H_2DIDS with membrane proteins was con-

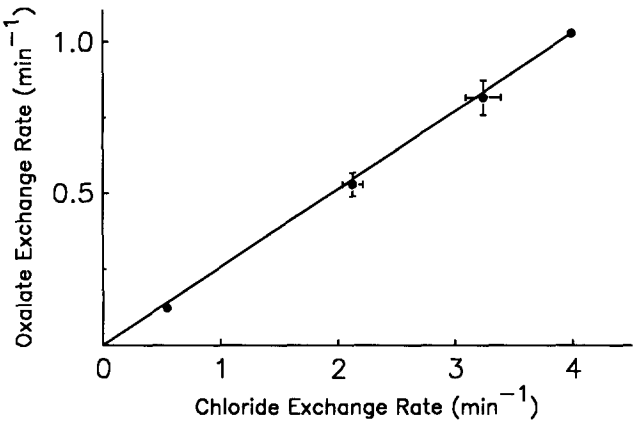

FIGURE 1. Irreversible inhibition of Cl^-–Cl^- and oxalate–oxalate exchange by H_2DIDS. Cells were preincubated with 0, 0.25, 0.5, and 1 μM H_2DIDS as described in Materials and Methods and were then washed and split into half for measurement of Cl^-–Cl^- exchange at 0°C in 150 mM KCl, 10 mM Hepes, pH 7.4, or oxalate–oxalate exchange at 20°C in 135 mM KCl, 10 mM Na_2oxalate, 10 mM MOPS, pH 7.0. For each H_2DIDS concentration, the rate constant for Cl^-–Cl^- exchange and oxalate–oxalate exchange are plotted. Error bars represent the range of two determinations, using a single preparation of cells.

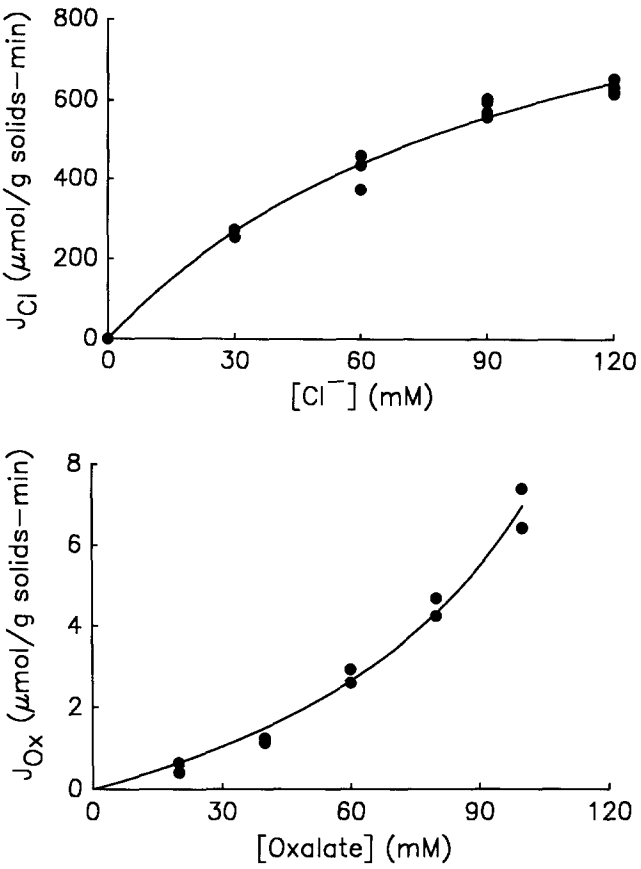

FIGURE 2. Competition between Cl^- and oxalate. Cells were washed and equilibrated in media consisting of mixtures of 150 mM NaCl and 100 mM Na_2oxalate, buffered at pH 7.0 with 10 mM MOPS and 10 mM citrate. In each medium, e.g., 60 mM Cl^-, 60 mM oxalate, the equilibrium exchange fluxes of Cl^- and oxalate were measured at 0°C. The data for both Cl^- (*top*; two separate experiments) and oxalate (*bottom*; one experiment) are fit to a model (Appendix 1) in which the apparent affinity for Cl^- (i.e., the $K_{1/2}$ in a self-exchange experiment with symmetric anion concentrations and no competitors) is 30 mM (Fröhlich and Gunn, 1986), and that for oxalate is 67 mM.

fined mainly to band 3. After sufficient time for covalent reaction with H_2DIDS, cells were washed and split into two aliquots for measurement of Cl^-–Cl^- and oxalate–oxalate exchange. As shown in Fig. 1, the extent of irreversible inhibition of oxalate exchange is indistinguishable from that of Cl^- exchange.

Mutual Competition between Oxalate and Chloride

If Cl^- and oxalate share a common transporter, then the affinity of oxalate as a substrate should be equal to its affinity as a competitive inhibitor of Cl^- transport, and vice versa. For an obligatory exchanger such as band 3, it is very difficult to determine absolute substrate affinities, because of intrinsic asymmetries in the translocation event, spectator anion effects, or noncompetitive inhibitory effects of the substrate anion (see Knauf, 1979; Gunn and Fröhlich, 1979; Knauf et al., 1984, 1989; Passow, 1986; Fröhlich and Gunn, 1986).

Although true affinities are hard to measure, the relative affinities of oxalate and Cl^- can be estimated in equilibrium exchange experiments in which each substrate is varied at the expense of the other. The flux of the substrate with the higher affinity will be a convex function of its concentration (negative second derivative of the flux vs concentration curve). The flux of the substrate with the lower affinity will have a concave flux vs concentration curve (positive second derivative). If the affinities are the same, the flux will be a linear function of concentration. This kind of experiment has shown, for example, that the HCO_3^- affinity is higher than that of Cl^- (Gunn et al., 1973; Wieth, 1979).

Fig. 2 shows the concentration dependence of the tracer oxalate and Cl^- fluxes measured under identical conditions. In both cases, cells were equilibrated with media containing 0–120 mM NaCl and 100–20 mM Na_2oxalate at 20°C, pH 7.0. This pH was chosen because the Donnan ratio is close to unity (Gunn et al., 1973). The Cl^- and the oxalate data in Fig. 2 have been fit by a model (see Appendix A) in which Cl^- and oxalate compete for a common site, and the apparent affinity for Cl^- is 2.2 times that for oxalate. It is significant that independent measurement of Cl^- and oxalate transport give the same relative affinities of the two anions. These data, taken together with the extensive study of inhibitors by Cousin and Motais (1976, 1979),

are strong evidence for a common transport pathway for Cl^- and oxalate.

Uphill Proton Fluxes during Net Cl^-–Oxalate Exchange

Band 3 has a titratable group (pK ~5.5) that is exposed to the extracellular medium. When this group is protonated, the influx of divalent anions is stimulated and the transport of monovalent anions is inhibited (Milanick and Gunn, 1982, 1984; Wieth et al., 1982; Labotka and Omachi, 1988). A proton, presumably the one bound by this titratable group, is cotransported with SO_4^{2-} during the net exchange of Cl^- for SO_4^{2-} (Jennings, 1976; Milanick and Gunn, 1984; Berghout et al., 1988; Jennings and Al-Rhaiyel, 1988). Fig. 3 shows that the net exchange of Cl^- for oxalate takes place in a manner analogous to Cl^-–SO_4^{2-} exchange. When Cl^--loaded cells are suspended in a weakly buffered oxalate medium, the extracellular pH rises rapidly, indicating an influx of acid (Fig. 3, *left*). Conversely, when oxalate-loaded cells at pH 7.2 are suspended in a weakly buffered Cl^- medium, the extracellular pH drops rapidly (Fig. 3, *right*).

When the Cl^-–oxalate exchange is nearly complete, the cells and medium are not at Donnan equilibrium with respect to anions and pH. For example, at 22°C the Cl^- efflux into an oxalate medium is essentially complete after 1 min (extrapolated from the measured Cl^- efflux at lower temperatures), but the extracellular pH is nearly a full unit above the final equilibrium value (Fig. 3, *left*). At this point, there is an inward HCO_3^- gradient (from atmospheric CO_2), which drives HCO_3^- influx in exchange for intracellular oxalate (presumably cotransported with H^+). This exchange continues, with recycling of HCO_3^- and CO_2 (Jacobs and Stewart, 1942), until the intracellular and extracellular pH, Cl^-, and oxalate are again at Donnan equilibrium.

The pH changes associated with Cl^-–oxalate exchange are qualitatively similar to those observed during Cl^-–SO_4^{2-} exchange (Jennings, 1976; Milanick and

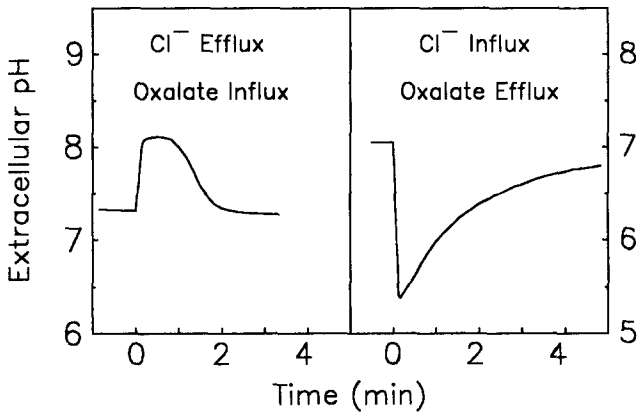

FIGURE 3. H^+ movement with oxalate during net exchange of Cl^- for oxalate. (*Left*) Cells were equilibrated with 150 mM KCl, 10 mM MOPS, pH 7.2, and were resuspended in 100 mM Na_2oxalate, buffered with 0.1 mM MOPS at pH 7.3. (*Right*) Cells were washed and equilibrated with 100 mM Na_2oxalate, 10 mM MOPS, pH 7.2, and were resuspended in 150 mM NaCl, buffered with 0.1 mM MOPS, pH 7.2. For Cl^-–oxalate exchange in both directions, there is rapid initial movement of H^+ in the direction of the oxalate flux followed by a gradual equilibration of the extracellular pH to 7.2. Hematocrit 2%. Temperature 22°C. Solutions were not bubbled to remove atmospheric CO_2. However, to stabilize the pH in the weakly buffered medium before adding cells, the air space above the solution was flushed with inert gas (helium). Traces shown are each representative of at least three experiments.

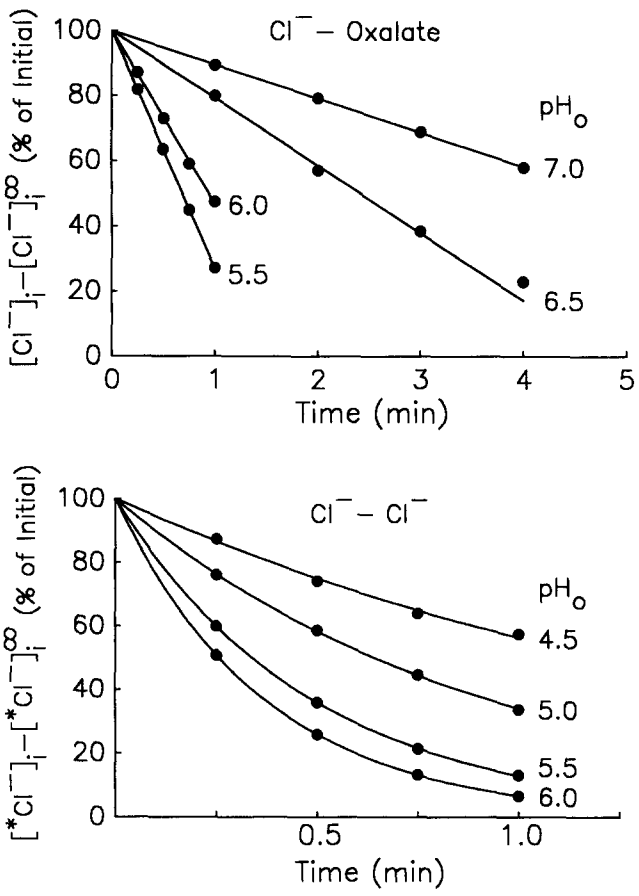

FIGURE 4. Nonexponential time course of Cl^- efflux into oxalate media. Cells were loaded with $^{36}Cl^-$ in 150 mM KCl, 10 mM Hepes, pH 7.5, and resuspended at 0°C in 100 mM Na_2oxalate (*top*) or 150 mM NaCl (*bottom*), 10 mM citrate, 10 mM MOPS, titrated with NaOH to the indicated pH values. Different ranges of extracellular pH were chosen for each part of the figure to illustrate the time courses for widely differing transport rates. In an oxalate medium (*top*), the intracellular Cl^- is a linear function of time until the flux is ~75% complete. The initial flux can be determined from the slope of the lines for data taken during the first 75% of the efflux. Each flux is one of a total of five performed on three preparations of cells. Data for $^{36}Cl^-$ efflux into Cl^- media can be fit very well by single exponential curves, which have been drawn through each data set (*bottom*; one of two fluxes on a single preparation of cells).

Gunn, 1984; Berghout et al., 1988), but they are far easier to detect. The SO_4^{2-}–H^+ cotransport flux at neutral pH can only be observed if care is taken to remove atmospheric CO_2 (Jennings, 1976). In the experiment shown in Fig. 3, the solutions were not bubbled with inert gas to remove atmospheric CO_2. In spite of the presence of traces of CO_2, the H^+ efflux and influx cause very large pH changes, which in both cases are in the opposite direction from that expected to be produced by Cl^- exchange for atmospheric HCO_3^-.

Kinetics of Cl^-–Oxalate Exchange

The study of net transport is potentially complicated by the fact that the ion concentrations (and in the present experiments, the pH) are not constant during the flux determination. Nonetheless, net anion transport can reveal information that is complementary to that obtained from equilibrium exchange measurements. Fig. 4 shows the time course of $^{36}Cl^-$ efflux from human red cells suspended in either 100 mM oxalate or 150 mM Cl^- media at various extracellular pH. (The effect of pH is discussed separately below.) The time courses of tracer efflux into the Cl^- media can be fit well to single exponentials. The Cl^- efflux into oxalate, however, is nearly linear for over half the efflux. A similar phenomenon was observed previously for Cl^-–SO_4^{2-} and Cl^-–phosphate exchange (Jennings, 1982) and is consistent with the idea that the catalytic cycle for net Cl^-–oxalate exchange is limited mainly by oxalate influx rather than Cl^- efflux.

To examine in more detail the effect of internal Cl^- concentration on Cl^-–oxalate exchange, cells were equilibrated with mixtures of 150 mM NaCl and 100 mM Na_2oxalate, each buffered at pH 7 with 10 mM MOPS, and then loaded with $^{36}Cl^-$ in the same medium. The net efflux of Cl^- was then measured in Cl^--free 100 mM Na_2oxalate at extracellular pH 6. The time course is much more rapid in cells initially containing lower concentrations of Cl^- (Fig. 5, *top*). The flux is a saturable function of the intracellular Cl^- concentration (Fig. 5, *bottom*). Estimation of the internal $K_{1/2}$ is difficult because very low Cl^- concentrations were not examined. It is clear, however, that the flux is half-maximal at an intracellular Cl^- concentration of less than 10 mM. This low apparent $K_{1/2}$ is consistent with a ping-pong model (Gunn and Fröhlich, 1979; Knauf, 1979; Jennings, 1982) in which the Cl^- efflux event is considerably faster than the oxalate influx event.

Extracellular pH Dependence of Cl^-–Cl^- and Cl^-–Oxalate Exchange

Fig. 6 shows the extracellular pH dependence of the efflux of $^{36}Cl^-$ into either Cl^- or oxalate media. The intracellular and extracellular pH are not constant during net Cl^-–oxalate exchange, but the rise in extracellular pH is < 0.1 unit over the time course of the flux measurement at 0°C. The intracellular pH is also expected to change (decrease) during Cl^-–oxalate exchange, because of the acid influx with oxalate. These pH changes do not have a detectable effect on the flux, as indicated by the approximately linear time course over the first half of the efflux (Figs. 4 and 5).

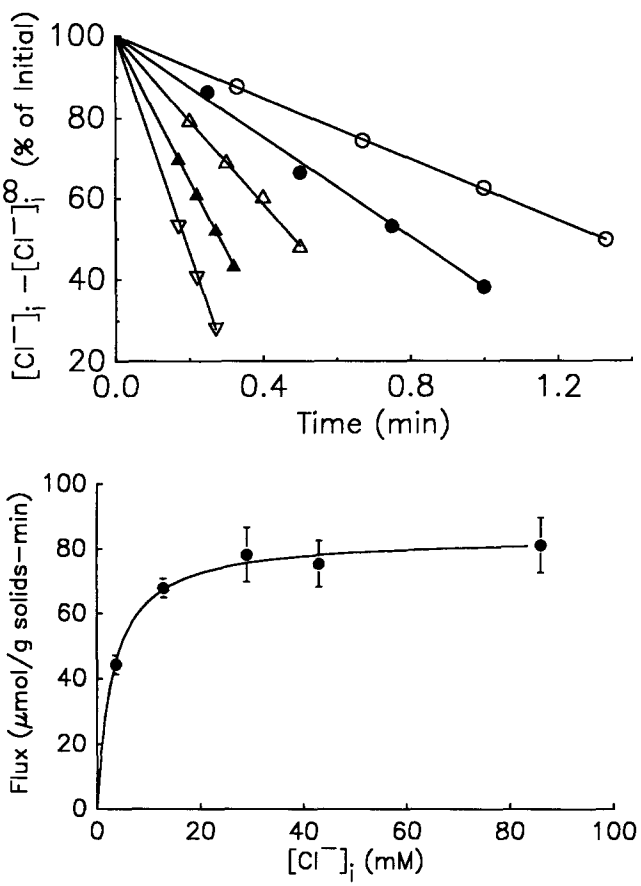

FIGURE 5. Efflux of Cl^- into oxalate media from cells loaded with mixtures of Cl^- and oxalate. Cells were equilibrated and loaded with $^{36}Cl^-$ in mixtures of 100 mM Na_2oxalate and 150 mM NaCl, each buffered with 10 mM MOPS, pH 7. Efflux was measured in 100 mM Na_2oxalate, 10 mM citrate, 10 mM MOPS, pH 6.0, 0°C. (*Top*) Time course of Cl^- efflux. Intracellular Cl^- was determined at each time point as in Fig. 4. The Cl^- concentration in the loading media was 150 mM (*open circles*), 90 mM (*filled circles*), 60 mM (*open triangles*), 30 mM (*filled triangles*), or 15 mM (*open inverted triangles*). The lines through the data are linear regressions. Each data point is the average of two determinations on a single preparation of cells. (*Bottom*) Cl^-–oxalate exchange flux (μmol/g solids-min) as a function of intracellular Cl^- concentration, from the data in the top part of the figure and an additional similar experiment. The intracellular Cl^- concentration of course varies throughout each flux measurement. The intracellular Cl^- concentration for each data point was chosen as the Cl^- concentration at the mid-point of the efflux data. Each data point represents the mean ± SD of a total of four fluxes with two preparations of cells.

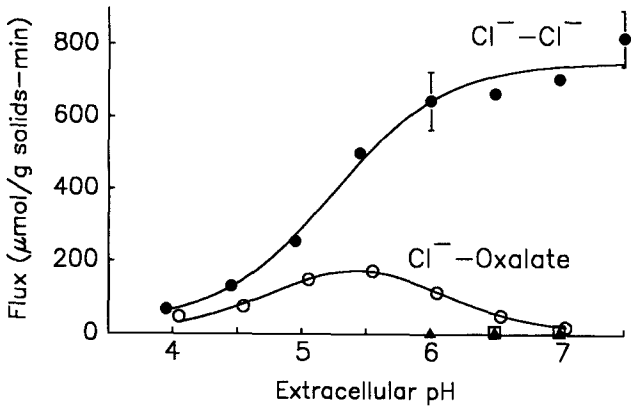

FIGURE 6. Efflux of $^{36}Cl^-$ at 0°C into Cl^- or oxalate media of varying extracellular pH. Cells were loaded with $^{36}Cl^-$ in 150 mM KCl, 10 mM Hepes, pH 7.5. Efflux was measured in either 150 mM NaCl, or 100 mM Na_2oxalate, buffered at the indicated pH value with 10 mM citrate and 10 mM MOPS. The efflux into Cl^- (Cl^-–Cl^- exchange; *filled circles*) has been fit by a curve describing a single inhibitory titration with a pK of 5.3, and a limiting flux at very low pH equal to 2% of that at neutral pH. Data are from single measurements at pH 6.5 and 7 and from two to three measurements at the other pH values. The Cl^-–oxalate exchange data (*open circles*; mean of two to seven determinations) were fit to a function that is equivalent to equation 13 or 14 in Appendix B, with a pH optimum of 5.4. The Cl^- effluxes into media containing 150 mM glycolate or glyoxylate at the indicated extracellular pH are shown in the open squares and filled triangles, respectively. Error bars are ± SD and are not shown unless larger than the symbol.

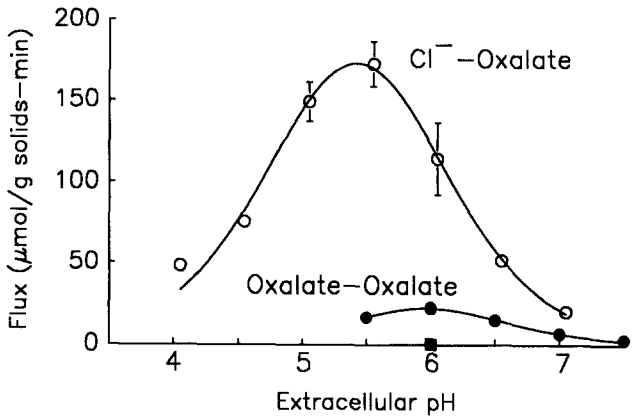

FIGURE 7. Effect of pH on the oxalate-oxalate equilibrium exchange flux. Cells were equilibrated in media containing 100 mM Na_2oxalate, 10 mM MOPS, 10 mM citrate at the indicated pH, and the oxalate–oxalate exchange flux was measured at 0°C. Data represent two flux determinations at each pH. For comparison, the Cl^-–oxalate exchange data from Fig. 6 are redrawn on the same scale (mean ± SD). Also for comparison, a flux was performed with cells loaded in oxalate at pH 6 exactly as for the oxalate–oxalate exchange experiments, except that the efflux was measured in 150 mM Na-gluconate, 10 mM MOPS, 10 mM citrate, pH 6. The efflux is very small in the absence of rapidly transported *trans* anions (filled square at pH 6).

Low extracellular pH (at an intracellular pH of 7.3) inhibits Cl^-–Cl^- exchange, with an apparent pK of ~5.2 (Fig. 6), in excellent agreement with previous work by Milanick and Gunn (1982) and Wieth et al. (1982). The initial efflux of Cl^- from the same cells into 100 mM oxalate media has a completely different dependence on extracellular pH. The efflux into oxalate media is stimulated 10-fold by lowering the extracellular pH from 7 to 5.5; further acidification inhibits oxalate influx. At extracellular pH below 5.5, Cl^-–oxalate exchange is nearly as rapid as Cl^-–Cl^- exchange.

Oxalate Self-Exchange

The pH dependence of oxalate–oxalate equilibrium exchange is shown in Fig. 7, with the Cl^-–oxalate exchange flux (oxalate influx) replotted from Fig. 6 for comparison. Oxalate–oxalate equilibrium exchange was examined over a more limited pH range because an intracellular pH below ~5.5 causes significant anion fluxes by pathways other than band 3 (Gunn et al., 1975). As with net oxalate influx into Cl^--loaded cells, oxalate self-exchange is activated by low pH; the pH optimum for self-exchange is ~6.0, although not enough data were obtained to define a precise pH optimum. In the same 100 mM oxalate media, the net Cl^-–oxalate exchange flux is larger than the oxalate–oxalate exchange flux, i.e., intracellular Cl^- has a substantial *trans* accelerating effect on oxalate transport. As expected, the initial efflux of labeled oxalate into a gluconate medium (square data point in Fig. 7) is negligible compared with the efflux into an oxalate medium, illustrating that oxalate efflux requires a rapidly transported *trans* anion.

Exchange of Glyoxylate and Glycolate for Cl^-

The extracellular pH dependence of Cl^-–oxalate exchange as well as the H^+ cotransport with oxalate could be caused by either of two different processes. One possibility is that divalent oxalate is transported on the protonated (divalent) form of band 3; lowering the pH would increase the flux by increasing the number of divalent transporters. Alternatively, monovalent oxalate transport on the normal (monovalent) form of the transporter would also cause an acid cotransport with oxalate. Lowering the pH would activate this mode of transport by increasing the concentration of monovalent substrate. The pK_a of the titration from monovalent to divalent oxalate is about 4.2 (Dean, 1979); therefore, the concentration of monovalent oxalate is fairly low even at pH 6. However, monovalent anion transport in red cells is generally much more rapid than that of divalent anions (see Passow, 1986),

TABLE I

Maximum Exchange Flux of Cl^- for Various Inorganic and Organic Anions, 0°C

Anion	Formula	pH_o	Flux*	Reference
Chloride	Cl^-	7.8	750	Gunn et al., 1973
Bicarbonate	HCO_3^-	8.7	1000	Wieth, 1976
Formate	$HCOO^-$	7.0	600	This work, not shown‡
Bromide	Br^-	7.8	360	Gunn and Frölich, 1979
Oxalate	$^-OOCCOO^-$	5.5	180	This work, Fig. 6
Phosphite	$HPO_3^{2-}/H_2PO_3^-$	6.0	20	Galanter and Labotka, 1990§
Glyoxylate	$OCHCOO^-$	7.0	9	This work, Fig. 6
Glycolate	$HOCH_2COO^-$	7.0	9	This work, Fig. 6
Hypophosphite	PO_2^-	7.0	8	Galanter and Labotka, 1990§
Iodide	I^-	7.8	8	Milanick, 1981
Acetate	H_3CCOO^-	7.9	2	Gunn et al., 1973
Phosphate	$HPO_4^{2-}/H_2PO_4^-$	6.5	0.5	Galanter and Labotka, 1990§
Sulfate	SO_4^{2-}	3.3	0.5	Milanick and Gunn, 1984

*Units are mmol/kg solids-min. Values are the observed maximum flux, with no attempt to correct for substrate inhibition. The intracellular pH was 7.2–7.5 except for Cl^-–HCO_3^- exchange, in which the pH on both sides of the membrane was 8.7. For HCO_3^-, the transport was measured as HCO_3^- efflux into Cl^-; for all the other entries, the direction of transport was Cl^- efflux, X^- or X^{2-} influx. The initial intracellular Cl^- concentration was about 100 mM.

‡Two $^{36}Cl^-$ efflux measurements in 150 mM Na-formate on a single preparation of cells. The data of Halestrap (1976) indicate that Cl^-–formate exchange is about three- to fourfold slower than Cl^-–HCO_3^- exchange at 2.5°C and 20 mM extracellular formate or HCO_3^-.

§Influx data at low temperature, pH 7.0, for phosphate, phosphite, and hypophosphite were extrapolated to 0°C and then multiplied by a factor of two to five to estimate the flux at the pH optimum (Labotka and Omachi, 1988). The values are of course approximate because the pH optimum may be temperature dependent.

and it is possible that monovalent oxalate ($HOOCCOO^-$) is translocated on the monovalent form of the transporter much more rapidly than divalent oxalate is transported on the divalent transporter.

As an independent estimate of the possible contribution of monovalent oxalate influx to Cl^-–oxalate exchange, the Cl^- efflux was measured in media containing anions that are structurally similar to monovalent oxalate ($HOOCCOO^-$), i.e., glyoxylate ($OCHCOO^-$) or glycolate ($HOCH_2COO^-$). The efflux of Cl^- into media containing either of these anions is considerably slower than that into oxalate (Fig. 6; Table I). The Cl^- efflux into glycolate is similar to that into glyoxylate, in agreement with Aubert and Motais (1975), indicating that minor differences in structure (alcohol vs aldehyde) have little effect on the exchange rate of these anions.

Temperature Dependence

The temperature dependence of Cl^- efflux into an oxalate medium at pH 7 is shown in Fig. 8. The temperature dependence corresponds to an activation energy of 35 kCal/mol, which is slightly higher than that for Cl^-–Cl^- exchange over the same temperature range (Brahm, 1977) but lower than that for Cl^- exchange with either phosphite or hypophosphite below 10°C (Galanter and Labotka, 1990). The fluxes in Fig. 8 were measured in media that were at pH 7.0 at 22°C, without further titration at other temperatures. Essentially the same temperature dependence was observed in a medium buffered with 10 mM citrate/10 mM phosphate as with 10 mM citrate/10 mM MOPS. The pH of the citrate/phosphate medium is less temperature dependent than the citrate/MOPS medium. Although a small fraction of the apparent activation energy may be

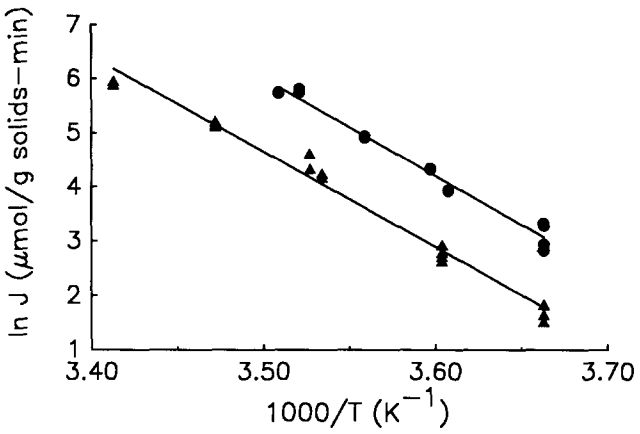

FIGURE 8. Temperature dependence of Cl^-–oxalate and oxalate–oxalate exchange. Efflux of Cl^- into 100 mM Na_2oxalate medium, pH 7.0, was measured as in Fig. 6 at various temperatures between 0°C and 12°C (*circles*). The media were buffered with 10 mM citrate and either 10 mM phosphate or 10 mM MOPS. The efflux of [^{14}C] oxalate was measured at Donnan equilibrium in 100 mM Na_2oxalate, 10 mM citrate, 10 mM MOPS, pH 7 (*triangles*). The natural logarithm of the flux is plotted vs 1,000/T, where T is the absolute temperature. The line drawn through both sets of data has a slope corresponding to an activation energy of 35 kCal/mol.

caused by pH shifts, it is clear that oxalate transport across the red cell membrane has a rather high activation energy.

The temperature dependence of oxalate self-exchange in the same 100 mM oxalate medium, pH 7, is also plotted in Fig. 8. At all temperatures, the Cl^-–oxalate exchange flux is larger than the oxalate–oxalate exchange flux, but the oxalate self-exchange flux also has a very high apparent activation energy. The high activation energy for oxalate–oxalate exchange is in agreement with the data of Cousin and Motais (1976, 1979). For both oxalate–oxalate and Cl^-–oxalate exchange, only a limited temperature range was examined, and it is difficult to judge whether there is a distinct break in the Arrhenius plot, as has been found for some other types of anion exchange (Brahm, 1977; Obaid et al., 1980; Glibowicka et al., 1988; Galanter and Labotka, 1990).

Effect of ATP Depletion and Okadaic Acid

Motais et al. (1981) originally showed that ATP depletion causes a moderate (~40%) inhibition of Cl^-–Cl^- exchange in red cells in a Ca^{2+}-free medium; there is a further 25% inhibition of anion exchange attributable to accumulation of intracellular Ca^{2+} (Low, 1978). Bursaux et al. (1984) found that ATP depletion has a similar inhibitory effect on SO_4^{2-} transport. In agreement with these laboratories, we find that overnight ATP depletion inhibits the Cl^-–Cl^- exchange flux by 40%. The same ATP depletion inhibits the oxalate-oxalate exchange flux by very nearly the same amount (50 ± 8%; four pairs of fluxes on two cell preparations). It should be pointed out that ATP depletion affects the rate constant for oxalate–oxalate exchange more strongly than the flux, because ATP depletion shifts the Donnan ratio and raises the intracellular oxalate content. Precise interpretation of the ATP depletion data for oxalate transport is difficult because of the shifts in the Donnan ratio, but it is clear that both Cl^-–Cl^- and oxalate–oxalate exchange are moderately inhibited by overnight ATP depletion.

Baggio et al. (1993) have reported that incubation of red cells with 1 μM okadaic acid, an inhibitor of Ser/Thr protein phosphatases type 1 and 2A (Cohen et al., 1990), caused a greater than threefold increase in the rate constant for oxalate–oxalate exchange at 4°C and an extracellular pH of 7.4. These results were interpreted as evidence that oxalate transport is stimulated by protein phosphorylation. An implication of this finding is that, under physiological conditions, the state of Ser/Thr phosphorylation of either band 3 or some unknown modulator protein is less than optimal for oxalate transport. Okadaic acid presumably increases the level of phosphorylation by inhibiting protein phosphatases.

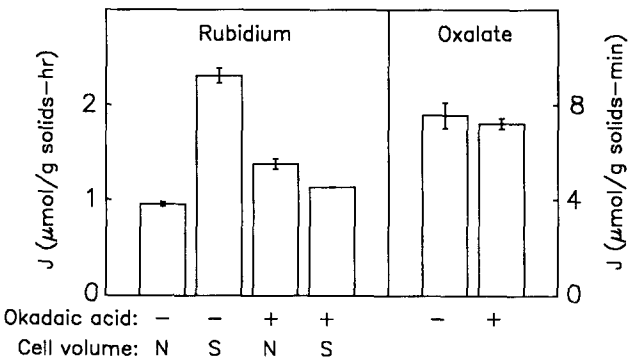

FIGURE 9. Lack of effect of 1 μM okadaic acid on oxalate-oxalate exchange measured at pH 7.0. Cells were incubated ± 1 μM okadaic acid in 1% dimethylformamide for 90 min at 37°C, washed, and the oxalate–oxalate exchange rate at 0°C and swelling-stimulated $^{86}Rb^+$ influx at 37°C were measured (see Materials and Methods). After the okadaic acid incubation, the oxalate and $^{86}Rb^+$ transport measurements were done in parallel and completed within 2 h. The $^{86}Rb^+$ influxes were performed in cells of normal volume (*N*) and swollen (*S*). There is no swelling-stimulated flux in okadaic acid-treated cells. Bars represent the mean and range of two determinations.

We have found, in contrast to the result reported by Baggio et al. (1993), that there is no detectable effect of a 90-min incubation with 1 μM okadaic acid on oxalate–oxalate exchange (Fig. 9). To determine whether okadaic acid had the expected effect on red cell protein phosphatases, the swelling-stimulated $^{86}Rb^+$ influx was measured in an aliquot of the same cells as were used for oxalate fluxes (Fig. 9). As found previously by Kaji and Tsukitani (1991) in human red cells and by Jennings and Schulz (1991) in rabbit red cells, okadaic acid strongly inhibits the swelling-stimulated $^{86}Rb^+$ flux. Also, measurement of protein phosphatase activity (using ^{32}P phosphorylase a as a substrate) in lysates showed that okadaic acid strongly inhibits red cell phosphorylase phosphatase activity (data not shown) and that the inhibition is not reversible over the times (less than 2 h) used to measure transport after the okadaic acid incubation.

The $^{86}Rb^+$ fluxes were performed on only one preparation of human red cells, but in numerous (over 10) separate experiments with rabbit red cells, okadaic acid inhibited $^{86}Rb^+$ transport and protein phosphatase activity. Therefore, the okadaic acid used in these experiments was active. In a total of 16 flux measurements on three preparations of cells, we find no effect of a 90-min preincubation with 10–1,000 nM okadaic acid on human red cell oxalate transport (OKA/control flux = 0.99 ± 0.06, SD). Eight of these fluxes were performed at 100 mM oxalate concentration, and eight were performed under the conditions described by Baggio et al. (1993): 10 mM oxalate, pH 7.4, 4°C in a predominantly

Cl^- medium. In no case do we find any stimulatory or inhibitory effect of okadaic acid.

DISCUSSION

Band 3 Is the Main Transport Pathway for Oxalate in Red Cells

All the data in this paper are consistent with the idea that band 3 is the main transport pathway for oxalate in human red blood cells, in agreement with the studies by Cousin and Motais (1976, 1979) using reversibly acting inhibitors. Irreversible inhibition by low concentrations of H_2DIDS is identical for Cl^-–Cl^- and oxalate–oxalate exchange (Fig. 1). Moreover, the effects of oxalate on Cl^- transport and of Cl^- on oxalate transport strongly suggest competition for a common site (Fig. 2), with Cl^- having a two- to threefold higher apparent affinity. These experiments do not address the issue of the possible asymmetry of the apparent affinity for oxalate binding to inward-facing and outward-facing transport sites.

Although estimates of the rate of red cell Cl^-–oxalate exchange have been published (Giebel and Passow, 1960; Aubert and Motais, 1975), the present results demonstrate that oxalate influx at low extracellular pH is far more rapid than previously believed. For comparison, Table I lists exchange fluxes of Cl^- for various monovalent and divalent anions in human red blood cells at 0°C at the pH optimum for each divalent anion and at neutral to alkaline pH for monovalent anions (which have no well-defined pH optimum). It is clear that oxalate transport is much faster than that of any other divalent anion and is also faster than many monovalent anions. The fastest divalent anion listed is phosphite (HPO_3^{2-}), but the pK for titration of phosphite to monovalent form is 6.4 (Labotka and Omachi, 1988), and it is reasonable to expect that the monovalent form is the main transported species. Dithionite ($S_2O_4^{2-}$) is a band 3 substrate (Salhany and Swanson, 1978), but it is not included in Table I because flux data are not available for Cl^-–$S_2O_4^{2-}$ exchange at 0°C.

The rapid translocation of oxalate compared with that of other divalent anions does not appear to be a consequence of a lower activation energy; the temperature dependence of Cl^-–oxalate exchange is comparable to that of SO_4^{2-} self-exchange (Glibowicka et al., 1988) and Cl^-–SO_4^{2-} exchange (Milanick and Gunn, 1984), which have activation energies of ~32 kCal/mol.

Extracellular pH Dependence of Cl^-–oxalate Exchange

The extracellular pH dependences of red cell Cl^- and SO_4^{2-} transport in the acid range can be explained reasonably well by the titratable carrier model (Gunn, 1972; Milanick and Gunn, 1982, 1984; Wieth et al., 1982), which can be summarized as follows. Band 3, at physiological pH, is in the appropriate charge state for monovalent anion transport. At pH 7.4, if a divalent anion is bound, transport is slow because the protein has an extra negative charge (the second charge on the substrate) in the anion-binding region. At low pH, a carboxyl group (probably the side chain of Glu 681; see Jennings and Smith, 1992) is protonated to convert band 3 to the appropriate charge state for divalent anion transport. Monovalent anion transport is inhibited at low pH because the proton bound to Glu 681 adds an extra positive charge.

Interpretation of transport data in terms of the titratable carrier model is more complex when the substrate itself (e.g., phosphate, oxalate) is titratable (see Berghout et al., 1985; Matsuyama et al., 1985; Labotka and Omachi, 1988). As pointed out by Labotka and Omachi (1988), the influx of a titratable anion on a titratable transporter, at saturating substrate concentration, should be maximal at a pH midway between the pK_a of the substrate and that of the transporter. The reason for this expectation is that the translocation event requires either a monovalent anion complexed with a monovalent carrier, or a divalent anion complexed with a divalent carrier. As the pH is lowered, the proportion of divalent carrier increases, but the proportion of divalent substrate decreases.

It should be pointed out that the pH optimum for influx should be midway between the pK values of substrate and transporter (Labotka and Omachi, 1988) only if the binding of divalent substrate to monovalent transporter has the same affinity as that of the monovalent substrate for the divalent transporter (see Appendix B). We do not know the actual affinities for these binding events, and the affinities may not be identical. It is therefore not possible to make a detailed interpretation of the extracellular pH dependence of Cl^-–oxalate exchange. The observed pH optimum of 5–5.5, however, is certainly consistent with the titratable carrier model (Gunn, 1972), in which the only inward transport events are monovalent oxalate bound to monovalent transporter and/or divalent oxalate bound to divalent transporter.

Monovalent vs Divalent Oxalate Transport

One way to estimate the contribution of monovalent oxalate to the total oxalate flux is to ask what proportion of the transporters are in the complex between monovalent transporter and monovalent oxalate. Consider, for example, the oxalate influx at an extracellular pH of 6.2 (two units above the pK_a of oxalate). Only 1% of the free oxalate is in the monovalent form. The most abundant complexes are therefore expected to be divalent oxalate with either the monovalent or divalent transporter. The influx of oxalate at pH 6.2 is over 10

times as large as that of three other two-carbon monocarboxylates (glycolate, glyoxylate, or acetate; Table I), in spite of the fact that free monovalent oxalate is less than 1% of the total. From these considerations it seems unlikely that monovalent oxalate accounts for a significant portion of the oxalate influx.

The above argument about the abundance of monovalent oxalate is based on the pK_a (4.2) of the titration of divalent oxalate to monovalent oxalate in free aqueous solution. It is possible, however, that oxalate bound to the transport site has a higher pK_a because the binding site may represent a less polar environment than free solution. If so, then the amount of bound monovalent oxalate could represent more than 1% of the total at pH 6.2. It is not possible, therefore, to exclude rigorously the possibility that monovalent oxalate contributes substantially to the observed flux. In any case, the transport characteristics of oxalate are unique. If it is transported as a monovalent anion, its flux on band 3 is much larger than that of structurally similar monocarboxylates. If it is transported as a divalent anion, its flux is much larger than any other divalent anion.

The equilibrium exchange flux of oxalate exhibits a pH optimum at about pH 6, which is higher than that for Cl^-–oxalate exchange. A similar situation exists for SO_4^{2-}, in which the equilibrium exchange flux is maximal at pH 6.5 (Schnell et al., 1977), whereas net SO_4^{2-} influx into Cl^--loaded cells is maximal at much lower pH (Milanick and Gunn, 1984). A detailed interpretation of the pH dependence of self-exchange is not possible at present, because low pH could inhibit both the efflux and influx translocation events.

Variability of Red Cell Oxalate Transport among Individuals

In population studies of human red cell oxalate transport in relation to kidney stone formation, the measured rate constant for oxalate transport has been found to be quite variable among different individuals (Baggio et al., 1986; Motola et al., 1992); the highest measured rate constant in a sample of normal donors is several times larger than the lowest; the variability among stone-formers is larger than among normal donors. A large-scale study of variability of Cl^-–Cl^- exchange in the normal human population has not been done. However, in a study of a small population, Cl^-–Cl^- exchange is quite similar among red cells of different (normal) donors (Fröhlich and Gunn, 1981). With the exception of genetic variants with either high (Bruce et al., 1993) or low (Schofield et al., 1992) transport rates, the Cl^-–Cl^- exchange flux does not vary by more than about ± 20% in the human population.

In considering the variability of oxalate transport in published studies, it is important to point out that in the present work, blood from only three donors was used, and the variability among individuals could be larger than is evident in this very small sample. We also emphasize that the data in this paper do not address the question of whether red cell oxalate transport is abnormal in individuals with nephrolithiasis.

Physiological Regulation of Red Cell Anion Exchange?

As mentioned above, we find that overnight ATP depletion causes 40–50% inhibition of the Cl^-–Cl^- and oxalate–oxalate exchange fluxes, very similar to results reported by Motais et al. (1981) for Cl^-Cl^- exchange and by Bursaux et al. (1984) for SO_4^{2-}–SO_4^{2-} exchange. One possible interpretation of the effect of ATP depletion is that anion transport is affected by the phosphorylation state of band 3 or some regulatory factor. As discussed by Motais et al. (1981), there are other possible mechanisms for the effect of ATP depletion, including rearrangement and/or aggregation of membrane proteins or loss of spectrin-free vesicles containing band 3.

With regard to the role of band 3 phosphorylation in anion transport it is relevant to ask whether the state of band 3 phosphorylation is optimal for transport in red cells that have never been depleted of ATP. The results of Baggio et al. (1993) suggested that the level of phosphorylation of band 3 (or some modulator) can be increased by okadaic acid, with a resultant increase in oxalate transport. Using essentially identical conditions, we could not reproduce the stimulatory effect of okadaic acid on oxalate transport. Our data therefore do not support the idea that, in normal red cells, an increase in band 3 phosphorylation causes an increase in transport.

We believe that, under physiological conditions, the transport function of band 3 is maximally active and is not regulated by phosphorylation. It is nonetheless possible that, under conditions of slow ATP depletion, the dephosphorylation of some site may affect transport. Band 3 certainly contains several sites for phosphorylation (Waxman, 1979; Dekowski et al., 1983; Low et al., 1987; Yannoukakos et al., 1991), with by far the majority of the phosphorylation on the NH_2-terminal cytoplasmic domain. Given that the effects of ATP depletion are moderate (40–50% inhibition of transport), it would be difficult to demonstrate a causal relationship between phosphorylation of a particular site and modulation of anion transport. In any case, this site, if it exists, is presumably fully phosphorylated in the normal mature cell, because Cl^- transport in freshly drawn blood has not been demonstrated to be activated further by manipulations that would be expected to increase phosphorylation.

Oxalate as a Substrate for AE1 Expression Systems

A potentially useful implication of the present results is that oxalate, at low extracellular pH, is transported by band 3 nearly as rapidly as Cl^- is at neutral pH. The

finding that oxalate is transported so rapidly by AE1 at low pH (and reasonably rapidly at physiological pH) implies that oxalate may be a useful alternative substrate for functional studies of band 3 expression in oocytes or other cells. Although oxalate transport has not been nearly as well characterized as Cl^- transport in most cells, it is possible that the background oxalate permeability is smaller than that of Cl^- and therefore would facilitate the study of oxalate transport by exogenously expressed AE1. In addition, the pH movements associated with Cl^-–oxalate exchange provide an independent way to detect and possibly quantify the anion exchange. The same H^+ cotransport is observed during Cl^-–SO_4^{2-} exchange, but the fluxes are 100-fold smaller and therefore much more difficult to detect. A disadvantage of oxalate as a substrate in expression experiments is that the flux is maximal at low pH, which may not be tolerated by some expression systems.

It is not clear whether oxalate will be a useful substrate in cells in which anion exchangers do not reach the surface membrane. For example, Ruetz et al. (1993) have found that the AE1 protein expressed in HEK 293 cells catalyzes Cl^-–SO_4^{2-} exchange in microsomes but not in whole cells, indicating that the transporter is synthesized and functional but that it does not reach the plasma membrane. In at least some subset of the endoplasmic reticulum, the membrane has a high oxalate permeability, although the oxalate permeability appears to be variable in different organelles (Gill et al., 1988). The high oxalate permeability of most of the endoplasmic reticulum will probably make it difficult to use oxalate in functional studies of AE1 expressed in microsomes.

Implications for Non-Erythroid Cells

The gene encoding red cell band 3 is known as AE1; two closely related proteins, AE2 and AE3, are expressed in a variety of tissues (see Kopito, 1990; Alper, 1991). Given the similarity of the membrane domains of these proteins, it seems likely that oxalate transport via AE2 and AE3 resembles that mediated by band 3. However, there are no experimental data on this point, and it is premature to draw conclusions about the contribution of these proteins to oxalate transport. Voltage-insensitive, DIDS-inhibitable oxalate–Cl^- and oxalate–OH^- exchange processes have been identified in the luminal membrane of rabbit ileum (Knickelbein et al., 1986). AE2 is known to be expressed in this membrane (Chow et al., 1992), and it is possible that AE2 mediates some of the observed oxalate exchange flux.

In the kidney, it is clear that oxalate transport in the proximal tubule takes place through pathways that are distinct from AE1 or other Cl^-–HCO_3^- exchangers (see Aronson, 1989). Oxalate exchange for Cl^- across the proximal tubular brush border membrane is electrogenic rather than the neutral exchange of Cl^- for H^+ + oxalate as we have observed in the red cell. There is also a SO_4^{2-}–oxalate exchange process in the proximal tubule that is different from band 3-like anion exchangers in that it has low affinity for Cl^- (Aronson, 1989). In the distal nephron, AE1 and (very likely) AE2 are expressed (Kopito, 1990; Alper, 1991), but it is not yet clear whether these proteins contribute significantly to oxalate transport.

APPENDIX A

Mutual Competition between Oxalate and Cl^-

The expression for the equilibrium tracer flux of an anion A^-, in the presence of symmetric A^- and no competing anions, can be written as follows:

$$J_A = J_{\text{maxA}}[\text{A}]/(K_{1/2} + [\text{A}]). \tag{A1}$$

If the mechanism is ping-pong, the half-saturation constant $K_{1/2}$ is a function of the true internal and external dissociation constants and the translocation rate constants (Knauf, 1979; Fröhlich and Gunn, 1986):

$$K_{1/2} = K_o/(1 + N) = K_i N/(1 + N), \tag{A2}$$

where N is the ratio of inward to outward rate constants for translocation of the A^--loaded transporter, and K_o and K_i are, respectively, the dissociation constants for binding to outward-facing and inward-facing transport sites.

In the presence of both Cl^- and oxalate on both sides of the membrane, there are multiple equilibria, i.e., between each anion and inward- and outward-facing sites, and between the inward-facing and outward-facing transporter with bound anion. The expression for the tracer flux of Cl^- at equilibrium is (see Gunn et al., 1973):

$$J_{\text{Cl}} = J_{\text{maxCl}}[\text{Cl}]/([\text{Cl}] + K_{1/2\text{Cl}} + [\text{Ox}]\,K_{1/2\text{Cl}}/K_{1/2\text{Ox}}]), \tag{A3}$$

where $K_{1/2\text{Ox}}$ and $K_{1/2\text{Cl}}$ are, respectively, the concentrations of oxalate and Cl^- (each symmetrically distributed) that would half-saturate the transporter in the absence of competitor. An analogous expression for the tracer oxalate flux is:

$$J_{\text{Ox}} = J_{\text{maxOx}}[\text{Ox}]/([\text{Ox}] + K_{1/2\text{Ox}} + [\text{Cl}]\,K_{1/2\text{Ox}}/K_{1/2\text{Cl}}) \tag{A4}$$

In the experiment in Fig. 2, the Cl^- concentration is varied from 0 to 120 mM as the oxalate concentration is varied from 100 to 20 mM. Cell volume varies slightly as a function of oxalate concentration, but the combined charge on intracellular Cl^- plus oxalate is constant, because the incubations are too brief to allow significant cation shifts or changes in cellular organic

phosphates, and the Donnan ratio is not distinguishable from unity. Therefore, the cellular oxalate (or Cl^-) content (μmol/g solids) is proportional to the oxalate (or Cl^-) concentration in the loading medium. The flux can therefore be calculated from the substrate concentration and the rate constant for tracer efflux.

The data in Fig. 2 are fit to Eq. A3 (Cl^-) and Eq. A4 (oxalate), with a $K_{1/2}$ of 30 mM for Cl^- and 67 mM for oxalate. The $K_{1/2}$ of 30 mM for Cl^- is in the lower end of the range of values in the literature for Cl^-–Cl^- exchange (Knauf, 1979; Fröhlich and Gunn, 1986). We do not claim that these data provide information about the absolute values of the $K_{1/2}$ for Cl^-; the data can be fit with a variety of combinations of $K_{1/2}$ for Cl^- and oxalate, as long as the $K_{1/2}$ for oxalate is two to three times higher than that for Cl^-. The important point is that both the Cl^- and oxalate data indicate that the two anions share a common transport site.

APPENDIX B

Extracellular pH Dependence of Oxalate Influx

To attempt to interpret the extracellular pH dependence of oxalate influx into Cl^--loaded cells, we assume that the outward-facing conformation of band 3 can be titrated from a monovalent form E^+ to a divalent form HE^{2+}, with a pK of 5.2 (see above). The substrate oxalate is titratable from divalent $[Ox^{2-}]$ to monovalent $[HOx^-]$ with pK 4.2 (Dean, 1979). The equilibria between extracellular substrate and outward-facing transporter are as follows:

Binding event	Dissociation constant
$E^+ + Ox^{2-} = EOx^-$	K_{12} (monovalent site, divalent substrate)
$HE^{2+} + Ox^{2-} = HEOx$	K_{22} (divalent site, divalent substrate)
$E^+ + HOx^- = EHOx$	K_{11} (monovalent site, monovalent substrate)
$HE^{2+} + HOx^- = HEHOx^+$	K_{21} (divalent site, monovalent substrate)
$E^+ + H^+ = HE^{2+}$	$K_H = 10^{-5.2}$
$Ox^{2-} + H^+ = HOx^-$	$K_{aOx} = 10^{-4.2}$

The concentrations of monovalent and divalent oxalate are

$$[HOx^-] = [Ox_t]\,[H^+]/(K_{aOx} + [H^+])\,. \quad \text{(B1)}$$

$$[Ox^{2-}] = [Ox_t]\,[K_{aOx}]/(K_{aOx} + [H^+])\,, \quad \text{(B2)}$$

where Ox_t is the total concentration of oxalate. It is assumed that the outward Cl^- gradient recruits all the transporters into the outward-facing configuration. This assumption is only approximately true, because maximum oxalate influx is about 15–20% as large as the Cl^-–Cl^- exchange flux. The fact that the $K_{1/2}$ for intracellular Cl^- is relatively low for Cl^- efflux into oxalate (Fig. 5) indicates that the catalytic cycle is limited mainly by oxalate influx rather than Cl^- efflux. Accordingly, it is assumed that, during oxalate influx from a Cl^--free medium into Cl^--loaded cells, the sum of all the forms of outward-facing transporter is equal to E_t, the total number of transporters. Therefore,

$$[E_t] = [E^+] + [HE^{2+}] + [EOx^-] + [HEOx] + [EHOx] + [HEHOx^+]\,. \quad \text{(B3)}$$

Experimentally, the oxalate influx into Cl^--loaded cells was measured as a function of oxalate concentration (20–100 mM; gluconate substitution) at extracellular pH 5.5 and 7.0. At both pH values the flux is half-maximal at an extracellular oxalate of between 10 and 25 mM (data not shown); therefore, the extracellular sites are nearly saturated at 100 mM total oxalate. The total concentration of transporters may then be approximated by the oxalate-bound forms:

$$[E_t] = [EOx^-] + [HEOx] + [EHOx] + [HEHOx^+]\,. \quad \text{(B4)}$$

According to the titratable carrier model (Gunn, 1972), only two forms of the transporter (HEOx and EHOx) contribute to the oxalate influx; the others (EOx^- and $HEHOx^+$) are in the wrong charge state because of a mismatch between the valence of the transporter and the substrate. Although [HEOx] and [EHOx] have the same net charge, they are not identical; the former has a proton bound to the transporter and the latter has a proton bound to the substrate. The concentrations of EOx^-, HEOx, EHOx, and $HEHOx^+$, relative to E^+, follow directly from the definitions of the dissociation constants K_{11}, etc.

$$[EOx^-] = [E^+]\,[Ox^{2-}]/K_{12} \quad \text{(B5)}$$

$$[HEOx] = [E^+]\,[H^+]\,[Ox^{2-}]/K_H K_{22} \quad \text{(B6)}$$

$$[EHOx] = [E^+]\,[HOx^-]/K_{11} \quad \text{(B7)}$$

$$[HEHOx^+] = [E^+]\,[H^+]\,[HOx^-]/K_H K_{21}\,. \quad \text{(B8)}$$

Expressions for each form of the transporter can be derived by substituting Eqs. B1, B2, B5–B8 into the conservation of mass Eq. B4. For example, the concentration of HEOx, i.e., divalent site complexed with divalent oxalate, is

$$[HEOx] = \frac{E_t K_{aOx} H^+/K_H K_{22}}{\{K_{aOx}/K_{12} + K_{aOx}[H^+]/(K_H K_{22}) + [H^+]/K_{11} + [H^+]^2/K_H K_2\}}. \quad \text{(B9)}$$

A similar expression can be written for the concentration of EHOx, the monovalent site complexed with monovalent oxalate:

$$[EHOx] = \frac{[E_t]\,[H^+]/K_{11}}{\{K_{aOx}/K_{12} + K_{aOx}[H^+]/K_H K_{22} + [H^+]/K_{11} + [H^+]^2/K_H K_{22}\}}. \quad \text{(B10)}$$

An interesting implication of Eqs. B9 and B10 is that the pH dependences of the concentrations of HEOx and EHOx should be identical.

At all pH values, the ratio between [HEOx] (divalent/divalent) and [EHOx] (monovalent/monovalent) is

$$[HEOx]/[EHOx] = K_{aOx}K_{11}/K_H K_{22}. \quad \text{(B11)}$$

The pH at which both [HEOx] and [EHOx] should be maximal (pH_{max}) can be derived by taking the first derivative of the right side of Eqs. B9 or B10 with respect to $[H^+]$ and solving for the $[H^+]$ at which the derivative is 0:

$$pH_{max} = 0.5\,(pK_{aOx} + pK_H + \log K_{12} - \log K_{21}). \quad \text{(B12)}$$

If the dissociation constants K_{12} and K_{21} are equal, then the pH optimum is midway between the pK of the transporter and that of the substrate. It is clear from the above derivations that precise evaluation of the relative amounts of EOx^-, HEOx, EHOx, and $HEHOx^+$ require quantitative information about all the binding affinities. As mentioned above, the oxalate concentration dependence of oxalate influx into Cl^--loaded cells was measured at pH 5.5 and 7, but we cannot draw conclusions about the dissociation constants from these measurements, because the half-saturation constant will depend on all four dissociation constants K_{11}, K_{12}, etc., as well as possible pH effects on the spectator anion gluconate, which does have some inhibitory activity (Knauf and Mann, 1984).

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