Asymmetry in the Mechanism for Anion Exchange in Human Red Blood Cell Membranes

Evidence for Reciprocating Sites That React with One Transported Anion at a Time

ROBERT B. GUNN and OTTO FRÖHLICH

From the Departments of Pharmacological and Physiological Sciences and Medicine, University of Chicago, Chicago, Illinois 60637

ABSTRACT   The kinetics of chloride and bromide transport were examined in intact human red blood cells and resealed ghosts. Because the influx and efflux of halide ions are almost equal (<0.01% difference), the stimulation of the exchange flux by external halides could be determined by measuring ^{36}Cl or ^{82}Br efflux. When the external halide concentration was increased by replacement of isoionic, isotonic solutions of sucrose and the nontransported anion citrate, the stimulation of the exchange flux was hyperbolic and was maximum at 20 mM halide externally. The $K_{1/2\text{-out}}$, the external concentration of chloride or bromide which stimulated the efflux to half of its maximum value, was 3 and 1 mM respectively, 15-fold smaller than $K_{1/2\text{-in}}$ which we found to be about equal to the $K_{1/2}$ of halide self-exchange with nearly equal internal and external concentrations. Thus, the transport mechanism behaves asymmetrically with respect to these transported halides. Bromide flux was two-fold greater in bromide-chloride heteroexchange than in bromide-bromide self-exchange but it was still much smaller than the chloride self-exchange flux. The maximum influx and efflux of bromide in exchange for chloride were roughly eqal. Thus, since the maximum transport rates in the two directions are nearly equal, the kinetics of bromide equilibrium exchange with equal concentrations on the two sides are controlled on the inside where $K_{1/2}$ is greatest. The $K_{1/2\text{-out}}^{Cl}$ was a hyperbolic function of internal chloride concentration and was proportional to the maximum flux at each internal chloride concentration. These results are evaluated in terms of two broad categories of kinetic models. We conclude that, in contrast to other ion transport systems which have been shown to have kinetics of a sequential mechanism, anion exchange is compatible with a ping-pong mechanism in which a single site reciprocates between inside- and outside-facing orientations with asymmetric $K_{1/2}$ values.

INTRODUCTION

Anion transport is a crucial fuction of the red cell membrane. While the intrinsic band 3 protein has been implicated in anion transport (Cabantchik and Rothstein, 1974), the molecular mechanism of this anion transport is unknown (Gunn, 1979). An important characteristic of the transport is that it is coupled so that there is a one-for-one exchange of monovalent inorganic anions. How this coupling is achieved is unknown. Two general schemes are possible: (a) mechanical coupling through the formation of a tertiary complex between the transport mechanism and one transported anion from each side with the simultaneous translocation of the two ions in opposite directions (sequential reaction [Cleland, 1963] of the ions with the mechanism followed by transport of both) and (b) mechanical coupling through formation of binary complexes between the membrane mechanism and only one transported ion at a time. This involves complex formation on the *cis* side, transport and decomplexation on the *trans* side; alternating with complex formation on the *trans* side, transport in the opposite direction, and decomplexation on the *cis* side (ping-pong reaction [Cleland, 1963] of ions alternating between the two sides of the membrane). We present data here to show that this coupling is by a mechanism with ping-pong kinetics in which single sites reciprocate between the inside and outside of the cell. We also demonstrate an asymmetry of the mechanism with respect to the transported anions.

A report of some preliminary experiments for this paper has been presented (Gunn and Fröhlich, 1978).

MATERIALS AND METHODS

Preparation of Cells

Fresh, heparinized whole blood from normal adults was centrifuged (Sorval RC-5, DuPont Instruments—Sorvall, Newtown, Conn.) at 12,000 g for 5 min. The plasma and buffy coat were aspirated and the cells were washed five times by repeated resuspension and centrifugation in a 27 mM glycylglycine-buffered medium at pH 7.8 at 0°C. At this pH the chloride self-exchange is maximal and variations in pH have the least effect on the flux.

Terminology

We use the expression "self-exchange" to signify measurements of the exchange of isotopes of a given halide when the halide is in chemical but not tracer equilibrium. We use the expression "homoexchange" to signify the measurements of the exchange of isotopes of a given halide when the total halide is not in equilibrium, but because net halide flux is slow, the exchange is measured under a nearly steady state for halide and at quasi-equilibrium. The term "heteroexchange" signifies measurement of a tracer flux when there is a net flux of halide in exchange for an equal but opposite net flux of another halide.

Self-Exchange: Equilibrium Exchange of Halide Isotopes

We used previously published methods (Gunn et al., 1973, 1975) to measure the self-exchange of halide isotopes at equilibrium. First, we equilibrated cells with the isotope (0.6 μCi/ml suspension) in a 30% (vol/vol) suspension, packed them in nylon tubes in

a centrifuge (Sorval RC-5; SS-34 rotor) at 17,000 g for 10 min, cut the nylon tubes, and separately saved the packed cells and supernate. We used duplicate samples of packed cells with a trapped extracellular fluid of 2.7% (wt/wt) to determine the efflux rate coefficient, cell water, and cell anion contents. The specific activity of the supernate was measured and always agreed with the specific activity of the packed cells within the 2% error caused by intracellular reducing substances (see Gunn et al., 1973). To measure the efflux rate coefficient, we injected packed cells into a well-stirred thermostatted solution of the same composition as the extracellular fluid, except without isotope. The supernatant fluid was sampled by rapid filtration at known time intervals after injection of the cells; a final sample was taken by centrifugation after 1 h at room teperature. The water content was determined by drying a known weight of packed cells to constant weight (>24 h at 95°C). The anion content was measured by coulometric titration of perchloric acid (7% wt/vol) extracts of known weights of packed cells and was corrected for trapped extracellular fluid.

Heteroexchange and Homoexchange of Anions

Nonequilibrium heteroexchange of a halide isotope with a different halide on the *trans* side and quasi-equilibrium steady-state homoexchange of a halide isotope with the same halide on the *trans* side were measured by first preparing cells or resealed ghosts (see below) loaded with isotope and packed in nylon tubes. Cells were injected into well-stirred, thermostatted solutions buffered to the same pH as the supernate of the packed cells or ghosts but with different anion compositions. The initial efflux rate coefficient was measured in duplicate by taking samples as described above. The initial halide content of intact cells was measured in two ways: first, by titration of perchloric acid extracts of packed cells and their supernate, with correction for 2.7% (wt/wt) trapped extracellular supernatant fluid and assuming 0.9 g hemoglobin/gram dry cell solids to convert dry weights to cell hemoglobin content; and second, by using the counts per minute/gram hemoglobin in the efflux suspension of each flux determination and the specific activity of the supernate of packed cells after loading with isotope. Since duplicate fluxes in 6–10 different media were determined using a single preparation of cells, the average of 12–20 values of the micromoles anion/gram hemoglobin was calculated. There was no difference in the results with intact cells using the two methods. The initial anion content of ghosts was measured using only the second method. Since the extracellular trapped volume in packed ghosts is quite variable (Funder and Wieth, 1976) the intracellular counts per minute $(cpm)/10^7$ ghosts from each suspension was calculated by taking the product of four factors: the $cpm/0.1$ ml of efflux suspension, the reciprocal of the number of ghosts per 0.1 ml of efflux, 10^7, the fraction of total counts initially intracellular (1 - trapped fraction) estimated from the extrapolated zero time intercept of the efflux graph of $\ln[1 - a(t)/a(T)]$ vs. time.

$$\frac{Intracellular\ cpm}{10^7\ ghosts} = \left(\frac{cpm}{0.1\ ml\ suspension}\right)\left(\frac{0.1\ ml\ suspension}{ghosts}\right)(10^7)\left(\frac{cpm\ intracellular}{cpm\ total}\right).$$

The product of the $cpm/10^7$ ghosts and the extracellular specific activity ($\mu mol/cpm$) gives the micromoles of anion within 10^7 ghosts. The number of erythrocyte ghosts was measured within a few hours using a Coulter Counter (model ZB, Coulter Electronics, Inc., Hialeah, Fla.). We determined the initial rate coefficient, b, from the first two or three points on a graph of $\ln[1 - a(t)/a(T)]$ vs. time where $a(t)$ was the radioactivity in a known volume of supernate at time t, and $a(T)$ was the total radioactivity in an equal volume of the supernate after the suspension was at room temperature for one hour.

Both extracellular and intracellular chloride concentrations were altered by substitution with citrate or in preliminary experiments with acetate. The extracellular solutions were buffered with 27 mM glycylglycine. We substituted 25 mM Na₃ citrate or K₃ citrate and 200 mM sucrose for equal volumes of 150 mM NaCl to obtain isotonic solutions with a range of chloride concentrations all with the same ionic strength, $I = 1/2\sum z^2 C_i$, where z is the valence and C_i the molar concentration of the ith ion. In some early experiments the intracellular halide concentration in intact erythrocytes was altered by substituting Na acetate for NaCl in the wash solutions. We found that four washes with four volumes per volume of cells in acetate solutions with 15-min incubations at 37°C were sufficient to reduce intracellular chloride concentration to a steady-state concentration approximately that of the wash solution.

Since citrate is not permeant, we altered the intracellular chloride with citrate solutions by preparing resealed ghosts by a modification of the procedure of Bodemann and Passow (1972) and Schwoch and Passow (1973). We washed fresh red cells with 165 mM KCl or KBr and hemolyzed them in 3.8 mM acetic acid, 4 mM MgSO₄ solution at 0°C. After 5 min, a concentrated resealing solution was added to restore isotonicity with citrate, sucrose, and chloride (or bromide) in the desired concentrations. After a further 10 min at 0°C, the ghosts were annealed by incubation for 45 min at 37°C and then washed. We packed the ghosts in nylon tubes in a centrifuge (RC-5) at 48,000 g for 20 min. Ghost volumes were calculated from the fractional cellular volume of packed ghosts and the number of ghosts per volume in the same suspension. Ghosts resealed in halide-citrate solutions had smaller volumes (45–58 μm^3) than ghosts resealed in halide solutions (71–85 μm^3). This presumably is because citrate is a larger anion and has a greater reflection coefficient than the 0.7 value estimated by Funder and Wieth (1976) for KCl in open ghost membranes.

Calculations

The formally correct calculation for the flux, M is given by Eq. 1:

$$M = b'S_1 S_2 / (S_1 + S_2),\qquad(1)$$

where b' is the rate coefficient equal to the negative of the slope of a graph of $\ln[1-a(t)/a(\infty)]$ vs. time and S_1 and S_2 are the total number of moles of the traced (nonradioactive) species in the intracellular (S_1) and extracellular (S_2) compartments per unit of cells or ghosts. The extracellular radioactivity at time t is $a(t)$.

A practical problem is the measurement of $a(\infty)$, the extracellular radioactivity when specific activity of the tracer has equalized in the two compartments but no net flux of the traced material has occurred. If there is very little extracellular traced (nonradioactive) material, $a(\infty)$ will have a small value after tracer equilibration. But if net efflux of traced material and tracer occurs, the radioactivity will increase in the extracellular supernate until total equilibrium with respect to traced anions is obtained, $a(T)$. The net loss of chloride at 0°C by the nonexchange-restricted pathway (conductance pathway) is slow ($t_{1/2} = 10$ h; extrapolated from Hunter, 1977), but traces of HCO₃ in the external medium in contact with atmospheric CO₂ are rapidly exchanged for internal halide and facilitate the net loss of intracellular halide even when extracellular halide is absent. This exchange greatly hampers the measurement of $a(\infty)$. We routinely have set aside a sample of each efflux suspension for at least 1 h at room temperature before collecting the supernate and measuring the concentration of radioactivity which we believe to be the parameter $a(T)$.

The evidence we have used to determine that a value of $a(T)$ was measured includes the observations that (a) the radioactivity in the supernate from experiments in which there was not extracellular chloride could be calculated from the product of the hematocrit of the efflux suspension, the cell chloride content, and the specific

activity of the injected cells (the initial extracellular radioactivity, estimated from the zero time intercept of the efflux graph, was 6–10% and was corrected for); (*b*) the equilibrium radioactivity per gram hemoglobin or per number of ghosts was calculated for each efflux suspension and was constant for each series of experiments over the entire range of initial extracellular chloride concentrations. In both cases, the results are only consistent with the measured equilibrium being $a(T)$ corresponding to the redistribution of nonradioactive anions into the extracellular phase. Thus, since $a(T)$ equals the total radioactivity in the suspension divided by the total volume (V_1 + V_2) and $a(\infty)$ equals the fraction of radioactivity in the external compartment (total radioactivity in the suspension times $S_2/[S_1 + S_2]$) divided by the external volume (V_2), the relationship between the two quantities is:

$$a(T) = a_\infty(S_1 + S_2)S_2^{-1} V_2(V_1 + V_2)^{-1}.$$

If the hematocrit is 1% and $V_1 = 0.67$ of the cell volume, the term $V_2/(V_1 + V_2)$ = 0.993 and can always be assumed to be unity in these experiments. The calculated rate coefficient when $a(T)$ is used in place of $a(\infty)$ is denoted as b. It differs from b' and can be shown in most cases to be approximately equal to $b'S_2/(S_1 + S_2)$ or M/S_1 where M is the flux and S_1 is the chloride content of red cells (μeq/g cell solids = 0.9 × μeq/g hemoglobin) or ghosts (μeq/3.1 × 10^{10} ghosts).

The range of validity of the approximation $b = b'S_2(S_1 + S_2)$ was calculated. Since $e^{-b't} = 1 - a(t)/a(\infty)$ and $e^{-bt} = 1 - a(t)/a(T) = 1 - a(t)S_2 \cdot [a(\infty)(S_1 + S_2)]^{-1}$, we can expand the exponential in its power series and compare terms. If only the first term is considered, we obtain directly the approximation $b = b'S_2(S_1 + S_2)^{-1}$. The second terms differ depending on the values of S_1 and S_2 and the size of the term bt. The greater the external anion concentration, the better the approximation. If the external concentration of chloride is only 0.7% of internal chloride so that $S_2/(S_1 + S_2) \approx 0.5$ and if the time points are less than 15% of the half-time, then the error in the approximation will be smaller than 3%. The later the time of the data points one must use, the greater the deviation from initial rates and the less accurate the approximation. In our experiments, the error in the approximation was always less than 20% of the rate. This procedure permits one to estimate the initial rate coefficient, b, when some of the data values are obtained after the half-time of the exchange. In the extreme case of no extracellular halide ($S_2 = 0$) the approximation is invalid and was not used; the efflux was calculated directly from the initial rate of tracer appearance in the extracellular phase and the constant specific activity.

Data Presentation

The maximum flux that was measured is called the peak flux. The concentration of anion at which the flux is half the peak flux is called $K_{1/2}^{peak}$ and is a value taken from the graphs. In contrast, when V_{max} and $K_{1/2}$ are given with standard errors (standard deviation), the values have been generated by an analysis of the data from an entire curve with at least 13 data points using the iterative least squares method of Wilkinson (1961) calculated on a Texas Instruments TI 59 (Houston, Texas). The calculation of these best estimates and their standard deviations assumes that the concentration dependence of anion-stimulated efflux obeys Michaelis-Menten kinetics.
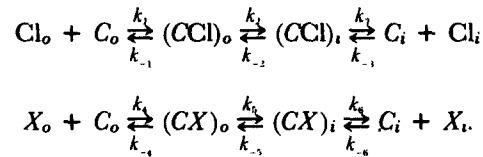
The fluxes in resealed ghosts and intact erythrocytes were calculated in equivalent units if 3.1 × 10^{13} cells had 1 kg dry cell solids and 0.9 kg of hemoglobin.

Theory

In order to distinguish between ping-pong and sequential kinetics, the apparent Michaelis constant ($K_{1/2}$) of the sites facing each compartment and the apparent
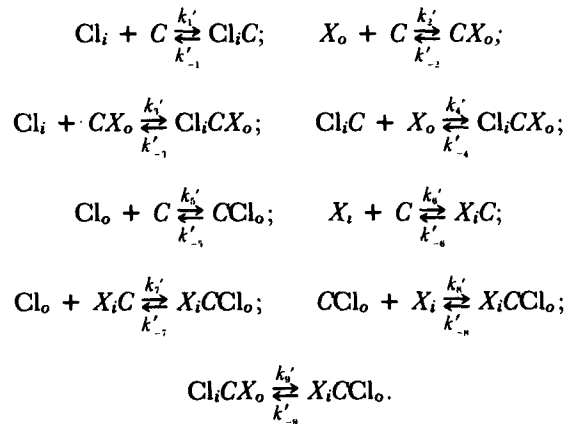
maximum flux (V_{max}) in each direction must be measured. This may be done by varying the anion concentration in one compartment and measuring the initial flux rate at several fixed concentrations of anion in the *trans* compartment (Sachs, 1977). In this paper, we report the initial efflux of chloride-36 or bromide-82 at several concentrations into solutions with different chloride or bromide concentrations. Because the net flux of chloride is negligible during these flux measurements, V_{max}^{influx} (= V_{max}^{efflux}) and $K_{1/2\text{-out}}$ may be determined as a function of Cl_{in}.

A simple ping-pong reaction mechanism for the coupled efflux of Cl_{in} and influx of X_{out} may be described by the following equations where C_o and C_i are the sites at the two membrane surfaces to which the anions bind.

$$Cl_o + C_o \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (CCl)_o \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (CCl)_i \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} C_i + Cl_i$$

$$X_o + C_o \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} (CX)_o \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} (CX)_i \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} C_i + X_i.$$

If $Cl_o = X_i = 0$, the reciprocal of the efflux, $V_{Cl}^{-1} = A + BCl_i^{-1} + DX_o^{-1}$ where A, B, and D are composite constants of several k_i values. $V_{max} = Cl_i(B + ACl_i)^{-1}$; $K_{1/2\text{-out}} = DCl_i(B + ACl_i)^{-1}$. The ratio $V_{max}/K_{1/2\text{-out}} = D^{-1}$ and is independent of Cl_i. Thus, the double reciprocal graph of V_{Cl}^{-1} vs. X_o^{-1} at several fixed Cl_i will be a family of parallel straight lines.

A simple sequential reaction mechanism involves the following reactions (The order of the reactions with the transport sites is ignored for simplicity):

$$Cl_i + C \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} Cl_iC; \qquad X_o + C \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} CX_o;$$

$$Cl_i + CX_o \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} Cl_iCX_o; \qquad Cl_iC + X_o \underset{k'_{-4}}{\overset{k'_4}{\rightleftharpoons}} Cl_iCX_o;$$

$$Cl_o + C \underset{k'_{-5}}{\overset{k'_5}{\rightleftharpoons}} CCl_o; \qquad X_i + C \underset{k'_{-6}}{\overset{k'_6}{\rightleftharpoons}} X_iC;$$

$$Cl_o + X_iC \underset{k'_{-7}}{\overset{k'_7}{\rightleftharpoons}} X_iCCl_o; \qquad CCl_o + X_i \underset{k'_{-8}}{\overset{k'_8}{\rightleftharpoons}} X_iCCl_o;$$

$$Cl_iCX_o \underset{k'_{-9}}{\overset{k'_9}{\rightleftharpoons}} X_iCCl_o.$$

If $Cl_o = X_i = 0$, the reciprocal of the efflux, $V_{Cl}^{-1} = A' + B'Cl_i^{-1} + (D' + E'Cl_i^{-1})(X_o)^{-1}$ where A', B', D', and E' are composite constants of the k_i' values. $V_{max} = Cl_i(B' + A'Cl_i)^{-1}$, $K_{1/2\text{-out}} = (E' + D'Cl_i)(B' + A'Cl_i)^{-1}$. The ratio $V_{max}/K_{1/2\text{-out}} = (D' + E'Cl_i^{-1})^{-1}$ is dependent on Cl_i and increases as Cl_i increases. Thus the double reciprocal graph of V_{Cl}^{-1} vs. X_o^{-1} at several fixed Cl_i values will be a family of converging straight lines; and the ratio of $V_{max}/K_{1/2\text{-out}}$ will be a hyperbolic function of Cl_i.

RESULTS

The data presented will be used in the kinetic analysis of mechanisms of anion exchange. We first measured the concentration dependence of the self-ex-

change fluxes of bromide in order to compare those values with chloride fluxes previously published (Gunn et al., 1973) and to provide a benchmark for the measurements of Br-Cl heteroexchange. We next measured Br-Cl heteroexchange in both directions in order to examine the symmetry or asymmetry of V_{max} in the two directions and the $K^{Br}_{1/2}$ on the two sides of the membrane. Finally, we measured the homoexchange of chloride under conditions which may distinguish the two classes of mechanisms, ping-pong and sequential, and which would demonstrate any asymmetry in $K^{Cl}_{1/2}$. In preliminary experiments we tested the effect of several anions on chloride self-exchange and found that citrate concentrations up to 25 mM had no significant impact at pH 7.8 and 0°C.
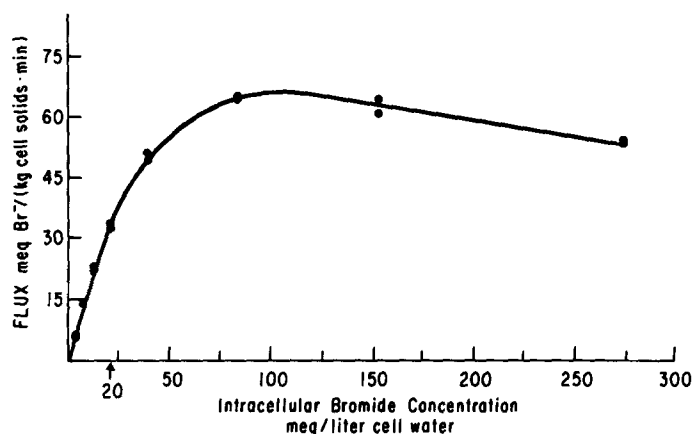
FIGURE 1.   Bromide equilibrium self-exchange flux as a function of intracellular bromide concentration in intact cells in 141 mM Na-acetate and different NH₄Br solutions.

We first measured the equilibrium self-exchange of bromide as a function of cellular bromide concentration (Fig. 1) as previously measured for chloride (Gunn et al., 1973). The extracellular Br concentration was approximately equal to the intracellular concentration and 141 mM acetate was present in the solutions. The flux was (a) a hyperbolic function of bromide at low concentrations; (b) half of peak flux at 19 mM Br; (c) peak flux equal to 65 meq Br/(kg cell solids·min) (or 71 meq/kg hemoglobin·min) at 80–90 mM Br; and (d) inhibited at high bromide concentrations. These four features of Br self-exchange flux were qualitatively similar to those of Cl, except that quantitatively (a) the maximum flux was only 14% of that of Cl; (b) the $K_{1/2}$ was slightly reduced ($K^{Cl\text{-}peak}_{1/2}$ = 26 mM); and (c) the peak flux was observed at a lower Br concentration than with Cl (Gunn et al., 1973).

In order to have parameters of bromide flux which could be compared with subsequent experiments, we also measured the self-exchange of bromide in resealed ghosts in isotonic, isoionic bromide-citrate-sucrose solutions (Fig. 2). The $K^{Br}_{1/2}$ was 7.1 ± 1.5 mM and V_{max} was 172 ± 7 meq/(3.1 × 10^{13} ghosts·

min). The bromide homoexchange was also measured by using duplicate samples of these ghosts placed into 144 mM Br solutions. The efflux as a function of Br_{in} was hyperbolic with $K^{Br}_{1/2-in}$ = 8.1 ± 1.6 mM and V_{max} = 163 ± 7 meq/(3 × 10^{13} ghosts·min). The central point of these results is that the $K^{Br}_{1/2}$ measured under self-exchange conditions is nearly the same as $K^{Br}_{1/2-in}$ measured under homoexchange with changing internal Br concentration.

In a separate experiment (Fig. 3) similarly prepared ghosts with different internal bromide concentrations were effluxed into 0.96, 22, and 147 mM chloride solutions. The $K^{Br}_{1/2-in}$ values were 8.0 ± 1.1, 18.1 ± 1.4, and 17.4 ± 1.4 mM; and V_{max} values were 152 ± 7, 301 ± 7, and 295 ± 7 meq/(3.1
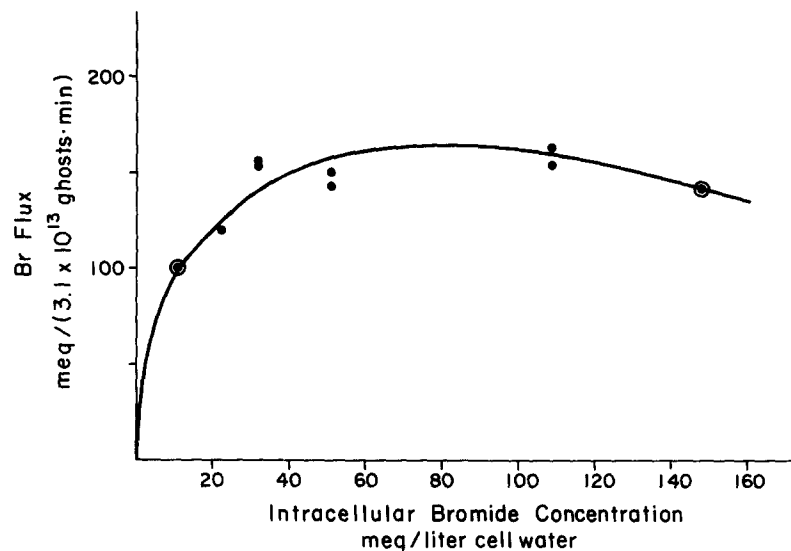
FIGURE 2.   Bromide equilibrium self-exchange flux as a function of intracellular bromide concentration in resealed ghosts in isotonic, isoionic bromide-citrate-sucrose solutions. $K_{1/2}$ = 7.1 ± 1.5 mM. V_{max} = 172 ± 7 meq/(3.1 × 10^{13} ghosts·min). The flux values at the highest Br concentration were excluded from this analysis.

× 10^{13} ghosts·min), respectively. Both $K^{Br}_{1/2-in}$ and V_{max} were increased by increased external chloride concentrations up to 22 mM but not by a further increase to 147 mM. No decline in flux at higher internal bromide concentrations was observed, nor did the highest external chloride concentration diminish V_{max}. The efflux from these same ghosts was measured into solutions with 1.0, 21.9, and 149 mM bromide (Fig. 4). The $K^{Br}_{1/2-in}$ values were 7.7 ± 1.0, 7.6 ± 0.6, and 6.5 ± 0.6 mM; and V_{max} values were 105 ± 5, 134 ± 3, 117 ± 3 meq/(3.1 × 10^{13} ghosts·min), respectively. The $K^{Br}_{1/2-in}$ values were insensitive to external Br concentrations in contrast to the increase with external chloride. The changes in V_{max} were also less striking with external bromide. The decline in flux at higher internal bromide concentrations was

evident at all three external bromide concentrations and is evidence for the presence of a modifier site on the cytoplasmic side of the membrane.

To determine the $K^{Br}_{1/2\text{-out}}$ we measured Cl-Br heteroexchange. The efflux of chloride from cells containing 110 mM Cl/(liter cell water) is shown in Fig. 5 as a function of external Br when ionic and osmotic strength are maintained by substituting citrate/sucrose solutions for Br. The $K^{Br}_{1/2\text{-out}}$ was 0.9 ± 0.3
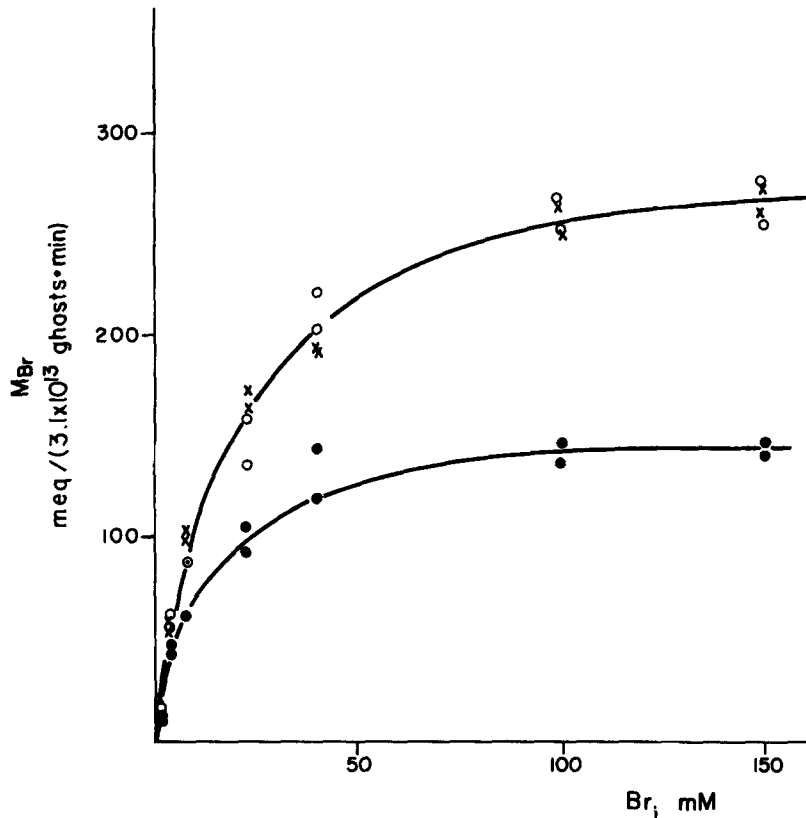

FIGURE 3.   Bromide efflux into-three chloride solutions. Resealed ghosts with different internal bromide concentrations (citrate-sucrose substitution) were loaded with ^{82}Br and effluxed into chloride media. When Cl_o = 0.96 mM, $K^{Br}_{1/2\text{-in}}$ = 8.0 ± 1.1 mM, V_{max} = 152 ± 7; when Cl_o = 22 mM, $K^{Br}_{1/2\text{-in}}$ = 17.2 ± 1.4 mM, V_{max} = 301 ± 7; when Cl_o = 147 mM, $K^{Br}_{1/2\text{-in}}$ = 18.1 ± 1.4 mM, V_{max} = 295 ± 7 meq/(3.1 × 10^{13} ghosts·min).

mM, and V^{influx}_{max} was 360 ± 20 meq/(kg hemoglobin·min). We have previously shown that chloride efflux into solutions with only acetate salts is negligible if scrupulous care is taken to remove HCO_3^- from the system (Brazy and Gunn, 1976). The presence of measureable fluxes at zero external Br in Fig. 5 probably reflects HCO_3^- in the system and net flux accompanying cations which are more permeable at high membrane potentials (Donlon and Roth-

stein, 1969). The spread in the duplicate determinations in the heteroexchange experiments is greater than in the self-exchange experiments in Figs. 1 and 2. This, in part, reflects the net transfer of the two anion species and error inherent in estimating the initial flux rates.
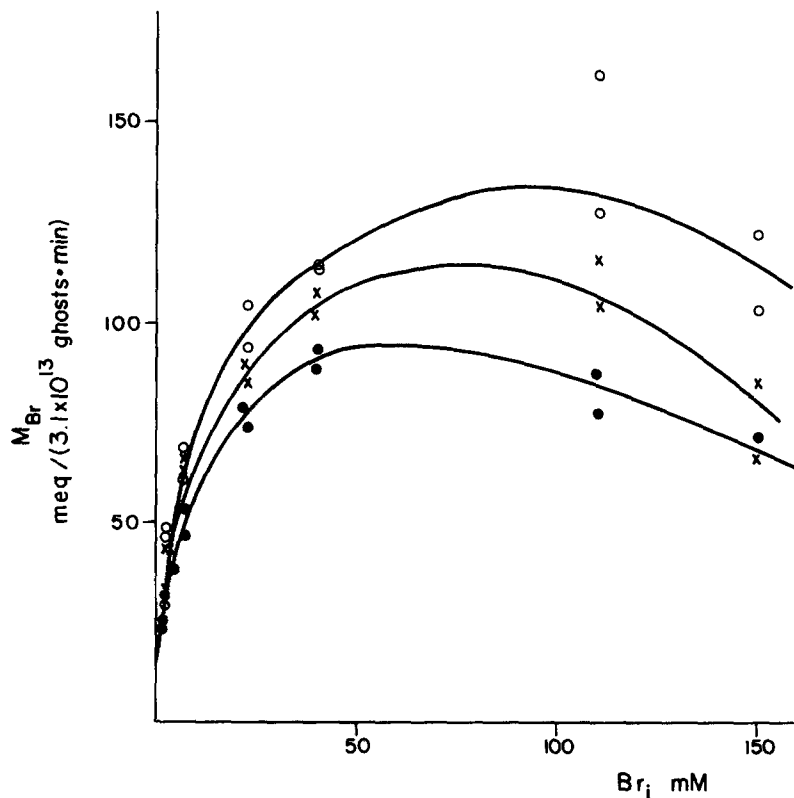

FIGURE 4.   Bromide efflux into three bromide solutions. Duplicate samples of the same ghosts used in Fig. 3 experiments were effluxed into bromide solutions. When Br_o = 1.0 mM, $K^{Br}_{1/2\text{-in}}$ = 7.7 ± 1.1 mM, V_{max} = 105 + 5; when Br_o = 22 mM, $K^{Br}_{1/2\text{-in}}$ = 7.6 ± 0.6 mM, V_{max} = 134 ± 3; when Br_o = 149 mM, $K^{Br}_{1/2\text{-in}}$ = 6.5 ± 0.6 mM, V_{max} = 117 ± 3 meq/(3.1 × 10^{13} ghosts·min). Data from reduced fluxes at higher Br_{in} concentrations were excluded in this analysis. Inhibition by internal Br was prominent at all three concentrations of Br_o indicating an internal modifier site.

Fig. 5 makes several points. First, Br can enter cells in exchange for chloride faster than in exchange for Br. Thus, the maximum influx for Br was greater than the maximum self-exchange flux. Secondly, the $K^{Br}_{1/2}$ value was lower in this experiment, in which the entry of Br was rate-limiting, than in either a self-exchange experiment with intact erythrocytes (Fig. 1) or lower than in a self-exchange experiment with ghosts with citrate substitution (Fig. 2) or when the exit of bromide was rate-limiting (Fig. 3). Thirdly, the flux declined when
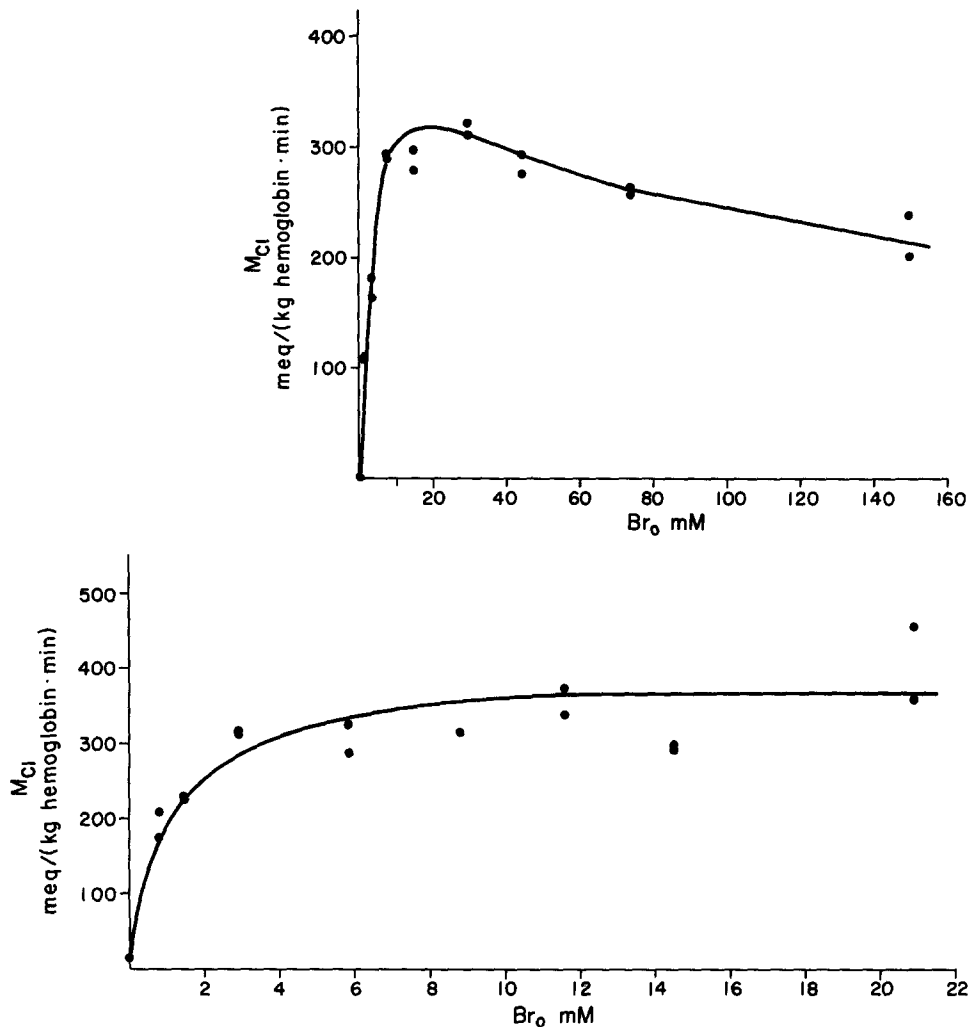
FIGURE 5. Chloride efflux vs. external Br (citrate-sucrose substitution). The ^{36}Cl efflux from cells with an initially normal chloride content (177 meq/kg cell solids) and concentration (109 mmol/liter cell water) and initial water content (1.586 kg/kg cell solids) was measured into solutions of bromide and citrate at constant osmolarity and ionic strength. Using all of the data points V_{max} = 360 ± 20 and $K^{Br}_{1/2\text{-out}}$ = 0.9 ± 0.3. If the data pair at 14.5 mM Br$_o$ is omitted, V_{max} = 406 ± 37 and $K^{Br}_{1/2\text{-out}}$ = 1.6 ± 0.5. In another similar experiment (*inset*), V_{max} = 356 ± 17 and $K^{Br}_{1/2\text{-out}}$ = 3.0 ± 0.5 when initial cell chloride was 213 meq/kg cell solids and 117 mmol/liter cell water and initial water content was 1.796 kg/kg cell solids.

external Br was greater than 22 mM (*inset* of Fig. 5). The activation of the flux shown in Fig. 5 reflects, in part, the binding of Br at the outer surface of the cells and is quantified by the parameter $K^{Br}_{1/2\text{-out}}$.

As suggested earlier, the efflux steps appear to be rate-limiting in a self-exchange experiment in which the same anion species is entering and leaving. In Fig. 5, however, bromide entry steps appear to be rate-limiting since chloride can normally exit three to four times faster. The $K^{Br}_{1/2\text{-out}}$ values (0.9–3.0 mM) are significantly lower than the $K^{Br}_{1/2\text{-in}}$ values (8.0–18.1 mM) shown in Fig. 3. The $K^{Br}_{1/2}$ value (7.1 mM measured in self-exchange experiments [Fig. 2]) is more nearly equal to the $K^{Br}_{1/2\text{-in}}$ values. If one considers that in the self-exchange experiment significant inhibition at the external modifier site is evident above 60 mM Br_o (Fig. 5, *inset*) which causes an underestimation of V_{max} and $K^{Br}_{1/2}$, then the true $K^{Br}_{1/2}$ of self -exchange is probably within the observed range of $K^{Br}_{1/2\text{-in}}$ values. These data show that the anion exchange system is kinetically asymmetric with different $K_{1/2}$ values on the two sides of the membrane. Whether the V_{max} values are asymmetric cannot be concluded from the observations that the maximum self-exchange flux is less than the maximum influx rate of bromide.
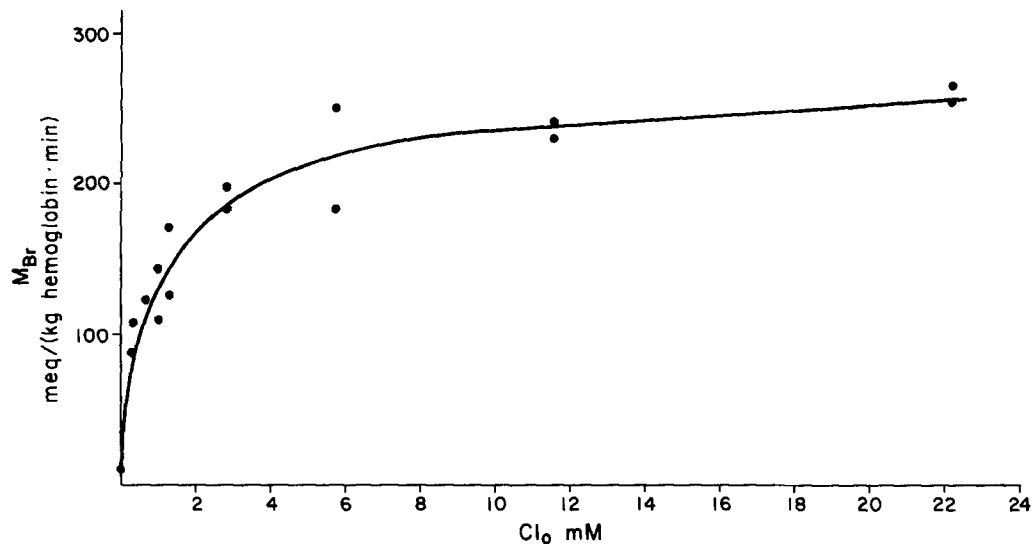

FIGURE 6.   Bromide efflux into chloride-citrate solutions. Cells preequilibrated with 151.5 mM Br and ^{82}Br were injected into isotonic, isoionic media with different chloride concentrations to determine the flux. Initial cellular bromide was 118 mM or 178 meq/kg cell solids and water content was 1.55 kg/kg cell solids. Efflux as meq/(kg hemoglobin·min) was stimulated by additions of external chloride along a hyperbolic curve. The estimates of $K^{Cl}_{1/2\text{-out}}$ and $V^{Br\text{-efflux}}_{max}$ are given in the text.

In order to examine the difference between V^{influx}_{max} and $V^{self\text{-exchange}}_{max}$ values, we measured the ^{82}Br efflux as a function of external chloride to determine the maximum rate of bromide efflux, V^{efflux}_{max}, when the return step (chloride entry) was not rate limiting (Fig. 6). The stimulation of Br efflux by chloride was hyperbolic; V^{efflux}_{max} was 324 ± 15 meq/(kg hemoglobin·min). When

compared with Fig. 5, these findings demonstrate that there is little difference in the maximum entry and the maximum exit flux when Br is exchanged for a more rapidly transported anion, Cl. However, the maximum rate of self-exchange was much slower than either the maximum rate of entry or exit. The internal consistency between experiments can be appreciated by comparing values in Fig. 6 with those in Fig. 3. In Fig. 6, cells with 118 mM internal bromide were effluxed into solutions with different chloride concentrations including 0.96 and 22 mM, the same solutions as in Fig. 3. The flux at 0.96 mM was about 125 meq/$(3.1 \times 10^{13}$ ghosts·min) in Fig. 6 and 125 meq/(kg cell solids·min) in Fig. 3 using 0.9 kg hemoglobin per kg cell solids to make the units nearly the same. Similarly, the fluxes at 22 mM were about 250 meq/$(3.1 \times 10^{13}$ ghosts·min) and 270 meq/(kg cell solids·min). These data support an underlying assumption in this study that resealed ghosts and intact erythrocytes do not have significantly different anion transport characteristics when internal ion compositions and ion gradients are matched in the two preparations.

In Fig. 6, $K^{Cl}_{1/2\text{-out}}$ (= 0.84 ± 0.14 mM) is much smaller than the $K^{peak}_{1/2}$ of chloride self-exchange, which ranges from 26 mM (Gunn et al., 1973) to 44 mM (Cass and Dalmark, 1973; Dalmark, 1975) and is much smaller than $K^{self-exchange}_{1/2}$ = 65 ± 5 mM from statistical analysis (Brazy and Gunn, 1976). This suggested to us that like bromide, the higher $K_{1/2}$ of chloride self-exchange compared to $K^{Cl}_{1/2\text{-out}}$ may be a reflection of a high $K^{Cl}_{1/2\text{-in}}$. Therefore, we measured the homoexchange of chloride between resealed ghosts with four different Cl concentrations and solutions with 2 and 23 mM Cl_{out}. In the first case $K^{Cl}_{1/2\text{-in}}$ equalled 27 ± 7 mM and in the second case $K^{Cl}_{1/2\text{-in}}$ equalled 64 ± 30 mM Cl. In both cases $K^{Cl}_{1/2\text{-in}}$ for inward facing sites was much greater than for the outward-facing sites measured in the experiments shown in Fig. 6. Altogether, these experiments show that anion transport is asymmetric for both chloride and bromide.

Activation of Chloride Efflux by External Chloride

In experiments using isoionic-isotonic replacement of chloride with citrate/sucrose in the external solutions (Fig. 7) the chloride efflux increased rapidly with increasing external chloride. The $K^{Cl}_{1/2\text{-out}}$ was 2.2 ± 0.3 mM, the peak flux was 855 meq/(kg cell solid·min) at only 21.2 mM Cl_o, and the calculated V_{max} was 941 ± 30 meq/(kg cell solids·min). The flux declined only slightly when the external chloride was increased to 160 mM (not shown).

Dependence of External Activation on Internal Anion Concentration

We next measured the stimulation by external chloride of chloride efflux from cells with different fixed internal chloride concentrations. In Fig. 8, the stimulation of chloride efflux from erythrocyte ghosts containing 13.9 mM Cl complemented with citrate-sucrose was measured into a series of external Cl concentrations with isoionic and isotonic substitution with citrate-sucrose. The maximal flux was 308 ± 12 meq/$(3.1 \times 10^{13}$ ghosts·min) and the $K^{Cl}_{1/2\text{-out}}$ was 1.25 ± 0.14 mM. Thus both V_{max} and $K^{Cl}_{1/2\text{-out}}$ were reduced by decreasing intracellular chloride from 110 mM (Fig. 7) to 13.9 mM (Fig. 8).
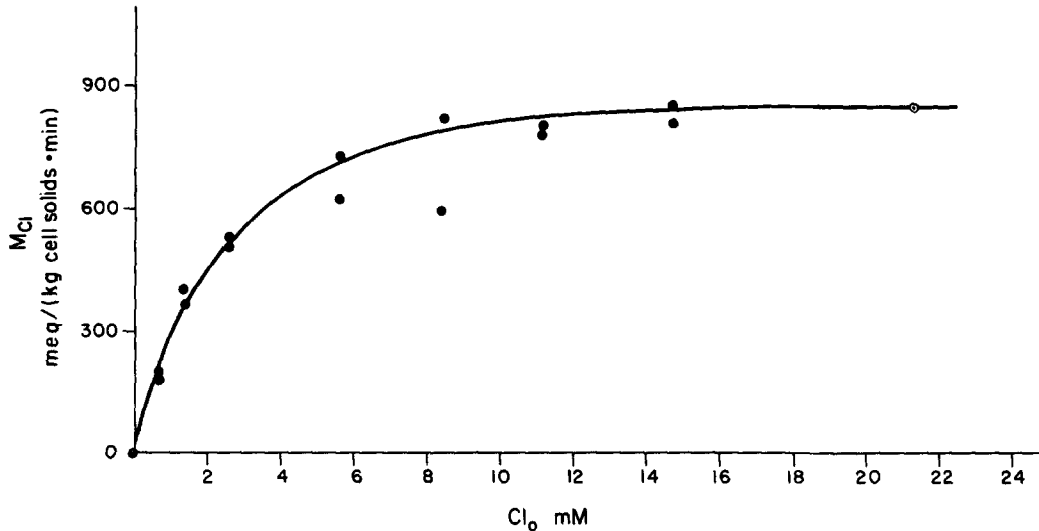
FIGURE 7.   Activation of chloride-36 efflux by external chloride (citrate-sucrose substitution). $K^{Cl}_{1/2\text{-out}}$ = 2.2 ± 0.3 mM. V_{max} = 941 ± 30 meq/(kg cell solids· min). Cl_{in} = 110 mmol/liter cell water.
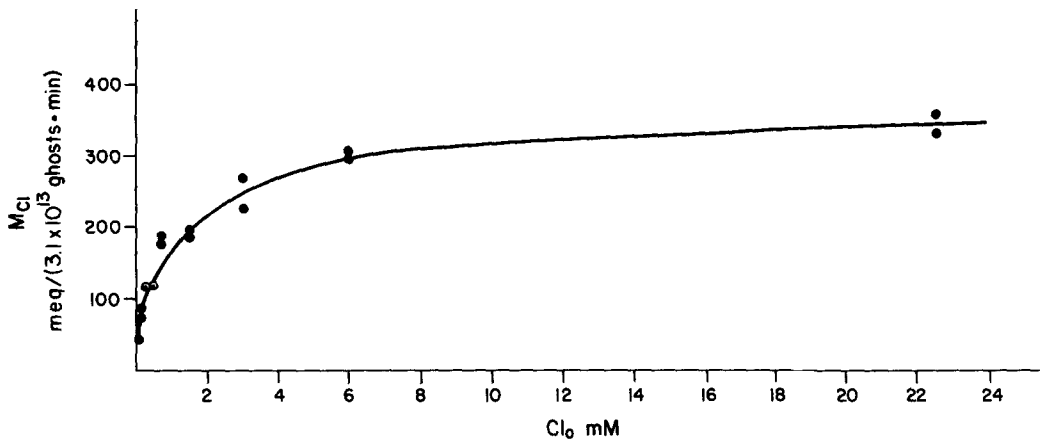

FIGURE 8.   Activation of chloride-36 efflux from ghosts with low (13.9 mM) intracellular chloride complemented with citrate-sucrose, by external chloride solutions (citrate-sucrose substitution). $K^{Cl}_{1/2\text{-out}}$ = 1.25 ± 0.14 mM; V_{max} = 308 ± 12 meq/(3.1 × 10^{13} ghosts·min).

A series of similar experiments were performed in which external chloride-stimulated chloride efflux was measured from ghosts and intact cells with different internal cell chloride concentrations. The calculated parameters for each series are given in Table I. The equations describing a ping-pong mechanism (see Theory) predict parallel lines on a double-reciprocal graph with a constant ratio of $K_{1/2}$ and V_{max}.

The Effect of Membrane Potential on $K^{Cl}_{1/2\text{-out}}$

Erythrocytes placed in low chloride solutions rapidly establish a membrane potential, inside positive, close to the chloride equilibrium potential, E_{Cl}. To ascertain whether the very low $K^{Cl}_{1/2\text{-out}}$ which we measured was caused by a shift in membrane potential, we measured chloride efflux into KCl-K_3-citrate-sucrose solutions in the presence of 1% ethanol or 1% ethanol and 10 μM valinomycin (Fig. 9). This concentration of valinomycin is sufficient to make chloride conductance rate-limiting for net KCl efflux (Hunter, 1977) and the membrane potential approximately equal to $E_K(= -14$ mV) which was calculated from the measured K concentrations in the cell water and medium. In the absence of valinomycin, $V_m \simeq 113$ mV if $P_{Cl}/P_K = 100$ (calculated from the Goldman equation), and $E_{Cl} = +190$ mV in the lowest external

TABLE I

PARAMETERS OF EXTERNAL CHLORIDE STIMULATION OF
CHLORIDE EFFLUX FROM RESEALED GHOSTS AND INTACT
CELLS WITH DIFFERENT INTERNAL CHLORIDE
CONCENTRATIONS

Cl_{in}	$K^{Cl}_{1/2\text{-out}}$	V^*_{max}	$V_{max}/K^{Cl}_{1/2\text{-out}}$
mM	*mM*		
5.5	0.47±0.10	123±9*	260±60
13.9	1.25±0.14	308±12*	250±30
19.0	0.86±0.12	343±17*	400±60
47.7	1.25±0.23	366±22*	290±60
51.5	1.19±0.15	618±28*	520±70
51.8	1.58±0.29	893±68*	565±110
90.0	1.77±0.20	883±39*	500±60
108.0	2.3±0.6	952±40‡	415±110
110.0	2.2±0.3	941±30‡	430±60
133.0§	3.2±0.3	1002±30‡	315±30
152.0§	3.0±0.4	1053±40‡	350±50

* meq/(3.1×10^{13} ghosts·min).

‡ meq/(kg cell solids·min).

§ Cl_{in} was increased by increasing external osmolarity with sucrose.

chloride solution ($Cl_o \simeq 0.03$ mM); and $V_m = 46$ mV and $E_{Cl} = +47$ mV when $Cl_{out} = 14$ mM. The $K^{Cl}_{1/2\text{-out}}$ and the V_{max} were only slightly decreased by valinomycin relative to the control values (with ethanol), which in turn were reduced relative to experiments without ethanol. We conclude that the systematic changes in the membrane potential in the range of chloride concentration ratios we have studied have no substantial effect on the measured fluxes or the calculated parameters in Figs. 3–8. Further experiments are needed to determine if there is any dependence of the exchange flux on the membrane potential uncomplicated by ionophores, solvent, and secondary pH gradients.

Temperature Dependence of Bromide Influx

The temperature dependence of bromide (22 mM) stimulated chloride efflux was measured (Fig. 10). The apparent activation energy for bromide influx was 155 ± 21 kJ/mol (37 ± 5 kcal/mol) which is not different from that for bromide self-exchange (Dalmark and Wieth, 1972). This result will be used in the discussion to exclude one possible explanation for the sharp change in the temperature dependence of bromide self-exchange at $25°C$ (Brahm, 1977).

Acetate Substituion for Chloride

In preliminary experiments we substituted acetate for chloride, one for one, in order to vary internal or external chloride concentrations (Gunn and Fröhlich, 1978). These experiments confirm the findings presented here. Qualitatively $K_{1/2\text{-out}}$ was less than $K_{1/2}$ of self-exchange or $K_{1/2\text{-in}}$, and the mechanism followed ping-pong kinetics. The quantitative interpretation was, however, hampered by the systematic alterations in the concentration of acetate, a noncompetitive inhibitor of anion self-exchange.
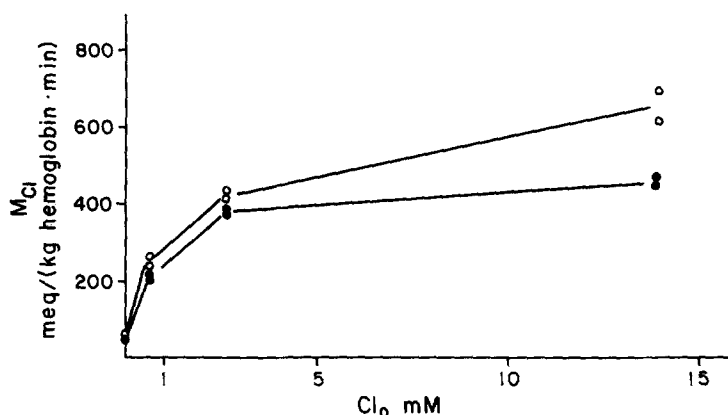

FIGURE 9. External chloride stimulated chloride efflux into 75 mM K^+ solutions with 1% ethanol in the absence (O) and presence (●) of $10\,\mu M$ valinomycin. In the absence of valinomycin, $K^{Cl}_{1/2\text{-out}} = 1.8 \pm 0.4$, $V_{max} = 735 \pm 45$; in the presence of valinomycin, $K^{Cl}_{1/2\text{-out}} = 0.9 \pm 0.1$, $V_{max} = 490 \pm 10$. Initially, $Cl_{in} = 106$ mM and $K_{in} = 140$ mM. $E_K = -14$ mV at all external chloride concentrations. $E_{Cl} = +190$ mV, $+118$ mV, $+85$ mV, $+47$ mV from lower to higher external chloride concentrations.

DISCUSSION

These experiments demonstrate that (a) the erythrocyte anion transport mechanism is asymmetric with respect to the rapidly transported anions chloride and bromide; (b) that the exchange is probably mediated by a mechanism with ping-pong kinetics which alternately forms binary complexes between the membrane protein and anions on the two sides of the membrane; and (c) that modifier sites are both on the outside and on the cytoplasmic side of the cell membrane.

Asymmetry

The erythrocyte membrane has an asymmetric distribution of phospholipids between the two lipid monolayers (Zwaal et al., 1975). The disposition of the intrinsic membrane proteins which span the lipid bilayer (some of which probably mediate transport) is also asymmetric (Steck, 1974). These proteins are glycosylated only on the external surface and have differential reactivities
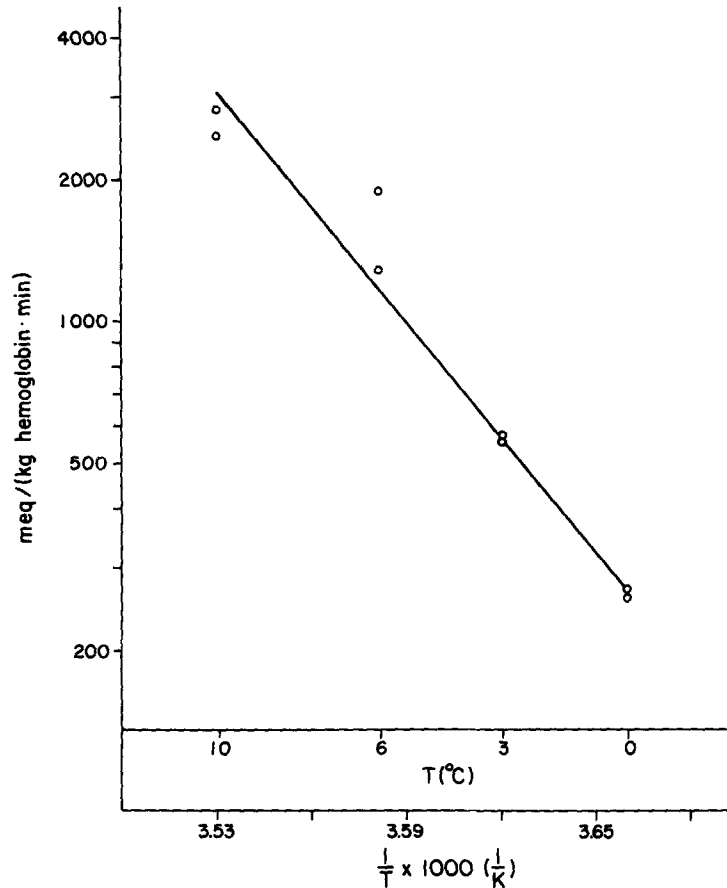

FIGURE 10.   Temperature dependence of chloride efflux into 22 mM bromide complemented with citrate-sucrose. The Arrhenius activation energy (E_a = 37 ± 5 kcal/mol) is for the entry of bromide which is rate limiting in these heteroexchange fluxes.

to modifying reagents added to the two sides. Anion transport inhibitors such as phlorizin (Schnell, 1973; Kaplan et al., 1976), persantin (Rice and Steck, 1977), and the stilbenes (SITS and DIDS) (Kaplan et al., 1976) act differentially on the two sides. But these experiments which show asymmetries in inhibition may only reflect asymmetry of the protein in the membrane and do not address the question of asymmetry of the transport mechanism. Up until

now the functional asymmetry of the transport mechanism remained to be established.

Schnell et al. (1977, 1978) reported that chloride transport is asymmetric and $K_{1/2}$ for chloride is lower on the inside (21 mM) than on the outside (53 mM) and that there is also asymmetry of sulfate inhibition of chloride transport. This $K_{1/2\text{-out}}^{Cl}$ value is the same as reported in self-exchange experiments (Brazy and Gunn, 1976; Dalmark, 1976) which measure an overall $K_{1/2}^{Cl}$ for entry and exit together. These $K_{1/2}$ values on the two sides do not agree with those presented in this paper. We found that both $K_{1/2}^{Br}$ and $K_{1/2}^{Cl}$ are much lower on the outside than on the inside and are 1–3 mM. Schnell et al. used resealed ghosts made in double isotonic solutions with 224 mM K_3 citrate to substitute for 330 mM KCl in order to vary external or internal chloride. At first we believed that the greater ionic strength and variable ionic strength in these experiments might explain the discrepancy, but we have performed a similar series of experiments in our laboratory and found $K_{1/2\text{-out}}^{Cl} = 3$ mM, the same value as reported here in isoionic isotonic solutions. The difference in ionic strength between 300 and 150 mM does not appear to affect the $K_{1/2\text{-out}}^{Cl}$ that is observed.

The $K_{1/2}$ of chloride transport is about 3 mM on the outside (Figs. 6, 7, and 8) and is about 60 mM on the inside.[1] The sum of these two $K_{1/2}$ values for chloride is in good agreement with $K_{1/2}$ for self-exchange of 65 mM (Brazy and Gunn, 1976); and if the mechanism has ping-pong kinetics, this agreement can be shown theoretically.[2] $K_{1/2}$ for bromide, another transported halide, is 1–3 mM on the outside (Fig. 3) which is smaller than both the $K_{1/2}$ (7–19 mM) for bromide self-exchange (Figs. 1 and 2) and $K_{1/2\text{-in}}^{Br}$ (6–8 mM; Fig. 4).

This inequality in $K_{1/2}$ values for the transport sites facing the internal and external solutions may arise from one or a combination of several factors. In a simple ping-pong mechanism the $K_{1/2}^{-1}$ values approximate the binding affinity constant for the ion-carrier complex if the binding of anions to the transport sites is rapid compared to the transport step. However, the present and previously published data do not permit us to distinguish between possible rate-limiting steps in the transport reactions (Passow and Wood, 1974). Another factor which may influence the $K_{1/2}$ values differently on the two surfaces is the surface charge. It is interesting to note that the charge asymmetry of the phospholipids (more negative phospholipids on the inside) is consistent with the greater $K_{1/2}$ for transported anions on the inside. The dissection of these possible factors will require a further series of experiments directed specifically at these questions.

Molecular Mechanism: Ping-Pong vs. Sequential

The data we have obtained both in sucrose-citrate solutions and in acetate solutions indicate that the molecular mechanism has ping-pong kinetics. A

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[1] This value of internal $K_{1/2}$ for chloride may be independently estimated from previously published self-exchange experiments and some recent measurements above pH 8. If we assume that the decline in flux at high pH values is only due to desaturation of the transport system as intracellular chloride decreases (Funder and Wieth, 1976), we calculate that $K_{1/2\text{-in}}^{Cl} = 50 \pm 15$ mM would be needed to fit the observed decline in fluxes.

[2] Fröhlich, O., and R. B. Gunn. Manuscript in preparation.

critical difference in the two classes of possible mechanisms is the dependence of $V_{max}/K_{1/2\text{-}cis}$ on the *trans* anion concentration. This ratio is independent of the *trans* anion concentration in all simple ping-pong mechanisms but is a hyperbolic function of *trans* anion concentration in all simple sequential mechanisms. Shown in Fig. 11 are the values of $V^{Cl}_{max}/K^{Cl}_{1/2\text{-}out}$ as a function of intracellular chloride concentration, Cl_{in}. The wide standard deviations reflect experimental errors in both $K_{1/2}$ and V_{max} and reflect very small errors in the primary data. Our primary data, which is the time-course of radioactivity in the extracellular phase, were used to calculate fluxes which were secondarily analyzed as a hyperbolic function of the initial extracellular chloride concentration, to give $K_{1/2\text{-}out}$ and $V_{max\text{-}out}$ which, in turn, were analyzed as a function
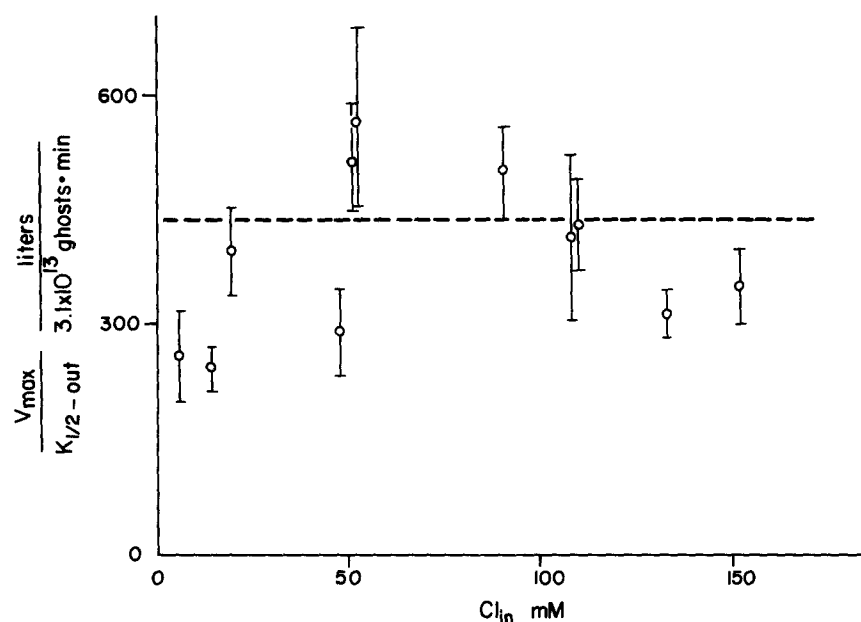

FIGURE 11. Dependence of $V_{max}/K^{Cl}_{1/2\text{-}out}$ on Cl_i. The data is from Table I. The dotted line is the best least-squares line from regression analysis using the squares of the standard deviation as weighting factors. The equation of the line is $V_{max}/K_{1/2\text{-}out} = (437 \pm 69) + (0.02 \pm 0.8) Cl_i$.

of internal chloride concentration. It is, therefore, a ratio of the results of this third level of analysis which is graphed in Fig. 11. Increasing scatter at each level of analysis is expected for nonperfect primary data. The weighted least squares analysis for a straight line is shown and does not have a slope significantly different from zero. This data analysis is consistent with the conclusion that the mechanism does have ping-pong kinetics.

Alternately, one can argue that the data in Fig. 11 follow a hyperbolic curve but that data have only been obtained after the $V_{max}/K_{1/2}$ values have saturated. If this interpretation is correct the then half-maximal value of $V_{max}/K_{1/2\text{-}out}$ would have to be below 1 mM Cl_i. In terms of the simple sequential

model given above (see Theory) (E') $\ll$ 0.0022 mmol·kg solids·min/liter^2. This small value of E' would not permit us to distinguish between the underlying mechanisms by this analysis. Nor do the present data provide a basis for excluding such a small value of E'. However, a sequential model with a small E' can be shown to have a slow dissociation rate for one of its two transport sites. This transport site will not always unload the anion on that site on each exchange cycle. The anion at this site behaves as a cofactor which is translocated back and forth but is rarely exchanged with other anions in the solution. The second site behaves as the single site in a ping-pong mechanism both translocating and exchanging. In this manner, an underlying sequential mechanism may degenerate to ping-pong kinetics like those demonstrated in Fig. 11. We maintain that the mechanism behaves as if it followed ping-pong kinetics when Cl_i is greater than 1 mM at pH 7.8 and 0°C.

The values of V_{max} in Table I are best fit to a hyperbolic function of Cl_i by V_{max}^{max} = 1,450 ± 170 meq(kg cell solids·min) at pH 7.8 and 0°C. The half V_{max}^{max} is reached when Cl_i = 57 ± 13 mM. In a ping-pong model the Cl_i concentration at half V_{max}^{max} equals $K_{1/2\text{-in}}^{Cl\text{-max}}$. The best fit of a hyperbolic curve to the data values $K_{1/2\text{-out}}^{Cl}$ vs. Cl_i gives $K_{1/2\text{-out}}^{Cl\text{-max}}$ = 3.9 ± 0.6 mM and the Cl_i at which half of this maximum is obtained is 65 ± 18 mM. For a mechanism with ping-pong kinetics this value is also $K_{1/2\text{-in}}^{Cl\text{-max}}$. There is agreement between these two estimates of $K_{1/2\text{-in}}^{Cl}$, the 65 ± 5 mM value calculated from self-exchange experiments at high pH[1] and the 64 ± 30 mM value measured in ghosts in 23 mM Cl_o. This agreement supports the conclusion that the molecular mechanism has ping-pong kinetics and therefore, functions by forming a complex with one anion at a time on alternate sides of the membrane with intervening transport steps.

The stimulation of halide efflux at even very low concentrations (0.5–10 mM) of external halides is hyperbolic. Previous measurements of self-exchange were performed only at concentrations greater than 10 mM Cl (Gunn et al., 1973; Dalmark, 1976; Brahm, 1977) which did not permit one to evaluate whether there was a sigmoid dependence at very low chloride concentrations. The absence of any sigmoid dependence is evidence that cotransport of two chlorides, (on perhaps the protonated mechanism which handles sulfate (Gunn, 1972, 1973; Jennings, 1976), is negligible compared with the transport of single chloride ions.

Lambert and Lowe (1978) reported the concentrations of external anions, which inhibit half of the chloride flux, $K_{I\text{-out}}^{HCO_3}$, equals 0.39 mM; $K_{I\text{-out}}^{F}$ = 38 mM; and $K_{I\text{-out}}^{Br}$ = 6.5 mM at 10°C and pH 7.4. This latter value is different from $K_{1/2\text{-out}}^{Br}$ $\approx$ 1 mM at 0°C and pH 7.8 reported here. In preliminary experiments we have found that $K_{1/2\text{-out}}$ in citrate media increased as expected (Gunn, 1972) at lower pH values sufficiently to explain this difference. We found a value of $K_{1/2\text{-out}}^{F}$ = 6.8 ± 0.6 mM (pH 7.8 at 0°C), which also is sixfold lower than their value at pH 7.4.

Modifier Sites

Dalmark (1976) has characterized the inhibition of chloride self-exchange by other halides and has shown competitive and noncompetitive inhibition. The

sequence of anions for increasing noncompetitive inhibition is the same as the sequence for competitive inhibition, and the reduction of chloride self-exchange at high chloride concentration can be explained most easily by assuming chloride is a noncompetitive inhibitor of its own flux through its reaction with a modifier site. The modifier site is evidenced by self-inhibition at high anion concentrations.

As shown in Fig. 5 *inset*, a modifier site is present on the outside surface since only the external bromide was varied. Similarly, a modifier site is detected on the inside surface when only internal bromide is varied (Fig. 4). Acetate and furosemide are also modifiers in the sense that they reduce self-inhibition (Brazy and Gunn, 1976). Comparison of the data from Fig. 8 with data (not shown) from measurements of the efflux of chloride into chloride-citrate solutions from red cells with 12.7 mM Cl and ~100 mM acetate inside indicate that acetate is an inhibitor of anion transport at an internal (modifier) site. A similar comparison of Fig. 7 with data on chloride efflux into acetate-chloride solutions indicates that acetate is an inhibitor of anion transport at an external (modifier) site. Therefore, there appear to be modifier sites on both the inside and outside of the erythrocyte membrane.

Differences between chloride and bromide affinity or effectiveness at the modifier sites can be seen by comparing Figs. 3 and 4. External chloride at 147 mM had no inhibition on bromide-chloride exchange compared with 22 mM Cl_o. But external bromide at 149 mM decreased Br-Br exchange compared with 21.9 mM at all internal bromide concentrations. The potency of external Br compared with external Cl can also be judged from Fig. 5 in which bromide self-inhibition is prominent. In addition, self-inhibition at internal sites is evident with bromide (Fig. 4) but not chloride at internal concentrations below 160 mM.

Schnell (1977) has suggested that self-inhibition is due to occupancy of sites on the *trans* side of a diffusion pathway. The blockade of the transport pathway would be greater at greater *trans* ion concentrations of halides. This was not found in experiments with Br_o >22 mM in Fig. 5. Internal sites along the bromide influx pathway are probably complexed to chloride to the same or lesser extent (Cl_i is constant) and to bromide to a lesser extent (smaller influx and smaller Br_{in} at any given time during the initial exchange) when Br_o concentrations were increased to self-inhibiting concentrations (Br_o >22 mM). Therefore, increased anion occupancy of a site more inside than the transport barrier cannot account for the inhibition of bromide influx. We prefer Dalmark's description (1976) of the modifier site with the addition that sites are present on both sides of the membrane.

Membrane Potential Effects on $K_{1/2}$

The evaluation of $K^{Cl}_{1/2\text{-out}}$ was made from a series of experiments in which the chloride concentration ratio (Cl_{in}/Cl_{out}) was not constant. Since the conductance of the erythrocyte membrane to chloride is much greater than that to any other ion present in the system, the membrane potential is weighted toward the equilibrium potential for chloride, E_{Cl}. Thus each experiment in which cells with a given internal chloride concentration are added to isotonic

solutions with a different external chloride concentration will be performed at a different membrane potential. Although the flux of chloride through the conductance mechanism is 10^4-10^5 times smaller than the exchange fluxes reported here, the membrane potential resulting from this conductance flux may have a systematic influence on the exchange flux and the evaluation of $K_{1/2\text{-out}}^{Cl}$. Fig. 9 shows that shifting the membrane potential by increasing the permeability to K^+ did not greatly alter the $K_{1/2\text{-out}}^{Cl}$ compared to that under control conditions. However, it may be significant that high concentrations of valinomycin (10 μM) did reduce the flux in comparison with ethanol in which it was dissolved. That the overall anion exchange is electrically silent has been established (Hunter, 1971, 1977; Knauf et al., 1977), but whether the individual partial reactions of the coupled cyclic mechanism are potential dependent is not established. These studies show that the concentration dependence of the rate-limiting steps in homoexchange which dominate the observed $K_{1/2\text{-out}}$ are not significantly dependent on the membrane potential. These studies do not exclude individual reactions from being membrane potential dependent (Knauf and Marchant, 1977).

Temperature Dependence of Br Influx

The nonlinearity of the Arrhenius diagram for the self-exchange fluxes of the rapid anions chloride, bromide (Brahm, 1977), and bicarbonate (Chow et al., 1976) is an unexplained phenomenon. One possible explanation is that at low temperatures, the efflux was rate-limiting for self-exchange and had the high activation energy of ~30 kcal/mol while at higher temperatures the influx became rate-limiting with a lower activation energy of ~20 kcal/mol. The present observation that E_a for bromide influx is 37 kcal/mol and is not 20 kcal/mol between 0°C and 10°C effectively rules out this explanation for the nonlinearity of the Arrhenius diagram.

Conclusion

This study demonstrates that the outside chloride transport site is half-saturated (~3 mM) at a much lower concentration than the internal chloride transport site (~50 mM). Thus anion transport is asymmetric. Since the translocation rates are about equal in the two directions across the membrane, the asymmetry in saturation determines that the self-exchange flux is limited by the efflux mechanism. This study also demonstrates that the dependence of the $K_{1/2\text{-out}}^{Cl}$ and V_{max} on internal chloride concentration at pH 7.8 and 0°C is like that predicted by a ping-pong mechanism with a single reciprocating transport site. This is in contrast to a sequential loading of anions on both sides with a simultaneous exchange involving two transport sites. We have also shown that modifier sites exist on both sides of the membrane.

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