Characteristics of Chloride Transport in Human Red Blood Cells

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ABSTRACT   The efflux of chloride-36 from human erythrocytes under steady-
state conditions is a saturable process that is competitively inhibited by bicar-
bonate and noncompetitively inhibited by acetate. This chloride self-exchange
flux is reversibly dependent on the pH of the medium between 5.7 and 9.6
with a maximum flux at pH 7.8. The increase in chloride flux between pH
5.7 and 7.8 is inexplicable by the fixed charge hypothesis. The interpretations
are made that chloride transport in human erythrocytes is carrier mediated,
that bicarbonate utilizes the same transport mechanism, and that the mecha-
nism can be titrated with hydrogen ions into less functional forms for chloride
transport.

INTRODUCTION

The primary physiological function of red blood cells is the transport of
oxygen to the tissues followed by removal of CO_2. The main fraction of the
CO_2 (approximately 85%) is carried to the lungs in the form of bicarbonate
ions, while most of the remainder is carried as carbamino-compounds (Rossi-
Bernardi et al., 1972). CO_2 formed by metabolism diffuses into the red cells
and is rapidly hydrated in the presence of intracellular carbonic anhydrase.
Two-thirds of the resulting bicarbonate ions immediately leave the red cells
in exchange for chloride as first described by Nasse (1878) and later by
Hamburger (1891). In a study of monovalent anion exchange Tosteson
(1959) gave some indication that an exchange diffusion mechanism may
be involved. Until recently it has not been clear whether these movements of
Cl^- and HCO_3^- occur by the diffusion of free ions or whether a chemical
interaction with components of the membrane is involved. The data pre-
sented in this paper indicate that a specific interaction of these anions with a
component of the erythrocyte membrane is involved in their transport and in
the exchange process which is a part of physiologic gas transport.

In this work we measured the exchange of chloride isotopes in human

erythrocytes at $0°C$ as a function of pH, chloride concentration, bicarbonate concentration, and acetate concentration in order to clarify the mechanism of chloride self-exchange in these cells.

MATERIALS, METHODS, AND CALCULATIONS

Solutions

Media with the following millimolar concentrations were prepared from reagent grade chemicals. Medium A: 145 NaCl, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 5 D-glucose, 27 glycylglycine (total $Cl^- = 150$). Medium B: 120 NaCl, 1.1 K_2HPO_4, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 22 $NaHCO_3$, 1.5 KCl, 5 D-glucose (total $Cl^- = 126.5$; $HCO_3^- = 22$ meq/liter). Medium C: 141 Na-acetate, 1.5 Ca(acetate)$_2$, 1.0 Mg(acetate)$_2$, 27 glycylglycine, 5 D-glucose, (Total $Cl^- = 0$).

At $0°C$ media A, C, and media made from these by the addition of NH_4Cl, or NH_4-acetate, were titrated to pH 7.4 from ~6.3 with NaOH. Medium B and other media containing bicarbonate were titrated with CO_2 to the desired pH.

Freshly drawn, heparinized human blood was centrifuged and washed three times in medium A at room temperature. The buffy coat and plasma were removed after the first centrifugation. In the different classes of experiments the cells were prepared differently, but in each, the cells were finally washed and resuspended to a hematocrit of ~30% in a medium in which the cells were in steady state with respect to chloride, water, and hydrogen ions at $0°C$; $Na^{36}Cl$ was added to this resuspension and allowed time to distribute between the water phases. The cells, packed by centrifugation in nylon tubes, were used to determine the efflux rate coefficient, cell water fraction, and chloride content. The details of the different preparative procedures are given below.

Methods for Cell Preparation

TITRATION OF CELLS IN A GLYCYLGLYCINE BUFFER   After the initial washing, cells from 4 ml of whole blood were resuspended to 20 ml and placed in a thermostated chamber. The cells were then titrated to the desired pH at $0°C$ using either 300 mM HCl or 300 mM NaOH. Because the buffer capacity of human red cells is approximately 50 meq/(liter of cells·pH U) (Harris and Maizels, 1952) and the pH equilibration proceeds more slowly at $0°C$, approximately two-thirds of the titration was carried out at 15–20°C. Then the suspension was cooled to $0°C$ and titration continued to the desired end point. The titrated suspension was then centrifuged and the cells washed three times at $0°C$ with a portion of medium A which had been titrated at $0°C$ to the same end point (±0.1 pH) as the cell suspension and which would be subsequently used for the efflux media.

TITRATION OF CELLS IN A BICARBONATE BUFFER   These methods are more fully elaborated by Funder and Wieth (1967 a). Briefly, cells were washed three times in medium B, cooled to $0°C$, and titrated with CO_2 to the desired pH (±0.02), using a Radiometer titrator (London Co., Cleveland, Ohio) to control the flow of CO_2. Despite the fact that HCO_3^- should leave the cells as the medium becomes more acid, control experiments in which the cells were washed after titration with

fresh medium B then replaced in the pH-stat and titration chamber, showed no effect of this small addition of cellular HCO_3^- on the subsequent chloride fluxes. In order to obtain pH's below 6.6 the rewashing procedure was required.

BICARBONATE-CHLORIDE SUBSTITUTION EXPERIMENTS Cells were washed three times in media like medium B except that both chloride and bicarbonate concentrations were varied, but the sum of their concentrations in the medium kept constant at 148.5 meq/liter. The cells were then titrated with CO_2 to pH 7.40 at 0°C.

MODIFICATION OF INTRACELLULAR CHLORIDE Three methods were employed to alter the intracellular chloride concentration. (a) After washing, cells were shrunk or swollen by washing again in media with increased or decreased tonicity. These solutions contained either sucrose (40 or 100 mM) or NaCl (20 or 50 mM) added to medium A or were constituted like medium A but with only 115 mM or 130 mM NaCl rather than 145 mM NaCl. The chloride content of these cells was constant (190–210 meq/kg cell solids), but the water fraction varied between 57.6 and 69.3 % and the intracellular chloride concentrations ranged from 94 to 158 meq/liter cell water. (b) After the initial washing, cells were resuspended and incubated at 37°C in a phosphate medium in order to promote intracellular synthesis of 2,3-diphosphoglycerate (McManus and Borgese, 1962; Parker, 1971). The increase of this impermeable polyanion displaced chloride from the cells. The incubation medium contained (millimolar): 115 NaCl, 20 Na_2HPO_4, 5 KCl, 10 adenosine, 5 pyruvate. Cell samples were incubated for 15, 60, 100, 140, and 180 min, then washed three times in portions of medium A at 0°C. Intracellular chloride ranged from 102 to 73 meq/liter cell water. (c) Cells were directly washed in media constituted like medium A but with added NH_4Cl. Four washes were performed at room temperature over a 2 h period and two final washes at 0°C over a 3rd h. At this time the cells had returned to their original volume and the NH_4Cl content was assumed to be in steady state. Cell chloride concentration was increased from normal (120 meq/liter cell water) up to 275 meq/liter cell water using this method.

SATURATION OF CHLORIDE FLUXES IN SODIUM ACETATE Cells were washed in medium C at room temperature in order to remove cellular chloride. The cells were then washed in the acetate media with various amounts of added NH_4Cl at 0°C until the cell water fraction returned to normal.

Method of Labeling Red Blood Cells with [36]*Cl*

After the last wash the cells were resuspended in the same medium to 5 ml total volume (hematocrit ~30%). The addition of 25 or 50 μl of 150–400 mM $Na^{36}Cl$ (0.6–0.1 mCi/mmole) to this cell suspension increased the extracellular chloride concentration 2–4 mM. The tracer was allowed to distribute between cells and medium for a period of time which was always more than 10 times longer than the half time of the subsequent chloride exchange.

Isolation of Packed and Labeled Erythrocytes

After the appropriate period of exposure to the tracer chloride, the cell suspension, still at 0°C was pipetted into nylon tubes (70 mm long, ID 3 mm) which had been

precooled in the centrifuge rotor. A portion of the suspension designated whole blood was saved. The cells were centrifuged at 0°C in a MSE Superspeed 40 refrigerated centrifuge (MSE Inc., Cleveland, Ohio) until a 85,000 g force was achieved ($\sim$10 min). After centrifugation the column of packed cells (100–200 mg wet weight) was isolated by cutting the tube 1 mm below the cell-medium interface. The trapped extracellular volume estimated with ^{22}Na has been reported to be 2% of the wet cell weight in this procedure (Funder and Wieth, 1967 b). The extracellular phase ("plasma") was saved, and the packed and labeled erythrocyte samples were kept cold until used (up to 4 h later).

Materials for Cell Filtration

The filtration technique was the same as that first described by Dalmark and Wieth (1970, 1972). Cell-free medium was isolated from cell suspensions as described below using Swinnex-25 filter holders (Millipore Corp., Bedford, Mass.) in which the filter support had been inverted. A prefilter (Millipore, AP 2502500) and filter (Millipore, RAWP 02500) were mounted with a rubber gasket. A small amount of silicone grease on the threads of the two halves of the holder helped make a better seal. The filter holders with a 13 gauge needle attached and 0.5 cm³ of water in the trough of the holder were frozen in a deep freeze and removed 2–5 min before use. The ice in the trough maintained the filters near 0°C during the filtration of the cell suspension.

The Efflux of Tracer Chloride under Steady-State Conditions for Total Chloride

25 ml of medium identical to that with which the cells had been equilibrated at 0°C, before addition of isotope, was placed in a thermostated (and pH-stated in the case of HCO_3^- buffered experiments) chamber. If the chloride concentration of these media was less than 100 meq/liter, NaCl was added in order to compensate for the carrier NaCl added with the isotope. The chloride concentration in the efflux medium and the final equilibrating medium ("plasma") never differed by more than 2% in any experiment. When the efflux medium was cooled to 0°C, 3 ml was withdrawn into a precooled 5 ml plastic syringe, 0.5 ml of this was saved for a radioactive blank, and a column of packed cells in a nylon sleeve was mounted on the end of the syringe. The tracer efflux was initiated when these cells were injected, followed by the 2.5 ml of medium, into the well stirred, thermostated, and pH-stated chamber. Serial samples of cell-free supernatant were removed using a 10 ml plastic syringe mounted on a filter holder with a needle which had been removed from the freezer several minutes earlier.

DETERMINATION OF CELL WATER, CHLORIDE, AND PLASMA CHLORIDE   The percent of wet cell weight which is intracellular water, was determined by drying to constant weight a column of packed cells from one of the nylon tubes. This apparent water fraction was then corrected assuming 2% extracellular trapped water (Funder and Wieth, 1967 b). Duplicate cell columns of known wet weight were added to 1 ml of 7% perchloric acid. Similarly, duplicate samples of "plasma" and whole blood were precipitated in perchloric acid. Chloride ion concentrations were determined on the clear supernatant by titration with $AgNO_3$ or by coulometric titration using

a Buchler Cotlove (Buchler Instruments Inc., Fort Lee, N.J.) or Radiometer (London Co.) (CMT) chloride titrator.

In some of the early experiments using a bicarbonate buffer, only the chloride concentration of the medium was known. In all experiments the intracellular chloride concentration was calculated as the product of the tracer chloride ratio between cell water and media and the medium chloride concentration. The average ratio of specific activity (counts per minute per equivalent) in cell water to that of plasma was 0.984 with standard error of the mean 0.004 (n = 49). This systematic deviation is due to the titration of reduced glutathione, which is found in normal erythrocytes at a concentration of 1.5–2 mmoles/liter cell water (Koj, 1962). Control experiments showed that reduced glutathione and cysteine added to perchloric acid extracts are refound with a recovery of 100%. Therefore, the titrations of intracellular chlorides are systematically elevated by approximately 2% at normal cell chloride concentrations.

DETERMINATION OF RADIOACTIVITY Duplicate samples of blanks, cell-free supernatants, and equilibrium samples (200 or 300 μl) from the efflux experiments as well as 100 μl of supernatant from plasma, erythrocytes, and whole blood precipitations were added to 10 ml of scintillation fluid and counted in a Beckman (Beckman Instruments, Inc., Fullerton, Calif.) or Packard Tricarb (33-10) (Packard Instrument Co., Downers Grove, Ill.) liquid scintillation spectrometer.

CALCULATIONS The efflux of tracer chloride in these experiments, where the cells are in steady state with respect to water content, pH, and chloride content during the tracer flux, behaved as an ideal two-compartment system (Dalmark and Wieth, 1972). The rate coefficient, k_{Cl}, for efflux was calculated from the slope of the graph of ln $(1 - a_t/a_\infty)$ vs. time (t) by linear regression where a_t is the specific activity of the extracellular compartment at time (t) and a_∞ is the specific activity of the same compartment at isotopic equilibrium. This slope was taken as the rate coefficient itself since the extracellular compartment constituted over 99% of the total chloride in the efflux chamber (0.2 g of cells in 25 ml of fluid and cell chloride concentration was usually less than in the medium) (Sheppard, 1962). The tracer flux M_{Cl}, was calculated from the equation

$$M_{Cl} = k_{Cl} \cdot (Cl)_{cell} \cdot d$$

$$\frac{meq}{(kg \ cell \ solids) \cdot min} = \frac{1}{min} \cdot \frac{meq}{kg \ cell \ water} \cdot \frac{kg \ cell \ water}{kg \ cell \ solids},$$

where $(Cl)_{cell}$ is the intracellular chloride concentration and d is the kilograms of cell water per kilogram of cell solids calculated from the corrected water fraction, $\%H_2O$, as $\%H_2O/(100-\%H_2O)$. By expressing the flux per kilogram of cell solids the flux is normalized to ca. 3×10^{13} cells and a constant cell surface area ca. 4.9×10^7 cm^2 (Funder and Wieth, 1967 a). Since cell water is a function of pH (see Fig. 3), temperature, and tonicity of the medium, fluxes per liter of cell water or per gram of wet cells are not at all comparable if these parameters are varied.

RESULTS

In all experiments presented in this paper the outflux of chloride-36 was measured at 0°C ± 0.1 from human red blood cells under conditions designed to minimize the net flux of chloride, hydrogen or hydroxyl ions, and water. This flux we will denote as the chloride self-exchange flux in order to emphasize that there is no net movement of chloride, only the exchange of a labeled chloride ion for unlabeled nonradioactive chloride during the efflux measurement.

A. The pH Dependence of Chloride Self-Exchange Flux

Chloride self-exchange was a function of the extracellular pH as is shown in Fig. 1 for cells in medium A. The flux had a maximum value of approximately 780 meq/(kg cell solids·min) near pH 7.8 and decreased 15% when the pH was 7 or 8.9. Outside these limits the flux sharply decreased. This pH dependence between pH 6.3 and 7.8 is opposite to that found for the divalent anions sulphate and phosphate (Passow, 1965; Deuticke, 1967; Schnell, 1972). The flux of these divalent anions decreased as the pH was increased above pH 6.3, while chloride self-exchange flux in the experiments reported here increased in this pH range up to the peak flux at pH 7.8.

In medium B, which contained 22 meq/liter HCO_3^- the pH dependence of chloride self-exchange flux was examined over a more limited range of pH (Fig. 2). The flux increased with pH to a peak near pH 7.8, the same as
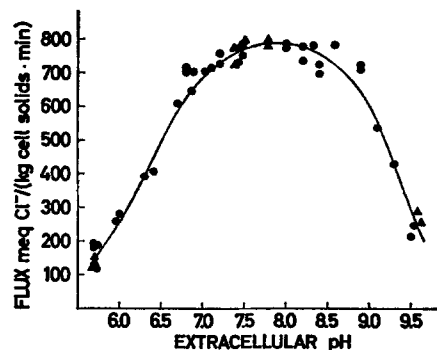

FIGURE 1. pH dependence of chloride self-exchange flux. The self-exchange flux of chloride was measured at 0°C in medium A which was titrated to different pH's with NaOH or HCl. The cells were titrated to the same pH at 0°C before being loaded with ³⁶Cl. Each point is a single efflux measurement. (▲) indicates values from cells titrated first to pH 5.7 or 9.6, the self-exchange flux measured on a portion of cells, then the remainder of cells titrated back to pH 7.4 or 7.8, respectively before measuring the chloride self-exchange flux in media at that pH. The maximal flux was 780 meq Cl^-/(kg cell solids·min) when pH was 7.8.
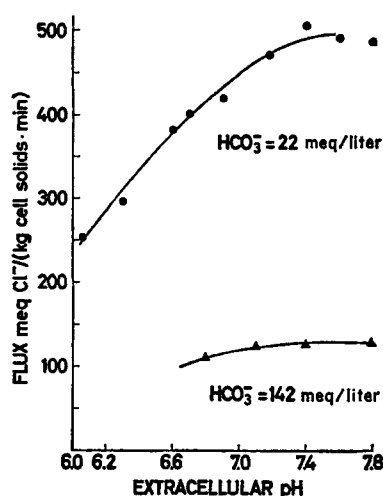
Figure 2. pH dependence of chloride self-exchange flux at two bicarbonate concentrations. The self-exchange flux of chloride was measured in medium B which contained 22 meq HCO_3^-/liter and in a medium like B except that 120 meq Cl^-/liter was replaced by HCO_3^- making the HCO_3^- concentration 142 meq/liter and the chloride concentration 6.5 meq/liter in the medium. Cells and media were titrated with CO_2 and maintained at the desired pH (see Methods) during the loading with [36]Cl and during the efflux measurements. The maximum flux in both media was near pH 7.8, the highest pH used in this series of experiments. The chloride self-exchange flux was reduced at all pH's by the presence of HCO_3^-. The fluxes in 142 meq/liter HCO_3^- have been multiplied by 10 in order to clarify the graphical presentation.

in medium A, however, the magnitude of the fluxes was depressed at all pH's with a peak flux of only 500 meq/(kg cell solids·min) as compared with 780 meq/(kg cell solids·min) in medium A. Fig. 2 also includes the pH dependence of the chloride self-exchange in the presence of 142 meq/liter bicarbonate and 6.5 meq/liter chloride. The flux was very much reduced, but showed a slight increase as the pH increased from 6.8 up to pH 7.8. The fractional reduction of the flux from its maximal level by an increased H^+ ion concentration was decreased by the addition of bicarbonate ions and removal of an equivalent amount of chloride. For example, in medium A (without bicarbonate) there was $\sim$12% reduction of the flux at pH 7 from the maximum flux, while in medium B (22 mM bicarbonate) a 10% reduction was observed and in 142 mM bicarbonate only $\sim$6% reduction. On the other hand comparing the flux in medium B with that in medium A, the inhibition caused by 22 mM bicarbonate ions is numerically less and as a fraction of the total flux smaller when the pH decreased. Therefore, the inhibitory effect of H^+ ions was decreased by substituting bicarbonate ions for chloride ions and the inhibitory effect of bicarbonate ions was decreased by increasing hydrogen ion concentration.

This inhibition of chloride self-exchange by changes of cellular and medium pH was reversible. Cells were titrated with HCl or NaOH to pH 5.7 or 9.6. The chloride self-exchange flux was determined on a portion of these cells while the remainder of the cells were titrated back to pH 7.4 or 7.8 and the exchange flux determined at that pH. The data points from these experiments are denoted by (▲) in Figs. 1 and 3.

Since all three factors of the chloride self-exchange flux, namely r_{Cl}, d, and k_{Cl} are functions of pH, the components of the fluxes given in Fig. 1 together with other data obtained from red cells titrated in medium A are presented in Fig. 3. The chloride distribution ratio, r_{Cl}, in medium A was a linear function of pH: r_{Cl} = 3.18 (SD 0.05) − 0.312 (SD 0.007) · pH, where the slope and intercept are from a least squares regression line and the standard deviations of the slope and intercept are given. The kilograms of cell water per kilogram of cell solids, d, was also a linear function of pH: d = 3.56 (SD 0.09) − 0.241 (SD 0.012) · pH. The rate coefficient, k_{Cl}, was a nonlinear, monotone, convex function of pH. At higher pH values of the medium the rate coefficient was greater and therefore the half-time of self-exchange was smaller and the scatter in the data increased. The lower values of k_{Cl} at the high pH's tended to be from experiments where the cells were stored for longer periods (1–2 h) before the efflux measurements, suggesting some time-dependent decrease in the flux at pH's greater than 9.4. Whether this time-dependent decrease is reversible is not known since the back titration of cells in experiments designed to test reversibility was begun as soon as the cells had adjusted to pH 9.6 and the sample for the flux at pH 9.6 had been removed. Thus, reversibility has been demonstrated only for relatively short periods (∼30 min) at pH 9.6.

B. The Dependence of Chloride Self-Exchange Flux on Chloride Concentration

Cells were prepared with intracellular chloride concentrations between 60 and 285 meq/liter cell water at pH 7.4 ± 0.1 by three different techniques. (a) cell water content was changed by altering the tonicity of the media by adding sucrose or NaCl or by removing NaCl from the external medium, (b) cell chloride content was reduced by a preincubation which promoted the synthesis of intracellular 2,3-diphosphoglycerate, a nonpenetrating polyanion, and (c) cells were preequilibrated with medium A plus various amounts of NH_4Cl. The NH_4Cl which can enter the cells distributes itself between the medium and cell aqueous phases (see Methods for details). The results of these experiments are shown in Fig. 4. Although the scatter of the data is large, in part due to differences in pH, the flux is nearly independent of intracellular chloride concentration in all three types of experiments and the results are presented in a single figure. The slope of the linear regression line obtained by the method of least squares is not significantly different from zero (dM_{Cl}/dCl = −0.12, SD 0.20, n = 56). However, considering only the
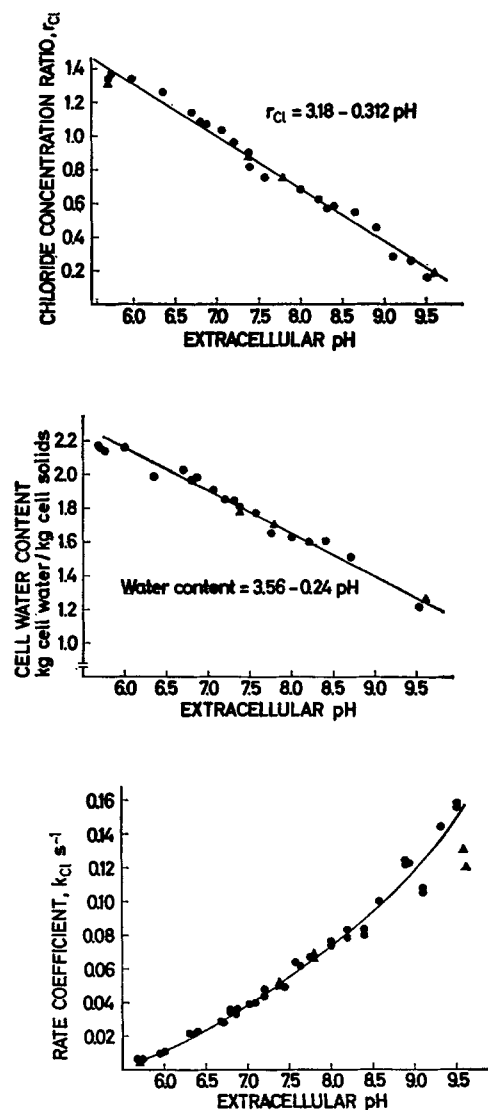
FIGURE 3. The component measurements of the fluxes in Fig. 1 and other measurements in medium A are shown as a function of extracellular pH. (▲) indicates values from cells titrated to pH 5.7 or 9.6 then titrated back to pH 7.4 or 7.8, respectively (see legend of Fig. 1). (*a*) r_{Cl} = 3.18 (SD 0.05) − 0.312 (SD 0.007) pH. (*b*) Water content, d = 3.56 (SD 0.09) − 0.241 (SD 0.012) pH. Measurements of water content of cells in media with Cl^- less than 150 meq/liter due to dilution with NaOH were not included in this figure since water content was a sensitive function of tonicity as well as pH. (*c*) The rate coefficient for chloride self-exchange is a function of pH and chloride concentration. When pH was increased the intracellular chloride concentration was lowered and k_{Cl} was increased partly on this basis, thus the pH dependence of k_{Cl} at constant chloride concentration can not be inferred directly from this figure.
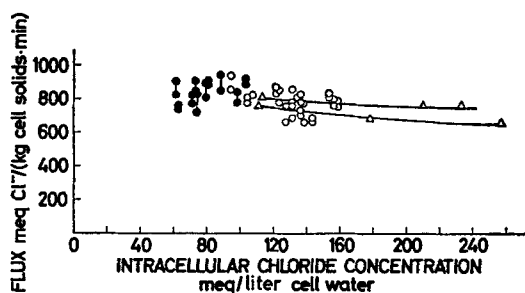
FIGURE 4. Intracellular chloride concentration was altered before measurement of the self-exchange flux of chloride by three methods: (●) preincubation to promote intracellular 2,3-diphosphoglycerate synthesis (Parker, 1971); (○) alteration of cell water content by variation of the tonicity of the medium; (△) addition of NH_4Cl to medium A. Extracellular pH = 7.4 ± 0.2. The slope of the regression line obtained by the method of least squares is not significantly different from zero. This indicates that chloride self-exchange flux was independent of chloride concentration under these conditions.

NH_4Cl experiments there was a slight progressive decrease of chloride self-exchange flux as NH_4Cl concentration increased which may have been an effect of increasing NH_4^+ concentration or ionic strength. The inhibition of the chloride self-exchange flux produced by addition of NH_4Cl varied in the four series of experiments between a reduction of 40 and 80 meq Cl/(kg cell solids·min) for each 100 meq/liter cell H_2O increase in cellular chloride. The independence of chloride self-exchange from the chloride concentration is equivalent to saying that the transport mechanism is a zero order process or that the mechanism is saturated at these chloride concentrations.

In order to demonstrate the dependence of chloride self-exchange flux on chloride at very low intracellular chloride concentrations, cells were first washed in isotonic Na-acetate (medium C), to remove chloride, then portions were washed in isotonic Na-acetate with different amounts of NH_4Cl in addition. In this way we avoided the substitution of various amounts of another anion for chloride, which is important since the sequence HCO_3^-, Br^-, I^-, and SCN^- increasingly inhibit chloride self-exchange (Dalmark and Wieth, 1972). In this experiment the inhibiting acetate ion was present at a constant concentration, although as a variable fraction of the total anion concentration, and only the effects of increasing NH_4^+ concentration and ionic strength were superimposed on the chloride dependence of the self-exchange. The results are shown in Fig. 5. The self-exchange flux increased linearly at low concentrations then increased more slowly and finally became independent of chloride concentration at a flux of ~460 meq/(kg cell solids·min). The Cl^- concentration at which the flux is half maximal, $K_{1/2}$ was 26 meq/liter cell water. The best smooth curve through these data does not have the functional form which results from Michaelis-Menten
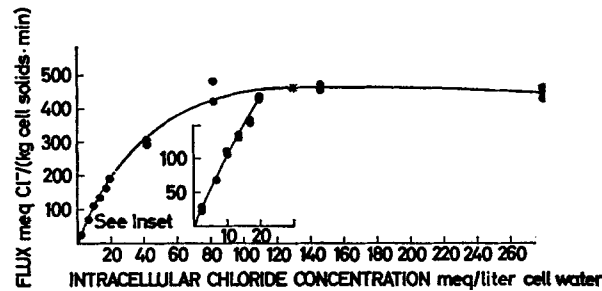
FIGURE 5. Chloride self-exchange flux as a function of chloride concentration. Red cells were washed in media containing (millimolar) 141 Na-acetate, 1.5 Ca (acetate)$_2$, 1.0 Mg (acetate)$_2$, 27 glycylglycine, and variable NH$_4$Cl which determined the final intracellular chloride concentration. The water content of the cells was constant, d = 1.97 − 1.88 kg water/kg cell solids, except in the cells with Cl$_{cell}$ = 279 meq/liter when d = 1.63. Extracellular pH = 7.2–7.4 and temperature = 0°C. Duplicate flux measurements were made at each chloride concentration. (*) indicates a benchmark which is shown also in Fig. 6. The maximal flux was 460 meq Cl$^-$/(kg cell solids·min). The chloride concentration at which the flux was half maximal was 26 meq/(liter cell water). The data curve indicates that chloride transport included the interaction of chloride with a membrane component whose availability was limited.

kinetics. The initial slope at low chloride concentrations is lower than the Michaelis-Menten curve with the same maximal flux and $K_{1/2}$. The data curve also tends to reach saturation at a lower chloride concentration than the comparable Michaelis-Menten function. There is no evidence of sigmoidicity in the curve at low chloride concentrations. The value of the saturated flux in the presence of 146 meq/liter acetate was lower than the saturated flux at the same pH in medium A (460 as compared to 780 meq/(kg cell solids·min).

This inhibition of chloride exchange by acetate was further explored by measuring the effect of NH$_4$-acetate additions on chloride self-exchange in medium A and in medium A plus 160 mM NH$_4$Cl. The results are shown in Fig. 6. There, the chloride self-exchange flux is graphed as a function of the total NH$_4^+$ concentration of the medium. Therefore, (NH$_4^+$) = 0 corresponds to the flux in medium A at pH 7.4 and (NH$_4^+$) = 160 corresponds to the flux in medium A plus 160 mM NH$_4$Cl. The addition of NH$_4$-acetate to medium A decreased the chloride self-exchange flux almost linearly (slope: −2.1/mM NH$_4$-acetate), not significantly different from the effect of addition of NH$_4$-acetate to medium A plus 160 mM NH$_4$Cl (slope: −1.8/mM NH$_4$-acetate). The slope of the relation between chloride self-exchange flux and NH$_4$-acetate concentration became slightly less negative at the highest acetate concentrations in both series of experiments. In medium A the intracellular chloride concentration was 131–117 (meq/liter cell water), while in the second set of experiments, chloride concentration was 266–230 (meq/ liter cell water) with increasing NH$_4$-acetate additions. At all of these con-
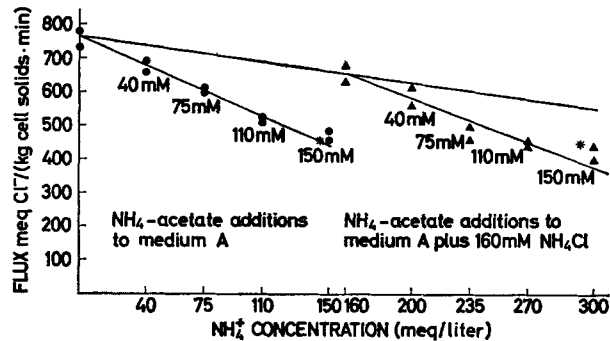
FIGURE 6. The effects of NH₄-acetate additions to medium A as compared with NH₄Cl additions on the chloride self-exchange flux at pH 7.4, 0°C. The flux in medium A (760 meq Cl⁻/[kg cell solids·min]) was reduced by the addition of NH₄Cl as indicated by the uppermost line in the figure. The addition of 40, 75, 110, and 150 mM NH₄-acetate (●) to medium A caused a nearly linear decrease of chloride self-exchange flux. The addition of similar concentrations of NH₄-acetate to medium A plus 160 mM NH₄Cl (▲) also caused a linear decrease of chloride self-exchange flux. The two slopes are not significantly different although the intracellular chloride concentration in one set of experiments (▲) (266–230 meq/[liter cell water]) was twice that in the other set of experiments (●) (130–117 meq/[liter cell water]).

centrations the transport mechanism was saturated (cf. Fig. 4) and only the inhibition of the saturated transport system was measured.

C. The Inhibition of Chloride Self-Exchange by Bicarbonate

As demonstrated in Figs. 1 and 2, the self-exchange of chloride in 22 meq/liter bicarbonate was lower than that in glycylglycine buffered medium A without bicarbonate. This inhibitory effect was further examined by measuring the chloride self-exchange in a medium like B but with the 120 mM NaCl partially or completely substituted by NaHCO₃. Therefore in Fig. 7 both the bicarbonate concentration decreased and the chloride concentration increased as one considers media on the abscissa from left to right. An analysis of this type of substitution experiment is presented in an appendix to this paper, but it must be pointed out here that the higher order dependency of chloride self-exchange on chloride concentration in Fig. 7 is only apparent and is not incompatible with the saturation curves in Figs. 4 and 5. Fig. 7 shows that at constant pH chloride self-exchange was progressively inhibited by increasing bicarbonate concentration and that this inhibition was most marked when the change in chloride concentration should have the least effect on chloride self-exchange, i.e. when the chloride concentration was high.

The data in Fig. 7 are consistent with bicarbonate being a competitive inhibitor of chloride self-exchange. To further document this point, the

effect of NH_4HCO_3 on chloride self-exchange in medium A was examined in an analogous set of experiments to those presented in Fig. 6 which showed the effect of NH_4 acetate. The results in Fig. 8 show that NH_4HCO_3 was a much less effective inhibitor when the chloride concentration was increased.

FLUX meq Cl⁻/(kg cell solids·min)

500
400
300
200
100

Cl⁻   10 20 30 40 50 60 70 80 90 100 110
HCO₃⁻ 108   98.8   80.6   68.7 48.4   38.4   19.3
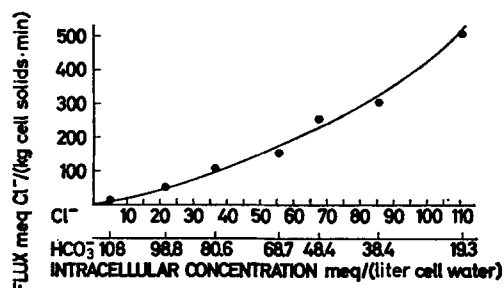INTRACELLULAR CONCENTRATION meq/(liter cell water)

FIG. 7. An equivalent substitution of chloride for bicarbonate in the extracellular medium accelerated the self-exchange flux of chloride at pH 7.4, 0°C. Intracellular concentrations were calculated from the product of the tracer distribution coefficient of chloride, r_{Cl}, and the extracellular concentrations of each ion. The sum of extracellular Cl^- and HCO_3^- concentrations was a constant, 148.5 meq/liter. The sum of intracellular Cl^- and HCO_3^- varied between 113 and 130 meq/(liter cell water). In experiments from left to right the r_{Cl} equalled 0.759, 0.810, 0.790, 0.838, 0.780, 0.804, 0.875. The scale of HCO_3^- concentration is nonlinear due to this variability in r_{Cl}. An analysis of this type of substitution experiment is given in the Appendix.
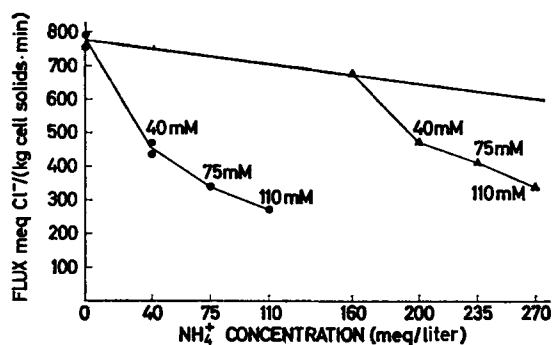
FLUX meq Cl⁻/(kg cell solids·min)

800
700
600
500   40mM   40mM
400   75mM   75mM
300   110mM   110mM
200
100

    0   40   75 110   160   200 235   270
         NH₄⁺ CONCENTRATION (meq/liter)

FIGURE 8. The effects of NH_4HCO_3 additions to medium A as compared with NH_4Cl additions on the chloride self-exchange flux at pH 7.4, 0°C. Each flux was performed in duplicate. Single symbols were used when duplicate values overlapped. The effect of NH_4Cl additions shown in the top line was not different from that shown in Fig. 6. The additions of 40, 75, and 110 mM NH_4HCO_3 to medium A (●) caused a nonlinear reduction of chloride self-exchange with a slope of −8 flux U/mM between 0 and 40 mM NH_4HCO_3. The additions of similar concentrations of NH_4HCO_3 to medium A plus 160 mM NH_4Cl (▲) produced a diminished though still nonlinear inhibition of chloride self-exchange flux. The slope was −5 flux U/mM between 160 and 200 meq/liter NH_4^+ due to the inhibitory effect of the added 40 mM NH_4HCO_3. Intracellular chloride was 140 meq/(liter cell water) in the first series of experiments (●), and 288 meq/(liter cell water) in the second series of experiments (▲).
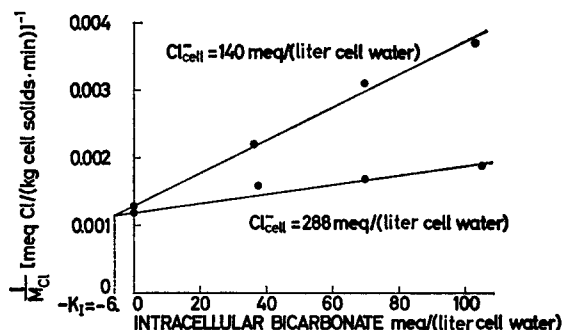
FIGURE 9. The reciprocal of the self-exchange flux (reaction velocity) is graphed as a function of bicarbonate (inhibitor) concentration at two intracellular chloride (substrate) concentrations. The data from Fig. 8 were used after correction for the NH_4^+/ ionic strength effects. In that correction, the fractional inhibition by bicarbonate was determined from the reduction of chloride self-exchange flux as compared with the NH_4Cl base line. The self-exchange flux at 288 meq Cl^-/(liter cell water) in the absence of NH_4^+/ionic strength effects was taken as 830 meq Cl^-/(kg cell solids·min) based on $K_{1/2}$ of 26 meq/liter from Fig. 5. The concentration of intracellular HCO_3^- was calculated as the product of r_{Cl} and extracellular HCO_3^-. The concentration at which the transport system was half saturated with HCO_3 was 6 ± 3 meq/liter by this analysis.

The initial slope of NH_4HCO_3 inhibition was -8.0/mM NH_4HCO_3 when intracellular chloride concentration was 140 meq/liter cell water (medium A) while the initial slope was reduced to -5.0/mM NH_4HCO_3 when intracellular chloride concentration was 288 meq/liter cell water (medium A plus 160 mM NH_4Cl). This data is regraphed in Fig. 9, after correction for the NH_4^+/ionic strength effects have been made.

DISCUSSION

We will limit our discussion to the general properties of chloride exchange, which lead to the conclusion that chloride transport is "carrier mediated." We define the term "carrier mediated" as any transport mechanism utilizing a limited number of membrane sites with which the transported species must chemically react in order to be transported. The kinetic consequences of such mechanisms include saturability, competitive and noncompetitive inhibition, and a discrepancy between the magnitudes of the tracer-determined flux and the net flux. This paper reports experiments designed to evaluate these aspects of chloride transport. The characterization includes studies of the effect of the concentrations of chloride and other anions, pH, and temperature on chloride self-exchange flux.

Saturation

The kinetics of chloride transport presented in Figs. 4 and 5 indicate that chloride interacts with a limited number of membrane sites and that the

transport of chloride is saturated when the chloride concentration is greater than 120 meq/liter. If transport were limited by the concentration of chloride ions, the flux would increase when chloride concentration was raised. This was not observed. The transport mechanism was at a maximal capacity and was limited by the availability of loci for transport. This saturation phenomenon is not peculiar to the experimental conditions of NH_4Cl additions to an Na-acetate medium, since Cass and Dalmark[1] have also obtained very similar results using red cells with different KCl contents prepared with nystatin treatment. The saturation data in Fig. 4 are subject to a number of considerations not present in the experiments in Fig. 5. For example, cells with low intracellular chloride concentrations had lower chloride distribution ratios and, therefore, lower intracellular pH values and more negative membrane potentials (as calculated from r_{Cl}) than the other cells in Fig. 4. A decrease in internal pH should tend to decrease chloride self-exchange (cf. Fig. 1), while a more negative membrane potential should promote tracer efflux if the transported species has a net negative charge (Hodgkin and Katz, 1949). In the other experiments (Figs. 4 and 5) where additions of NH_4Cl were used to increase internal and external chloride concentration, the ionic strength and NH_4^+ concentration also increase in parallel. Nevertheless, the curve in Fig. 5 clearly demonstrates the saturation of the chloride self-exchange flux in human red blood cells. At low chloride concentrations the self-exchange flux was apparently limited by the availability of chloride to the transport system and therefore, it increased proportionally with chloride concentration. When chloride concentration was increased, the transport system became progressively independent of chloride concentration, i.e. saturated. The maximum transport was 460 meq Cl/(kg cell solids·min) (460 flux U) under these conditions which included a constant background concentration of Na-acetate and variable NH_4Cl concentration. Since NH_4Cl additions inhibit chloride self-exchange by 40–80 flux U per 100 mM due to either the accompanying increase of the ionic strength or ammonium ion concentration, the value of the maximal flux may be low by 60–100 flux U in the presence of 150 mM NH_4Cl. The apparent $K_{1/2}$ in Fig. 5 is 26 meq Cl/liter, but if the correction is made for the increasing inhibition by ionic strength/NH_4^+, $K_{1/2}$ is increased to 35 meq Cl/liter. The major reduction of the flux from that observed in medium A was due to the inhibition by acetate. As seen by comparing the bench marks (*) on the left-hand sides of Figs. 5 and 6, where the composition of the media in the two series of experiments was nearly the same, the inhibition by acetate ions (141 meq/liter) alone was 200 flux U. For reasons dealt with below we believe that the acetate is a noncompetitive inhibitor of the chloride self-exchange mecha-

[1] Cass, A., and M. Dalmark. 1972. Equilibrium dialysis of the ions in red cells: high and low ionic contents obtained using the polyene antibiotic nystatin. Submitted for publication.

nism. Therefore, the shape of the saturation curve shown in Fig. 5 should be the same if the acetate concentrations were zero. That is, the only effect of acetate ions should be a reduction in the ordinate scale, not a shift in the apparent $K_{1/2}$.

Inhibition of Chloride Self-Exchange by Acetate and Bicarbonate

Implicit in our conception of carrier-mediated transport is a reaction in which a complex is formed between the transported species and the transport mechanism. If this reaction is not perfectly specific for chloride, other anions may react with the mechanism and thereby occupy transport loci and effect a reduction in the transport of chloride. This inhibition may be competitive or noncompetitive depending on whether the inhibiting ion reacts with only the unloaded carrier at the chloride reaction site, or whether it reacts with both the unloaded and loaded carrier at some distant site. In the case of a competitive inhibitor, an increase in the chloride concentration will diminish its effectiveness, whereas the inhibition by a noncompetitive agent will not be altered. The reversibility of the inhibition upon removal of the inhibitor does not influence this distinction.

The noncompetitive nature of acetate inhibition and the competitive nature of bicarbonate inhibition was demonstrated by the addition of these ions as ammonium salts to the standard medium A, in comparison with the effects of similar amounts of ammonium chloride. Since the chloride self-exchange flux was approximately 85% ($K_{1/2}$ = 26) saturated when the cells were in medium A alone, the addition of 100 meq Cl^-/liter cell water should increase the degree of saturation by approximately 6% or the flux by approximately 55 meq Cl/(kg cell solids·min). Consequently, the inhibition by NH_4Cl was probably greater than that calculated from the negative slope of the graphs in Figs. 6 and 8. However, by using the NH_4Cl inhibition line as the basis for the NH_4^+/ionic strength effects, we could evaluate the specific effects of acetate and bicarbonate by adding them also as the ammonium salt.

Acetate appeared to be a noncompetitive inhibitor of chloride self-exchange. In support of this we found that the inhibition by NH_4-acetate was nearly linear at two chloride concentrations (Fig. 6). The slope of the graph of self-exchange flux vs. NH_4^+ concentration was not significantly different when intracellular chloride was increased (-1.8 compared with -2.1 flux U/mM NH_4-acetate). The deviations from linearity at high NH_4-acetate concentrations, which were more pronounced when the chloride (and NH_4^+) concentration was elevated, may be due to the interference of the NH_4^+/ionic strength effect with the acetate effect. If acetate were a competitive inhibitor with a $K_{1/2}$ less than 75 meq/liter, the nonlinearity of the inhibition curve would be more evident at the NH_4-acetate concentrations used in these ex-

periments (Fig. 6). Further evidence in support of the noncompetitive nature of acetate inhibition of chloride transport is that acetate transport itself does not seem to be mediated by this carrier system. Firstly, strong inhibitors of chloride transport such as trinitro-m-cresolate and phloretin have no inhibitory effect on [^{14}C] acetate self-exchange flux. Secondly, the temperature dependence of acetate self-exchange is low (Arrhenius activation energy is $\sim$16 kcal/mol). Thirdly, the pH dependence of acetate self-exchange is very different from that of chloride. [^{14}C]acetate self-exchange increases linearly with H$^+$ concentration suggesting that acetate is transported primarily in the form of the nonionized acetic acid. (Gunn, Wieth, and Tosteson, unpublished observations). These data all lead to the conclusion that acetate inhibition of chloride self-exchange is noncompetitive.

Besides acetate, several other examples of noncompetitive inhibitors of chloride transport are known, though less fully evaluated. For example, trinitro-m-cresolate (Gunn and Tosteson, 1971) and phloretin (Wieth et al., 1972) are among the most potent, while L-anilino-8-naphthaline sulfonate (Fortes and Hoffman, 1972), 2,4-dinitrophenol, picrate (Gunn, Wieth, and Tosteson, 1971), and salicylate (Wieth, 1970) are less potent and acetate is the least potent noncompetitive inhibitor.

In contrast to acetate, the bicarbonate anion appeared to be a competitive inhibitor of chloride self-exchange. NH$_4$HCO$_3$ added to medium A as shown in Fig. 8, caused a nonlinear decrease in chloride self-exchange and the inhibitory effect of a given concentration of HCO$_3^-$ was reduced when the chloride concentration was elevated. As shown, the chloride self-exchange flux in the presence of 110 meq/liter of HCO$_3^-$ increased from 270 to 335 meq Cl/(kg cell solids·min) when the intracellular chloride increased from 140 to 288 meq/(liter cell water); therefore the chloride flux as a function of chloride concentration was not yet maximal under these conditions. This reflects the behavior of a competitive inhibitor which shifts the saturation curve toward the right without altering the maximum transport rate. If there were not the accompanying inhibition by NH$_4^+$ and/or ionic strength one would expect the chloride flux finally to reach 780 meq Cl/(kg cell solids·min) at infinite chloride concentration, if bicarbonate is a competitive inhibitor.

Although bicarbonate inhibits chloride self-exchange, the net exchange of intracellular chloride and extracellular bicarbonate, each crossing the membrane in a direction opposite to the other, is not slower than the self-exchange of chloride at comparable bicarbonate concentrations (Dalmark, 1972. Fig. 6). Therefore, bicarbonate is as good as exchange partner for chloride as chloride itself. In other words, the chloride self-exchange system seems well adapted to the physiological chloride for bicarbonate exchange because bicarbonate competes for the same transport system.

The activation energy for the free diffusion of anions in aqueous solutions is low, but the apparent activation energy for the self-exchange of anions across red cell membranes is high. Dalmark and Wieth (1970, 1972) found a $Q_{10} = 8$ between $0°$ and $10°C$ for chloride self-exchange which is equivalent to an apparent activation energy of 33 kcal/mol. In addition bromide, iodide, thiocyanate, sulfate, and phosphate self-exchange have activation energies of this magnitude although their rates of penetration differ by as much as 10^4 (Dalmark and Wieth, 1972; Passow, 1969; Deuticke, 1970). While the similarity of the activation energies for the self-exchange of these anions does not itself imply that these processes are carrier mediated or that these anions share a common mechanism of transport, the surprisingly high value of the apparent activation energies is consistent with a significant interaction between the transported species and some component of the permeation barrier. Such a component could be carriers.

Comparison between Net and Unidirectional Fluxes

The final evidence to be discussed which supports a carrier mechanism for chloride transport concerns the difference between net and tracer-measured chloride fluxes and the electrical resistance of red cell membrane which appears to be considerably higher than values calculated with the assumption that the rate of tracer exchange is a measure of the membrane permeability to free chloride ions. The sum of the evidence cited below has made us postulate that chloride may penetrate the membrane by two pathways: as free ions through "the conductance pathway," or as a neutral complex through the "anion exchange pathway" (Tosteson et al., 1972; Wieth et al., 1972). Qualitative evidence showing that the net movement of *chloride ions* through the conductance pathway occur at a significantly lower rate than the exchange of chloride was found in gramicidin-treated red cells by Chappell and Crofts (1966), by Harris and Pressman (1967), and by Scarpa et al. (1968, 1970). Quantitative determination of the relative magnitudes of the two pathways on valinomycin-treated human red cells (Hunter, 1971) showed that the *chloride ion* permeation is 10^4–10^5 times slower at $38°C$ than one would expect if the rate of chloride tracer exchange were a measure of true chloride ion permeability. Similar discrepancies between *chloride ion* permeabilities and rates of exchange have been found on gramicidin-treated human red cells at $25°C$ by Cass and Dalmark[2] and on valinomycin-treated sheep red cells at $37°C$ by Tosteson et al. (1972).

The low permeability to *chloride ions* is further substantiated by the finding of a high electrical membrane resistance (Lassen, 1972; Lassen et al., 1972). Due to the technical difficulties connected with direct measurements of the membrane potential of small cells (Lassen et al., 1971) quantitative com-

[2] Cass, A., and M. Dalmark. 1972. Personal communication.

parisons between the electrical resistance and the value calculated from the rate of chloride exchange are not available for small mammalian red cells, but recent measurements of both membrane resistance and chloride exchange in the giant red cells of *Amphiuma* lead Lassen et al. (1972) to the conclusion that only 10^{-5} of the chloride crossing the membrane moves across the membrane as free ion.

The pH Dependence of Chloride Self-Exchange

The pH dependence of chloride self-exchange shown in Fig. 1 is neither affirmative nor negative evidence for carrier-mediated transport of chloride. In general terms the curve only implies that the transport capacity for chloride under conditions where the system is nearly saturated, can be reduced by extremes of pH. However, the similarity between this curve and the pH dependency of the activity of many enzymes is striking. Even though reservations must be made because of factors such as the desaturation of the transport mechanism at alkaline pH values (see Fig. 3 *a*), it does appear reasonable to postulate the existance of less functional forms of the transport system at the extremes of pH. That the depression of chloride transport observed at extracellular values of pH 5.7 and 9.6 can be reversed by back titration to pH 7.4 or 7.8, seems to support this contention. Gunn (1972 *a*, *b*) suggested that the chloride carrier is titrated into the carrier for divalent anions as the pH is reduced below pH 7.8.

On the other hand, the pH dependence of chloride self-exchange in Fig. 1 is inexplicable by the fixed charge hypothesis (Passow, 1961, 1965, 1969). According to that hypothesis, anion flux should decrease monotonically as the hydrogen ion concentration of the medium decreases and concomitantly the fixed positive charge and mobile counter anion concentrations within the membrane decrease. Not only the pH dependence of chloride self-exchange, but also the pH dependence of iodide (Wood and Passow, 1971; Gunn, 1972 *b*), sulfate (Schnell, 1972), and phosphate (Deuticke et al., 1968) fluxes show pH maxima and thus over some pH range the fluxes of these anions also increase as hydrogen ion concentration decreases in direct conflict with that predicted by the fixed charge hypothesis.

As an alternative Wieth (1972; Wieth et al., 1972) has suggested that the transport of monovalent inorganic anions is mediated by anion exchange molecules which behave in the erythrocyte membrane as organic amines behave in the artificial membranes studied by Shean and Sollner (1966). This idea has been incorporated into a model by Gunn (1972 *a*) and the data in this report used to estimate the parameters of the model (Gunn, 1972 *b*). The essential features of the model are the following: monovalent and divalent inorganic anions permeate the erythrocyte membrane primarily as neutral complexes with a carrier whose valence before complexation de-

pends on the degree of titration with hydrogen ions. More comparative data on mono- and divalent anion transport are needed before quantitative details of this titratable carrier model can be critically evaluated.

APPENDIX

Analysis of Substitution Experiments

In an experiment such as that shown in Fig. 7, where the self-exchange flux of chloride is determined as a function of chloride concentration, but the variation in chloride is accompanied by the variation of bicarbonate concentration, a known competitive inhibitor, the true concentration dependence of chloride flux is obscured. This type of substitution experiment is commonly performed on biological systems because ionic strength and osmolality of the medium can remain nearly constant if one anion is substituted for another. However, an analysis of this type of substitution in a carrier system has not previously been put forward. For this analysis let us assume that chloride self-exchange is competitively inhibited by bicarbonate (I) and the chloride flux, M_{Cl}, is of the form:

$$M_{Cl} = \frac{M_{max}}{1 + \frac{k_{\frac{1}{2}}}{Cl}\left(1 + \frac{I}{k_I}\right)},$$

where M_{max}, $k_{1/2}$, and k_I are the maximum flux, the concentration of chloride for half maximal flux in the absence of inhibitors, and the inhibitory constant, respectively, and all are constants at a given pH and temperature. If, further, we have the constraint as in the experiments in Fig. 7 that the total concentration of chloride and bicarbonate (I) is constant: $Cl^- + I^- = S$ (in Fig. 7 intracellular $Cl + HCO_3 = $ 113–130 meq/liter), then by simple calculus the following can be proven:

(a) If $k_{1/2} = k_I$, $d^2M_{Cl}/d\ Cl^2 = 0$ and M_{Cl} is a linear function of Cl (or $S - I$).

(b) If $k_{1/2} > k_I$, $d^2M_{Cl}/d\ Cl^2 > 0$ and M_{Cl} is a convex function of Cl (or $S - I$) as shown in Fig. 7.

(c) If $k_{1/2} < k_I$, $d^2M_{Cl}/d\ Cl^2 < 0$ and M_{Cl} is a concave function of Cl (or $S - I$), with the limiting case that as k_I becomes very large the function becomes a Michaelis-Menten function. The $k_{1/2}^{-1}$ and k_I^{-1} are a measure of the affinity of the carrier for chloride and the inhibitor, respectively. From the data in Fig. 7 the affinity of the carrier for bicarbonate is 2–3 times that for chloride, a result we have shown more directly in other experiments presented in this paper.

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