

# Some Effects of Low pH on Chloride Exchange in Human Red Blood Cells

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**ABSTRACT** In order to test the range of pH values over which the titratable carrier model for inorganic anion exchange is valid, chloride self-exchange across human red blood cells was examined between pH 4.75 and 5.7 at 0°C. It was found that chloride self-exchange flux had a minimum near pH 5 and increased again with further increase in hydrogen ion activity. The Arrhenius activation energy for chloride exchange was greatly reduced at low pH values. The chloride flux at pH 5.1 did not show the saturation kinetics reported at higher pH values but was proportional to the value of the chloride concentration squared. In addition, the extent of inhibition of chloride self-exchange flux by phloretin was reduced at low pH. Our interpretation of these findings is that the carrier-mediated flux becomes a progressively smaller fraction of the total flux at lower pH values and that a different transport mode requiring two chloride ions to form the permeant species and having a low specificity and temperature dependence becomes significant below pH 5. A possible mechanism for this transport is that chloride crosses red cell membranes as dimers of HCl at these very low pH values.

## INTRODUCTION

The characteristics of chloride self-exchange in human erythrocytes including the dependence of chloride fluxes on pH between 5.7 and 9.6 have been recently described (Gunn et al., 1973). Chloride fluxes were shown to increase with pH up to a maximum flux at pH 7.8 above which chloride fluxes declined. In addition, chloride fluxes were saturated at physiologic chloride concentrations, and were competitively inhibited by bicarbonate anions. Based on this data and the characteristics of sulfate (Passow, 1969) and phosphate (Deuticke, 1970) transport, a titratable carrier model for inorganic anion transfer has been developed (Gunn, 1972; 1973) which describes the behavior of an electrically silent exchange mechanism for monovalent and divalent anions in erythrocyte membranes. Briefly, the model is that the rate-limiting process for self-exchange is the translocation of a carrier-ion complex which is inde-

pendent of pH and ion concentrations. These latter variables influence transport through mass action equilibrium reactions at the membrane surfaces between the ions of the medium and a carrier confined to the membrane. To explain the observed decrease in chloride tracer fluxes when pH decreased below 7.8, the postulate was made that the anion carrier mechanism is titratable into a more positively charged form at low pH which does not transport chloride to an appreciable extent, but is responsible for sulfate and phosphate ( $\text{HPO}_4^{2-}$ ) transport, and below pH 6 the carrier is titratable to a further conformation which is unsuited for both monovalent and divalent inorganic anion transport.

In this paper, we report some of the characteristics of chloride transport below pH 5.7 and examine some of the limitations of this model. In particular a loss of saturation kinetics below pH 5.5 suggests that this simple model is inadequate at lower pH values. We considered the possibility that the normal carrier mechanism binds two chloride ions to form the permeating species at low pH. However, this idea is inconsistent with the low temperature dependence and loss of specificity of the monovalent anion transport at low pH. We propose that the most reasonable mechanism for chloride transport at low pH is nonionic transport of HCl.

The detailed molecular mechanisms by which chloride is transported across red cell membranes are unknown but two phenomenologically separable pathways are usually considered. The first is a forced exchange of equal equivalents of anions in the two directions. The second permits the net movement of anion equivalents and therefore is called a conductance pathway since electrical current may be carried through the membrane by it. The first mechanism dominates the self-exchange of chloride isotopes at physiologic pH values while both mechanisms contribute significantly to the self-exchange of sulfate. In this paper a partial dissection of these pathways at low pH values suggests nonionic diffusion may contribute yet a third pathway under these conditions.

#### METHODS

With the exception of the titration of cell suspensions the methods we have used here are the same as those detailed elsewhere (Gunn et al., 1973). In brief, we measured the exchange of chloride isotopes when the cells were in steady state with respect to the total chloride, water, and hydrogen ion content. A cell suspension was titrated to the desired pH (detailed below) and the cells washed into a medium at the same pH. Isotope ( $^{36}\text{Cl}$ ) added to the suspension ( $0.6 \mu\text{Ci/ml}$ ) was allowed to distribute between cells and medium for at least six half times of the subsequent exchange before the suspension was centrifuged in small nylon tubes. Water and chloride content of the packed cells was determined and corrected for a trapped extracellular space of 2% (wt/wt). The rate coefficient of  $^{36}\text{Cl}$  efflux ( $k_{\text{Cl}}$ ) was measured by injecting packed cells into a stirred isotope-free medium chemically identical to the preequilibration medium. From this suspension, samples of cell-free supernatant were serially taken using the

filtration technique of Dalmark and Wieth (1972). The isotopic efflux was calculated from the product of the tracer efflux rate coefficient,  $k_{Cl}$ , the tracer chloride distribution ratio between cell water and medium,  $r_{Cl}$ , the medium chloride concentration,  $C_{Cl}$  (meq/kg medium), and the cell water content (kg cell water/kg cell solids) corrected for trapped extracellular fluid. See Gunn et al., 1973 for details.

#### *Titration of Cell Suspensions*

We have found that the use of strong bases or acids either alone (0.3 M) or in combination with isotonic saline (e.g. 0.15 M NaCl in 0.15 M HCl) resulted in some hemolysis since some cells were exposed to very strong acid or base before mixing could occur. More importantly this method of titration was very slow at extreme pH values. At 0°C, where most of our measurements were made, the titration of red cell suspensions (hematocrit = 0.2–0.4) would require hours to titrate to pH 5 with HCl as judged by the time for the suspension to reach a stable pH. To avoid these difficulties in the present experiments, red cells were repeatedly centrifuged and resuspended in 170 mM NaCl saturated with 1 atm CO<sub>2</sub> (pH 4.3–4.5) until the cell suspensions were titrated to the desired pH at 0°C. The final titration was performed in a thermostated chamber using a Radiometer Titrator (London Co., Cleveland, Ohio) to control the flow of CO<sub>2</sub> as described by Funder and Wieth (1967). This process, which required four to six washes each with 20 vol of CO<sub>2</sub>-saturated salt solution, could be performed in 20–30 min. Cells, once titrated to the desired pH, were washed four times with the appropriate glycylglycine-buffered medium at the same pH and temperature for a time sufficiently long to allow the anions to reach a steady-state distribution. The resulting suspension always had a pH value within 0.04 pH units of the value to which the cells and medium had been separately titrated. Titrations toward the alkaline range were performed in an analogous way using 150 mM NaHCO<sub>3</sub>. At pH values above 8.3, additions of 0.15 M NaOH were required. The presence of HCO<sub>3</sub><sup>-</sup> in the medium appeared to greatly facilitate these titrations.

The behavior of erythrocytes at pH <5.7 was not as reproducible as we reported at higher pH values. Ion fluxes in fresh cells from the same donor showed greater variation from day to day and on a given day there was a deterioration of the cells with time. In the control chloride fluxes measured on duplicate samples of cells, fluxes increased by as much as 10%/h at pH 5.1 at 0°C. For this reason, the experiments of a given day were carried out in the particular sequence to minimize a misinterpretation of the results. For example, flux measurements were made at lowest pH first, thus the minimum in fluxes at pH 5 was not due to deterioration of the cells which would be expected to increase the fluxes with time. In no case did the slow increase in the flux with time or the variation from day to day interfere with the interpretations we offer.

#### *Solutions*

Media were prepared with reagent grade chemicals to make the following millimolar concentrations. Medium A: 145 NaCl, 1.5 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 5 D-glucose, 27 glycylglycine (20 sucrose) titrated with HCl or NaOH. Medium C: 141 Na-acetate, 1.5 Ca (acetate)<sub>2</sub>, 1 Mg (acetate)<sub>2</sub>, 5 D-glucose, 27 glycylglycine, (20 sucrose) titrated with glacial acetic acid or NaOH.

Because erythrocytes swell upon acidification due to an increased content of osmotically active anions, 20 mM sucrose was added to all media with a pH below pH 5.7. This increase in external osmolarity was sufficient to prevent cells from reaching hemolytic volume even at pH 4.7 at 0°C. Variation in intracellular (and extracellular) chloride concentration without substitution of chloride for another anion was effected by adding NH<sub>4</sub>Cl to the Na-acetate medium (C).

#### *Heteroexchange Measurements*

We measured the appearance of <sup>36</sup>Cl (from cells preloaded in medium A at the corresponding pH) in a series of sodium acetate media containing 20 mM sodium salts of different monovalent anions. These are the only nonsteady state, non-self-exchange experiments reported here. The fraction of <sup>36</sup>Cl remaining in the cells was graphed versus time and the slope of the straight line portion of this graph reported as  $k'$  (s<sup>-1</sup>), a rate coefficient. We believe that the non-steady-state nature of these experiments vitiates any precise interpretation of these rate coefficients, but their relative values do correspond to the relative ease with which the external anions can exchange for internal chloride.

#### *Phloretin Experiments*

Phloretin (K and K Laboratories, Inc., Plainview, N. Y.) was dissolved in absolute alcohol to make a 0.25 M stock solution. Because of the slow equilibration of isotope at 0°C during the loading of cells in the presence of phloretin, these cell suspensions were warmed at 23°C during a part of the tracer loading phase, then cooled to 0°C for at least 1 h before the cells were packed and isolated.

#### *Calculations of Apparent Chloride Permeability*

The values for apparent chloride permeability,  $P_{Cl}$  (cm/s) were calculated using the equation  $P_{Cl}(\text{cm/s}) = M_{Cl}(\text{mol/cm}^2 \cdot \text{s})/C_{Cl}(\text{mol/cm}^3)$  by multiplying the quotient of the flux and ion concentration ( $M_{Cl}/C_{Cl}$ ) which in this paper has the units (liter cell water) (kg cell solids · min)<sup>-1</sup> by  $(3.4 \times 10^{-7} \text{ kg cell solids} \cdot \text{min} \cdot \text{cm})$  (liter cell water · s)<sup>-1</sup> which assumes  $4.9 \times 10^7 \text{ cm}^2$  red cell surface area in the cells whose dry weight is 1 kg (Funder and Wieth, 1967). No corrections were made for the membrane potential component of the driving force on the efflux of chloride. The membrane potential as estimated from the chloride equilibrium ratio is small (<10 mV); and the correction, assuming a monovalent negative permeant and assuming that the Goldman flux equation is valid (Katz, 1966), would only increase the calculated  $P_{Cl}$  by 11% if the transmembrane potential was +6 mV. The  $P_{Cl}$  calculated here is an operational factor, namely the proportionality coefficient between flux and concentration when that relationship is linear. Without knowing the chemical form of the permeating species this proportionality coefficient cannot be identified as the permeability coefficient in Fick's law. Also, without knowing the charge on the permeant species it cannot be properly corrected for the electrical driving force, using, for example, the Goldman flux equation.

## RESULTS

*Chloride Self-Exchange at pH < 5.7*

In Fig. 1 the pH dependence of chloride self-exchange at low pH and high external  $\text{Cl}^-$  is shown together with the data previously reported at higher pH values and the component data are given in Table I. The chloride flux reached a minimum at pH 5.1 and 5.3 in these two studies. The increased flux at very

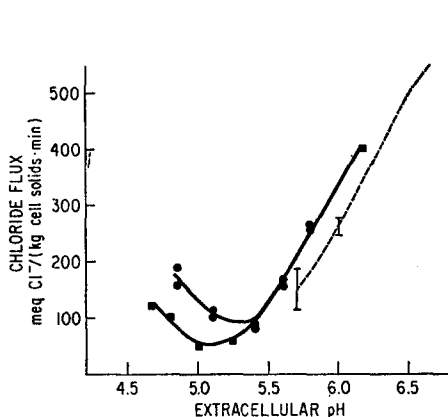


FIGURE 1

FIGURE 1. Chloride self-exchange flux as a function of extracellular pH in two experiments. The underlying data comprising these measurements is given in Table I. The dashed line is data taken from Gunn et al. (1973) and shows the range of values in a similar medium but without sucrose. Intracellular chloride concentrations at the same pH values were slightly higher with sucrose added to the medium (208–210 meq/liter at pH 5.7 in earlier experiments and 219–223 meq/liter at pH 5.6 and 5.8 in the present experiments). In this and subsequent figures duplicate flux values are graphed as single points where they would overlap, but graphed separately when differences could be shown on the graph.

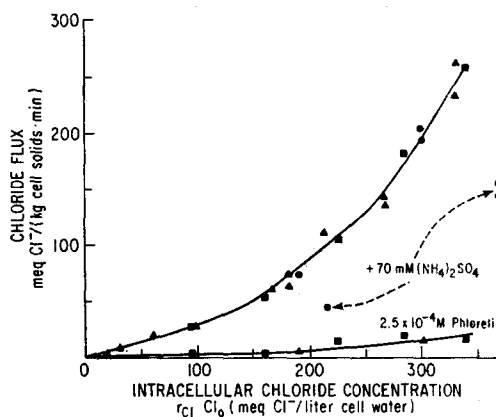


FIGURE 2

FIGURE 2. Chloride self-exchange flux as a function of chloride concentration. Red cells were washed in medium C with different  $\text{NH}_4\text{Cl}$  concentrations which determined the final intracellular chloride concentrations. Extracellular pH = 5.14–5.18 and temperature =  $0^\circ\text{C}$ . The intracellular chloride concentration was determined from the product of extracellular chloride concentration,  $\text{Cl}_o$ , and the ratio radioactive chloride in the water phases between cell interior and medium at equilibrium of the tracer. The residual flux after addition of  $2.5 \times 10^{-4}$  M phloretin to the efflux medium (cells not pre-washed with phloretin-containing medium) is shown by the lower data. At two chloride concentrations 70 mM  $(\text{NH}_4)_2\text{SO}_4$  was added to the wash and efflux medium and cells were prewashed at  $23^\circ\text{C}$  to facilitate ammonium sulfate equilibration. The line segments were drawn by eye through the data. The different symbols represent the values obtained from different experiments on separate days. The squares (■) represent only single determinations.

TABLE I  
COMPONENT MEASUREMENTS OF CHLORIDE FLUXES IN FIG. 1

Exp.	pH	$k$ $s^{-1}$	$\frac{r_{Cl}}{Cl_{in}^{36}/Cl_{out}^{36}}$	$Cl_o$ meq/liter	$d$ kg cell water/kg cell solid	$M$ meq $Cl^-$ /kg cell solids · min
K <sub>50</sub>	4.66	0.0043	1.55	151	2.04	124
	4.80	0.0035	1.54	151	2.07	101
	4.99	0.0017	1.51	150	2.16	50
	5.23	0.0023	1.52	149	2.14	67
	6.20	0.0166	1.27	151	2.10	401
K <sub>46</sub>	4.84	0.0057	1.56	155	1.94	159
	4.86	0.0067	1.56	155	1.94	187
	5.09	0.0041	1.55	156	1.95	116
	5.09	0.0040	1.55	156	1.95	112
	5.38	0.0034	1.48	150	2.09	95
	5.39	0.0035	1.48	150	2.09	96
	5.61	0.0060	1.46	153	2.07	167
	5.60	0.0060	1.46	153	2.07	170
	5.80	0.0100	1.44	153	1.98	262
	5.80	0.0104	1.44	153	1.98	271

The product of  $k$ ,  $r_{Cl}$ ,  $Cl_o$ , and  $d$  and 60 s/min is  $M_{Cl}$ , the flux. Intracellular chloride concentrations meq/(liter cell water) are equal to  $r_{Cl}$  times  $Cl_o$ . In experiment K<sub>46</sub> the variation in the pH and rate coefficient for efflux for duplicate samples of medium and packed cells can be seen.

low pH was unexpected and many of the subsequent experiments were carried out to characterize this transport.

The possibility that this increased flux reflected a general deterioration of membrane permeability properties is not excluded. However, the decreased chloride flux as hydrogen ion activity increased up to  $10^{-5}$  M precluded such a nonspecific deterioration up to this point. Furthermore, we observed low chloride fluxes at pH 5 even though cells had been first titrated to pH 4.6 at 0°C then titrated back to pH 5 and their chloride flux measured. This would indicate that the alterations of the red cell membrane were reversible and that the alteration of the membrane may have involved only specific titratable groups or the formation of a new permeant species at very low pH values.

#### *Dependence of Chloride Flux on Chloride Concentration*

Previously we reported that at pH 7.2 chloride self-exchange fluxes showed saturation when intracellular and extracellular chloride concentration were increased by adding  $NH_4Cl$  to isotonic Na-acetate media (Gunn et al., 1973). At pH 7.2, half saturation was reached at 26 meq  $Cl^-$ /liter cell water. This saturation phenomenon has been observed at pH values down to 5.5 but not below (see Fig. 5). At pH 5.15 (Fig. 2; Table II) chloride fluxes increased as the square of the chloride concentration (Fig. 3 a) and showed no tendency to

TABLE II  
COMPONENT DATA FOR FLUXES GRAPHED IN FIG. 2

Exp.	Cl <sub>out</sub>	Cl <sub>in</sub>	<i>d</i>	<i>r</i> <sub>Cl</sub>	<i>M</i> <sub>Cl</sub>	
K <sub>37</sub>	12	19	2.30	1.57	6.2	6.2
	21	30	2.28	1.46	9.6	10.5
	46	62	2.25	1.34	21	24
	149	180	1.90	1.21	65	77
C <sub>54</sub>	64	98	2.03	1.52	29	29
	119	167	1.93	1.40	64	61
	164	212	1.87	1.29	114	113
	218	266	1.87	1.22	139	145
	272	330	1.78	1.21	262	235
C <sub>55</sub>	60	97	2.03	1.63	29	<u><i>M</i><sub>Cl</sub> + phloretin</u> 2.1
	112	160	1.92	1.44	55	4.6
	163	226	1.87	1.38	106	16.2
	217	283	1.78	1.30	173	21.2
	264	339	1.72	1.28	259	17.9
C <sub>58</sub>	162	196	1.93	1.21	75	77
	271	311	1.80	1.15	205	196
						<u><i>M</i><sub>Cl</sub> + 70 mM(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub></u>
	161	217	1.64	1.35		46
	279	369	1.51	1.32		153
K <sub>57</sub>	18	30	1.49	1.70	12	19

Cl<sub>o</sub> and Cl<sub>i</sub> are the extracellular and intracellular chloride concentrations per kilogram water; *d* is the water content, kilogram cell water per kilogram cell solids; *r*<sub>Cl</sub> is the ratio of intracellular and extracellular chloride tracer concentrations; *M*<sub>Cl</sub> is the chloride flux in milliequivalents of chloride per kilogram cell solids per minute. Experiment K<sub>57</sub> was performed in 242 mM Na-acetate plus 18 mM NH<sub>4</sub>Cl; see text for discussion.

saturate even though the chloride concentration reached 330 meq Cl<sup>-</sup>/liter cell water. Assuming cell surface area to be  $4.9 \times 10^7$  cm<sup>2</sup>/kg cell solids (Funder and Wieth, 1967), the slope of the graph at low chloride concentrations in Fig. 2 would correspond to an apparent erythrocyte membrane permeability to chloride of  $1.3 \times 10^{-7}$  cm/s. This value is 10 times greater than previous estimates of the chloride permeability of the "conductance pathway" to chloride at pH 7.2 (Hunter, 1971). The nonsaturability of chloride fluxes and particularly the cooperative effect of chloride on its own flux indicated at a different mode of chloride transport was present. This second transport mechanism was inhibited by phloretin and ammonium sulfate (Fig. 2). The membrane permeability to chloride in the presence of phloretin calculated from the slope of the graph is  $7 \times 10^{-9}$  cm/s at pH 5.15, 0°C, *r*<sub>Cl</sub> = 1.6 which

agrees rather well with the permeability of the conductance pathway at this temperature.

In Fig. 3 *b* the chloride flux at very low pH is graphed as a function of the hydrogen ion concentration squared. The few data points available suggest that below pH 5 the chloride flux increased as the square of hydronium ion concentration. If so, the permeant species may require two hydronium (hydrogen) ions as well as two chloride ions in its formation.

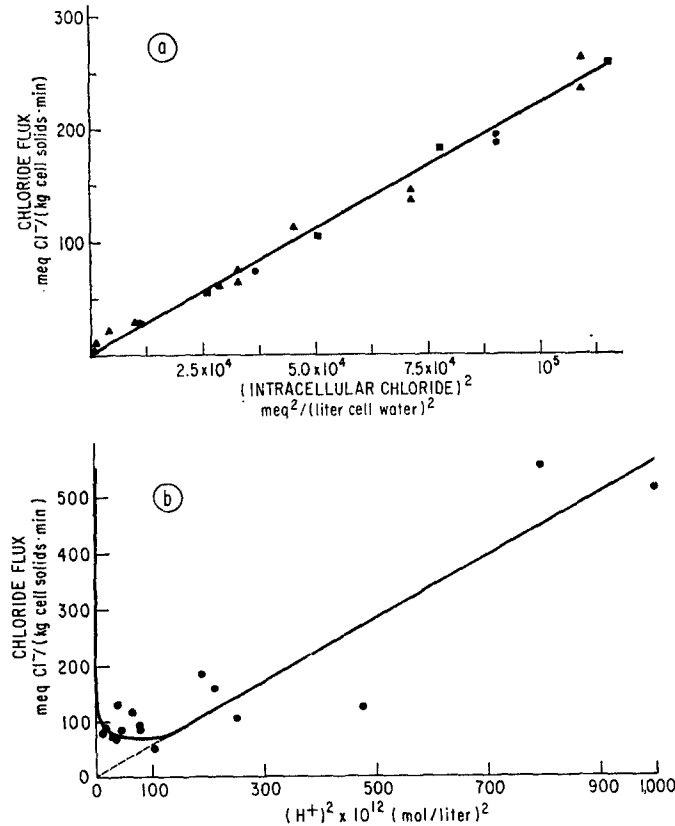


FIGURE 3. (a) Chloride self-exchange flux as function of the intracellular chloride concentration squared. The data is the same as in Fig. 2: extracellular pH 5.14–5.18 and temperature = 0°C. The indicated line with the least square deviation has slope =  $0.00220 \pm 0.00008$  (SD) and intercept =  $-1.5 \pm 5.8$  (SD). In these calculations data from experiments with  $(Cl) < 100$  meq/liter cell water were excluded. If all data points are included in the least squares analysis, chloride flux  $M = (6 \pm 6) + 0.0021 \pm 0.00005 (Cl)^2$ . (b) Chloride self-exchange flux at low pH values as a function of the square of the hydrogen ion concentration in the medium. The value 100 on the abscissa corresponds to pH 5.0, the value 1,000 corresponds to pH 4.5. The curve was drawn by eye. The general relationship between  $(H^+)^2$  and chloride flux is linear between pH 5 and pH 4.5. The technical difficulties of measuring chloride fluxes at these extreme pH values make this relationship uncertain and further clarification arduous.



*Phloretin Inhibition of Chloride Transport*

Table III which shows the residual ( $2.5 \times 10^{-4}$  M) phloretin-insensitive chloride self-exchange flux as a function of pH, demonstrates several important points. First at pH values below 7.7, the residual chloride flux increased monotonically with a marked increase about pH 5. At all pH values, the chloride self-exchange was inhibited by phloretin but the fractional inhibition progressively decreased as the pH of the red cell suspension was reduced below 7.7 (Fig. 4). The fluxes below pH 5.1 were particularly less sensitive to inhibition by phloretin. Second at high pH values in Table III there appeared to be

TABLE III  
CHLORIDE FLUXES IN THE ABSENCE AND PRESENCE OF PHLORETIN AT  
DIFFERENT pH VALUES AT 0°C meqCl<sup>-</sup>/(kg cell solids·min)

pH	Chloride flux, $M_{Cl}$		Inhibition %
	Phloretin (0)	Phloretin ( $2.5 \times 10^{-4}$ M)	
4.7	120-200	250.0 (170-350)	~0
5.1	110	37.0 (32-42)	66.0
5.7	200	1.8	99.1
6.6	550	1.3	99.7
7.3	750	1.2	99.8
7.7	780	0.82	99.9
8.4	750	2.3	99.7
8.8	675	6.1	99.1

At low pH the effectiveness of phloretin as an inhibitor of  $^{36}Cl$  self-exchange was lost. At high pH a slight decrease in the percent inhibition was also observed. The one higher flux value in the presence of phloretin at pH 4.7 only reflects the great variability of the chloride flux at these extreme pH values and probably not a stimulation of chloride flux by phloretin. Average of duplicate flux values are given with a range when wide variations were observed.

a release from the phloretin inhibition. LeFevre and Marshall (1959) reported a complete desorption of bound phloretin at high pH and an accompanying release of inhibition of hexose transport. Their explanation was that phloretin at these high pH values changed to an inactive enol form which could not bind to the membrane from an active keto form which could bind. Alternatively, equilibrium between these forms may shift toward an inactive form at higher pH values due to the ionization of the phenol group. If this mechanism is correct, our data suggest that the pK for the ionization of phloretin should be greater than 7.7 at 0°C. At pH 7.7, 0°C, and  $r_{Cl} = 0.85$ , the residual chloride flux in the presence of 0.25 mM phloretin corresponds to an apparent permeability for chloride of  $2.0 \times 10^{-9}$  cm/s.

Chloride fluxes in the presence and absence of phloretin at pH 5.5 and 5.15 (from Fig. 2) are given in Fig. 5. Although the control fluxes below 240 meq

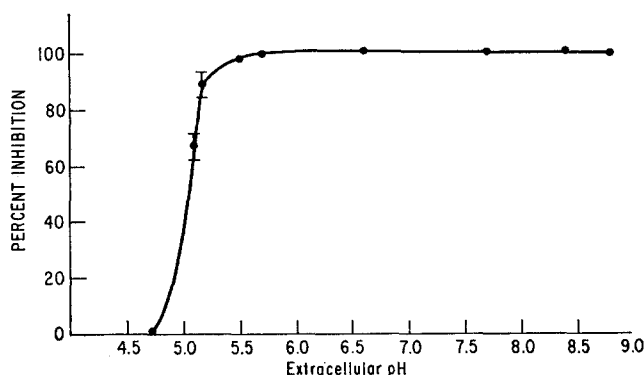


FIGURE 4. Percent of chloride self-exchange flux inhibited by phloretin,  $2.5 \times 10^{-4}$  M. The graph includes data from Table III, the range of inhibition at pH 5.15 from Fig. 2, and the inhibition at pH 5.5 from Fig. 5.

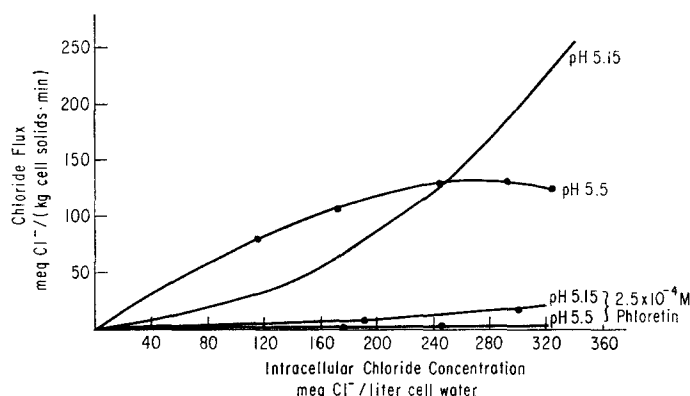


FIGURE 5. Saturation of chloride self-exchange flux at pH 5.5 in contrast to the square dependence at pH 5.15 taken from Fig. 2. At pH 5.5,  $M_{Cl}$  maximum is  $130 \text{ meq Cl}^- / (\text{kg cell solid} \cdot \text{min})$  and  $K_{1/2}$  is  $80 \pm 10 \text{ meq/liter cell water}$ . At  $Cl_{cell} = 230\text{--}240$  the fluxes at the two pH values are of equal magnitude as also shown in Fig. 1 by equal values on either side of the minimum. The residual flux in the presence of phloretin is larger at all chloride concentrations at pH 5.15 than at pH 5.5.

$\text{Cl}^-$ /liter cell water were reduced at the lower pH value, the phloretin-insensitive flux increased with lower pH at all chloride concentrations. In one experiment a 10-fold increase in the phloretin-insensitive flux caused by lowering the pH was restored to control levels when the pH was raised back to pH 7.7, which suggests the pH effect is reversible.

#### *Temperature Sensitivity of Chloride Fluxes at low pH Values*

In Fig. 6 the pH dependence of chloride self-exchange at 0 and  $10^\circ\text{C}$  is shown. The minimum flux shifted from near pH 5.4 to near pH 5 as the temperature was changed from 0 to  $10^\circ\text{C}$ . In another experiment the minimum flux was at

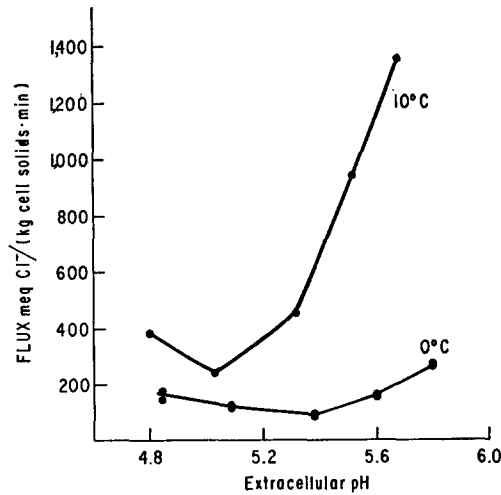


FIGURE 6. pH dependence of chloride self-exchange at 0 and 10°C. The apparent  $Q_{10}$  for chloride transport was greatly reduced by decreasing extracellular pH below 5.7. The pH value at which the self-exchange was minimum decreased as the temperature increased.

pH 5.1 at 0°C and shifted to pH 4.7 to 20°C. From Fig. 6 alone it is clear that the temperature activation (0–10°C) of the flux was less at low pH values. In contrast to the high Arrhenius activation energy of 33.2 kcal/mol found by Dalmark and Wieth (1972) at pH 7.4, the Arrhenius activation energy at low pH was below 10 kcal/mol.

The temperature activation was examined as a function of intracellular chloride to evaluate the possibility that near pH 5 the relationship between the flux and temperature was due to comparing different intracellular chloride concentrations. The erythrocyte fluxes showed greater variability at very low pH as the temperature increased. However, as shown in Fig. 7, the general relationship between flux and concentration was not altered at 5 and 10°C, and more importantly the temperature activation at comparable intracellular chloride concentrations was very small.

#### *Heteroexchange Selectivity at pH 7.82 and 5.04*

The net loss of chloride from red cells is not easily interpreted because either net cation movements or the entrance of the anion substituted for chloride in the external medium may be rate limiting. In both cases, there may be an additional unknown alteration of the red cell membrane potential during the flux measurements. We have nevertheless made some preliminary observations in such a system which bears on the selectivity of this exchange mechanism within the red cell membrane.

In Table IV are presented the initial rate coefficients for  $^{36}\text{Cl}$  efflux from

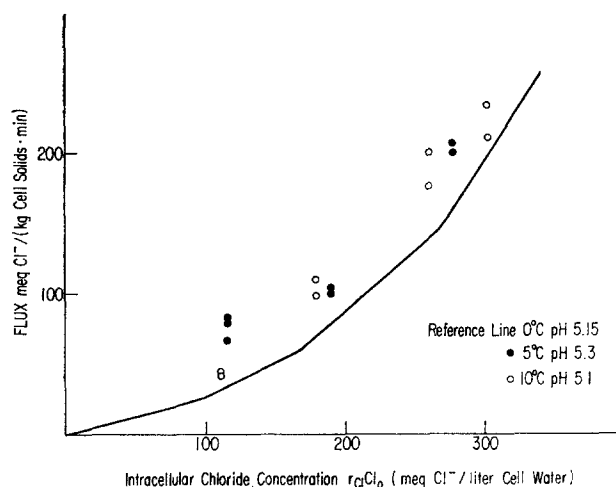


FIGURE 7. Chloride self-exchange flux as a function of chloride concentration at 5 and 10°C compared to the reference line at 0°C. All fluxes were measured at low pH 5.1–5.3. Cells were prepared by equilibration in different  $\text{NH}_4\text{Cl}$  concentrations added to a sodium acetate medium.

TABLE IV  
THE INITIAL RATE COEFFICIENT,  $k'$  ( $\text{s}^{-1}$ ), FOR THE EFFLUX OF  $^{36}\text{Cl}$  FROM ERYTHROCYTES INTO Na-ACETATE MEDIA TO WHICH SODIUM SALTS OF DIFFERENT ANIONS HAD BEEN ADDED

$X^-$	$k' \text{ s}^{-1}$	
	pH 7.82	pH 5.04
Acetate $^-$	0.00016*	0.0046
Cl $^-$	0.0250	0.0041
NO $_3^-$	0.0210	0.0041
Br $^-$	0.0089	0.0050
I $^-$	0.0012	0.0057

\* The rate coefficient after 180 s was used because of a transient rapid efflux, sensitive to the  $\text{pCO}_2$  of the medium, probably due to  $\text{HCO}_3^-$  exchange for  $^{36}\text{Cl}$ .

The erythrocytes were titrated with  $\text{CO}_2$  in 170 mM NaCl, then washed thrice in medium A which had the same pH as the cell suspension.  $^{36}\text{Cl}$  added to the suspension was allowed to equilibrate for 10 half times of the  $^{36}\text{Cl}$  self-exchange under these conditions. The packed cells (200–300 mg) were injected into 20 ml of Na-acetate medium (C) to which 200  $\mu\text{l}$  of 1 M Na-salt had been added. The data are the average of two experiments.

cells preequilibrated in a high chloride medium (medium A) into a Na-acetate medium (medium C) with added 20 mM sodium salts of different anions. The rate coefficients observed for efflux into medium C alone and medium C with 20 mM Na-acetate were the same. Therefore, the change in ionic strength and

osmolarity caused by the Na salt additions had no significant effect on the rate coefficients. At pH 7.82, the addition of NaCl greatly accelerated the tracer efflux. This rate coefficient,  $0.025 \text{ s}^{-1}$ , was slower than the self-exchange rate coefficient at steady state in medium A ( $k_{c1} = 0.068 \text{ s}^{-1}$ ). However, this was consistent with the reduced external chloride concentration (20 meq/liter compared with medium A, 150 meq/liter) and the inhibition by acetate (Gunn et al., 1973). Nitrate appeared to be an equally good exchange partner for the chloride as chloride itself, while bromide and iodide were less efficient, perhaps reflecting their slower rates of self-exchange using the carrier system (Dalmark and Wieth, 1972). When similar experiments were made with cells and media at pH 5.04, the rate of chloride efflux was independent of the counterion to the sodium. The marked increase in the rate of chloride loss in acetate medium at low pH was unexpected. From these results we cannot say whether HCl, acetic acid and/or HI, etc., were being exchanged or whether  $\text{Cl}^-$ , acetate $^-$ , and/or  $\text{I}^-$ , etc., were being exchanged but as a consequence the effective anion selectivity of the erythrocyte membrane was lost. Data on  $^{131}\text{I}$  fluxes support this loss of selectivity. The self-exchange flux of iodide tracers in 150 mM iodide medium is greatly increased at low pH and exceeds the chloride self-exchange in 150 mM chloride medium by 10- to 20-fold between pH 5.1 and 4.9 at  $0^\circ\text{C}$  (Gunn, in preparation).

#### DISCUSSION

The characteristics of chloride self-exchange at physiologic pH values, including a high temperature coefficient, a high degree of selectivity among monovalent anions, a high sensitivity to phloretin inhibition and saturation kinetics, are all lost or greatly diminished at very low pH values. Therefore, we believe that another mechanism, different from that of the normal carrier, is the predominant mode of transport for chloride under these conditions.

One possible explanation for the increased flux of chloride tracer with hydrogen ion activity (at very low pH values) which does not involve a carrier is that the penetrating species is HCl as a nonionized acid. It has been inferred from the pH dependence of weaker acids such as salicylate (Dalmark and Wieth, 1972) that the major form of organic anion transport in red cells is due to the trace amounts of the nonionized acid even three or four pH units above the pK values. However, the extension of this notion to HCl has not been made previously. While the pK value for this strong acid in water is  $-8$  to  $-9$  (Sillén, 1971) and the nonionized species is for all practical purposes nonexistent in aqueous solution, that is not to say that HCl might not form at the surface of a low dielectric phase such as the cell membrane. In aprotic solvents the dissociation constant for (HCl) has been measured by freezing point depression, conductivity, distribution between phases, solubility, and infrared spectroscopy techniques. The pKa depended on the basicity of the

medium and its dielectric constant. Values range from  $pK_a = 3.6$  in  $C_6H_5CN$  at  $25^\circ C$  to  $pK_a = 7.52$  in nitrobenzene saturated with water at  $25^\circ C$  (McCorkell et al., 1968). Particularly interesting is the measurement in 75% benzene 25% ethanol where  $pK_a = 5.51$  at  $25^\circ$  since this may approximate the characteristics of the hydrophobic core of a biological membrane (Aleksandrov et al., 1968). In dry 2-propanol the  $pK_a$  of HCl was 4.43 (Kreshkov et al., 1966) while in a liquid ion-exchange membrane containing 2-propanol and traces of water the dissociation constant for HCl has been reported to be  $3.8 \times 10^{-4}$  mol/liter ( $pK = 3.42$ ; Walker et al., 1968). Here HCl behaved as a weak acid in a membrane medium with a dielectric constant of 18.3 and might be expected to have an even lower dissociation constant in a red cell membrane with an effective dielectric constant of approximately 2. Following this notion further, the square dependence of the flux on chloride concentration would suggest that  $(HCl)_2$ , a dimer of the ion pair, is the permeant species. This would in turn require that the flux be proportional to  $H_3O^+$  concentration squared and this appears to be true given the few data points below pH 5 in Fig. 3 *b*. Dimers of HCl in dioxane (Koskikallio and Syrjälä, 1965) and dimers of  $HNO_3$  complexed with organic amines (Knoch, 1965) have been reported as well as evidence for  $HCl_2^-$  and  $ClHB_7^-$  as salts with  $R_4N^+$  in solids (Sillén, 1971).

However, it should be remembered that at physiologic pH values, this mechanism would not be expected to make a significant contribution to the flux of  $^{36}Cl$  because of both the decrease in  $H_3O^+$  concentration above pH 5.0 and the six- or morefold increase in the chloride flux by the usual carrier mechanism.

#### *Loss of Saturation Kinetics*

The stimulatory effect of chloride on its own transport shown in Fig. 2 is a newly observed phenomenon in erythrocytes. It is important to note that the chloride concentration in these experiments was not altered by substituting another small inorganic anion for chloride, because of the difficulties in interpreting experiments in which both substrate and competitor are simultaneously varied (see Appendix in Gunn, et al., 1973). On the other hand, the addition of ammonium salts in these experiments may not have been without secondary consequences. We may be reasonably certain that neither the accompanying ammonium ions themselves nor the increased ionic strength resulting from these additions were the cause for the stimulation of chloride efflux since the addition of 140 meq/liter of ammonium ions with sulfate resulted in inhibition of the chloride flux. Therefore, on this basis we may reject the possibility that ammonium ions neutralized membrane negative surface charges, and thereby enhanced the effective chloride concentration at transport loci and consequently the chloride flux.

Consideration must be briefly given to the possibility that the sulfate carrier, which itself was postulated to be a form of the chloride carrier (Gunn, 1972), mediated the flux of two chloride ions at a time below pH 5.1. In addition to the square dependence of the chloride flux of chloride concentration which would be a straightforward consequence of such a mechanism, this chloride flux might be expected to be sensitive to competitive inhibition by sulfate like that shown in Fig. 2. However, we have also found that 70 mM  $(\text{NH}_4)_2\text{SO}_4$  inhibited chloride fluxes at physiologic and at very low pH values, but not at intermediate values, and we may speculate that the mechanism of inhibition is different at low and high pH values. At normal pH values, the inhibition may be due to the common basis of chloride and sulfate carriers (Gunn, 1972) while at very low pH the inhibition by sulfate may result from competition for co-transported species, possibly  $\text{H}^+$ . Sulfate flux decreases with pH below a peak at pH 6.3 (Schnell et al., 1973) and it may be presumed that the number of sulfate carriers also decreases below pH 6.3. In a like manner any chloride flux via the sulfate carriers would be expected to decrease with pH, but this was observed only down to pH 5.1 and not below.

The effects of inconstant internal pH are probably not responsible for the unusual concentration dependence of the flux shown in Fig. 2, nor are the changes in internal chloride concentration sufficient to account for the increasing flux below pH 5. The intracellular pH of the cells during the experiments shown in Fig. 2 may be estimated from the chloride ratio between cell water and medium in the steady state. In contrast to cells at physiologic pH where the net charge of intracellular buffers is negative, at low pH the net charge is positive and these cells have a higher concentration of chloride in the cell water than in the medium and thus, a higher intracellular pH than the medium if hydroxyl and chloride ratios are equal in the steady state. Specifically  $\text{pH}_{\text{in}} - \text{pH}_{\text{out}} = \log r_{\text{Cl}}$ . The chloride ratio ranged from 1.21 at highest chloride concentration  $\{(\text{Cl}_{\text{in}}) = 330 \text{ mmol/liter}\}$  to 1.57 at  $(\text{Cl}_{\text{in}}) = 19 \text{ mmol/liter}$  cell water and so the  $\text{pH}_{\text{in}} - \text{pH}_{\text{out}}$  ranged from 0.2 at 19 mM to 0.08 at the highest chloride concentrations. Using the pH dependence from Fig. 1, which reflects the parallel changes of both internal and external pH, the shift of  $\text{pH}_{\text{in}}$  from 5.23 at the highest chloride concentration in Fig. 2 to 5.25 at 19 mM chloride would not be expected to cause more than 10–20% change in the flux and would enhance the flux rather than reduce it from 270 to 10 meq/kg cell solid  $\cdot$  min. Thus, these variations in internal pH are both in the wrong direction and too small to influence the square dependence of the flux seen in Fig. 2. A similar calculation using the chloride ratios in Table I shows that the elevation in intracellular chloride with decreasing extracellular pH is insufficient to account for the increased flux below pH 5 shown in Fig. 1 if the proportionality coefficient between the flux and  $(\text{Cl}_{\text{in}})^2$  at pH 5.15 (Fig. 3) is the same at lower pH values.

The chloride fluxes in this paper have a rough correlation with cell water content,  $d$ . The data show that, for the most part, smaller cells have larger chloride fluxes, the exception being the results in Table I,  $K_{50}$ , pH 6.2. This relationship is probably fortuitous and arises from the relations between pH (Table I) and cell volume, or anion contents at different  $\text{NH}_4\text{Cl}$  concentrations (Table II) and cell volume, rather than from volume-dependent chloride fluxes. To examine this point the chloride efflux was measured from cells in 242 mM Na-acetate and 18 mM  $\text{NH}_4\text{Cl}$  which had very small volumes,  $d = 1.49$  kg cell water/kg cell solids (Table II,  $K_{57}$ ). These flux values were also low like those from cells with 20 mM  $\text{NH}_4\text{Cl}$  in  $K_{37}$  with much larger volumes,  $d = 2.28$ . A careful examination of the chloride flux at saturating chloride concentration and different cell water contents at physiologic pH by M. Dalmark (personal communication) showed no volume dependence of chloride self-exchange when  $d$  varied from 1.2 to 2.6 kg cell water/kg cell solids. The carrier-mediated chloride self-exchange and the new transport mechanism at low pH in human erythrocytes appear to be independent of cell volume in contrast with the volume-dependent cation conductances found in feline (Lieb, 1967) and canine erythrocytes (Parker and Hoffman, 1965). Since sulfate fluxes in cat erythrocytes were dependent on chloride concentration, the reported decrease in sulfate flux in shrunken cells may only reflect the larger NaCl concentrations concomitantly used to reduce their volume (Sha'afi and Pascoe, 1972).

#### *Phloretin*

Phloretin, the aglycone of phlorizin, is an inhibitor of many carrier transport systems in red blood cells. Hexose (LeFevre and Marshall, 1959), phosphate (Gerlach et al., 1964), urea, methyl urea, glycerol (Macey et al., 1972), and sulfate (Lepke and Passow, 1973) fluxes are all inhibited by phloretin. In addition, the rapid  $\text{Na}^+$  exchange fluxes of cat erythrocytes are inhibited by phloretin (Romualdez et al., 1972). Recently we reported that phloretin was a potent inhibitor of chloride self-exchange (Wieth et al., 1973). In addition, direct measurements of the membrane potential in amphiuma red cells, have shown that phloretin decreases chloride conductance in these particular red cells (Vestergaard-Bogind and Lassen, 1974).

The data in Fig. 4 show that the fractional inhibition of the flux by phloretin progressively decreased in low pH media. We have in fact observed negligible phloretin inhibition below pH 4.9. This is consonant with our interpretation that a non-carrier-mediated, phloretin-insensitive mechanism is an increasingly important component of the total self-exchange flux at lower pH values and particularly below pH 5. It is difficult to understand the effects of phloretin in a quantitative sense without some definitions and calculations. In this discussion we may consider chloride transport in erythrocytes as the sum result



of three parallel pathways: a carrier-mediated, electrically silent exchange pathway; a current-carrying or conductance pathway; and nonionic diffusion of HCl. The first two pathways may have overlapping molecular mechanisms. For example, current might be carried via the carrier system if unloaded charged carriers could cross the membrane. This would be a conductance pathway due to "slippage" of the usual carrier-mediated system. In addition to this complication, the carrier-mediated system appears to have at least two forms, one in which only a single anion equivalent (a monovalent anion) is loaded to a positive locus and a second in which two anion equivalents (a divalent anion) are loaded before transfer occurs. Whether in this latter state the transport is completely electrically silent (pure exchange pathway), or carries a net flux of charge equal to the anion flux across the membrane (pure conductance pathway), or carries a net flux of charge less than the anion flux (mixed pathway), remains to be determined. In the definition of the pure exchange pathway, it is not necessary that the charge of the chloride-carrier complex be zero, but only that no net charge (except a capacitance charge) can be transported by the carriers. The self-exchange flux of tracers which includes, of course, tracer flux through the electrically silent carrier mechanism as well as through the conductance pathway is approximately 600 meq Cl<sup>-</sup>/(kg cell solids · min) at pH 6.8 0°C. The conductance pathway contributes very little to this flux. If one extrapolates the measurements of Dalmark and Cass (personal communication), who determined net KCl fluxes of gramicidin-treated human red cells in isotonic media at 25 and 10°C, to 0°C, the apparent permeability to chloride ions would be  $4 \times 10^{-9}$  cm/s, which corresponds to a flux of 1.9 meq Cl<sup>-</sup>/(kg cell solids · min) in a medium with 160 meq Cl/liter. As shown in Fig. 4, the residual chloride flux in cells equilibrated with a saturated solution of phloretin at pH 6.8 was 1.3 meq Cl<sup>-</sup>/(kg cell solids · min). Although most of the reduction of the flux by phloretin was due to inhibition of the carrier-mediated transport, one cannot conclude from this that the fractional inhibition of the carrier-mediated transport is greater than the fractional inhibition of the conductance pathway. On the other hand, from three indirect clues we believe the fractional inhibition, if any, of the noncarrier pathway of human red cells is much less than that of the carrier pathway. These clues are the absence of phloretin inhibition of net flux of KCl from gramicidin-treated cells into a low ionic strength medium where admittedly the chloride conductance pathway was abnormally large (Wieth et al., 1973) and the small phloretin inhibition (2- to 10-fold) of the linearly independent portion of the flux of slowly transported anions such as sulfate (Gunn et al., 1974) and iodide (Gunn, in preparation). Consequently, the residual chloride flux in the presence of phloretin may largely reflect the characteristics of the noncarrier pathways. If this is the case, the data in Fig. 4 show that at pH 5.1 the noncarrier pathways were increased compared with pH 7.7 and suggest

that below pH 5.1 these pathways might have been the dominant transport modes for chloride crossing the erythrocyte membrane. Again, whether this flux at very low pH is a conductance pathway for free halide ions or a non-carrier-mediated HCl or (HCl)<sub>2</sub> transport cannot be fully resolved at this time.

One limitation of the titratable carrier model can be deduced from Fig. 5. The titration of the chloride carrier mechanism into a form which does not exchange chloride is effected in two ways. The maximum transport capacity at pH 5.5 (Fig. 5) compared with pH 7.4 (Gunn et al., 1973) is reduced and the chloride concentration at which the flux is half maximum is increased. Phenomenologically the interaction of hydronium ions with the transport appears to be by uncompetitive inhibition i.e. causing both alteration of  $M_{\text{maximum}}$  and  $K_{1/2}$ . In this respect the competitive inhibition postulated in the titratable carrier model is a simplification and the pH range of applicability for that model has been exceeded at pH 5.5 and lower values.

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