

# Titration of Transport and Modifier Sites in the Red Cell Anion Transport System

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**ABSTRACT** This work demonstrates the existence of titratable transport and modifier sites in the anion transport system of human red cells. Effects of alkaline extracellular pH on chloride exchange were studied up to pH 13 at 0°C. The studies revealed two sets of reversibly titratable groups. One set, having a  $pK$  of  $\sim 11$ , appeared to be identical with the inhibitory halide-binding modifier site. Deprotonation of this site stimulated anion transport. The apparent dissociation constants of chloride and iodide at this modifier site were 0.3 and 0.06 M, respectively, and it was confirmed that the organic sulfonate NAP-taurine inhibits anion transport reversibly by a high-affinity interaction with halide-binding modifier sites at the extracellular side of the membrane. Other groups, with apparent  $pK$  of  $\sim 12$  at chloride concentrations above 0.1 M, were named as "transport sites" because transport function depended totally on their protonation. The apparent  $pK$  decreased when extracellular halide concentration was lowered below 0.1 M. It was independent of the intracellular chloride concentration, and was equally sensitive to extracellular chloride and iodide. Inhibition by deprotonation, which was followed to an extracellular pH of 13, was fully reversible. Hydroxyl ions were not transported to an appreciable extent by the anion exchange system. The  $pK$  values of both sets of groups make it likely that they are both arginyl residues, functioning as anion recognition sites similar to the role of functionally essential arginyl residues observed with numerous enzymes.

## INTRODUCTION

The exchange of monovalent anions through the red cell membrane is mediated by a large transmembrane protein with a molecular mass of  $\sim 10^5$ . The main evidence for the participation of specific amino acid residues in the translocation process has been derived from covalent modification of the transport protein (Cabantchik and Rothstein, 1974; Passow et al., 1975; cf. the review by Knauf, 1979). Although it is generally assumed that titratable

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groups of amino acid residues must play an essential role for anion translocation, conclusive information about the nature of the titratable residues in the region of the transport site has not yet been obtained from pH studies of anion exchange (Gunn and Fröhlich, 1980; Wieth et al., 1980). We now report that anion exchange can be studied at extremely alkaline extracellular conditions, provided the intracellular pH is initially neutral, and we demonstrate the existence of two classes of functionally important titratable groups, both being titrated at very alkaline extracellular pH values.

One set of groups has an apparent  $pK$  of 12 at  $0^{\circ}\text{C}$  and physiological ionic strength. The high  $pK$  value makes it likely that we are dealing with a guanidino group of an arginyl residue, as previously inferred from indirect evidence (Funder and Wieth, 1976). In the following article (Wieth et al., 1982), this hypothesis is further strengthened by studies of the irreversible modification of anion transport with phenylglyoxal, a reagent specific for the modification of arginyl residues in proteins (Takahashi, 1968).

A second class of groups with an apparent  $pK$  of  $\sim 11$  seems to be identical with the transport-modulating modifier site described by Dalmark (1976). Under conditions where transport is believed to be inhibited by binding of anions to the alleged modifier sites, removal of protons from these groups with an apparent  $pK$  of 11 stimulated chloride exchange. By titration we were thus able both to eliminate the self-inhibition of chloride transport, found at high chloride concentrations, and to eliminate inhibition ascribed to the binding of ions with higher affinity for the modifier sites like iodide (Dalmark, 1976) and extracellular NAP-aurine (Knauf et al., 1978a). Our findings suggest that the modifier sites must be positively charged to interact with the inhibitory anions.

#### METHODS

##### *Preparation of Red Cells and Resealed Ghosts*

Freshly drawn heparinized human blood was centrifuged, the plasma and buffy coat were removed, and the red cells were resuspended and washed in a 165-mM KCl solution. Erythrocytes for experiments were titrated with  $\text{CO}_2$  to pH 7.2 at  $0^{\circ}\text{C}$  in the KCl medium. When the desired pH had been reached, the bicarbonate formed during titration was removed by repeated washings in the unbuffered KCl medium. The preparation of resealed ghosts with intracellular KCl concentration varying from 16.5 to 600 mM followed the protocol given by Funder and Wieth (1976). The intracellular phase of the ghosts, containing  $\sim 10$  g hemoglobin per liter and 2 mM Tris, had an intracellular pH of 7.2 and a buffer capacity of  $3.5 \times 10^{-3} \text{ mol} \cdot \text{l}^{-1} \cdot \text{pH}^{-1}$ . After the final washings, cells or ghosts were resuspended to a hematocrit of 30–40%, labeled with  $^{36}\text{Cl}$  ( $0.5 \mu\text{Ci}/\text{ml}$  suspension), and packed by centrifugation for 10 min at 40,000  $g$  in slender nylon tubes (3-mm inside diameter). The extracellular volume trapped in the packed cell column was  $\sim 2\%$  (erythrocytes) and  $\sim 8\%$  (ghosts) as estimated by the inulin space.

##### *Electrolyte Media and Chemicals*

Chloride efflux experiments were carried out in a 165-mM KCl medium, buffered with 1 mM  $\text{KH}_2\text{PO}_4$  and 5 mM CHES (cyclohexylaminoethanesulfonic acid; Cal-

biochem-Behring Corp., Switzerland). The buffers were not needed to keep the pH constant at high hydroxyl ion concentrations (cf. Fig. 3). Therefore, we were able to demonstrate that the chloride fluxes were not affected by the presence or absence of the extracellular buffer anions (cf. Fig. 2). The experiments of Fig. 4 were carried out in a medium containing 330 mM KCl. In the experiment of Fig. 5, the flux medium contained 60 mM KI and 105 mM KCl. The ghosts used contained 165 mM KCl, but the iodide influx is so slow compared with the rate of chloride exchange that virtually no chloride-iodide exchange took place during the short experiments.

In the experiments of Figs. 7 and 8, the chloride concentration was varied at the external side of the membrane only, whereas the intracellular chloride concentration was kept at 165 mM. The media for these experiments were prepared by substituting appropriate fractions of the 165-mM KCl medium with a chloride-free citrate-sucrose medium containing 25 mM potassium citrate, 200 mM sucrose, and 5 mM CHES. This medium is isotonic with, and has approximately the same ionic strength as, the 165-mM KCl medium. In other experiments (Fig. 9), the KCl concentration was varied between 16.5 and 82.5 mM KCl on both sides of the membrane. The media for these experiments were buffered with 2 mM Tris (the same concentration as found in the ghosts after resealing).

NAP-aurine [*N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate; Pierce Chemical Co., Rockford, Ill.] was kept in the dark, and the flux experiments of Fig. 7 were carried out in a light-shielded vessel to prevent photoactivation of the reactive aryl azide group.

<sup>36</sup>Cl with a specific activity of 0.4–0.6 mCi/mmol was purchased from The Radiochemical Centre, Amersham, England.

#### *pH Measurements*

To ensure precise measurements at pH values as high as 13, the glass electrode (G202C, Radiometer, Copenhagen, Denmark) was calibrated at 0°C with certified National Bureau of Standards buffers: phosphate buffers (pH 6.98 and 7.53) and 0.01 M borax buffer (pH 9.46). In the extreme alkaline range the electrode was tested in a solution of Ca(OH)<sub>2</sub> and saturated at 25°C, having a pH of 13.42 at 0°C (Bates, 1964). During the study we have noted that several calomel electrodes with a ceramic plug liquid junction have a considerable diffusion potential (up to 5 mV) in the borate buffer and in alkaline citrate solutions. The diffusion potential could easily be recognized by its sensitivity to stirring, and electrodes with a minimum error were selected by measuring them against a calomel electrode with an open liquid junction (K102, Radiometer, Copenhagen, Denmark).

#### *Flux Experiments*

The efflux of radioactive chloride from labeled cells or ghosts was initiated by injecting a packed cell sample (usually ~200 mg) into 30 or 40 ml of the well-stirred, thermostated electrolyte medium, which had been titrated to the appropriate pH with 1 M KOH immediately before use. Serial sampling of the cell-free extracellular solution was done with the Millipore-Swinnex technique described by Dalmark and Wieth (1972). Seven samples (including an equilibrium sample) were taken at appropriate intervals for the determination of the extracellular accumulation of radioactivity as a function of time. The rate coefficient of chloride efflux ( $k$ , s<sup>-1</sup>) was calculated by linear regression analysis of  $\ln(1 - a_t/a_\infty)$  vs. time ( $t$ ),  $a_t$  being the activity in the extracellular compartment at time ( $t$ ), and  $a_\infty$  the activity at isotopic equilibrium (counts per minute per milliliter). The unidirectional chloride exchange

flux was calculated from the relation

$$J = k \cdot (V/A) \cdot C_A \text{ mol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}, \quad (1)$$

where  $V$  is the solvent volume of the red cells or ghosts,  $A$  is the mean surface area ( $1.42 \times 10^{-6} \text{ cm}^2 (\text{cell})^{-1}$ ; cf. Funder and Wieth, 1976), and  $C_A$  is the intracellular chloride concentration in mol ( $\text{cm}^3 \text{ cell water})^{-1}$ . Eq. 1 is only correct in a case where intracellular chloride is an insignificant amount of the total chloride content of the two compartments. In experiments where the efflux of chloride was measured from ghosts containing 165 mM chloride into media containing 16.5 mM halide (Figs. 7, 8, and 11), intracellular chloride amounts to 5–10% of the total, and appropriate corrections of the rate coefficient were made before calculating the flux.

Fluxes have been conveniently related to dry weight of erythrocytes and expressed in millimoles per kilogram cell solids $^{-1} \cdot \text{min}^{-1}$  in work with red cells. The amount of red cells containing 1 kg cell solids corresponds to 2.7 liters of red cells with a normal volume of  $87 \mu\text{m}^3$ , i.e.,  $3.1 \times 10^{13}$  cells. This reference unit was introduced to allow comparison of the transport in ghosts with the transport of the original red cells (Funder and Wieth, 1976). When working with fluxes in ghosts without the need of comparing the results to red cell fluxes, the reference unit of  $3.1 \times 10^{13}$  cells is awkward. In such work, therefore, we have used the unit  $\text{mol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  (Wieth, 1979). For the convenience of the reader we have listed appropriate interconversion factors between the different flux units in Table I. A typical chloride exchange flux at  $0^\circ\text{C}$  of  $300 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  equals  $792 \text{ mmol} (3.1 \times 10^{13} \text{ cells} \cdot \text{min})^{-1}$ .

TABLE I  
FACTORS FOR INTERCONVERSION OF ERYTHROCYTE FLUX UNITS

	$\text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$	$\frac{\text{mmol}}{(3.1 \times 10^{13} \text{ cells} \cdot \text{min})^{-1}}$	$\frac{\text{mmol}}{(\text{liter cells} \cdot \text{min})^{-1}}$
$\text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$	1	2.64	0.98
$\text{mmol} (3.1 \times 10^{13} \text{ cells} \cdot \text{min})^{-1}$	0.38	1	0.37
$\text{mmol} (\text{liter cells} \cdot \text{min})^{-1}$	1.02	2.69	1

To convert a value of flux given in a unit recorded in the extreme lefthand column of the table, find the desired unit in the upper row. At the junction of row and column is the factor by which the given value must be multiplied to convert it into the new unit.

### Model Calculations

Two classes of titratable sites were identified by our kinetic studies of red cell anion transport: (a) transport sites essential for the transfer of anions, and (b) modifier sites, which regulate transport capacity by arresting the anion flux when occupied by regulatory anions (Dalmark, 1976). In this work we present the evidence that both these functionally characterized sites in fact exist as separate titratable entities. The pH dependence of the chloride exchange is characterized by apparent dissociation constants for transport and modifier sites,  $K_{ts}$  and  $K_{ms}$ , respectively. Our results show that protonation of the so-called transport site is essential for the function of the anion transport system. Although we have found that  $\text{p}K_{ts}$  is very sensitive to the extracellular anion concentration (Fig. 10), our studies do not permit conclusions regarding the precise functional role of the titratable group. It is even likely that other groups in the transport site region of the band 3 protein participate in the anion recognition and translocation at the transport site.

The titration of the two classes of groups is exemplified in the Discussion section (Fig. 12). Here we present the phenomenological equations, extending the transport model of Dalmark (1976) to comprise the titration of the two alkaline groups, thus describing the exchange flux at pH values above 7.5:

$$J^{\text{Cl}} = J^{\circ} \left\{ 1 + \frac{K_A}{\text{Cl}} \left( 1 + \frac{\text{I}}{K_i} \right) \right\}^{-1} \frac{\text{H}^+}{\text{H}^+ + K_{\text{ts}}} \cdot \left\{ 1 + \left( \frac{\text{Cl}}{K_{\text{AA}}} + \frac{\text{I}}{K_{\text{ii}}} \right) \frac{K_{\text{ms}}}{K_{\text{ms}} + \text{H}^+} \right\} \left\{ 1 + \frac{\text{Cl}}{K_{\text{AA}}} + \frac{\text{I}}{K_{\text{ii}}} \right\}^{-1}, \quad (2)$$

where  $J^{\text{Cl}}$  is the experimentally measured flux,  $J^{\circ}$  is a theoretical maximum flux defined below, and  $\text{H}^+$  is the extracellular hydrogen ion concentration. The degrees of protonation of the transport and modifier sites at a given pH are determined by the dissociation constants  $K_{\text{ts}}$  and  $K_{\text{ms}}$ . The affinities of chloride (Cl) and of an inhibitor anion (I) for the transport site are expressed by the dissociation constants  $K_A$  and  $K_i$ , and the corresponding affinities for the modifier site by the apparent dissociation constants are written as  $K_{\text{AA}}$  and  $K_{\text{ii}}$ , following the principles of the nomenclature of Dalmark (1976).

$J^{\circ}$  is the theoretical maximum flux that would be seen in a hypothetical situation where all transport sites are protonated and saturated with chloride in the absence of any binding of anions to the modifier sites. The equally theoretical flux,  $J_{\text{Cl}}^{\circ}$ , characterizes the unsaturated transport system, when chloride ions, due to their low concentration or to the presence of a competitive inhibitor, occupy only a fraction of the transport sites.  $J_{\text{Cl}}^{\circ}$  is defined by Eq. 3:

$$J_{\text{Cl}}^{\circ} = J^{\circ} \left\{ 1 + \frac{K_A}{\text{Cl}} \left( 1 + \frac{\text{I}}{K_i} \right) \right\}^{-1}. \quad (3)$$

By comparison of Eqs. 3 and 2, one can see that  $J_{\text{Cl}}^{\circ}$  is the theoretical chloride flux that would be obtained at a given chloride and inhibitor concentration if all transport sites were protonated and no modifier sites were occupied by inhibitory anions. When  $K_{\text{AA}}$  and  $K_{\text{ii}}$  are known,  $J_{\text{Cl}}^{\circ}$  can be calculated from the flux ( $J_{\text{H}}^{\text{Cl}}$ ) measured at a pH that is sufficiently low to ensure that the transport sites for all practical purposes are fully protonated:

$$J_{\text{Cl}}^{\circ} = J_{\text{H}}^{\text{Cl}} \left( 1 + \frac{\text{Cl}}{K_{\text{AA}}} + \frac{\text{I}}{K_{\text{ii}}} \right). \quad (4)$$

In the experiments shown in Figs. 1, 4, 5, and 6, where a significant increase of the chloride flux is assumed to be due to the release of hydrogen ions from the purported modifier site,  $J_{\text{Cl}}^{\circ}$  is the maximum value that would be attained at the given chloride concentration after a complete release of anions from the modifier sites. As expounded in the Discussion (Fig. 12), the maximum is not attained because dissociation of hydrogen ions at the transport site converts the latter into a nontransporting form at high pH values.

Evaluation of the values of  $K_{\text{ts}}$  and of  $K_{\text{ms}}$  from the experimental results was done as follows. Defining  $\text{p}K_{\text{ts}}$  and  $\text{p}K_{\text{ms}}$  as the negative  $\log_{10}$  of the  $K$  values,  $\text{p}K_{\text{ts}}$  could be estimated directly from a graph depicting  $J^{\text{Cl}}$  vs. pH. It was clear that  $\text{p}K_{\text{ms}}$  was at least one pH unit lower than  $\text{p}K_{\text{ts}}$ . Therefore, chloride transport could be assumed not to be significantly affected by anion binding to modifier sites in the most alkaline

pH range, and only a small error was introduced by taking as the  $pK_{ts}$  that pH value where  $J^{Cl} = J^{Cl}_{max} \cdot 0.5$ . Using the value for  $K_{AA}$  reported by Dalmark (1976), which in the case of chloride has since been confirmed by several other research groups,  $pK_{ms}$  could be calculated in the cases where the flux stimulation at alkaline pH values is sufficiently large to give a distinct maximum of the exchange flux before it decreases due to the titration of the transport site. By differentiating  $J^{Cl}$  vs.  $H^+$  in Eq. 2, it was found that the hydrogen ion concentration at which  $J^{Cl}$  has its maximum,  $H^+ (J^{Cl}_{max})$ , is a function of  $K_{ts}$ ,  $K_{ms}$ , and of the ratio  $Cl/K_{AA} = R$ ,

$$H^+ (J^{Cl}_{max}) = \frac{K_{ts} K_{ms}}{RK_{ms} - K_{ts}} \pm \left\{ R(K_{ts} K_{ms} + RK_{ts} K_{ms} - K_{ts}^2) \right\}^{1/2} \left( \frac{K_{ms}}{RK_{ms} - K_{ts}} \right). \quad (5)$$

Therefore, when as in the experiments of Figs. 4 and 5 the maximum was not too broad, the value of  $pK_{ms}$  could be found when the flux maximum was located. In a case such as that of Fig. 1, where the maximum is shallow and not well defined by the experimental data, a  $pK_{ms}$  value was estimated using the knowledge that the flux increase starts one pH unit below  $pK_{ms}$ . The graph fitting the experimental data was subsequently constructed by changing the  $pK_{ms}$  value stepwise until the best fit was obtained.

The value of  $pK_{ts}$  could be determined in all types of experiments reported in this study, whereas determination of  $pK_{ms}$  was only feasible under conditions where inhibition by anion binding to modifier sites was fairly pronounced. The reproducibility of repeated determinations of  $pK_{ts}$  on the same batch of cells was about 0.1 pH unit, whereas a day-to-day variation of up to 0.3 pH was found with different preparations of cells from the same donor.

#### RESULTS

It has been previously shown that the chloride exchange flux between resealed ghosts and a 165-mM KCl medium is constant between pH 7.2 and 10.8 at 0°C. Irreversible membrane damage took place when the pH was increased further in those experiments where hydroxyl ions are at equilibrium across the red cell membrane (Funder and Wieth, 1976). In the present study, resealed ghosts (or intact red cells) with an initial intracellular pH of 7.2 were exposed to alkaline electrolyte media, thus establishing a hydroxyl ion gradient across the membrane. It was found that the membranes tolerated exposure to extremely alkaline pH values long enough to permit the determination of anion exchange fluxes, as evidenced by the complete reversibility of the pH effects shown below (Fig. 2).

Virtually identical results were obtained with intact red cells and resealed ghosts. Fig. 1 illustrates the results of a typical series of pH experiments. Chloride self-exchange was determined at 0°C as a function of *extracellular* pH in resealed ghosts suspended in a 165-mM KCl medium. The flux was constant between pH 7.2 and 10 and increased by 15% at pH 11 before decreasing steeply at even higher pH values in a fashion resembling a titration curve. A small increase of the flux at pH values of 11–11.5 was observed in repeated experiments and formed the basis for the following working hypothesis: the transport system contains two types of anion-binding groups that can be titrated in the alkaline pH range from the extracellular side, modifier sites,

and transport sites. The chloride exchange flux is stimulated when the modifier site with a  $pK$  of  $\sim 11$  releases a proton and loses its ability to bind an inhibitory anion. Titration of another group in the transport system with a  $pK$  of  $> 11$  inhibits transport function by removing a proton that is necessary for normal transport function. The titration ranges of the two groups overlap, so that the full stimulation due to the removal of a proton from the group with the lowest  $pK$  is not seen, because transport decreases when the high- $pK$

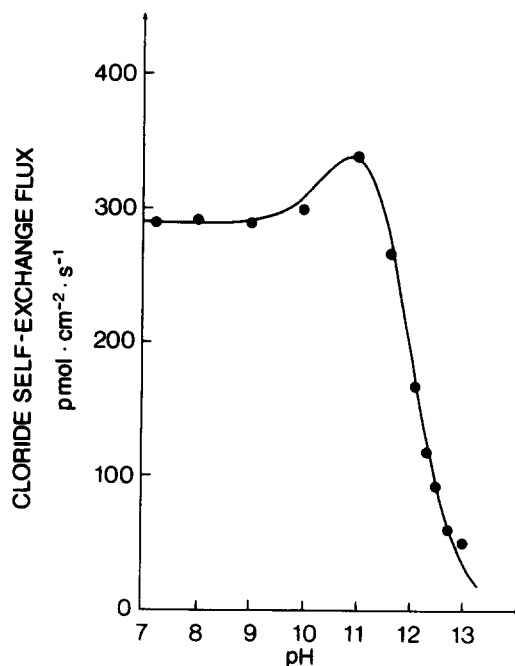


FIGURE 1. Extracellular titration of chloride self-exchange in resealed ghosts at  $0^{\circ}\text{C}$  in the presence of 165 mM chloride. Ghosts: initial intracellular pH 7.2, KCl 165 mM. Medium: 165 mM KCl buffered with phosphate and CHES. The curve is calculated from Eq. 2 with  $pK_{ts}$  11.9 and  $pK_{ms}$  10.8, as found by analysis as described in Methods. The theoretical maximum chloride exchange flux at 165 mM ( $J_{Cl}^0$ ) was  $435 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ , using the value of Dalmark (1976) for the chloride affinity for a modifier site ( $K_{AA} = 335 \text{ mM}$ ). The pH optimum of the flux,  $J^{Cl}$ , was 10.9 according to Eq. 5.

group is titrated. Assuming that the group with the lowest  $pK$  is identical with the halide-binding modifier site introduced by Dalmark (1976), this hypothesis is amenable to experimental testing. Before doing so, it was necessary to document the reversibility of the pH effect on transport, and to examine the membrane permeability to hydroxyl ions under the extreme gradients used in the present study.

Fig. 2 illustrates the complete reversibility of the inhibitory alkaline extracellular titration effect. The figure shows two control experiments performed

with intact red cells at extracellular pH values of 7.8 and 13. At the latter pH value, the rate coefficient of chloride exchange was only 7% of the value found at pH 7.8, in agreement with the results reported in Fig. 1. The experiment at pH 13 was repeated, but after ~30 s the pH was changed from 13 to 7.7 by the addition of HCl. The rate of chloride exchange increased instantaneously to a value within 5% of that of the cells that had not been exposed to the alkaline medium, permitting the conclusion that the extracellular alkaline titration effect on chloride transport is fully reversible.

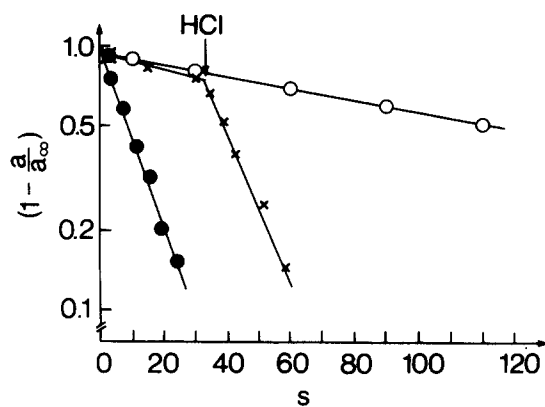


FIGURE 2. Complete reversibility of the alkaline inhibition of chloride exchange in intact erythrocytes at 0°C.  $^{36}\text{Cl}$  efflux into a 165-mM KCl medium buffered with 2 mM phosphate was followed at 0°C as a function of time at pH 7.8 (●) and pH 13 (○). In the third experiment (×), the efflux was followed at pH 13 for 33 s, then the pH was suddenly changed to 7.7 by the addition of 0.35 ml 1 M HCl to 30 ml of the suspension (arrow marked HCl on the graph). The rate coefficient changed immediately to a value of  $0.07 \text{ s}^{-1}$ , identical to that found with cells that had not been exposed to the alkaline pH. The ordinate scale is logarithmic,  $a$  is the concentration of radioactivity in the extracellular medium at the time of sampling, and  $a_{\infty}$  is the corresponding concentration after isotonic equilibrium has been attained.

It is not possible to decide from the present experiment whether the slow  $^{36}\text{Cl}^{-}$  efflux at pH 13 represents an exchange of intracellular chloride for extracellular hydroxyl ions. A bicarbonate-facilitated transfer of hydroxyl ions through the so-called Jacobs-Stewart cycle (Jacobs and Stewart, 1942) can be excluded at pH 13. The  $pK_2$  of carbonic acid at 0°C and an ionic strength of 0.17 is 10.3, so almost all (99.8%) of the  $\text{CO}_2$  taken up from the atmosphere will be present in the solution as carbonate ions. However, it is still possible that a fraction of the radioactive chloride released slowly at the alkaline pH is in fact exchanged with extracellular hydroxyl ions. Therefore, we measured the hydroxyl ion influx under similar conditions in a separate experiment. The result is shown in Fig. 3. Red cells with an intracellular pH of 7.1 were suspended in an unbuffered 165-mM KCl solution with an extracellular pH of 12.4, corresponding to a hydroxyl ion concentration of 2.9 mM. The



hematocrit of the suspension was 1.3%, and the hydroxyl ion influx was calculated from the change of extracellular pH with time (Fig. 3 A). Fig. 3 B shows that the amount of hydroxyl ions taken up by the cells was an almost linear function of time, and corresponded to an influx of  $3.4 \text{ pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ , only  $\sim 1\%$  of the maximum chloride exchange found in the experiment shown in Fig. 1. One can calculate from the data of Fig. 3 how much the intracellular

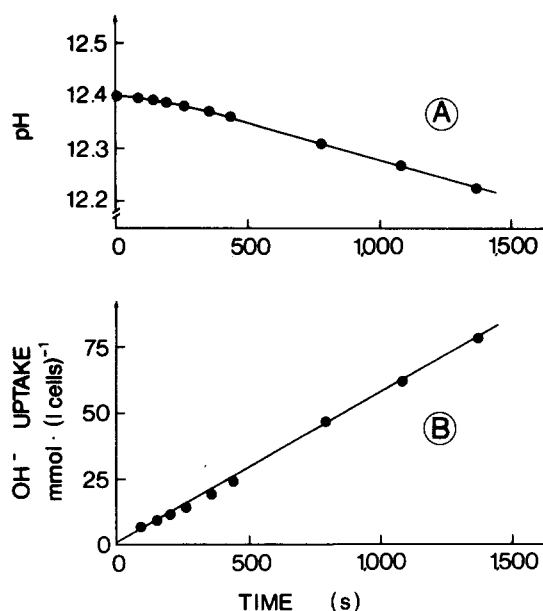


FIGURE 3. Hydroxyl ion uptake into intact erythrocytes at pH 12.4. Human erythrocytes (with an intracellular pH of 7.1) were suspended at a hematocrit of 1.3% in an unbuffered 165-mM KCl medium at  $0^{\circ}\text{C}$ , initial extracellular pH 12.4. (A) pH change vs. time. (B) Calculated hydroxyl ion uptake per liter cells. The buffer capacity of the cells was  $44 \text{ mmol} (\text{liter cells} \cdot \text{pH})^{-1}$  in the range 7–11.5. The equilibration pH measured with a similar sample of lysed cells was 11.5, which indicates that the approach of pH towards equilibrium is very slow. The hydroxyl ion uptake was fairly linear with time:  $5.6 \times 10^{-5} \text{ mol} (\text{liter cells})^{-1} \cdot \text{s}^{-1} = 3.44 \times 10^{-12} \text{ mol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ .

pH will change with time. The hydroxyl ion influx corresponds to an uptake of  $3.4 \times 10^{-3} \text{ mol} (\text{1 cell min})^{-1}$ . With a cellular buffer capacity of  $4.4 \times 10^{-2} \text{ mol} \cdot \text{l}^{-1} \cdot \text{pH}^{-1}$  (determined by titrating the lysed cells), the intracellular pH change of intact cells is  $<0.1 \text{ pH unit per minute}$ . However, in resealed Tris-buffered ghosts with a buffer capacity per liter of  $3.5 \times 10^{-3} \text{ mol} \cdot \text{pH}^{-1}$ , the intracellular pH will change proportionately more when resealed ghosts are suspended in the alkaline solutions. However, even with the weakly buffered ghosts a few minutes will elapse before the intracellular pH is increased to the critical level above pH 10.8, where irreversible membrane damage occurs.

Having established that the titration effects are reversible, and that the chloride/hydroxyl exchange is not detrimental to the determination of chlo-

ride self-exchange fluxes, we proceeded to analyze the effect of pH on transport function. First of all, we subjected the stimulation of transport (thought to be caused by titration of a halide-binding modifier site) to experimental testing. We examined whether transport responds in a predictable way to an increase of chloride concentration and to the partial substitution of chloride with iodide. Fig. 4 shows that there was a marked increase of the stimulation of anion transport around pH 11 when the chloride concentration in ghosts and medium was increased to 330 mM. As described in the Methods section, the solid line in Fig. 4 was constructed with the following assumptions: the value

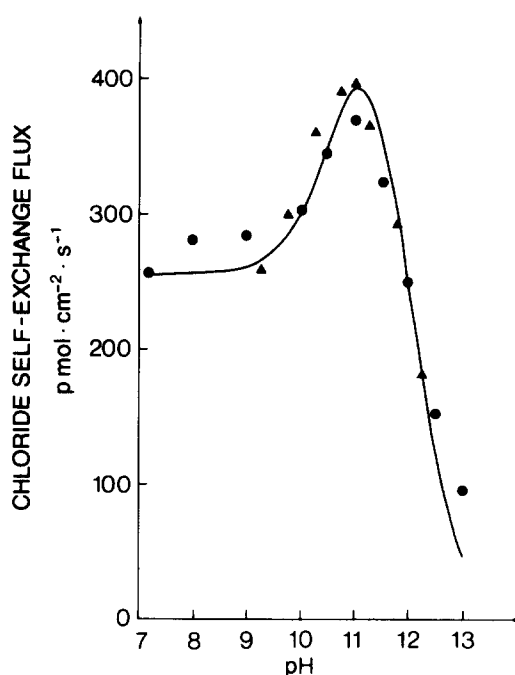


FIGURE 4. Extracellular titration of chloride self-exchange at 0°C in the presence of 330 mM chloride. Ghosts: initial intracellular pH 7.2, 330 mM KCl. Medium: 330 mM KCl buffered with phosphate and CHES. The curve was calculated from Eq. 2 with  $pK_{ts}$  12.0 and  $pK_{ms}$  10.7, as found by analysis as described in Methods. The theoretical maximum flux at 330 mM ( $J_{Cl}^0$ ) was 520  $\text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$  ( $K_{AA} = 335 \text{ mM}$ ). The pH optimum of the flux,  $J_{Cl}$ , was at pH 11.1 according to Eq. 5. Results, obtained on different days with two cell samples from the same donor, are represented by the different data symbols.

for the dissociation constant of chloride for the reaction with the modifier site ( $K_{AA}$ ) of 335 mM was taken from the results of Dalmark (1976), and the apparent  $pK$  values of the modifier ( $pK_{ms}$ ) and of the transport site ( $pK_{ts}$ ) were subsequently determined by calculating the best fit to the experimental results. The values determined for  $pK_{ms}$  of 10.7 and of  $pK_{ts}$  of 12 were not significantly different from the values of 10.8 and 11.9 used for the curve-fitting in Fig. 1.

The stimulation of chloride exchange with increasing pH seen in Fig. 4 is not due simply to the increase of ionic strength. This was shown by performing chloride-exchange experiments in media containing 105 mM chloride plus 60 mM iodide, the idea being that chloride self-exchange would be stimulated when iodide is released from the deprotonated modifier site. Iodide has a sevenfold higher affinity for the modifier site than chloride (Dalmark, 1976). As shown in Fig. 5, the experimental results were in agreement with the hypothesis. The theoretical curve in the figure was fitted with apparent  $pK$

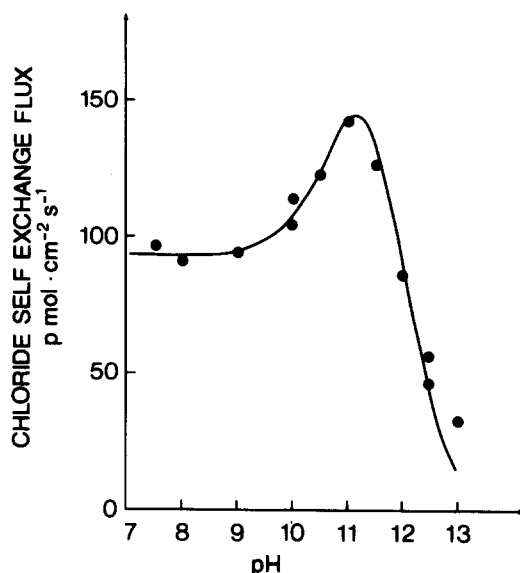


FIGURE 5. Extracellular titration of chloride self exchange at 0°C in the presence of 60 mM iodide and 105 mM chloride. Ghosts: initial intracellular pH 7.2, 165 mM KCl. Medium: 60 mM KI, 105 mM KCl, buffered with phosphate and CHES. The curve was calculated from Eq. 2 with  $pK_{ts}$  12.0 and  $pK_{ms}$  11.0 as found by analysis as described in Methods. The theoretical maximum flux ( $J_{Cl}^0$ ) was 216  $\text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ , assuming  $K_{AA}$  for chloride = 335 mM and  $K_{ii}$  for iodide = 60 mM (Dalmark, 1976). The value of  $J_{Cl}^0$  is low because of competition between chloride and iodide for transport sites.  $J_{Cl}^0$  in the absence of iodide was 400  $\text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ , as calculated from the chloride exchange of the same ghosts suspended at pH 7.5 in the 165-mM KCl medium.

values of 11.0 ( $pK_{ms}$ ) and 12.0 ( $pK_{ts}$ ), assuming dissociation constants of 335 and 60 mM, respectively, for the chloride and iodide interaction with the modifier site.

Knauf et al. (1978a) have shown that the organic sulfonate NAP-aurine has a strong inhibitory effect when interacting reversibly with the external side of the red cell membrane. Studies of the inhibitory effect as a function of chloride concentration permitted the conclusion that NAP-aurine inhibits anion exchange by binding reversibly to a halide-binding modifier site exposed to the extracellular medium. If this hypothesis is correct, removal of modifier

sites by titration ought to reduce the inhibitory effect of extracellular NAP-taurine on anion exchange. As shown in Fig. 6, this was in fact found to be the case. The inhibited flux increased markedly when the extracellular pH was increased from 7.5 to 11, as predicted by theory. The apparent  $pK$  values were 11.0 ( $pK_{ms}$ ) and 11.8 ( $pK_{ts}$ ), similar to those of the iodide experiment.

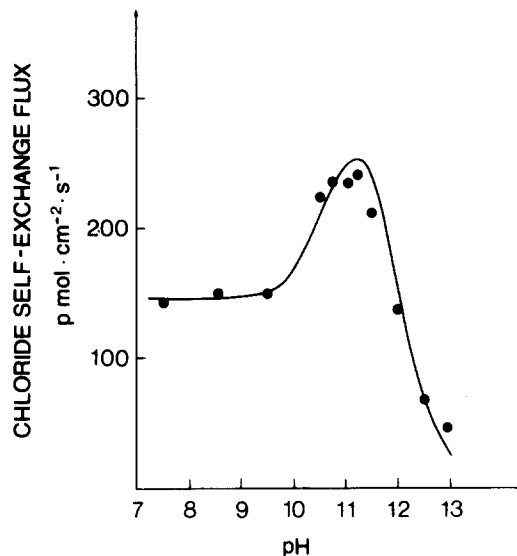


FIGURE 6. Extracellular titration of chloride self-exchange at 0°C in the presence of 100  $\mu$ M NAP-taurine and 165 mM chloride. Ghosts: initial intracellular pH 7.2, 165 mM KCl. Medium: 165 mM KCl buffered with phosphate and CHES, 100  $\mu$ M NAP-taurine. The curve was fitted to Eq. 2 with  $pK_{ts}$  11.8 and  $pK_{ms}$  11.0. The chloride self-exchange in the absence of NAP-taurine was 317  $\text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ , corresponding to a theoretical maximum flux,  $J_{Cl}^0$ , of 473  $\text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ . The pH optimum of the flux at pH 11.2 in the presence of NAP-taurine was used to determine  $pK_{ms}$  by means of Eq. 5. Knowing  $pK_{ts}$  and  $pK_{ms}$ , we could find the value of  $J_{Cl}^0$ , which would give the peak value found at pH 11.2. Thus determined,  $J_{Cl}^0$  in the presence of NAP-taurine was 430  $\text{pmol} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ , only slightly reduced compared with the value in the absence of NAP-taurine, confirming that the affinity of the organic sulfonate for the transport site is weak.  $K_{ii}$  for NAP-taurine calculated from the ratio of  $J_{Cl}^0/J_{Cl}$  at pH 7.5 was 66  $\mu$ M.

According to Dalmark (1976), the dissociation constant of chloride from the modifier site is 0.33 M. Consequently, interaction between chloride and modifier site should be minimal at low external chloride concentrations, and stimulation of chloride exchange should not be observed when extracellular pH is increased. The results of Fig. 7 show that this prediction was, indeed, fulfilled. The figure reveals another characteristic feature, a marked shift of the titration curve towards lower pH values when the extracellular chloride concentration was lowered to 16.5 mM, while maintaining an intracellular concentration of 165 mM KCl. The curve of Fig. 7 was fitted with a  $pK_{ts}$  of

10.8, which suggests that the apparent dissociation constant is a function of extracellular chloride. This was examined more closely in the experiments of Figs. 8 and 9. Fig. 8 shows the chloride exchange flux at various extracellular concentrations of KCl as a fraction of the flux found at pH 7.5. Ghosts containing 165 mM KCl were suspended in media containing either 165, 82.5, 33, or 16.5 mM chloride, KCl being gradually replaced with sucrose-citrate. The apparent  $pK$  of the titratable transport function decreased gradually when the chloride concentration of the medium was lowered, going from a value of 12.0 at 165 mM KCl to a value of 11.1 at 16.5 mM KCl. The chloride distribution across the membrane was asymmetrical when the external chlo-

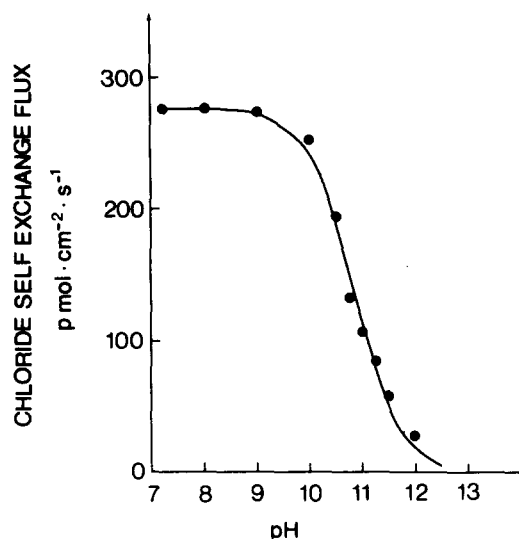


FIGURE 7. Extracellular titration of chloride self-exchange at 0°C in the presence of 16.5 mM extracellular chloride. Ghosts: initial intracellular pH 7.2, 165 mM KCl. Medium: isotonic citrate-sucrose medium with 16.5 mM KCl (cf. Methods). The curve was calculated from Eq. 2 with a  $pK_{ts}$  of 10.8. At the low-chloride concentration, the modifier site-mediated inhibition is negligible, and accordingly no estimate of  $pK_{ms}$  can be made. Note that there is no pH optimum of  $f^{Cl}$ .

ride concentration was below 165 mM. To examine whether this asymmetrical anion distribution was a determining factor for the anion dependence of the apparent  $pK$ , we also determined the pH dependence of the exchange fluxes under conditions where intra- and extracellular chloride concentrations were equal. The results, obtained with ghosts containing 16.5 and 82.5 mM chloride and shown in Fig. 9, demonstrate that the effect of external chloride on the apparent  $pK_{ts}$  was independent of both the concentration of internal chloride and of the asymmetrical anion distribution. The variation of  $pK_{ts}$  with anion concentration is summarized in Fig. 10. The figure shows that  $pK_{ts}$  increased linearly with  $\log(Cl)_o$  below an extracellular chloride concentration of  $\sim 100$  mM, with a slope of unity. The same slope was found whether the chloride

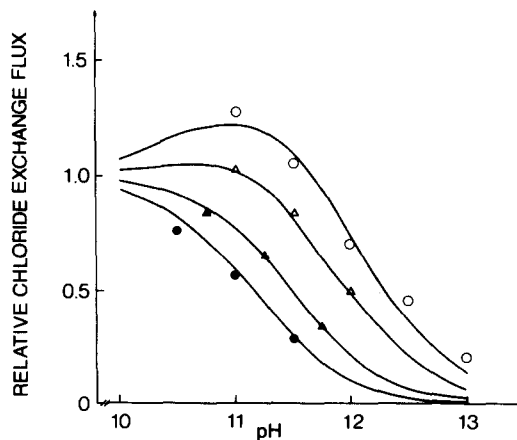


FIGURE 8. Extracellular titration of chloride self-exchange at 0°C at four extracellular chloride concentrations: 16.5 (●), 33 (▲), 82.5 (△), and 165 mM (○). Ghosts: initial intracellular pH 7.2, 165 mM KCl. The magnitudes of the fluxes were calculated relative to the flux at each chloride concentration at pH 7.5, taken to be unity. As predicted by Eq. 2, stimulation of the chloride exchange above this level is only seen at extracellular chloride concentrations high enough to affect the modifier site significantly. All graphs were fitted with a  $pK_{ms}$  of 10.7; the  $pK_{ts}$  values fitting the data were 11.1 (16.5 mM  $Cl^-$ ), 11.4 (33 mM  $Cl^-$ ), 11.8 (82.5 mM  $Cl^-$ ), and 12.0 (165 mM  $Cl^-$ ).

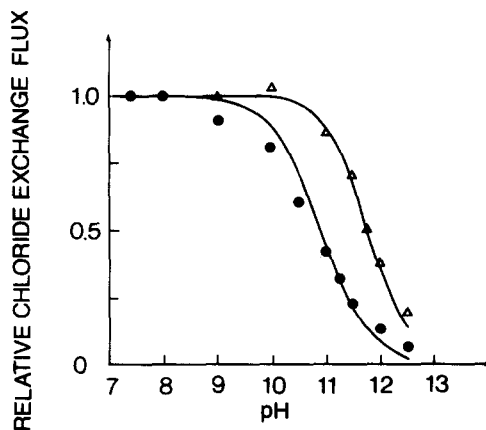


FIGURE 9. Extracellular titration of chloride self-exchange at 0°C at identical intra- and extracellular chloride concentrations. Ghosts: initial intracellular pH 7.2, 16.5 mM KCl (●); 82.5 mM KCl (△). Media: KCl concentrations as in ghosts, buffering with 2 mM Tris. The magnitudes of the chloride fluxes were calculated relative to the values found at pH 7.2: 102  $\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$  (at 16.5 mM chloride), and 251  $\mu\text{mol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$  (at 82.5 mM chloride). Both graphs were fitted with a  $pK_{ms}$  of 10.7; the  $pK_{ts}$  values fitting the data were 10.9 (16.5 mM chloride) and 11.6 (82.5 mM chloride).

distribution was symmetrical or asymmetrical. No further increase of  $pK_{ts}$  above a value of 12 was observed when the chloride concentration was raised from 165 to 600 mM on both sides of the membrane.

We finally turn to the possibility that the effect of chloride on the apparent  $pK$  of the transport function may be related to the affinity of the anions for the transport system, i.e., being caused by halide binding to the protonated group. If this was so, anions with a greater affinity than chloride ought to cause a larger displacement of the apparent  $pK$  under appropriate conditions. Dalmark (1976) showed that the apparent affinity of iodide is sevenfold larger than that of chloride. We have already demonstrated in Fig. 5 that the  $pK_{ts}$

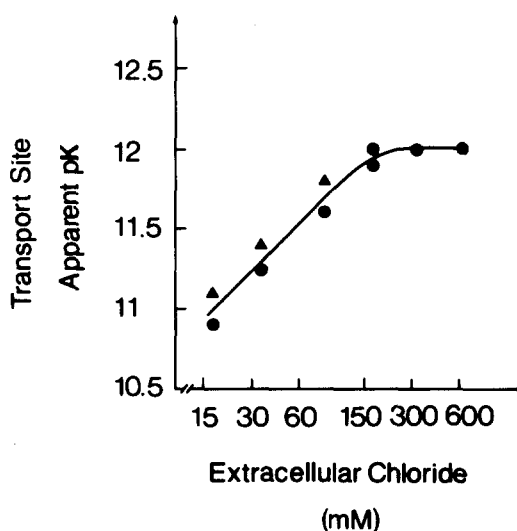


FIGURE 10. Variation of the apparent  $pK$  of the transport site ( $pK_{ts}$ ) with extracellular chloride concentration. The data indicated by triangles are results obtained with asymmetrical chloride distribution (Fig. 8). The data indicated by the filled circles are results obtained with identical KCl concentrations in ghosts and media (cf. Figs. 1 and 9). The scale of the abscissa is logarithmic. The solid line was fitted by hand with a slope of unity below an extracellular chloride concentration of 100 mM.

of  $\sim 12$  found in the presence of 165 mM chloride is not affected when 60 mM chloride in the extracellular medium is replaced by iodide. However, no conclusion can be made from this finding because the results of Fig. 10 demonstrated that the effect on  $pK_{ts}$  levels off at high anion concentrations. Therefore, it was necessary to perform experiments with iodide at low anion concentrations. If a chemical adsorption term were of decisive importance for the position of the titration curve, 8.25 mM iodide might cause a shift comparable to that caused by 50 mM chloride. Another problem to be dealt with is the recent finding by Milanick and Gunn (1981) that differences between apparent halide affinities disappear when anion transport is studied under conditions where the intracellular halide concentration is larger than

that of the extracellular phase. We have dealt with the above-mentioned problems, first by examining the effects of the halides at a total halide concentration of 16.5 mM, where the  $pK_{ts}$  is sensitive to changes of anion concentration, and secondly by performing experiments both with symmetrical and with asymmetrical anion distribution. The results are shown in Fig. 11. The extracellular halide concentration was 16.5 mM in all three experiments. The  $pK_{ts}$  was not appreciably changed from a value of  $\sim 10.9$ , when half of the chloride was replaced by iodide. This insensitivity of the  $pK$  did not depend on the anion distribution: the effects of pH on the relative

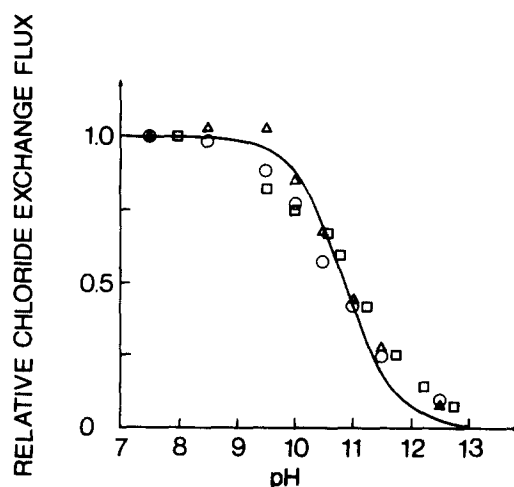


FIGURE 11. Lack of effect of iodide on the extracellular titration of chloride self exchange at  $0^{\circ}\text{C}$ . Ghosts: two of the experiments ( $\Delta$ ,  $\circ$ ) were performed with an intracellular KCl concentration of 165 mM, the ghosts for the third experiment ( $\square$ ) contained 8.3 mM KCl plus 8.3 mM KI. Media: 16.5 mM KCl plus citrate sucrose ( $\Delta$ ); 8.3 mM KCl and 8.3 mM KI plus citrate sucrose ( $\circ$ ); 8.3 mM KCl plus 8.3 mM KI ( $\square$ ). Note that the intra- and extracellular anion concentrations were identical in the latter experiment. The magnitude of the fluxes were calculated relative to the values found at pH 7.5. These reference fluxes were 187 ( $\Delta$ ), 70 ( $\circ$ ), and 29 ( $\square$ )  $\text{pmol}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ . The solid line shows the titration of a single group with a  $pK$  of 10.9.

exchange fluxes were identical in the experiment where the ghosts contained 165 mM chloride and in the one where both the intra- and extracellular chloride and iodide concentrations were 8 mM. The results of Fig. 11 thus demonstrate that it is the extracellular concentration rather than the chemical nature of the transported halide or the anion gradient across the membrane that determines the apparent  $pK$  of the titratable transport function.

#### DISCUSSION

The present report demonstrates the existence of two classes of functionally important chemical groups in the erythrocyte anion transport system that are titrated at alkaline extracellular pH values. The pH range above 11 has



previously been considered inaccessible to experimentation, but our results show that the frailty of resealed erythrocyte membranes to pH values above 10.8 at 0°C (Funder and Wieth, 1976) is due exclusively to the effect of alkalinity on membrane components facing the intracellular phase. Treatment of red cell membranes with NaOH releases a number of polypeptides localized to the cytoplasmic side of the membrane (Steck and Yu, 1973). The functional deterioration seen at intracellular pH values above 11 seems related to the "alkali-stripping" of peripheral membrane proteins.

It was possible to explore the effects of extracellular pH on transport functions between pH 7 and 13 when using erythrocytes and ghosts with an initial neutral intracellular pH. A pH of 13 corresponds to a hydroxyl ion concentration of almost  $10^{-2}$  M,  $K_w = C_{OH^-} \cdot C_{H^+}$  being  $10^{-14.94}$  at 0°C. The huge hydroxyl gradients create measurable fluxes of hydroxyl ions across the membranes, as shown in Fig. 3, but it is clear from experiments such as that shown in Fig. 2 that no irreversible membrane damage occurs during experiments of a few minutes' duration.

Our interpretative working hypothesis is illustrated by the titration from alkaline to neutral pH values in the schematic diagram of Fig. 12. The figure illustrates titration at a high extracellular chloride concentration (330 mM), similar to the experiments shown in Fig. 4. As pH is decreased from pH 13, a proton acceptor with a  $pK$  of 12 binds hydrogen ions, forming a positively charged group by the reaction indicated by line 1 in Fig. 12. The flux increases proportionally to the protonation of this group, which is termed the transport site without any implication other than its necessity for transport functioning. The flux would continue to increase towards a theoretical maximum value of 1.0 ( $J_{Cl}^0$ ) if transport were not modulated by another group, the modifier site. This second group has an apparent  $pK$  of 11. It can bind an anion only when it is positively charged, and transport is blocked when the inhibitory anion has been bound. The titration of the modifier site is illustrated in line 2 in Fig. 12. In the present example we chose a chloride concentration of 0.33 M, the value of the dissociation constant for the functional interaction of chloride with a halide-binding modifier site reported by Dalmark (1976). When all modifier sites are protonated, i.e., below pH 9 in the example, the chloride flux is accordingly inhibited by 50% of the theoretical maximum value. The experimentally observable flux (line 3 in Fig. 11) is the product of the two other functions. It exhibits a maximum (in the present example at pH 11.3). The pH optimum is only conspicuous when the extracellular medium contains a sufficiently high concentration of anions that interact with the inhibitory modifier sites.

We only deal with the titration of the chloride flux above pH 7.5 in this article. If titration is carried on to more acid pH values, chloride transport decreases along with the titration of a third group with a  $pK$  of 6.1. This group is located at the intracellular side of the membrane (Wieth et al., 1980). In unpublished experiments (not shown), we have found yet another functionally important group that is titrated at low pH from the extracellular side of the membrane. The apparent  $pK$  of the latter group is 5.2 at 0°C. In the

present report we focus on the two sets of groups with apparent  $pK$  values of 12 and 11.

#### *A Titratable Anion Transport System*

The first extensive model of red cell anion transport, the fixed charge hypothesis, was based on evidence from studies of sulfate transport of the

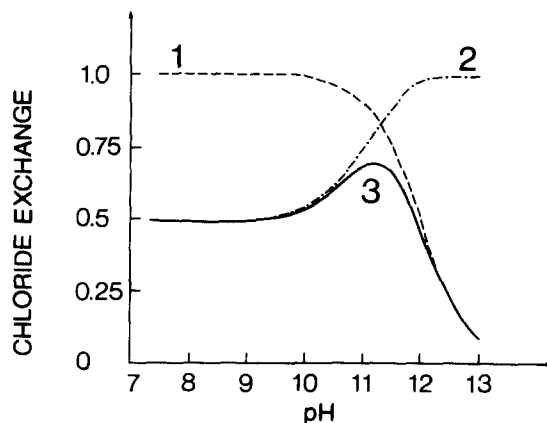


FIGURE 12. Schematic diagram interpreting the pH dependence of chloride exchange by means of two pH dependent functions: (1) titration of a transport site that permits transport only when protonated, and (2) titration of an inhibitory anion-binding modifier site, inhibiting transport only when anions are found to the protonated form. The ordinate shows the magnitude of the chloride exchange flux ( $J^{\text{Cl}}$ ) relative to a theoretical maximum flux,  $J^{\text{Cl}}_0 = 1$ . The following values were chosen for illustration: chloride concentration, 335 mM =  $K_{\text{AA}}$  (causing 50% inhibition, when all modifier sites are protonated),  $pK_{\text{ms}} = 11$ , and  $pK_{\text{ts}} = 12$ . The graph marked (1) illustrates titration of the transport site:

$$Y = \frac{H^+}{K_{\text{ts}} + H^+}$$

The graph marked (2) illustrates the titration of the modifier site:

$$Y = \frac{1 + \frac{\text{Cl}}{K_{\text{AA}}} \frac{K_{\text{ms}}}{K_{\text{ms}} + H^+}}{1 + \frac{\text{Cl}}{K_{\text{AA}}}}$$

The product of these two functions forms the normalized experimental flux ( $J^{\text{Cl}}/J^{\text{Cl}}_0$ ); cf. Eq. 2.

existence of titratable functional groups in the transport system (Passow, 1969). The demonstration of the inability of the red cell anion transport system to carry a net current of chloride ions (Chappel and Crofts, 1966; Hunter, 1971; Lassen, 1972), together with the first detailed information about the kinetics of monovalent anion transport, gave rise to a mobile carrier

model that accounted for the selectivity and the exchange diffusion properties of the transport system (Wieth, 1971, 1972). The basic ideas of the model were derived from the carrier function of amphiphilic secondary amines in artificial membranes (Sollner, 1970). A hydrophobic membrane barrier had already been assumed in the latest version of the fixed charge hypothesis (Passow, 1969). The mobile carrier was now proposed to transfer the anion through the hydrophobic lipid membrane core after forming an electrically neutral pair with a cationic group of the carrier molecule, transfer of the unloaded carrier being prevented by the electrostatic work of introducing a charge in a low dielectric phase. Shortly thereafter, the mobile carrier model was expanded to account for the pH dependence of anion transport (Gunn, 1972; Gunn et al., 1973), and the bell-shaped graph relating chloride exchange flux in intact erythrocytes to pH was interpreted to indicate the existence of a transport site for monovalent anions, binding anions when protonated ( $pK$  8.8) and being inactivated after binding a second proton to a group with a  $pK$  of 6.9 (Gunn, 1973). It has since become clear that the titration curve of chloride transport in intact erythrocytes does not give a simple clue to the acid-base properties of the transport system (Gunn and Fröhlich, 1980; Wieth et al., 1980). Dalmark (1975) found that there was no decrease of flux with increasing pH in nystatin-treated, KCl-loaded erythrocytes, and finally studies of chloride transport in resealed ghosts conclusively demonstrated the absence of a functional titratable group between pH 7.5 and 10.8 (Funder and Wieth, 1976). It became clear that the apparent titratability of chloride transport in intact erythrocytes below pH 10 was due solely to the decrease of intracellular chloride concentration accompanying the titration of intracellular buffers, and it was pointed out that if the anion binding site is a positively charged amino acid side chain, a guanidino group of an arginyl residue would be the likely candidate (Funder and Wieth, 1976). Today only the basic features of the mobile titratable carrier model remain. The role of the small mobile carrier molecule has been taken over by the large transmembrane band 3 protein. Many essential features of the fixed charge and the mobile carrier hypotheses have been amalgamated in recent models. Current models assume that single anions are alternately transported from one compartment to the other in a reciprocal cycle, involving a minor conformational change in the transport protein molecule in shifts making the transport pathway accessible to extra- and intracellular anions.

#### *The Modifier Site*

The experiments of Figs. 1 and 4 show that chloride transport can be stimulated at alkaline extracellular pH values. The increase represents a stimulation of chloride self-exchange, rather than the induction of a chloride-hydroxyl exchange imposed by the hydroxyl ion disequilibrium. The small influx of  $\text{OH}^-$  at pH 12.4 (Fig. 3) thus supports previous, more indirect evidence indicating that hydroxyl ions are not significantly transported by the exchange mechanism (Wieth et al., 1980).

We therefore adopted the working hypothesis that stimulation of anion

exchange is caused by the deprotonation and consequent inactivation of a modifier site, and we examined its possible relation to the halide-binding modifier site reported by Dalmark (1976). The inhibition of chloride transport at high chloride concentration has been ascribed to the existence of modifier sites, inactivating the transport mechanism when binding an anion. The kinetics of inhibition are analogous to the substrate inhibition frequently found in enzyme kinetics. The phenomenon of substrate inhibition is not in itself proof that an enzyme possesses a specific inhibitory binding site. Inhibition of urease activity at high substrate concentration may for instance be caused by the exclusion of a water molecule needed for the interaction at the catalytic site (Laidler, 1958). The present data, however, give strong evidence that separate modifier sites of the anion transport system, having an apparent  $pK$  of  $\sim 11$ , are exposed to and can be titrated from the extracellular side of the membrane.

It is conceivable that the large anion transport protein may contain a multitude of modifier sites that affect transport function indirectly, e.g., by conformational changes induced by the adsorption of ions to charged side chains of the protein. However, it is likely that the titratable modifier sites we studied are identical with the halide-binding modifier site described by Dalmark (1976), which again appears to be identical with the NAP-aurine binding modifier site described by Knauf et al. (1978*a* and *b*). In the case of halide inhibition, the dissociation constants determined by Dalmark for chloride (335 mM) and iodide (60 mM) are in good quantitative agreement with the data shown in the Figs. 4, 5, and 7. The present results also confirm and extend the conclusions of Knauf et al. (1978*a*) that NAP-aurine interacts with an external modifier site, which may be identical with the inhibitory halide-binding site. NAP-aurine is a potent inhibitor of chloride exchange, acting on the extracellular side of the membrane under conditions similar to those used in our experiments. The inhibitor site had a relatively low chloride affinity (Knauf et al., 1978*a*), which suggests that it might in fact be identical with the chloride modifier site, although the reported chloride affinity was slightly higher than that reported by Dalmark (1976). Our results (Fig. 6) show that the titration curve of the NAP-aurine modifier site is quite similar to that of the halide site, indicating at least that the sites have the same  $pK$ . The fact that the theoretical maximal chloride flux ( $J_{Cl}^0$ ) in the experiment of Fig. 6 was 80–90% of the value found in the absence of NAP-aurine confirms the notion that the affinity of NAP-aurine for the transport site is weak. Accepting a depression of  $J_{Cl}^0$  by 20% as a reasonable maximum estimate, the apparent dissociation constant of NAP-aurine for transport is not lower than 400  $\mu M$ , in good agreement with the value reported by Knauf et al. (1978*a*). The affinity for the modifier site is  $\sim 10$ -fold higher, with an apparent  $K_I$  of  $\sim 50 \mu M$  in the presence of 165 mM chloride (cf. legend to Fig. 6).

Knauf et al. (1978*b*) studied the location of covalently bound NAP-aurine after photoactivation of the azide. After proteolysis of covalently labeled band 3, the inhibitor was found in the same transmembrane 17,000 mol wt fragment that contains the covalent DIDS binding site. Reduced binding of NAP-

taurine is observed in DIDS-treated membranes, and, conversely, pretreatment with NAP-taurine largely prevents the binding of DIDS to band 3 (Cabantchik et al., 1976), which suggests that the two inhibitors either bind to the same site in the anion transport system or to two adjacent interacting sites. Our functional titration studies do not give any information pertaining to the location of the inhibitors, but certainly weigh in favor of the idea that the two classes of sites are discrete functional entities.

A natural question is whether the modifier site has any functional role. In the following section we discuss the possibility that both the titratable transport site and the modifier sites are side chains of arginyl residues. In line with the hypothesis "that arginyl residues constitute positively charged binding sites for enzymes acting on anionic substrates and for all proteins that interact with anions" (Riordan, 1979), it is of interest to note that separate arginyl residues function both at anion-binding regulatory sites and at anion-binding catalytic sites, respectively, in fructose-1,6-*bis*-phosphatase (Riordan et al., 1977). A further basis for speculations concerning the possible functional role of arginines as modifier sites is provided by studies of the enzymic dynamics and of the roles of arginines in the catalytic functioning of carboxypeptidase A (Nakagawa and Umeyama, 1978). This enzyme contains several functionally important arginyl residues. Arginine-145 functions as an anion-binding site in the catalytic center, whereas two other arginine residues (127 and 71) may act as initial binding sites, ensuring that the negatively charged substrate "slides" smoothly to the catalytic arginine-145 through the initial binding sites. As proposed by Knauf (1979), the topographically close relation of modifier sites and transport sites in band 3 may form the basis for a related mechanism, the modifier site directing the transported anion towards the translocation site. This verifies the prediction of Passow (1969) that fixed charges regulate the anion concentration at the rate-limiting barrier. Passow's hypothesis thus assigns a functional role to the modifier sites, and explains why transport and modifier sites are located to close to one another that binding of a probe to one site interferes with the binding of a different reagent to the other. The proposed arrangement may also provide structural basis for the functional asymmetry of the anion transport system as discussed in the following section.

#### *The Titratable Transport Site*

By our use of the term "transport site" we wish to imply only that functioning of the anion exchange mechanism is totally dependent upon the protonation of a group (or a class of groups) with an apparent  $pK$  of  $\sim 12$  at physiological anion concentration (Fig. 1). The amount of information available from the present type of experiments is limited by the fact that the alkaline titration can be carried out only at the extracellular side of the membrane. So although it is obvious that the titratable group is essential for transport, there is no evidence that we are dealing with a mobile anion-binding site. In fact it seems unlikely that anion translocation depends on the interaction of the transported anion with a single group. Thus, in the model of Passow et al. (1980), an

assembly of five positively charged amino acid residues is thought to constitute a nonconducting anion gate that permits the ion transfer during a concerted conformational change.

The acid-base properties of the titratable group may contain a clue to the nature of its interaction with anions. Below an extracellular chloride concentration of 0.1 M, the apparent  $pK$  decreased linearly with  $\log(Cl)_o$  when extracellular chloride was lowered (Fig. 10). Similar anion dependent equilibria have been observed with many proteins (Steinhardt and Reynolds, 1969). Most studies have been carried out on soluble proteins, whereas we are dealing with the phase equilibrium of groups located in the interface between a membrane and the extracellular medium. Therefore the anion-dependent acid-base properties of insoluble protein molecules, like those of the wool keratin fibers studied by Steinhardt and Harris (1940), may be particularly relevant to our problem.

Steinhardt and Harris (1940) observed that the binding of hydrogen ions by the wool protein depended strongly on the presence of anions. There was a close approach to stoichiometry in the relationship. The titration curve was displaced one pH unit for a 10-fold change of chloride concentration. The authors pointed out that there were two possible causes to be considered: (a) an effect on the chemical equilibria due to the coupling of chloride and of hydrogen ion binding through the mass-action law; and alternatively, (b) an effect of the external anions on an interfacial potential, determining the local hydrogen ion concentration in the environment of the titrated groups.

**INTERDEPENDENCE OF ANION AND PROTON BINDING** To be able to choose between the above-mentioned possible causes of the anion dependence of  $pK_{ts}$ , we will examine how the acid-base properties of a titratable chloride-binding group are affected by changes in anion concentration. The reactions to be considered are the chemical equilibria of a titratable carrier (Gunn, 1972, 1973). A group (S) is titrated with a proton ( $H^+$ ) to form the anion-binding group:



The reaction being characterized by its dissociation constant  $K_H$  ( $pK_H = -\log K_H$ ).

The protonated site can react with the transported anion ( $Cl^-$ ):

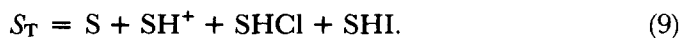


characterized by the dissociation constant  $K_A$ . Alternatively, the site can bind a competing anion ( $I^-$ ):



the latter reaction having the dissociation constant  $K_i$ .

The total number of sites ( $S_T$ ) is given by the sum of the four configurations:



The magnitude of the chloride-exchange flux is assumed to be proportional to

the fraction ( $F$ ) of  $S_T$  being in the transport-facilitating configuration:

$$F = \text{SHCl}/S_T = \left\{ 1 + \frac{K_A}{\text{Cl}} \left( 1 + \frac{I}{K_i} + \frac{K_H}{H^+} \right) \right\}^{-1}. \quad (10)$$

In analogy to Eq. 3 we define  $F_{\text{Cl}}^{\circ}$  as the maximum value of  $F$  obtained at given anion concentrations under conditions where the proton concentration is sufficiently high to eliminate a significant contribution by the term ( $K_H/H^+$ ) to the value of  $F$ :

$$F_{\text{Cl}}^{\circ} = \left\{ 1 + \frac{K_A}{\text{Cl}} \left( 1 + \frac{I}{K_i} \right) \right\}^{-1}. \quad (11)$$

Because  $pK_{ts}$  is defined as the pH at which  $F$  equals  $0.5 F_{\text{Cl}}^{\circ}$ , the relation between  $pK_{ts}$  and  $pK_H$  can now be derived from Eqs. 10 and 11:

$$pK_{ts} - pK_H = \log \left\{ \frac{2 \text{Cl}}{F_{\text{Cl}}^{\circ} K_A} - \left( 1 + \frac{\text{Cl}}{K_A} + \frac{I}{K_i} \right) \right\}. \quad (12)$$

It was not possible to demonstrate a satisfactory agreement between the experimental results and the predictions of Eq. 12. The results of Fig. 10

TABLE II  
EFFECT OF CHLORIDE BINDING ON  $pK_{ts}$  ACCORDING TO  
THE ANION-BINDING TITRATABLE CARRIER MODEL

Relative chloride concentration, $\text{Cl}/K_A$	$F_{\text{Cl}}^{\circ}$	$pK_{ts} - pK_H$
0.001	0.00099	0
0.01	0.0099	0.004
0.1	0.0909	0.04
1	0.50	0.30
10	0.909	1.04
100	0.990	2.00
1,000	0.999	3.00

Values of  $pK_{ts} - pK_H$  in the absence of inhibitory anions were calculated according to Eq. 12. Note that  $pK_{ts}$  approaches  $pK_H$  when the relative chloride concentration is low, and that there is a stoichiometric relation between  $\log(\text{Cl}/K_A)$  and  $pK_{ts} - pK_H$ , only when  $\text{Cl}/K_A$  is above 10.  $F_{\text{Cl}}^{\circ}$ , the maximum number of sites that can be combined with Cl, is defined in Eq. 11.

showed that  $pK_{ts}$  varied linearly with  $\log(\text{Cl})_0$  at low anion concentrations, and that the anion effect diminished above 0.1 M chloride. In Table II we have calculated the difference  $pK_{ts} - pK_H$  for varying values of the ratio  $\text{Cl}/K_A$ : the chloride concentration normalized with its dissociation constant. The result is contrary to that shown in Fig. 10. The  $pK_{ts}$  varies with the chloride concentration only at high concentrations, and a stoichiometric relation between  $pK_{ts}$  and  $\log \text{Cl}_0$  is not seen until the ration ( $\text{Cl}/K_A$ ) is  $\sim 10$ . It is also apparent that Eq. 12 does not explain the leveling off of the anion effect on  $pK_{ts}$  at the higher anion concentrations (Fig. 10).

According to Eq. 12,  $pK_{ts}$  should be sensitive to the presence of competing anions having a high affinity for the transport site. Such an effect of iodide was not observed in the experiments of Figs. 5 and 11. The supposed ratio of about seven between the apparent affinities of  $I^-$  and  $Cl^-$  (Dalmark, 1976) ought to cause a considerable displacement of  $pK_{ts}$  at low chloride concentrations, according to a hypothesis based on chemical equilibria. The lack of effect of iodide substitution would seem to argue strongly that chloride and iodide do not have widely differing affinities for binding if they bind to the titratable site. Milanick and Gunn (1981) have recently reported that the apparent affinity for external iodide is the same as that for external chloride under conditions where the anion distribution across the membrane is asymmetrical. Therefore, we have performed experiments with 8 mM chloride and iodide on both sides of the membrane (Fig. 11). The lack of effect of iodide also under these conditions makes us conclude that it seems to be the halide concentration, rather than the chemical nature of the anion, that determines the effect on the  $pK_{ts}$ . It would be of interest to compare the titration of chloride and iodide transport as a function of anion concentration. Such experiments, which must be done at the same temperature, have not been carried out. Both the present results and those of Milanick and Gunn (1981) raise the question of whether the apparent affinities of anions for transport are a true measure of chemical affinities for binding, or whether the asymmetry of the transport system may be related to differences in the rate constants for inward and outward translocation (Knauf et al., 1980). At this point one can safely conclude that the titratable group studied here cannot alone be responsible for the chemical specificity of the anion transport system towards chloride and iodide.

It follows from Eq. 12 that a hypothesis based on anion binding predicts that the intrinsic  $pK$  of the titratable group ( $pK_H$ ) is approached at low anion concentrations. If the variation of  $pK_{ts}$  with anion concentration were a reflection of the chemical equilibria,  $pK_H$  would accordingly be lower than the value of  $\sim 11$  found at the lowest anion concentrations (Fig. 10). In the following article (Wieth et al., 1982), we present evidence that function of the anion transport system is dependent on the integrity of one or more arginyl residues. The kinetics of the chemical reaction with phenylglyoxal suggest that the modified groups are guanidino groups of arginyl residues, having  $pK$  values of  $\sim 12$ . We also present evidence supporting the notion that the groups that can be chemically modified may be identical with the titratable groups of the transport system. If this assumption is correct, the intrinsic  $pK$  of the titratable group must be higher than the value predicted by an interpretation of the present data based on Eq. 12.

**ANION EFFECTS ON AN INTERFACIAL POTENTIAL** Lack of evidence that variations of  $pK_{ts}$  with anion concentration are related to chemical affinities of halides for binding to a titratable group makes it necessary to consider the alternative hypothesis that  $pK_{ts}$  is the apparent  $pK$  of a group that is indispensable for normal transport function, although it does not interact with anions in reactions like those described by Eqs. 7 and 8 above. It is likely that



the interfacial regions of the band 3 protein have a high density of fixed positive charges, including the modifier sites. The local pH in these regions will be determined not only by the hydrogen ion concentration of the buffered extracellular medium, but also by the interfacial surface potential, which varies with the concentration of mobile counter ions. A positive surface potential will decrease the local hydrogen ion concentration and the difference between the measurable bulk pH ( $\text{pH}_b$ ) and the surface pH ( $\text{pH}_s$ ) will vary with the potential and therefore with the concentrations of "permeant" ion species, i.e., ions that are not excluded from the charged region.

Assuming a Boltzmann distribution of hydrogen ions from the solution up to the surface of the site,  $\text{pH}_s$  differs from that measured in the bulk phase:

$$\text{pH}_s = \text{pH}_b + \frac{q_e \cdot \psi_s}{2.3 kT}$$

where  $q_e$  is the numerical value of electronic charge,  $\psi_s$  is the interfacial surface potential relative to that of the bulk solution,  $k$  is the Boltzmann constant, and  $T$  is the absolute temperature. Only when  $\psi_s = 0$  will the surface and the bulk pH be equal. If  $\psi_s$  is positive,  $\text{pH}_s > \text{pH}_b$  because hydrogen ions are repelled by the electric field. One can calculate the changes of surface potential that would result in the variation of  $\text{p}K_{ts}$  values found in experiments like those shown in Figs. 8 and 9, if it is assumed that the changes of  $\text{p}K$  are apparent only. Thus a decrease of  $\text{p}K_{ts}$  by 0.7 unit after a fivefold decrease of chloride concentration from 82.5 to 16.5 mM corresponds to an increase of a positive surface potential by 38 mV.

The interfacial potential of a charged surface varies with the concentration of mobile counter ions in a way that is in qualitative agreement with the observed anion dependence of  $\text{p}K_{ts}$  (Fig. 10). Useful approximations have been obtained both by a Gibbs-Donnan model and by the Gouy model of the diffuse double layer (Davies and Rideal, 1963). At surface potentials above 100 mV the Gouy model simplifies to

$$\delta\psi_s/\partial \log C = -2.3 kT/q_e$$

(Davies and Rideal, 1963, p. 76), a 10-fold decrease of chloride concentration, causing an increase of the interfacial potential by 54 mV at 0°C. This agrees with the stoichiometric variation of  $\text{p}K_{ts}$  with  $\log \text{Cl}_o$  at the lowest chloride concentrations (Fig. 10). The variation is similar whether or not the ionic strength of the bulk medium is kept constant with citrate, which suggests that citrate is excluded from the transport domain of the band 3 protein. The highest positive surface potentials are generated at the lowest chloride (or iodide) concentrations, and it is in agreement with theory that the effect of increasing anion concentrations on surface potential and on  $\text{p}K_{ts}$  levels off when the concentration of mobile counter ions becomes sufficiently high (Fig. 10). These considerations, based solely on electrostatics, represent an extreme simplification, e.g., neglecting geometrical factors and energy contributions due to chemically specific ion adsorption as implied in the Stern adsorption theory (Davies and Rideal, 1963, pp. 84–90; Steinhardt and Reynolds, 1969).

We have already presented evidence that the chemical affinities of chloride and iodide do not play a significant role for the shift of  $pK_{ts}$ . In future work it will be of great interest to investigate whether amphiphilic anionic inhibitors influence  $pK_{ts}$  through chemical binding to the titratable group.

The proposed arrangement of positive fixed charges in the region of the transport site is supported by current models of anion transport (Passow et al., 1980). If confined to the extracellular facing region, the proposed arrangement may contribute to the pronounced asymmetry of apparent affinities of transported anions like chloride, bromide (Gunn and Fröhlich, 1979), and bicarbonate (Lambert and Lowe, 1978; Wieth, 1979). These works have demonstrated that the apparent affinities for monovalent inorganic anions increased dramatically when extracellular KCl was gradually replaced by citrate or gluconate solutions. The results of Fig. 8 show that the apparent chloride affinity in such experiments decreases with increasing pH, exemplified by the observation that at pH 11.5 the chloride flux relative to that found at pH 7.5 is hardly affected at 165 mM external chloride, whereas the relative chloride transport is decreased by 70% at pH 11.5 when the extracellular chloride is 16.5 mM. According to an electrostatic model, the apparent affinity of external chloride ions for transport will increase if a decreased concentration of transported anions causes an increase of the positive surface potential. Deprotonation of positively charged titratable groups at the transport site region will, on the other hand, decrease both the charge density and the surface potential and will therefore cause a decrease of the apparent chloride affinity, as is seen at alkaline pH values in Fig. 8.

#### *Identity of the Titratable Groups*

It is possible to limit the number of likely candidates by virtue of the acid-base properties of the two classes of titratable groups dealt with here. If we are dealing with positively charged amino acid side chains,  $\epsilon$ -amino groups of lysine and guanidino groups of arginyl residues seem to be the obvious candidates. Passow et al. (1980) have suggested that the transport site region contains a cluster of at least five positively charged amino acid residues. Estimated from their chemical reactivities, two of these were reported to be lysines with abnormally low  $pK$  values of 8.8 and 8.5, respectively. Other positively charged groups may belong to arginines with appreciably higher  $pK$  values than those reported for the lysyl residues.

In view of the general role assigned to arginyl residues at anion recognition sites in proteins (Riordan et al., 1977; Riordan, 1979), it is quite probable that one or both of the titratable groups dealt with here are in fact arginines. According to Cohn and Edsall (1943), typical  $pK$  values of arginyl residues in water soluble protein are 11.6–12.6 at 25°C. The ionization enthalpy of 10–13 kcal·mol<sup>-1</sup> increases these values by ~0.8 pH unit at 0°C. The  $pK$  values of the groups located in an integral membrane protein are likely to be lower: there is strong evidence from inhibitor studies that the anion transport region of band 3 is relatively hydrophobic. This would tend to decrease the  $pK$  of a positively charged group because the electroneutral form of the titratable group is stabilized in a low dielectric environment. Accordingly,  $pK$  values of

11 for the modifier site and 12 for the transport site are both within the expected range. We are currently pursuing the possible essential role of arginyl residues by studying the irreversible inactivation of the anion transport system using the pronounced reactivity of phenylglyoxal towards uncharged guanidino group (Borders et al., 1981; Wieth et al., 1982).

Earlier in this article we proposed that the modifier site(s) may play a functional role by providing a positively charged slide, guiding extracellular anions to the transport site by a molecular arrangement similar to that proposed to lead a negatively charged substrate into the active center of the enzyme carboxypeptidase A (Nakagawa and Uneyama, 1978). A similar speculative hypothesis can be proposed for the role of a guanidino group in a transport site. The idea is based on the molecular function of another enzyme, aconitase (Gawron and Jones, 1977). An arginyl residue in the active center of aconitase is proposed to provide a mobile sidearm, which supposedly rotates  $180^\circ$  around the carbon of the guanidino group when binding citrate, thereby catalyzing its conversion to D-isocitrate. In terms of membrane transport, a similar anion-induced rotation of a guanidino group could be involved in the conformational change accompanying the exchange diffusion process, e.g., if the group becomes free to rotate only when interacting with a transportable anion. In the absence of a negatively charged "transport substrate" the conformation of the transport protein is retained in a stable *cis* or *trans* conformation by intramolecular salt linkages. Interaction with anions induces rapid oscillations, resulting in turnover numbers of at least  $40,000\text{ s}^{-1}$  at a physiological temperature level, as can be calculated from the magnitudes of chloride or bicarbonate fluxes (Brahm, 1977; Wieth and Brahm, 1980).

Our results show that protonation of the titratable transport site is essential for transport function. The properties of this site alone cannot account for the chemical discrimination of the transport system between chloride and iodide (Figs. 5 and 11), and its acid-base properties are not affected by conditions that supposedly change the equilibrium between *cis* and *trans* configurations of the system (Fig. 10). The apparent chloride affinity of the transport system is markedly decreased at alkaline pH values (Fig. 8). These observations underline that the kinetics of anion exchange are not determined by the interaction of a transported anion with a single amino acid side chain in the band 3 protein. Rather the properties of the transport system at any given pH are influenced by the state of protonation of several titratable groups, two of which have revealed themselves in the present study.

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