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HCO_3^-/Cl^- Exchange across the Human Erythrocyte Membrane: Effects of pH and Temperature

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Summary. Changes in extracellular pH (pH_o) in red cell suspensions were monitored in a stopped-flow rapid reaction apparatus under conditions where dpH_o/dt was determined by the rate of HCO_3^-/Cl^- exchange across the membrane. Experiments were performed at 5 °C < T < 40 °C using either untreated cells or cells exposed to 0.11 mM SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid). Although SITS exposure reduced the rate of exchange by 90%, both untreated and SITS-treated cells are similarly affected by changes in pH_o and temperature. The rate of HCO_3^-/Cl^- exchange exhibits a minimum at about pH_o 5 and a maximum at about pH_o 7.4 at all temperatures. A transition temperature of 17 °C was observed in the Arrhenius relationship for all pH_o. The activation energies (E_a) in kcal/mol are 19.6 below and 11.7 above 17 °C for $5 < pH_o < 8$. These findings, similar to those reported for Cl^- self-exchange, suggest that: (i) a change in the rate-limiting step for HCO_3^-/Cl^- exchange occurs at 17 °C, possibly due to an altered interaction between the transport pathway and membrane lipids; (ii) the carrier system can be titrated by either H^+ or SITS from the outside of the membrane, but the untitrated sites continue to transport normally; (iii) the pH_o dependence of the rate of exchange is consistent with the titratable carrier having its most alkaline pK in the range expected for amino groups; and (iv) below pH_o 5, the nature of the exchange is markedly altered.

The most prominent feature of erythrocyte ionic permeability is its selectivity for anions: small hydrophilic anions such as Cl^- or HCO_3^- penetrate the membrane about one million times faster than hydrophilic cations of similar size such as Na^+ or K^+ (Tosteson, 1959). Since the high permeability to both Cl^- and HCO_3^- is necessary for CO_2 exchange and provides an effective regulatory system for helping to control acid-base balance in body fluids (Rothstein, 1968), this property may represent a favorable physiological specialization of erythrocytes.

Despite the fact that the red cell membrane permeability for monovalent anions is relatively high, the actual rate of Cl^- transport across the membrane is much less than that in a water layer of the same thickness (Tosteson, 1959). This suggests that either there is a severe restriction to the mobility of Cl^- in the membrane and/or that only a small fraction of the membrane is directly involved in Cl^- passage. Mond

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(1927) had observed that SO_4^{2-} influx in red cells decreased in response to increasing extracellular pH, and concluded that the observed pH dependence could be explained by assuming titratable positive charges in the membrane. Passow (1964) refined the hypothesis of fixed charges, which also satisfies the requirement that transport takes place through only a small fraction of the membrane surface.

Recently, new experimental data have appeared which essentially rule out the possibility that rapid anion exchange takes place by simple diffusion through positively-charged channels. The findings of a high activation energy for Cl^-/Cl^- (Dalmark & Wieth, 1970; Brahm, 1977) and HCO_3^-/Cl^- (Chow, Crandall & Forster, 1976; Crandall, Obaid & Forster, 1978) exchange, an electrical resistance of the *Amphiuma* red cell membrane that is unexpectedly high (Lassen, 1972), saturation of Cl^-/Cl^- exchange (Gunn *et al.*, 1973; Brahm, 1977), and the unusual dependence of Cl^-/Cl^- (Gunn, 1972; Gunn *et al.*, 1973; Brahm, 1977) and SO_4^{2-}/SO_4^{2-} (Schnell, Gerhardt & Schöppe-Fredenburg, 1977) exchange on pH, could best be explained by postulating a carrier system for anion exchange across the erythrocyte membrane (Gunn, 1972; Gunn *et al.*, 1973; Gunn, 1977) that might function like certain amines in artificial membranes (Gutknecht, Graves & Tosteson, 1978). Furthermore, the anion transport mechanism has been shown to be related to the Band 3 protein in the erythrocyte membrane (Ho & Guidotti, 1975; Passow, 1977; Cherry *et al.*, 1976; Lepke *et al.*, 1976; Rothstein, Cabantchik & Knauf, 1976). Some experimental evidence has been obtained suggesting that the ionic selectivity of the red cell membrane is due in part to the role of protein amino groups at or near the transport site (Knauf & Rothstein, 1971; Obaid, Rega & Garrahan, 1972).

With the exception of Brahm (1977), most of the data on rapid anion transport have been obtained by studying halide self-exchange, particularly Cl^-/Cl^- exchange, at very low temperatures, in order to decrease the rate of the process to a level that could be studied by available techniques (Dalmark & Wieth, 1970; Gunn *et al.*, 1973; Gunn, Wieth & Tosteson, 1975). Little work has been reported on the physiologically important process of erythrocyte HCO_3^-/Cl^- exchange, and almost none is available on this exchange in the physiological temperature range. Since the half time of this process *in vivo* may be of the same order of magnitude as the transit time of red cells through capillary beds, the exchange kinetics might limit the amount of CO_2 that can be transferred between blood and tissue or between blood and alveolar gas. Recently, Chow *et al.* (1976) demonstrated that the activation energy

for HCO_3^-/Cl^- exchange between 24 and 42 °C was significantly different from that between 2 and 12 °C. Crandall *et al.* (1978) were able to detect a transition temperature at 17 °C. Lambert and Lowe (1978) found that the dependence of HCO_3^-/Cl^- exchange on the extracellular HCO_3^- concentration was consistent with the existence of a saturable membrane anion carrier exhibiting Michaelis-Menten kinetics.

In the present work we have studied HCO_3^-/Cl^- exchange in human red cell suspensions over a wide range of conditions using a method based on observing the rate of extracellular pH change under conditions where the HCO_3^-/Cl^- exchange is rate-limiting. When a large quantity of H^+ is added to a red cell suspension which contains HCO_3^- and carbonic anhydrase in its extracellular fluid, some H^+ ions rapidly combine with HCO_3^- in the extracellular fluid to form CO_2, which quickly enters the red cells and rehydrates to form $H^+ + HCO_3^-$. This process, which takes place in about 20 msec (Chow *et al.*, 1976) has been called a "redistribution" phase and can be thought of as being equivalent to a sudden injection of HCO_3^- into the cells. As a result of these rapid adjustments, the intracellular concentration of HCO_3^- increases and HCO_3^- begins to flow out of the cells in exchange for Cl^-. As HCO_3^- enters the extracellular fluid, it combines there with H^+ ions to form CO_2 (rapidly under the influence of the extracellular carbonic anhydrase), raising the extracellular pH. The CO_2 re-enters the cells and re-forms H^+ and HCO_3^- there. The net result of a complete cycle of HCO_3^- leaving the cells and CO_2 entering to re-form HCO_3^- results in the transfer of HCl from outside to inside the cell. This process, which takes place after the initial rapid redistribution phase, has been called the Jacobs-Stewart cycle (Jacobs & Stewart, 1942). Due to flux through the Jacobs-Stewart cycle, $d\text{pH}/dt$ in the extracellular fluid is rate limited by the exchange of HCO_3^- for Cl^- across the red cell membrane.

We have studied HCO_3^-/Cl^- exchange at temperatures from 5 to 40 °C, at extracellular pH (pH_o) from 3.8 to 8.0, at intracellular pH (pH_{in}) from 7.0 to 8.0, and in the presence and absence of SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid), a potent anion exchange inhibitor. The results indicate that: (i) at $3.8 < pH_o < 8.0$, HCO_3^-/Cl^- exchange has a minimum at about pH 5 and maximum at about pH 7.4, for all T; (ii) variation in intracellular pH between pH 7.0 and 8.0 has no effect on the exchange; (iii) in normal cells, and for $5 < pH_o < 8$, the activation energy for HCO_3^-/Cl^- exchange changes at a transition temperature of 17 °C ($E_a = 11.7$ kcal/mol above, and 19.6 kcal/mol below, 17 °C); and (iv) SITS-pretreatment markedly de-

creases HCO_3^-/Cl^- exchange, but pH dependence, transition temperature, and activation energies are not significantly different from those for untreated cells. On the basis of these findings, we conclude that HCO_3^-/Cl^- and Cl^-/Cl^- exchange occur via the same transport pathway across the human red blood cell membrane. The data also suggest that the most alkaline titratable site on the carrier may involve an amino group, and that lipid-protein interactions may be in part responsible for the temperature dependence of the exchange process.

Materials and Methods

Apparatus

The stopped-flow rapid reaction apparatus used in these experiments has been described previously (Crandall, Klocke & Forster, 1971; Chow *et al.*, 1976; Crandall *et al.*, 1978). In the apparatus, equal volumes of a red cell suspension (*A*) and phosphate-buffered saline solution (*B*) are forced through a four-jet mixer (0.004 ml) into a 0.1-ml measuring chamber. A pH-sensitive glass electrode (Leeds and Northrup 117145) is used to follow the pH of the mixture as a function of time, both before and after flow stops. The reference electrode liquid junction is a KCl-saturated cotton wick bridging a snug-fitting Teflon plug, and is pressure- and flow-insensitive (Crandall *et al.*, 1971). The voltage across the electrodes is amplified (Transidyne General MPA-6 with its own power source MPS-15) and monitored on a storage oscilloscope screen (Tektronix 5103N). A measure of flow velocity is simultaneously monitored on the oscillocope screen by recording voltage output from a magnet-in-coil device mounted on the stopped-flow apparatus drive block. The entire apparatus is water-jacketed, and the experiments reported here were carried out between 5 and 40 °C. The response time of the electrode system has been estimated to be less than 5 msec, using a ramp change in pH due to carbonic acid dehydration as a test reaction. The lag time of the apparatus (elapsed time between mixing and reaching the glass electrode) is less than 20 msec at the linear flow rates used in these experiments (25–50 cm/sec).

Preparation of Solutions

Freshly drawn human blood from hematologically normal adults was used; coagulation was prevented with heparin (2 U/ml). The blood was centrifuged at $1,750 \times g$ for 10 min and the plasma and buffy coat were removed by aspiration. The remaining cells were washed three times with large volumes of 146.5 mM NaCl, 3.5 mM KCl. After the last wash the cells were spun down for 10 min at $10,000 \times g$ and resuspended in the same solution to about 20% hematocrit. NaOH or HCl was added to the suspending medium to reach a final pH between 7.1 and 8.4. Bovine carbonic anhydrase (Sigma Chem. Co., #C-7500, St. Louis, Mo.) was added to the suspension to a concentration of 800 Wilbur-Anderson U/ml of suspension. This suspension was degassed and equilibrated with a 10% O_2, 90% N_2 gas mixture. Freshly prepared $NaHCO_3$ was then added to a final concentration of 1 to 15 mM, and the suspension (*A*) maintained in a closed tonometer thereafter. For the experiments in which cells were treated with SITS (4-acetamido-4′-isothiocyanostilbene-

2,2′-disulfonic acid) (Polyscience Corp., Niles, Ill.) the red cells were washed three times as described above and resuspended to 10% hematocrit in a solution containing 146.5 mM NaCl, 20 mM tris buffer (pH 7.4), 0.2% ethanol and 0.11 mM SITS. This suspension was incubated at 37 °C for 10 min with constant agitation. The cells were then rewashed three times with 146.5 mM NaCl, 3.5 mM KCl, resuspended in this medium to 20% hematocrit, and treated further as described above (suspension A_s).

The phosphate-buffered solution (B) was made up of 112.5 mM NaCl, 15 mM KH_2PO_4, 15 mM Na_2HPO_4, adjusted to $3.2 < pH < 8.4$ with HCl or NaOH. Solution B was degassed and equilibrated with 10% O_2, 90% N_2. All suspensions and solutions were prepared at room temperature.

Experimental Procedure

A calibration curve for the stopped-flow pH electrode apparatus was obtained before each experiment by passing standard buffer solutions (SO-B-X, Fisher Scientific Co., N.J.) through the electrode chamber. Solution B was then passed through the electrode chamber and the output stored on the oscilloscope screen as a reference value for a particular experimental run. Subsequently, equal volumes (2.5 ml) of A and B were driven through the mixing chamber at approximately constant velocity until flow was abruptly halted. pH equilibration in the mixture was recorded on the storage oscilloscope screen. When the reaction was complete, a photograph was taken of the experimental record (*see* below).

Experimental runs were performed at temperatures between 5 and 40 °C and with pH_B between 3.2 and 8.4. In those experiments for which $3.2 < pH_B < 6.7$, pH_A was adjusted to 7.7 to yield an extracellular pH of the mixture immediately after redistribution (pH_P) from 3.8 to 6.7. Under these conditions, the subsequent pH equilibration is due to the Jacobs-Stewart cycle moving HCO_3^- from inside to outside the cells. When $7.5 < pH_B < 8.4$, pH_A was adjusted to 7.1, leading to pH_P between 7.3 and 8.0. These conditions cause HCO_3^- to move from outside to inside the cells in the Jacobs-Stewart cycle. In all these experiments, the total CO_2 content of A was adjusted such that, after redistribution, the bicarbonate concentration difference across the red cell membrane was about 5 mM.

To investigate the effects of inhibitors on HCO_3^-/Cl^- exchange, the above experiments were repeated using cells pretreated with SITS. Experiments were performed over the same range of temperature and pH_B to determine the effects of SITS on the temperature and pH_o dependence of the anion exchange.

Because the intracellular pH in the experiments described above varied between 7.0 and 7.6, a group of experiments was performed to study the effects of changes in intracellular pH on the rate of HCO_3^-/Cl^- exchange. After adjusting pH_A to a value between 7.1 and 8.3, equal volumes of A and B (pH_B 6.7) were rapidly mixed. pH_P was about 6.7 in all these experimental runs. Intracellular pH after redistribution was calculated to be between 7.0 and 8.0. Experiments were performed between 5 and 37 °C.

At the conclusion of each experiment, hematocrit of suspension A was measured using standard Wintrobe tubes, after centrifugation at approximately $2{,}500 \times g$ for 15 min. Intracellular pH (pH_{lys}) was measured on a lysate of cells from suspension A, prepared by rapidly freezing and thawing packed cells twice in dry ice and acetone. pH_{lys}, as well as pH_A and pH_B, were determined anaerobically (Radiometer BMS3 Mk2 blood gas machine). A mixture of equal volumes of A and B was collected after having passed through the rapid reaction apparatus. After centrifugation, the supernatant from the mixture was titrated anaerobically (Radiometer ABU 13 autoburette with TTA 60 titration assembly). The buffer capacity of the extracellular fluid of the mixture as a function of pH was obtained by differentiation of the titration curve.

Computations

The initial flux ϕ of HCO_3^- either into or out of the red cells per unit of membrane surface area was determined from the dpH/dt observed in the mixture immediately after stopping flow in the rapid reaction apparatus:

$$\phi = \beta \cdot (d\text{pH}/dt) \cdot (1 - \text{Hct})/(\text{Hct} \cdot A/V)$$

where V = volume/cell and A = surface area/cell. The intracellular and extracellular HCO_3^- concentration immediately after the "redistribution" phase were computed from the measured extracellular pH at the time of stopping flow ("plateau" pH) using the equilibrium constant for carbonic acid, the assumption that CO_2 concentration is the same intra- and extracellularly, and that total CO_2 content remains constant. The equations, given previously (Chow *et al.*, 1976), were solved on a DEC PDP-10 digital computer using an iterative procedure. A velocity constant for HCO_3^-/Cl^- exchange was then calculated to normalize for small differences in concentration gradient:

$$k = \phi/([HCO_3^-]_i - [HCO_3^-]_o).$$

The Arrhenius activation energy E_a of HCO_3^-/Cl^- exchange was calculated by linear regression analysis of the relation between the natural logarithm of k and the reciprocal of the absolute temperature.

Results

Typical Experimental Record

Figure 1 shows a typical experimental record obtained when solution B (pH 6.7) was mixed with suspension A (hematocrit = 16%, $pH_A = 7.7$) containing 800 U carbonic anhydrase (CA)/ml and 4.4 mM $NaHCO_3$. The upper tracing represents the pH of the fluid in the measuring chamber as a function of time. The lower trace indicates when flow of reactants starts and stops. Before flow starts, the buffer solution B is in the measuring chamber. During flow, the "plateau" pH (pH_P) is that of the extracellular fluid of the mixture after the rapid redistribution of HCO_3^- (which takes place in about 20 msec), but before significant HCO_3^-/Cl^- exchange has had time to occur. After flow stops, extracellular fluid pH (pH_o) of the mixture in the measuring chamber rises toward its equilibrium value as the Jacobs-Stewart cycle effects the transfer of H^+-equivalents from outside to inside the erythrocytes. From the slope at time zero, it is possible to measure $d\text{pH}_o/dt$, and by knowing the buffer capacity of the medium and the hematocrit of the suspension, the initial flux of HCO_3^- can be determined. The rate constant k calculated from the initial HCO_3^- flux and HCO_3^- concentration difference across the

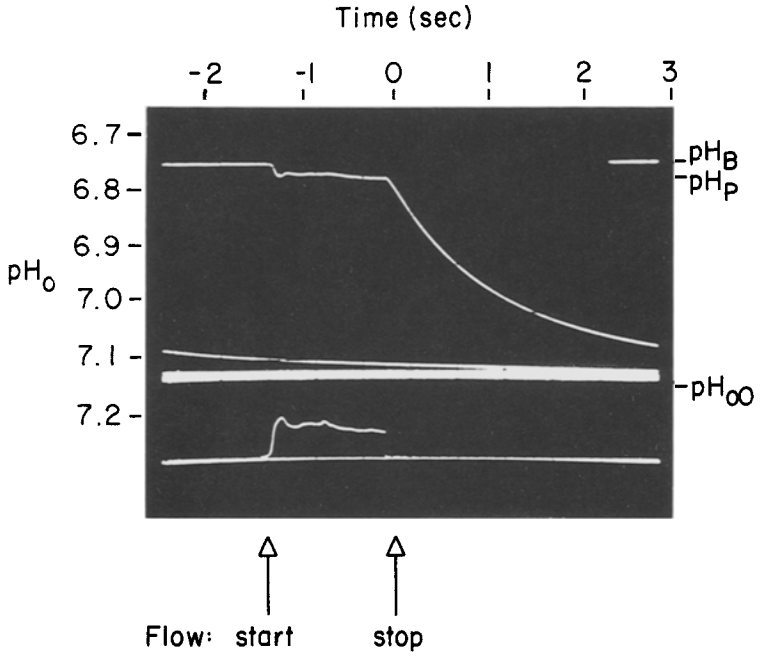

Fig. 1. Oscilloscope tracing of a typical experimental record at 37 °C. In this experiment, equal volumes of suspension *A* (hematocrit = 16%, pH_A 7.7) containing 800 U CA/ml and 4.4 mM $NaHCO_3$, and solution *B* (pH_B 6.7), were mixed in the stopped-flow rapid reaction apparatus. The times of starting and stopping flow are indicated by the lower trace. The upper trace represents the extracellular pH of the fluid in the measuring chamber as a function of time. Each trace was swept across the screen several times. *See* text for further details of the experimental records

red cell membrane after redistribution normalizes the fluxes for small differences in HCO_3^- concentration gradient.

pH Dependence of HCO_3^-/Cl^- Exchange

(a) Intracellular pH. HCO_3^-/Cl^- exchange is essentially independent of internal H^+ concentration in the range of intracellular pH from 7.0 to 8.0, as shown in Fig. 2. Changes in temperature between 5 and 37 °C alter the magnitude of the flux, but have no effect on the intracellular pH dependence.

(b) Extracellular pH. The effect of extracellular pH on the rate of HCO_3^-/Cl^- exchange is shown in Fig. 3. The rate constant has a maximum value at about pH_o 7.4 and a minimum at about pH_o 5 for all temperatures between 5 and 40 °C. Below pH_o 5, k increases dramatically.

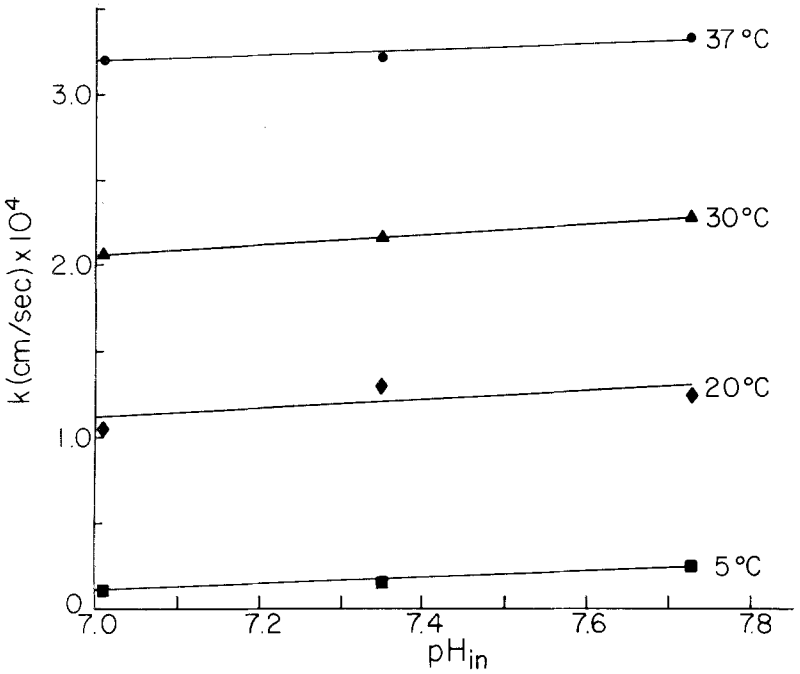

Fig. 2. Effect of intracellular pH (pH_{in}) on HCO_3^-/Cl^- exchange. After adjusting pH_A to a value between 7.1 and 8.4, equal volumes of *A* (hematocrit=20%) containing 800 U CA/ml and 1.0 to 13.0 mM $NaHCO_3$, and *B* (pH_B 6.7), were rapidly mixed. After redistribution, intracellular pH was calculated to be between 7.0 and 8.0, and HCO_3^- concentration difference across the membrane was about 5 mM. Experiments were performed between 5 and 37 °C. Each point represents the average rate constant *k* from several experimental runs on each of two populations of cells

Fig. 3. Effect of extracellular pH (pH_o) on HCO_3^-/Cl^- exchange. Equal volumes of *A* (hematocrit=20%), containing 800 U CA/ml and 1.2 to 15.0 mM $NaHCO_3$, and adjusted to pH_A 7.7 for $pH_B \leqq 6.7$ or to pH_A 7.1 for $pH_B \geqq 7.5$, and *B* ($3.2 < pH_B < 8.3$) were mixed in the stopped-flow rapid reaction apparatus. After redistribution, HCO_3^- concentration difference across the membrane was calculated to be about 5 mM. Each point represents the average rate constant *k* from several experimental runs on each of two to nine different populations of cells

Fig. 4. Effect of SITS on pH_o dependence of HCO_3^-/Cl^- exchange. Before preparing suspension *A*, red cells were pretreated at hematocrit=10% with 0.11 mM SITS. Otherwise, suspension *A* and solution *B* were prepared and experiments run as described for Fig. 3. Each point represents the average rate constant *k* from several experimental runs on each of one to three different populations of cells

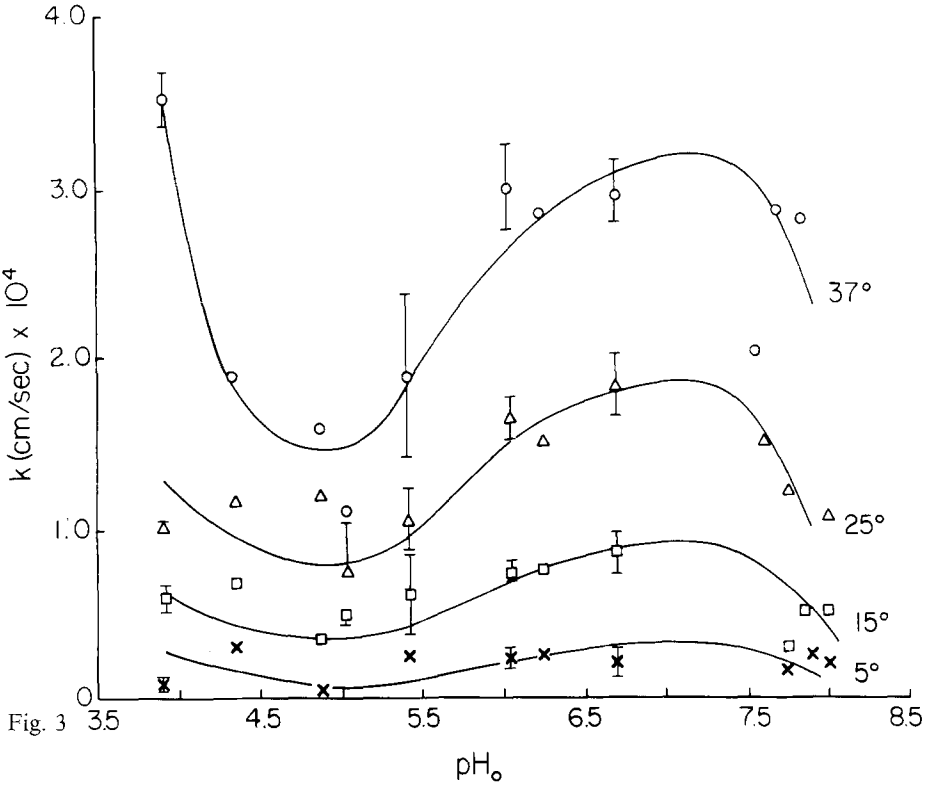
4.0
3.0
2.0
1.0
0
k (cm/sec) x 10^4
37°
25°
15°
5°
3.5
4.5
5.5
6.5
7.5
8.5
pH_o

Fig. 3

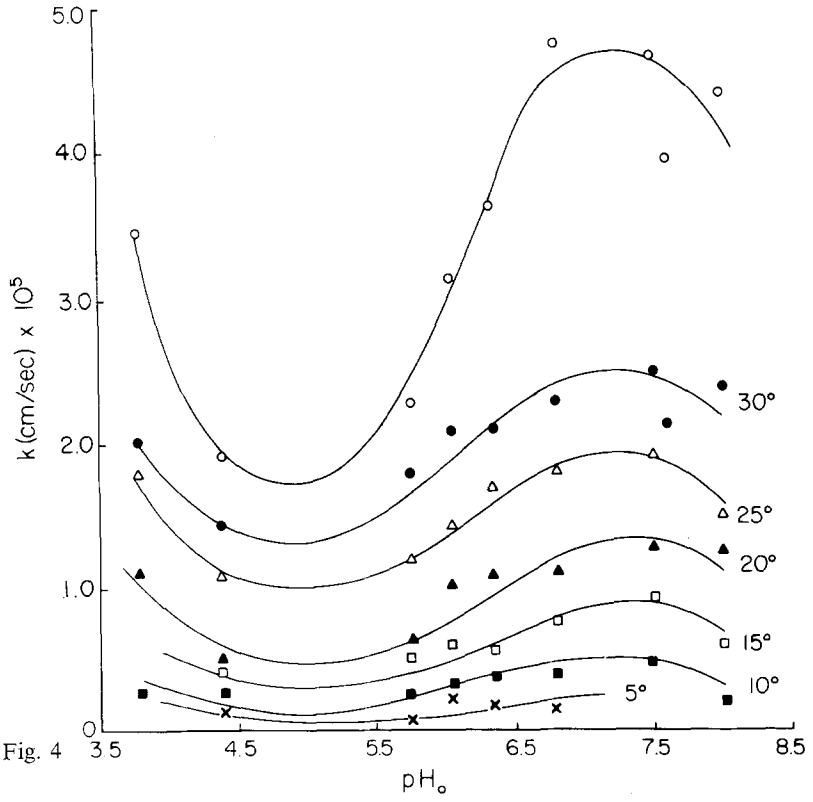
5.0
4.0
3.0
2.0
1.0
0
k (cm/sec) x 10^5
30°
25°
20°
15°
10°
5°
3.5
4.5
5.5
6.5
7.5
8.5
pH_o

Fig. 4

These results are similar to those reported previously for Cl^- self-exchange (Gunn *et al.*, 1973, 1975). The rate constant decreases above pH_o 7.5, consistent with previous results in intact red cells (Gunn *et al.*, 1973; Brahm, 1977) for Cl^- self-exchange, but different from those obtained in ghost preparations (Funder & Wieth, 1976).

Figure 4 shows the effect of SITS, a potent inhibitor of anion exchange that does not itself penetrate the membrane, on the pH_o dependence of HCO_3^-/Cl^- exchange. Under conditions in which SITS reduces the flux by almost 90%, the pH dependence of the anion exchange remains unaltered, in that the maxima and minima for all temperatures occur at the same pH_o.

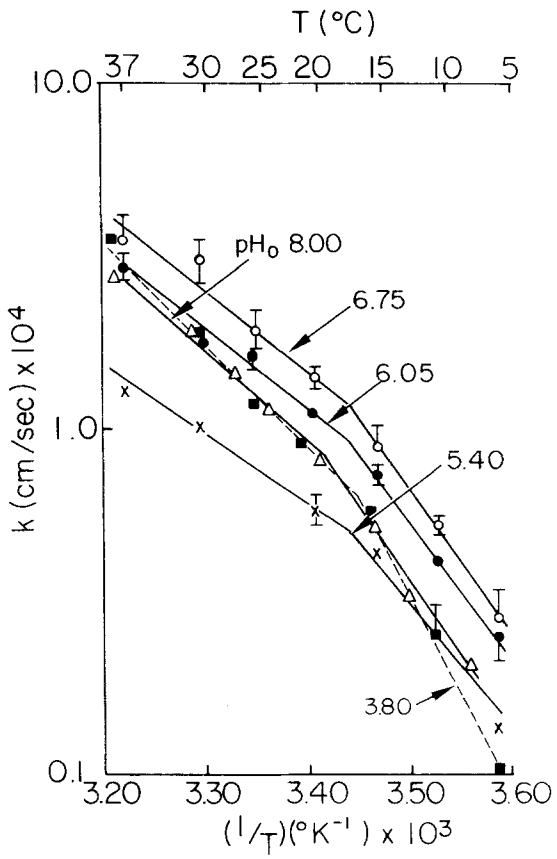

Fig. 5. Temperature dependence of HCO_3^-/Cl^- exchange. The logarithm of the rate constant for HCO_3^-/Cl^- exchange (log k) is shown plotted against the reciprocal of the absolute temperature ($1/T$). Experiments were performed between 5 and 40 °C. *A* and *B* were prepared as described for Fig. 3. Each point represents the average rate constant k from several experimental runs on each of two to nine different cell populations

Temperature Dependence of HCO_3^-/Cl^- Exchange

Figure 5 shows an Arrhenius diagram of the relationship between the rate constant for HCO_3^-/Cl^- exchange and the reciprocal of absolute temperature. The curves have been plotted for different values of pH_o between 3.8 and 8.0. For all pH_o, the Arrhenius plots exhibit a transition temperature at 17 °C. Above pH_o 5, the calculated activation energies below and above 17 °C are independent of pH_o. However, the values for activation energies increase below pH_o 5.

The effects of SITS on the Arrhenius plots of the HCO_3^-/Cl^- exchange at different pH_o are shown in Fig. 6. Although SITS inhibits 90% of the anion exchange, the transition temperature and the activation energies obtained with SITS-pretreated cells are not different from those shown

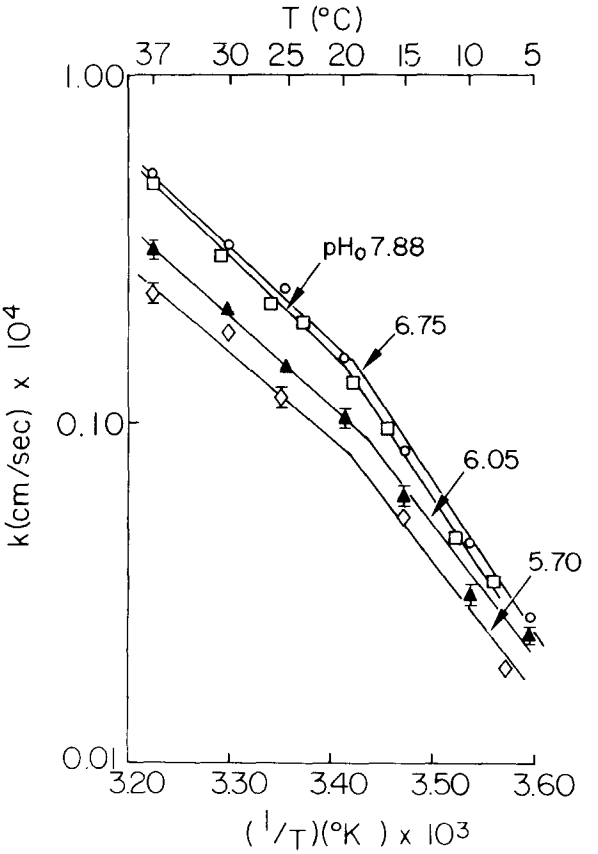

Fig. 6. Effects of SITS on temperature dependence of HCO_3^-/Cl^- exchange. Experiments were performed between 5 and 40 °C. *A* and *B* were prepared as described for Fig. 4. Each point represents the average rate constant *k* from several experimental runs on each of one to three different populations of cells

Table 1. Effect of pH_o on activation energies for HCO_3^-/Cl^- exchange in human erythrocytes

pH_o	E_{a_I} (kcal/mol)[a]		$E_{a_{II}}$ (kcal/mol)[a]	
	Control	SITS	Control	SITS
<4	15.8		29.2	
5.40	10.4		16.9	
5.70		12.2		17.1
6.05	9.8	13.1	17.2	15.8
6.25	10.0		17.0	
6.40	10.3	13.1	17.1	16.9
6.75	11.4	12.2	18.6	17.0
8.00	12.8	14.1	17.7	19.3

[a] E_{a_I} is activation energy above 17 °C; $E_{a_{II}}$ is activation energy below 17 °C.

Table 2. Effect of pH_{in} on activation energies for HCO_3^-/Cl^- exchange in human erythrocytes ($pH_o = 6.7$)

pH_{in}	E_{a_I} (kcal/mol)[a]	$E_{a_{II}}$ (kcal/mol)[a]
7.03	12.6	(23.0)
7.35	11.8	19.1
7.80	11.0	18.9

[a] E_{a_I} is activation energy above 17 °C; $E_{a_{II}}$ is activation energy below 17 °C.

in Figure 5 for untreated cells. These results are similar to those obtained with DIDS for Cl^- self-exchange (Brahm, 1977).

Table 1 summarizes the values of the activation energies for untreated and SITS-pretreated cells, both above and below the transition temperature, for the entire range of pH_o studied. Table 2 summarizes the values of the activation energies for untreated cells at pH_o 6.7, with intracellular pH ranging from 7.0 to 8.0. The Arrhenius plots from which these activation energies were calculated also showed a transition temperature at about 17 °C.

Discussion

The experiments reported above were performed in a stopped-flow rapid reaction apparatus, under conditions where HCO_3^-/Cl^- exchange is the rate-limiting step in the Jacobs-Stewart cycle. This approach has

allowed us to study HCO_3^- fluxes in the physiological temperature and concentration ranges, and to use the same technique for all conditions studied. In order to use the Jacobs-Stewart cycle to investigate HCO_3^-/Cl^- exchange, it is necessary that the remaining steps in the cycle be relatively rapid. Chow *et al.* (1976) demonstrated that bovine carbonic anhydrase at concentrations greater than that used here (800 U/ml suspension *A*) did not increase the rate of change of extracellular pH, suggesting that the extracellular CO_2 hydration-dehydration reactions are not rate-limiting. Recently, it has been confirmed that the intracellular carbonic anhydrase accelerates the hydration-dehydration reactions by 15,000 times in intact red cells (Silverman, Tu & Wynns, 1976; Itada & Forster, 1977). It is extremely unlikely that these reactions could ever rate-limit the Jacobs-Stewart cycle. The other step involved is CO_2 diffusion across the red blood cell membrane. The erythrocyte has been shown to have very low resistance to the diffusion of this gas (Gros & Moll, 1971), despite the possibility that a significant fraction of the resistance resides in an unstirred layer (Gutknecht, Bisson & Tosteson, 1977). The presence of an unstirred layer adjacent to the membrane, however, might have influenced the HCO_3^-/Cl^- exchange rates measured in our experiments. Sha'afi *et al.* (1967) estimated the unstirred layer around red cells in a stopped-flow apparatus to be about 6 μm. With an estimated diffusion coefficient for Cl^- and HCO_3^- of about $1–2 \times 10^{-5}$ cm^2/sec, the permeability ($D/\Delta x$) of these anions through the unstirred layer ($1.6–3.3 \times 10^{-2}$ cm/sec) is two orders of magnitude greater than that for HCO_3^-/Cl^- exchange (*see* above and Chow *et al.*, 1976). It is clear, therefore, that under our experimental conditions, HCO_3^-/Cl^- exchange kinetics are determined primarily by the red cell membrane itself.

pH Dependence of HCO_3^-/Cl^- Exchange

As shown in Fig. 2 and Table 1, little change was found either in the magnitude of the fluxes or in the values of activation energies below and above 17 °C as a function of pH_{in}. These results allow evaluation of the effects of extracellular pH on HCO_3^-/Cl^- exchange without having to consider changes in intracellular pH as a separate independent variable.

Changes in extracellular pH have a dramatic and immediate effect on HCO_3^-/Cl^- exchange, as shown in Fig. 3. It is interesting to note that the maximum for the HCO_3^-/Cl^- exchange rate constant appears to be in the vicinity of pH_o 7.4, close to the pH of blood plasma *in vivo*.

Changes in temperature alter the magnitude of the exchange, but the pH_o dependence remains the same. The pH_o-dependent behavior of HCO_3^-/Cl^- exchange across the red cell membrane is remarkably similar to that reported for Cl^-/Cl^- exchange in intact cells (Gunn *et al.*, 1973, 1975; Dalmark, 1975; Brahm, 1977), and is generally consistent with the kinetic titratable carrier model proposed initially by Gunn (1972).

The ratio of maximum to minimum k for HCO_3^-/Cl^- exchange at 37 °C (Fig. 3) is about four, similar to that reported for Cl^- self-exchange at 38 °C (Brahm, 1977). The turnover number associated with the highest k under the conditions of our experiments (at pH 7.4) is 1.4×10^9 ions/cell/sec, or 1.4×10^3 ions/site/sec based on 10^6 transport sites/cell (Zaki *et al.*, 1975; Lepke *et al.*, 1976). This turnover number is fifty times smaller than that reported for Cl^- self-exchange of 5×10^{10} ions/cell/sec (Brahm, 1977), probably because the HCO_3^- concentrations used in these experiments are below the K_m for the exchange process. Chow *et al.* (1976) showed a linear relationship between HCO_3^-/Cl^- exchange rate and HCO_3^- concentrations up to 20 mM $NaHCO_3$, suggesting a $K_m > 10$ mM. Lambert and Lowe (1978) recently reported a K_m for HCO_3^- transport of 0.55 mM under somewhat different experimental conditions.

Because the effects of pH_o on mono- and divalent anion fluxes are different (Deuticke, 1970; Schnell *et al.*, 1977), it was suggested (Gunn, 1972) that three equilibria are established between the carrier system and hydrogen ions at each membrane-solution interface:

$$(1)\quad C_0 + H^+ \overset{K_1}{\rightleftharpoons} C_1 \qquad (2)\quad C_1 + H^+ \overset{K_2}{\rightleftharpoons} C_2 \qquad (3)\quad C_2 + H^+ \overset{K_3}{\rightleftharpoons} C_3$$

where C_0, C_1, C_2 and C_3 are the various forms of the carrier in the membrane (C_0 is uncharged). The opposite effects of pH_o in the range of 6 to 8 on mono- and divalent anion fluxes can be explained by assuming that the form of the carrier which transports univalent anions such as Cl^- or HCO_3^- (C_1) can be titrated by H^+ to a form which carries divalent anions such as SO_4^{2-} (C_2). The $C_1 \rightleftharpoons C_2$ equilibrium has an apparent pK of about 6.7. The titration of C_2 to C_3, a form which does not carry anions, would account for the inhibition of SO_4^{2-} or HPO_4^{2-} fluxes at values below pH_o 6.3 (Gunn, 1972).

The fall in HCO_3^-/Cl^- exchange rate constant as pH_o rises above 7.4 (Fig. 3) has also been observed for Cl^- self-exchange in intact cells above pH_o 7.8, at 0 °C (Gunn *et al.*, 1973) and 38 °C (Brahm, 1977). These results are consistent with a titratable site for the exchange system ($C_0 \rightleftharpoons C_1$) having a pK in the range expected for amino groups. The

data on Cl^-/Cl^- exchange have been interpreted as possibly being due to the decrease in internal Cl^- concentration which occurs as a result of the increased negative charge of hemoglobin at alkaline pH (Dalmark, 1975). In the present HCO_3^-/Cl^- exchange experiments, however, the concentration gradient was maintained approximately constant over the entire pH_o range and the data in Fig. 3 have been normalized for any small differences that remained. Furthermore, "upstream" HCO_3^- concentration was about 6.1 mM (range: 5.1–7.7 mM) in all experiments. These findings differ from those obtained for Cl^-/Cl^- flux in red cell ghosts (Funder & Wieth, 1976) and in cells with high Cl^- concentrations (Dalmark, 1975). In those conditions, the flux remains at its maximum value between pH 7.0 and 10.0, results which have been interpreted as suggesting a guanidino group as the most alkaline titratable site in the carrier. It is unlikely that the different results can be explained by the conversion of HCO_3^- to CO_3^{2-} at $pH_o \simeq 8.0$, since the pK for this equilibrium is about 10.2 and the HCO_3^- concentration gradient would be at most 1% less than that used in our calculations. It should be noted, however, that our experiments involved HCO_3^- efflux for pH_o 6.7 and HCO_3^- influx for $pH_o > 7.5$, and therefore asymmetrical membrane properties may contribute to the discrepancy. This possibility cannot be ruled out on the basis of our data alone, but seems unlikely since the curve in Fig. 3 is not discontinuous at pH_o 6.7–7.5. Furthermore, major asymmetric properties for HCO_3^-/Cl^- exchange would make little sense teleologically because CO_2 transport *in vivo* requires flux of HCO_3^- in opposite directions in lung and systemic capillaries.

Temperature Dependence of HCO_3^-/Cl^- Exchange

Figure 5 shows the temperature dependence of the rate constant for HCO_3^-/Cl^- exchange over the entire range of pH_o studied. All the Arrhenius relationships exhibit a transition temperature at about 17 °C. Below and above 17 °C, the activation energies (Tables 1 and 2) are high and in the range predicted by Parsegian (1969) for the energy barrier in a carrier mediated transport model. These findings are qualitatively similar to those reported previously for Cl^- self-exchange (Dalmark & Wieth, 1970; Brahm, 1977), but our values for activation energies are considerably lower. The activation energies are independent of intracellular pH in the range of 7.0 to 8.0 (Table 2) and are also unchanged in the range of pH_o from 5.0 to 8.0 (Table 1). Since k changes with pH_o in this

range these data indicate that hydrogen ions are titrating carrier sites accessible from the outside of the cell membrane, without altering the rate-limiting step of the process. Below pH_o 5, however, the activation energy values are higher, suggesting either a different transport mechanism or a dramatic change in the usual transport pathway kinetics. These very low pH_o data are consistent with previous findings for Cl^-/Cl^- exchange (Gunn *et al.*, 1975). Transition temperatures are not an exclusive feature of the anion exchange process in the red cell membrane. They have been observed at similar temperatures for other transport processes in red cells and in other biological membranes (Sen & Widdas, 1962; Lyons & Raison, 1970; Lacko, Witte & Geck, 1973; Blatt, 1974; Dubinsky & Racker, 1978). Since the common element in all these systems seems to be the lipid bilayer, it suggests that lipid state changes may be responsible for the transition temperature (Chapman, 1975). In this regard, it is interesting that the viscosity of red cell membrane particle suspensions and extracted membrane lipids exhibit a transition temperature at about 18 °C (Zimmer, Schirmer & Bastian, 1975). On the basis of this evidence, we are inclined to suggest that it is the state of the red cell membrane lipids and their influence on the function of membrane proteins that is responsible for our observations of a transition temperature at 17 °C for all the conditions studied here.

We cannot rule out, however, the possibility that the change in the rate-limiting step is due to either a primary change in the transport proteins or another transport pathway becoming involved in the temperature dependence of the exchange. The data of Ross and McConnell (1978), showing that red cell Band 3 proteins reconstituted into vesicles transport SO_4^{2-} with a very low activation energy, suggest that the environment of the Band 3 proteins is critical to the temperature dependence of the transport process. The possibility that a second "reaction" involved in the transport mechanism is important in determining the transition temperature is supported by the results of Brahm (1977) on Cl^- and Br^- self-exchange, who found that the transition temperature for both anion exchanges is associated with an identical turnover number despite occurring at 15 °C for Cl^- and 24 °C for Br^- ($3.5–3.7 \times 10^3$ ions/site/sec). Our data, however, show that the transition temperature for HCO_3^-/Cl^- exchange occurs at a turnover number one order of magnitude less (3.9×10^2 ions/site/sec). While we cannot explain at this time the high transition temperature for Br^-, it seems unlikely that a critical turnover number determines the transition temperature for anion exchange in general. The likelihood that variable K_m is responsible

for the transition temperature (Silvius, Read & McElhaney, 1978) is remote, since Brahm (1977) showed a transition temperature of 17 °C despite sometimes operating at 100% saturation over the entire range of temperature.

Effects of SITS on Temperature and pH_o-Dependence of HCO_3^-/Cl^- Exchange

The results in Figs. 4 and 6 indicate that SITS, a potent inhibitor of anion transport which does not enter the cells (Knauf & Rothstein, 1971), reduces the magnitude of HCO_3^-/Cl^- exchange without altering either its temperature or its pH_o dependence. This means that SITS functionally eliminates the carrier sites to which it is bound, but that the transport units which continue to exchange anions retain all their normal properties. In this sense, SITS and extracellular hydrogen ions affect the transport in a similar fashion. The effect of pH_o on HCO_3^-/Cl^- exchange, as well as its similarity to the effect of SITS, show that the titratable transport sites or groups closely related to them are accessible from the outside of the cell surface. The influence of SITS on HCO_3^-/Cl^- exchange is in general the same as the effect of this class of inhibitors on other anion exchanges.

In summary, a number of similarities between HCO_3^-/Cl^- exchange across the erythrocyte membrane, and the more easily and extensively studied Cl^- self-exchange, has been shown in this work. These findings are fortuitous, in that they allow an extrapolation of our knowledge of anion exchange in general to the physiologically relevant HCO_3^-/Cl^- exchange. New insights into the behavior of the anion transport system include the suggestion that the most alkaline titratable site on the carrier system may involve an amino group, and that lipid-protein interaction may play a major role in determining its temperature dependence.

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