Kinetics of Bicarbonate-Chloride Exchange across the Human Red Blood Cell Membrane

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ABSTRACT The kinetics of bicarbonate-chloride exchange across the human red cell membrane was studied by following the time course of extracellular pH in a stopped-flow rapid-reaction apparatus during transfer of H^+ into the cell by the CO_2 hydration-dehydration cycle, under conditions where the rate of the process was determined by HCO_3^--Cl^- exchange flux across the membrane. The flux of bicarbonate increased linearly with [HCO_3^-] gradient from 0.6 to 20 mM across the red cell membrane at both 37°C and 2°C, and decreased as transmembrane potential was increased by decreasing extracellular [Cl^-]. An Arrhenius plot of the rate constants for the exchange indicates that the Q_{10} is strongly dependent on temperature, being about 1.7 between 24°C and 42°C and about 7 between 2°C and 12°C. These data agree well with the published values for Q_{10} of 1.2 between 24°C and 40°C and of 8 between 0°C and 10°C. The results suggest that different processes may determine the rate of HCO_3^--Cl^- exchange at low vs. physiological temperatures, and that the functional (and/or structural) properties of the red cell membrane vary markedly with temperature.

INTRODUCTION

The movement of anions across the red blood cell membrane, in particular the exchange of bicarbonate for chloride during and after CO_2 outflow in the lungs or uptake in the tissues, is an important if not rate-determining step in the net transport of metabolic waste products from cells to environment. From the classical experimental data of Roughton (1964) one can calculate that approximately half of the total CO_2 exchanged in the lungs in a resting human has to come from HCO_3^- that moves into the red cell from plasma during passage of blood through the lungs. However, the basic measurements were made under conditions of internal chemical equilibrium in the blood. On the reasonable assumption that rapidity of HCO_3^--Cl^- exchange is the same as that for Cl^--Cl^- exchange, and taking values for the latter from the literature, Forster and Crandall (1975) calculated that the cell-plasma exchange processes would not

achieve equilibrium by the end of the capillary and that in normal lungs the capillary blood would give up 25% more CO_2 to the alveolar gas if intra- and extracellular [HCO_3^-] and [H^+] were in equilibrium when the blood left the alveolar capillary. Since the experimental half-time for Cl^--Cl^- (or HCO_3^--Cl^-) exchange across the red cell membrane is about 0.2 s (Tosteson, 1959) and H^+ equilibration is on the order of tens of seconds (Forster and Crandall, 1975), while the average time spent by red cells in the pulmonary capillaries (pulmonary transit time) has been estimated to range from 0.1 to 2 s (Roughton, 1945; Roughton and Forster, 1957; see review by Piiper, 1969), it appears that red cell transmembrane exchanges limit the amount of CO_2 which can be eliminated from blood in the lung capillary.

The kinetics of HCO_3^--Cl^- exchange between red blood cells and the extracellular fluid has been studied by several techniques. Luckner (1939), using essentially a Cl^- electrode, found a half-time for the exchange of 0.11 s at 37°C, and later (Luckner, 1948) reported for the process a Q_{10} of 1.2 between 40°C and 24°C and of 1.5 between 24°C and 10°C. Luckner's apparatus, however, suffered from the possible presence of a significant boundary layer effect. Dirken and Mook (1931), Piiper (1964), and Hemingway et al. (1970), using continuous-flow rapid-mixing filtration techniques, reported half-times for HCO_3^--CL^- exchange that ranged from 0.04 to 0.2 s.

In recent years, with the advent of radioactive tracer techniques, the kinetics of Cl^--Cl^- self-exchange at electrochemical equilibrium has been examined. Tosteson (1959), using isotopic methods with the continuous-flow filtration technique, found a half-time of 0.2 s at room temperature. Dalmark and Wieth (1972) found that the exchange of radioactive Cl^- with human red cells was so slow at 0°C that it was practical to follow its time course by collecting successive extracellular fluid samples with a syringe-filter from a stirred reaction vessel. They found a Q_{10} of 8 between 0°C and 10°C. Gunn et al. (1973) and Cass and Dalmark (1973) reported that Cl^- self-exchange flux exhibited saturation kinetics at 0°C, and that the process was competitively inhibited by HCO_3^-. It should be noted that in red blood cells exposed to valinomycin, the net movement of Cl^- and cation is slower than the self-exchange of Cl^- (Harris and Pressman, 1967; Scarpa et al., 1968, 1970; Hunter, 1971; Tosteson et al., 1973).

We have developed a method for the determination of the kinetics of HCO_3^--Cl^- exchange of human red cells which requires only 2 ml of reactant and is convenient to use over a wide range of temperatures (2–42°C). If acid is added to a red cell suspension (Fig. 1) containing a low concentration of CO_2 and a high concentration of extracellular carbonic anhydrase, H_2CO_3 dehydrates extracellularly and the resulting CO_2 hydrates intracellularly, forming H^+ and HCO_3^-. This anion then moves out of the cell in exchange for Cl^-, resulting in an equivalent net transfer of H^+ from outside to inside (Jacobs and Stewart, 1942). The speed of the exchange of HCO_3^- for Cl^- across the membrane under these circumstances determines the rate of transfer of H^+, which can be followed by monitoring dpH/dt. Using this technique, we have examined the characteristics of bicarbonate-chloride exchange across the red cell membrane as a function of several important parameters.

MATERIALS AND METHODS

Instruments

The stopped-flow glass pH electrode rapid-reaction apparatus (Crandall et al., 1971) was used to follow the extracellular pH change after mixing cell suspension A with an equal volume of an acidic buffer solution B. A schematic diagram of the apparatus is shown in Fig. 2. The output from the glass electrode is fed into a high input impedance differential

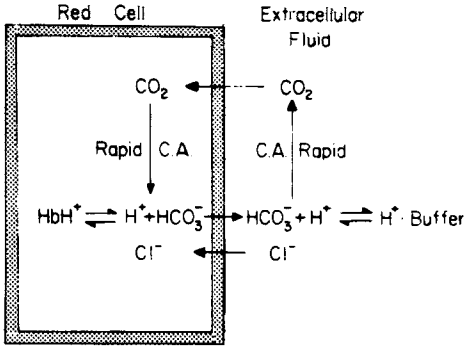

FIGURE 1. Jacobs-Stewart cycle in the presence of extracellular carbonic anhydrase.

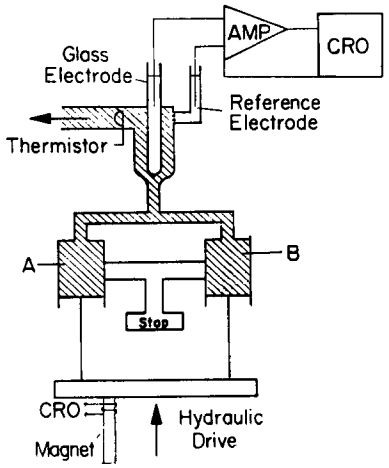

FIGURE 2. Schematic diagram of the stopped-flow glass pH electrode rapid reaction apparatus (see text).

DC preamplifier (Transidyne General Corp., Ann Arbor, Mich., model MPA-6). A magnet is attached to the hydraulic drive bar and moves along with the hydraulic drive through a copper coil fixed in space. The electrical output from the copper coil is recorded on the storage oscilloscope and gives an estimate of the linear flow speed of the reaction mixture in the mixing chamber as well as a stop indication. The temperature of the reaction mixture is monitored by a thermistor probe (Yellow Springs Instrument Co., Yellow Springs, Ohio, type 421) placed in the effluent stream beside the glass electrode. The precision of the measurement is about ± 0.002 pH unit.

A volume of <1 ml is sufficient to wash out residual fluid in the electrode chamber at

the flow rates (about 50 cm/s) used in these experiments. This washout volume is the same whether the cell suspension or the acid buffer is in the chamber before flow starts.

Lag time (time between instant of mixing and impingement of the mixture on the glass electrode) was determined by measuring the extent to which known reactions, the hydration-dehydration of CO_2-bicarbonate, had proceeded during flow (Crandall et al., 1971; Gros et al., 1976). At flow rates of about 50 cm/s, lag time is less than 50 ms.

Crandall et al. (1971) have shown that this electrode apparatus responds to a ramp change in pH produced by the same reaction in less than 5 ms. This includes the time required for the change in pH of the bulk fluid to be transmitted through any possible stagnant layer at the electrode tip to the surface of the glass, plus the time for the potential across the glass itself to change.

The measured dpH/dt after this 5-ms delay is that expected from the absolute values of the CO_2-bicarbonate hydration-dehydration rates calculated from reaction velocity constants reported in the literature. In later work, Chow (1975) showed that if the buffer capacity of the fluid being measured becomes very low, the ability of the stopped-flow electrode to follow rapid changes in pH may become compromised. This problem was investigated by Gros et al. (1976) who showed that in the presence of 2 mM imidazole buffer the electrode correctly followed the dpH/dt due to CO_2 hydration in reaction mixtures containing different concentrations of carbonic anhydrase, up to rates of 6 s^{-1}. The conditions of the present experiments (dpH/dt < 1 s^{-1} at buffer concentrations ≥ 5 mM) were well within the reliable operating range of the electrode apparatus.

Preparation of Solutions

30 ml of fresh human blood were drawn into a heparin-rinsed syringe and immediately centrifuged for 10 min at 2,000 *g* at 4°C. The cells were separated, washed with 10 vol of a degassed solution consisting of 146.5 mM NaCl and 3.5 mM KCl in distilled water, and recentrifuged, this whole procedure being carried out three times. After the last wash, the cells were resuspended in an aliquot of this medium which had been equilibrated with 10% O_2 and 90% N_2 to give a hematocrit of about 16%, and stored in a 110-ml tonometer. The total CO_2 content in the suspension was <3 μM, and the hemoglobin was >95% oxygenated.

The pH of the cell suspension was adjusted slowly to about 8.0 with freshly prepared 0.1 N NaOH. Fixed amounts of $NaHCO_3$ solution (also freshly prepared) and bovine carbonic anhydrase (carbonate hydrolyase, E.C. 4.2.1.1., Sigma Chemical Co., St. Louis, Mo., no. C-7500, 4,000 Wilbur-Anderson [W-A] U/mg solid) were added to the suspension in the tonometer or in glass syringes so that the final concentrations in the suspensions were 2 mM $NaHCO_3$ and 40,000–80,000 W-A U/100 ml. The suspensions were equilibrated to the desired temperature in closed glass syringes. The preparation of this red cell suspension required about 4 h.

The normal acidic buffer solution (B) contained 15 mM Na_2HPO_4, 15 mM KH_2PO_4, and 112.5 mM NaCl in distilled water. It was thoroughly evacuated, CO_2 being removed, and then equilibrated with 10% O_2 and 90% N_2 at atmospheric pressure at the same temperature at which the experiment was to be performed. The resulting solution had a pH of about 6.7.

These reactant solutions were modified for different experiments as follows.

EXPERIMENTS ON THE EFFECT OF CHANGING EXTRACELLULAR CARBONIC ANHYDRASE CONCENTRATION The enzyme concentration in the cell suspensions (A) was varied from 0 to 80,000 W-A U/100 ml. In the measurements at 2°C, the acidic buffer solution (B) was made up with a lower buffer capacity (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 137.5 mM NaCl) in order to speed up the pH change.

EXPERIMENTS ON THE TEMPERATURE DEPENDENCE OF HCO_3^--CL^- EXCHANGE For the experiments at 2°C, acidic buffer solution (B) of low buffer capacity (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 137.5 mM NaCl) was used agin.

EXPERIMENTS ON THE EFFECT OF CHANGING [HCO_3^-] GRADIENT Different amounts of $NaHCO_3$ were added to the cell suspensions to vary the total added CO_2 content from 0 to 20 mM. The osmolarity was kept at about 310 mosmol by varying NaCl content. In those experiments at 37°C with 10 mM total added CO_2 content, the pH of the acidic buffer was reduced to 6.5 in order to maintain relatively constant pH after mixing.

EXPERIMENTS ON THE EFFECT OF VARYING EXTRACELLULAR [CL^-] NaCl in the acidic buffer solution (B) was replaced by Na isethionate[1] to vary the extracellular [Cl^-] from 0 to 112.5 mM. In order to reduce the [Cl^-] in the mixture further, we mixed one part of the cell suspension (A) which contained 150 mM Cl^- with five parts of the acidic buffer solution (B). The composition of the cell suspension (A) was adjusted to give a hematocrit of 48% [$NaHCO_3$] of 6 mM, and [carbonic anhydrase] about 3,000 W-A U/ml so that the suspension after mixing had the same hematocrit, same total CO_2 concentration, and same [carbonic anhydrase] as in those experiments where A and B were mixed 1:1. At 37°C, the phosphate buffer concentration of B was decreased to 16.5 mM so as to keep the extracellular buffer concentration after mixing approximately the same as that in the 1:1 mixing ratio experiment. In addition, at 2°C, the buffer concentration of B was again decreased, this time to 5.5 mM.

Procedure

All experiments were performed with 2.5 ml of cell suspension mixed with equal volumes of the acidic buffer unless otherwise stated. The electrode was first exposed to the acidic buffer solution (B) and the oscilloscope trace of the glass electrode output was recorded. Cell suspension (A) and acidic buffer solution (B) were then driven through the mixing chamber at approximately constant speed (50 cm/s) until the driving piston hit the "stop" and the flow was brought to an abrupt halt. The subsequent pH changes in the reacting mixture[2] were followed on a storage oscilloscope and photographed. The flow rate was recorded on a separate channel of the oscilloscope. The glass pH electrode was calibrated by flowing standard buffer solutions through the mixing chamber before the experiments were carried out. The temperature of the apparatus was regulated at a preset value from 2° to 42°C by using a constant temperature bath. Lysis was always less than 2% as judged by the hemoglobin concentrations in the effluent. The hemoglobin released contributes only about 1% of the buffer power of the extracellular fluid of the mixture, and its effect can be neglected since changes of this magnitude did not affect the computations.

The hematocrit of suspension (A) was determined by using standard Wintrobe tubes after centrifugation at 2,740 *g* for 15 min. pH of suspension (A), cell lysate, and the acidic buffer solution (B) were measured independently at the temperature at which the experiments were performed with an anaerobic pH electrode (Instrumentation Laboratory, Inc., Lexington, Mass., model 113). Lysate of suspension (A) was prepared by freezing and thawing the packed cells.

[1] The effect of Na isethionate on the enzymatic activity of carbonic anhydrase was checked. In the presence of Na isethionate, carbonic anhydrase catalyzed HCO_3^- dehydration at least as well as in NaCl of the same concentration.

[2] "The mixture" always means the mixed fluid of suspension A and the acidic buffer solution B in the measuring chamber of the stopped-flow rapid-reaction apparatus. All the calculations on kinetics were carried out on the mixture.

RESULTS

Experimental Record

A typical record of an experiment at 37°C, in which the acidic buffer solution (B) at pH 6.68 was mixed with suspension (A) (hematocrit 16.5%) at pH 7.8 containing 2 mM total CO_2 and 80,000 W-A U carbonic anhydrase per 100 ml suspension is reproduced in Fig. 3. At this concentration of total CO_2, as discussed below, the contribution of OH^- and phosphate fluxes to the pH change is negligible and the amount of carbonic anhydrase is more than sufficient to speed up the extracellular hydration-dehydration reaction so that it does not determine the overall H^+ transfer rate. The topmost trace indicates the linear flow speed through the mixing chamber. A downward deflection represents an increase of flow rate. The scale is roughly 80 cm/s per vertical scale division.

The bottom three traces indicate the pH of the fluid in the measuring chamber. The topmost of this group of lines indicates the pH of the acidic buffer solution (B), which is 6.68. The bottom line is the pH of the mixture after the reaction is complete, equal to 7.0. The middle curve is the pH of the mixture during the reaction. As flow starts, shown by the initial downward deflection of the flow trace, the pH rises abruptly from the pH of the acidic buffer solution (B) to about 6.72, the pH of the mixture about 50 ms after mixing ("plateau" pH). Immediately after the cell suspension is mixed with acidic buffer solution, the following processes will occur in order of decreasing speed.

NEUTRALIZATION IN THE EXTRACELLULAR FLUID This process includes the reaction of H^+ with buffer and with OH^- and is complete in microseconds (Eigen and Hammes, 1963). The resulting pH is within 0.001 pH of that of solution (B) because of the high buffer power of (B).

CO_2 REDISTRIBUTION This is the establishment of CO_2 equilibrium between the intra- and extracellular fluid. The sudden increase of extracellular $[H^+]$ causes a rapid dehydration of extracellular HCO_3^- (under the influence of added carbonic anhydrase) to form CO_2, which then diffuses into the cells and hydrates rapidly into H^+ and HCO_3^-. Most of the H^+ produced intracellularly will associate with the hemoglobin buffer. HCO_3^- will accumulate inside and a quasi-steady state is reached in which the dissolved CO_2 is in chemical equilibrium with HCO_3^- and H^+ in both intra- and extracellular spaces due to the presence of carbonic anhydrase and the rapidity of CO_2 transport (Gros and Moll, 1971):

$$\frac{[H^+]_o \cdot [HCO_3^-]_o}{[CO_2]} = \frac{[H^+]_i \cdot [HCO_3^-]_i}{[CO_2]} = K_{CO_2},$$

where subscripts o and i mean extracellular and intracellular, respectively. This CO_2 redistribution phase is almost complete within the dead time of the rapid mixing apparatus (0.050 s), and accounts for almost all the increase in pH above that of solution (B) seen during flow (the plateau). In the experiment illustrated in Fig. 3, by calculation, CO_2 redistribution should increase pH 0.033 U whereas the plateau was 0.040 pH above solution (B). The slight difference can easily be accounted for by some transfer of H^+ into the cell by the Jacobs-Stewart cycle (see below) during the dead time.

CHANGE IN INTRACELLULAR HEMOGLOBIN CARBAMATE This results from the change of intracellular [CO_2] and [H^+]. These reactions have a half-time of about 0.05 s (Forster et al., 1968). The total amount of CO_2 involved is <5% of that in the suspension.

JACOBS-STEWART CYCLE (FIG. 1) After the redistribution phase, CO_2 is in chemical equilibrium in both intra- and extracellular spaces. The electrochemical potential gradient of HCO_3^- drives it out of the cells into the extracellular fluid in exchange for Cl^-, where it reacts with H^+ and is rapidly dehydrated to CO_2, which will enter the cell and form HCO_3^- again, completing the cycle, having transferred an H^+ into the cell. This cycle operates continuously, pro-

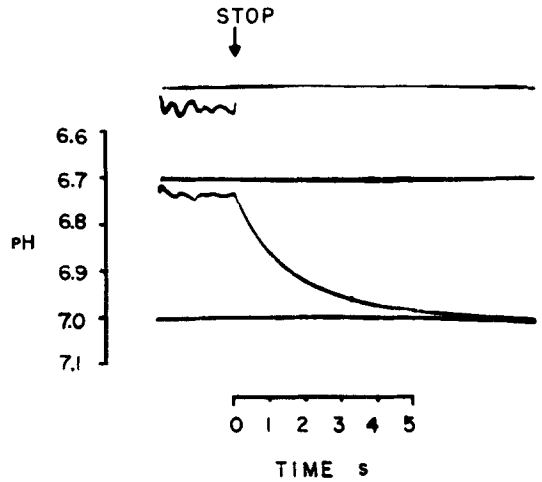

FIGURE 3. Experimental record of the measurement of kinetics of bicarbonate transport at 37°C. Suspension A = washed human red blood cells. Hematocrit 0.165, pH 7.8, total CO_2 2 mM, carbonic anhydrase 80,000 W-A U/100 ml. Suspending medium = 146.5 mM NaCl, 3.5 mM KCl. Solution B = pH 6.68. 30 mM phosphate buffer, 112.5 mM NaCl. The suspending media of suspension A and solution B were de-aerated and equilibrated with 10% O_2, 90% N_2 gas mixture.

ducing an equivalent flux[3] of H^+ into the cell until the pH of the buffered extracellular solution is in electrochemical equilibrium with the cell contents, a process with a half-time of about 1 s in Fig. 3. The greater the concentration of extracellular buffer, the slower this equilibration; if there were no such buffer the process would be complete in less than 0.2 s. The total H^+ (and therefore HCO_3^-) flux in moles-second^{-1} (ϕ_{H^+}) in 1 liter of mixture at the start of the Jacobs-Stewart cycle is obtained from the initial slope of the Jacobs-Stewart cycle phase of the experimental record by the relation

$$\phi_{H^+} = \frac{dpH}{dt} \times B_o \times V_o \approx \phi_{HCO_3^-}, \quad (1)$$

[3] "H^+ flux" or "H^+ transfer" used throughout this paper does not imply the actual movement of H^+ as ions across the membrane. The transfer is apparent. It is effected mainly by CO_2 movement with subsequent hydration (Jacobs-Stewart cycle).

where B_o and V_o are the buffer capacity and volume of the extracellular fluid in MpH^{-1} and liters/liter mixture respectively. B_o is determined by the total phosphate concentration and pK at the temperature of the given experiment.

WATER MOVEMENT The accumulation of intracellular HCO_3^- leads to an increase in osmolarity which causes water to enter the cell and the cell to swell. At least several seconds are required for this process (Blum and Forster, 1970) and its effect is not large. The subsequent HCO_3^--Cl^- exchange will not alter the total electrolyte content of the cell.

Effect of Extracellular Carbonic Anhydrase Concentration

The ratio of catalyzed H^+ flux to the uncatalyzed flux is plotted against the carbonic anhydrase concentration in the mixture in Fig. 4. The results show that at both 37°C and 2°C, the flux was accelerated gradually with the addition of carbonic anhydrase, until it reached about 20,000 W-A U/100 ml mixture (about 1.7 μM). The rate of transfer leveled off beyond this point, indicating that the transfer of H^+ ions was rate determined by H_2CO_3 dehydration extracellularly at low carbonic anydrase concentrations, that further acceleration of the extracellular hydration-dehydration of CO_2 could not speed up the rate of H^+ transfer any further, and that therefore the overall rate is determined by some other process(es).

Calculation of Initial Values

The intra- and extracellular bicarbonate concentrations after the rapid redistribution of CO_2 (start of the Jacobs-Stewart cycle) are calculated as follows.

(*a*) The dissolved CO_2 is in equilibrium with HCO_3^- and H^+ in both intra- and extracellular spaces due to the presence of carbonic anhydrase. Therefore,

$$[H^+]_o[HCO_3^-]_o = [H^+]_i[HCO_3^-]_i, \tag{2}$$

where the intracellular concentrations are in cell water. Also, recall

$$\frac{[H^+]_o[HCO_3^-]_o}{[CO_2]} = K'_{CO_2}. \tag{3}$$

pK′ of this reaction changes from 6.1 to 6.3 as temperature changes from 37°C to 0°C (Siggaard-Anderson, 1962).

(*b*) Total CO_2 has to remain constant, i.e.

$$\Sigma CO_2 = [HCO_3^-]_o \times (1\text{-}Hct) + [HCO_3^-]_i \times Hct \times \alpha + [\text{dissolved } CO_2] + [\text{carbamino } CO_2] \times Hct + [H_2CO_3],$$

where ΣCO_2 and *Hct* are, respectively, the total CO_2 content and hematocrit in the mixture, and α, the fraction of water inside the red blood cell, is 0.72 (Savitz et al., 1964). [Carbonic acid] present is negligible because both pH_i and pH_o in the system are much higher than the pK of this acid. The greatest effect of the carbamino reaction of CO_2 with intracellular hemoglobin is to reduce the total amount of CO_2 entering the Jacobs-Stewart cycle. When one uses the equilibrium constants obtained by Ferguson and Roughton (1934 *a*, *b*), the estimated amount of CO_2 taken up by the carbamino reaction is less than 5% of the total

CO_2 present in our system; therefore, it is neglected. The conservation relation then becomes

$$\Sigma CO_2 = ([HCO_3^-]_o + [CO_2]) \times (1\text{-}Hct) + [HCO_3^-]_i \times Hct \times \alpha,$$

$[CO_2]$ can be related to $[HCO_3^-]_o$ by equation (3); therefore,

$$\Sigma CO_2 = [HCO_3^-]_o \times (1 + 10^{pK'-pH_o}) \times (1\text{-}Hct) + [HCO_3^-]_i \times Hct \times \alpha. \quad (4)$$

(*c*) Intracellular pH can be calculated from the initial intracellular pH_{iA} of suspension (A) plus the pH change resulting from the increased intracellular $[H^+]$ and $[HCO_3^-]$ produced by the hydration of CO_2 inside the cell during the redistribution period before the exchange of HCO_3^- for Cl^- across the cell

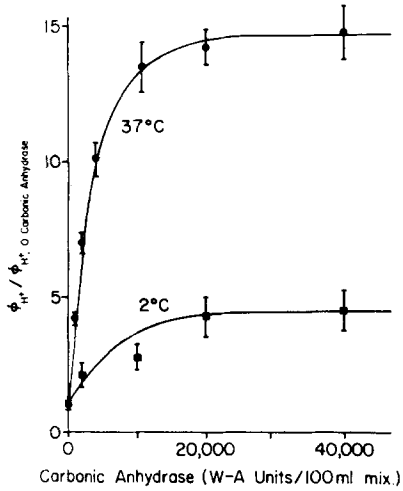

FIGURE 4. The effect of extracellular carbonic anhydrase concentration on the rate of H^+ transfer by the Jacobs-Stewart cycle. The ordinate is the ratio of the flux of H^+ ions in the presence of carbonic anhydrase to the uncatalyzed flux. The abscissa is the extracellular carbon anhydrase concentration in the mixture in Wilbur-Anderson units per 100 ml.

membrane. The H^+ ions are buffered by the 27.8 mM intracellular hemoglobin (in relation to cell water) (Horrobin, 1968), of buffer capacity 2.54 mM $[H^+]$/(pH × mM [Hb]) (German and Wyman, 1937; Rossi-Bernardi and Roughton, 1967). Therefore,

$$pH_i = pH_{iA} - ([HCO_3^-]_i - [HCO_3^-]_{iA})/B, \quad (5)$$

where B = 27.8 mM [Hb] × 2.54 mM $[H^+]$/(pH × mM [Hb]). pH_{iA} can be measured from the lysate of the packed cells of suspension (A). $[HCO_3^-]_{iA}$ can be calculated from the hematocrit and the Donnan ratio (see Funder and Wieth, 1966):

$$\frac{[HCO_3^-]_{iA}}{[HCO_3^-]_{oA}} = \frac{[Cl^-]_{iA}}{[Cl^-]_{oA}} = \frac{[H^+]_{oA}}{[H^+]_{iA}} = 10^{(pH_{iA} - pH_{oA})}. \quad (6)$$

The three equations (2), (4), and (5) were solved for the unknowns $[HCO_3^-]_i$,

$[HCO_3^-]_o$, and $[H^+]_i$ at the specific extracellular pH where the Jacobs-Stewart cycle begins.

In order to compare our data with results reported in the literature, it is convenient to define a phenomenological exponential rate constant k for the exchange processes analogous to previous definitions:

$$k = \frac{\phi_{HCO_3^-}}{([HCO_3^-]_i = [HCO_3^-]_o)V_i}, \quad (7)$$

where V_i is the intracellular water volume in liters/liter mixture and the values of $\phi_{HCO_3^-}$, $[HCO_3^-]_i$, and $[HCO_3^-]_o$ are obtained at the start of the Jacobs-Stewart cycle. This definition assumes that HCO_3^- flux is always proportional to $[HCO_3^-]$ gradient and does not include the influence of an electrical potential gradient across the membrane, a possibility which may become important during measurements under nonequilibrium conditions such as ours.

Temperature Dependence of HCO_3^--Cl^- Exchange

The HCO_3^- flux increased 60-fold (Table I) as the temperature increased from 2°C to 42°C, in spite of the fact that the $[HCO_3^-]$ gradient across the wall decreased 30% and the Cl^- concentrations were maintained approximately constant. The initial intracellular pH averaged 7.9 ± 0.4 and the extracellular pH after the establishment of the Jacobs-Stewart cycle averaged 6.8 ± 0.1.

These results are plotted as the $\log_{10}$ of the phenomenological exponential constants k against 1/T in Fig. 5, along with comparable data from the literature. Interestingly, these data do not lie on a straight line. The absolute value of the slope decreases as the temperature rises, which means that the activation energy of the transfer processes decreases as the temperature rises. By linear regression analysis, the Q_{10} between 2°C and 12°C was 7 ($r = -0.958$) and the Q_{10} between 24°C and 42°C was 1.7 ($r = -0.936$). Over the same temperature ranges, activation energies were 30 kcal/mol and 8.8 kcal/mol, respectively. In the experiments included in Fig. 5, the membrane potential was kept relatively constant at −10 ± 3 mV over the entire temperature range.

Effect of Changing $[HCO_3^-]$ Gradient

Flux increased approximately linearly with the transmembrane $[HCO_3^-]$ gradient from 0 to about 20 mM (Fig. 6) at both 37°C and 2°C. $[HCO_3^-]$ gradient was that at the end of the redistribution phase. The pH and $[Cl^-]$ of the cell suspensions and the acidic buffer solutions were adjusted so as to maintain the intra- and extracellular pH and the transmembrane potential initially in the mixture within relatively narrow ranges ($\Delta pH_i < 0.4$, $\Delta pH_o < 0.2$, and $\Delta E < 8$ mV). The flux observed in the absence of a transmembrane $[HCO_3^-]$ gradient (obtained by attempting to remove all CO_2 from the solution) is assumed to be due to the movements of anions such as OH^- and phosphate across the membrane, and possibly H^+ transport by residual and/or metabolic CO_2 via the Jacobs/Stewart cycle. Because of the small magnitude of this flux and the general scatter of the data, the HCO_3^- flux is assumed equal to the measured H^+ flux when the total CO_2 in the mixture is ≥1 mM, which was the case in these experiments. It is difficult to study HCO_3^- flux at $[HCO_3^-] > 20$–30 mM because the pH change due to the Jacobs-Stewart cycle then becomes too small to

measure accurately in comparison to the large pH change that takes place during the preceding CO_2 redistribution phase.

Effect of Varying Extracellular [Cl^-] on HCO_3^- Flux

The flux of HCO_3^- out of the red cells ($J_{HCO_3^-}$) increased as the extracellular [Cl^-] increased under conditions where the [HCO_3^-] gradient from inside to

TABLE I

EFFECT OF TEMPERATURE ON THE RATE OF HCO_3^-/Cl^- EXCHANGE

Donor	T	J ± S.E. (n)	$[HCO_3^-]_i$	$[HCO_3^-]_o$	k	$P_{HCO_3^-}$ ± SE
		$\frac{nmol}{cm^2\ s}$				
	°C		*mM*	*mM*	s^{-1}	*cm/s*
WG	2	0.021 ± 0.002 (5)	5.85	0.55	0.119 ± 0.006	(3.8 ± 0.2) × 10^{-6}
WG	2	0.024 ± 0.004 (2)	6.15	0.52	*n* = 4	*n* = 8
TW	2	0.042 ± 0.002 (6)	9.07	0.36		
LP	2	0.041 ± 0.001 (5)	8.56	0.47		
TZ	5	0.093 ± 0.002 (6)	7.94	0.46	0.340	1.10 × 10^{-5}
TW	6	0.113 ± 0.007 (4)	7.55	0.48	0.423	1.38 × 10^{-5}
TW	10	0.200 ± 0.010 (3)	7.58	0.48	0.750	2.43 × 10^{-5}
TZ	12	0.300 ± 0.002 (6)	7.30	0.60	1.160	3.64 × 10^{-5}
TW	15	0.440 ± 0.007 (6)	6.63	0.54	1.980	6.49 × 10^{-5}
LC	20	0.700 ± 0.010 (6)	6.84	0.53	3.010	9.41 × 10^{-5}
TZ	24	0.790 ± 0.020 (6)	5.58	0.59		
TW	25	0.870 ± 0.010 (6)	5.51	0.59	[4.2 ± 0.4]	(1.2 ± 0.2) × 10^{-4}
GH	26	0.670 ± 0.010 (3)	6.40	0.51		
LC	31	1.070 ± 0.030 (6)	5.34	0.61	6.150	1.99 × 10^{-4}
GH	37	1.030 ± 0.070 (3)	4.92	0.59		
GH	37	1.109 ± 0.030 (6)	4.71	0.66		
TW	37	0.850 ± 0.020 (4)	4.29	0.65		
LC	37	1.420 ± 0.020 (4)	4.19	0.69	7.3 ± 0.7	(2.2 ± 0.2) × 10^{-4}
GH	37	0.957 ± 0.040 (4)	4.85	0.64	*n* = 8	*n* = 8
GH	37	1.350 ± 0.040 (6)	4.62	0.65		
LC	37	1.130 ± 0.010 (4)	4.79	0.62		
GH	37	1.200 ± 0.040 (5)	4.68	0.63		
LC	42	1.240 ± 0.020 (6)	4.11	0.66	9.500	3.27 × 10^{-4}

Suspension A: washed human red blood cells. Hematocrit 0.16. Intracellular pH 7.9 ± 0.4. Carbonic anhydrase 80,000 W-A U/100 ml, $NaHCO_3$ 2 mM. Suspending medium: NaCl 146.5 mM, KCl 3.5 mM equilibrated with 10% O_2, 90% N_2 gas mixture at the specific temperature.

Solution B: pH 6.8 ± 0.1. Phosphate buffer 30 mM, NaCl 112.5 mM equilibrated at the specific temperature, or for 2°C, phosphate buffer 10 mM, NaCl 137.5 mM equilibrated with 10% O_2, 90% N_2 gas mixture.

J ± standard error is the HCO_3^- flux carried by the Jacobs-Stewart cycle. $[HCO_3^-]_i$, $[HCO_3^-]_o$, $P_{HCO_3^-}$, and k are calculated intra- and extracellular [HCO_3^-], permeability, and rate constant, respectively.

outside was constant at about 4 mM at 37°C and 5 mM at 2°C (Fig. 7). The intracellular [Cl^-] was kept constant at about 86 mM. The slopes of the curves decrease with increasing extracellular [Cl^-] concentration (or with [Cl^-] gradient), more obviously at the lower temperature.

DISCUSSION

Rate-Determining Step

The major assumptions in the study of the kinetics of bicarbonate-chloride exchange by the present technique are that (*a*) the rate-determining step for the

transfer of H^+ in the Jacobs-Stewart cycle is HCO_3^--Cl^- exchange across the membrane under conditions where the extracellular CO_2 hydration-dehydration reaction is accelerated and; (*b*) total CO_2 concentration is much less than [Cl^-] and much larger than [OH^-], and no other freely movable ions are present in significant concentrations. This implies that in the Jacobs-Stewart cycle, neither the extracellular or the intracellular CO_2 hydration-dehydration reaction, nor CO_2 diffusion across the membrane, is rate-determining. The expression "rate-determining" is used here in the usual sense of a "bottleneck" in a series of steps in a sequence (e.g., in consecutive chemical reactions) (Moore, 1962). That the

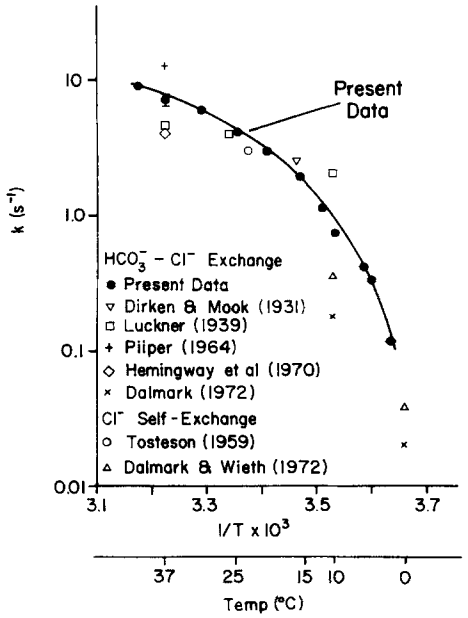

FIGURE 5. Arrhenius plot for the rate constant of bicarbonate transport. Present data are represented by ●. The solid curve was fitted by eye. Data calculated from the literature are represented by different open symbols. In order to make the rate constants comparable, all data in the literature have been corrected for differences in packed cell volume.

extracellular CO_2 hydration-dehydration reactions are not rate determining under our experimental conditions is shown in the data in Fig. 4. As the carbonic anhydrase concentration in the extracellular fluid is increased, the rate of CO_2 reaction will increase in proportion (Roughton and Booth, 1946; Kernohan et al., 1963), but above a concentration of 20,000 W-A U/100 ml mixture (~1.7 μM, which would accelerate the CO_2 reactions about 200-fold), addition of further carbonic anhydrase had no effect on the rate of H^+ transfer. We conclude that the overall process is no longer rate determined by the chemical reaction rate in the extracellular fluid. As a precaution, we routinely employed carbonic anhydrase concentrations above this value. The concentration of carbonic anhydrase inside the red cell is sufficient to accelerate the CO_2 reaction about 10,000 times (Kernohan et al., 1963). We assume therefore that the intracellular CO_2 reactions are also not rate determining.

CO_2 gas diffusion is believed to be at least three orders of magnitude more rapid than HCO_3^--Cl^- exchange across the erythrocyte membrane (Gros and Moll, 1971), and therefore should not be rate determining. When extracellular

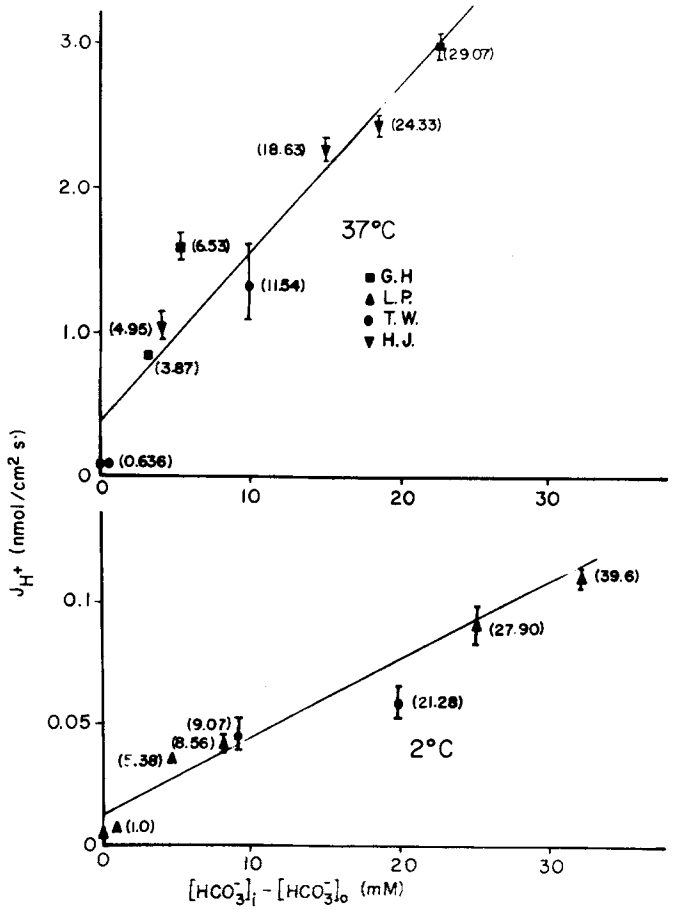

FIGURE 6. Variation of H^+ flux with bicarbonate gradient at 37°C and 2°C. The intracellular pH of suspension A was 7.7 ± 0.1 at 37°C and 8.26 ± 0.07 at 2°C. The extracellular pH at the beginning of the Jacobs-Stewart cycle was 6.74 ± 0.06 at 37°C and 6.9 ± 0.1 at 2°C. The numbers in parentheses are the intracellular $[HCO_3^-]$. At 37°C, the linear regression equation is J_{H^+} (nmol/cm² s) = 0.117 gradient + 0.409 (r = 0.954). At 2°C, the linear regression equation is J_{H^+} (nmol/cm² s) = 0.0031 gradient + 0.012 (r = 0.994).

$[Cl^-]$ was decreased, this decreased the rate of H^+ transfer from outside to inside (Fig. 7). This change in $[Cl^-]$ gradient should not significantly influence the diffusion of CO_2 or the rate of CO_2 hydration-dehydration reaction. Thus, it may be concluded that the rate-determining step in transferring H^+ out of the red cell under the conditions of our experiments is the exchange of chloride for bicarbonate ions across the membrane.

Mechanisms of Bicarbonate-Chloride Exchange

The most simple mechanism for movement of a particle down an electrochemical gradient is that of simple diffusion. Most data on univalent anion movements across erythrocyte membranes have been interpreted in this way by use of a constant field assumption, slightly modified (see, for example, the review by Passow, 1969). Recently, this interpretation has been questioned for chloride-

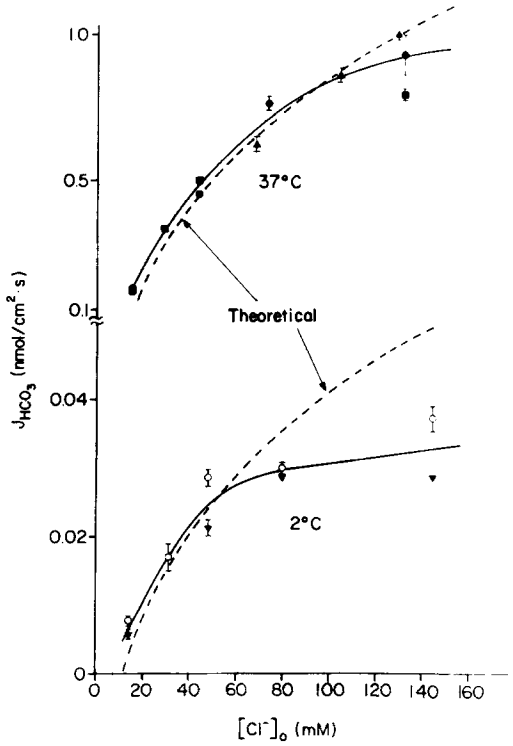

FIGURE 7. Variation of bicarbonate flux with extracellular Cl^- concentration. 37°C. Donor: GH. The theoretical line is bicarbonate flux calculated from Eq. (7) for the same experimental conditions: $[HCO_3^-]_i$ = 4.35 mM, $[HCO_3^-]_o$ = 0.67 mM, $[Cl^-]_i$ = 86 mM, assuming HCO_3^- permeability of 2.1×10^{-4} cm/s. 2°C. Donor: LP. The theoretical line is calculated by assuming HCO_3^- permeability of 8×10^{-6} cm/s. $[HCO_3^-]_i$ = 5.38 mM, $[HCO_3^-]_o$ = 0.71 mM, $[Cl^-]_i$ = 86 mM. The different symbols indicate data from different sets of experiments.

chloride self-exchange kinetics across the red cell membrane (see Sachs et al., 1975), and by implication for Cl^-/HCO_3^- exchange. Cation fluxes in red blood cells exposed to cation ionophores, which are postulated specifically to increase cation permeability, appear to be limited by anion fluxes, which suggests that the net movement of Cl^- across the red cell membrane is slower by four orders of magnitude than self exchanges (Harris and Pressman, 1967; Hunter, 1971; Tosteson et al., 1973). Membrane conductance in Amphiuma red blood cells is much less than it should be if the specific rate of net movements of Cl^- were the same as the specific rate of Cl^- self exchange (Lassen et al., 1974). Both these

lines of evidence have been interpreted to mean that net movements of Cl^- across the membrane do not occur through the same mechanism as Cl^- self exchange. Gunn et al. (1973) have reported that the efflux of $^{36}Cl^-$ from loaded cells at 0°C exhibited saturation kinetics, showed inhibition of chloride self exchange by various substances (including HCO_3^-), and was influenced by intracellular pH. These data have been interpreted to mean that Cl^- exchange is mediated by a carrier mechanism (Gunn, 1972). Little comparable data exist for HCO_3^--Cl^- exchange kinetics at any temperature, or for Cl^--Cl^- exchange kinetics at temperatures above 0°C.

The data we have presented, unfortunately, do not unambiguously elucidate the mechanism of Cl^--HCO_3^- exchange, but can be interpreted equally well by assuming either carrier-mediated or diffusional exchange transport. However, our flux measurements over a wide range of temperature (see discussions below) do place requirements on the properties of the membrane exchange mechanism.

Bicarbonate Permeability Computations

Since our data are compatible with a diffusion model, we have used the constant field assumption to compute a value for bicarbonate permeability under different conditions, both to take into account the possible effects of membrane potential and to be able to compare our data with those of previous workers. If the mechanism, after further investigations, turns out to be dependent on carrier mediation (a conclusion not now possible, at least at high temperatures, for HCO_3^--Cl^- exchange), then these permeability values are only "effective" and must be interpreted accordingly.

Permeability ($P_{HCO_3^-}$) in centimeters/second is calculated according to the constant field passive diffusion theory (Goldman, 1943; Crandall et al., 1971). A Cl^- permeability value of 1×10^{-4} cm/s at 23°C (calculated from Tosteson, 1959) was used in most computations.

Computed values of $P_{HCO_3^-}$ increased two orders of magnitude from 3.8×10^{-6} cm/s at 2°C to 3.27×10^{-4} cm/s at 42°C, consistent with values computed from data reported for other monovalent anions with respect to the erythrocyte membrane. At 37°C, intracellular pH of about 7.6 and extracellular pH of 6.7, the average value of 2.2×10^{-4} cm/s (Table I) is within an order of magnitude of the Cl^- permeability (at 23°C) of 1×10^{-4} cm/s (Tosteson, 1959), or OH^- permeability of 2.2×10^{-3} cm/s (Crandall et al., 1971). It is also of the same order of magnitude as the reported HCO_3^- permeability of rat erythrocyte membrane (Silverman, 1974) obtained under alkaline conditions and of the human erythrocyte membrane under physiological conditions (37°C, pH 7.4, 0.025 M [HCO_3^-]) using ^{18}O exchange between CO_2 and H_2O (Itada et al., 1976). The rate of HCO_3^- ion movement across the red cell membrane is roughly 10^{-5} its own mobility in water (Moore, 1962) if one calculates the mobility of HCO_3^- in a layer equivalent to the membrane thickness 0.01 μm.

Effects of Temperature on HCO_3^--Cl^- Exchange Kinetics

Our data in Fig. 5 show that the rate constants of HCO_3^--Cl^- exchange varied greatly with temperature, increasing 80-fold from 0.12 to 9.5 s^{-1} in the tempera-

ture range from 2°C to 42°C. The Arrhenius plot also indicates that the Q_{10} and the activation energy (E_A) both change continuously with temperature. Between 2°C and 12°C, our measured Q_{10} is 7, decreasing gradually to 1.7 between 24°C and 42°C. The transport process thus seems to be markedly dependent on temperature in the cold, and much less dependent on temperature in the physiological temperature range.

Several other investigators have studied the temperature dependence of HCO_3^- and/or Cl^- exchange in human red cells. Our measured Q_{10} of 7 between 2°C and 12°C agrees well with the reported value of 8 between 0°C and 10°C for HCO_3^--Cl^- exchange and Cl^- self exchange (Dalmark and Wieth, 1972). Previous data on temperature dependence of bicarbonate-chloride transport at high temperatures were obtained by Luckner, yielding a Q_{10} of 1.2 between 24°C and 40°C (in close agreement with our value of 1.7). Brahm (1975), using isotope techniques, obtained values for k at 38°C of 13.1 s^{-1} and for E_A between 15°C and 38°C of 22 kcal/mol, both remarkably close to our own data. Dalmark (1972) concluded from the kinetic data previously available for HCO_3^--Cl^- exchange that the Q_{10} of 8 measured between 0°C and 10°C also applied to the exchange at high temperatures. However, the data available at that time were not precise enough to allow accurate determination of the relationship between Q_{10} and temperature.

Self exchange of other inorganic anions so far studied, such as bromide, iodide, thiocyanate (Dalmark and Wieth, 1972), sulfate (Passow, 1969), and phosphate (Deuticke and Duhm, 1964), all have shown a constant Q_{10} of 5–8, although the only one of these monovalent anions studied over an adequate range of temperature was iodide. HCO_3^--Cl^- exchange kinetics (and perhaps Cl^--Cl^- exchange as well) thus seem to have a qualitatively different temperature dependence from these other anion exchanges. Glucose transport exhibits a similar variation in Q_{10} in human red cells (Sen and Widdas, 1962; Lacko et al., 1973), and in *Escherichia coli* (Linden et al., 1973). Viscosity studies (Zimmer and Schirmer, 1974) support the possibility that there is a phase transition of the erythrocyte membrane lipids at 18–19°C, although this interpretation is subject to question (Gottlieb and Eanes, 1974).

The nonlinear behavior of HCO_3^--Cl^- exchange in the Arrhenius plot suggests that two processes may be involved in the exchange, one with low activation energy (perhaps a diffusion process) and the other with high activation energy (perhaps a chemical process). The two processes can be either consecutive or simultaneous, with the low activation energy process determining the exchange rate at physiological temperature and the high activation energy process determining the rate at low temperatures (Stearn, 1949). The latter mechanism (simultaneous processes) would have to be accompanied by functional (± structural) alterations in the red cell membrane with temperature to explain the data. It is also possible that the changes in HCO_3^- exchange with temperature are produced by variations in a carrier transport mechanism (for example, a change in K_m with temperature). However the large variation in activation energy for the process, as well as its low absolute value at higher temperature, is not consistent with usual chemical processes involving binding. We conclude that caution is necessary when extrapolating data obtained at low temperatures or for

other ionic species to the behavior of physiologically important processes under in vivo conditions.

Relationship of HCO_3^- Flux to Driving Gradient

[HCO_3^-] GRADIENT The data presented in Fig. 6 show that HCO_3^- flux increases linearly with increasing [HCO_3^-] gradient at both 37°C and 2°C, total osmolarity being kept constant by decreasing [Cl^-] appropriately (130–110 mM range). As discussed above, the present technique did not allow accurate measurements at higher gradients. These data are consistent with passive diffusion of HCO_3^- across the membrane or with competitive inhibition between HCO_3^- and Cl^- (Gunn et al., 1973). However, Dirken and Mook (1931), Luckner (1939), and Hemingway et al., (1970) did measure the kinetics of HCO_3^--Cl^- exchange at high bicarbonate gradients (up to 125 mM) at 37°C by different techniques. Their calculated rate constants are not significantly lower than those determined in the present experiments at low bicarbonate gradient (Fig. 5), suggesting that the relationship may remain linear for HCO_3^- transport up to 125 mM [HCO_3^-] gradient in the presence of about 100 mM [Cl^-] at 37°C. The rate constant (0.02 s^{-1}) reported by Damark (1972) for HCO_3^--Cl^- exchange at 0°C ([HCO_3^-] gradient ~150 mM) is lower than the value (0.1 s^{-1}) at 2°C obtained with the present technique ([HCO_3^-] gradient ~30 mM), suggesting the possibility of saturation kinetics at high [HCO_3^-] gradients at low temperature.

EXTRACELLULAR [CL^-] When the extracellular [Cl^-] is reduced from its normal value of 150 mM, HCO_3^- flux decreases monotonically in spite of the fact that HCO_3^- concentrations remain unchanged (Fig. 7). These data, especially those at 2°C, may be interpreted as exhibiting a saturation phenomenon. The [Cl^-] at which the flux appears half-maximal at 2°C was about 30 mM, very close to the value reported by Gunn et al. (1972) at 0°C. However, since HCO_3^- efflux, which equals in value the Cl^- influx, was influenced by both the [HCO_3^-] gradient and the [Cl^-] gradient, it is also logical to interpret our data as an interaction through transmembrane potential. By using a constant value for $P_{HCO_3^-}$ and the constant field assumption, the theoretical relationship between HCO_3^- flux and extracellular [Cl^-] shown in Fig. 7 was computed. Values of HCO_3^- permeability used were 2.1×10^{-4} cm/s at 37°C, and 8×10^{-6} cm/s at 2°C. The fluxes predicted by the constant field equation fit the data adequately at 37°C, but there is some disagreement at 2°C.

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