

Kinetics of Bicarbonate Transport in Human Red Blood Cell Membranes at Body Temperature

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ABSTRACT We studied unidirectional [¹⁴C]HCO₃⁻ efflux from human resealed red cell ghosts with 1 mM acetazolamide under self-exchange conditions at pH = pH^(i=o) 7.4–9.0 and 0–38°C by means of the Millipore-Swinnex and continuous flow tube filtering techniques. ¹⁴CO₂ loss from cells to efflux medium and further to the atmosphere was insignificant. [¹⁴C]HCO₃⁻ efflux was determined at pH 7.8, 38°C under symmetric variation of the HCO₃⁻ concentrations ($C^{(i=o)}$), and asymmetric conditions: $C^{(i)}$ varied, $C^{(o)}$ constant, or $C^{(o)}$ varied, $C^{(i)}$ constant. MM-fit, $J^{ff} = J_{max}^{ff} \cdot C \cdot (C + K_{1/2})^{-1}$, used to describe the concentration dependence of $J^{ff,o}$ when only $C^{(o)}$ varied, yields at $C^{(i)} = 50$ mM: $K_{1/2}^o = 3.8$ mM, $J_{max}^{ff,o} = 20$ nmol cm⁻² s⁻¹; at $C^{(i)} = 165$ mM: $K_{1/2}^o = 10$ mM, $J_{max}^{ff,o} = 32$ nmol cm⁻² s⁻¹. When $C^{(i)}$ varied, noncompetitive self inhibition by HCO₃⁻ binding (inhibitor constant K_I) to an intracellular site was included (MS-fit). Under conditions of (a) symmetry: $C^{(i=o)} = 9$ –600 mM, $K_{1/2}^s = 173$ mM, $K_I = 172$ mM, and $J_{max}^{ff,s} = 120$ nmol cm⁻² s⁻¹, (b) asymmetry: $C^{(o)} = 50$ mM, $K_{1/2}^s = 116$ mM, $K_I = 136$ mM, and $J_{max}^{ff,i} = 92$ nmol cm⁻² s⁻¹. All flux parameters accord with the ping-pong model for anion exchange. The data for $C^{(i)} < 200$ mM also fit well to the MM equation, but $K_{1/2}$ and J_{max}^{ff} are different from the MS-fit and are inconsistent with the ping-pong model. Thus, self-inhibition (MS-fit) must be included even at low concentrations. As at 0°C, the system is asymmetric: 8–10 times more unloaded transport sites face inward than outward when $C^{(i=o)}$. $J^{ff,s}$ was not monoexponentially dependent on temperature at 0–38°C, indicating that the transmembrane anion transport is controlled by several rate constants with different temperature dependencies. $J^{ff,s}$ was not significantly affected by increasing pH^(i=o) from 7.4 to 7.8, but it decreased by 50% when pH was raised to 9.0.

KEY WORDS: band 3 • anion exchange • asymmetry • red cell ghosts • ping-pong model

INTRODUCTION

Red blood cells play an important role in transport of metabolic gases in the human body. One important step in the removal of carbon dioxide from oxygen-consuming tissues to the lungs is the exchange of bicarbonate for chloride across the erythrocyte membrane, referred to as the “Hamburger Shift,” that involves the transmembrane protein, called band 3 based on its electrophoretic characteristics (Fairbanks et al., 1971); capnophorin, i.e., “smoke carrier” (Wieth and Bjerrum, 1983) based on the physiological role of the transport protein in the removal of CO₂, the “smoke” that results from metabolism; or AE1 to emphasize its membership in a genetically related family of anion exchangers (Kopito, 1990).

Though the detailed molecular basis for the Hamburger Shift is unknown, a high level of understanding of anion transport kinetics has been attained through studies with radioactive tracers. Most studies measure chloride self exchange, predominantly at 0°C where simple filtration methods can be used (e.g., Dalmark

and Wieth, 1972; Gunn et al., 1973; Funder and Wieth, 1976; Gunn and Fröhlich, 1979; Hautmann and Schnell, 1985; Gasbjerg and Brahm, 1991; Bjerrum, 1992; for reviews see Knauf, 1986; and Passow, 1986). By exploiting the high time resolution of the continuous flow tube technique, Brahm (1977) extended the temperature range for studies of chloride transport up to body temperature. By comparing chloride exchange flux at body temperature with the flux determined as net current of anions (Hunter, 1977) it became clear that anion exchange is a highly electroneutral process and that net transport of anions, possibly performed by capnophorin, is about five orders of magnitude slower than the exchange transport.

On the basis of studies of chloride transport mainly at 0°C, the “ping-pong model” for anion exchange was proposed (Gunn and Fröhlich, 1979; Fröhlich and Gunn, 1986). According to this model, a transport site in the protein, unloaded or loaded with an anion, may either face the internal or the external compartment. Anion transport is achieved by a conformational change in the loaded form of the protein, resulting in translocation of the substrate. Translocation of the unloaded form of the transport site, which represents a potential pathway for conductive anion transport (see Fröhlich, 1984), is negligible compared to the translocation of the loaded forms, and is therefore not included in the

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model. This simple transport model is in accordance with most experimental results obtained in tracer experiments using monovalent anions at 0°C. However, at high intracellular anion concentrations the model needs modification because experimental data indicate that anion binding to an internally located allosteric site prevents anion translocation (Dalmark, 1976; Wieth, 1979; Knauf and Mann, 1986; Gasbjerg and Brahm, 1991).

Studies of bicarbonate self-exchange were initiated in the late seventies by Wieth (1979), who showed that unidirectional efflux of radioactively labeled bicarbonate could be studied at alkaline pH (pH 8.7) at 0°C without significant errors caused by efflux or loss to the atmosphere of $^{14}\text{CO}_2$. Subsequent studies, still at alkaline pH, were done at body temperature in order to characterize physiological aspects of bicarbonate transport in human red blood cells (Wieth and Brahm, 1980; Wieth et al., 1982). Recently Gasbjerg and Brahm (1991) showed that bicarbonate tracer experiments can be carried out at more physiological pH values and that the ping-pong model adequately describes bicarbonate transport at 0°C, pH 7.8.

Here we present data showing that it is possible to study the exchange of bicarbonate at 0–38°C and near physiological pH (pH 7.8). Using these methods, we have asked if either the simple or modified ping-pong model is adequate to describe HCO_3^- exchange at body temperature, and have used HCO_3^- exchange to measure the asymmetry of the system at physiological temperature. We have also examined the pH dependence of HCO_3^- exchange at 38°C, as well as the temperature dependence over the range from 0 to 38°C.

MATERIALS AND METHODS

Media

All media were made from reagent grade chemicals. Bicarbonate containing media were made shortly before the experiments and were stored at $\sim 4^\circ\text{C}$ until use in capped bottles with very small air volumes above the solution surface. When used for efflux experiments at higher temperature, the media were heated, and pH was adjusted by adding 1 N H_3PO_4 or KOH a few minutes before the experiments. The flux media had the following composition (mM): 1–600 KHCO_3 , 1 acetazolamide. Sucrose was used as an osmotic substituent under conditions with asymmetric anion distribution, and as an osmotic additive when the intracellular bicarbonate < 100 mM with the purpose of adjusting mean cell volumes as close as possible to 90 fl $4,4'$ -diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) prepared as the sodium salt (Gasbjerg et al., 1993) was used to make a 10 mM stock solution in water and was stored frozen until use. Osmolarity of all efflux media was checked by using a Roebing Micro Osmometer (Messtechnik, Berlin, Germany).

The physical chemistry of bicarbonate solutions at 0°C has been described previously (Wieth, 1979; Gasbjerg and Brahm, 1991). Following the strategy applied previously for media at 0°C (Gasbjerg and Brahm, 1991) and using data from the literature

(Harned and Scholes, 1941; Harned and Bonner, 1945) at 38°C we obtained a pK value of 6.1 for the $\text{CO}_2 \rightleftharpoons \text{HCO}_3^-$ equilibrium, and a pK value of 9.8 for the $\text{HCO}_3^- \rightleftharpoons \text{CO}_3^{2-}$ equilibrium. At pH 7.8, 38°C, a nominal 165 mM bicarbonate solution contains about 2% CO_2 and 1% CO_3^{2-} . In the following we have used the nominal HCO_3^- concentration.

Preparation of Cells

Resealed human red cell ghosts were used for all efflux experiments. The preparation of the cells, the loading with $[^{14}\text{C}]\text{HCO}_3^-$ (Amersham Corp., Buckinghamshire, UK), and the determination of radioactivity, intracellular bicarbonate concentration, and cell water volume were carried out as described by Gasbjerg and Brahm (1991).

Efflux Experiments, Calculation of Unidirectional Efflux

The Millipore-Swinnex filtering technique (Dalmark and Wieth, 1972) and the Continuous Flow Tube Method (Brahm, 1977; 1989) were used for determination of the rate constants for $[^{14}\text{C}]\text{HCO}_3^-$ efflux. At all temperatures $[^{14}\text{C}]\text{HCO}_3^-$ efflux followed a monoexponential time course for solute transport in a closed two-compartment system, and the rate constant, k (s^{-1}) was determined by linear regression analysis. Under asymmetric conditions with $C^{(i)}$ high and $C^{(o)}$ very low (< 10 mM), the "equilibrium" sample that is needed for the calculation of k was taken when the tracer had reached nearly the same distribution as unlabeled HCO_3^- , and before a significant net flow of HCO_3^- had taken place (for details of this procedure and the calculation of k , see Gasbjerg and Brahm, 1991). k is related to $T_{1/2}$, the half time for isotopic equilibrium, P_{app} (cm s^{-1}), the apparent permeability,¹ and J^{eff} ($\text{mol cm}^{-2} \text{s}^{-1}$), the unidirectional bicarbonate efflux by:

$$J^{eff} = k \cdot V_c \cdot A_c^{-1} \cdot C^{(i)} = \ln 2 \cdot T_{1/2}^{-1} \cdot V_c \cdot A_c^{-1} \cdot C^{(i)} = P_{app} \cdot C^{(i)}, \quad (1)$$

where $V_c \cdot A_c^{-1}$ is the ratio of the cell water volume to the cell membrane area. V_c was determined in each experiment, whereas A_c was assumed to be constant, $1.42 \times 10^{-6} \text{ cm}^2 \text{ cell}^{-1}$. (For details, see Gasbjerg and Brahm, 1991).

The resealed red cell ghosts behave as perfect osmometers (any relative change in the osmolality of the medium is followed by an inverse change in cell volume). Hence, if the osmolality of the flux medium was different from the wash medium, the intracellular concentration was corrected according to the difference in osmolality. This correction was made in order to assign the calculated flux (cf. Eq. 1) to the actual intracellular concentration (the flux value needs no correction, because the intracellular amount of bicarbonate, $C^{(i)} \cdot V_c$, is not changed). In cases where $C^{(o)}$ was very low, the rate constant was corrected for the effect of intracellular and extracellular compartment sizes as described previously (Gasbjerg and Brahm, 1991).

¹Abbreviations used in this paper: $C^{(i)}$, intracellular bicarbonate concentration; $C^{(o)}$, extracellular bicarbonate concentration; $C^{(i=o)}$, bicarbonate concentration when internal bicarbonate equals external bicarbonate concentration; $J_{max}^{ff,s}$, maximum unidirectional efflux (symmetric conditions); $J^{ff,s}$, actual unidirectional efflux (symmetric conditions); $K_{1/2}$, overall half-saturation constant (symmetric conditions); K_i , self-inhibition dissociation constant (symmetric conditions); $J_{max}^{ff,o}$, maximum unidirectional efflux (asymmetric conditions where $C^{(o)}$ varies); $J^{ff,o}$, actual unidirectional efflux (asymmetric conditions where $C^{(o)}$ varies); $K_{1/2}^o$, outside half-saturation constant (asymmetric conditions where $C^{(o)}$ varies); $K_{1/2}^i$, inside half-saturation constant (asymmetric conditions where $C^{(i)}$ varies).

Curve Fitting

All curve fittings except for straight lines were done by using the Enzfitter computer program (Elsevier Biosoft) using a nonlinear least-squares regression analysis with simple weighting. All data obtained by curve fitting are presented with the standard error calculated by the program.

RESULTS

Control Experiments

To study the stability of the bicarbonate media and to investigate if loss of radioactivity in any of the experimental steps may affect determination of unidirectional bicarbonate efflux, we performed the following experiment which mimics all steps in the standard experimental procedure, except that the volume of packed resealed red cell ghosts normally used for efflux experiments was replaced with a volume of radioactive bicarbonate buffer. At each step radioactive samples were taken and counted in a cooled (5°C) scintillation counter.

Test of bicarbonate media stability. Radioactive bicarbonate was added to 1 liter of double-distilled water at room temperature. KHCO_3 was added to a final concentration of 165 mM and was stirred with a magnet for 5 min. The solution was heated to 38°C in a totally filled capped bottle and titrated from the spontaneous pH of 8.2 to 7.8 with H_3PO_4 . There was no loss of radioactivity during the different steps.

Test for loss of radioactivity to the atmosphere during efflux experiments, pipetting and scintillation counting. (a) Millipore-Swinnex filtration method: 40 ml of $^{14}\text{C}]\text{HCO}_3^-$ medium was poured into the flux chamber and stirred with a magnet during the experiment. Six filtered samples of ~ 1 ml were obtained within 30 s. We assured that no air was left in the syringes used to obtain the filtered samples before they were sealed with a rubber cap.

(b) Continuous flow tube method: $^{14}\text{C}]\text{HCO}_3^-$ medium stored in a syringe was mixed with non-radioactive medium in the flow tube mixing chamber, and six filtered samples and one total sample ("equilibrium sample") were obtained. From each of these samples 200 μl was pipetted into scintillation fluid either 2 or 10 min after filtration. The pipetted samples were counted three times: within 1 h after pipetting, and after being stored 24 and 48 h in the scintillation counter.

No loss of radioactivity was detected during any of these experimental procedures.

Amount of $^{14}\text{CO}_2$ efflux at 38°C, pH 7.8. Radioactive carbon, ^{14}C , may leave the cell in the form of either $^{14}\text{CO}_2$, $^{14}\text{C}]\text{H}_2\text{CO}_3$, $^{14}\text{C}]\text{HCO}_3^-$ or $^{14}\text{CO}_3^{2-}$. Fig. 1 shows two experiments, carried out under symmetric conditions with $C^{(i)} = C^{(o)} = 165$ mM, which demonstrate that efflux of carbon dioxide can be ignored in the calculation of unidirectional bicarbonate flux even at 38°C. The experiments were carried out at pH 7.8, 38°C us-

ing resealed red cell ghosts that, as in all experiments in this study, were resealed in a medium containing 1 mM acetazolamide, which effectively inhibits carbonic anhydrase activity. The graph depicts the fraction of tracer that is left in the intracellular compartment as a function of time. The results depicted by circles (see also inset) were obtained by using the continuous flow tube method, and demonstrate that ^{14}C efflux follows a monoexponential time course. The rate constant for bicarbonate efflux under control conditions is 2.5 s^{-1} and the half time ($T_{1/2} = \ln 2 k^{-1}$) is 280 ms. The upper plot (diamonds) was obtained by the Millipore-Swinnex filtration method and shows unidirectional ^{14}C efflux after combined irreversible and reversible (50 μM) DIDS inhibition (see Gasbjerg et al., 1993). Under these conditions, the rate constant is reduced to 0.019 s^{-1} ($T_{1/2} = 37\text{ s}$), i.e., the efflux is inhibited by 99.2%. From these results we conclude that the contribution from $^{14}\text{CO}_2$ and $^{14}\text{C}]\text{H}_2\text{CO}_3$ is $< 1\%$ of the total ^{14}C -efflux and can be ignored.

Effects of pH on $^{14}\text{CO}_2$ efflux at 38°C. To determine the "background" of nonbicarbonate tracer efflux in the pH-range 7.4–9.0 where we characterize the pH dependence of bicarbonate transport, we performed experi-

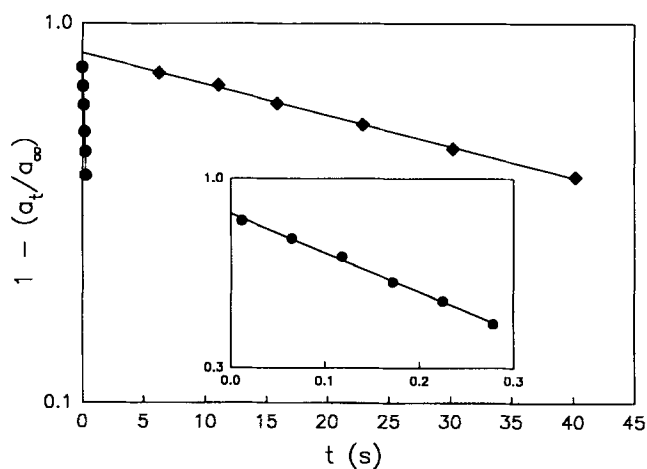


FIGURE 1. Inhibition of unidirectional ^{14}C bicarbonate efflux at 38°C, pH 7.8 with the specific anion transport inhibitor DIDS. The plots show the fraction of tracer in the intracellular compartment as a function of time (t , s), calculated by subtracting from 1 the ratio of a_t , the radioactivity in a sample of the extracellular medium at time t , to a_{∞} , the radioactivity in an equilibrium sample; for details, see Gasbjerg and Brahm (1991). Resealed human red cell ghosts were used with $C^{(i)} = C^{(o)} = 165$ mM KHCO_3 ; carbonic anhydrase activity was inhibited by addition of 1 mM acetazolamide to cells and media. The control data with no inhibitor present (circles) were obtained by using the continuous flow tube method; the inset shows the same data plotted with an expanded time axis. The rate constant is 2.5 s^{-1} and the half time ($T_{1/2} = \ln 2 k^{-1}$) is 280 ms. The data after combined irreversible and reversible (50 μM) inhibition with DIDS (diamonds) were obtained with the Millipore-Swinnex filtration method. The rate constant for tracer efflux is 0.019 s^{-1} ($T_{1/2} = 37\text{ s}$).

ments like that shown in Fig. 1 with cells and media titrated to the pH values concerned, and plotted the rate constants as a function of pH as shown in Fig. 2. The inhibition obtained after irreversible DIDS binding to the transporter (*triangles*) was further increased by adding DIDS to the efflux medium, either 50 μM (*diamonds*) or 200 μM (*squares*), as indicated by the smaller rate constants at the different pH values. The solid curve is the expected rate constant of spontaneous bicarbonate dehydration as determined from data obtained from the literature (Magid and Turbeck, 1968; Magid, 1970). At pH 7.8, 38°C the calculated rate constant for conversion of HCO_3^- to CO_2 is $\sim 0.004 \text{ s}^{-1}$, i.e. $T_{1/2} \sim 3 \text{ min}$. The tracer efflux rate constant for DIDS-inhibited cells was higher, $\sim 0.02 \text{ s}^{-1}$, still far below that for the uninhibited bicarbonate self exchange of $\sim 2.5 \text{ s}^{-1}$.

Donor variation. We determined unidirectional bicarbonate efflux under control conditions ($C^{(i)} = C^{(o)} = 165 \text{ mM KHCO}_3$) for 6 donors (A through F). Two donors (C and E) had a slightly, but statistically significantly lower efflux rate ($\sim 10\%$ lower, t test: $P = 0.0002$) than the other four donors who had almost identical efflux rates. Only blood cells from A, B and C were used in the following experiments. In cases where data obtained with C as donor were combined with data obtained with blood cells from the other two donors, the flux values obtained with C as a donor were multiplied by 1.11.

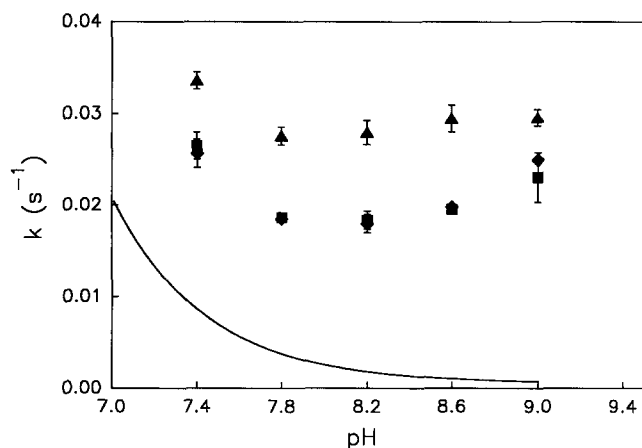


FIGURE 2. pH dependence of residual ^{14}C efflux after irreversible and reversible inhibition with DIDS. All experiments were performed using ghosts with $C^{(i)} = C^{(o)} = 165 \text{ mM KHCO}_3$. The plot shows the measured rate constant for ^{14}C efflux as a function of pH at 38°C. The triangles show the rate constants obtained for ghosts with DIDS irreversibly bound to the cell membrane. Addition of either 50 (*diamonds*) or 200 μM (*squares*) DIDS to the efflux medium further decreased the rate constants. Bars indicate standard errors, if larger than the size of the points. The solid curve shows the calculated rate of spontaneous bicarbonate dehydration at 38°C in the pH range 7–9, based on data from Magid and Turbeck (1968) and Magid (1970).

Temperature Dependence of Unidirectional Bicarbonate Efflux

We determined the temperature dependence of unidirectional bicarbonate efflux at $C^{(i)} = C^{(o)} = 165 \text{ mM KHCO}_3$ in the temperature range 0–38°C at pH 7.8. In the temperature range 0–15°C we used the Millipore-Swinnex filtering method, and at 15–38°C the continuous flow tube method was applied. At 15°C we used both methods and obtained the same flux value. This result demonstrates that both techniques, even at the limit of their performance, are well suited for determination of unidirectional bicarbonate efflux in isolated cells, in accordance with previous results obtained with rapidly transported halides (Brahm, 1977; Wieth and Brahm, 1985).

Fig. 3 depicts an Arrhenius plot of the natural logarithm of the unidirectional anion efflux vs. the reciprocal absolute temperature. If one activation energy dominates, a linear relationship is expected in this type of plot, and the numerical value of the slope of the line equals the Arrhenius activation energy for the process. Fig. 3 shows, however, a nonlinear dependence for unidirectional bicarbonate efflux, indicating complicated transport kinetics (see DISCUSSION). The flux at 38°C is about four times smaller than predicted from linear extrapolation of the data at 0–10°C.

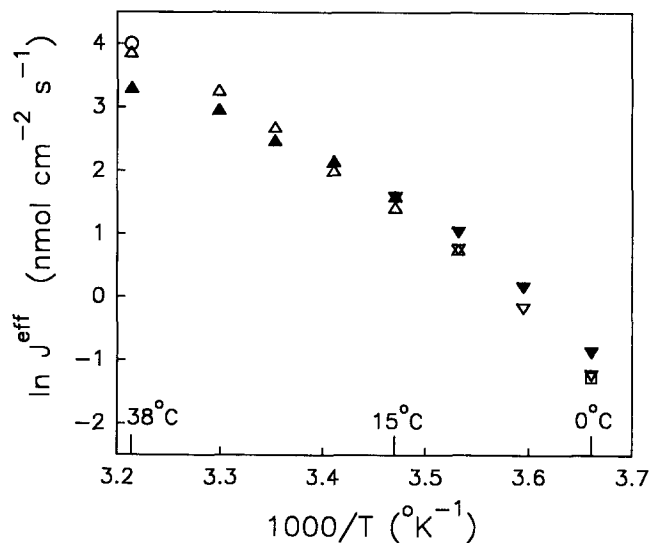


FIGURE 3. Arrhenius plot of $\ln J^{\text{eff}}$ ($\text{nmol cm}^{-2} \text{ s}^{-1}$) against the reciprocal absolute temperature at 0–38°C for bicarbonate (*solid triangles*) and chloride (*open symbols*) transport in resealed red cell ghosts. Bicarbonate data were obtained with $C^{(i)} = C^{(o)} = 165 \text{ mM KHCO}_3$ and pH = 7.8. The chloride data are from Brahm (1977, *open triangles*, pH 7.2), Gasbjerg and Brahm (1991, *square*, pH 7.8), and Knauf et al. (1996, *circle*, pH 7.8). Fluxes were measured by the Millipore-Swinnex filtering method (*inverted triangles and square*), or by the continuous flow tube method (*circle and upright triangles*). For discussion, see text.

Unidirectional Bicarbonate Efflux as a Function of $pH^{(o)} = pH^{(i)}$

Fig. 4 shows the unidirectional bicarbonate efflux in the pH range 7.4–9.0 under conditions with identical external and internal pH. With increasing pH the fraction of carbonate in the cells and in the efflux medium gradually increases. At pH = 9.0 the ratio of carbonate to bicarbonate is ~ 0.16 . Carbonate may be translocated at a slow rate like sulfate (see Passow, 1986). Because intracellular CO_3^{2-} and HCO_3^- are in rapid equilibrium, however, they can be regarded as a single compartment in terms of isotope exchange, equal in size to the sum of the amounts of CO_3^{2-} and HCO_3^- . Since this is the same as the nominal amount of HCO_3^- , use of Eq. 1 with the nominal HCO_3^- concentration gives an accurate value for the unidirectional HCO_3^- efflux.

External, Internal, and Overall Apparent Affinity for Bicarbonate

By fitting Michaelis-Menten like equations to the experimental data at 38°C, pH 7.8, we determined $K_{1/2}$ and J_{max}^{eff} under three fundamentally different conditions as shown in Fig. 5, A–D.

$C^{(i)}$ is constant, $C^{(o)}$ varies (external apparent affinity). Figs. 5, A and B show the results obtained under asymmetric conditions with $C^{(i)} = 50$ mM (Fig. 5 A) or $C^{(i)} = 165$ mM (Fig. 5 B) while $C^{(o)} = 2$ –50 mM (Fig. 5 A) or $C^{(o)} = 2$ –245 mM (Fig. 5 B). In the experiments shown in Fig. 5 A, sucrose was added to the intracellular compartment during the ghost cell resealing period to adjust cell volume and to the extracellular medium in all experiments to balance internal and external osmolarities. A simple Michaelis-Menten like function (MM-fit:

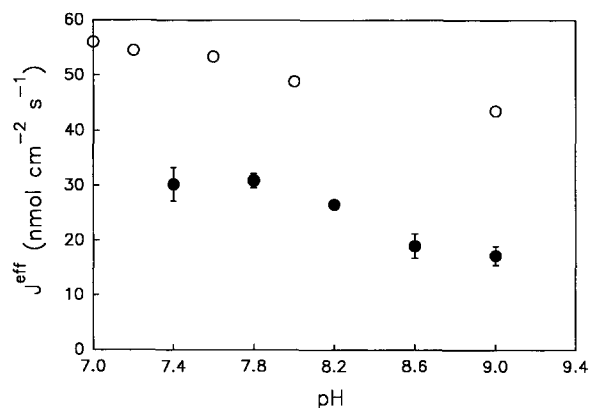


FIGURE 4. pH dependence of J^{eff} for bicarbonate (solid circles) and chloride (open circles) in ghosts at 38°C. Data were obtained using the continuous flow tube method with $C^{(i)} = C^{(o)} = 165$ mM $KHCO_3$ or KCl. Bicarbonate ghosts were titrated with either H_3PO_4 or KOH; chloride ghosts were titrated with either HCl or KOH. The chloride data are from Brahm (1977). Bars indicate standard errors for the bicarbonate data.



FIGURE 5. Concentration dependence of unidirectional bicarbonate efflux at 38°C, pH 7.8. All results were obtained with resealed red cell ghosts using the continuous flow tube method. Bars indicate standard errors if larger than the points. (A–B) $C^{(o)}$ is varied. The flux parameters given in the text were obtained by using the Enzfitter computer program and the Michaelis-Menten equation (MM-fit: $J^{eff} = J_{max}^{eff} \cdot C \cdot (C + K_{1/2})^{-1}$) (A) $C^{(i)} = 50$ mM, and $C^{(o)}$ varied from 2 to 50 mM. Sucrose was added to ghosts to adjust volume and to media to balance internal and external osmolarities. (B) $C^{(i)} = 165$ mM, and $C^{(o)}$ varied from 2 to 245 mM. Sucrose was added to ghosts if $C^{(o)} > 165$ mM $KHCO_3$ and to media if $C^{(o)} < 165$ mM $KHCO_3$. (C) $C^{(i)}$ varied from 8 to 666 mM, $C^{(o)} = 50$ mM. A modified Michaelis-Menten like equation (MS-fit: $J^{eff} = \frac{J_{max}^{eff} \cdot C}{[(C + K_{1/2}) \cdot (1 + C^{(i)} K_1^{-1})]^{-1}}$) was used to obtain the flux parameters given in the text. Sucrose was added to ghosts if $C^{(i)} < 100$ mM $KHCO_3$ and to media to adjust cell volume. (D) $C^{(i)} = C^{(o)}$ varied from 9 to 600 mM. MS-fit was used to obtain the flux parameters. Sucrose was added to ghosts and media if $C^{(i)} = C^{(o)} < 100$ mM $KHCO_3$.

$J^{eff} = \frac{J_{max}^{eff} \cdot C \cdot (C + K_{1/2})^{-1}}$ fitted well to the experimental data and yielded $K_{1/2}^o = 3.8 \pm 0.6$ mM, and $J_{max}^{eff,o} = 20.0 \pm 0.5$ $nmol\ cm^{-2}\ s^{-1}$. In Fig. 5 B sucrose was added to the flux medium in experiments where $C^{(o)} < C^{(i)} = 165$ mM, and to the intracellular medium in experiments with $C^{(o)} > 165$ mM. The MM-equation also fitted well to the experimental data and gave $K_{1/2}^o = 10 \pm 1$ mM and $J_{max}^{eff,o} = 32.0 \pm 0.5$ $nmol\ cm^{-2}\ s^{-1}$.

$C^{(o)}$ is constant, $C^{(i)}$ varies (internal apparent affinity). Fig. 5 C shows the results obtained under asymmetric conditions with $C^{(i)} = 8$ –666 mM, and $C^{(o)} = 50$ mM. As a stabilizing step in the ghost preparation sucrose was added to the ghosts when $C^{(i)} < 100$ mM, and to maintain osmotic equilibrium sucrose was added to the efflux medium in all experiments. As the MM-fit does not suffice to describe the experimental results in the whole concentration range, the effect of bicarbonate binding to an intracellular noncompetitive self inhibition site was included in the MS-fit ($J^{eff} = \frac{J_{max}^{eff} \cdot C}{[(C + K_{1/2}) \cdot (1 + C^{(i)} K_1^{-1})]^{-1}}$). A MS-fit gave $K_{1/2}^i = 116.4 \pm 21.6$ mM, $J_{max}^{eff,i} = 92.4 \pm 15.7$ $nmol\ cm^{-2}\ s^{-1}$, and $K_1 = 139.1 \pm 24.0$ mM.

It should be noted that in this case the parameters determined by the Enzfitter program strongly depend on the initial guesses of the transport parameters. We here present the parameters that gave the best fit to the experimental data (i.e., the residual sum of squares was the smallest), but in this case notably different parameters that describe the experimental data almost as well could be obtained by changing the initial guesses for the parameters.² If intracellular self inhibition was ignored, the use of the MM-fit gave a good fit in a limited range ($C^{(i)} < 200$ mM) with $K_{1/2}^s = 13.4 \pm 2.1$ mM and $J_{max}^{eff,i} = 25.4 \pm 0.9$ nmol cm⁻² s⁻¹.

$C^{(o)} = C^{(i)}$ varies (overall apparent affinity). Fig. 5 D shows the results under conditions with symmetrical variation of the bicarbonate concentration in cells and efflux media ($C^{(i)} = C^{(o)} = 9$ –600 mM). Since $C^{(i)}$ varied in this series and because inhibition is apparent at high concentrations, we chose the MS-fit to describe the experimental data. The flux parameters obtained for the symmetric condition are: $K_{1/2}^s = 173 \pm 46$ mM, $K_I = 172 \pm 44$ mM, and $J_{max}^{eff,s} = 122 \pm 32$ nmol cm⁻² s⁻¹. Using the MM-fit for $C^{(i)} = C^{(o)} < 200$ mM, we obtained $K_{1/2}^s = 46.2 \pm 4.3$ mM, and $J_{max}^{eff,s} = 39.2 \pm 1.1$ nmol cm⁻² s⁻¹.

As discussed in the following paper (Knauf et al., 1996), variations in ionic strength in the symmetric experiments or changes in membrane potential in the asymmetric series may have unexpected effects on the

system. Thus, the reported parameters should be regarded as “apparent” ones that strictly pertain only to the particular experimental conditions used here.

Asymmetry of bicarbonate transport at 38°C and pH 7.8. The asymmetry factor (Knauf et al., 1984) designates the ratio of unloaded outward-facing transport sites to unloaded inward-facing transport sites under conditions with equal intracellular and extracellular anion concentrations. By combining the half saturation constants obtained from the symmetric and one asymmetric series, the asymmetry factor for bicarbonate transport, A , can be calculated according to Knauf and Brahm (1989):

$$A = \left(\frac{K_{1/2}^s}{C^{(i)}} + 1 \right) \left(\frac{K_{1/2}^s}{K_{1/2}^o} - 1 \right)^{-1}. \quad (2)$$

In fact any combination of two half saturation constants can be used to determine the asymmetry factor, but the combination in Eq. 2 is probably least affected by experimental variation. According to the ping-pong model, the asymmetry factor is independent of which monovalent transported anion is used because it is determined by the change in molar Gibbs free energy (ΔG_m^0) for the transition between the anion-free forms of the transport proteins:

$$\Delta G_m^0 = -R \cdot T \cdot \ln(A). \quad (3)$$

By combining $K_{1/2}^o = 10$ mM from Fig. 5 B and $K_{1/2}^s = 173$ mM from Fig. 5 D we obtained $A = 0.13$. Using $K_{1/2}^o = 3.8$ mM from Fig. 5 A and $K_{1/2}^s = 173$ mM from Fig. 5 D yields $A = 0.10$. Straight-line fits to the flux versus concentration data at low concentrations, as described in the following paper (Knauf et al., 1996), give almost the same values for A ($A = 0.10$ –0.11). Hence, our data strongly indicate that 8–10 times more sites face inward than outward when the bicarbonate concentrations on the two sides of the membrane are equal. The free energy difference between the two unloaded forms (Eq. 3) is 5.3–6 kJ mol⁻¹.

DISCUSSION

Control Experiments

The permeability calculated from the irreversible-DIDS-inhibited bicarbonate efflux (Fig. 2) is 1.7×10^{-6} cm s⁻¹ at pH 7.8, almost twice as high as the permeability for irreversible DIDS-inhibited chloride efflux from intact red blood cells ($P = 1.0 \times 10^{-6}$ cm s⁻¹, Gasbjerg et al., 1993). When DIDS-treated resealed red cell ghosts (irreversible inhibition) were suspended in media with up to 200 μM DIDS (reversible inhibition, cf. Fig. 2), the apparent permeability was reduced to 1.2×10^{-6} cm s⁻¹. Similar experiments with intact red blood cells (Gasbjerg et al., 1993) show that chloride permeability

²The experimental results (summarized in Table I) appear to be in accordance with the modified ping-pong model. However, the transport parameters were influenced by the initial guesses supplied to the Enzfitter program which of course is a potential source of error. The dependency on initial guesses is likely due to the fact that $K_{1/2}$ and K_I do not differ much at 38°C. On the other hand this lessens a symmetry problem that is encountered by the use of the MS-fit to determine the kinetic parameters. This symmetry problem is often ignored, but is substantial at 0°C for both chloride and bicarbonate transport (see Gasbjerg and Brahm, 1991). The problem becomes evident by rearranging the MS-equation:

$$J^{eff} = \frac{J_{max}^{eff} \cdot K_I \cdot C^{(i)}}{(K_{1/2} + C^{(i)}) \cdot (K_I + C^{(i)})}.$$

The equation shows that two sets of solutions (=estimated parameters) can be obtained by swapping the estimated values of $K_{1/2}$ and K_I if J_{max}^{eff} is adjusted accordingly. For the symmetric series at 38°C the two solutions are essentially identical because $K_{1/2}$ and K_I have very similar values, and J_{max}^{eff} thus assumes essentially the same value in the two solutions. For the asymmetric series where only $C^{(i)}$ is varied two sets of parameters were also obtained that from a mathematical point of view are indistinguishable. To some extent, this ambiguity is resolved by ³⁵Cl NMR measurements, which give a dissociation constant for binding to the transport site at 0–3°C of <100 mM (Falke et al., 1984; Glibowicka et al., 1988), indicating that the transport site has a lower dissociation constant than does the modifier inhibitory site. We chose one set of parameters ($K_{1/2}^s = 116.4$ mM, $K_I = 139.1$ mM, $J_{max}^{eff,s} = 92.4$ nmol cm⁻² s⁻¹) because this set fits with the prediction based on the experimental values for the symmetric series and $K_{1/2}^o$ (cf. Table I).

can be 99.999% inhibited to $6 \times 10^{-9} \text{ cm s}^{-1}$ by 50 μM DIDS, i.e., to a level as low as in lipid bilayers. This low anion permeability could not be reached for bicarbonate, in the first place because the inescapable spontaneous dehydration equals an apparent bicarbonate permeability of $2.4 \times 10^{-7} \text{ cm s}^{-1}$ (pH 7.8); and secondly, because there is an additional contribution that increased the permeability fivefold to $1.2 \times 10^{-6} \text{ cm s}^{-1}$.

We do not know why bicarbonate permeability after maximum (irreversible + reversible) DIDS inhibition did not reach the level expected for spontaneous dehydration. Similar inhibition experiments demonstrated that the rate constant for chloride transport in resealed red cell ghosts was over a hundred times smaller than the measured rate for maximally inhibited bicarbonate efflux at pH 7.8 (data not shown). Hence, additional efflux due to leaks in the ghost membrane is not likely. One possibility is that some residual catalytic activity, due to carbonic anhydrase or some other catalyst, remains in the ghosts. Another possibility is that ^{14}C is translocated across the membrane as $[^{14}\text{C}]\text{H}_2\text{CO}_3$. Both the catalytic activity of carbonic anhydrase (see Magid, 1970) and the protonation of $[^{14}\text{C}]\text{HCO}_3^-$ decrease significantly with increasing pH, but Fig. 2 shows no such decrease in the rate constant for bicarbonate efflux. This seems to rule out residual carbonic anhydrase or $[^{14}\text{C}]\text{H}_2\text{CO}_3$ flux, but leaves the possibility of other unidentified catalytic activity. The fraction of ^{14}C efflux after maximum irreversible and reversible inhibition, however, amounts to $<0.5\%$ of the efflux of ^{14}C from control ghosts, so the efflux of $[^{14}\text{C}]\text{HCO}_3^-$ overwhelmingly predominates.³

Temperature Dependence of Unidirectional Bicarbonate Efflux

An Arrhenius plot (Fig. 3) of HCO_3^- transport shows a decreasing slope as temperature is raised. This result, like similar data for Cl^- , Br^- , and F^- (Brahm, 1977; Wieth and Brahm, 1985), indicates that the temperature dependence is not constant in the temperature range 0–38°C. Qualitatively, the temperature dependencies for Cl^- and HCO_3^- (Brahm, 1977; Fig. 3) are

³It should be noted that the control experiments, which showed that loss of CO_2 to the atmosphere is insignificant during all experimental procedures, do not fully apply to the studies of residual tracer efflux after DIDS inhibition. When bicarbonate efflux is inhibited by DIDS, a larger fraction of $^{14}\text{CO}_2$ (compared to control conditions) leaves the cells. The excess $^{14}\text{CO}_2$ in the efflux medium will be converted to $[^{14}\text{C}]\text{HCO}_3^-$ with a half time of 3.6 s. Hence, the fraction of $^{14}\text{CO}_2$ in the efflux medium under these conditions is higher than under the test conditions, and probably more $^{14}\text{CO}_2$ is released to the atmosphere under these conditions especially at lower pH values. However, in all efflux experiments in this study semilog plots of tracer efflux were strictly linear with correlation coefficients >0.99 supporting the conclusion that loss of radioactivity to the atmosphere was not a major problem in any of the experiments.

similar, but they differ quantitatively: with $C^{\text{a}} = C^{\text{o}} = 165 \text{ mM}$, unidirectional Cl^- efflux at 0°C is 0.28–0.29 $\text{nmol cm}^{-2} \text{ s}^{-1}$ (Brahm, 1977; Gasbjerg and Brahm, 1991) while HCO_3^- is 0.42 $\text{nmol cm}^{-2} \text{ s}^{-1}$ (Gasbjerg and Brahm, 1991). At 38°C Cl^- efflux increases ~ 200 times to 48–55 $\text{nmol cm}^{-2} \text{ s}^{-1}$ (Brahm, 1977; Knauf et al., 1996) and HCO_3^- only ~ 75 times to 31 $\text{nmol cm}^{-2} \text{ s}^{-1}$ (Fig. 4). The unidirectional fluxes of Cl^- and HCO_3^- are the same around 22°C.

When the Arrhenius plots for Cl^- , Br^- , and F^- were fitted to two intersecting regression lines, the “break” in the lines occurred at different temperatures for Cl^- (15°C) and Br^- and F^- (23–25°C), but at the same “critical” turnover number of $\sim 4 \times 10^9 \text{ ions cell}^{-1} \text{ s}^{-1}$ (Brahm, 1977; Wieth and Brahm, 1985). The fact that the break occurs at different temperatures for different anions indicates that the nonlinear temperature dependence is not related to a phase transition in the membrane, which would occur at the same temperature regardless of the anion substrate. If two straight lines are used to fit the Arrhenius plot for HCO_3^- transport (Fig. 3), they intersect around 13°C. Thus, for monovalent anions, the break temperature ranges from 13 to 25°C, supporting the concept that the nonlinear temperature dependence of anion transport is not related to a phase transition in the membrane. The turnover number for HCO_3^- transport at 13°C is $\sim 4 \times 10^9 \text{ ions cell}^{-1} \text{ s}^{-1}$, similar to the critical turnover number for Cl^- , Br^- , and F^- .

Ions such as iodide, salicylate, and thiocyanate, which never reach the critical turnover number, show a constant apparent activation energy (Dalmark and Wieth, 1972). The divalent anion sulfate showed a decrease in the apparent Arrhenius activation energy when temperature is raised above 30°C (Glibowicka et al., 1988), but the critical turnover number for sulfate was only $\sim 7 \times 10^6 \text{ ions cell}^{-1} \text{ s}^{-1}$, 500–600 times lower than that for chloride. Similar activation energy changes at low critical turnover numbers have also been seen with other “nonspherical” anions, such as phosphate, phosphite, and even hypophosphite, a monovalent anion (Galanter and Labotka, 1980).⁴ Galanter and Labotka suggested that the difference between these ions and the halides might be related to the shape (spherical or nonspherical) and chemical nature (with or without oxygen atoms) of the anions. According to this hypothesis, bicar-

⁴Interpretation of these data is complicated by the fact that transport was measured by heteroexchange experiments in media with some Cl^- , at concentrations of the oxyanions that were not saturating in all cases. This, together with the effects of pH on the titration state of some of the anions, means that the turnover numbers are probably considerably underestimated and that the measured fluxes may not accurately reflect the temperature dependence of the maximum flux for each anion.

bonate would be expected to behave like phosphite, rather than like Cl^- . The data with HCO_3^- thus provide evidence against this otherwise attractive explanation.

Of course, the concept of a critical turnover number is not the only explanation for the observed nonlinearity, because the Arrhenius plot reflects a complex interplay of several factors. Falke et al. (1985) have argued from ^{35}Cl and ^{37}Cl NMR data that anion binding and release are not the rate-limiting steps, even at physiological temperatures, so the temperature effect on $J_{\text{max}}^{\text{eff}}$ should be determined by the rate constants for anion translocation in each direction (see Glibowicka et al., 1988; Gasbjerg and Brahm, 1991). However, in our experiments J^{eff} with $C^{(i)} = C^{(o)} = 165 \text{ mM KHCO}_3$, rather than $J_{\text{max}}^{\text{eff}}$, was measured. For HCO_3^- , the combined effect of incomplete saturation and self inhibition at $C^{(i)} = 165 \text{ mM}$ results in an unidirectional flux that is $\sim 65\%$ of $J_{\text{max}}^{\text{eff}}$ at 0°C (Gaspberg and Brahm, 1991) and $\sim 25\%$ of $J_{\text{max}}^{\text{eff}}$ at 38°C . Between 0 and 38°C we anticipate values between 25 and 65% , but very little is known about the parameters at intermediate temperatures. Thus, the effects of varying saturation and self inhibition may explain about half of the fourfold difference between the measured flux at 38°C and that expected by extrapolating the data at 0 – 10°C . For Cl^- , at 0°C the flux with 165 mM KCl is only 54% of $J_{\text{max}}^{\text{eff}}$; because of the uncertainty in the values for K_j and $K_{i/2}$ at 38°C (Knauf et al., 1996), however, J^{eff} could lie between 21 and 73% of $J_{\text{max}}^{\text{eff}}$, so the effect on the temperature dependence may be negligible or as large as that seen for HCO_3^- .

Effects of Temperature on Asymmetry

To explain the break in the Arrhenius plots for Cl^- and SO_4^{2-} , Glibowicka et al. (1988) began by assuming that the dissociation constants for chloride and for sulfate are equal at the two sides of the membrane from 0 to 60°C . This simplifies the model so that the asymmetry factor is equal to the ratio of the rate constants k and k' . They further suggested that the rate is limited by either k or k' respectively below or above the break point. On the basis of these two assumptions, they calculated that the asymmetry factor for chloride should be 0.43 at 0°C , and that it should increase to 3.12 at 38°C .⁵ These values indicate that in the low temperature range one rate constant is about two to three times higher than the other, but at 38°C it is two to three times lower than the other. The asymmetry factor at 0°C of 0.43 in their study is somewhat higher than previous findings (0.06 –

0.32 with a mean of 0.15 , see Knauf and Brahm, 1989). An asymmetry factor of 3.12 at 38°C is exorbitantly greater than the values of 0.01 – 0.11 for chloride (Knauf et al., 1996) and 0.10 – 0.13 for bicarbonate in the present study. Hence, at least one of their two assumptions that (a) the ratio of the two rate constants for translocation gives the asymmetry factor and (b) the change of the ratio with temperature causes the deflection in the Arrhenius plot must be incorrect.

The observation that the asymmetry factor, A , remains $\ll 1$ at both 0 and 38°C could only be reconciled with a change in the rate-limiting translocation step (from k to k' or vice versa) if there were compensating changes in the dissociation constants for bicarbonate at the inside and outside. We therefore prefer the simpler model, in which the rate-limiting translocation step remains the same. If this step actually consists of at least two processes with different temperature dependencies, the change in activation energy could be explained without a change in the ratio of the translocation rate constants. The fact that changes in activation energy are seen in heteroexchange experiments with phosphate, phosphite, and hypophosphite (Galanter and Labotka, 1990), where k' for the particular ion is always rate-limiting, supports this alternative.

Unidirectional Bicarbonate Efflux as a Function of $\text{pH}^{(o)} = \text{pH}^{(i)}$

The pH-dependence of bicarbonate transport is more complex than for other monovalent anions because both the transport protein and the concentration of bicarbonate are affected by changing pH. By shifting pH from 7.4 to 9.0 the actual bicarbonate concentration in a nominal 165 mM medium changes from $\sim 157 \text{ mM}$ at pH 7.4 through its maximum value of 160 mM at pH 7.95 to 139 mM at pH 9.0 . Because the bicarbonate flux is nearly independent of concentration near 165 mM (Fig. 5 D), desaturation of the transport system should not have a noticeable effect on the unidirectional efflux. The ~ 10 -fold increase in carbonate concentration might contribute to the observed flux decrease at pH 9 , because carbonate may have a significant affinity for the transport site and/or the modifier inhibitory site and, therefore, may inhibit the unidirectional bicarbonate efflux. However, the pH dependence of bicarbonate transport in ghosts at 38°C in the present study is similar to that for chloride transport in ghosts at 38°C (Brahm, 1977; Fig. 4), suggesting that the pH dependence of bicarbonate transport at pH 7.4 – 9.0 is dominated by a direct pH effect on the transport protein.

External, Internal, and Overall Apparent Affinity to Bicarbonate

The ping-pong model for anion transport across the red cell membrane provides a useful description of the

⁵The authors could as well have obtained an asymmetry factor of 3.12 at 0°C and of 0.43 at 38°C by assuming that the rate constant for the reverse process was rate limiting at the two temperatures. This, however, would be inconsistent with observations that $A < 1$ at 0°C , if the authors' assumption of equal dissociation constants at the inside and outside is valid.

concentration dependence of chloride and bicarbonate self-exchange at 0°C (Gunn and Fröhlich, 1979; Gasbjerg and Brahm, 1991). In the present study at 38°C we used the same two strategies as Gasbjerg and Brahm (1991) at 0°C. First, we used the modified ping pong model (MS-fit whenever $C^{(i)}$ was varied; MM-fit when only $C^{(o)}$ was varied) to characterize the concentration dependence of J^{eff} over the whole experimental concentration range (Fig. 5, A–D). Alternately, we restricted the range of $C^{(i)}$ to <200 mM and used the simple ping pong model without the modifier site (MM-fit) to describe the concentration dependence in all four experimental series. Tables I and II respectively show the results of the fitting procedure with the two models. In each case, we used the fitted flux parameters for the data in Fig. 5, B and D to predict the parameters for the experiments shown in Fig. 5, A and C. (For details of the calculations, see Fröhlich and Gunn [1986] or Gasbjerg and Brahm [1991].)

The much smaller deviations between predictions and experiments in Table I as compared to Table II show that the modified ping pong model provides a more internally consistent description of the experimental data. The simple ping pong model (Table II) fails to account for the decrease in $K_{1/2}^o$ from 10 to 3.8 mM when $C^{(i)}$ is

TABLE I
Kinetic Parameters for Bicarbonate Transport at pH 7.8, 38°C
Modified Ping Pong Model (Self Inhibition Included)

Parameter	Unit	Experimental	Calculated	% dev.
$K_{1/2}^o(165)$	mM	10	—	—
$K_{1/2}^i$	mM	173	—	—
K_i	mM	172	—	—
J_{max}^{eff}	nmol cm ⁻² s ⁻¹	122	—	—
A	—	—	0.13	—
$J_{max}^{eff,o}(165)$	nmol cm ⁻² s ⁻¹	63*	63	0
$K_{1/2}^o(50)$	mM	3.8	4.9	29
$J_{max}^{eff,o}(50)$	nmol cm ⁻² s ⁻¹	25*	30	20
$K_{1/2}^i(50)$	mM	116	110	5
$J_{max}^{eff,i}(50)$	nmol cm ⁻² s ⁻¹	92	87	5

*Corrected for self inhibition. Four experimentally obtained parameters were used as input to the modified ping pong model. The model only needs two half saturation constants for a calculation of any other half saturation constant (if obtained from asymmetric series, the fixed concentrations of course must be different). As input values we chose $K_{1/2}^o$ (obtained for $C^{(i)} = 165$ mM, $C^{(o)}$ varied: $K_{1/2}^o(165)$ and $K_{1/2}^i$). The third input parameter is K_i that accounts for self inhibition. We chose $K_i = 172$ mM obtained in the symmetric series. As we know the asymmetry factor and one asymmetric half saturation constant (which may also be one of the half saturation constants used to calculate A, e.g. $K_{1/2}^o(165)$) we only need one J_{max}^{eff} value to calculate the J_{max}^{eff} value under any other conditions. We chose J_{max}^{eff} . In the lower five rows we compare experimentally obtained flux parameters with those calculated by using the input parameters and the modified ping pong model. The last column shows the deviation of the calculated parameters (%dev. = 100 |(experimental – calculated)| / experimental).

TABLE II
Kinetic Parameters for Bicarbonate Transport at pH 7.8, 38°C
Simple Ping Pong Model (No Self Inhibition)

Parameter	Unit	Experimental	Calculated	% dev.
$K_{1/2}^o(165)$	mM	10	—	—
$K_{1/2}^i$	mM	46	—	—
J_{max}^{eff}	nmol cm ⁻² s ⁻¹	39	—	—
A	—	—	0.37	—
$J_{max}^{eff,o}(165)$	nmol cm ⁻² s ⁻¹	32	32	0
$K_{1/2}^o(50)$	mM	3.8	7.4	95
$J_{max}^{eff,o}(50)$	nmol cm ⁻² s ⁻¹	20	23	15
$K_{1/2}^i(50)$	mM	13	27	107
$J_{max}^{eff,i}(50)$	nmol cm ⁻² s ⁻¹	26	31	19

The strategy of Table I was also used in Table II except that self inhibition was ignored, and therefore K_i was not included in the calculations.

lowered from 165 to 50 mM. It should be noted that we used the calculated $K_{1/2}^o$ to determine the expected values of the half saturation constant when $C^{(i)} = 50$ mM, $K_{1/2}^o(50)$. The observed MM-fit $K_{1/2}^o$ (13 mM, for $C^{(i)} < 200$ mM) predicts an even smaller effect of $C^{(i)}$ on $K_{1/2}^o$, confirming that the model without modifier site inhibition is inconsistent with the experimental values. This contrasts with the situation at 0°C, where a simple ping-pong model provided a self-consistent result for a limited chloride concentration range. At 38°C, the modified ping-pong model is required to give consistent results even at low Cl⁻ concentrations in the physiological range.

Effect of Transport Site Conformation on Modifier Site Inhibition

The simple ping-pong model predicts that when $C^{(i)}$ is kept constant and $C^{(o)}$ is varied, $J_{max}^{eff}/K_{1/2}^o$ should be constant. If internal bicarbonate can only bind to the modifier site when the transport site faces inward, the same relationship is observed for the extended ping-pong model with modifier site inhibition because the self-inhibition affects J_{max}^{eff} and $K_{1/2}^o$ to exactly the same extent. If, on the other hand, bicarbonate can bind to the modifier site regardless of the conformation of the transport site, this leaves $K_{1/2}^o$ unaffected, but causes J_{max}^{eff} to be divided by $(1 + C^{(i)}/K_i)$. Thus, $J_{max}^{eff}/K_{1/2}^o$ will only be constant if J_{max}^{eff} is multiplied by this factor. Table III

TABLE III
The Effect of Self Inhibition on $J_{max}^{eff}/K_{1/2}^o$

$C^{(i)}$ (mM)	$J_{max}^{eff}/K_{1/2}^o$ ($\cdot 10^4$ cm s ⁻¹)	
	Corrected for self-inhibition	Not corrected (with self-inhibition)
165	63	32
50	68	53

shows that $J_{max}^{eff}/K_{1/2}^o$ is the same at two different internal bicarbonate concentrations only if this factor is taken into account. Thus, within the framework of the ping-pong model, which provides a self-consistent interpretation of the rest of the bicarbonate data, the $J_{max}^{eff}/K_{1/2}^o$ data provide evidence that the conformation of the transport site does not affect the ability of bicarbonate to bind to the modifier site. An implication of the MS ping-pong model, therefore, is that $J_{max}^{eff}/K_{1/2}^o$ data must be corrected for self-inhibition; otherwise a variation in $J_{max}^{eff}/K_{1/2}^o$ with $C^{(i)}$ will be observed, in apparent contradiction to the predictions of the ping-pong model.

Concluding Remarks

The present study demonstrates that bicarbonate exchange mediated by the anion transport system in human red blood cells can be studied at physiological temperatures and near physiological pH. Only when the kinetics include self-inhibition by internal bicarbonate is a self-consistent fit to the ping-pong model obtained. The asymmetric distribution of unloaded transport sites is similar to that seen at 0°C. The asymmetry is also similar to that seen at 38°C with Cl⁻ as substrate in the following paper (Knauf, et al., 1996), as predicted by the ping-pong model.

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