Secondary CO₂ Diffusion Following HCO₃⁻ Shift across the Red Blood Cell Membrane

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Abstract In order to clarify the interaction between CO₂ diffusion and HCO₃⁻ shift in the red blood cell (RBC), HCO₃⁻ shift was measured by using a stopped flow method combined with fluorometry. When HCO₃⁻ entered the RBC, the intracellular P_{CO₂} increased, causing a secondary outflow of CO₂. Conversely, when HCO₃⁻ ions flowed out of the RBC, the resulting decrease of P_{CO₂} caused an inward CO₂ diffusion. The P_{CO₂} change caused by the inward HCO₃⁻ shift was about 3- to 4-fold that of the outward shift. During the respective in- and outward-shifts, the mean half-times of the extracellular pH changes were 0.15 and 0.13 sec. These were approximately twice as long as those of the primary CO₂ diffusion. The permeability of HCO₃⁻ across the RBC membrane was obtained by comparing the experimental extracellular pH curve with a numerical solution for CO₂ and HCO₃⁻ diffusions accompanied by the hydration and dehydration reactions. Thus the HCO₃⁻ permeability was determined to be 5 \times 10^{-4} and 7 \times 10^{-4} cm/sec, in the in- and outward-HCO₃⁻ shifts, respectively. The influence of Cl⁻ concentration on HCO₃⁻ permeability was tested by reducing the initial Cl⁻ gradient across the RBC membrane. In a physiological Cl⁻ concentration range the HCO₃⁻ permeability was not affected by the Cl⁻ gradient.

Key Words: HCO₃⁻ shift, CO₂ diffusion, HCO₃⁻ permeability, stopped flow method, Cl⁻ shift.

In studies to elucidate the rate of gas exchange in vivo, the HCO₃⁻ permeability across the red blood cell (RBC) membrane is an important parameters. KLOCKE (1976) measured the HCO₃⁻ shift in RBC suspension by inhibiting the activity of carbonic anhydrase by using acetazolamide. However, because this enzyme is very active inside the RBC (KERNOHAN et al., 1963), the rate of HCO₃⁻ shift in the normal RBC seemed not to obey the rate measured in the suspension containing acetazolamide. Thus, we attempted to measure the rate of HCO₃⁻ shift through extracellular pH by adding carbonic anhydrase into the normal RBC suspension.
During the course of the pH measurement using a stopped flow apparatus, it was found that the intracellular HCO₃⁻ change resulted in the P CO₂ change, causing a secondary CO₂ diffusion. Thus, from a recorded extracellular pH change which resulted from both the changes in P CO₂ and HCO₃⁻, we attempted to determine HCO₃⁻ permeability. First, by varying the permeability a numerical solution for extracellular pH change was derived from the CO₂ diffusion equations accompanying the HCO₃⁻ shift. Then, by comparing the computed pattern with the measured one, HCO₃⁻ permeability was determined in terms of the transfer coefficient, γ-(HCO₃⁻). To obtain a numerical solution of the diffusion equation, UCHIDA et al. (1983) estimated the diffusion coefficients for CO₂ and HCO₃⁻ inside the RBC, and NIIZEKI et al. (1983) measured the transfer coefficient for CO₂, γ(CO₂) across the RBC membrane. SHIMOUCHI et al. (1984) clarified that the P CO₂ change caused by the HCO₃⁻ shift could be computed by a modified Henderson-Hasselbalch equation. Using these parameter values, KAGAWA and MOCHIZUKI (1984) obtained a program for the numerical solution. Based upon the above results, together with other necessary parameters, the extracellular pH change was computed. Good agreement was observed between the computed and measured pH changes when γ(HCO₃⁻)=5x10⁻⁴ and 7x10⁻⁴ cm/sec were used for the permeabilities of the in- and outward-HCO₃⁻ shifts, respectively.

Generally, the HCO₃⁻ shift occurs in exchange for Cl⁻ ions in order to secure an electrical neutrality within the RBC (TAKIWAKE et al., 1983). Thus, it seems probable that the rate of the HCO₃⁻ shift depends on the rate of the counter shift of Cl⁻ ions. In order to verify the dependency of the HCO₃⁻ permeability on the Cl⁻ gradient, we also attempted to measure the rate of HCO₃⁻ shift by lowering the initial Cl⁻ gradient without substituting any other chemical agents. The rate of HCO₃⁻ shift was not decreased despite the lowered Cl⁻ gradient. In the present study we attempted to determine the permeability of HCO₃⁻ ion across the RBC membrane, as well as to clarify the influence of the physiological Cl⁻ concentration gradient to the HCO₃⁻ shift.

METHODS

Experimental procedure. A stopped flow apparatus combined with a fluorometer was used in this experiment, and was previously described in detail (NIIZEKI et al., 1983). In this apparatus equal volume (2.5 ml) of RBC suspension and solution were injected through a multi-jet mixing chamber into an observation tube. The change of pH in extracellular fluid was measured at 37°C by using pH sensitive fluorescence of 4-methylumbelliferone. At a flow rate of 15 ml/sec the dead time was about 10 msec.

The RBC sample was separated from freshly drawn human blood of 4 normal subjects, and washed three times with saline solution as described in Table 1. Then, it was diluted with the same solution to attain a hematocrit ranging from 14 to 15%.

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Table 1. Experimental conditions for $\text{HCO}_3^-$ shift before mixing.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Inward shift</th>
<th>Outward shift</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>14.0 0.0</td>
<td>15.2 0.0</td>
</tr>
<tr>
<td>Extra. $\text{HCO}_3^-$, mM</td>
<td>7.6 40.0</td>
<td>7.4 40.0</td>
</tr>
<tr>
<td>Intra. $\text{HCO}_3^-$, mM</td>
<td>5.0 4.1</td>
<td>5.0 4.1</td>
</tr>
<tr>
<td>$\text{Cl}^-$, mM</td>
<td>148.0 110.0</td>
<td>138.0 130.0</td>
</tr>
<tr>
<td>pH</td>
<td>6.86 7.67</td>
<td>6.85 7.62</td>
</tr>
<tr>
<td>$P_{\text{CO}_2}$, Torr</td>
<td>38.4 38.4</td>
<td>38.4 38.4</td>
</tr>
</tbody>
</table>

The experimental conditions are also shown in Table 1. All the suspension and solution were equilibrated with gas mixture of 38.4 Torr $P_{\text{CO}_2}$ and 150 Torr $P_{\text{O}_2}$. In experiment 1 of the inward shift, the $\text{Cl}^-$ concentrations in the suspension and solution were 148 and 110 mM, and in experiment 2 were 138 and 130 mM, respectively. The osmotic pressure in the suspension of experiment 2 was slightly hypotonic before mixing, whereas that of experiment 1 was isotonic. In the outward $\text{HCO}_3^-$ shift, the $\text{Cl}^-$ concentrations in the suspension and solution were 110 and 148 mM, so that the osmotic pressure was kept in a normal range, as seen in experiment 1 of the inward shift. In order to quantitatively describe the $P_{\text{CO}_2}$ change following the $\text{HCO}_3^-$ shift, we measured the $\text{HCO}_3^-$ content, pH, and $P_{\text{CO}_2}$ before and after the mixing. The $\text{HCO}_3^-$ content was measured by Natelson's analyzer (Natelson, 1951), and pH and $P_{\text{CO}_2}$ were measured by use of the respective electrodes (I-L Meter, model 213). Total $\text{HCO}_3^-$ content was obtained by subtracting the dissolved $\text{CO}_2$ and carbamate fraction from the total $\text{CO}_2$ content. Then the intracellular $\text{HCO}_3^-$ content was determined by subtracting the extracellular $\text{HCO}_3^-$ from the total $\text{HCO}_3^-$ content. The $P_{\text{CO}_2}$ values at the final stage of diffusion were evaluated from a log $P_{\text{CO}_2}$-pH line measured in the same RBC suspension after observing the reaction, and also checked with a $P_{\text{CO}_2}$-electrode. All the experiments were carried out in the presence of 0.2 g/l of carbonic anhydrase (Boehringer Mannheim, EC 4.2.1.1) and 40 $\mu$M of 4-methylumbelliferone (Wako Chemical) in extracellular fluid.

Simulation of extracellular pH curve. A computer program made by KAGAWA and MOCHIZUKI (1984) was used for solving a partial differential equations for the $\text{CO}_2$ diffusion and the $\text{HCO}_3^-$ shift in a disc model with 3.5 $\mu$m radius and 1.6 $\mu$m thickness. From the data of UCHIDA et al. (1983), the intracellular diffusion coefficients of $\text{CO}_2$ and $\text{HCO}_3^-$ were assumed to be $0.34 \times 10^{-4}$ and $0.14 \times 10^{-5}$ cm$^2$/sec, respectively. The transfer coefficient for $\text{CO}_2$ was newly obtained by KAGAWA and MOCHIZUKI (1984) by referring to both the data of NIHZEKI et al. (1983) and the present study as $2.5 \times 10^{-9}$ cm/sec/Torr. In the computer program, the differential equations for the $\text{HCO}_3^-$ and $\text{CO}_2$ diffusions were first solved without taking the chemical reactions into account. Then, we computed the intracellular changes of
HC0₃⁻ and P₄CO₂ caused by dehydration and hydration reactions by using a modified Henderson-Hasselbalch equation as follows

\[
\log \left( 1 + \frac{AP}{P_0} \right) = -\frac{\alpha \cdot AP}{\bar{P}_c \cdot Ht} + \log \left( 1 + \frac{\Delta (HC0₃⁻)}{(HC0₃⁻)(P_0)} \right),
\]

(Shimouchi et al. (1984), Eq. (15)).

Throughout the computation it was assumed that the chemical reactions were completed in the RBC within 1 msec. Moreover, the above computation was carried out for a time increment of 1 msec successively for both radial and vertical directions. Repeating the above steps, we estimated the time course of extracellular pH by varying HCO₃⁻ permeability across the RBC membrane. The simulation was continued until the computed extracellular pH curve coincided with the measured one. Details of programming and the transfer coefficient of CO₂ will be described in a forth coming paper (Kagawa and Mochizuki, 1984).

RESULTS

The relationship between fluorescence intensity of 4-methylumbelliferone and pH is shown by a calibration curve in Fig. 1A. The plotted points were obtained in effluent RBC suspension equilibrated at various P₄CO₂ levels. The output current was 25.2±0.6 mm per 0.1 pH (mean±S.D.) on the recorded scale. The error caused by using the calibration curve was less than 0.025 in pH. The log P₄CO₂-PH equilibrium curve is shown in Fig. 1B. The plotted points were obtained from the

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summarized data of 11 experiments. The correlation coefficient in the individual experiment was higher than 0.99. Figure 2A and B represent the pH changes recorded during the inward (Exp. 1) and outward shifts, respectively. In Fig. 2A, the extracellular pH during the flowing stage was about 7.4, and after the mixing decreased to 7.15 with the half-time being 0.16 sec. In Fig. 2B, the pH value increased from the initial level of 7.39 to 7.5, the half-time being 0.17 sec. In both the HCO₃⁻ shifts, 90% saturation time was about 0.5 sec. In general, when the extracellular HCO₃⁻ concentration decreases by approximately 1 mM with constant $P_{\text{CO}_2}$ arround 40 Torr, pH should decrease by about 0.02, as calculated from the Henderson-Hasselbalch equation. However, in the inward shift the pH change was about 10 times as great as the predicted pH change, as shown in Fig. 2A. In Fig. 2B, the measured pH change was about 5 times as great as the predicted change. That is, the result shown in Fig. 2 revealed that the extracellular pH change was ascribed mainly to the $P_{\text{CO}_2}$ change.

When two saline solutions with different HCO₃⁻ contents were mixed at a ratio of 1:1, the HCO₃⁻ content after mixing became an algebraic mean of two HCO₃⁻ contents, and no $P_{\text{CO}_2}$ change was observed thereafter. As shown in Table 1, the $P_{\text{CO}_2}$ gradient across the RBC membrane was initially zero. Thus, it was obvious that HCO₃⁻ ions entering the RBC were dehydrated, producing CO₂ molecules that secondarily diffused from the RBC. When HCO₃⁻ ions flowed out, the CO₂ hydration occurred inside the RBC, resulting in a decrease in intracellular $P_{\text{CO}_2}$, and a secondary inward CO₂ diffusion. The changes of pH, $P_{\text{CO}_2}$,
and \( \Delta pH \) due to \( \Delta HC03^- \) shift are summarized in Table 2, together with the half-times and the transfer coefficients. All the changes (\( \Delta pH \), \( \Delta P_{CO2} \), and \( \Delta HC03^- \)) were greater during the inward shift than during the outward shift. During the

Fig. 3. Summarized extracellular pH changes. (A) represents the normalized pH changes during the inward shift. The open and closed circles are the measured values with S.D. in experiments 1 and 2, respectively, and (B) the pH change during the outward shift. The solid lines show numerical solutions obtained by using \( \gamma(HCO3^-) \) values figured on the lines. The pH(0) is extracellular pH during the flowing stage and pH(\( \infty \)) is equilibrated pH after mixing.
inward shift, the $\Delta P_{\text{CO}_2}$ was about 3- to 4-fold that of the outward shift.

In order to compare the rate of pH change among individual measurements, the pH change was normalized and plotted on a semilogarithmic graph. The plotted points and bars in Fig. 3A and B represent the mean and S.D. of the measured values, and the solid lines show the simulated pH curves computed with three respective $\gamma(\text{HCO}_3^-)$ values. The open and closed points in Fig. 3A were obtained from experiments 1 and 2 of the inward shift, showing good coincidence with the pH curves computed with $\gamma(\text{HCO}_3^-)=5 \times 10^{-4}$ and $7 \times 10^{-4}$ cm/sec, respectively. The rate of the latter shift was significantly faster than the former ($p<0.01$), despite the lowered Cl$^-$ gradient. In other words, the $\gamma(\text{HCO}_3^-)$ was not reduced in proportion to the Cl$^-$ concentration gradient. In the outward shift, good agreement between the calculated and measured pH curves was observed when $\gamma(\text{HCO}_3^-)=7 \times 10^{-4}$ cm/sec was used. That is, the permeability of HCO$_3^-$ in the outward direction was 20 to 30% higher than that in the inward direction.

**DISCUSSION**

For computing the partial differential equation, the hydration and dehydration reactions within the RBC were assumed to be completed in 1 msec. Thus, the ratio of the dehydrated (or hydrated) HCO$_3^-$ quantity to the total HCO$_3^-$ gain (or loss) could be calculated, in each volume element and each increment time of 1 msec, by using Eq. (1). The relation of produced (or eliminated) CO$_2$ quantity to the CO$_2$ eflux (or influx) could also be evaluated by using a similar equation. As shown in Fig. 3A and B, the computed pH pattern fitted well with the measured pattern. In addition, the permeability or transfer coefficient for HCO$_3^-$ in the present study ranged from 5 to $7 \times 10^{-4}$ cm/sec, which was within the limit of the values of $0.5 \times 10^{-2}$ to $10^{-2}$ cm/sec, as measured by KLOCKE (1976). Therefore, the assumption that the CO$_2$ dehydration and hydration reactions could be completed within 1 msec seemed to be reasonable.

The patterns of the HCO$_3^-$ content and $P_{\text{CO}_2}$ illustrated in Figs. 4 and 5 were obtained during the course of computation of the extracellular pH shown in Fig. 3A and B, respectively. The difference between the initial and final extracellular HCO$_3^-$ concentrations, $\Delta$HCO$_3^-$, and the difference between the initial and final $P_{\text{CO}_2}$'s, $\Delta P_{\text{CO}_2}$, are consistent with the respective measured values in Table 2. The half-times of the intracellular HCO$_3^-$ change are about 0.12 sec during the inward shift and significantly longer than that during the outward shift of about 0.03 sec. On the other hand, the half-times of the extracellular $P_{\text{CO}_2}$ change during the inward and outward-shifts are about 0.18 and 0.15 sec, respectively. In both the shifts, the intracellular $P_{\text{CO}_2}$ curve shows a shorter half-time than the extracellular $P_{\text{CO}_2}$, demonstrating the existence of a secondary CO$_2$ diffusion.

Chow et al. (1976) measured the rate of HCO$_3^-$/Cl$^-$ exchange by utilizing a Jacobs-Stewart cycle in a RBC suspension with a phosphate buffer. The HCO$_3^-$...
permeability calculated by them was $2.2 \times 10^{-4}$ cm/sec at 37°C, which corresponded to half of our values. The extracellular HCO$_3^-$ concentration used by them was less than 1 mM, and in addition, the CO$_2$ diffusion accompanying dehydration reaction should take place not only across the RBC membrane, but also in the extra-

Fig. 4. Intra- and extracellular HCO$_3^-$ and $P_{CO_2}$ patterns during the inward shift computed in a disc model by using $\gamma$(HCO$_3^-$)=$5 \times 10^{-4}$ cm/sec.

Fig. 5. Intra- and extracellular HCO$_3^-$ and $P_{CO_2}$ patterns during the outward HCO$_3^-$ shift computed by using $\gamma$(HCO$_3^-$)=$7 \times 10^{-4}$ cm/sec.
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Fig. 6. Intracellular \( P_{\text{CO}_2} \) patterns computed under the conditions that normal blood with a hematocrit of 40\% and a \( P_{\text{CO}_2} \) of 40 Torr enters a tissue capillary and is exposed to a tissue \( P_{\text{CO}_2} \) of 46 Torr for 0.6 sec, after which no further exchanges take place between tissue and blood. The upper curve was obtained by FORSTER and CRANDALL (1975) with a two-compartment model. The lower curve was computed in a diffusion model with \( \eta(\text{HCO}_3^-) = 7 \times 10^{-4} \text{ cm/sec} \), \( \eta(\text{CO}_2) = 2.5 \times 10^{-8} \text{ cm/(sec·Torr)} \).

Forster and Crandall (1975) computed the time course of the \( P_{\text{CO}_2}, \) pH, and \( \text{HCO}_3^- \) changes in intra- and extracellular fluid by using a peripheral capillary model. In order to compare the influences of the parameter values to the computed pattern we calculated the intracellular \( P_{\text{CO}_2} \), according to their conditions, whereas other parameter values used were similar to those used in Figs. 3 and 5. The initial extra- and intracellular \( P_{\text{CO}_2} \) were assumed to be 46 and 40 Torr. Initial extra- and intracellular pH and \( \text{HCO}_3^- \) contents were taken to be 7.4 and 7.137, and 23.1 mmol/l, plasma and 11.4 mmol/l, RBC, respectively. The Donnan ratio, (mmol/
\( \frac{1}{l} \text{RBC} / (\text{mmol} / l, \text{plasma}) \), was assumed according to Kagawa and Mochizuki (1984) to be

\[
\gamma = 0.7(7.137 - \text{pH}_e) + 0.494,
\]

where \( \text{pH}_e \) is the intracellular \( \text{pH} \). The upper curve of Fig. 6 (F & C) is the pattern obtained by Forster and Crandall, and the lower was obtained by using \( \gamma(\text{HCO}_3^-) = 5 \times 10^{-4} \text{cm/sec} \) in a diffusion model having the fractional hematocrit of 0.4. Because of low \( \gamma(\text{CO}_2) \) value and outward \( \text{HCO}_3^- \) shift, the intracellular \( \text{P}_{\text{co}_2} \) was lowered and the equilibration time became longer in our model than in theirs. In our model the half-time was 80 msec and in a contact time of 0.6 sec equilibration was completed by about 97%. In the postcapillary, the extracellular \( \text{P}_{\text{co}_2} \), dropped, slightly as the inward \( \text{CO}_2 \) diffusion proceeded, attaining the equilibrium in 0.2 sec.

Figure 3A shows that the extracellular \( \text{pH} \) change was faster when the \( \text{Cl}^- \) gradient across the RBC membrane was reduced (Exp. 2) than when the \( \text{Cl}^- \) gradient was counterbalanced with the \( \text{HCO}_3^- \) gradient (Exp. 1). In experiment 2 the osmotic pressure of extracellular fluid was hypotonic \((260 \text{ mOsm}) \) before mixing and turned isotonic \((290 \text{ mOsm}) \) after mixing, suggesting the occurrence of water shift during the \( \text{HCO}_3^- \) shift. Therefore, the high \( \gamma(\text{HCO}_3^-) \) value of experiment 2 may be explained by the influence of water shift to the extracellular \( \text{pH} \) change which apparently increased the \( \gamma(\text{HCO}_3^-) \) value. At all events, the inward \( \gamma(\text{HCO}_3^-) \) value in vivo may be in a range of 5 to \( 7 \times 10^{-4} \text{ cm/sec} \).

As shown in Fig. 3A and B, the \( \text{HCO}_3^- \) permeability was significantly higher during the outward shift than during the inward shift. According to Crandall et al. (1971) the permeability of \( \text{OH}^- \) ion across the RBC membrane was inversely related to the extracellular \( \text{pH} \), though the \( \text{HCO}_3^- \) permeability measured by Klocke (1976) was independent of the \( \text{pH} \) value. The \( \text{pH} \) recordings shown in Fig. 1A and B demonstrate that the extracellular \( \text{pH} \) values were both about 7.4 at the initial stage. It increased to 7.5 during the outward \( \text{HCO}_3^- \) shift and decreased to 7.15 during the inward shift. No great \( \text{pH} \) difference was observed between the in- and outward-shifts. Therefore, the difference in \( \text{HCO}_3^- \) permeability between the in- and outward-shifts, as tabulated in Table 2, may be ascribed to the difference in the direction of the \( \text{HCO}_3^- \) shift rather than that in the extracellular \( \text{pH} \).

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