Carbonic Anhydrase Activity in Intact Red Blood Cells Measured with $^{18}$O Exchange*

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We have used a stirred, temperature-regulated, reaction vessel separated by a Teflon membrane from the ion source of a mass spectrometer to monitor continuously the time course of disappearance of C$^{18}$O$^{36}$O, mass 46, at chemical equilibrium as the $^{18}$O exchanges with $^{16}$O in water. This instrument is sensitive to less than 0.01 mm Hg of partial pressure of C$^{18}$O$^{36}$O with a response time of less than 3 s. The equation of Mills and Urey was used to calculate the hydration velocity constant for uncatalyzed or catalyzed homogeneous solutions from the exponential disappearance of mass 46. Addition of red blood cells to the reaction mixture produced biphasic (double exponential) disappearance curve for mass 46. A theory of this process has been developed which describes the time course of disappearance of C$^{18}$O$^{36}$O, mass 46, at chemical equilibrium as a function of the catalytic factor for intracellular carbonic anhydrase (A) and the permeability of the cell membrane to HCO$_3^-$ ($P$) in addition to the known values; water volume of the cells in the suspension, extracellular pH, the extracellular hydration reaction velocity constant, $k_a$, and dehydration reaction velocity constant, $k_d$. Using this theory, A and P were estimated from the disappearance curve for mass 46 at different values of hemocrit in the reaction mixture, both by a trial and error curve fitting procedure and by a more convenient graphical linearization method. The values of A and P obtained were very sensitive to small amounts of lysis (less than 1%), but the graphical method of analysis minimized this effect. For the blood cells of five normal subjects suspended in 24 mM bicarbonate in 145 mM NaCl at pH 7.4 and 37°C, using the graphical method we obtained an average value of 9,906 for A as compared to 19,900 for a comparable concentration of hemolysate. Correcting for a lower pH and chloride concentration inside the cell the latter figure would reduce to 17,500, still 80% higher than the intracellular value. The reason for this discrepancy is not clear. The average permeability of the red cell to bicarbonate ion was 8 $\times$ 10$^{-4}$ cm/s.

In 1940, Mills and Urey (1) first used the $^{18}$O exchange reaction between CO$_2$ and water to determine the rate constants for the hydration and dehydration reactions of CO$_2$ and bicarbonate. The practical basis of their method was the fact that $^{18}$O exchange approaches isotopic equilibrium nearly 2 orders of magnitude more slowly than the chemical reactions approach elemental equilibrium. This $^{18}$O exchange rate is slow enough, in relation to the time required for sampling, to permit measurements of the course of the process from which it is possible to compute the individual dehydration and hydration constants if we assume chemical identity of the isotopes. This method can be applied to estimate the rates of catalyzed reactions as well. The human red blood cell contains carbonic anhydrase (EC 4.2.1.1) which catalyzes the reversible hydration of CO$_2$. This enzyme is highly active and exhibits the highest turnover number of any known enzyme (2). Because of the rapidity of the catalyzed rate, enzyme activity has not been measured in intact red blood cells, where supply of substrate and removal of products may be rate-limiting. The velocity of CO$_2$ hydration in human red cells, determined from the speed of CO$_2$ uptake in a cell suspension, is about half that calculated from the carbonic anhydrase activity of diluted hemolyzate (3). While this discrepancy may result in part from compartmentalization, the peculiar chemical environment of the intracellular enzyme may contribute as well. The catalytic activity of carbonic anhydrase is known to be highly sensitive to such factors as concentration of buffer, ionic strength, anion concentration (4), pH, and protein concentration, conditions which are hard to reproduce extracellularly. The $^{18}$O exchange method provides a useful technique for the measurement of intracellular carbonic anhydrase activity because both the substrate and products move rapidly across the red cell membrane and because the overall exchange process is much slower than the chemical process. We have followed the time course of the exchange between C$^{18}$O$^{36}$O and water in a red cell suspension by means of a continuously sampling inlet system for a mass spectrometer, which was designed for this purpose. We reported earlier (5) that the disappearance of $^{18}$O in C$^{18}$O-$^{18}$O is biphasic, i.e., the sum of two experimental functions, and we speculated that this is caused by the separation of the extracellular bicarbonate pool from the intracellular enzyme. A theory has been developed describing the process and permitting the calculation of the CO$_2$ reaction velocity constants intra- and extracellularly. In addition, the theory permits us to estimate the permeability of the red cell membrane to bicarbonate ion.

METHODS

Preparation of Materials—Sodium bicarbonate labeled with $^{18}$O was prepared by exchange reaction of nonlabeled bicarbonate and $^{18}$O water. One gram of unlabeled sodium bicarbonate (Fisher Scien-

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The intracellular bicarbonate was recovered from the solution by lyophilization. Freshly drawn blood from healthy adults was washed immediately with 145 mM NaCl three times and suspended in the same solution containing 1% bovine serum albumin (Sigma Chemical Co.) at a hematocrit approximating 40%. Hemolysates were prepared by freezing and thawing the cell suspension or by adding an equal volume of distilled water. Both methods, with or without removal of cell ghosts by centrifugation, gave identical values for enzyme activity. Hemoglobin concentration of both cell suspension and lysate is determined spectrophotometrically as oxyhemoglobin, the former after lysis. Concentration of carbonic anhydrase C (Sigma Chemical Co.) was determined spectrophotometrically using a molecular extinction coefficient of the enzyme of 0.193 absorbance units per mg per ml. Hemoglobin concentration of both cell suspension and lysate is determined spectrophotometrically as oxyhemoglobin, the former after lysis. Concentration of carbonic anhydrase C (Sigma Chemical Co.) was determined spectrophotometrically using a molecular extinction coefficient of the enzyme of 0.193 absorbance units per mg per ml.

Apparatus—A glass reaction vessel of approximately 10 ml was constructed (Fig. 1) in the bottom of which is a 0.6-cm diameter Teflon membrane, 0.0012 cm thick, separated from the ion source of the mass spectrometer ( Consolidated Electrodynamics Corp., model 21-620 A) by a sintered glass disc of the same diameter (5, 6). The reaction vessel is continuously monitored by a combination pH electrode (Radionet meter model G 2641-C) and one for a thermostir, permitting continuous monitoring of pH and temperature of the contents. The Teflon film separates the reaction mixture at atmospheric pressure from the high vacuum of the mass spectrometer and the sintered glass disc is necessary for mechanical support. Teflon is extremely impermeable to water and the H_2O mass peak height is generally equivalent to a CO_2 partial pressure of less than 3% of an atmosphere. For a given area, the thinner the Teflon membrane, the greater the sensitivity to dissolved gas in the reaction mixture, but the greater the relative diffusion resistance of the inevitable stagnant layer of fluid next to the film, which last makes the mass spectrometer unsatisfactory with small amounts of gas. Therefore the proportional decrease in mass 46 peak caused by the addition of hemolysate was determined in standard 1 mm capillary tubes centrifuged at 4700 g for 5 min. The hematocrit of the final reaction mixture was calculated as hematocrit = 0.61. The factor 0.61 represents the water content of normal human red cells at an osmotic pressure of 288 mosm (7, 8) and at 37°C, corrected for the osmolarity of the reaction mixture. The amount of hemolysate in the reaction mixture is also expressed as v, the equivalent amount of cell water before lysis.

In Fig. 3 is shown a typical trace of the mass 46 peak (C^18O^18O) catalyzed by intact (A) and lysed (B) red blood cells. At the start of the record in Fig. 2A before the addition of the suspension the 46 peak decreases slowly with a half-time of about 5 min. The addition of the cell suspension at the arrow produces an extremely rapid fall in C^18O^18O abundance followed by a continued slow decrease as described earlier (5). At the second arrow, excess enzyme is added to produce an equilibrium rapidly. In Fig. 2B the disappearance is accelerated by an equivalent amount of hemolysate. In contrast to the reaction catalyzed by cells, a biphasic decrease is not evident, even when the sensitivity is increased 3.5-fold and the paper speed doubled (Fig. 2C). However, if in addition the amount of hemolysate added was decreased to one-tenth, a small rapid initial phase becomes evident (Fig. 2C). This initial rapid drop can be expected from O exchange theory established by Mills and Urey (1) as shown later. In the present study this initial phase is ignored in experiments with enzyme without cell suspension since its absolute value is small compared to that caused by intact cells. The mass 44 peak height (not shown in the figure) did not appear to change during the experiment. Since C^18O^18O is converted to C^18O, as the mass 46 peak decreases the mass 44 peak should increase, the total pco_2 remaining constant. The initial abundance of ^18O in CO_2 was less than 1.5% and was diluted practically to the level of natural abundance because of the large excess of oxygen in water (55.5 m). According to the binomial distribution the increase of mass 44 peak height (C^18O^18O) at final equilibrium should have been less than 3% of its initial value, a change that can be ignored, and the 44 peak height can be considered constant throughout the run. Therefore the proportional decrease in mass 46 peak height can be considered equal to the proportional decrease in the ratio of peak heights at mass 46 to 44.

### RESULTS

In Fig. 3 is shown a typical trace of the mass 46 peak (C^18O^18O) catalyzed by intact (A) and lysed (B) red blood cells. At the start of the record in Fig. 2A before the addition of the suspension the 46 peak decreases slowly with a half-time of about 5 min. The addition of the cell suspension at the arrow produces an extremely rapid fall in C^18O^18O abundance followed by a continued slow decrease as described earlier (5). At the second arrow, excess enzyme is added to produce an equilibrium rapidly. In Fig. 2B the disappearance is accelerated by an equivalent amount of hemolysate. In contrast to the reaction catalyzed by cells, a biphasic decrease is not evident, even when the sensitivity is increased 3.5-fold and the paper speed doubled (Fig. 2C). However, if in addition the amount of hemolysate added was decreased to one-tenth, a small rapid initial phase becomes evident (Fig. 2C). This initial rapid drop can be expected from O exchange theory established by Mills and Urey (1) as shown later. In the present study this initial phase is ignored in experiments with enzyme without cell suspension since its absolute value is small compared to that caused by intact cells. The mass 44 peak height (not shown in the figure) did not appear to change during the experiment. Since C^18O^18O is converted to C^18O, as the mass 46 peak decreases the mass 44 peak should increase, the total pco_2 remaining constant. The initial abundance of ^18O in CO_2 was less than 1.5% and was diluted practically to the level of natural abundance because of the large excess of oxygen in water (55.5 m).

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Fig. 2. Continuous trace of mass 46 ($^{13}$C$^{18}$O$^{16}$O) peak height in scale divisions against time. Temperature, 37°C, pH 7.4; NaCl, 145 mm; total NaHCO$_3$, 25 mm. A, initially uncatalyzed. At the arrow, 10 µl of cell suspension were added; fractional cell water volume, $v$, is 0.0002. B, initially uncatalyzed. At the arrow, 10 µl of hemolysate were added; $v$ of original cells equal to 0.0003. C (inset), magnification (3-fold increase in sensitivity and twice chart speed) of record B at the time of addition of hemolysate (I) and similar record for addition of equal volume of hemolysate diluted 10 times (II). Excess enzyme was added at the end of the experiment to produce complete isotopic equilibrium.

Fig. 3 is a logarithmic plot of mass 46 peak height less its value at final isotopic equilibrium against time. Prior to the addition of the cells, the graph is linear, that is, the mass 46 peak decreases exponentially with time. The uncatalyzed CO$_2$ hydration rate constant can be calculated from the half-time of this exchange according to the $^{18}$O exchange theory of Mills and Urey (1) (see Equation 13). The addition of cell suspension produces an initial rapid disappearance phase (II) followed by a slower decrease of the 46 peak, which is again linear. As a comparison, the exchange catalyzed by lysed cells is shown as a dashed line. Addition of the hemolysate accelerates the exchange but the process is exponential as expected. This represents the catalytic effect of the enzyme in the lysate, which increases the apparent value of $k_v$ by a factor that is proportional to the amount of enzyme added to the reaction mixture (see Fig. 4). In the presence of acetazolamide (1 mm) no change is observed upon the addition of either intact or lysed cells. The cells were preincubated in the same concentration of inhibitor before addition to the reaction mixture in order to assure the presence of the inhibitor inside the cell. The temperature and pH of the medium were monitored and maintained constant throughout each experiment.

The cells were generally suspended in isotonic saline (145 mm NaCl solution) containing 1% bovine serum albumin. The volume of the aliquot of suspension was so small it should not have changed the chemical composition of the standard medium. Preadjustment of pH, bicarbonate concentration, and temperature of the suspension to match the composition of the reaction medium did not alter the disappearance curve of the mass 46 peak. Therefore, this preadjustment was omitted. In order to provide a measure of the magnitude of the initial
membrane to \( \text{HCO}_3^- \), a \(^{18}O \) exchange theory was formulated which is presented in the next section.

Theory of Exchange of \(^{18}O \) between \( \text{CO}_2 \) and Water in Red Cell Suspension—The basic chemical reactions describing the exchange of \(^{18}O \) in single labeled \( \text{CO}_2 \) with unlabeled water were given by Mills and Urey (1).

\[
\begin{align*}
\text{CO}_2 + \text{H}_2\text{O} &\rightarrow \frac{1}{3}\text{H}_2\text{CO}_3 + \frac{2}{3}\text{H}_2\text{O} \\
\text{CO}_2 + \text{H}_2\text{O} &\rightarrow \frac{1}{3}\text{H}_2\text{CO}_3 + \frac{2}{3}\text{H}_2\text{O}
\end{align*}
\]

The singly labeled carbonic acid has a two-thirds probability of forming labeled \( \text{CO}_2 \) and unlabeled water, and a one-third probability of forming labeled water. Kinetic isotope effects are ignored. According to the reaction schema of Eigen et al. (9) \( \text{CO}_2 \) also reacts to form \( \text{HCO}_3^- \) directly. However the ionization reactions are so fast on the time scale of the present measurements that we could not separate this process from the formation of \( \text{H}_2\text{CO}_3 \) with subsequent ionization. The chemical reactions of \( \text{CO}_2 \) and water analogous to those above achieve chemical equilibrium in less than 10 s (10) at the pH and temperature of these experiments and it is assumed that elemental equilibrium pertains throughout the isotopic exchange processes. This is substantiated by the constant values of the mass 44 peak.

The processes included in the same \(^{18}O \) exchange in a red cell suspension are shown in Fig. 6 and include not only those reactions described in Equation 1, but analogous chemical reactions catalyzed by carbonic anhydrase within the red cell, plus the diffusion of labeled \( \text{CO}_2, \text{HCO}_3^- \), and water across the cell membrane. The concentration of carbonic acid is considered negligible. The rate of decrease of \( ^{18}\text{O}^{16}\text{O} \), on the assumption that \( \text{CO}_2 \) is instantaneously equilibrated between the intra- and extracellular space, is described by

\[
\frac{d[\text{H}_2\text{CO}_3]}{dt} = -k_v(w + 1)\frac{[\text{H}_2\text{CO}_3]}{[\text{CO}_2]}
\]

where \( w \) is the water volume of the red cells expressed as a fraction of the total solution volume. This figure is generally less than 0.004 so that the remaining volume, that of the extracellular volume can be assumed unity. \( k_v \) and \( k_v' \) are the extracellular hydration and dehydration velocity constants, respectively, in \( s^{-1} \). \( i \) and \( o \) indicate intra- and extracellular spaces, respectively. \( A \) is the ratio of the total intracellular
specific CO₂ hydration (or dehydration) reaction rate to the specific extracellular rate. Note that the intracellular rate includes catalyzed and uncatalyzed reactions.

The rate of change of labeled carbonic acid and carbonate inside the red cell is

\[
\frac{\text{d}[\text{HCO}_3^{18}O]}{\text{dt}} = -k_u[\text{HCO}_3^{18}O]_o + \frac{[\text{HCO}_3^{18}O]_o}{1 + P_a[H^+]_o} - k_a[\text{HCO}_3^{18}O]_0 
- P_a \cdot V \cdot [\text{HCO}_3^{18}O]_o - [\text{HCO}_3^{18}O]_o
\]

where \(P\) is the permeability of the red cell membrane to HCO₃⁻ in cm/s and \(a\) is the area of red cell membrane in cm²/cm³ of cell water. The rate of change of labeled carbonate + bicarbonate in the extracellular fluid is

\[
\frac{\text{d}[\text{HCO}_3^{18}O]_o}{\text{dt}} = k_u[\text{HCO}_3^{18}O]_o + \frac{[\text{HCO}_3^{18}O]_o}{1 + P_a[H^+]_o} - k_a[\text{HCO}_3^{18}O]_0
- P_a \cdot V \cdot [\text{HCO}_3^{18}O]_o - [\text{HCO}_3^{18}O]_o
\]

The abundance of ^{18}O in the several pertinent molecular species is defined as follows:

\[
a = \frac{[\text{HCO}_3^{18}O]_o}{1 + 2[\text{HCO}_3^{18}O]_o}
\]

\[
b = \frac{[\text{HCO}_3^{18}O]_o}{[\text{HCO}_3^{18}O]_o + [\text{HCO}_3^{18}O]_o}
\]

\[
c = \frac{3[\text{HCO}_3^{18}O]_o}{3[\text{HCO}_3^{18}O]_o + [\text{HCO}_3^{18}O]_o}
\]

The concentration of doubly or triply labeled species is always less than 0.02 of the corresponding singly labeled species because of the low levels of ^{18}O abundance (<2%) used experimentally. The abundances of ^{18}O in bicarbonate ion and carbonic acid in one phase are always equal and the protonation reaction is always in chemical equilibrium because of the rapidity of the ionization (11). Therefore, bicarbonate and carbonic acid can be treated as a single pool. The abundance of ^{18}O in water, \(\beta\), considered unchanged during the exchange process because of the large water pool, 55.5 m.

Since elemental chemical equilibrium is assumed throughout,

\[
k_u - \frac{[\text{HCO}_3^{18}O]_o}{[\text{CO}_2]_o} = k_a - \frac{[\text{HCO}_3^{18}O]_o}{[\text{CO}_2]_o}
\]

where \(K_{\text{HCO}_3}\) is the ionization constant for carbonic acid equal to 3.4 x 10⁻⁴ (12).

Substituting Equations 4 through 7 in Equations 1, 2, and 3, and rearranging, we obtain the simultaneous differential equations:

\[
\frac{\text{d}[\text{HCO}_3^{18}O]_o}{\text{dt}} = -k_u[\text{HCO}_3^{18}O]_o - k_a[\text{HCO}_3^{18}O]_0
- P_a \cdot V \cdot [\text{HCO}_3^{18}O]_o - [\text{HCO}_3^{18}O]_o
\]

\[
\frac{\text{d}[\text{HCO}_3^{18}O]_o}{\text{dt}} = k_u[\text{HCO}_3^{18}O]_o + k_a[\text{HCO}_3^{18}O]_0
- P_a \cdot V \cdot [\text{HCO}_3^{18}O]_o - [\text{HCO}_3^{18}O]_o
\]

\[
\frac{\text{d}[\text{HCO}_3^{18}O]_o}{\text{dt}} = k_u[\text{HCO}_3^{18}O]_o + \frac{[\text{HCO}_3^{18}O]_o}{1 + P_a[H^+]_o} - k_a[\text{HCO}_3^{18}O]_0
- P_a \cdot V \cdot [\text{HCO}_3^{18}O]_o - [\text{HCO}_3^{18}O]_o
\]

Since \(a\), \(\gamma_i\), and \(\gamma_\beta\) approach \(\beta\) at the end of the exchange process, the differential equations can be simplified by introducing the new variables \(\alpha = \alpha - \beta\), \(\gamma_{i'\beta} = \gamma_i - \beta\), and \(\gamma_{i'\beta'} = \gamma_i - \beta\), eliminating \(\beta\). We have been unable to obtain an analytical solution for the three simultaneous differential equations. However, Equation 9 can be simplified and solutions obtained as follows. The rates of the intracellular chemical reactions of CO₂ and the red cell bicarbonate exchanges by diffusion are so great that any changes in the labeled ^{18}O stored in the intracellular HCO₃⁻ can be ignored by comparison. In other words we assume that the rate of dehydration of intracellular labeled bicarbonate equals its rate of formation + the net rate of its diffusion into the cell at any instant because the rate of storage in cellular bicarbonate can be neglected. Thus, we can obtain \(\gamma_{i'\beta'}\) as a function of \(\alpha\) and \(\gamma_{i'\beta}\).

\[
\frac{\text{d}[\text{HCO}_3^{18}O]_o}{\text{dt}} = 2\alpha \gamma_{i'\beta'} + \gamma_{i'\beta'\beta'} \cdot \gamma_{i'\beta'} \cdot (k_a[H^+]_o)
1 + P_a \cdot V \cdot [\text{HCO}_3^{18}O]_o
\]
Intracellular Carbonic Anhydrase Activity

The larger exponential constant, that with the positive radical, applies to the transient change in $I^{80}$ enrichment of CO$_2$. When pH is high, the size of the dissolved CO$_2$ pool is much less than that of the HCO$_3^-$ so that the transient phase is extremely rapid with a half-time of 5 to 10 s (see Fig. 3). The smaller exponential, with the negative radical, applies to the quasi-steady state exchange of $^18O$ in CO$_2$ and HCO$_3^-$ intracellularly and extracellularly with water (see Fig. 3).

The solutions for $y_{1}$ and $y_{2}$ are of similar form to Equation 12 for $a'$. The exponential constants $m$ and $n$ are the same so that the half-time of disappearance of $y_{1}$, $y_{2}$, and $a'$ are equal in Phase III.

Silverman (13) has independently reported an equation describing the exchange of labeled oxygen in CO$_2$ with water in a red cell suspension for the situation in which CO$_2$ flux across the membrane is zero in comparison to the bicarbonate flux. Equation 12 assumes that the permeability of the membrane to CO$_2$ is essentially infinite, that assumption is defended under "Discussion." Gerster (14) has derived an analogous equation for the exchange of $^18$O in CO$_2$ of a gas phase with water of a liquid phase. If the volume of red cells in the suspension vanishes, that is $u$ goes to zero, or if the carbonic anhydrase activity disappears, that is $A$ goes to zero, at a pH high enough that $K_{ih} > > 1$, Equation 12 becomes the following:

\[ C = -k_{2} \frac{(1 + AV) + 2k_{2}u/3}{1 + 8} \]

The larger exponential constant, that with the positive radical, applies to the transient readjustment of the smaller C$^{'18}$O$^{'18}$O pool, while the smaller exponential constant with the negative radical applies to the slower quasi-steady state disappearance of $^18$O from CO$_2$ and HCO$_3^-$ (see Fig. 2C).

If the hydration-dehydration reactions of CO$_2$ are catalyzed by an enzyme in homogeneous solution, as in a hemolysate, the apparent value of $h_{c}$ determined experimentally, using Equation 13 to interpret the disappearance of C$^{'18}$O$^{'18}$O, will equal the sum of the uncatalyzed ($h_{c}$) and the catalyzed rates. Thus, Equation 13 also permits us to determine catalytic activity in solutions (16).

We have been unable to obtain an explicit solution of Equation 12 for $A$ and $P$ in terms of the experimentally determined variation in $y_{1}$ (mass 46 peak height - final mass 46 peak height) with time. Therefore, Equation 12 was fitted by trial and error to the biphasic disappearance curve for $C$ in Fig. 3 inserting the following data corresponding to a temperature of 37° in standard medium at [H$^{+}$]o of 10$^{-7}$ M, $h_{c}$ = 0.18 s$^{-1}$, $K_{ih}$ = 3.4 x 10$^{-4}$ M (12), $h_{c}$ = 64.2 s$^{-1}$, and $a$ = 20,000 cm$^{-1}$ (17).

The value of $h_{c}$ was calculated from the rate of disappearance of the mass 46 peak in uncatalyzed standard medium at 37° and pH 7.4 using Equation 13. The value of $h_{c}$ was calculated from Equation 14 using 9.33 x 10$^{-7}$ M for $K'$ from the results of Harned and Bonner (15) for 37° and the given ionic strength. The equation was solved for the two remaining constants, $A$ and $P$, by trial and error using a series of experimental exchange curves for different concentrations of cell suspension (18).

If Equation 11 is substituted in Equation 8 and rearranged, we obtain the following:

\[ \frac{1 + C}{1 + \frac{1}{c'B(A + 1)}} - \frac{1}{c'B(A + 1)} = \frac{1}{A} \frac{1}{\gamma'} + \frac{1}{\gamma} + \frac{1}{\gamma} \]

where $C$ equals

\[ \frac{1}{k_{s} \frac{d\gamma}{dt}} - \frac{1}{k_{s} \frac{d\gamma}{dt}} = \frac{1}{A} \frac{1}{\gamma} + \frac{1}{\gamma} \]

This relation corresponds to that first described by Mille and Urey (1) (with [H$_2$CO$_3$] neglected in comparison to [CO$_2$]) and permits one to calculate the uncatalyzed reaction velocity constant $k_{s}$ from experimental measurements of the exponential rate constant ($\gamma$) of $^18$O disappearance, pH, and an independently determined value of $K'$. $K'$ can be determined at equilibrium and accurate values for various temperatures and ionic strengths are available (15).

In analogy with Equation 12, the larger exponential constant with the radical positive applies to the transient readjustment of the smaller C$^{'18}$O$^{'18}$O pool, while the smaller exponential constant with the radical negative applies to the slower quasi-steady state disappearance of $^18$O from CO$_2$ and HCO$_3^-$ (see Fig. 2C).

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The function of the equivalent volume, \( v \), of intact cells. Extrapolating the least squares regression of \( 1/t_{1/2} \) on \( v \) to one gives a value of 3738 min\(^{-1}\) or 62.3 s\(^{-1}\). The corresponding exponential constant, \( n \), equals 62.3 \( \times 0.093 \) or 34.2 s\(^{-1}\). An apparent value of hydration reaction velocity constant at the intracellular enzyme concentration can be calculated from Equation 13 substituting in the values of 3.98 \( \times 10^{-4} \) M for hydrogen ion concentration in the reaction mixture and 9.53 \( \times 10^{-2} \) M for \( K' \) (15), giving a value of 3200 s\(^{-1}\). Thus, the \( CO_2 \) reactions at this concentration of carbonic anhydrase are faster than the uncatalyzed rate in the ratio 3200/0.18 = 17,777. This calculation makes the implicit assumption that the turnover number of the carbonic anhydrase is proportional to the concentration of the enzyme over at least 4 orders of magnitude. This assumption is supported by the work of Ker-nohan and Roughton (19) and Donaldson and Quinn (20). In order to compare the measurements of enzyme activity in hemolysate at pH 7.4 and [Cl\(^-\)] equal to 145 mM with estimations of intracellular activity at pH 7.2 and [Cl\(^-\)] about 70 mM, the calculated enzyme activity has been multiplied by the factor 0.88 as described under "Discussion," giving 15,643.

Table I represents the results of similar measurements and calculations of \( A \) and \( P \) in the cells of five normal healthy subjects. The average value of \( A \) is 9,906 representing an acceleration of approximately 10,000 by the carbonic anhydrase in the red cell. The average value of enzyme activity extrapolated to intracellular conditions is 17,500, 76% greater than the value estimated for the red cell interior. The average...
value of red cell membrane permeability to bicarbonate is 3.0 \times 10^{-4} \text{ cm/s}.

**DISCUSSION**

The time course of C^{18}O^{18}O disappearance in solutions without catalysis, in solutions containing carbonic anhydrase, either as hemolysate or purified enzyme, and in cell suspensions appears to be described quantitatively by the double exponential diffusion and chemical reaction theory presented in Equation 12. The special case of uncatalyzed solution has previously been described by Mills and Urey (1) and that of the homogeneous catalyzed solution by Silverman (16).

When the effective value of $k_1$ in the extracellular fluid is suddenly increased by adding carbonic anhydrase or lysate according to Equation 13, the time course of the labeled CO$_2$ should be the sum of the two exponential terms. This is not apparent in the logarithmic plot for the addition of hemolysate in Fig. 3, but an early rapid phase of C^{18}O^{18}O disappearance is easily seen in Fig. 2C where the chart speed has been increased and the concentration of the lysate added decreased to one-tenth. The early rapid phase occurs because the sudden increase in the rate of loss of labeled oxygen from CO$_2$ is not balanced by an equally sudden rise in the rate of formation of labeled CO$_2$, so that the concentration of C^{18}O^{18}O falls rapidly to a new quasi-steady state.

When red cells are added to an uncatalyzed solution of labeled bicarbonate, $\alpha$, the $^{18}$O abundance in CO$_2$, which had been greater than $\gamma_0$, the abundance of extracellular bicarbonate, falls rapidly (Phase II) to approximate equilibrium with the intracellular bicarbonate, and thereafter falls more slowly (Phase III). The fact that $\alpha$ is less than $\gamma_0$ during Phase III is demonstrated by the transient rise in $\alpha$ produced by the addition of carbonic anhydrase during Phase III (Fig. 5B). The increase in the extracellular reaction rates caused $\alpha$ to approach nearer to $\gamma_0$ and since $\alpha$ is much less than $\gamma_0$, it first rises and later falls along with $\gamma_0$. The second addition of the same amount of cell suspension (Fig. 5C) causes a second biphase acceleration in the labeled CO$_2$ disappearance. However, the step ratio for the second aliquot (1.33) is much less than for the first (1.76). This decrease in step ratio produced by successively adding cells is the same phenomenon as the leveling off of the step ratio with increasing $v$ seen in Figs. 4C and 7 (upper) and is described by Equation 15. In Fig 5D cells are added to produce the same hematocrit as in Fig. 5, B and C, but in this case the extracellular CO$_2$ reaction rate had been doubled by the prior addition of carbonic anhydrase. The usual biphase decrease in C^{18}O^{18}O is seen but the step ratio is reduced by 20% to 1.24 as compared to 1.51 in Fig. 5C and the exponential constant, $\alpha$, in Phase III is increased by 26%. $\alpha$ is defined as the total intracellular CO$_2$ reaction rate/extracellular rate and therefore an increase in the extracellular rate causes a decrease in $\alpha$ (although not in $A_k$) which reduces the magnitude of the step ratio as shown by Equation 15.

One difficulty encountered in applying the method was the presence of an irreducible trace amount of hemolysis in the reaction mixture which increased $h_1$, the extracellular hydration reaction velocity constant, in proportion to $v$. In order to determine the extent of this lytic process in the extracellular fluid during an experiment, 200 $\mu$L of cell suspension freshly prepared as described under "Methods" were diluted in 20 ml of the standard reaction mixture and centrifuged. To 10 ml of the supernatant additional $^{18}$O-labeled bicarbonate was added and the rate of C^{18}O^{18}O depletion was determined and compared with that in equivalent standard medium. The $t_{1/2}$ was reduced to 69% of the uncatalyzed control value corresponding to a 45% acceleration of the CO$_2$ reactions, as a result of carbonic anhydrase liberated into the extracellular fluid by hemolysis.

A home concentration of 0.45 $\mu$M in the supernatant increased 3-fold, indicating progressive hemolysis on standing.

The effect of extracellular carbonic anhydrase is to increase $k_1$ and $k_2$ (which are defined as the respective extracellular reaction velocity constants, rather than the uncatalyzed values) and to decrease $A$, because the intracellular reaction velocity is considered unchanged; $A_k$ and $A_k$, remain unchanged. It is important to note that the acceleration of $k_1$ will increase with $v$ presuming the same proportion of cells are lysed. When $v = 0.0032$, the fractional increase in $k_1$, after 5 h is 150%, at $v = 0.00032$ this increase would be only 15%. Since the experimental runs were generally carried out 1 to 5 h after the blood was drawn, the effect of hemolysis at low values of $v$ is minimal, but cannot be ignored at high $v$. The value of $\beta$ and the intercept of the plot of $1/(\text{step ratio} - 1)$ versus $1/v$ in Equation 15 are theoretically unaffected by an increase in $k_1$ because $A_k$ is constant. However, the slope increases as $A$ decreases, so the value of $1/(\text{step ratio} - 1)$ will increase. Fortunately, in the reciprocal plot (see Fig. 8) the values of step ratio at high $v$ have less effect on the least mean squares slope than those at low $v$, so the value of $A$ obtained should be less influenced by changes in the degree of hemolysis found experimentally than the value obtained by fitting Equation 12 to data at high $v$. Similar considerations demonstrate that the experimental value of $v$ is changed less than 10% by hemolysis of the degree seen experimentally. In order to calculate the intracellular rate of the reaction, $A_k$, it is necessary to know the actual experimental value of $k_1$, and not to assume that it is equal to the uncatalyzed value.

The problem produced by hemolysis is illustrated in Fig. 7, where the values for $A$, 9620, and for $P$, 2.58 $\times 10^{-4}$ cm/s, obtained from the reciprocal plot in Fig. 8 were introduced into Equation 12 and the step ratio and $1/v_{1/2}$ of Phase III computed.

The values calculated for the step ratio (open circles, Fig. 7) agree with the experimental points (closed circles, Fig. 7) in the range where $v$ is relatively small, but do not agree at the highest $v$. The calculated values for $1/v_{1/2}$ of Phase III are consistently less than the experimental values.

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**Table I**

**Carbonic anhydrase activity and bicarbonate permeability of normal red blood cells**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Intracellular Enzyme Activity</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated* from lysate</td>
<td>Measured (A)</td>
</tr>
<tr>
<td>D. W.</td>
<td>18,900</td>
<td>9,160</td>
</tr>
<tr>
<td>N. I.</td>
<td>17,900</td>
<td>8,150</td>
</tr>
<tr>
<td>H. J.</td>
<td>15,600</td>
<td>9,620</td>
</tr>
<tr>
<td>S. L.</td>
<td>17,200</td>
<td>11,200</td>
</tr>
<tr>
<td>G. M.</td>
<td>17,900</td>
<td>11,400</td>
</tr>
<tr>
<td>Mean</td>
<td>17,500</td>
<td>9,806</td>
</tr>
</tbody>
</table>

* Extrapolated from measurements on hemolysate to intracellular conditions on the assumption that cell water equals 61% of cell volume. Estimated activity was decreased by the factor 0.88 to correct for the lower intracellular pH and chloride ion concentration.
Intracellular Carbonic Anhydrase Activity

It is possible to choose values of \( A \) and \( P \) by trial and error so that the calculated step ratio and reciprocal of \( t_{1/2} \) for Phase III agree with the experimental measurements at a given value of \( v \), but not for all \( v \). It is also possible to choose values of \( A \) and \( P \), for example 16,000 and 0.0007 cm/s, respectively, which yield computations of 1/\( t_{1/2} \) that fit at all \( v \) in Fig. 7, but yield computations of step ratio that are less than the experimental data at high \( v \).

Because of hemolysis, as \( v \) increases, carbong anhydrase activity in the extracellular fluid increases and \( k \), increases, theoretically resulting in a decrease in \( A \) and in 1/\( t_{1/2} \) (\( n \)) but an increase in step ratio. This is shown to be true by the experiment illustrated in Fig. 5D, in which carbonic anhydrase was added to the extracellular fluid prior to the addition of the cells. The step ratio decreased and 1/\( t_{1/2} \) in Phase III increased in comparison to experiments (Fig. 5, B and C) in which no carbonic anhydrase was added.

Although the theory of CO\(_2\)/O\(_2\) exchange describes the general experimental results, there is a discrepancy between the numerical values of \( A \) and \( P \) obtained by the least means squares reciprocal plot method (as in Fig. 8) and those that can be obtained by a trial and error curve fitting of Equation 12 to the biphasic mass 46 disappearance curve. For example, when the data on the cells of H. J. were analyzed by the reciprocal plot method, values of 9,620 for \( A \) and 0.00026 cm/s \( P \) were obtained. When Equation 12 is fitted by trial and error to 1/\( t_{1/2} \) of Phase III, values of \( A \) = 16,000 and \( P \) = 0.0007 cm/s were obtained.

We place more reliance on the linearized reciprocal plot because it provides a convenient statistical technique of analyzing data. Furthermore, the reciprocal plot method is weighted less by measurements at higher \( v \), which are considered less reliable for the following reasons. At higher \( v \) there is greater error produced by hemolysis. At higher \( v \) the disappearance of labeled CO\(_2\) can become so rapid that the instrument cannot respond accurately. Finally, at higher \( v \) the mass 46 peak may approach the noise level before 1/\( t_{1/2} \) of Phase III is defined accurately.

The values of step ratio and \( n \) vary with \( v \) in a similar manner for the cells of all five subjects (Fig. 4). Therefore, although the cells of H. J. have been used in these examples, the conclusions are generally applicable.

The results in Table I obtained from the reciprocal plot method indicate that the intracellular carbonic anhydrase activity is about 50% of the value one would expect from the measurements of its activity in lysate. Although if we were to use the estimate of 16,000 for \( A \) obtained on the cells of H. J. by the trial and error curve fitting method, the intracellular value is approximately the same as the lysate.

This discrepancy between the experimentally determined intracellular CO\(_2\) reaction rate and the rate extrapolated from measurements of carbonic anhydrase turnover in lysate could be caused by the fact that the intracellular reactions are rate-limited by processes other than the enzyme turnover rate. It could also be produced by an effect of the chemical environment within the red cell upon enzyme function.

**Exchange of \(^{18}\)O within Red Cell May Be Rate-limited by Some Process Other Than Enzyme Turnover**

**Diffusion Resistance of Red Cell Membrane to CO\(_2\), May Cause Significant Drop in \(\alpha\) across Cell Wall**

Constantine et al. (3) measured the time course of [CO\(_2\)] when a red cell suspension was suddenly mixed with a solution containing CO\(_2\) in a continuous flow rapid reaction apparatus using a \( p_{CO_2} \) electrode to monitor the progress of the reaction. They found that the initial rate of CO\(_2\) disappearance in the mixture represented a rate of hydration of CO\(_2\) that was half the value expected from experimental measurements of the rate constant in a dilute lysate of the same cell suspension. Kernohan and Roughton (19) obtained similar experimental results in a continuous flow rapid reaction apparatus using thermal detection. If we assume that all this discrepancy is produced by a drop in [CO\(_2\)] across the cell membrane, we can calculate an upper limit for the relative diffusion resistance of the cell membrane to CO\(_2\). In the present experiments we are dealing with the exchange of \(^{18}\)O in CO\(_2\) which is about 1/5 as fast as the elemental reaction which Constantine et al. studied. This means that the drop in concentration of labeled CO\(_2\) across the membrane of the red cell is reduced to 1/5 of the extracellular value, a matter of 1.6%. This estimate of relative diffusion resistance of the red cell membrane is an upper limit, because there are other factors that might contribute to the discrepancy between the measured and expected rate of CO\(_2\) reaction inside the red cell, such as the effect of a different chemical environment for the carbonic anhydrase inside as compared to outside the cell, and CO\(_2\) gradients within the cell. We conclude that a significant drop in \(\alpha\) across the red cell membrane in our experiments is unlikely.

**Abundance of Labeled Water, \(\beta\), May Become Greater Intracellular Than Extracellularly Because of Membrane Resistance to Water Movement—The end product of the exchange process, \(^{18}\)O-H\(_2\)O, is diluted in the large pool of oxygen in intracellular water which exchanges easily with extracellular water. A maximal value for build-up of \(\beta\) inside the cell can be computed by setting the rate of disappearance of labeled CO\(_2\) inside the cell equal to the flux of labeled water through the cell membrane. Thus,**

\[
\frac{\partial (\text{water})}{\partial t} = -\beta \frac{\partial (\text{H}_2\text{O})}{\partial n} = \frac{\partial (\text{H}_2\text{O})}{\partial n} \cdot \frac{\beta}{\alpha} = \frac{\beta}{\alpha} \left( p_{\text{H}_2\text{O}} + p_{\text{HCO}_3^-} \right) = \frac{\beta}{\alpha} \left( \frac{25}{2} \right) \quad \text{mM} \quad \text{cm/s}
\]

where \((\partial (\text{water})/\partial t) \cdot (1/\alpha)\) is the exponential disappearance constant, \(n, [\text{CO}_2] + [\text{HCO}_3^-] = 25 \text{ mM; } P \text{ for } \text{H}_2\text{O} = 0.0215 \text{ cm/s } (21), \alpha = 20,000 \text{ cm}^2, \text{ and } [\text{H}_2\text{O}] = 55.5 \text{ m.} \) The exponential constant \(n\) is linearly related to \(v\) so that the choice of \(v\) is not critical. Even during the rapid fall in \(n\) in Phase II (Fig. 5D), where \(v = 0.00032, t_{1/2} = 3.8s\) and \(m = 0.18, \beta/\alpha = 6 \times 10^6\), a negligible value. Therefore, we conclude that \(\beta\) is the same inside and outside cells.

**Diffusion Gradients May Develop within Red Cell—If the rate of disappearance of \(^{18}\)O from CO\(_2\) and HCO\(_3^-\) at different points within the cell is rapid compared to the rate of diffusion of CO\(_2\) and HCO\(_3^-\), significant gradients of the labeled species can develop, even over small distances within the red cell (17, 22). The rate of uptake of O\(_2\) by red cells is about 1/50 the rate of O\(_2\) combination with hemoglobin in a comparable homogeneous solution, owing to the rapidity of the chemical reactions as compared to the gaseous diffusion. The velocity of CO\(_2\)/O\(_2\) disappearance is several orders of magnitude slower than the reactions of O\(_2\) with hemoglobin, but the diffusion coefficient (cm/s) of CO\(_2\) is only 18% less than that of O\(_2\). Therefore, we can expect the gradients of labeled CO\(_2\) and HCO\(_3^-\) within the cell to be minimal. Significant diffusion gradients of labeled water, \(\beta\), within the cell appear unlikely for similar reasons.**

**Environment of Carbonic Anhydrase within Red Cell May Decrease Its Turnover Rate**

Intracellular pH measured in lysed packed normal cells is...
Intracellular Carbonic Anhydrase Activity

about 7.2, while the extracellular pH is 7.4. We found enzymic activity of lysate decreased by the factor 0.72 as pH decreased from 7.4 to 7.2 in standard medium. Chloride ion inhibits carbonic anhydrase, especially isozyme B (4). Intracellular [Cl\textsuperscript{-}] is about 70 mM while [Cl\textsuperscript{-}] of standard medium is 145 mM, which should increase the expected value of intracellular enzyme activity by 1.22. The product of these factors, 0.88, has been used in computing the value of intracellular carbonic anhydrase activity extrapolated from measurements on lysate (Table I), and yet the discrepancy between the intra- and extracellular carbonic anhydrase activity remains. There is no clear explanation at this time for our finding that intracellular carbonic anhydrase activity is less than would be expected from measurements on red cell lysate, although a difference in chemical environment is the most likely cause.

The average \( P \) in Table I equals 3.0 \( \times 10^{-4} \) cm/s which is similar to the value of 1.61 \( \times 10^{-4} \) cm/s obtained for the rat red blood cell membrane at 25\degree using \(^{18}\text{O}\) labeled bicarbonate as in this paper, but at very alkaline pH (13) and to the value of 2.2 \( \times 10^{-4} \) cm/s for the exchange of \( \text{HCO}_3^- - \text{Cl}^- \) in human red cells at 37\degree obtained by Chow et al. (24) and for OH\textsuperscript{-} of 2.2 \( \times 10^{-4} \) cm/s at 37\degree reported by Tosteson (24) and for OH\textsuperscript{-} of 2.2 \( \times 10^{-4} \) cm/s at 37\degree (25).

Recent experimental results suggest that Cl\textsuperscript{-}-Cl\textsuperscript{-} exchange across the red cell membrane takes place by a facilitated exchange mechanism which can be saturated (26) and a similar mechanism may obtain for \( \text{HCO}_3^- - \text{HCO}_3^- \) exchange. This means that the flux of bicarbonate ion across the red cell membrane would not be proportional to the differences in \( \text{HCO}_3^- \) concentration. In deriving Equation 12 it is assumed that the instantaneous flux of labeled bicarbonate is proportional to its gradient across the cell wall. This statement is true regardless of the mechanism of bicarbonate ion transport, provided the elemental conditions remain constant. The value of \( P \) obtained applies only to the given experimental conditions if bicarbonate flux across the red cell membrane is carried by an exchange mechanism.

The proportional variation in estimated \( P \) is somewhat large because it is mainly determined by the zero intercept in the plot of \( 1/(\text{step ratio} - 1) \) versus \( 1/\nu \), which is normally close to the origin.

Acknowledgments.—We thank Lydia Lin for help in the mathematical derivations and Henry B. John for skillful technical assistance.

Addendum.—After this manuscript was submitted, the paper of Silverman et al. (27) appeared reporting similar findings on the \(^{18}\text{O}\) exchange of CO\textsubscript{2} in suspensions of red cells, but analyzing their data on the assumption that the membrane diffusion resistance to CO\textsubscript{2} was the rate-limiting process.

REFERENCES
Carbonic anhydrase activity in intact red blood cells measured with 18O exchange.

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