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A New Approach to Measuring the Rate of Rapid Bicarbonate Exchange across Membranes

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SUMMARY

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This is a description of a new method for measuring the rate of rapid bicarbonate exchange across membranes, including a description of the inhibition of the bicarbonate exchange across the rat erythrocyte membrane by sodium picrate. The method is based on the measurement, by mass spectrometry, of the rate of depletion of ^{18}O from labeled bicarbonate dissolved in a solution in which red cells are suspended. The rate of this depletion depends (a) on the rate of diffusion of labeled bicarbonate into the cell, (b) on the rate of chemical reaction of labeled bicarbonate to form labeled carbon dioxide, which then diffuses across the membrane, and (c) on the rate of carbonic anhydrase-catalyzed depletion inside the cell. Expressions are derived for the permeability constant of the cell membrane to bicarbonate and the rate constant for the catalyzed, intracellular dehydration of bicarbonate in terms of the rates of depletion of ^{18}O from bicarbonate. The contribution of labeled CO_2 diffusion across the membrane, factor (b) above, is estimated by assuming that labeled CO_2 generated intracellularly does not accumulate in the suspending solution, but is depleted of its label inside the cell. This assumption is not rigorously verified and is a possible source of error in separating the bicarbonate flux from the CO_2 flux. This method is most accurate in regions above pH 9 and is restricted to cells containing carbonic anhydrase. At pH 9.6 and 25° the permeability constant of the rat erythrocyte membrane to bicarbonate is $(1.81 \pm 0.12) \times 10^{-4}$ cm/sec, giving a half-time of 0.13 ± 0.02 sec for the exchange of bicarbonate in the cell. The rate constant for the catalyzed, intracellular dehydration of bicarbonate is 3.3 ± 0.4 sec^{-1}. P, calculated in this way, was found to increase when the pH of the red cell suspension was decreased.

INTRODUCTION

This is a description of a new method for the measurement of the rate of rapid bicarbonate exchange across membranes. Its application to the passage of bicarbonate across the rat erythrocyte membrane and the inhibition of that process by sodium picrate is also described. The method is heavily based on two previous studies which elegantly describe the kinetics of ^{18}O depletion from labeled carbon dioxide. Mills and

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Urey (1) were the first to measure this depletion and from its velocity obtained the rate constant for the hydration of CO_2. Gerster (2) made similar measurements and, in addition, used the ^{18}O depletion to determine the diffusion constant of CO_2 between a gaseous phase and an aqueous phase. The notation of Gerster (2) is adopted in this work.

THEORY

Isotope depletion in a homogeneous medium. When enriched with ^{18}O, bicarbonate is present as three labeled species: $HC^{16}O^{16}O^{18}O^-$, $HC^{16}O^{18}O^{18}O^-$, and $HC^{18}O^{18}O^{18}O^-$. In aqueous solutions, depletion of this label from bicarbonate occurs by exchange of the label with the oxygen of water according to reactions such as

$$H^+ + HC^{16}O^{18}O^{18}O^- \rightleftarrows C^{16}O^{18}O + H_2{}^{18}O \quad (1)$$

$$C^{16}O^{18}O + H_2{}^{16}O \rightleftarrows HC^{16}O^{16}O^{18}O^- + H^+ \quad (2)$$

For convenience, this exchange is measured at chemical equilibrium, and the depletion of label from bicarbonate is based on the almost infinite dilution of the species $H_2{}^{18}O$ by $H_2{}^{16}O$. Thus reaction 2 occurs with unlabeled water and yields depleted bicarbonate. If carbonic anhydrase is present in the solution, the rate of depletion of ^{18}O from bicarbonate is enhanced, since reactions 1 and 2 are catalyzed by this enzyme. It is important to note that the cyclic chemical process represented by reactions 1 and 2 can deplete only 1 atom of ^{18}O from a bicarbonate molecule in one cycle of chemical reaction.

Actually, two mechanisms for the uncatalyzed dehydration of bicarbonate are important in the pH range 8–10 (3):

$$HCO_3^- + H^+ \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} CO_2 + H_2O$$

$$HCO_3^- \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} CO_2 + OH^-$$

The composite rate constant for uncatalyzed dehydration is k':

$$k' = k_{-2} + k_{-1}[H^+] \quad (3)$$

and the composite rate constant for uncatalyzed hydration is k:

$$k = k_1 + k_2[OH^-] \quad (4)$$

Using k and k', the following simplified expression gives the rates at equilibrium (Eq. 11 of ref. (2).

$$k'[HCO_3^-] = k[CO_2] \quad (5)$$

The rate equations for the depletion of ^{18}O from labeled bicarbonate in a homogenous phase are presented in complete form by Mills and Urey (1) and also by Gerster (2). The fractions of total oxygen atoms which are ^{18}O in CO_2 and HCO_3^- in solution are given by τ and τ', respectively.

$$\tau = \frac{[C^{16}O^{18}O] + 2[C^{18}O^{18}O]}{2[CO_2]}$$

$$\tau' = \frac{[HC^{16}O^{16}O^{18}O^-] + 2[HC^{16}O^{18}O^{18}O^-] + 3[HC^{18}O^{18}O^{18}O^-]}{3[HCO_3^-]}$$

The kinetics of depletion of ^{18}O from CO_2 and HCO_3^- is then measured by observing the decay of ^{18}O enrichment, $d(\tau - \tau_\infty)/dt$ and $d(\tau' - \tau'_\infty)/dt$, where τ_∞ and τ'_∞ are the atom fractions of ^{18}O in CO_2 and HCO_3^- at infinite time and are very close to the natural abundance of ^{18}O, which is 0.002. For conciseness in the derivations to follow, we write τ instead of $\tau - \tau_\infty$ and τ' instead of $\tau' - \tau'_\infty$, and reintroduce τ_∞ and τ'_∞ in the final equations.

The decay of ^{18}O from CO_2 and HCO_3^- is described as follows (1, 2):

$$-\frac{d\tau}{dt} = k(\tau - \tau') \quad (6)$$

$$-\frac{d\tau'}{dt} = k'\left(\tau' - \frac{2}{3}\tau\right) \quad (7)$$

with rate constants k and k' given by Eqs.

3 and 4. The solution of these simultaneous equations is of the form

$$\tau' = a_1 e^{-\theta_1 t} + a_2 e^{-\theta_2 t} \qquad (8)$$

with a similar expression for τ. The values of θ_1 and θ_2, the roots of the characteristic equation, are

$$2\theta_{1,2} = k + k' \pm \sqrt{(k + k')^2 - (4/3)kk'}$$

These roots, in the form $\sqrt{x^2 - y}$, are approximated by $| x - y/2x |$. The approximation is excellent for pH values greater than 7, for which $y/2x$ is less than 5×10^{-3} sec^{-1}.

This approximation gives the important root

$$\theta_1 = \frac{kk'}{3(k + k')} = \frac{k'}{3}\left[\frac{1}{1 + k'/k}\right]$$

The second root becomes unimportant very soon after the exchange begins (1). Using the rate expression, Eq. 5, we obtain

$$\theta_1 = \frac{k'}{3}\left[\frac{[HCO_3^-]}{[HCO_3^-] + [CO_2]}\right] = \frac{k'}{3} f \qquad (9)$$

where f is the ratio in brackets. Above pH 8 the equilibrium between HCO_3^- and CO_3^{2-} must be considered. The rate of proton transfer between these two species is nearly instantaneous in comparison with the time scale of ^{18}O exchange; however, its presence must be accounted for, since CO_3^{2-} cannot be depleted of ^{18}O but must be converted to HCO_3^- for depletion to occur. Considering this additional equilibrium in Eq. 5 and following the above derivation yields an expression identical with Eq. 9, but with

$$f = \frac{[HCO_3^-]}{[HCO_3^-] + [CO_2] + [CO_3^{2-}]}$$

Substituting Eq. 9 into Eq. 8, the solution to the differential equations describing the depletion of ^{18}O from bicarbonate can be written

$$\ln(\tau' - \tau'_\infty) = -\tfrac{1}{3}k'ft + \ln(\tau'_0 - \tau'_\infty) \qquad (10)$$

The atom fractions at infinite time, described earlier, have been included in this final expression. This same solution has also been obtained by the following route. In alkaline solution it is a good approximation to set $\tau = \tau'$ (4). Using this approximation in Eqs. 6 and 7 yields

$$-\frac{d\tau'}{dt} = \frac{fk'}{3}\tau' \qquad (11)$$

with the factor f included to account for the fraction of total carbonate species existing as bicarbonate. The rate of depletion in an intracellular solution containing carbonic anhydrase is described by replacing k' with k'_c, as shown in Fig. 1. A study, using the above equations, of carbonic anhydrase-catalyzed ^{18}O exchange in a homogeneous solution has been reported (4).

Gerster (2) has shown that similar expressions can be derived for the depletion of ^{18}O from CO_2 which is doubly labeled and from HCO_3^- which is doubly or triply labeled. The fraction of total oxygen atoms in CO_2 which appear in doubly labeled CO_2 is given by c.

$$c = \frac{[C^{18}O^{18}O]}{[CO_2]}$$

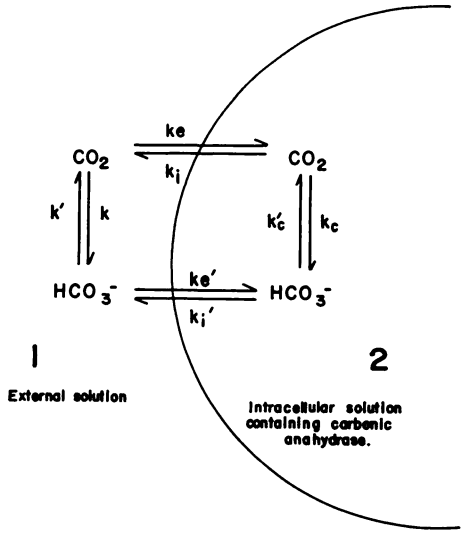

FIG. 1. *Chemical and diffusive processes of CO_2 and HCO_3^- as studied by isotope depletion technique*

Medium 1 represents extracellular fluid, and medium 2 intracellular fluid. k' and k_e' are chemical rate constants; k_e' and k_i' are diffusion constants as described in the text.

The fraction of total oxygen atoms in those molecules of HCO_3^- which yield doubly labeled CO_2 upon acidification is c'. This qualification is necessary because the enrichment of HCO_3^- in each experiment is determined by converting it into CO_2 with acid and measuring the enrichment of the CO_2 (see EXPERIMENTAL PROCEDURE).

$$c' = \frac{[HC^{16}O^{18}O^{18}O^-] + 3[HC^{18}O^{18}O^{18}O^-]}{3[HCO_3^-]}$$

The kinetics of depletion of ^{18}O from these multiply labeled species is then described as follows (2):

$$-\frac{dc}{dt} = k(c - c') \tag{12}$$

$$-\frac{dc'}{dt} = k'\left(c' - \frac{1}{3}c\right) \tag{13}$$

Following the same procedures used to solve Eqs. 6 and 7, it can be shown that a solution of these simultaneous equations for c' is of the form

$$c' = b_1 e^{-\gamma_1 t} + b_2 e^{-\gamma_2 t} \tag{14}$$

with the value of γ_1 given by

$$\gamma_1 = \frac{2k'}{3} f$$

The term in Eq. 14 containing γ_2 is not significant, and the solution to the differential equations describing the depletion of ^{18}O from multiply labeled bicarbonate can be written

$$\ln(c' - c'_\infty) = -\tfrac{2}{3}k'ft + \ln(c'_0 - c'_\infty) \tag{15}$$

The atom fractions at infinite time are included in this final expression. As was the case with Eq. 7, the equation describing the depletion of ^{18}O from multiply labeled bicarbonate, Eq. 13, can be written

$$-\frac{dc'}{dt} = \frac{2k'f}{3} c' \tag{16}$$

by making the approximation that $c = c'$ in alkaline solution. This also yields the solution, Eq. 15.

To summarize, in a homogeneous solution the rates of depletion of the total isotopic enrichment of bicarbonate, $\tau' - \tau'_\infty$, and of the isotopic enrichment of multiply labeled bicarbonate, $c' - c'_\infty$, are related to the rate constant for dehydration of bicarbonate in the solution, k'. However, these rates of depletion are not identical; rather, they depend on whether we measure $\tau' - \tau'_\infty$ or $c' - c'_\infty$, the rate of the latter being twice the rate of the former. That is, the slope of a plot of $\ln(c' - c'_\infty)$ vs. time should be twice the slope of a plot of $\ln(\tau' - \tau'_\infty)$ vs. time in a homogeneous solution.

Isotope depletion in two media separated by a membrane. In this section we consider the isotopic depletion of labeled bicarbonate in two solutions separated by a membrane permeable to carbon dioxide and bicarbonate ions. A further requirement is that the membrane be impermeable to carbonic anhydrase and that the concentration of this enzyme be different in the solutions separated by the membrane. Reference is again made to Fig. 1, in which k' represents the extracellular rate constant for dehydration of bicarbonate and k'_c represents the rate constant when catalyzed by intracellular carbonic anhydrase.

The approach taken in the following derivations is first to consider the case for very alkaline solutions in which the concentration of CO_2 is so small that the flux of labeled CO_2 across the membrane can be neglected. In another section the case for lower pH is discussed, in which the flux of labeled CO_2 across the membrane is considered.

When the intracellular and extracellular solutions are at very alkaline pH, only the diffusion of labeled bicarbonate and carbonate across the membrane is important. In this case we consider a form of Fick's first law of diffusion, Eq. 17, containing the permeability constant for bicarbonate, P, the volume of the cell, V_2, and surface area of the cell membrane, A (5). Alternatively, V_2 can be taken as the volume of all the cells in the suspension, and A as the total membrane

surface area of all the cells. V_1 is the volume of extracellular solution. In regions of high pH where the concentration of CO_3^{2-} is appreciable, P represents a constant describing the permeability of both HCO_3^- and CO_3^{2-}.

$$-\frac{d\tau'_2}{dt} = \frac{PA}{V_2}(\tau'_2 - \tau'_1) \qquad (17)$$

The subscripts 1 and 2 refer to the extracellular and intracellular fluids, respectively. This form of Fick's law is valid for simple passive diffusion of bicarbonate and for facilitated diffusion at bicarbonate concentrations far from saturation.

$$2\theta_{1,2} = \frac{k'f}{3} + k'_i + \frac{k'_cf}{3} + k'_e \pm \left\{\left[\left(\frac{k'_cf}{3} + k'_i\right) - \left(\frac{k'f}{3} + k'_e\right)\right]^2 + 4k'_ek'_i\right\}^{1/2}$$

Since the flux of labeled CO_2 across the membrane is neglected, it is more convenient to use here the expression for bicarbonate depletion given by Eq. 11 rather than the simultaneous equations, Eqs. 6 and 7, which describe the same depletion. By incorporating Eq. 17 into Eq. 11, the kinetic processes of Fig. 1 at very alkaline pH are described by the following:

$$-\frac{d\tau_1'}{dt} = \frac{k'f}{3}\tau'_1 + k'_e(\tau'_1 - \tau'_2) \qquad (18)$$

$$-\frac{d\tau'_2}{dt} = \frac{k'_cf}{3}\tau'_2 + k'_i(\tau'_2 - \tau'_1) \qquad (19)$$

where $k'_e = PA/V_1$ and $k'_i = PA/V_2$. These equations are based on the following assumptions. (a) Chemical equilibrium exists on both sides of the membrane, and kinetic isotope effects are negligible. (b) At very alkaline pH the concentration of CO_2 is so small that the flux of labeled CO_2 across the membrane is negligible. (c) The permeability for bicarbonate going into the cell is equal to the permeability for bicarbonate going out of the cell; that is, one P describes the bicarbonate permeability of the membrane. (d) There is no isotope effect for bicarbonate diffusion; that is, one P describes the permeability of all isotopic species of bicarbonate.

In the experiments the isotopic enrichments of the bicarbonate in the suspending solution, τ'_1 and c'_1, are measured. The solution of the simultaneous differential Eqs. 18 and 19 for τ'_1 has the following form:

$$\tau'_1 = a_1e^{-\theta_1 t} + a_2e^{-\theta_2 t} \qquad (20)$$

Without introducing any approximation, θ_1 and θ_2 can be obtained by solving the quadratic, characteristic equation obtained from Eqs. 18 and 19. These solutions, in the form $\sqrt{x^2 + y}$, are approximated by $|x + y/2x|$. The approximation is valid, since the experimental data give a very small value for $y/2x$ (less than 10^{-2} sec^{-1} in the red cell experiments) and the error which results is much smaller than the experimental errors.

This approximation gives

$$\theta_1 = \frac{k'f}{3} + k'_e - \frac{k'_ek'_i}{\frac{k'_cf}{3} + k'_i - \frac{k'f}{3} - k'_e} \qquad (21)$$

The expression for θ_2 contains the terms $k'_cf/3 + k'_i$ and, with the knowledge that $k'_c \gg k'$ and $k'_i \gg k'_e$, we can determine that $\theta_2 \gg \theta_1$. As a result, the second term on the right in Eq. 20 becomes unimportant soon after the experiment begins. The equation which describes the total ^{18}O enrichment in the external medium as a function of time can then be written

$$\tau'_1 - \tau'_\infty = (\tau'_0 - \tau'_\infty)e^{-\theta_1 t}$$

where θ_1 is given by Eq. 21 and τ'_∞ is introduced so that the equation represents isotopic enrichment rather than total isotopic content. Thus a plot of $\ln(\tau'_1 - \tau'_\infty)$ vs. time yields $-\theta_1$ as its slope.

However, the slope θ_1 contains the two unknowns k'_e and k'_c and cannot be solved.

For a second source of information we consider the time dependence of c'_1 and c'_2.

$$-\frac{dc'_1}{dt} = \frac{2k'f}{3}c'_1 + k'_e(c'_1 - c'_2) \quad (22)$$

$$-\frac{dc'_2}{dt} = \frac{2k'f}{3}c'_2 + k'_i(c'_2 - c'_1) \quad (23)$$

A comparison of Eqs. 22 and 23 with Eqs. 18 and 19 illustrates in terms of kinetic expressions a cardinal point to be repeated in the next section: The rate of depletion of label depends on whether one observes c' or τ', but the rate of diffusion of labeled bicarbonate through the membrane is the same for c' and τ'.

The solution of the simultaneous differential Eqs. 22 and 23 for c'_1 is of the form

$$c'_1 = b_1e^{-\gamma_1 t} + b_2e^{-\gamma_2 t} \quad (24)$$

with

$$2\gamma_{1,2} = \frac{2k'f}{3} + k'_i + \frac{2k'_cf}{3} + k'_e \pm \left\{\left[\left(\frac{2k'_cf}{3} + k'_i\right) - \left(\frac{2k'f}{3} + k'_e\right)\right]^2 + 4k'_ek'_i\right\}^{1/2}$$

Approximating the square root as above gives

$$\gamma_1 = \frac{2k'f}{3} + k'_e - \frac{k'_ek'_i}{\frac{2k'_cf}{3} + k'_i - \frac{2k'f}{3} - k'_e} \quad (25)$$

Finally, a plot of $\ln(c'_1 - c'_\infty)$ vs. time yields $-\gamma_1$ as its slope:

$$c'_1 - c'_\infty = (c'_0 - c'_\infty)e^{-\gamma_1 t} \quad (26)$$

The information gained from the two slopes θ_1 and γ_1 can now be used to obtain k'_c and k'_e. For this purpose we set $\theta = \theta_1 - \frac{1}{3}k'f$ and $\gamma = \gamma_1 - \frac{2}{3}k'f$ and note that $k'_i = V_1k'_e/V_2$. Solving Eqs. 21 and 25 simultaneously gives

$$k'_e = \frac{\gamma}{2 - \gamma/\theta} \quad (27)$$

$$k'_c = \frac{3}{2}\frac{V_1}{fV_2}\left(\frac{\gamma}{\gamma/\theta - 1}\right) \quad (28)$$

To obtain these solutions the term $k'f/3 + k'_e$, which is about 10^{-2} sec^{-1} in our red cell experiments, has been neglected with respect to $k'_cf/3 + k'_i$, which is about 10 sec^{-1} in our experiments. These solutions for k'_e and k'_c are very similar to the expressions derived by Gerster (2) for the diffusion of CO_2 between gaseous and aqueous phases. The term f appears in Eq. 28. It is necessary since a fraction $(1 - f)$ of the total carbonate and bicarbonate exists as enriched carbonate and cannot be depleted of its label until it is converted to the bicarbonate form.

In this section it has been shown that under certain restricted conditions, mainly a restriction to work with alkaline solutions, two kinetic constants can be obtained by measuring the rate of change of the isotopic contents of bicarbonate in the extracellular solution only. The kinetic constants are the diffusion rate constant k'_e, related to the permeability of the membrane to bicarbonate, and a rate constant for dehydration of bicarbonate in the cell, related to the intracellular activity of carbonic anhydrase.

Explanation of physical phenomena. In a suspension of cells at very alkaline pH, HCO_3^- diffuses through the cell membrane, but the flux of labeled CO_2 across the membrane is negligible because the concentration of CO_2 is very small. Furthermore, ^{18}O is depleted from the bicarbonate in the external medium in a relatively slow process and is depleted in the intracellular fluid more rapidly in a catalyzed process, provided that the cells contain carbonic anhydrase.

Since the isotopic depletion of bicarbonate depends on chemical reactions, such as Eqs. 1 and 2, the rate constants k, k', k_c, and k'_c can be obtained by measuring the rate of ^{18}O exchange between bicarbonate and water. But the diffusion constants k'_e and k'_i (defined by Eqs. 18 and 19) can also be obtained

by observing the same isotopic depletion rates, since access to the intracellular medium containing carbonic anhydrase is determined by these diffusion constants.

The rate constants k and k', k_c and k'_c are related by equilibrium constants. Furthermore, k'_e and k'_i can be related by assuming that the permeability constant for influx of HCO_3^- and for the efflux of HCO_3^- are the same. The uncatalyzed rate constant, k', can be easily determined by experiments carried out in the absence of any cells.

These considerations leave two unknowns, k'_c and k'_e, which can be determined, using the equations derived in the previous section, by obtaining two knowns from the rate of depletion of ^{18}O in the HCO_3^- of the external medium. These two knowns are the rate of depletion of the total ^{18}O content of the bicarbonate (the quantity τ') and the rate of depletion of ^{18}O from bicarbonate species which are doubly or triply labeled (the quantity c').

The fact that these two rates give independent information concerning the processes of Fig. 1 is the cardinal point in this technique. This independence arises because chemical reactions, such as in Eqs. 1 and 2, can deplete labeled bicarbonate only one label at a time. As a result, the rate of isotopic depletion of labeled bicarbonate depends on whether one observes bicarbonate which is singly or multiply labeled (2). However the diffusion process by which bicarbonate passes from the extracellular environment to the intracellular environment (or vice versa) cannot distinguish singly, doubly, or triply labeled bicarbonate and passes each through the membrane with equal facility. In the cell depletion is hundreds of times faster than in the external medium, and the bicarbonate passing out of the cell is greatly depleted of ^{18}O. Thus the isotopic depletion of bicarbonate in the external solution occurs by a combination of two processes, one dependent (the chemical mechanism) and one independent (the diffusion mechanism) of the degree to which each bicarbonate ion is labeled. How these two processes are measured to obtain k'_c and k'_e from experimental data is the main purpose of this report.

Isotope depletion in suspension of red cells. In order for this method to be applicable in regions of more physiological pH and to be applicable to red cells, the flux of labeled CO_2 across the cell membrane must be accounted for. This is particularly important for red cells, since the permeability of the red cell membrane to CO_2 is very large, so large that it has not yet been measured experimentally. Forster (6) estimated this permeability to be 7.5×10^{-3} cm/sec or 0.58 cm/sec, depending on whether CO_2 moves through membrane pores or passes through the membrane in a way dependent on its lipid solubility. These values can be contrasted with the permeability of the red cell membrane to water, 5.3×10^{-3} cm/sec (7), and to bicarbonate, about 10^{-4} cm/sec (8–10).

Labeled CO_2 generated by Eq. 1 in the suspending solution diffuses into the red cell membrane. Its subsequent catalyzed depletion in the cell contributes to the over-all depletion measured experimentally. This factor was neglected in previous derivations by assuming very alkaline pH and very small CO_2 concentrations. However, this contribution can be calculated by considering the rate of formation of labeled CO_2 in alkaline solution, which is slow compared to the diffusion of HCO_3^- into the cell. This is done below, using approximate permeability constants and rate constants at pH 8.

The experiment is begun by dissolving labeled bicarbonate in a suspension of red cells. The labeled bicarbonate in the suspending solution can be depleted of ^{18}O by two processes, both shown in Fig. 1: diffusion of labeled bicarbonate across the cell membrane, followed by very rapid depletion in the carbonic anhydrase-containing intracellular solution (process A), and chemical reaction of labeled bicarbonate in the extracellular solution, forming labeled CO_2 which

rapidly diffuses into the cell, where it is depleted to a certain extent and may diffuse back into extracellular solution (process B). The rate constant for process A is determined by the rate-limiting step, the diffusion of labeled HCO_3^- into the cell. The diffusion rate constant $k'_e = (P_{HCO_3^-})A/V_1$. Approximating $P_{HCO_3^-}$ by 10^{-4} cm/sec and $A/V_1 = 133$ cm^{-1}, a typical value for the red cell experiments, $k'_e = 1.3 \times 10^{-2}$ sec^{-1}, equivalent to a half-time for diffusion of 53 sec.

The rate constant for process B is determined by the rate-limiting step, the rate of disappearance of ^{18}O from HCO_3^- in forming labeled CO_2. The rate of disappearance of ^{18}O from HCO_3^- in process B is written

$$\frac{-3}{f}[HCO_3^-]\frac{d\tau'_1}{dt} = k'[HC^{16}O^{16}O^{18}O^-] + 2k'[HC^{16}O^{18}O^{18}O^-] + 3k'[HC^{18}O^{18}O^{18}O^-]$$

$$-\frac{d\tau'_1}{dt} = fk'\tau'_1 \quad (29)$$

Here the back-reaction of labeled CO_2 to form labeled HCO_3^- ($k = 3.85 \times 10^{-2}$ sec^{-1} for uncatalyzed hydration at pH 8) is neglected because of the rapid rate of CO_2 diffusion into the cell ($k_e = 13$ sec^{-1} using $P_{CO_2} = 10^{-1}$ cm/sec and $A/V_1 = 133$ cm^{-1}).

Consequently the rate constant for process B is approximated by $fk' = 7.4 \times 10^{-4}$ sec^{-1} at pH 8, giving a half-time for HCO_3^- depletion by process B of 16 min. This is to be compared with the rate and half-time of depletion by process A (1.3×10^{-2} sec^{-1} and 53 sec).

It is concluded that, under the conditions stated above, the contribution to the total rate of depletion by labeled CO_2 diffusion into the cell is small compared to the contribution of labeled HCO_3^- diffusion into the cell. Furthermore, the contribution to total depletion caused by diffusion of labeled CO_2 into the cell is approximated by fk'. This approximation is checked experimentally by using a passive anion diffusion inhibitor which blocks process A, leaving a total depletion rate constant of fk' due to process B (see RESULTS AND DISCUSSION). The magnitude of k' will be increased by the presence of any carbonic anhydrase in the extracellular solution arising as a result of red cell lysis.

These considerations modify only slightly the general approach taken earlier. The depletion of ^{18}O from bicarbonate in the extracellular solution is measured as before. Because of the rapid diffusion of labeled CO_2 into the red cell, followed by ^{18}O depletion, the back-reaction of labeled CO_2 to form labeled HCO_3^- is neglected in the suspending solution. Consequently, for red cell suspensions, in place of Eq. 18 we write

$$-\frac{d\tau'_1}{dt} = fk'\tau'_1 + k'_e(\tau'_1 - \tau'_2) \quad (30)$$

and in place of Eq. 22 we write

$$-\frac{dc'_1}{dt} = fk'c'_1 + k'_e(c'_1 - c'_2) \quad (31)$$

These equations are valid even if there is no chemical equilibrium in the extracellular solution.

Labeled CO_2 generated by Eq. 1 in the intracellular solution is now considered. The very large carbonic anhydrase content of the red cell favors back-reaction by Eq. 2 inside the cell rather than outside the cell. That is, diffusion of labeled CO_2 across the cell membrane occurs perhaps many times, but Eq. 2 occurs inside the cell. The differential equations for τ'_2 and c'_2 are written as Eqs. 19 and 23. This implies intracellular chemical equilibrium between CO_2 and HCO_3^-. Such an assumption is less firm than the assumptions leading to Eqs. 30 and 31. It is subject to experimental verification by comparing the value of k'_c obtained using these equations with the value of k'_c expected on the basis of the known carbonic anhydrase ac-

tivity in red cells (see RESULTS AND DISCUSSION).

The alternative assumption—that a significant fraction of labeled CO_2 formed intracellularly by Eq. 1 diffuses out of the red cell and remains in the suspending solution—is not compatible with Eqs. 19 and 23. The incorrect use of Eqs. 19 and 23 for this alternative assumption introduces an error into the determination of k'_c and does not permit the separation of CO_2 and HCO_3^- flux in the calculation of the permeability of the membrane to bicarbonate. Furthermore, accumulation of labeled CO_2 in the suspending medium, accompanied of course by accumulation of unlabeled CO_2, would lower the pH of the suspending solution, an effect not detected in the red cell experiments.

Equations 30, 31, 19, and 23 also lead to Eqs. 27 and 28, with the exception that in this case $\theta = \theta_1 - k'f$ and $\gamma = \gamma_1 - k'f$.

EXPERIMENTAL PROCEDURE

Preparation of labeled bicarbonate. ^{18}O-labeled potassium bicarbonate–potassium carbonate mixtures were prepared by dissolving these compounds in ^{18}O-enriched water (normalized, up to 60 atom % ^{18}O) obtained from Miles Laboratories. The molar ratio of carbonate to bicarbonate for this mixture was 0.34 when the mixture was to be used in isotonic solutions at pH 9.6; the ratio was 0.16 when the mixture was to be used in isotonic solution at pH 9.1; and pure potassium bicarbonate was enriched for use in isotonic solutions at pH 7.7. These compounds were allowed to react with $H_2{}^{18}O$ for 12 hr, following which the water was distilled off.

^{13}C-Enriched carbonate-bicarbonate mixture was prepared by the following procedure. Enriched CO_2 was released by the addition of acid to 1 mmole of ^{13}C-enriched $BaCO_3$ (90 atom % ^{13}C) obtained from Merck Sharp & Dohme. The CO_2 was absorbed in 1 ml of 1.0 N KOH, following which the water was removed by distillation.

Preparation of red cells. Erythrocytes were obtained from defibrinated rat blood (rats were female, Wistar strain) and washed twice at room temperature with the following solutions, referred to as the diluting media. For isotopic depletion experiments at pH 9.6 the following diluting medium was used: 22.5 mM $KHCO_3$, 7.5 mM K_2CO_3, 114 mM NaCl, and 5 mM glucose.

Experiments described in Table 4 were performed with and without sodium picrate; small amounts of sodium chloride were replaced by sodium picrate in such a way as to keep the osmolality of the solutions constant. For experiments at pH 9.1 and 7.7 the diluting medium contained (unless stated otherwise) 30 mM $KHCO_3$, 20 mM glycylglycine, 5 mM glucose, sufficient NaOH to adjust to the desired pH, and NaCl (about 94 mM) to bring the solution to 300 mOsM. In addition, each dilution medium contained 0.05% (w/v) albumin.

After the last washing, red cells were packed by centrifugation, and a measured volume (0.2 to 0.6 ml) of packed cells was resuspended in the appropriate diluting medium. The ratio of the volume of the diluting medium to the volume of cells, V_1/V_2, was carefully measured. This ratio varied from about 200 to 1500. Erythrocytes remained in this suspension for no longer than 2 hr until experiments were completed.

Performing exchange reaction. The exchange reactions were performed in a gastight 5.0-ml glass syringe fitted with a water circulation jacket to provide constant temperature (25.0° ± 0.1°, maintained with a Haake type F constant temperature circulator). With this apparatus the reaction solution was not in contact with any gas phase from which CO_2 could be absorbed. At zero time 1.0 ml of red cells suspended in diluting medium and 3.5 ml of ^{18}O-enriched diluting medium were mixed in the syringe. The contents of this reaction vessel were constantly stirred with a magnetic stirrer. The composition of the enriched diluting medium was identical with that of the diluting medium, with the exception that the en-

riched diluting medium was prepared with ^{18}O-labeled bicarbonate and carbonate. Consequently the chemical equilibrium established across the red cell membrane with the diluting medium was not disturbed by addition of the enriched diluting medium. Small adjustments of the pH of the diluting medium and enriched diluting medium were made immediately before each run, using a microsyringe and 0.1 N KOH or 0.1 N HCl.

Aliquots of this reaction mixture were taken at intervals of 1–5 min. These aliquots, approximately 0.5 ml in volume, were injected from the reaction vessel by a syringe needle through a serum stopper into an evacuated flask containing sulfuric acid (9 M). This immediately stopped the slow depletion of ^{18}O from bicarbonate by liberating CO_2 from the bicarbonate. This CO_2 was passed through a trap immersed in a Dry Ice–acetone bath to remove water vapor and was collected in sample vials.

At the end of each kinetic run the pH of the contents of the reaction vessel was measured.

Measuring isotopic enrichments. Isotopic ratios were measured on a Dupont CEC 21-491 or a Finnigan 3000 mass spectrometer at an ionizing voltage of 70 eV. Argon abundance (m/e 40) was monitored in each mass spectral scan to detect possible CO_2 contamination by inadvertent introduction of air during the experiments. The standard deviations for a minimum of five scans in the measurement of isotope ratios were less than 1.5% for ^{18}O enrichment greater than 1% and less than 2.5% for enrichments less than 1%.

At alkaline pH there is a negligibly small concentration of CO_2 in the reaction solution. As a result, the atom fraction of ^{18}O in the CO_2 liberated by acid is taken as equal to the atom fraction τ' of ^{18}O in the bicarbonate of the reaction solution. The ^{18}O enrichment in the CO_2 samples was determined by the following formula:

$$\tau' = \frac{\frac{1}{2}(46) + (48)}{(44) + (46) + (48)}$$

where (44), (46), and (48) are the heights of the corresponding mass peaks. Similarly, the atom fraction of ^{18}O in CO_2 containing a double oxygen label can be taken as equal to the atom fraction c' of bicarbonate. The isotope ratio c' in the CO_2 samples was determined by the following formula:

$$c' = \frac{(48)}{(44) + (46) + (48)}$$

Finally, in taking aliquots of the reaction mixture, red cells were sampled along with the suspending solution. As a result, the CO_2 liberated from HCO_3^- in each aliquot by treatment with acid contained CO_2 originating from the intracellular as well as the extracellular bicarbonate. Since V_2/V_1 is of the order of 10^{-3}–10^{-2}, and since the fraction of the CO_2 liberated by acid and originating from the intracellular solution is a comparable number, the error introduced by not separating red cells from the suspending solution in each aliquot is quite negligible.

RESULTS AND DISCUSSION

Homogeneous solutions. Experiments are described here to demonstrate that the rate of depletion of the total enrichment of bicarbonate, as shown in Eq. 10, is half the rate of depletion of the isotope enrichment of multiply labeled bicarbonate, as shown in Eq. 15, in homogeneous solutions. That is, it will be demonstrated that $\gamma/\theta = 2.0$ for homogeneous solutions.

Figure 2 shows the data obtained for the uncatalyzed depletion of ^{18}O from bicarbonate at pH 9.1 and 25° with a total carbonate and bicarbonate concentration of 108 mM. According to Eqs. 10 and 15, the rate constant for the uncatalyzed dehydration of bicarbonate can be determined from the slopes of Fig. 2. [This rate constant is, for example, the slope of the plot of log $(\tau' - \tau'_\infty)$ vs. time multiplied by $-3(2.303)/f$; f is determined to be 0.945 at pH 9.1, using a pK_a of 10.33 for bicarbonate.] The uncatalyzed rate constant for the dehydration $k' = (2.23 \pm 0.10) \times 10^{-4}$ sec^{-1}

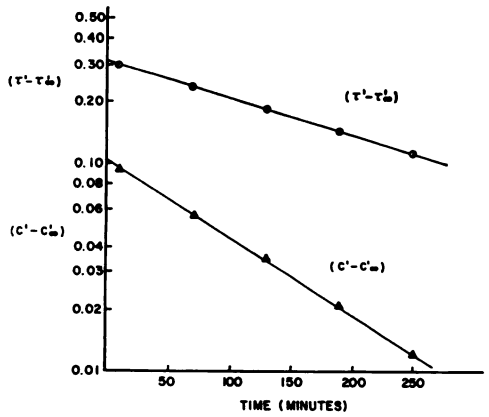

FIG. 2. *Rate of depletion of ^{18}O from labeled bicarbonate resulting from uncatalyzed dehydration*

The exchange was observed at pH 9.1 and 25° at a concentration of 102 mM $KHCO_3$ and 6 mM K_2CO_3. The reaction solution also contained 32 mM NaCl and 5 mM glucose.

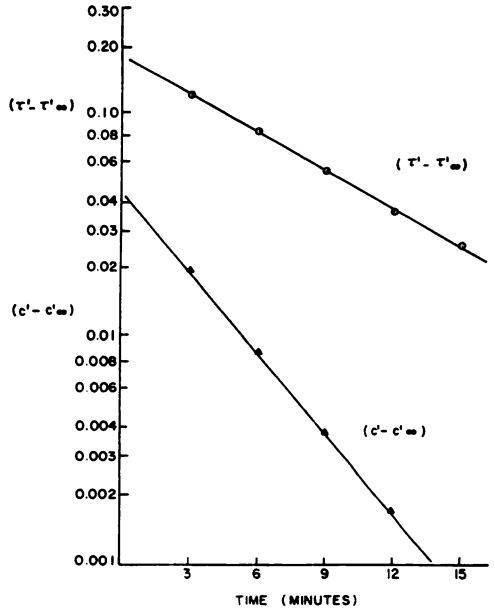

FIG. 3. *Rate of depletion of ^{18}O from labeled bicarbonate resulting from catalyzed dehydration*

This exchange was observed at pH 9.1 and 25°. The composition of the reaction solution was the same as in Fig. 2, except that lysed rat erythrocytes were added to the solution. Before lysis V_1/V_2 was 1.6×10^3.

at pH 9.1 and 25° is obtained from four runs similar to that of Fig. 2. This is to be compared with the value $k' = 2.34 \times 10^{-4}$ sec^{-1} calculated from the rate constants of Gibbons and Edsall (11). At pH 9.6 the slopes of plots similar to Fig. 2 give $k' = (2.00 \pm 0.08) \times 10^{-4}$ sec^{-1}. The rate constants of Gibbons and Edsall (11) at this pH yield $k' = 2.04 \times 10^{-4}$ sec^{-1}.

The ratio of the slope of the log $(c' - c'_\infty)$ plot to the slope of the log $(\tau' - \tau'_\infty)$ plot of Fig. 2 gives $\gamma/\theta = 2.02$. From four similar runs the value, including standard deviation, is 2.04 ± 0.03.

To find γ/θ in a homogeneous solution containing carbonic anhydrase, 0.25 ml of packed rat erythrocytes was diluted in 50 ml of distilled water, causing the cells to lyse. Then K_2CO_3, $KHCO_3$, glucose, and NaCl were added to give the solution the same composition as the diluting medium used for the uncatalyzed runs at pH 9.1. The isotope exchange reaction was carried out as described earlier (Fig. 3). The slope θ, taken directly from Fig. 3, is 9.45×10^{-4} sec^{-1}, and the slope γ is 1.91×10^{-3} sec^{-1}, yielding a ratio $\gamma/\theta = 2.02$. This result was easily reproducible, three runs yielding 2.01 ± 0.04.

Red cell suspensions. To find γ/θ in a suspension of intact rat erythrocytes, an experiment similar in detail to that described in the previous paragraph was performed. In this run, however, 0.25 ml of packed rat erythrocytes was diluted directly in 50 ml of the same diluting medium used for the experiments in homogeneous solution at pH 9.1, a solution isotonic with the cells. The isotopic exchange was then performed as described earlier, and the results are shown in Fig. 4. The slope θ_1, taken directly from Fig. 4, is 5.28×10^{-4} sec^{-1}, and the slope γ_1 is 9.51×10^{-4} sec^{-1}, giving $\gamma_1/\theta_1 = 1.80$. Three of these runs, all under identical conditions and dilutions and using erythrocytes from one rat, gave $\gamma_1/\theta_1 = 1.81 \pm 0.01$.

The equations, Eqs. 10 and 15, describing depletion in homogeneous solutions allow only $\gamma/\theta = 2.0$ and cannot explain the above result using intact red cells. However, Eqs.

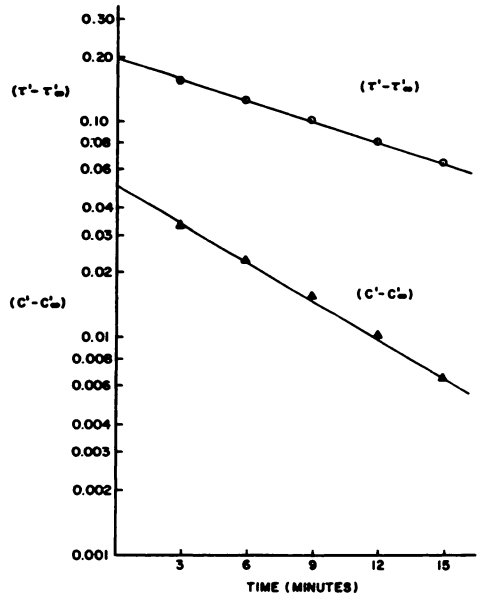

FIG. 4. *Rate of depletion of ^{18}O from labeled bicarbonate resulting from catalyzed dehydration*

This is identical with the experiment of Fig. 3 except that cells were not lysed. V_1/V_2 was 1.6×10^3.

21 and 25, which allow for diffusion as well as depletion in two aqueous solutions separated by a membrane, introduce factors k'_e and k'_i into θ and γ. As a result, γ/θ can be less than 2.0 in such media, the value of γ/θ less than 2.0 being a consequence of the diffusion of bicarbonate.

Equation 27 shows that γ/θ also approaches 2.0 for the case of very rapid diffusion, that is, when k'_e is very large. For no diffusion, as in the case of an impermeable membrane, the rate of depletion of labeled bicarbonate is the same in the presence and absence of cells. The ratio γ/θ also enters in the expression for k'_c, the rate constant for intracellular bicarbonate dehydration, as shown in Eq. 28. For very rapid diffusion of bicarbonate across the membrane, that is, when γ/θ equals 2.0, $k'_c = (3/2)(V_1/V_2)\gamma$. Substituting 2θ for γ results in $\theta = (k'_c/3)(V_2/V_1)$, and the catalyzed rate yields a slope θ as if carbonic anhydrase were spread homogeneously throughout the entire solution. In the case of no diffusion across the membrane, k'_c cannot be measured by the isotope exchange method described here.

TABLE 1

Rates of depletion of ^{18}O from HCO_3^- in suspensions of rat erythrocytes at 25°

Rat erythrocytes were suspended in diluting medium, and the ^{18}O depletion experiment was performed. θ_1 is the rate obtained directly from a plot of $\ln(\tau' - \tau'_\infty)$ vs. time. k' is the rate constant for dehydration in the suspending medium. It was obtained by centrifuging the suspension of red cells and performing the ^{18}O experiment on the resulting supernatant solution. f is the fraction of all carbonate species existing as bicarbonate.

pH	V_1/V_2	θ_1, no picrate	θ_1, 3.5 mM picrate	fk', supernatant	k'_{uncat}
		$sec^{-1} \times 10$		$sec^{-1} \times 10^3$	
9.6	180	4.72	0.93	0.92	0.20
8.5	1215	1.54	0.50	0.48	0.36
7.8	1485	5.58	2.52	2.26	1.06
7.7	1500	5.09	2.58	2.21	1.29
7.6	1485	5.85	3.12	2.90	1.57
7.5	1485	6.30	3.82	3.65	1.93

The kinetic significance of k'_c, a rate constant describing an enzyme-catalyzed reaction at equilibrium, is most adequately explained by Boyer (12). The kinetic expressions derived by Boyer (12) for the case of one enzyme-substrate intermediate have been applied to the carbonic anhydrase-catalyzed ^{18}O exchange between bicarbonate and water (4). Since the isotope exchange occurs at chemical equilibrium, in general cases k'_c is not simply related to the parameters of the Michaelis-Menten equation.

The application of Eqs. 27 and 28 to the rates of ^{18}O depletion in suspensions of red cells is now described. Experiments were performed in a pH range from 7.7 (CO_2 = 4.5%; CO_3^{2-} = 0.2%, HCO_3^- = 95.3%) to pH 9.6 (CO_2 = 0.05%, CO_3^{2-} = 16%, HCO_3^- = 84%). Table 1 shows the results of several experiments in this pH range, using rat erythrocytes. The values of θ_1 are depletion rates obtained directly from plots of $\ln(\tau' - \tau'_\infty)$ vs. time. k' is the rate constant in the extracellular solution, as shown in Fig. 1, and was obtained by centrifuging each red cell suspension and performing the

^{18}O experiment on the resulting supernatant fraction. As can be seen from Table 1, the values of fk' are larger than k'_{uncat}, the rate constant for the uncatalyzed dehydration of bicarbonate at 25°. This situation results from the presence of carbonic anhydrase in the external solution and is caused by the lysis of a small number of red cells during the experiment. The purpose of Table 1 is to verify Eq. 29, which approximates the contribution to the over-all rate of depletion caused by the flux of labeled CO_2 into the red cell membrane. Table 1 shows that in the presence of sufficient picrate to block passive anion exchange across the membrane (13) the rate of depletion remaining (θ_1 in the presence of 3.5 mM picrate) is about equal to fk', as predicted by Eq. 29. According to Table 4, inhibition of the depletion rate is maximal at 3.5 mM picrate. It is possible that picrate also inhibits CO_2 flux across the membrane, but the agreement between θ_1 in the presence of picrate and fk' in Table 1 is evidence that inhibition of CO_2 flux is not great. Also, at the concentrations used in this study, picrate does not inhibit carbonic anhydrase.

The results of one particular experiment at pH 9.6 on rat erythrocytes are presented in Table 2 and will be used to demonstrate the calculation of membrane permeability to bicarbonate. The rates of depletion by the bicarbonate diffusion pathway, θ and γ, are obtained by subtracting from the total rates the value fk', which approximates the rate of depletion by the CO_2 diffusion pathway in which the dehydration of bicarbonate is the rate-limiting step. Substituting the values of θ and γ from Table 2 into Eq. 27 gives $k'_e = 2.27 \times 10^{-2}$ sec^{-1}.

The permeability constant P is determined from k'_e by first relating k'_e to k'_i (see Eqs. 18 and 19), $k'_i = (V_1/V_2)k'_e$. Then P is related to k'_i, $P = k'_i V_2/A$. It follows that

$$P = \left(\frac{V_1}{V_2}\right) k'_e \left(\frac{V_2}{A}\right)$$

In this way the terms V_2 cancel in the calculation of P. This is a great advantage since it precludes complications caused by the fact that not all of the intracellular contents of the erythrocytes are available as solvent for bicarbonate. No error is introduced by considering V_2 to be simply the volume of the rat erythrocytes.

A/V_2 was taken to be 2.88×10^4 cm^{-1}. [Wintrobe *et al.* (14) reported the volume of the rat erythrocyte to be 41 μm^3. Ponder (15) reported the diameter of the rat erythrocyte to be 7.5 μm. The area of the cell can then be determined to be 118 μm^2 by

TABLE 2

Depletion rates in suspension of rat erythrocytes at pH 9.6 and 25°

Erythrocytes, all obtained from the same rat, were suspended ($V_1/V_2 = 225$) in diluting medium, and the ^{18}O exchange experiment was performed. θ_1 and γ_1 are rates obtained directly from plots of $\ln(\tau' - \tau'_\infty)$ and $\ln(c' - c'_\infty)$ vs. time. k' is the rate constant for dehydration in the suspending medium (see Fig. 1). It was obtained by centrifuging the suspension of red cells and performing the ^{18}O experiment on the resulting supernatant solution. f is 0.84 at pH 9.6.

Erythrocytes in diluting medium	$\theta_1 = 4.56 \times 10^{-3}$ sec^{-1}
	$\gamma_1 = 7.22 \times 10^{-3}$ sec^{-1}
	$fk' = 0.84 \times 10^{-3}$ sec^{-1}
Erythrocytes in diluting medium containing 3.5 mM picrate	$\theta_1 = 0.81 \times 10^{-3}$ sec^{-1}
	$\gamma_1 = 1.05 \times 10^{-3}$ sec^{-1}
	$fk' = 0.80 \times 10^{-3}$ sec^{-1}
Depletion rates by bicarbonate diffusion pathway	$\theta = \theta_1 - fk' = 3.72 \times 10^{-3}$ sec^{-1}
	$\gamma = \gamma_1 - fk' = 6.38 \times 10^{-3}$ sec^{-1}

TABLE 3

Results of isotope depletion measurements in suspensions of rat erythrocytes at pH 9.6 and 25°

Erythrocytes for each run were obtained from a different rat. The factor f at pH 9.6 is 0.84.

V_1/V_2	θ^a	γ/θ	k'_c	k'_e	P
$\times 10^{-2}$	$sec^{-1} \times 10^1$		sec^{-1}	$sec^{-1} \times 10^2$	$cm\ sec^{-1} \times 10^4$
2.25	3.31	1.75	3.11	2.32	1.81
2.25	4.11	1.72	3.93	2.52	1.97
1.98	3.52	1.76	2.88	2.58	1.77
1.80	4.37	1.72	3.36	2.69	1.68

[a] The over-all depletion rate, θ_1, minus the rate constant for depletion in the extracellular solution, fk'.

using Eq. 2.6 from Ponder (16).] The permeability constant calculated from the rates in Table 2 is $P = 1.78 \times 10^{-4}$ cm sec^{-1}. This represents diffusion of both HCO_3^- and CO_3^{2-} through the red cell membrane. However, since the carbonate concentration is small and since doubly charged ions generally diffuse more slowly through the red cell membrane than singly charged ions, this value of P reflects mostly the permeability of the membrane to bicarbonate. The value $k'_c = 3.56\ sec^{-1}$ is obtained from the data in Table 2 and Eq. 28.

Following these procedures, a mean value and standard deviation for P and k'_c at pH 9.6 and 25° are obtained using four experiments, the data from which are presented in Table 3:

$$P = (1.81 \pm 0.12) \times 10^{-4}\ \text{cm/sec}$$

$$k'_c = 3.32 \pm 0.45\ sec^{-1}$$

This value of the permeability constant corresponds to a half-time of 0.13 sec for the exchange of bicarbonate in the rat erythrocyte under the conditions of Table 3. At pH 9.1 and 25°, following the same procedure, $P = 1.98 \times 10^{-4}$ cm/sec and $k'_c = 6.17\ sec^{-1}$. The half-time for bicarbonate exchange in the cell in this case is 0.12 sec. At pH 7.7 and 25°, $P = 4.75 \times 10^{-4}$ cm/sec and $k'_c = 19.8\ sec^{-1}$, giving a half-time for exchange of 0.05 sec.

There are two main reasons why the value of P obtained by this technique increases as pH decreases. The first concerns the pH dependence of the permeability of the membrane to anions. According to the pore theory as described by Passow (17), charged channels lined with charged amino groups, for example, are present in the membrane. One can also postulate a charged, membrane-bound carrier molecule. At high pH, fewer of these amino groups are charged, decreasing the attractive forces between the channel or carrier and the anions. It is pertinent to note that the values of pH of our measurements are in the region of the pK of the charged amino groups.

The second reason arises from an error present at pH 7.7 which we have not corrected for. This error is caused by an intermolecular exchange of oxygen atoms between CO_2 and CO_3^{2-} not involving an oxygen from water (18). The effect of this exchange is to increase γ and hence cause our calculated values of P at pH 7.7 to be too large. The magnitude of the error is unknown, since we cannot, at this time, separate the various contributions to the rate γ. The presence of this exchange was confirmed in our experiments at pH 7.7 by introducing into the red cell suspensions a small amount of ^{13}C-enriched $KHCO_3$ that was not enriched in ^{18}O and ^{18}O-enriched $KHCO_3$ that was not enriched with ^{13}C. The appearance in the mass spectrum of a $^{13}C^{16}O^{18}O$ peak is indicative of an intermolecular oxygen exchange. One possible way to write this exchange is (18)

$$^{13}C^{16}O^{16}O + {}^{12}C^{16}O^{16}O^{18}O^{2-} \rightleftarrows {}^{13}C^{16}O^{18}O + {}^{12}C^{16}O^{16}O^{16}O^{2-} \quad (32)$$

Such an exchange was not observed in the red cell experiments at pH 9.6 and was observed to a negligibly small degree at pH 9.1, provided that the ratio V_1/V_2 was not small.

The above values of the half-time for bicarbonate exchange in the rat erythrocyte

can be compared with several measurements using different techniques. Dirken and Mook (8) obtained a value of 0.16 sec near pH 7 and room temperature for ox red cells, using a rapid flow technique. Also using rapid flow techniques, Piiper (9) measured the net influx and efflux of total CO_2 at 37° through red cell membranes in which the CO_2 exchange was mainly an exchange of bicarbonate ions. The half-time averaged 0.04 sec for cow, pig, and dog erythrocytes. Luckner (10) reported a half-time of 0.11 sec at 37°, using an electrochemical technique to measure the CO_2-chloride exchange in human, cow, and pig erythrocytes.

At pH 9.6 and 25° the ratio of the rate constant for catalyzed dehydration at equilibrium to the uncatalyzed rate constant for dehydration is 16,500 (k'_c/k'_{uncat} = 3.3 $sec^{-1}/2.0 \times 10^{-4}$ sec^{-1}). At pH 7.7 this ratio is 15,400 (k'_c/k'_{uncat} = 19.8 $sec^{-1}/1.28 \times 10^{-3}$ sec^{-1}).[1] These ratios for rat erythrocytes at 25° can be compared with the estimated value of 13,000 reported by Kernohan *et al.* (19) for the ratio of the catalyzed to uncatalyzed rates of CO_2 formation in human red cells at 37°. The carbonic anhydrase activity of rat red cells (2400 enzyme units/g) is known to be greater than that of human red cells [1470 enzyme units/g (ref. 20, pp. 670, 676)]. Although in rough agreement, these ratios cannot be compared rigorously. As stated previously, the use of Eqs. 19 and 23 must be verified experimentally by, for example, comparing the value of k'_c obtained by this ^{18}O method with the value of k'_c obtained independently. Hence the validity of Eqs. 19 and 23 for ^{18}O depletion in red cells can be established only roughly in this work, by comparison of our value of intracellular rate enhancement with that of Kernohan *et al.* (19). This uncertainty is a potential source of error in the calculation of k'_c by the ^{18}O method and also introduces an error into the separation of CO_2 flux and bicarbonate flux when calculating the permeability constant of the red cell membrane to bicarbonate.

It is pertinent to comment here on the effect of the possible accumulation in red cells of ^{18}O-labeled water, an accumulation which is likely to occur because of the rapidly catalyzed depletion of labeled HCO_3^- and CO_2 in the cell. Using $V_1/V_2 = 225$, a typical value for the experiments at pH 9.6, there is a total of approximately 1×10^{-3} g atom of oxygen in the water of all of the cells in 4.5 ml of red cell suspension. At 30 mM total carbonate with 20% initial enrichment of ^{18}O, there is a total of about 8×10^{-5} g atom of ^{18}O in each experiment. Considering the permeability of the red cell to water (7) and the fact that some depletion occurs outside the red cell, it is unlikely that accumulation of ^{18}O-labeled water presents a sizable error in the calculation of k'_c at pH 9.6. However, in suspensions containing few red cells, accumulation of $H_2{}^{18}O$ in the cell could give a value of k'_c which is too small.

Returning to other experiments at pH 9.6, Table 4 demonstrates that inhibition of depletion by picrate ion is maximal using 3.5 mM sodium picrate in the red cell suspensions.

In placing aliquots of the red cell suspension in acid to retrieve CO_2, it is possible that CO_2 is generated from the organic material of the red cells. If this is the case, the isotopic enrichments resulting from these samples and the ratio γ/θ would be affected. To test whether these isotopic measurements include such an error, experiments using a ^{13}C label were performed. Following the procedures described earlier, packed rat erythrocytes were suspended in diluting medium at pH 9.6. The bicarbonate in the diluting medium was enriched with ^{13}C. Aliquots at several different times were then placed in acid (9 M sulfuric), and the CO_2 liberated was measured for ^{13}C enrichment. The uniformity

[1] The ratio k_c'/k'_{uncat} has approximately the same value at pH 7.5 and 9.5, as determined by ^{18}O depletion studies on homogeneous solutions containing human carbonic anhydrase C. At intermediate values of pH this ratio is higher than at pH 7.5 or 9.5 (C. K. Tu and D. N. Silverman, unpublished observations).

TABLE 4

Results of isotope depletion measurements in suspensions of rat erythrocytes at pH 9.6 and 25°

The anion diffusion inhibitor sodium picrate was used. $V_1/V_2 = 225$. Erythrocytes for each run were obtained from the same rat. The factor f at pH 9.6 is 0.84.

Picrate	θ_1[a]	θ'[b]	γ/θ	k'_c	k'_e	P
mM	$sec^{-1} \times 10^3$	$sec^{-1} \times 10^3$		sec^{-1}	$sec^{-1} \times 10^3$	$cm\ sec^{-1} \times 10^4$
0	4.48	3.31	1.75	3.11	2.32	1.81
0.40	3.59	2.29	1.64	2.36	1.04	0.82
1.56	1.64	0.31	1.58	0.34	0.12	0.09
2.50	1.30	−0.03			0	0
3.50	1.33	0			0	0

[a] Depletion rate obtained directly from a plot of $\ln(\tau' - \tau'_\infty)$ vs. time.

[b] Over-all depletion rate, θ_1, minus the rate constant for depletion in the extracellular solution, fk'.

of the isotope ratios of Table 5 indicates that there is a negligible error in these measurements due to CO_2 originating from organic material in the samples.

This technique requires that carbonic anhydrase be present on one side of the membrane. In order to measure the permeability constant, Eqs. 27 and 28 demonstrate that the magnitude of k'_c should be approximately in the same range as $k'_i = (V_1/V_2)k'_e$. However, there is some experimental flexibility to arrange that k'_c and k'_i be of the same order of magnitude, since carbonic anhydrase can be partially inhibited to reduce the magnitude of k'_c, an option available for studies *in vivo*.

This isotope exchange method offers several advantages for measuring HCO_3^- diffusion through membranes when the rate of exchange is rapid, as in red cells. First, because of the high sensitivity of the mass spectrometer, this technique can be performed with relatively small numbers of cells and small volumes of ^{18}O-enriched bicarbonate solutions. Second, this technique can be used for the measurement of rapid CO_2 and HCO_3^- exchange *in vitro*. Double labeling with ^{13}C and ^{18}O is valuable in such experiments. Measuring ^{18}O depletion only from ^{13}C-containing CO_2 and HCO_3^- prevents errors arising from dilution by unlabeled CO_2 and HCO_3^- in the physiological fluid. Furthermore, such double label measurements would not be affected by the turnover of CO_2 or HCO_3^- by normal metabolic processes. Experimentally, this technique would involve inserting an amount of ^{13}C- and ^{18}O-labeled CO_2 or HCO_3^- into a physiological fluid and periodically taking aliquots of the fluid for measurement by mass spectrometry.

TABLE 5

^{13}C content of carbonate and bicarbonate in suspending medium before and at times after addition of rat erythrocytes

The pH of the suspension was 9.6, and temperature 25°. The composition of the suspending medium is given in the text.

Time	^{13}C content[a]
	%
Before addition	12.03
After addition	
1 min	11.87
10 min	11.81
100 min	11.91

[a] % ^{13}C = (45)/[(44) + (45)] × 100% for CO_2 obtained by treating the suspension with acid.

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