could be as high as 2. The inverse observed effect seems to require that the k_{-1} and k_2 steps have different mechanisms. For example, if the k_2 transition state were of the **1, 3, 4,** or **5** type, while the k_1 transition state had the **2** structure, then the solvent isotope effect on k_2 could be larger than that on k_{-1}, and the observed inverse effect on α could be explained. Perhaps such a dissymmetry in the mechanisms of the two paths open to the intermediate could also account for their observed[7] unsymmetric catalysis.

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Buffer Dependence of Carbonic Anhydrase Catalyzed Oxygen-18 Exchange at Equilibrium

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Abstract: The carbonic anhydrase (human C and bovine) catalyzed rate of exchange of oxygen-18 between bicarbonate and water was measured at chemical equilibrium with and without buffers. An increase of the buffer 1,2-dimethylimidazole (pK_a = 8.3) from 0 to 10 mM was accompanied by an enhancement of up to 80% in the rate of catalyzed oxygen-18 exchange near pH 8.3; this enhancement diminished as pH increased. However, the rate of exchange was not further enhanced by the addition of buffer beyond a 10 mM concentration. Similar effects were observed using tris sulfate. The addition of these buffers had no influence on the uncatalyzed rate of oxygen-18 exchange. Furthermore, 1,3-dimethylimidazolium sulfate and pyrrole, which are not buffers in the pH range studied, did not increase the carbonic anhydrase catalyzed exchange rate. Ionic strength was held constant in all experiments using Na_2SO_4; bicarbonate concentrations were as low as 0.5 mM, and enzyme was 5×10^{-9} M. We interpret these results as evidence for the involvement of a buffer-assisted proton transfer in the hydration–dehydration mechanism of carbonic anhydrase. In the absence of added buffers, the enzymatic rate is determined by alternating hydration–dehydration steps at equilibrium, which do not require proton transfer to the enzyme. In addition, bicarbonate itself may act as a buffer. As the buffer concentration is increased, the exchange data show a change in the rate-determining step. In the region of low buffer concentration, proton transfer is rate limiting and the catalytic exchange rate increases as buffer increases. Near 10 mM buffer, the maximum enzyme activity is reached and further buffer does not affect the catalytic rate.

Carbonic anhydrase (E.C. 4.2.1.1) catalyzes the reversible hydration of carbon dioxide with a very high turnover rate.[1,2] A likely mechanism for the carbonic anhydrase catalyzed hydration and dehydration of CO_2 is given in Figure 1. A similar scheme was thoroughly discussed by Lindskog and Coleman[3] and was found to be in agreement with the observed physicochemical properties of carbonic anhydrase. Of particular pertinence are the experimental observations that the activity of the enzyme depends on the state of ionization of a group in the active site with a pK_a near 7, that

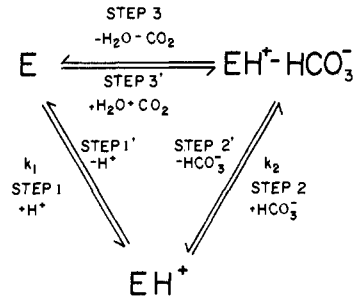

Figure 1. A possible mechanism for carbonic anhydrase showing that the basic form of the enzyme is active in the hydration of CO_2 and the acidic form is active in the dehydration of bicarbonate.

the acid form is active in the dehydration of HCO_3^-, and that the basic form is active in the hydration of CO_2.[4] According to such a scheme, the catalyzed dehydration measured far from equilibrium can proceed only as fast as the enzyme can be protonated. This is a problem because the observed turnover rate for dehydration ($\sim 10^5$ sec^{-1}) is greater than the rate at which solvent water can protonate the active site ($\sim 10^2$-10^3 sec^{-1}) and has led to the hypothesis of a buffer-mediated proton transfer to the enzyme.[3,5,6] That is, it is postulated that a buffer donates a proton to the active site and that this acid form of the enzyme is active in the dehydration of HCO_3^-. Another hypothesis attempts to explain this very efficient catalysis by identifying H_2CO_3 as substrate in the catalyzed dehydration.[7] The problem of the diffusion limit of H_2CO_3, which arises in this case, is dealt with by assuming a large reaction distance for the catalyzed process with diffusion of substrate over the surface of the enzyme to the active site.

To assess the buffer dependence of the catalyzed reaction, we have measured the dehydration activity of carbonic anhydrase using oxygen-18 exchange techniques under equilibrium conditions. This study was suggested by Khalifah[5] as one which can be carried out with relatively small substrate concentrations and no buffer. Another approach to this problem has been taken elsewhere: the measurement under equilibrium conditions of the carbon-13 NMR line widths of CO_2 and HCO_3^- in the presence of carbonic anhydrase.[8] The goal of such investigations is to lower the external buffer concentrations to a point where proton transfer from the buffer species to the active site of the enzyme becomes kinetically significant. This goal is complicated by the fact that the substrate itself, bicarbonate, has the ability to donate protons.

Methods

Kinetic Method. The measured variable in this technique is the rate of depletion of oxygen-18 from labeled bicarbonate. Such depletion can occur in a catalyzed or uncatalyzed process by the dehydration of labeled bicarbonate in water followed by hydration of the resulting carbon dioxide. Equations 1 and 2 demonstrate the uncatalyzed depletion for the case of doubly labeled bicarbonate in alkaline solution. If ^{18}O is released in the hydroxide ion, as shown in eq 1, it is essentially infinitely diluted by $^{16}OH^-$. Thus, eq 2 occurs with unlabeled hydroxide and yields ^{18}O-depleted bicarbonate. For simplicity and convenience, this depletion is measured at chemical equilibrium. If carbonic anhydrase is present in solution, the rate of depletion of oxygen-18 from bicarbonate is enhanced since the reactions represented by eq 1 and 2 are catalyzed by this enzyme.

$$HC^{16}O^{18}O^{18}O^- \longrightarrow C^{16}O^{18}O + {}^{18}OH^- \quad (1)$$

$$C^{16}O^{18}O + {}^{16}OH^- \longrightarrow HC^{16}O^{16}O^{18}O^- \quad (2)$$

The rate equations describing the depletion of ^{18}O from bicarbonate have been presented in complete form by Mills and Urey[9] and also by Gerster.[10] The complete approach considers the two mechanisms for the uncatalyzed dehydration[11] and the depletion of singly, doubly, and triply labeled bicarbonate. Consistent with the notation of previous work,[9,12] the atom fraction of oxygen-18 in bicarbonate is γ as defined by eq 3. The atom fraction of oxygen-18 in carbon dioxide, α, is defined by eq 4. The kinetics of depletion

$$\gamma = \frac{[HC^{16}O^{16}O^{18}O^-] + 2[HC^{16}O^{18}O^{18}O^-] + 3[HC^{18}O^{18}O^{18}O^-]}{3[HCO_3^-]} \quad (3)$$

$$\alpha = \frac{[C^{16}O^{18}O] + 2[C^{18}O^{18}O]}{2[CO_2]} \quad (4)$$

of ^{18}O from HCO_3^- and CO_2 is then measured by observing the decay $d(\gamma - \gamma_\infty)/dt$ and $d(\alpha - \alpha_\infty)/dt$, where γ_∞ and α_∞ are the atom fractions at infinite time and are close to the natural abundance of ^{18}O which is 0.002. For conciseness in the derivation, we write γ and α instead of $\gamma - \gamma_\infty$ and $\alpha - \alpha_\infty$ and reintroduce γ_∞ and α_∞ in the final result.

The rate of change of γ and α can then be expressed by eq 5 and 6 as shown by Mills and Urey.[9] R is the rate of de-

$$-3[HCO_3^-]\frac{d\gamma}{dt} = R(3\gamma - 2\alpha) \quad (5)$$

$$-2[CO_2]\frac{d\alpha}{dt} = R(2\alpha - 2\gamma) \quad (6)$$

hydration of HCO_3^- at equilibrium, which is equal to the rate of hydration of CO_2 at equilibrium. The solution of these equations for γ results in eq 7 where θ_1 is defined by eq 8. The second term on the right in eq 7 becomes negligible almost immediately after the isotope exchange begins.[9]

$$\gamma = a_1 e^{-\theta_1 t} + a_2 e^{-\theta_2 t} \quad (7)$$

$$\theta_1 = \frac{R}{3[HCO_3^-]}\left[\frac{[HCO_3^-]}{[HCO_3^-] + [CO_2]}\right] = \frac{fR}{3[HCO_3^-]} \quad (8)$$

The ratio in brackets is f. 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 6 yields an expression identical with eq 8 but with

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

Consequently, a plot of $\ln(\gamma - \gamma_\infty)$ vs. time for an uncatalyzed ^{18}O exchange yields $\theta_1 = fk_{uncat}/3$, where $k_{uncat} = R/[HCO_3^-]$. In the presence of carbonic anhydrase

$$\theta_1 = \frac{f}{3}(k_c + k_{uncat}) \quad (9)$$

where $k_c = (R/[HCO_3^-]) - k_{uncat}$. An interpretation of that part of R which represents the catalyzed process is given by Boyer.[13] It is the rate of the catalyzed dehydration (and hydration) at equilibrium and is dependent on the total enzyme concentration as well as on the kinetic and equilibrium constants of the enzyme-substrate system in a way that is determined by the number of enzyme-substrate intermediates in the catalytic mechanism. In the present work, the rate constant k_c is taken as a qualitative indication of enzyme activity, a procedure which does not require

that assumptions be made concerning the number of intermediates in the mechanism.

Materials. An oxygen-18 enriched mixture of potassium carbonate and potassium bicarbonate was prepared by dissolving these compounds in ^{18}O-enriched water (Miles Laboratories, up to 60 atom %, normalized). After 12 hr the water was removed by vacuum distillation.

Human carbonic anhydrase C (HCA-C) was prepared by the method of Armstrong[14] and stored in a saturated solution of ammonium sulfate at 3°. Before each run, enzyme was prepared by passing a small aliquot (about 5 ml) of the enzyme-$(NH_4)_2SO_4$ solution through a column (80 by 2.5 cm in diameter) containing Sephadex G-25. Enzyme was then used immediately in the ^{18}O-exchange runs. Bovine carbonic anhydrase (BCA) was obtained in lyophilized form from Worthington Biochemical Corp. and before each experiment was handled by the above procedure. Tris(hydroxymethyl)aminomethane sulfate (tris sulfate, Sigma) and sodium barbital (Merck) were obtained commercially and used without further purification as was imidazole (Eastman). 1,2-Dimethylimidazole (Aldrich) was purified by vacuum distillation; pyrrole was distilled. 1,3-Dimethylimidazolium iodide was prepared by the method of Overberger et al.[15] and recrystallized from ethyl acetate. 1,3-Dimethylimidazolium sulfate was prepared by passing the iodide through an ion-exchange resin pretreated with H_2SO_4.

Procedure. The experimental procedure and apparatus were similar to that described earlier.[12] The purified enzyme solution was diluted to the appropriate activity and divided into four to six portions; each was used in a different experiment within a 3-4-hr period. Carbonic anhydrase activity was measured by the changing pH method of Maren[16] in the procedure and was determined to be constant during the course of each series of experiments.

Approximately 15 ml of a buffered or unbuffered solution of carbonic anhydrase was placed in a flask containing the enriched carbonate-bicarbonate mixture. Ionic strength was maintained at 0.20 M by the addition of the noninhibitory Na_2SO_4. The pH was set roughly by the molar ratio of carbonate to bicarbonate in the enriched mixture. Fine adjustments in the pH were made with two microsyringes using either 0.05 N NaOH or 0.05 N H_2SO_4. This solution was then injected into the reaction vessel, a gas-tight, glass syringe fitted with a water circulation jacket to provide constant temperature (25.0 ± 0.05° maintained with a Haake Model FE constant temperature circulator).

Aliquots (2.5 ml) were removed at intervals and injected into an evacuated flask containing 9 M sulfuric acid. The CO_2 liberated by this procedure was passed through a trap immersed in a Dry Ice-acetone bath to remove water vapor and then collected in vials. Each CO_2 sample was then analyzed for ^{18}O enrichment on a Finnigan 3000 mass spectrometer at an ionizing voltage of 70 eV. At alkaline pH there is a negligibly small concentration of CO_2 in the reaction solution (for pH >8, % CO_2 is 2% or less). As a result, the atom fraction of ^{18}O in the CO_2 liberated by acid is taken as equal to the atom fraction γ 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

$$\gamma = \frac{{}^1/_2(46) + (48)}{(44) + (46) + (48)}$$

where (44), (46), and (48) are the heights of the corresponding mass peaks. The standard deviation obtained from four to ten measurements of γ was 1.5-2.0%.

At the end of each exchange experiment the pH of the contents of the reaction vessel was measured. This reading agreed, within 0.03 pH units, with the reading taken prior to the start of the experiment. For a series of experiments using different buffer concentration but the same pH, the values of pH agreed within 0.07 pH units.

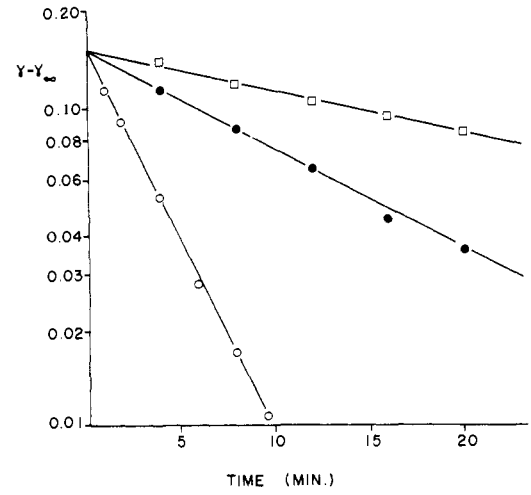

Figure 2. The rate of depletion of oxygen-18 from labeled bicarbonate at 25° in the presence of 5×10^{-9} M human carbonic anhydrase C. Total concentration of carbonate species was 7 mM with Na_2SO_4 added to make an ionic strength of 0.2 M. The atom fraction of ^{18}O in bicarbonate is γ: (□) pH 9.40, (●) pH 9.15, (○) pH 8.40.

Table I. Rates Derived from the Depletion of ^{18}O from Bicarbonate[a]

pH	θ_1,[b] $sec^{-1} \times 10^3$	k_{uncat},[c] $sec^{-1} \times 10^4$	k_c,[d] $sec^{-1} \times 10^3$
10.00	0.094	1.95	0.24
9.15	1.16	2.26	3.48
8.90	1.47	2.56	4.37
8.35	4.29	4.29	12.9

[a] Carbonic anhydrase (human C) was 5×10^{-9} M and total carbonate 7 mM at 25°. Ionic strength was made 0.2 M Na_2SO_4. [b] Observed rate of depletion, given in eq 9. [c] Rate constant for uncatalyzed dehydration at 25°. [d] Rate constant of the catalyzed dehydration at equilibrium and 25°.

The rate constant for catalyzed dehydration at equilibrium, k_c of eq 9, is a linear function of carbonic anhydrase concentration in the range of enzyme concentrations employed here (10^{-9}-10^{-8} M). In addition, the effects of buffers on θ_1 were the same when the ^{18}O-exchange reactions were carried out in vessels with and without a hydrophobic surface coating (Desicote, Beckman).

Results

^{18}O Depletion in Unbuffered Media. Carbonic anhydrase catalyzed ^{18}O depletion of bicarbonate was measured in the absence of added buffers. The reaction solutions contained 5×10^{-9} M HCA-C or BCA and from 5×10^{-4} to 10^{-2} M total carbonate species with an overall ionic strength adjusted to 0.2 M with sodium sulfate. These reaction solutions were buffered only to the extent that the enzyme and substrate themselves are buffers at the concentrations used and values of pH reported.

Experimental results at several values of pH using HCA-C are shown in Figure 2. The slopes of these plots are described in terms of the contributions of the catalyzed and uncatalyzed reaction rates to the values of θ_1 of eq 9. The relative contributions of k_{uncat} and k_c at several values of pH are given in Table I. Addition of a powerful carbonic anhydrase inhibitor such as ethoxzolamide ($K_I = 10^{-9}$ M) at a concentration of 10^{-5} M abolishes k_c leaving a depletion rate equal to that found in a solution lacking carbonic anhydrase. The magnitude of k_{uncat} at each pH was deter-

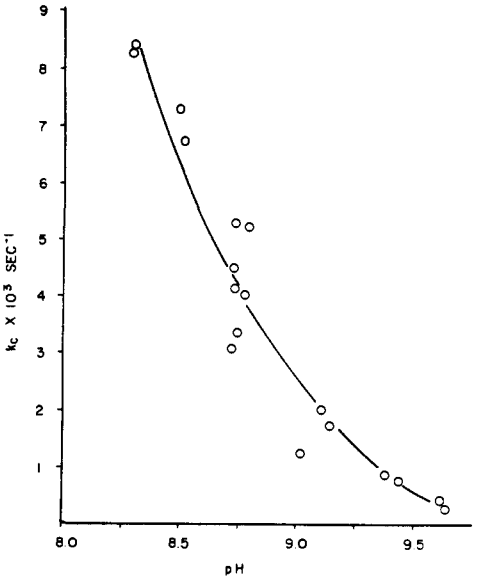

Figure 3. The first-order rate constant k_c for the catalyzed component of the dehydration of bicarbonate at equilibrium and 25° as a function of pH. The concentration of bovine carbonic anhydrase was 5×10^{-9} M with a total concentration of carbonate species of 0.5 mM and sufficient Na_2SO_4 to bring the ionic strength to 0.2 M.

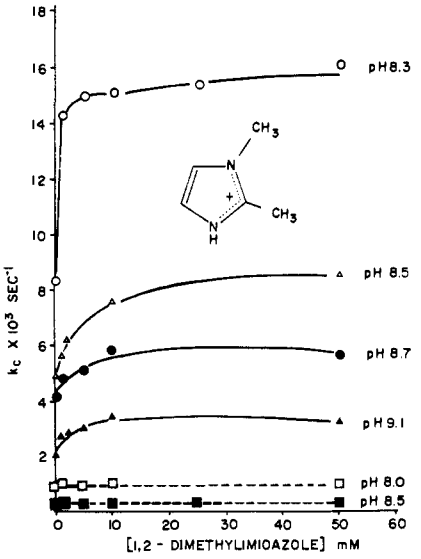

Figure 4. The first-order rate constant k_c for the catalyzed component of the dehydration of bicarbonate at equilibrium and 25° vs. the 1,2-dimethylimidazole concentration. The concentration of bovine carbonic anhydrase was 5×10^{-9} M with total carbonate at 0.5 mM and sufficient Na_2SO_4 to bring the ionic strength to 0.2 M. The dotted lines represent values of k_{uncat}, measured in the absence of enzyme.

mined from experiments identical with those described previously but without the addition of carbonic anhydrase. Values of k_{uncat} determined by this ^{18}O-exchange method are in good agreement with the dehydration rate constants determined by Gibbons and Edsall.[17] The plot of k_c vs. pH in Figure 3 gives the high pH extension of the pH profile for bovine carbonic anhydrase dehydration activity, determined also by Kernohan[18] for an alkaline pH region. A similar pH dependence was found for HCA-C in the absence of buffers.

^{18}O Depletion in Buffered Media. Following procedures identical with those described in the previous section, isotope depletion kinetics were measured as a function of increasing external buffer concentration, maintaining ionic strength at 0.2 M with Na_2SO_4. Two of the buffers used,

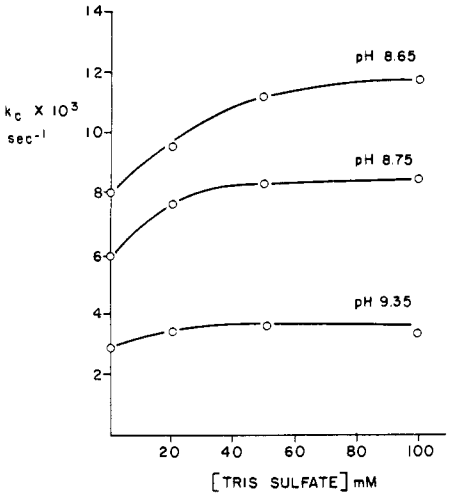

Figure 5. The rate constant k_c at 25° vs. the concentration of tris sulfate. The concentration of human carbonic anhydrase C was 5×10^{-9} M with total carbonate at 7 mM and sufficient Na_2SO_4 to bring the ionic strength to 0.2 M.

tris sulfate and sodium barbital, are primary or secondary amines, and the possibility exists that they will form carbamates with CO_2. This possibility was determined to be negligible with tris sulfate and barbital in the range of pH of these studies by adding barium chloride to the reaction solutions. Barium precipitates free carbonate but not carbamates. With tris sulfate and barbital all of the carbonate was accounted for by weighing the precipitate. Furthermore, no evolved CO_2 was detected when acid was added to the supernatant, decomposing any carbamates that might have formed. After addition of acid to the supernatant, gas pressure was monitored to detect any CO_2 evolved.

The effect on k_c of bovine carbonic anhydrase caused by increasing concentrations of the buffer 1,2-dimethylimidazole (pK_a = 8.3) is shown in Figure 4. Data obtained with HCA-C showed a similar dependence of k_c on concentration of 1,2-dimethylimidazole. The effect of tris sulfate (pK_a = 8.3) on k_c of HCA-C, using a different substrate concentration, is shown in Figure 5. Since k_c in these experiments is at least an order of magnitude greater than k_{uncat}, it is unlikely that the effects shown in Figures 4 and 5 are caused by buffer acting on the uncatalyzed depletion. This was demonstrated by performing ^{18}O-exchange experiments in the presence of 1,2-dimethylimidazole but in the absence of enzyme. The dotted lines on Figure 4 illustrate that the uncatalyzed rate of depletion of ^{18}O from labeled bicarbonate is relatively independent of the concentration of this buffer. This result was confirmed in subsequent runs taken in an alkaline pH region.

To test whether the buffer dependence exhibited in Figure 4 is caused by a solvent effect, the dependence of k_c on 1,3-dimethylimidazolium sulfate was determined as shown in Figure 6. This compound is structurally analogous to the buffer 1,2-dimethylimidazole and, like the buffer in protonated form, bears a positive charge. However, 1,3-dimethylimidazolium cannot donate or accept a proton. Results similar to those of Figure 6 were also obtained with pyrrole, which is also incapable of proton donation in the pH region of these studies.

Discussion

The main advantage afforded by the oxygen-18 exchange technique in studying the buffer dependence of carbonic anhydrase is that this is an equilibrium technique in which it is possible to measure catalysis of bicarbonate dehydration in the presence of very low concentration of buffers. In

view of the hypothesis of buffer involvement in the catalytic mechanism of carbonic anhydrase,[3,5,6] the main goal of this work is to determine whether buffer concentration can be decreased to a region in which the postulated proton transfer between buffer and enzyme active site limits the catalytic rate.

As a step toward this goal, the results of the isotope-exchange experiments using BCA in the absence of added buffers are given in Figure 3. A primary observation from these data is that even in the absence of added buffers, and at low substrate concentration (5×10^{-4} M total carbonate species), the catalytic dehydration rate constant at equilibrium, k_c, is substantial. Very similar results were obtained with HCA-C. From eq 9 and the data in Figure 3 at pH 8.5, the rate of catalyzed dehydration at equilibrium is $k_c[HCO_3^-] \simeq (7 \times 10^{-3}\ sec^{-1})(5 \times 10^{-4}\ M) = 3.5 \times 10^{-6}\ M\ sec^{-1}$. This dehydration rate is comparable to the rate of catalyzed dehydration measured far from equilibrium but at the same enzyme and initial bicarbonate concentrations. This high enzyme activity at equilibrium obtained without added buffers is compatible with the hypothesis of buffer aided catalysis because of the following considerations.

First, at equilibrium the need for a proton-transfer mechanism from buffer or water to the active site is less than in a nonequilibrium experiment. At equilibrium, an enzyme molecule can alternate between hydration and dehydration (i.e., between steps 3′,2′ and 2,3 of Figure 1) and avoid a need for the proton-transfer mechanism altogether. Second, the substrate molecule itself, bicarbonate, can donate a proton and carbonate can accept a proton. It is likely that, to a certain extent, bicarbonate acts as a buffer providing protons to the active site.

Figures 4 and 5 demonstrate that the catalytic dehydration rate constant at equilibrium, k_c, is large in the absence of added buffers and increases by up to 80% as buffer concentration is increased. Evidence that this is not a solvent effect is demonstrated in Figure 6 by using 1,3-dimethylimidazolium sulfate, a charged molecule similar to 1,2-dimethylimidazole but not able to donate or accept a proton. Results similar to Figure 6 were also obtained with increasing concentrations of pyrrole, which is also incapable of donating or accepting protons in the pH region of this study. Furthermore, the effect on k_c is not caused by the enhancement of the uncatalyzed rate by buffers, as shown by the dotted lines in Figure 4. A possible explanation of the behavior in Figures 4 and 5 is that the buffer, through a proton donating-proton accepting mechanism, enhances the catalytic rate. The proton transfer causing this enhancement could occur at a number of possible steps in the catalytic pathway. These ^{18}O-exchange experiments do not identify the step involved. If protonation of the zinc hydroxide is responsible for the pK_a of the enzyme, then E of Figure 1 is RZnOH. If bicarbonate forms an inner-sphere complex with zinc prior to the catalyzed dehydration, it is likely that $RZn^{18}OH$ forms to a certain extent. In order for ^{18}O depletion to occur, this label must be lost to solvent water. This occurs by protonation to $Zn^{18}OH_2^+$ followed by ligand exchange with bicarbonate, releasing $H_2{}^{18}O$. For this case, steps 1 and 1′ could be rate determining in the absence of buffers. It is also possible that proton transfer involving an enzyme-substrate complex is responsible for the observed enhancement of catalytic rate. The following step, not shown in Figure 1, could be involved in proton transfer involving buffer: $ECO_2 \rightleftharpoons EH^+CO_2$.

To explain these effects as due to buffer-assisted catalysis it is necessary to determine whether the buffer concentrations of Figures 4 and 5 required to bring about enhancement of catalysis give an estimate of the expected magnitude for the rate constant of proton transfer from the enzyme-active site to the buffer. The catalytic rate can be expected to increase when added buffers cause the rate of step 1 and the rate of step 1′ in Figure 1 to be comparable to or greater than the catalyzed hydration-dehydration rate at equilibrium given by steps 3′,2′ and 2,3. More specifically, when steps 1 and 1′ are slow, the enzyme molecule must alternate regularly between one hydration step and one dehydration step in the absence of a proton donor-acceptor. But if steps 1 and 1′ are comparable to or greater than steps 2,3 and 3′,2′, sequences of only hydration reactions or only dehydration reactions by the enzyme molecule are allowed. In this latter case, the rate of catalysis will be enhanced.

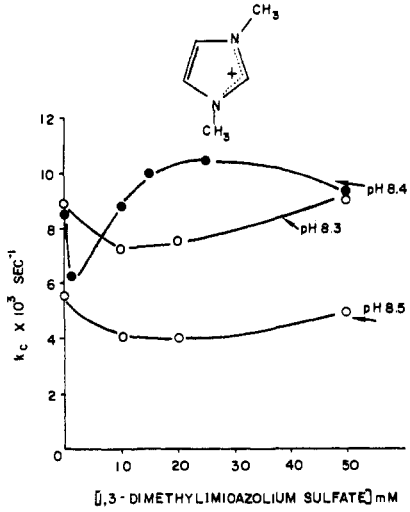

Figure 6. The rate constant k_c at 25° vs. the concentration of 1,3-dimethylimidazolium sulfate: (●) the concentration of bovine carbonic anhydrase was 5×10^{-9} M with total carbonate at 0.5 mM, (○) the concentration of human carbonic anhydrase C was 5×10^{-9} M with total carbonate at 7 mM. All solutions were made 0.2 M in ionic strength with Na_2SO_4.

Using data from Figure 4 for BCA at pH 8.3 and 1 mM 1,2-dimethylimidazole:

$$\text{rate of dehydration} = k_c[HCO_3^-] = (14.3 \times 10^{-3}\ sec^{-1})(5.0 \times 10^{-4}\ M) = 7.2 \times 10^{-6}\ M\ sec^{-1}$$

If the contribution of buffer to the catalysis is neglected, and H_3O^+ is taken as proton donor in step 1 of Figure 1 preceeding dehydration, a lower limit for the rate constant k_1 of step 1 can be calculated. K_a for the enzyme active site is 10^{-7}.

$$k_1[E][H^+] \geq \text{rate of dehydration}$$

$$k_1[E_{Tot}]\frac{K_a}{K_a + [H^+]}[H^+] =$$

$$k_1[5 \times 10^{-9}\ M][0.95][10^{-8.3}\ M] \geq 7.2 \times 10^{-6}\ M\ sec^{-1}$$

$$k_1 \geq 3 \times 10^{11}\ M^{-1}\ sec^{-1}$$

This rate constant is larger than the diffusion limit for the transfer of a proton from H_3O^+ to the enzyme.[19] The need to hypothesize some facilitated proton transfer exists under these experimental conditions.

Consider the proton transfer involving buffer and enzyme:

$$B + EH^+ \underset{k_r}{\overset{k_f}{\rightleftharpoons}} BH^+ + E$$

An estimate of k_f can be obtained by assuming that the rate of proton transfer from the enzyme to buffer is equal to or greater than the rate of dehydration at equilibrium calculated above. The pK_a of 1,2-dimethylimidazole is near 8.3 and the concentration of unprotonated buffer at pH 8.3 is taken as 5×10^{-4} M.

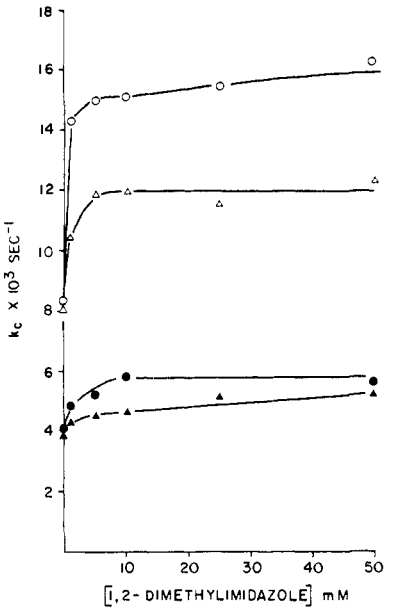

Figure 7. The rate constant k_c vs. the concentration of 1,2-dimethylimidazole at 25°. The concentration of bovine carbonic anhydrase was 5 $\times 10^{-9}$ M in each case. At pH 8.3 the concentration of total carbonate species was (○) 0.5 mM and (△) 5.0 mM. At pH 8.75 the concentration of total carbonate species was (●) 0.5 mM and (▲) 10.0 mM. Na_2SO_4 was added to bring all solutions to 0.2 M in ionic strength. For clarity, data at each pH are plotted to give the same intercept on the ordinate.

$$k_f[B][EH^+] \geq \text{rate of dehydration} = \text{rate of hydration}$$

$$k_f[B][E_{Tot}]\frac{[H^+]}{[H^+] + K_a} = k_f[5 \times 10^{-4}\ M] \times [5 \times 10^{-9}\ M](4.8 \times 10^{-2}) \geq 7.2 \times 10^{-6}\ M\ \text{sec}^{-1}$$

$$k_f \geq 6.0 \times 10^7\ M^{-1}\ \text{sec}^{-1}$$

This result is for the case in which the pK_a of the acceptor is greater than the pK_a of the donor. For small molecules with this ΔpK_a one anticipates a value of k_f near 10^9 M^{-1} sec^{-1}, the diffusion limit.[19] The rate of exchange of protons between *p*-nitrophenol (pK_a = 7.14) and imidazole (pK_a = 6.95) is near 5 × 10^8 M^{-1} sec^{-1} in both directions.[19] The lower limit for k_f obtained above involving the active site of carbonic anhydrase and 1,2-dimethylimidazole is smaller by an order of magnitude than the small molecule analogy. This is possibly a result of hindrance of the approach of buffer by the geometry of the enzyme active site.

Figures 4 and 5 demonstrate that increasing the buffer concentration causes an increase in k_c up to a maximum value and then is incapable of increasing k_c further. This behavior would be expected for a catalytic pathway consisting of at least two mechanistic steps, the first dependent and the second independent of buffer concentration.[20] At very low concentration of buffer, the first step is rate limiting and the overall catalytic rate constant k_c is controlled by this step. As buffer is increased, the rate of the first step eventually exceeds the rate of the second step. Then the second step becomes rate limiting and the overall catalytic rate constant is independent of further increases in buffer concentration. Such a scheme for carbonic anhydrase catalyzed dehydration can be rationalized in terms of Figure 1. Assume the protonation of the enzyme in step 1, prior to the binding of bicarbonate, is buffer mediated;[3,5,6] that is, the rate of this step is dependent on the concentration of the acidic buffer species in solution. The binding of substrate and subsequent chemical reaction in the active site, steps 2 and 3, are expected to be independent of buffer concentration for dehydration. Consequently, as buffer concentration is increased, the rate of step 1 increases to a point where it is faster than subsequent steps. At this stage, further increases in buffer concentration cannot cause corresponding increases in k_c. The extent to which k_c is enhanced by buffer increases as pH decreases (Figures 4 and 5), an expected result since the rate of step 1 depends upon concentration of the acidic buffer species. In this regard, addition of imidazole (pK_a = 6.95) and barbital (pK_a = 7.43) to the enzyme solution at a pH (pH >8.7) in which these compounds are protonated to a very small extent did not increase the catalytic rate.

In Figures 4 and 5, the values of k_c in the absence of added buffer represent catalysis along steps 2,3 and 3′,2′ of Figure 1 plus a possible contribution of steps 1 and 1′ by the bicarbonate and carbonate buffer system. To clarify the role of the substrate in the catalysis, catalytic rates at equilibrium were measured varying bicarbonate concentration from 10 to 0.5 mM in the absence of added buffers. With our apparatus we could not accurately measure k_c below 0.5 mM bicarbonate because of absorption of atmospheric CO_2. The results clearly showed no substantial decrease in catalytic rate when bicarbonate concentration was decreased to 0.5 mM in the pH region 8–9.[21] In fact, an increase in k_c with decreasing bicarbonate concentration was observed, as reported at higher bicarbonate concentration by Silverman.[12] Consequently, we were not able to decrease bicarbonate in the absence of buffers to the point where a possible contribution of protons in step 1 by HCO_3^- limits the catalytic rate. A careful study of k_c in regions of low bicarbonate concentrations is necessary to separate the possible effects on k_c of bicarbonate as a buffer and the effects on k_c of bicarbonate as substrate (the latter as described by Boyer[13]). However, in support of the idea that bicarbonate itself can affect k_c as a proton donor, Figure 7 demonstrates that the effect of added buffers on k_c is greater when the bicarbonate concentration is low. An alternative explanation of the data in Figure 7 is possible by consideration of the fact that step 3′ and step 2 become more favored at the higher substrate concentration. That is, at a higher substrate concentration a larger proportion of the catalysis occurs along the slower, buffer-unassisted pathway.

With the above considerations, this is one interpretation of the data in Figure 4. The values of k_c in the absence of added buffers are due in large part to alternating hydration and dehydration steps at equilibrium. At a bicarbonate concentration of 5 × 10^{-4} M there is possibly a contribution to k_c caused by this substrate acting as a buffer. The increase in k_c over this value as buffers are added represents an increase in catalysis at equilibrium caused by the availability of steps 1 and 1′ of Figure 1 or caused by the availability of steps which are enhanced by proton transfer to an enzyme-substrate complex.

The conclusion of these experiments is that the activity of carbonic anhydrase at equilibrium is enhanced, to a moderate extent, by the availability of protons as provided by a buffer. It is expected that this dependence extends into regions near neutral pH which cannot be effectively investigated by our apparatus because of the very rapid rate of ^{18}O exchange near neutral pH. These experiments provide evidence that, in the narrow pH region studied here, the maximal activity of carbonic anhydrase is dependent on the presence of buffers in the proximity of the active site, as has been proposed[3,5,6] to account for the large turnover number for this enzyme.

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(21) This may simply reflect the fact that CO_2, HCO_3^-, CO_3^{2-} is a poor buffer system at pH 8–9.