Comparative Studies of Bovine Carbonic Anhydrase in H_2O and D_2O. Stopped-Flow Studies of the Kinetics of Interconversion of CO_2 and HCO_3^-†

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ABSTRACT: The physiological importance of the enzyme bovine carbonic anhydrase is to maintain an equilibrium between carbon dioxide and bicarbonate. In the present study, the enzyme-catalyzed approach to equilibrium has been followed from the direction of CO_2 hydration and HCO_3^- dehydration by use of the stopped-flow indicator technique in H_2O as well as D_2O. The resulting rates were separated into a turnover and an apparent binding term following the method of Lineweaver and Burk. For CO_2 hydration K_M remains essentially constant (15 mM) over the pH range 5.9 to 10.2, rising only slightly at low values of pH. On the other hand, k_{cat} changes sigmoidally over the same pH range with a point of inflection at pH 6.85 in H_2O and 7.35 in D_2O. The basic form of the enzyme is required for maximum activity ($k_{cat} = 8.3 \times 10^5\ s^{-1}$). Up to pH 10.2, there is no indication for a second rise in activity which is so common for the enzyme acting as an esterase. The turnover number for HCO_3^- dehydration also varies sigmoidally with the acidic form of the enzyme needed for full activity ($k_{cat} = 3.8 \times 10^5\ s^{-1}$). Over the pH range 5.5 to 7.9, K_M remains constant (22 mM). The effect of D_2O on k_{cat} and K_M has also been measured. The ratio, $k_{cat}^{H_2O}/k_{cat}^{D_2O}$, for CO_2 hydration is 3.3 at the basic plateau, while the ratio, $k_{cat}^{HCO_3^-}/k_{cat}^{DCO_3^-}$, for bicarbonate dehydration is 4.3 at the acidic plateau. Values of this magnitude are consistent with mechanisms involving proton transfer in the turnover step. The pH-independent parameters, K_M, also exhibit significant solvent deuterium isotope effects, $K_M^{H_2O}/K_M^{D_2O} = 3.0$, and $K_M^{HCO_3^-}/K_M^{DCO_3^-} = 2.6$, while the corresponding k_{cat}/K_M ratios show much smaller isotope effects.

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a widely distributed zinc metalloenzyme whose physiological role is to catalyze the reversible hydration of CO_2. For many years, it was thought that the enzyme was specific for this function, but, over the last decade, many reports have demonstrated the enzyme's truly remarkable versatility. It is capable of catalyzing the hydration of certain aldehydes (Pocker and Meany, 1965, 1967; Pocker and Dickerson, 1968), as well as the hydrolysis of many different esters (Pocker and Stone, 1965, 1967; Thorslund and Lindskog, 1967; Verpoorte et al., 1967; Pocker and Storm, 1968; Kaiser and Lo, 1969; Pocker and Watamori, 1971; Pocker and Guilbert, 1972; Pocker and Beug, 1972; Pocker and Sarkanen, 1973, 1975; Pocker et al., 1977).

The enzyme from mammalian sources consists of a single polypeptide chain, has a molecular weight of 30 000, and contains one zinc cation per molecule of protein (Lindskog et al., 1971). In addition, the x-ray crystallographic work on human carbonic anhydrase C has illustrated that the zinc is located at the bottom of a crevice and is tetrahedrally coordinated to three histidyl residues and one water molecule (Liljas et al., 1972; Kannan et al., 1971). Besides the three histidyl moieties bound to the zinc, there is a fourth located near the entrance of the active site.

All available kinetic evidence suggests that the enzymatic activity at neutral pH values is linked to the titration of some acidic group in the active site. There are, at present, three different proposals regarding the identity of the acid and these proposals have led to six different mechanisms, Table I. The first group of mechanisms, Ia–c, involves the ionization of $Zn-OH_2$ complex to generate the active conjugate base, Zn–OH. The second group of mechanisms, IIa and IIb, involves the ionization of the imidazolium moiety of the histidine located near the entrance of the active site. The last mechanism, IIIa, also involves the titration of a histidine. However, in this instance, it is one of the histidines bound to zinc that is suggested to be the catalytically active species.

As should be noted, there are some obvious differences between the six mechanisms other than the involvement of the different bases. First, in mechanisms Ia, IIa, IIb, and IIIa, bicarbonate is bound directly to the zinc, whereas in Ib and Ic, it is not. Secondly, carbonic acid is the hydration product in only one of the mechanisms, Ic. Finally, mechanism Ia is the only one not involving proton transfer.

It appeared to us that these differences might be sufficient to enable a number of the proposed mechanisms to be eliminated. In regards to the first of the aforementioned differences, we felt that a detailed study of the enzyme-catalyzed dehydration of bicarbonate would be useful in determining how this anion binds in the active site of the enzyme. With respect to the latter point, we hoped that a thorough investigation of the solvent deuterium isotope effects would indicate whether proton transfer was part of the enzymatic turnover for its natural substrates. This paper describes results obtained for the bovine carbonic anhydrase catalyzed reversible hydration of CO_2 in H_2O as well as D_2O.

Experimental Section

Materials. Carbon dioxide was purchased from Airco (Research Grade, 99.99% pure), and sodium bicarbonate from Matheson Coleman and Bell (analytical grade). All buffers were prepared in either deionized, distilled H_2O or in D_2O

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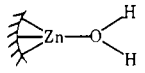
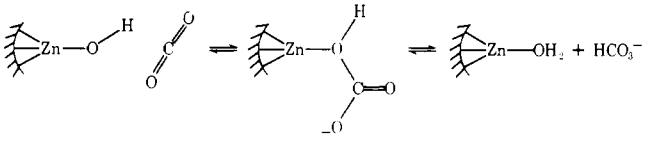
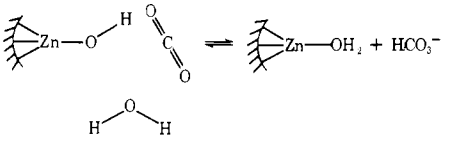
TABLE I: Proposed Mechanisms For Carbonic Anhydrase.[a]

I. Acidic group: 

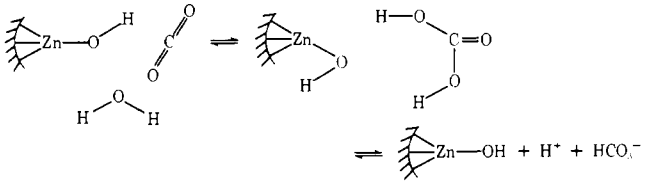
(a) Nucleophilic attack of Zn–OH on CO_2[b]

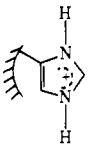


(b) General base assisted H_2O on CO_2[c]

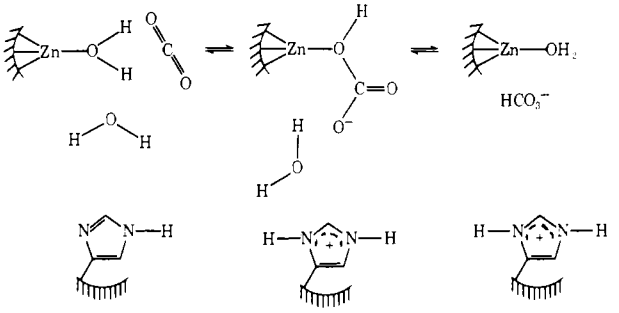


(c) Nucleophilic attack of Zn–OH on CO_2 with concomitant proton transfer[d]

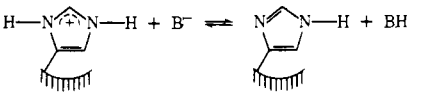


II. Acidic group: 

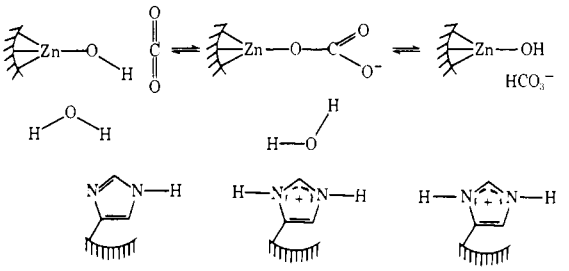
(a) General base assisted $Zn–OH_2$ on CO_2[e]

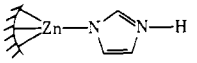


followed by

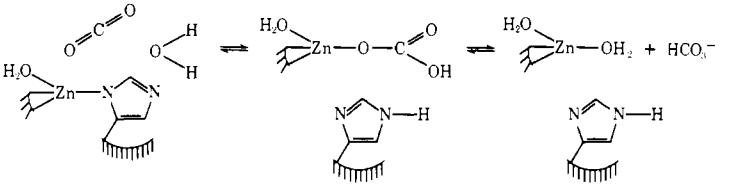


(b) General base assisted Zn–OH attack on CO_2[f]



III. Acidic group: 

(a) General base assisted H_2O attack on CO_2[g]



[a] Although some of the mechanisms have been suggested as the result of esterase studies, they will be illustrated with CO_2. [b] Coleman, 1967; Davies, 1961. [c] Khalifah, 1971. [d] Kaiser and Lo, 1969. [e] Pocker and Storm, 1968; the buffer assisted deprotonation of ImH^+ is in accord with the following observations:

$$ImH^+ + HPO_4^{2-} \underset{k_r \simeq 5 \times 10^8\ M^{-1}\ s^{-1}}{\overset{k_f \simeq 5 \times 10^8\ M^{-1}\ s^{-1}}{\rightleftharpoons}} Im + H_2PO_4^-$$

whereas

$$ImH^+ + H_2O \underset{k_r = 1.5 \times 10^{10}\ M^{-1}\ s^{-1}}{\overset{k_f = 1.5 \times 10^3\ s^{-1}}{\rightleftharpoons}} Im + H_3O^+$$

[f] Wang, 1970. [g] Pesando, 1975; Gupta and Pesando, 1975; Appleton and Sarkar, 1974 (see, however, Martin, 1974).

(Stohler, 99.8% D). In general, the total buffer concentration was held between 0.02 and 0.05 M and the ionic strength was maintained at 0.1 by the addition of Na_2SO_4. The buffer components sodium dihydrogen phosphate monohydrate (Merck), sodium monohydrogen phosphate (Baker and Adamson), sodium sulfate (Baker), and sulfuric acid were reagent grade and used without further purification. 3-Picoline (Aldrich) was shown to be 96% pure by titration with HCl and was used as obtained. Imidazole (Eastman) was recrystallized three times from benzene–Norite, while 1,2-dimethylimidazole (Aldrich) was distilled under vacuum, bp 57 °C (2 Torr). *N,N*-Dimethylglycine was prepared from *N,N*-dimethylglycine·HCl (Nutritional Biochemicals Corp.) by a previously published procedure (Pocker and Guilbert, 1972). Bovine carbonic anhydrase, BCA, was obtained from Mann Laboratories, and a single batch was used for all kinetic determinations. Sodium azide (Matheson Coleman and Bell) and acetazolamide (Lederle Laboratories) were used without further purification.

Solutions and Buffer Components. Saturated solutions of CO_2 (99.99% pure) were prepared by bubbling the gas through deionized, distilled water, or through D_2O (Stohler, 99.8% D) in a vessel fitted with a stopcock and thermostated at 25.0 °C. Portions of these saturated solutions were withdrawn by allowing them to flow by gravity from the vessel into a gas-tight Hamilton syringe. To determine the concentration of CO_2, a known volume of the saturated solution was added to an excess of standardized $Ba(OH)_2$ containing $BaCl_2$. The resulting solution was back titrated against standardized HCl with phenolphthalein as the indicator. The concentration of CO_2 saturated in H_2O at 25.0 °C was found to be 3.38×10^{-2} M, while in D_2O it was 3.81×10^{-2} M. Solutions of sodium bicarbonate (MC/B reagent grade) were prepared by carefully weighing the salt and dissolving it in deionized distilled water or in D_2O just prior to use. All indicators were used as obtained, except phenol which was sublimed, and *p*-nitrophenol which was recrystallized from water made slightly acidic with HCl.

Apparatus. All pH measurements were recorded on a Beckman 101900 research pH meter fitted with a Corning glass electrode (No. 476022) and a Beckman reference electrode (No. 39071). The pD was obtained by adding 0.41 to the observed pH meter reading (Glasoe and Long, 1960). The hydration and dehydration rates were monitored by the use of an indicator technique on a Durrum-Gibson stopped-flow spectrophotometer thermostated by a bath of our own design to 25.0 ± 0.05 °C. The instrument was equipped with two equal diameter glass drive syringe barrels to ensure that the mixing was one-to-one by volume. The old stainless steel mixing components were replaced with the new Kel-F flow system.

Kinetic Procedure and Calculations. All rates on the stopped-flow spectrophotometer were initiated by rapid 1:1 mixing of the substrate with a buffer containing the desired catalyst and monitored over a very short time interval so as to record only the initial linear portion of the reaction. The output on the oscilloscope was adjusted to read 2% transmittance full scale. The data were analyzed by first converting the percent transmittance readings to absorbance and then by plotting absorbance vs. time in the customary zero-order fashion. Usually nine kinetic runs were recorded per buffer.

Since there is no convenient technique available to directly monitor the disappearance of CO_2 or the appearance of HCO_3^-, an indicator technique has been developed which essentially follows changes in the proton concentration (DeVoe and Kistiakowsky, 1961; Gibbons and Edsall, 1964; Khalifah, 1971). An expression for the velocity of disappearance of CO_2 can then be written in terms of measurable quantities (eq 1).

$$V_{CO_2\ \text{hydration}} = \frac{d[CO_2]}{dt} = \frac{d[H^+]}{dt} = \frac{dOD}{dt}\frac{d[H^+]}{dOD} \quad (1)$$

The first quantity, dOD/dt, is a term which measures the change in optical density as a function of time. It can be evaluated as the slope of a zero-order rate plot. The second term, $d[H^+]/dOD$, which has been called the "buffer factor" and given the symbol Q_0, is simply an expression which relates changes in the optical density of the indicator to the number of protons released in the hydration reaction. The primary consideration in selecting an indicator for use with a buffer should go to choosing one whose pK_{Hln} is near the pK_a of the buffer. For this reason bromocresol purple was used with picoline; *p*-nitrophenol with phosphate and imidazole; metacresol purple with 1,2-dimethylimidazole; and phenol with *N,N*-dimethylglycine.

The hydration velocities when divided by the initial concentration of CO_2 yield k_{obsd}, eq 2

$$k_{obsd} = \frac{V_{CO_2\ \text{hydration}}}{[CO_2]_0} = \frac{(dOD/dt)(Q_0)}{[CO_2]_0} \quad (2)$$

This expression is valid only in the pH range where one proton is released per molecule of CO_2 hydrated. Certainly above pH 8.5 this is not the case, because additional protons are released upon the dissociation of HCO_3^- into CO_3^{2-}, and the appropriate adjustment to eq 2 must be made.

In studying the dehydration of bicarbonate, the same considerations hold as for CO_2 hydration. However, kinetic investigations of the two cobalt complexes $Co(NH_3)_5CO_3^+$ and $Co(NH_3)_5OH_2^{3+}$ were handled somewhat differently (Pocker and Bjorkquist, 1977).

The kinetic procedure and analysis of the zero-order rate data have been previously described (Khalifah, 1971; Pocker and Bjorkquist, 1977). Rates with the buffer alone were always performed just prior to the enzymatic studies. Consequently, k_{enz} could easily be determined from eq 2a, where [enz] is the active concentration of BCA as determined by acetazolamide inhibition (Kernohan, 1965; Pocker and Watamori, 1971).

$$k_{enz} = \frac{k_{obsd} - k_{buffer}}{[enz]} \quad (2a)$$

The method of Lineweaver and Burk was then employed to separate k_{enz} into its respective binding and turnover terms.

The inhibition studies were performed by premixing the inhibitor with the enzyme. Rates were then performed by either holding the concentration of the inhibitor constant while varying the substrate, or vice versa.

Results

BCA-Catalyzed Hydration of CO_2. A typical Lineweaver–Burk plot for the BCA-catalyzed hydration of CO_2 is shown in Figure 1. The variation of K_M and k_{cat} with pH was obtained from a series of such plots. The results for the hydration in both H_2O and D_2O are presented in Figure 2.

It is evident that K_M remains virtually independent of pH ($K_M^{H_2O}$ = 15 mM) over the pH range 10.3 to 7.0 and then rises only gradually at low pH. However, the extent of the increase seems to depend on the choice of buffer. In a phosphate buffer at pH 6.07, K_M was found to be 43 mM, whereas in a picoline buffer at pH 6.18, K_M was only 22 mM.

The turnover number of the enzyme substrate complex, k_{cat}, was found to increase as the pH was raised. There is, at least, an eightfold increase in going from pH 6 to 10 with a maximum

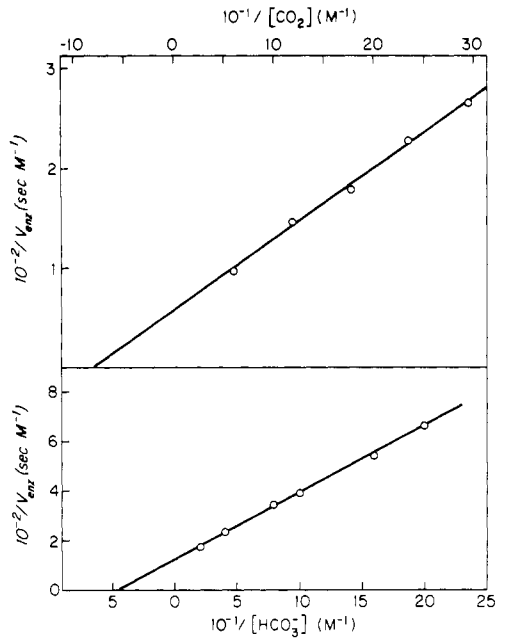


FIGURE 1: (Upper) Representative Lineweaver-Burk plot for the BCA-catalyzed hydration of CO_2; $[BCA]_0 = 2.26 \times 10^{-8}$ M; 0.05 M 1,2-dimethylimidazole buffer, pH 8.19; $t = 25.0$ °C, $I = 0.10$. (Lower) Representative Lineweaver-Burk plot for the BCA-catalyzed dehydration of bicarbonate: $[BCA]_0 = 4.01 \times 10^{-8}$ M, 0.02 M imidazole buffer, pH 6.99; $t = 25.0$ °C; $I = 0.10$.

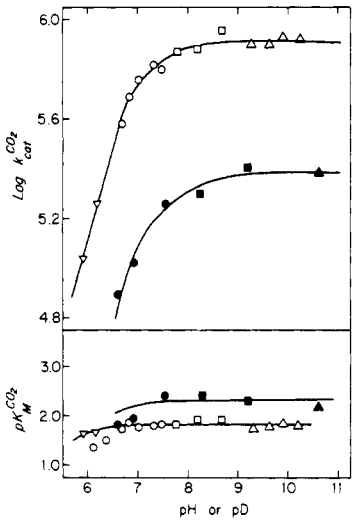


FIGURE 2: The pH dependence of k_{cat} (upper graph) and K_M (lower graph) for the BCA-catalyzed hydration of CO_2 in H_2O (open symbols) and D_2O (filled symbols) at 25.0 °C and $I = 0.10$. Buffers employed: (▽) 3-picoline; (○) phosphate; (□) 1,2-dimethylimidazole; and (△) *N,N*-dimethylglycine.

value of $8.3 \times 10^5\ s^{-1}$. In the pH region 6 to 8, k_{cat} can best be described by a sigmoid titration curve with an inflection occurring at pH 6.85. It should also be pointed out that at pH values up to 10.3, there is no indication of a second rise in activity which is so common in the profiles of esters (Pocker and Storm, 1968; Pocker and Watamori, 1973). At low pH there is also a difference between the k_{cat} data collected in phosphate and in picoline buffers.

The results for the hydration in D_2O indicate that the apparent binding is constant over the entire pD range, except for a small increase at low pD which seems primarily to be characteristic of the phosphate buffer. The value of the ratio, $K_M^{H_2O}/K_M^{D_2O}$, is 3. In comparing k_{cat} in H_2O vs. D_2O, Figure

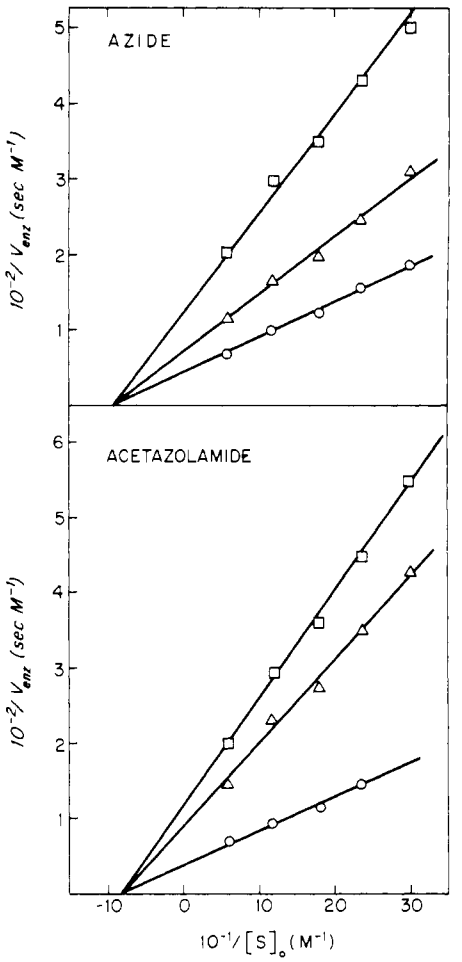


FIGURE 3: (Upper) Lineweaver-Burk plots for the BCA-catalyzed hydration of CO_2 in the presence of varying concentrations of azide: (○) [azide] = 0.00; (△) [azide] = 6.2×10^{-4} M; (□) [azide] = 18.6×10^{-4} M. For all three, [BCA] = 5.17×10^{-8} M. (Lower) Lineweaver-Burk plots for the BCA-catalyzed hydration of CO_2 in the presence of varying concentrations of acetazolamide: (○) [acetazolamide] = 0.00; (△) [acetazolamide] = 3.16×10^{-8} M; [□] [acetazolamide] = 4.21×10^{-8} M. For all three, [BCA] = 4.54×10^{-8} M. In both plots the data were collected by premixing the inhibitor and the enzyme in a phosphate buffer, pH 7.47, at $t = 25.0$ °C and $I = 0.10$.

2, two points are immediately obvious. First, the pK_a of the enzymatic group controlling the activity is 6.85 in H_2O, while it is shifted to 7.35 in D_2O. Secondly, the turnover of the active form of the enzyme is 3.3 times faster in H_2O than D_2O, i.e., $k_{cat}^{H_2O}/k_{cat}^{D_2O} = 3.3$, at the plateau.

The acetazolamide inhibition constant, K_i, for the hydration of CO_2 is 4×10^{-9} M at pH 7.47 and the Lineweaver-Burk plots in Figure 3 appear to demonstrate that CO_2 is inhibited noncompetitively by acetazolamide. It should be noted, though, that in this determination the inhibitor was premixed with the enzyme. As pointed out by Kernohan this technique actually leads to noncompetitive inhibition for the BCA-catalyzed hydration of CO_2, regardless of whether it is or not (Kernohan, 1966; Lindskog and Thorslund, 1968). This occurs because the rate of dissociation of the EI complex is so much slower than the breakdown of the ES complex that the binding of CO_2 appears to be unaffected by the presence of acetazolamide. Azide is also a fairly good inhibitor of CO_2 hydration. The inhibition was observed to be noncompetitive, Figure 3, but this could be caused in part bv a relatively slow breakdown of the EI complex as well.

BCA-Catalyzed Dehydration of HCO_3^-. A typical

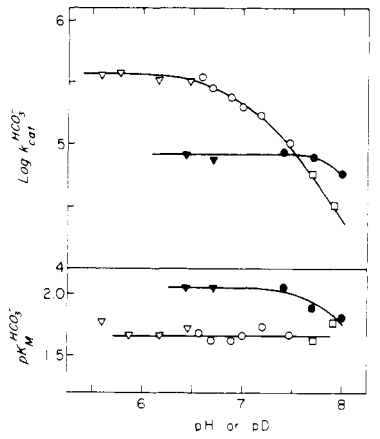


FIGURE 4: The pH dependence of k_{cat} (upper graph) and K_m (lower graph) for the BCA-catalyzed dehydration of bicarbonate in H_2O (open symbols) and D_2O (filled symbols) at 25.0 °C and $I = 0.10$. Buffers employed: (▽) 3-picoline; (○) imidazole; (□) 1,2-dimethylimidazole.

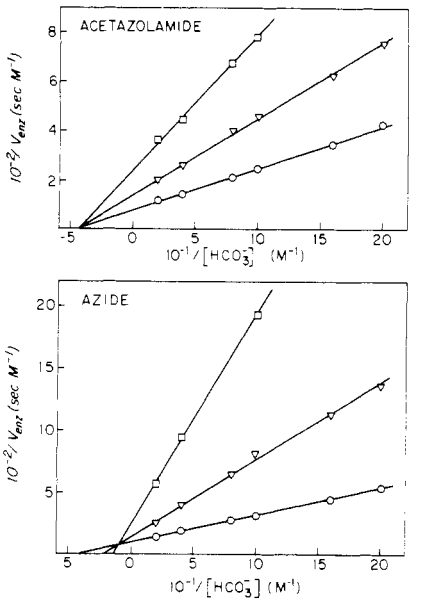


FIGURE 5: (Lower) Lineweaver-Burk plots for the BCA-catalyzed dehydration of bicarbonate in the presence of varying concentrations of azide: (○) [azide] = 0.00; (▽) [azide] = 7.48×10^{-4} M; (□) [azide] = 24.9×10^{-4} M. For all three concentrations of azide, [BCA] = 2.94×10^{-8} M. (Upper) Lineweaver-Burk plots for the BCA-catalyzed dehydration of bicarbonate in the presence of varying concentrations of acetazolamide: (○) [acetazolamide] = 0.00; (▽) [acetazolamide] = 1.71×10^{-8} M; (□) [acetazolamide] = 3.5×10^{-8} M. For all three concentrations of acetazolamide, [BCA] = 3.25×10^{-8} M. In both plots the data were collected by premixing the enzyme and inhibitor in an imidazole buffer, pH 6.57, at $t = 25.0$ °C and $I = 0.10$.

Lineweaver-Burk plot for the BCA-catalyzed dehydration of bicarbonate is shown in Figure 1. As was the case with CO_2, a profile of K_M and k_{cat} was obtained and the results are displayed graphically in Figure 4. Over the pH range 5.5 to 8.0, the apparent binding of HCO_3^- remained constant ($K_M^{H_2O}$ = 22 mM), while the turnover, k_{cat}, changed tenfold. The enzyme is most active at acidic pH values where k_{cat} is 3.8×10^5 s^{-1}. As the pH is raised, the turnover is reduced and passes through a point of inflection at pH 6.9. It is interesting to note that both k_{cat} and K_M determined in a phosphate buffer at pH 6.8 were dependent on buffer concentration. For example, K_M changed from 20 mM to 40 mM upon increasing the total buffer concentration from 10 mM to 30 mM. Christiansen and Magid (1970) observed similar effects with human carbonic anhydrase using a pH-stat technique. To reduce these large fluctuations, imidazole buffers were used in place of phosphate buffers for studying the dehydration rates.

The data collected for k_{cat} and K_M in D_2O, Figure 4, do not exactly correspond with that collected in H_2O. The Michaelis constant, K_M, seems to be constant at low pD, but as the pD is raised from 7 to 8, K_M increases from 9 mM to 16 mM. On the other hand, the turnover number, k_{cat}, remains relatively constant ($k_{cat}^{D_2O} = 8.5 \times 10^4$ s^{-1}) up to a pD value of 7.5 before it begins to drop in magnitude. It is evident, however, that at the plateau the turnover is much faster in H_2O than D_2O, with the ratio $k_{cat}^{H_2O}/k_{cat}^{D_2O}$ being 4.3.

Both acetazolamide and azide inhibit the dehydration of bicarbonate. Of the two, acetazolamide is the much more powerful inhibitor with a K_i of 7×10^{-9} M at pH 6.57. The Lineweaver-Burk plots in Figure 5 apparently demonstrate that the inhibition by acetazolamide is noncompetitive, but it should be noted again that these results were obtained by premixing the inhibitor with the enzyme.

In contrast to acetazolamide, the inhibition of HCO_3^- dehydration by azide is neither competitive nor noncompetitive, Figure 5. Rather, it seems to be of mixed order. This is an interesting result because it demonstrates that the rate of breakdown of the EI complex must at least be comparable to the rate of breakdown of the ES complex. This suggests that the noncompetitive inhibition observed for CO_2 may actually mean that CO_2 and azide have independent binding sites. Furthermore, the partially competitive inhibition observed for bicarbonate implies that the binding sites for bicarbonate and azide may be overlapping.

When the inhibition constant for azide was studied as a function of pH, Figure 6, it became clear that the inhibition is dependent on an acidic group in the enzyme whose pK_a is 6.9. Maximum inhibition ($K_i = 7 \times 10^{-4}$ M) occurred when this group was fully protonated.

Finally, in deliberating the role of carbonic anhydrase in the reversible hydration of CO_2, it is useful to remember that the enzyme must not affect the overall equilibrium constant. Therefore, at equilibrium the forward and reverse velocities must not only be equal (eq 3) but related to each other by the equilibrium constant for the reaction (eq 4).

$$\frac{k_{cat}^{CO_2}[E][CO_2]}{K_M^{CO_2}} = \frac{k_{cat}^{HCO_3^-}[E][HCO_3^-]}{K_M^{HCO_3^-}} \quad (3)$$

$$K_{eq} = \frac{[HCO_3^-][H^+]}{[CO_2]} \quad (4)$$

Combining eq 3 and 4 leads to an expression known as the Haldane relationship (eq 5).

$$\frac{k_{cat}^{CO_2}}{K_M^{CO_2}} = \left(\frac{k_{cat}^{HCO_3^-}}{K_M^{HCO_3^-}}\right)\left(\frac{K_{eq}}{[H^+]}\right) \quad (5)$$

Hence

$$\log\left(\frac{k_{cat}^{CO_2}}{K_M^{CO_2}}\frac{K_M^{HCO_3^-}}{k_{cat}^{HCO_3^-}}\right) = \log K_{eq} - \log [H^+] \quad (6)$$

A plot of the relationship in eq 6 is shown in Figure 7. The points constitute a straight line of slope unity as predicted by eq 6, and the value of 6.3 obtained for pK_{eq} from the intercept is in fair agreement with that obtained in the absence of enzyme at an ionic strength of 0.1 (Pocker and Bjorkquist, 1977; Harned and Davis, 1943).

Discussion

In considering the six different proposed mechanisms for CA, it is first necessary to establish that the conjugate acid of

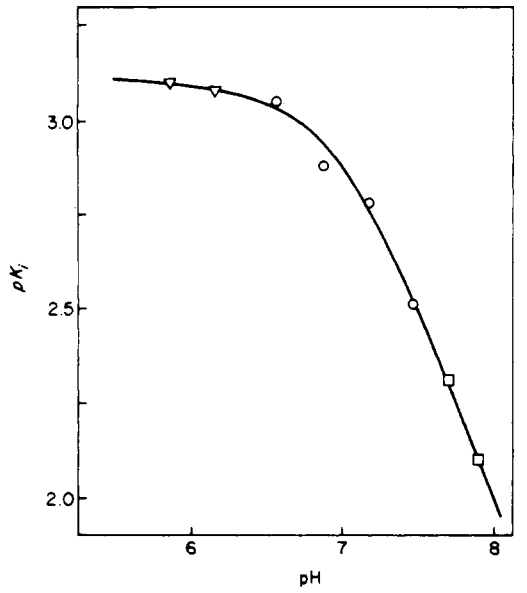
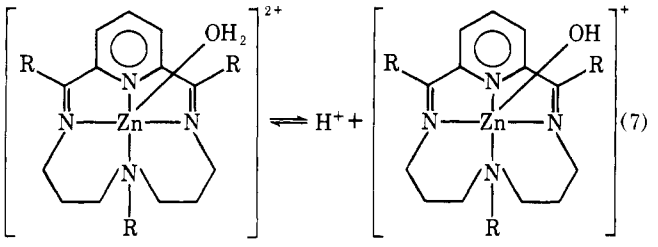


FIGURE 6: Plot of pK_i as a function of pH for the azide ion inhibition of the BCA-catalyzed dehydration of bicarbonate at t = 25.0 °C and I = 0.10. Buffers employed: (▽) 3-picoline; (○) imidazole; (□) 1,2-dimethylimidazole.

the necessary base does indeed ionize at pH 7. The acid in question for the three mechanisms grouped together in category I, Table I, is $\geq ZnOH_2$. Unfortunately, few, if any, reliable data are available for the acidity of aquotriamine zinc(II) complexes. About the closest any complex has come in mimicking the coordination of zinc in CA is the macrocyclic compound shown in eq 7 (Prince and Woolley, 1972; Woolley,



(7)

1975). A potentiometric titration revealed that there was only one acidic hydrogen and that its pK_a was 8.16, but it is still open to question whether the zinc-bound water in CA can attain a pK_a as low as seven.

The acid in the two mechanisms outlined under group II, Table I, is the imidazolium ion of a histidine residue. There is no question that its pK_a can be around seven. Indeed, the pK_a of the imidazolium ion of histidine residue 63 in human carbonic anhydrase C is 7.12 (Campbell et al., 1975). The mechanism in IIb, however, not only uses imidazole as the base, but it may also require that the zinc–aquo complex already be in its basic form before the imidazolium loses its proton. From what is known about the acidity of zinc–aquo complexes, an ionization at such a low pH seems unlikely. Moreover, when the catalytically important residue is protonated, the enzyme possesses no activity. Therefore, the zinc–hydroxo complex would have to be inactive toward CO_2; yet in contradistinction, it is known that cobalt–hydroxo complexes are highly active toward CO_2 (Chaffee et al., 1973; Bjorkquist, 1975; Pocker and Bjorkquist, 1977).

The acid utilized for the mechanism under group III is the zinc–imidazolate complex. There is no precedent in the literature for the ionization of this acid to occur around pH 7. In fact, Zn(histidine)$_2$ has been titrated and the pK_a was found

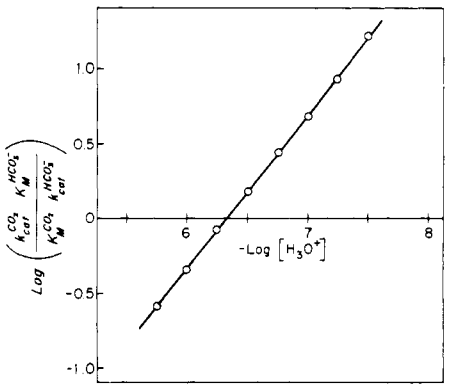


FIGURE 7: Haldane relationship for the BCA-catalyzed reversible hydration of CO_2. Data for k_{cat} taken from solid curves in Figures 2 and 4. A value of 15 mM was taken for $K_M^{CO_2}$ and 22 mM for $K_M^{HCO_3}$.

to be greater than 11.6 (Guilbert, 1971). Similarly, the pK_a values for both Co(II)(imidazole)$_4$ and Co(II)(L-histidine)$_2$ are 12.5 (Morris and Martin, 1970). Clearly, these results cast serious doubt as to whether zinc–imidazolate can be the active base in the enzyme at neutral values of pH.

Regardless of whether a nucleophile or a general base is the group controlling enzymic activity, all proposed mechanisms must first be able to explain the tremendous efficiency of the enzyme. If bicarbonate is the product of CO_2 hydration, eq 8, then the overall stoichiometry requires that the enzyme must end up protonated.

$$E + CO_2 + H_2O \rightleftharpoons EH^+ + HCO_3^- \qquad (8)$$

Consequently, before it can catalyze the hydration of another CO_2 molecule, it must first transfer its proton to the solvent. Similarly, in the dehydration reaction, the basic form of the enzyme must be supplied protons from the solvent. The point has been raised that these proton-transfer steps may actually be rate limiting (Koenig and Brown, 1972) and indeed recent evidence has suggested this to be so at low buffer concentrations (Silverman and Tu, 1975, 1976; Jonsson et al., 1976; N. Tanaka, unpublished observations). However, calculations have shown that buffer components, normally present in moderate concentration, can participate in the protonation/deprotonation of the enzyme and allow these processes to be safely below the diffusion-controlled limit (Prince and Woolley, 1973; Khalifah, 1973; Lindskog and Coleman, 1973). On the other hand, calculations utilizing carbonic acid as the substrate for dehydration (i.e., mechanism Ic, Table I) demonstrate that the value for k_{enz} exceeds the diffusion-controlled limit.[1] Normally, a calculation of this type would be sufficient evidence to dismiss mechanism Ic from further consideration. However, such a conclusion may be premature in this case. There exists the small, but finite, possibility that the pK_a of

[1] If carbonic acid is the substrate, then

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \xrightarrow[E]{k'_{enz}} H_2O + CO_2$$

Moreover, there exists the restriction that the calculated enzymatic velocity must keep pace with the empirically observed velocity, i.e.,

$$k'_{enz}[E][H_2CO_3] = k_{enz}[HCO_3^-][EH^+]$$

Therefore

$$k'_{enz} = k_{enz}\frac{[EH^+]}{[E]}\frac{[HCO_3^-]}{[H_2CO_3]} = \frac{k_{enz}K_a^{H_2CO_3}}{K_a^{EH}}$$

Taking $K_a^{H_2CO_3}$ as 2×10^{-4} (Wissburn et al., 1954), K_a^{EH} as 10^{-7}, and $k_{enz} = (k_{cat}/K_M)$ as 1.7×10^7 M^{-1} s^{-1}, then $k'_{enz} = 3 \times 10^{10}$ M^{-1} s^{-1} which exceeds the diffusion-controlled limit (Eigen, 1965).

TABLE II: Solvent Deuterium Isotope Effects.

Substrate	Catalyst[a]	Product[b]	Parameter	Units	Ratio H_2O/D_2O[c]
CO_2[d]	H_2O	$HCO_3^- + H_3O^+$	k_0	s^{-1}	1.8
	OH^-	HCO_3^-	k_{OH^-}	$M^{-1}\ s^{-1}$	0.63
	$Co(NH_3)_5OH^{2+}$	$Co(NH_3)_5OCO_2H^{2+}$	$k_{Co(III)OH}$	$M^{-1}\ s^{-1}$	1.0
	BCA	$HCO_3^- + H_3O^+$	k_{cat}	s^{-1}	3.3
			K_M	M	3.0
			k_{cat}/K_M	$M^{-1}\ s^{-1}$	1.1
HCO_3^-[d]	H_2O	$OH^- + CO_2$	k_0	s^{-1}	2.0[e]
	H_3O^+	$H_2O + CO_2$	$k_{H_3O^+}$	$M^{-1}\ s^{-1}$	0.56
$Co(NH_3)_5CO_3^+$[f]	H_3O^+	$Co(NH_3)_5OH_2^{3+} + CO_2$	$k_{Co(III)CO_3}$	$M^{-1}\ s^{-1}$	1.1
HCO_3^-	BCA	$H_2O + CO_2$	k_{cat}	s^{-1}	4.3
			K_M	M	2.6
			k_{cat}/K_M	$M^{-1}\ s^{-1}$	1.65
CH_3CHO[g]	BCA	$CH_3CH(OH)_2$	k_{cat}/K_M	$M^{-1}\ s^{-1}$	1.5-1.6
p-Nitrophenylpropionate[h]	BCA	CH_3CH_2COOH + *p*-nitrophenol	k_{cat}/K_M	$M^{-1}\ s^{-1}$	0.64-0.67

[a] Catalyst formula in H_2O. [b] Product formula in H_2O. [c] These ratios are unitless; values are reported at 25.0 °C, except where indicated otherwise. [d] $K_{eq}^{H_2O} = [HCO_3^-][H_3O^+]/[CO_2] = k_0^{CO_2(H_2O)}/k_{H_3O^+}^{HCO_3^-} = 2.9 \times 10^{-2}\ s^{-1}/4.1 \times 10^4\ M^{-1}\ s^{-1} = 7.1 \times 10^{-7}$ M at 25.0 °C, and ionic strength 0.10; $K_{eq}^{D_2O} = [DCO_3^-][D_3O^+]/[CO_2] = k_0^{CO_2(D_2O)}/k_{D_3O^+}^{DCO_3^-} = 1.6 \times 10^{-2}\ s^{-1}/7.3 \times 10^4\ M^{-1}\ s^{-1} = 2.2 \times 10^{-7}$ M at 25.0 °C, and ionic strength 0.10; $K_{eq}^{H_2O}/K_{eq}^{D_2O} = 3.23$; $pK_{eq}^{D_2O} = pK_{eq}^{H_2O} + 0.51$. [e] Taken from Pocker and Davison (1977); value obtained using Magid's (1968) pH-stat technique with N_2 flow at 1.4 L per min. [f] Carbonato complex rather than HCO_3^- is the real substrate. [g] At 0.0 °C (Pocker and Meany, 1965). [h] Ratio depends upon the detailed analysis of the values in the plateau region before the incursion of the second rise in activity at high pH (Pocker and Storm, 1968). With *p*-nitrophenyl acetate, one obtains $k_{enz}^{H_2O}/k_{enz}^{D_2O}$ values ranging between 0.65 and 0.9 depending upon the detailed analysis of the plateau region (Pocker and Stone, 1967).

carbonic acid is not 3.8 in the active site of the enzyme as assumed in the calculation. If, due to a partially hydrophobic environment, the acidity were to be reduced by only a factor of three to four, this would be just enough to circumvent the diffusion difficulties.

The discussion up to this point has shown that mechanisms IIb and IIIa are unlikely from the standpoint of having to suggest extremely anomalous pK_a values for an acid in the active site and that mechanism Ic may be unlikely for having to invoke carbonic acid as an intermediate in the enzymatic pathway. That leaves mechanisms Ia, Ib, and IIa as the most reasonable possibilities, and it is conceivable that mechanism Ia can be distinguished from the other two by considering the solvent deuterium isotope effect on k_{cat}. Since the active base in mechanism Ia functions as a nucleophile, the ratio, $k_{cat}^{H_2O}/k_{cat}^{D_2O}$, for the hydration of CO_2 should be about unity barring an unusually large secondary isotope effect. On the other hand, the active base in mechanism Ib and IIa functions as a general base to facilitate proton transfer via at least one water molecule. Hence, the solvent deuterium isotope effect on k_{cat} should be substantially greater than unity, since a proton is being transferred in the turnover step. The ratio, $k_{cat}^{H_2O}/k_{cat}^{D_2O}$, for the hydration of CO_2 was found to be 3.3 (Figure 2). A value of this magnitude seems to be clear evidence that a proton transfer is occurring during the turnover step. As further proof, the uptake of CO_2 by the conjugate base of the metal complex $Co(NH_3)_5OH_2^{3+}$ was followed both in H_2O and D_2O (Table II; see also, Pocker and Bjorkquist, 1977). This complex serves as a reasonably good model for the enzymatic mechanism Ia, because it is known that $Co(NH_3)_5OH^{2+}$ directly attacks CO_2 to form $Co(NH_3)_5CO_3H^{2+}$ (Dasgupta and Harris, 1968). The solvent deuterium isotope effect of unity indicates that there is not a significant secondary isotope effect for the nucleophilic attack of a metal-hydroxo complex on a CO_2 molecule. Therefore, mechanism Ia cannot be operating in the hydration of CO_2. An identical conclusion can be reached by comparing the isotope effect for the decarboxylation of $Co(NH_3)_5CO_3H^{2+}$ with that for the enzyme-catalyzed dehydration of bicarbonate. Perhaps this comparison is even more valid since both the chemical and enzymatic isotope effects involve ratios of first-order rate constants.

There are two differences between mechanisms Ib and IIa that may enable them to be distinguished. First, it should be noted that the location where bicarbonate binds is different in the two mechanisms. Whereas bicarbonate is positioned near the zinc in mechanism Ib, it is directly coordinated to the zinc in mechanism IIa. Coleman has obtained both the visible and ORD absorption spectra of Co(II)CA in the presence and absence of HCO_3^- (Coleman, 1965). His work indicates that these spectra are not perturbed nearly as much by HCO_3^- as by other anions, such as CN^- and acetazolamide, which are known to coordinate directly to the metal. Nevertheless, since HCO_3^- was able to alter the d orbitals surrounding the metal, Coleman felt that it must be bound within the metal's primary coordination sphere. The reason the various anions cause different effects can be explained by the spectrochemical series: $OH^- < HCO_3^- < CN^-$. Recent nuclear magnetic resonance (NMR) work by Lanir and Navon on acetate binding has demonstrated that two acetates can bind in the active site of CA, but that only one of the bound acetates inhibits the enzyme (Lanir and Navon, 1974a,b). Moreover, the distances between the metal in Mn(II)BCA and the methyl group of the acetate ion calculated using longitudinal and transverse relaxation times were 4.3 and 4.8 Å for the acetate bound to the inhibitory and noninhibitory sites, respectively. These distances are consistent with a model in which the inhibitory acetate binds directly to the metal through one of its negatively charged oxygens and the noninhibitory binds at some other site. Since acetate is quite similar in shape to bicarbonate, the intriguing possibility exists that two bicarbonates may also be binding in the active site: one to the metal and the other nearby. Evidence that this might be the case has been recently presented using ^{13}C NMR (Yeagle et al., 1975; Stein et al., 1977). The authors claim that two bicarbonates do indeed bind to CA, one of which is apparently coordinated to the zinc and the other which is separated from the metal by perhaps one water molecule. This raises the point as to which one of these sites is the "true" binding site for bicarbonate turnover.

The Michaelis constant, K_M, for HCO_3^- is essentially

TABLE III: Maximum Solubility of CO_2 in Various Solvents.

Solvent	Temp (°C)	$n_{CO_2}{}^a \times 10^4$	$M_{CO_2}{}^b \times 10^2$	$\chi_{CO_2}{}^c \times 10^4$	$\chi_{CO_2} \times 10^4$ lit. value[d]
H_2O	25	6.1	3.38	6.3	6.1
	0	14	7.77	14.1	
D_2O	25	6.9	3.81	7.1	
Acetone	25	143	20	209	207
	0	294			
Butanone	25	141	16	160	
3-Pentanone	25	148	14	152	
Acetophenone	25	120	10	120	
Propanal	0	154			
Formamide	25	64	16	64	
N-Methylformamide	25	82	14	82	
N,*N*-Dimethylformamide	25	140	18	140	
N,*N*-Dimethylacetamide	30	113	12	113	
Me_2SO	25	97	14	97	
Carbon tetrachloride	25	89	9.2	104	107
Toluene	25	82	9.1	85	

[a] n_{CO_2} is the mole fraction of CO_2 obtained by bubbling CO_2 through the solvent at atmospheric pressure. [b] M_{CO_2} is the molarity of a CO_2-saturated solution calculated from n_{CO_2}. [c] χ_{CO_2} is the mole fraction of CO_2 when its partial pressure is 1 atm. χ_{CO_2} was calculated from n_{CO_2} knowing the vapor pressure of the solvent at the desired temperature. [d] Hildebrand and Scott, 1964.

constant over the pH range 5.5 to 8.0, Figure 3. The independence of $K_M{}^{HCO_3^-}$ with pH is surprising, since the binding of another anion, azide, has a marked pH dependence, Figure 6. This apparent paradox, however, results from the different methods used to evaluate K_i and K_M. The inhibition constant, K_i, is truly a measure of the inhibitor's ability to bind to and inactivate the enzyme. The pH dependency simply arises because azide binds to the acidic and basic forms of the enzyme with different affinities. On the other hand, K_M was evaluated from kinetic data by the method of Lineweaver and Burk. In contrast to K_i, it is possible to observe a pH-independent K_M even though the substrate is binding to the acidic and basic form of the enzyme with a different affinity (Bjorkquist, 1975).

Clearly, the available data do not allow mechanisms Ib and IIa to be distinguished; yet the results from the solvent isotope effects strongly suggest that proton transfer is accompanying the enzymatic interconversion of CO_2 and HCO_3^-. Consequently, only those mechanisms which utilize proton transfer as part of the rate-determining step can be correct.

The value of pk_{enz}, $\Delta pk_{enz} = pk_{enz}{}^{D_2O} - pk_{enz}{}^{H_2O}$, is about 0.5 unit larger in D_2O than in H_2O. The observation that acids are weaker in deuterium oxide than they are in ordinary water has been exemplified before (McDougall and Long, 1962; Glasoe, 1965; Robinson et al., 1969) and it is in this respect that the enzyme appears to exhibit a normal solvent deuterium isotope effect for an acid having a pK_a around neutrality. A Δpk_{enz} value of 0.55 has been reported earlier both for the enzymatic hydration of acetaldehyde (Pocker and Meany, 1965) as well as for the enzymatic hydrolysis of *p*-nitrophenyl esters (Pocker and Stone, 1967; Pocker and Storm, 1968). Furthermore, the catalytic efficiency of bovine carbonic anhydrase, expressed in terms of k_{enz}, $k_{enz} = k_{cat}/(K_M + [S]) \simeq k_{cat}/K_M$, is not as greatly affected by a change of solvent from ordinary water to D_2O (Table II).

On the other hand, the interpretation of the isotope effect associated with the pH-independent Michaelis-Menten parameter, K_M, is rather difficult. There is still no clear-cut evidence as to where the CO_2 molecule fits into the active site. Many observations suggest (see in particular Notstrand et al., 1975) that the active site of carbonic anhydrase involves a combination of polar and hydrophobic regions. In this respect, our CO_2 solubility measurements in both H_2O and D_2O (Table III) indicate that the K_M values derived from kinetic measurements must represent a rather complex situation. Indeed, while CO_2 is somewhat more soluble in D_2O than in H_2O, $\chi_{CO_2}{}^{D_2O}/\chi_{CO_2}{}^{H_2O} = 7.1 \times 10^{-4}/6.3 \times 10^{-4} = 1.13$, a very large solvent deuterium isotope effect is exhibited by the Michaelis-Menten parameter K_M, $K_M{}^{H_2O}/K_M{}^{D_2O} = 3.0$. Similarly, for bicarbonate dehydration we find, $K_M{}^{HCO_3^-}/K_M{}^{DCO_3^-} = 2.6$. These large solvent deuterium isotope effects cannot be easily rationalized.[2] In addition, we are faced with the observation that K_M for dehydration is independent of pH in the region of the pK_a of the catalytic group, Figure 4 (Bjorkquist, 1975; Steiner et al., 1975).

The possibility exists that an internally located group, B_i or B_iH^+, may be involved in the dynamics of proton transport between the zinc-coordinated H_2O or OH^- and the buffer system of the medium (eq 9).

$$ZnOH_2|B_i|B \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} ZnOH|B_iH^+|B \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} ZnOH|B_i|BH^+ \quad (9)$$

Some of the dynamic advantages associated with the coexistence of two such ionizing groups, one catalytic—the other acting as a general acid-general base relay system—have been discussed earlier in papers from our laboratory (Pocker and Meany, 1965; Pocker and Storm, 1968; Pocker and Guilbert, 1972, 1974; Pocker and Bjorkquist, 1975). More recently, Steiner et al. (1975) have used an extended scheme to argue in favor of a complete separation of catalytic and proton-transfer roles. Using separate catalytic and proton-transfer steps, they were able to show that if stage a is rate limiting, a fairly complex expression can be derived leading to $K_M{}^{HCO_3^-}$ values which to a first approximation are independent of pH. Furthermore, the proton relay depicted by eq 9 occurs in two

[2] The most serious uncertainty in the interpretation of these isotope effects arises from the possibility that the solvent change from H_2O and D_2O changes the properties of hydrogen bonds, hydrophobic interactions, and other factors (Jencks, 1969). These effects are individually small but they may combine or be amplified to cause sufficiently large overall solvent deuterium isotope effects to be mistaken for proton-transfer catalysis (Schowen, 1975; Kresge, 1975). Clearly, solvent changes can affect enzyme conformation (Hermans and Scheraga, 1959) as well as intramolecular isomerization rates (Cathou and Hammes, 1965; French and Hammes, 1965).

distinct stages: an intramolecular stage a and an intermolecular stage b. Recent studies show that at low buffer concentrations, stage b becomes partially rate limiting (Silverman and Tu, 1975, 1976; Jonsson et al., 1976; Pocker and Tanaka, submitted for publication). Reaction mechanisms are elucidated in successive approximations; the relative timing of proton relay and of bond changes associated with hydration-dehydration should represent the next major stage in the study of carbonic anhydrase catalysis.

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Polypeptides in the Succinate–Coenzyme Q Reductase Segment of the Respiratory Chain†

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ABSTRACT: Complex II (succinate–coenzyme Q reductase) was resolved into ten different polypeptides by polyacrylamide gel electrophoresis. Four polypeptides, C_{II-1}, C_{II-2}, C_{II-3}, and C_{II-4} with molecular weights of 70 000, 24 000, 13 500, and 7000, were present in large amounts in all preparations examined. C_{II-1} and C_{II-2} are the flavoprotein and iron–sulfur protein, respectively, of succinate dehydrogenase; C_{II-3} and C_{II-4} have not been functionally identified. Six polypeptides were present in much smaller amounts as judged by staining intensity, and each of these comigrated with components in complex III. The amino acid compositions of several of the minor components in complex II were identical with that of an equivalently migrating polypeptide in complex III. We conclude that succinate–coenzyme Q reductase contains four different polypeptides and is contaminated with variable amounts of complex III when isolated as complex II.

Succinate–coenzyme Q reductase or complex II provides one of the two major entry points for electrons into the cytochrome containing portion of the electron transfer chain (for review, see Hatefi, 1976). The question of how many components function in transferring electrons from succinate via coenzyme Q to cytochrome *b* and the structural relationship between these components and the bc_1 segment of the respiratory chain remain obscure. Complex II but not purified succinate dehydrogenase will recombine with purified complex III to reconstitute succinate cytochrome *c* reductase activity (Davis and Hatefi, 1971a,b). This indicates that there are components in complex II other than succinate dehydrogenase which are required for efficient electron transfer from succinate to oxygen. However, these components have not been identified nor their role established. As a first step in this direction we have examined the polypeptide composition of complex II and here identify two polypeptides as likely candidates for the functional and/or structural link between succinate dehydrogenase and the cytochrome portion of the respiratory chain.

Experimental Procedure

Complex II was prepared from beef heart mitochondria as described by Ziegler and Rieske (1967). Protein concentrations were determined by the method of Lowry et al. (1951). Heme *b* and c_1 concentrations were determined as described by Williams (1964). Flavin analysis was performed as described by Singer et al. (1971).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed as described by Weber and Osborn (1969) and by Swank and Munkres (1971). Two-dimensional gel electrophoresis was performed as described by Smith and Capaldi (1977), using the Weber–Osborn buffer system in the first dimension and the Swank–Munkres system in the second dimension.

For preparative gel electrophoresis, a slab gel (0.5 cm × 15 cm × 12 cm) of 10% acrylamide and 0.67% bisacrylamide was used. Complex II (8 mg), dissociated in 2% sodium dodecyl sulfate, 1% β-mercaptoethanol, 4 M urea by boiling for 1 min, was applied to the top of the gel in a volume of 500 μL and the sample was electrophoresed through the gel in the Swank–

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