PROTON TRANSFER IN THE CATALYTIC MECHANISM OF CARBONIC ANHYDRASE

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I. INTRODUCTION

The goal of this review is to answer three crucial and timely questions concerning the catalysis by carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) of the hydration of CO_2 and dehydration of bicarbonate.

1. What is the role of intermolecular proton transfer in the catalysis?
2. What is the rate-limiting event in the catalytic pathway?
3. What is the mechanism of interconversion of CO_2 and HCO_3^-?

That such questions remain, in significant part, unsettled for an enzyme discovered a half-century ago[1] is a reflection of the difficulty in assigning specific roles in the catalysis to residues, the position and chemical properties of which are known. Moreover, this comment is made for an enzyme which has a simple substrate, CO_2, and catalyzes a reaction which is relatively straightforward, the hydration of CO_2 to form a proton and bicarbonate.

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

The unifying theme of this review is the role of proton transfers, which are becoming recognized as important and sometimes rate-limiting steps in the catalytic pathway of carbonic anhydrase. With a turnover number that is one of the largest known for any enzyme (as great as 10^6 sec^{-1}), the hydration of CO_2 catalyzed by carbonic anhydrase has a mechanism in which intra- and inter-molecular proton transfers appear to be rate limiting. This provides a very useful opportunity to study the nature of these proton transfer processes which might then help to understand such processes in other enzymes.

We are pleased that the scope of this review can be narrowed significantly because of many excellent reviews which have already been written. Accordingly, we have emphasized the most recent advances in the three crucial questions listed above. Pocker and Sarkanen[2] have reviewed extensively the catalytic properties of the two isozymes of red cell carbonic anhydrase with a useful emphasis on the ability of these isozymes to catalyze the hydrolysis of certain esters, cyclic sulfonates, and the hydration of a variety of aldehydes. A review by Lindskog[3] stresses the function of the zinc, which is an essential component of this enzyme, and the properties of many metallocarbonic anhydrases

which result when zinc is replaced by other metals. The spectroscopic properties of many metallocarbonic anhydrases are reviewed and related to structural features of the enzymes by Bertini et al.[4] Several earlier reviews stress structural and chemical properties of carbonic anhydrase.[5-10] Tashian[11] has written an excellent review comparing the isozymes of carbonic anhydrase and tracing the evolutionary origins of some mammalian carbonic anhydrases. The best review of the physiological function of carbonic anhydrase is that of Maren which remains unexcelled in scholarship and scope.[12]

A. Distribution

Carbonic anhydrase is immensely widespread in nature occurring in animals (vertebrates and invertebrates), higher plants, algae, and bacteria. All of the known physiological functions of this enzyme are related to the hydration of CO_2 to form bicarbonate and a proton and the reverse reaction, the dehydration of bicarbonate: in red cells the role of carbonic anhydrase is in respiration;[13] the production and transport of bicarbonate and protons plays a role in secretory tissues such as the ciliary body of the eye, the salivary glands, the choroid plexus, the gastric mucosa, and the renal tubules;[12] carbonic anhydrase is involved in the calcification of shells of birds; and this enzyme plays a role in photosynthesis.

Three genetically distinct isozymes of carbonic anhydrase have been discovered and designated I, II, and III, superseding the older designations of B (for I) and C (for II) and muscle (for III). Complete amino acid sequences are available for isozyme I from humans,[14-16] rhesus monkey,[17] horse,[17a] and cattle,[18] and for isozyme II from humans,[19,20] cattle,[21] sheep,[22] horse,[22a] and rabbit.[23] The sequence for the more recently discovered isozyme III from cattle is also known.[18] The high degree of homology between isozyme I and II[11] and their very similar structures as determined by X-ray diffraction[24] certainly indicates a common ancestral gene. Tashian,[11,18,24a] in fact, discusses evidence that the gene duplication leading to separation of isozymes I, II, and III occurred at least 300 million years ago.

Carbonic anhydrase I is found in the red cells of man, where it is the second most abundant protein in erythrocytes. It is also found in primates and rodents, but not in the red cells of ruminants and felines. The predominate form of carbonic anhydrase in secretory tissue is isozyme II.[12] Moreover, isozyme II is present in all mammalian red cells.

Siffert and Gros[24b] have found that white skeletal muscle of the rabbit contains carbonic anhydrase with the kinetic and inhibition properties of type II isozyme. Carbonic anhydrase III has been found in the cytoplasm of skeletal muscle of man, rabbit, sheep, ox, and other species.[25-31,42] Carter et al.[32] have discovered that the sulfonamide insensitive carbonic anhydrase in the livers of male rats is indistinguishable from isozyme III.

All of these isozymes have a molecular weight near 30,000; isozyme I and II are monomers but isozyme III can dimerize through formation of a disulfide bond.[30] There is one zinc atom per monomer which is essential for catalysis of CO_2 hydration. However, the isozymes differ in activities,[2,42] sulfonamide and binding affinities,[33,42] and immunologic properties.[34]

B. Structure

The crystal structures of human carbonic anhydrase II[35] and human carbonic anhydrase I[36] have been determined to about 2 Å resolution. These structures have been compared, particularly in the active site region.[37,38] A schematic drawing of the tertiary structure of carbonic anhydrase is given in Figure 1; a central, twisted, pleated sheet of 10 chain segments comprises about one third of the total number of residues (259 total residues in human type II and 260 in human type I). ORD, CD, and Raman scattering

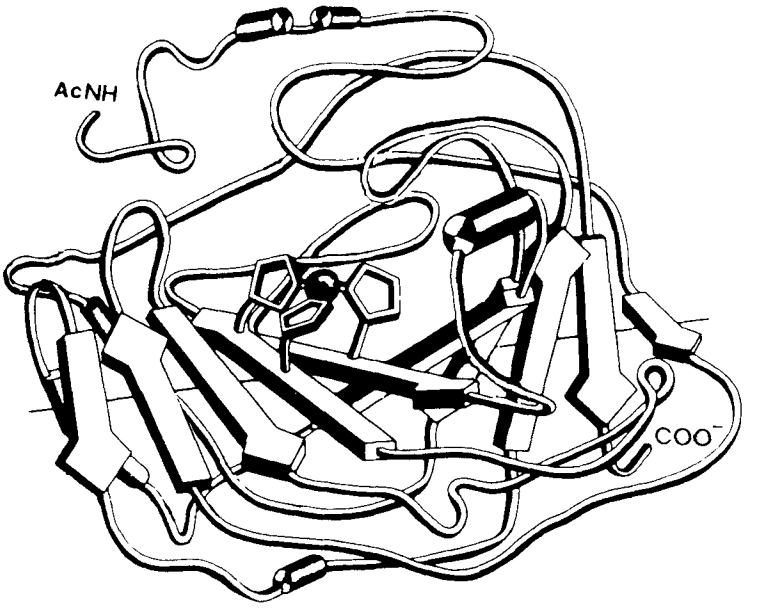


FIGURE 1. The secondary and tertiary structure of carbonic anhydrase. The cylinders designate helices and the arrows β-structure. The sphere in the center is a zinc ion bound to three histidyl side-chains. (From Kannan, K. K., Nostrand, B., Fridborg, K., Lövgren, S., Ohlsson, A., and Petef, M., *Proc. Natl. Acad. Sci. U.S.A.*, 72, 51, 1975. With permission.)

studies[39,40] confirm about 40% β-pleated sheet and 20% helix in both the type I and II human isozymes which are highly homologous in structure. These isozymes share 159 identical amino acid residues (61% of total); those residues not identical are mostly similar in polarity and hydrophobic character. The essential zinc is located at the bottom of a conical cavity about 12 Å deep and 10 Å wide at the surface of the enzyme. The ligands of zinc are the same in type I and type II isozymes. Histidines 94 and 96 are coordinated through their N_τ atoms, while histidine 119 is coordinated through N_π.[24] The three histidine ligands are involved in a hydrogen-bonding network as shown in Figure 2, and being neutral do not compensate the positive charge on the Zn^{2+} ion. This is in contrast to other zinc-containing enzymes such as carboxypeptidase and liver alcohol dehydrogenase in which one of the protein ligands is a negatively charged side chain. A useful comparison of the geometry of the zinc-binding centers of these enzymes is presented by Argos et al.[41] A fourth coordination site on the zinc, which has a tetrahedral geometry, is occupied by water or possibly a hydroxide ion. There has been increasing discussion of a fifth coordination site also,[3] which we discuss in Section IV.

Residues in the active site cavity can be divided into a hydrophobic region and a hydrophilic region as is done in Table 1. Some of these residues are shown in Figure 2. Histidine 64, which is well suited to participate in proton transfer because of its pK_a, is present in all type I and II isozymes sequenced to date, but this position is occupied by a lysine in bovine type III carbonic anhydrase.[18] Type I isozymes which have been sequenced have two more histidines near the active site, at positions 67 and 200, which are not present in type II (Table 1).

An exception is isozyme I from the horse which has Gln 67.[17a] These histidines make the active site cavity somewhat smaller for the type I compared to type II enzymes. Khalifah[43] has identified a strong interaction of His 200 with the ligands of zinc. This suggests that His 200 may influence the binding of HCO_3^- to the active site in type I and

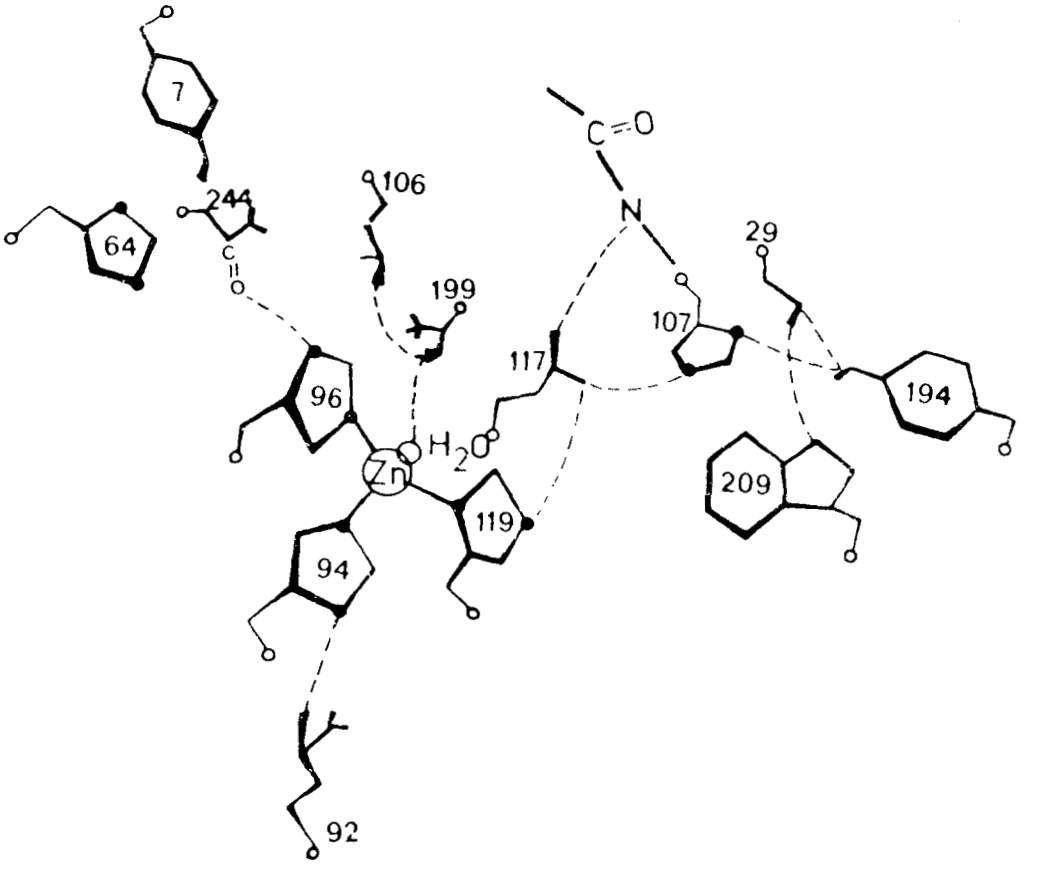


FIGURE 2. A schematic drawing of the side chains and hydrogen-bonding near the zinc in the crystal structure of human red cell carbonic anhydrase I. (From Kannan, K. K., Nostrand, B., Fridborg, K., Lövgren, S., Ohlsson, A., and Petef, M., *Proc. Natl. Acad. Sci. U.S.A.*, 72, 51, 1975. With permission.)

possibly account for some of the difference in activity between I and II isozymes.[43] Other invariant residues are Glu 106 which is hydrogen bonded in the X-ray structures to Thr 199 which is, in turn, hydrogen bonded to the water ligand of the zinc.[37] Threonine 199 appears to be the only residue in hydrogen bond contact with the water ligand of zinc and may have a role in proton transfer in the catalysis.[44]

The zinc ion can be removed from carbonic anhydrase by dialysis using metal complexing agents,[45-47] and replaced by a number of divalent metal ions and also Co^{3+} and VO^{2+}. The order of decreasing stability of the complexes formed is: $Hg >> Cu > Zn > Cd,Ni > Co > Mn$ for human type I.[46] Difference electron density studies of the complexes in which Co^{2+}, Cu^{2+}, Mn^{2+}, and Hg^{2+} replace zinc in human carbonic anhydrase II showed that these metals, except Hg^{2+}, bind at the same position as Zn^{2+}. Hg^{2+} is displaced about 0.8 Å from the zinc site and the geometry around the Hg^{2+} is changed to that of a distorted octahedron (two chloride ions are also ligands of Hg^{2+}).[48] Other than the native enzyme, significant catalytic activity is found with Co(II)-carbonic anhydrase which has both k_{cat} and K_m for hydration about 20% of that found for native enzyme.[49] The high pH form of Cd(II)-carbonic anhydrase I has about 30% of the activity of its native counterpart in the hydrolysis of *p*-nitrophenylacetate.[50] The apoenzyme is completely inactive and has a structure very similar to the native enzyme according to spectroscopic studies.[40,51-53]

Table 1
ACTIVE SITE RESIDUES IN HUMAN CARBONIC ANHYDRASE I AND II

Hydrophilic region		Hydrophobic region	
HCA I	HCA II (if different)	HCA I	HCA II (if different)
TYR 7		SER 65	ALA 65
ASN 61		PHE 91	ILE 91
HIS 64		ALA 121	VAL 121
HIS 67	ASN 67	LEU 131	PHE 131
ASN 69	GLU 69	LEU 141	
GLU 92		VAL 143	
HIS 94		GLY 145	
HIS 96		PRO 201	
HIS 119		CIS-PRO 202	
THR 199		THR 204	LEU 204
HIS 200	THR 200	SER 206	CYS 206 (shielded)
		VAL 207	
		ILE 211	VAL 211

From Kannan, K.K., in *Biophysics and Physiology of Carbon Dioxide*, Bauer, C., Gros, G., and Bartels, H., Eds., Springer-Verlag, New York, 1980, 184. With permission.

C. Activity

We concentrate here on the catalyzed hydration of CO_2 and dehydration of bicarbonate since this reaction, of the reactions catalyzed by carbonic anhydrase, is the most rapid and the most taxing on proton transfer. In addition to the hydration of CO_2 and dehydration of HCO_3^-, carbonic anhydrase also catalyzes a number of other reactions which have in common the apparent addition of hydroxide to a carbon-oxygen double bond or an analogous structure. Such reactions are listed rather completely by Pocker and Sarkanen[2] and include the hydration of aliphatic aldehydes, the hydrolysis of aryl carboxylate esters, and the hydrolysis of alkyl, aryl, and diaryl carbonate esters. A few kinetic constants are compared in Table 2. The pH-rate profiles for these reactions are similar to the catalyzed hydration of CO_2 in that all appear to depend predominantly on the ionization of a single residue with a pK_a near 7 under conditions of physiological ionic strength. This statement must be qualified by many comments concerning the influence of secondary ionizing groups which have an effect on the catalysis of CO_2 hydration by the type I isozymes,[54] on the hydrolysis of *p*-nitrophenylacetate by type II in the absence of inhibitory ions,[55] and on the esterase function of bovine carbonic anhydrase for a variety of substrates.[2,56] It is also important to point out that the apparent pK_a of the activity-controlling group depends on the particular isozyme studied, the identity of the metal at the active site, and the presence of anions. Many monovalent anions inhibit carbonic anhydrase and shift the pK_a to higher values.

Table 2 shows a simple comparison of kinetic constants for type I, II, and III isozymes. The Michaelis parameters for the hydration of CO_2 and dehydration of HCO_3^- by bovine red cell carbonic anhydrase, a type II isozyme, are given in Figures 3 and 4. The active site structure and kinetic properties of bovine red cell carbonic anhydrase II are very similar to those of human isozyme II.[2,3] The influence of the activity-controlling group on k_{cat} for hydration is apparent; K_m is independent of pH. The inverse relationship between the pH profiles for k_{cat} for hydration and k_{cat} for dehydration results from the fact that a proton is

Table 2
COMPARISON OF THE HYDRATION OF CO_2 AND ACETALDEHYDE, AND HYDROLYSIS OF *p*-NITROPHENYLACETATE CATALYZED BY CARBONIC ANHYDRASE

Enzyme	Substrate	pH	k_{cat}	K_m
			sec^{-1}	m*M*
Human carbonic anhydrase I[a]	CO_2	7.4	4×10^4	4
Human carbonic anhydrase II[a]	CO_2	7.4	7×10^5	9
Feline carbonic anhydrase III[b]	CO_2	7.5—7.6	4.2×10^3	37
Bovine carbonic anhydrase II[c]	*p*-Nitrophenyl acetate	7.6	7	12
Bovine carbonic anhydrase II[d]	Acetaldehyde	7.2	800	650

[a] Khalifah;[54] 0.2 ionic strength, 25°C.
[b] Sanyal *et al.*,[42] 0.005 to 0.01 ionic strength, 25°C.
[c] Pocker and Sarkanen;[2] aqueous 10% (v/v) acetone, 0.10 ionic strength, 25°C.
[d] Pocker and Dickerson;[89] ionic strength 0.01 to 0.1, 0.0°C.

a substrate in the dehydration reaction. The maximal value of k_{cat}/K_m for hydration catalyzed by carbonic anhydrase II is near 10^8 $M^{-1}sec^{-1}$, a value approaching diffusion control. The rate constants for the association of small molecules, inhibitors and proton-transfer agents, with carbonic are in the range of 10^8 to 10^9 M^{-1} sec^{-1}.[57,58] These values can be compared to the rate constant for the uncatalyzed reaction between OH^- and CO_2 of 8.5×10^3 $M^{-1}sec^{-1}$; the uncatalyzed reaction between CO_2 and H_2O proceeds with a rate constant of 3.5×10^{-2} sec^{-1} at 25°C.[59]

II. THE ROLE OF INTERMOLECULAR PROTON TRANSFER IN THE CATALYSIS

A. The Dilemma: Diffusion-Limited Rates and an Unusually Rapid Catalysis

A dilemma concerning the identity of the products of the hydration of CO_2 in the catalyzed reaction arose when it was necessary to explain the large turnover number for catalytic hydration, as great as 10^6 sec^{-1} for carbonic anhydrase II. The problem was to identify the products as carbonic acid or bicarbonate and a proton. A direct solution to the problem was not possible because of the very rapid ionization of H_2CO_3 and because H_2CO_3 is not a significant fraction of all CO_2 species at any pH at equilibrium. The dilemma was that both carbonic acid as product and bicarbonate and a proton as products appeared to be inconsistent with the known maximum for bimolecular rate constants describing the diffusion-limited encounter between enzyme and substrate.

Considering carbonic acid as the substrate for the dehydration involves the reaction of a neutral substrate to yield neutral products and requires no ionization change on the enzyme:

$$E + H_2CO_3 \rightleftharpoons E + CO_2 + H_2O \quad (1)$$

This view of the catalysis is still consistent with the observed inverse relation of the pH

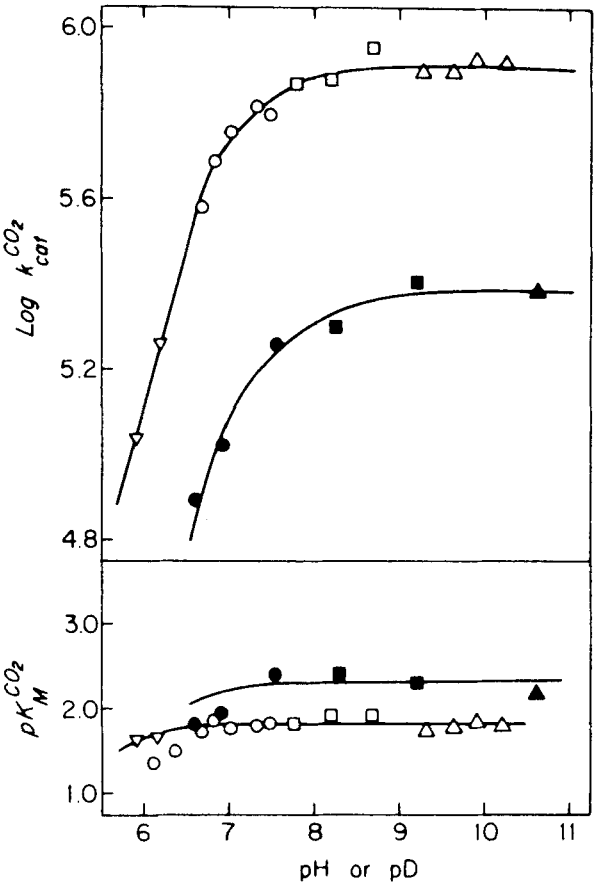


FIGURE 3. The pH dependence of k_{cat} and K_m for the hydration of CO_2 catalyzed by bovine red cell carbonic anhydrase (type II isozyme) in H_2O (open symbols) and D_2O (filled symbols). Temperature was 25°C and ionic strength was maintained at 0.1 with Na_2SO_4. The following buffers were present at concentrations between 0.02 and 0.05 *M*: (▽) 3-picoline; (○) phosphate; (□) 1,2-dimethylimidazole; and (△) *N,N*-dimethylglycine. (From Pocker, Y. and Bjorkquist, D. W., *Biochemistry*, 16, 5698, 1977. With permission.)

profile for hydration and dehydration described in Figures 3 and 4 since H_2CO_3 is not observed directly as a substrate or product. In the pH range in which this catalysis is usually observed, pH 6 to 9, HCO_3^- is the predominant species and the pH dependence of its concentration is the inverse of that of H_2CO_3.

But from another point of view the catalysis as described by Equation 1 is unacceptable because it requires the bimolecular rate constant for the association of enzyme and carbonic acid to exceed known diffusion-controlled limits. This was first recognized by DeVoe and Kistiakowsky[60] and described further by Khalifah[61] both of whom used the following argument. Assuming that the substrate in dehydration is carbonic acid, the value of $k_{cat}^{H_2CO_3}/K_m^{H_2CO_3}$ would be $5 \times 10^{10}\ M^{-1}\ sec^{-1}$ based on kinetic experiments using the type II isozyme of carbonic anhydrase. This is a lower limit for the rate constant of association of enzyme and H_2CO_3 considered as substrate[62] and exceeds that which can be expected for a diffusion-controlled reaction between H_2CO_3 and enzyme as calculated

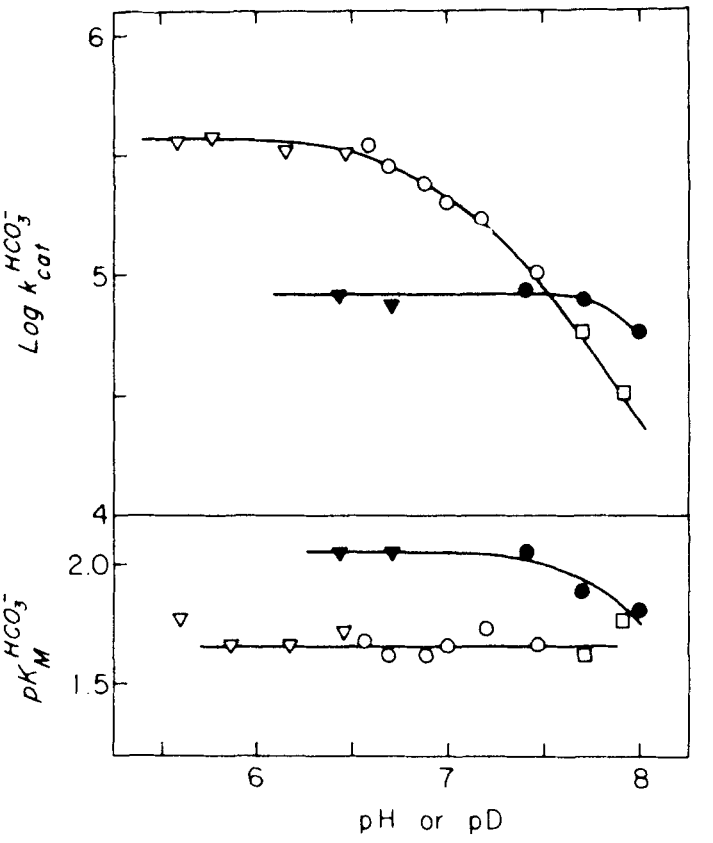


FIGURE 4. The pH dependence of k_{cat} and K_m for the dehydration of HCO^-_3 catalyzed by bovine red cell carbonic anhydrase in H_2O (open symbols) and D_2O (filled symbols). The following buffers were present at concentrations between 0.02 and 0.05 *M*: (▽) 3-picoline; (○) imidazole; (□) 1,2-dimethylimidazole. Other conditions as in Figure 3. (From Pocker, Y. and Bjorkquist, D. W., *Biochemistry*, 16, 5698, 1977. With permission.)

from the model of Alberty and Hammes[63] which assumes diffusion of substrate into a hemispherical cavity without electrostatic interactions. Assuming a reaction distance of 5Å, the distance between substrate and enzyme when reaction occurs, and the appropriate diffusion coefficients for enzyme and H_2CO_3, the estimate from the model of Alberty and Hammes is near $1 \times 10^9\ M^{-1}\ sec^{-1}$ for the diffusion-limited bimolecular rate constant, smaller than the minimum value obtained from the kinetic data. This minimum value of $5 \times 10^{10}\ M^{-1}sec^{-1}$ is also larger by at least a factor of about 10^2 than the rate constants for formation of the initial enzyme-substrate complexes for many reactions obtained by fast reaction techniques and listed by Hammes and Schimmel (Table 1 of Reference 64). The argument has been made that the surface of carbonic anhydrase may provide a large target area for H_2CO_3 as substrate and provide a surface for rapid diffusion to the active site.[65] But this idea has not been tested experimentally, and the arguments concerning diffusion-controlled limits of encounter remain the strongest evidence against H_2CO_3 as substrate or product. Jönsson and Wennerström[112] have presented a thorough discussion of the problem of diffusion control and possible mechanisms for carbonic anhydrase.

The alternative possibility is that bicarbonate is a substrate or product in the catalysis.

Since this possibility requires that an acid (CO_2) be the product of the catalysis of a base (HCO_3^-), the ionization state of enzyme must be altered during the catalysis, as shown in Equation 2. Continued dehydration steps then require the return of the enzyme to its original, protonated state, Equation 3. The inverse pH-rate profiles for hydration and dehydration (Figures 3 and 4) are consistent with this scheme since a proton is consumed in the dehydration direction and must be included in the kinetic expressions.

$$EH^+ + HCO_3^- \rightleftharpoons E + CO_2 + H_2O \quad (2)$$

$$E + H_3O^+ \rightleftharpoons EH^+ + H_2O \quad (3)$$

However, it was readily recognized that Equations 2 and 3 as they stand are not acceptable because they also require a rate exceeding that for a diffusion-controlled bimolecular reaction. The protonation in Equation 3 must proceed at least as fast as the overall catalytic rate, which has a maximal turnover number at pH near 6 of 6×10^5 sec^{-1} for dehydration of HCO_3^- catalyzed by human carbonic anhydrase II (Figure 4). The rate of Equation 3 is given by $k[H_3O^+][E]$; in order for this protonation to keep up with the maximal rate of catalysis, $k[H_3O^+]$ must equal or exceed 6×10^5 sec^{-1} at pH 6. But this requires that k equal or exceed 6×10^{11} M^{-1} sec^{-1}, which again violates the known diffusion-controlled limit near 10^{10} M^{-1} sec^{-1}. It does not help to consider water as a proton donor since its pK_a (15.7) is so much greater than that of the proton acceptor (7 for the ionizable group on the enzyme) that the rate constant for proton transfer would not be much larger than 10^3 sec^{-1}.[66]

B. A Role for External Buffer

It is probably no surprise that a solution to the dilemma lay, rather undisguised, in the literature since 1962 when Alberty stated: "At pH 8, let's say, the hydrogen ion concentration is 10^{-8} *M*, and, even if the bimolecular rate constant is 10^{10} M^{-1} sec^{-1}, the reaction of a proton with the site is not going to happen very fast. What is really happening in a step like this is that ES reacts with the acid form of the buffer HA to form ESH plus A, and this reaction, as Eigen's work has shown, is usually a diffusion controlled reaction. This step, then, can occur very rapidly because the concentration of the acid constituent of the buffer is much greater than the concentration of free protons".[67] And again, Eigen and Hammes in 1964: "The general acid-base mechanism then must involve a proton transfer between enzyme and substrate followed by the protonation or deprotonation of the enzyme in order to reform its initial state. Since the concentration of H^+ and OH^- is very small (pH 7) de- or reprotonation may be mediated by H_2O in combination with buffers".[68]

These general comments were applied to the specific case of carbonic anhydrase in 1973 by Khalifah,[61] Lindskog and Coleman,[69] and Prince and Woolley[70] who recognized that proton transfer between buffer and enzyme could be an important process. Then, in addition to Equation 3, one must consider

$$E + BH^+ \underset{k_5}{\overset{k_{-5}}{\rightleftharpoons}} EH^+ + B \quad (4)$$

The rate of this proton transfer is given by $k_{-5}[BH^+][E]$. It is required that this rate must equal or exceed the maximal turnover number of 6×10^5 sec^{-1} for dehydration of HCO_3^- catalyzed by type II isozyme in the presence of, let us say, 10^{-2} *M* protonated buffer. For the reaction in Equation 4, this requirement can be met with $k_{-5} = 6 \times 10^7$ M^{-1} sec^{-1}, well within the possibility of diffusion-controlled processes. This is the situation in kinetic

experiments with carbonic anhydrase which are usually carried out using buffers at concentrations exceeding 20 m*M*, conditions for which intermolecular proton transfer is not as rate limiting as at lower buffer concentrations. At sufficiently low buffer concentration, however, this hypothesis predicts that the catalytic rate will be limited by the proton transfers of Equations 3 and 4, and the fraction of enzyme protonated at the active site will not be in equilibrium with solution.

Verification of the hypothesis presented some problems since the catalyzed reaction produces or consumes protons and the turnover numbers are highly pH dependent in the range of pH 6 to 8. Khalifah suggested measuring isotope exchange catalyzed by carbonic anhydrase carried out at chemical equilibrium and, hence, avoiding the necessity of large concentrations of buffers.[61] The goal of such an investigation is to lower the external buffer concentration to a point for which the proton transfer between the buffer species and active site of the enzyme becomes rate limiting.

C. The Evidence — a Buffer-Dependent Catalysis

1. Equilibrium Velocities

The first experimental evidence for the role of buffer in the catalyzed hydration of CO_2 was obtained by Tu and Silverman[71-73] who, taking the suggestion of Khalifah,[61] measured the effect of buffer on an isotope exchange catalyzed by carbonic anhydrase II. Addition of buffer caused the enhancement of the rate of exchange at chemical equilibrium of ^{18}O between CO_2 and water. The exchange occurs because of the hydration-dehydration cycle and the measured variable is the ^{18}O content of CO_2 as determined by a mass spectrometer.

$$CO^{18}O + H_2O \rightleftharpoons HCOO^{18}O^- + H^+ \rightleftharpoons COO + H_2{}^{18}O \qquad (5)$$

There is a review of this ^{18}O method,[74] which was originated by Mills and Urey[75] in order to measure the rate constant for the uncatalyzed hydration of CO_2. The first-order rate constant describing the decrease in ^{18}O content of CO_2 is θ which has a catalyzed and uncatalyzed component: $\theta = \theta_{cat} + \theta_{uncat}$. Figure 5 shows the increase in θ_{cat} upon addition of the buffer imidazole ($pK_a = 7.1$).

A second type of ^{18}O exchange is also dependent on buffer concentration and has helped to understand the meaning of these results: the exchange of ^{18}O between ^{13}C- and ^{12}C-containing species of CO_2, discovered by Gerster et al.[76]

$$^{12}C^{16}O^{16}O^{18}O^{-2} + {}^{13}C^{16}O^{16}O \rightleftharpoons {}^{12}C^{16}O^{16}O + {}^{13}C^{16}O^{16}O^{18}O^{-2} \qquad (6)$$

Equation 6 shows the mechanism for the uncatalyzed exchange; this reaction has been determined to occur with a rate constant of $114 \pm 11\ M^{-1}\ sec^{-1}$ at 25°C.[77] The mechanism for this exchange when catalyzed by carbonic anhydrase is different, involving the labeling of the active site with ^{18}O as described below. The first-order rate constant ϕ describes the exchange of ^{18}O between ^{12}C- and ^{13}C-containing species of CO_2 and is also the sum of catalyzed and uncatalyzed components: $\phi = \phi_{cat} + \phi_{uncat}$. Figure 5 shows the change in ϕ_{cat} upon addition of buffer. The main advantage of this ^{18}O method is that, as an equilibrium technique, it can be performed in the absence of buffers. The main disadvantage is that it is a slow kinetic method being used to measure a very rapid catalysis. As a result, ^{18}O-exchange experiments must be carried out at low concentrations of enzyme, near 10^{-9} *M*, at which the catalyzed rate of ^{18}O exchange approaches that of the uncatalyzed rate.

Patterns similar to Figure 5 were observed using the buffers *N*-methylimidazole ($pK_a = 7.3$), 2,4-lutidine ($pK_a = 6.8$), and *N*-methylmorpholine ($pK_a = 7.7$), whereas compounds

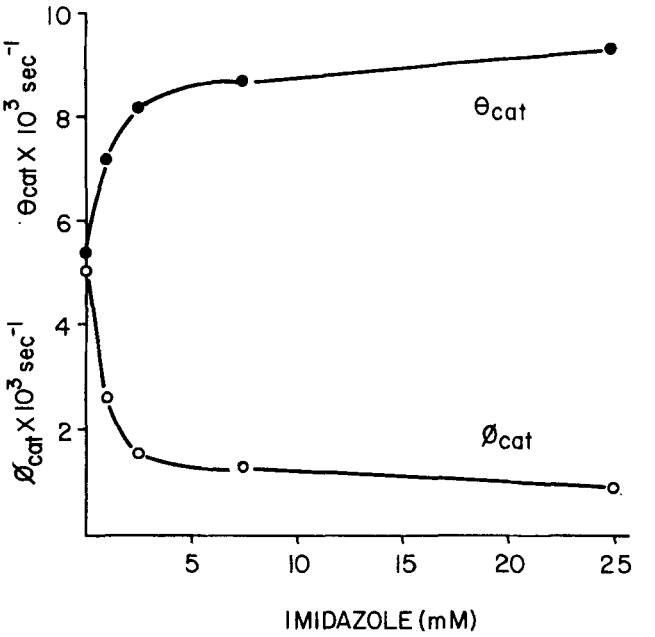
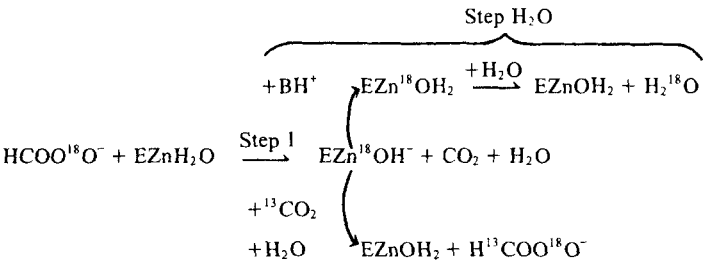


FIGURE 5. The rate constants for the catalyzed exchange of ^{18}O between CO_2 and water, θ_{cat} (●); and for the catalyzed exchange of ^{18}O between ^{12}C- and ^{13}C-containing species of CO_2, ϕ_{cat} (○), as a function of concentration of imidazole. Bovine red cell carbonic anhydrase was present at 1.6×10^{-9} *M* and the total concentration of all CO_2 species was 10 m*M*. Ionic strength was maintained at 0.2 with Na_2SO_4. Temperature was 25° C and pH 7.0. (From Tu, C. K. and Silverman, D. N., *J. Am. Chem. Soc.*, 97, 5935, 1975. With permission.)

similar to imidazole in structure but lacking in proton transfer capability such as 1,3-dimethylimidazolium sulfate and pyrrole up to concentrations of 50 m*M* had no effect on θ_{cat} and ϕ_{cat}.[72] From this it appears that the buffer effect shown in Figure 5 is not a solvent effect, a consequence of binding of buffer without proton transfer, or an effect on the uncatalyzed ^{18}O exchange. The shape of the curves are characteristic of a change in rate-limiting step[78] and consistent with a buffer-enhanced release of ^{18}O to solvent. The symmetrical pattern of Figure 5 suggests that the effects of buffer on the rate constants θ_{cat} and ϕ_{cat} are related, a high buffer concentration favoring the release of ^{18}O to solvent and a low buffer concentration causing more ^{18}O to be retained in the CO_2-HCO_3^- system.

These observations are consistent with a scheme in which ^{18}O labels the active site of carbonic anhydrase with a residence time for ^{18}O comparable to the time for a catalytic hydration-dehydration cycle. ^{18}O is eventually either released to solvent as $H_2{}^{18}O$ where it is infinitely diluted by $H_2{}^{16}O$, or it is reincorporated into CO_2 and HCO_3^- by a subsequent hydration reaction. In the presence of $^{13}CO_2$ this reincorporation produces the doubly labeled $H^{13}COO^{18}O^-$. These features are shown in Scheme 1, a simple twofold pathway for ^{18}O exchange. Although this scheme can be presented in a general form not specifying a particular mechanism,[74] we have chosen to present it in terms of the zinc-hydroxide mechanism because the presence of a buffer effect strongly suggests a change in ionization state of the enzyme during the catalysis. Other roles of the buffer are possible, within the zinc-water mechanism for example.[79] This point is discussed specifically in Section IV.



Scheme (1)

Step 1 is the dehydration of ^{18}O-labeled bicarbonate which yields CO_2 and has a 1/3 probability of leaving ^{18}O at the active site. The step in which $^{13}CO_2$ reacts with labeled active site to give $H^{13}COO^{18}O^-$ is a hydration step which has the same rate as Step 1 at chemical equilibrium. Step H_2O is the release of labeled oxygen to solvent water, a step assumed to be independent of Step 1. This scheme shows that although the experiment is performed at chemical equilibrium, there is a flow of ^{18}O in the system from HCO_3^- to H_2O. This flow is enhanced by buffer and is probably related to the lifetime of ^{18}O at the active site. That is, the buffer through proton transfer causes an increase in the rate of release of ^{18}O to solvent and decreases the amount of time ^{18}O is available at the active site for reincorporation in the CO_2-HCO_3^- system. These experiments do not reveal the site on the enzyme involved in proton transfer; in the zinc-hydroxide hypothesis the ultimate proton acceptor would be $EZn^{18}OH^-$ which upon protonation would rapidly exchange $H_2{}^{18}O$ with solvent. In terms of Equation 4, we identify $EZnOH^-$ with E and $EZnOH_2$ with EH^+. A slowly exchanging ^{18}O in $EZn^{18}OH^-$ is consistent with many tightly-bound, anionic inhibitors of carbonic anhydrase.

It is necessary here to describe the further analysis of the ^{18}O exchange data. Extending the kinetic analysis of Mills and Urey,[75] the five rate equations that describe the exchange of ^{18}O in Scheme 1 were written and solved by Silverman et al.[73] using the hypothesis that zinc-bound hydroxide is active in the hydration of CO_2 and by Koenig and Brown[80] using the hypothesis that zinc-bound water is the only active form in the enzyme. Except for the definition of one term, the solutions are identical and allow two rates to be calculated from the ^{18}O-exchange parameters: (1) R_1 is the catalyzed rate of interconversion of CO_2 and HCO_3^-, (2) R_{H_2O} is the rate of release from the enzyme of water bearing substrate oxygen, both pertaining to equilibrium processes.

$$R_1 = 3[S_{tot}](\theta_{cat} + \phi_{cat}) \tag{7}$$

$$R_{H_2O} = \frac{R_1 \theta_{cat}}{\phi_{cat}} \left(1 - \frac{[CO_2]}{3[S_{tot}]}\right) \tag{8}$$

Here, $[S_{tot}] = [CO_2] + [HCO_3^-]$. In the experimental protocol, $[S_{tot}]$ is held constant as pH or buffer is varied. In these experiments the kinetic isotope effects caused by the enrichment in ^{18}O and ^{13}C are neglected. A small value of ϕ_{cat} is a characteristic of many of these experiments, especially those carried out in the presence of buffer. With ϕ_{cat} small, the experimental error amounts to a sizeable fraction of ϕ_{cat}, 10 to 20% in worst cases. Division by ϕ_{cat} in Equation 8 causes these errors to propagate into R_{H_2O}. Oxygen-17 magnetic resonance studies have not been able to determine properties of oxygen at the active site of native carbonic anhydrase.[81] However, Bertini et al.[82] have observed exchangeable $H_2{}^{17}O$ in the first coordination sphere of the metal in human Cu(II)-carbonic anhydrase I over the entire range of pH from 5 to 10. Apparently, this bound

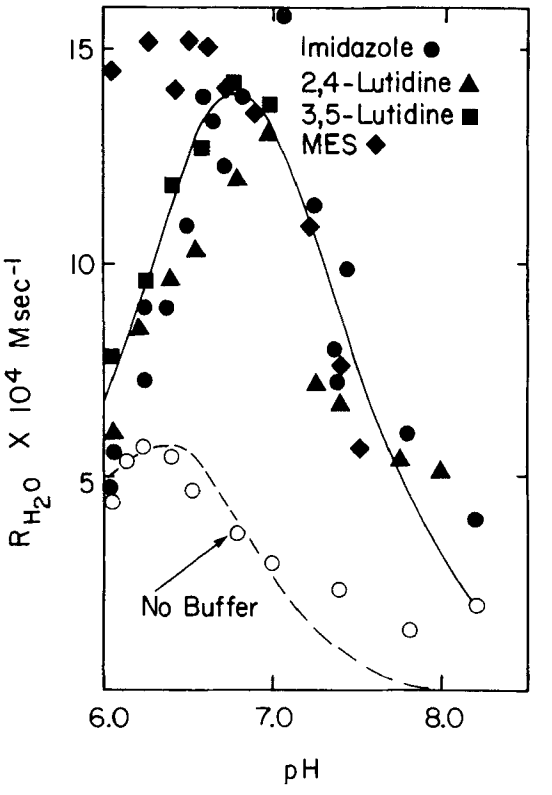


FIGURE 6. R_{H_2O} at various values of pH calculated from θ_{cat} and ϕ_{cat} using Equation 8. The following data were obtained using Na_2SO_4 to maintain an ionic strength at 0.2: (○) no buffer present; (●) 50 m*M* imidazole; (▲) 50 m*M* 2,4-lutidine; (■) 50 m*M* 3,5-lutidine. The following data were obtained in the absence of Na_2SO_4: (◆) 30 m*M* 4-morpholineethane sulfonic acid (MES). Human carbonic anhydrase II was present at 1.6×10^{-9} *M* and the total concentration of all CO_2 species was 15 m*M*, 25°C. (From Silverman, D. N., Tu, C. K. Lindskog, S., and Wynns, G. C., *J. Am. Chem. Soc.*, 101, 6734, 1979. With permission.)

water remains in the presence of monodentate inhibitors of the enzyme but is removed by the binding of oxalate and *p*-toluene sulfonamide.

It is clear that R_1 measured by the ^{18}O method is the same equilibrium rate as measured by the broadening of the ^{13}C resonance peaks of $H^{13}CO_3^-$ and $^{13}CO_2$ caused by rapid interconversion between these two species in the presence of carbonic anhydrase.[83] It is important to comment that the values of R_1 obtained by these two equilibrium methods, ^{18}O exchange and ^{13}C resonance broadening, are in agreement for the catalysis by human carbonic anhydrase II.[73] Both equilibrium methods are also in agreement that the value of R_1 is not different in the presence or absence of noninhibitory buffers.[73,83]

An important conclusion from these observations is that the effect of buffer on the catalysis of the hydration of CO_2 is measurable only in the step of Scheme 1 designated Step H_2O, the rate of which is given by R_{H_2O}. Figure 6 presents values of R_{H_2O} in the range of pH 6 to 8 measured in the absence of buffer and in the presence of a concentration of several buffers (50 m*M*) sufficient to maximize the buffer effect, as

demonstrated in Figure 5. To ascribe these effects to buffer-assisted catalysis it is necessary to explain several features in Figure 6. The first is to demonstrate that the value of R_{H_2O} is large enough to require buffer-facilitated proton transfer, and the second that the buffer concentration required to bring about enhancement of R_{H_2O} gives an estimate of the expected magnitude for the rate constant for proton transfer from enzyme to buffer. If the contribution of buffer is neglected and H_3O^+ alone is considered as a proton donor, then we can calculate the bimolecular rate constant for proton transfer that would be necessary to explain the value of $R_{H_2O} = 1.5 \times 10^{-3}\ M\text{sec}^{-1}$ at pH 7 in Figure 6. In this calculation we assume that the release of $H_2{}^{18}O$ from the active site (Scheme 1) is not rate limiting.

$$R_{H_2O} = k[H_3O^+][EZn(OH)]$$

$$1.5 \times 10^{-3}\ M\text{sec}^{-1} = k\,(10^{-7}\ M)\,(8 \times 10^{-10}\ M)$$

$$k = 1.9 \times 10^{13}\ M^{-1}\text{sec}^{-1}$$

Here we have assumed an ionization constant of $10^{-7}\ M$ for the equilibrium between zinc-bound water and zinc-bound hydroxide at the active site. The bimolecular rate constant obtained from this calculation greatly exceeds that of the diffusion-controlled limit of about 10^9 to $10^{10}\ M^{-1}\text{sec}^{-1}$. Consequently, we consider the mechanism in which buffer acts as a proton-transfer agent. According to Figure 5 and Equation 8, R_{H_2O} reaches its maximal value near 5 mM of the buffer imidazole, at pH 7, a similar result being obtained for other buffers. With 2.5 mM protonated imidazole ($pK_a = 7.1$) at pH near 7,

$$R_{H_2O} = k_{-5}[BH^+][EZn(OH)]$$

$$1.5 \times 10^{-3}\ M\text{sec}^{-1} = k_{-5}\,[2.5 \times 10^{-3}\ M]\,[8 \times 10^{-10}\ M]$$

$$k_{-5} = 7.5 \times 10^{8}\ M^{-1}\text{sec}^{-1}$$

This calculation demonstrates that consideration of buffer as a source of protons yields a bimolecular rate constant for proton transfer within the maximum limits of encounter-controlled processes and that buffer-facilitated proton transfer is a viable hypothesis for the experimental observations.

Another feature of Figure 6 that needs to be explained is the large value of R_{H_2O} in the absence of external buffer. According to the argument in the preceding paragraph, one expects a very small value of R_{H_2O} when H_3O^+ is the only proton source at pH 7. We exclude the substrate itself as buffer at pH near 7. This is far from the pK_a of the HCO_3^--CO_3^{2-} buffer system which is 10.3, and far from the HCO_3^--H_2CO_3 system which is 3.8. Under the conditions of Figure 6, with 15 mM total concentration of CO_2 species, the concentration of CO_3^{2-} at pH 8 is near $10^{-4}\ M$, and the concentration of H_2CO_3 at pH 6 is similar. These concentrations are below the millimolar range in which buffers such as imidazole and 3,5-lutidine cause enhancement of ^{18}O exchange activity. The most convincing evidence that substrate itself is not a buffer is that near neutral pH in the absence of external buffers R_{H_2O} does not change as the total concentration of CO_2 species increases from 2.5 to 30 mM.[73] These are conditions for which all of the buffer effect on ^{18}O exchange is manifested in R_{H_2O}, which is very sensitive to buffers in the range of 0 to 10 mM. Since there is no external buffer that can account for the observed behavior in Figure 6, it is a reasonable conclusion that there is one or more internal proton transfer groups, residues of the enzyme itself, that cause the large values of R_{H_2O}. That is, an internal group B'H in the enzyme, EZn(^{18}OH)B'H, permits an intramolecular

proton transfer which results in a form of enzyme, $EZn(^{18}OH_2)B'$, from which $H_2{}^{18}O$ can be rapidly released to solvent. This explains qualitatively the observations in Figure 6. A quantitative explanation of Figure 6 has been attempted[73] based on the assumed presence of a single proton transfer group of pK_a near 7 (possibly His 64). This topic is discussed further in Section IV.

Finally, millimolar concentrations of many types of buffers with values of pK_a between 6 and 8 are capable of enhancing ^{18}O exchange with water near neutral pH: imidazole and its methylated derivations,[71] tris(hydroxymethyl) aminomethane,[71] 2-4 lutidine,[72] *N*-methylmorpholine.[72] Silverman et al.[84] reported that micromolar concentrations of bovine oxyhemoglobin caused an increase in θ_{cat} and decrease in ϕ_{cat} in the presence of bovine carbonic anhydrase similar in pattern to that in Figure 5. The same effect was observed with L-histidine. Albumin, glycine, and alanine as controls had little or no effect on θ_{cat} and ϕ_{cat}. These results were explained by suggesting that hemoglobin and histidine bind tightly to carbonic anhydrase and function in a manner similar to an internal buffer. Subsequent evidence to support the binding of hemoglobin and carbonic anhydrase II was obtained using the technique of counter-current distribution in aqueous/aqueous biphasic systems[85,86] and suggested an association constant of $4 \times 10^5\ M^{-1}$ at pH 8.0.[86] Of significant interest here is Backman's finding that human carbonic anhydrase II interacts with human CO-hemoglobin to affect the counter-current distribution pattern, but human carbonic anhydrase I does not.[86] However, Tu et al.[87] have found that the enhancement of the catalyzed ^{18}O exchange between CO_2 and water reported to be caused by complex formation with hemoglobin is instead due to a metal ion contamination, probably copper: the addition of hemoglobin or histidine increased ^{18}O exchange rates by extracting metal ions from inhibitory sites on the enzyme. Hence, there is no evidence from ^{18}O studies that hemoglobin acts as a proton transfer agent to carbonic anhydrase in a bound complex.

2. Steady-State Velocities

A more straightforward assessment of the role of external buffer in the catalysis can be made simply by measuring the buffer dependence of the initial velocity of the catalyzed hydration of CO_2. Jonsson et al.[88] found that this initial velocity catalyzed by human carbonic anhydrase II is dependent on buffer for concentrations of buffer less than about 15 m*M*. This was found to be a general effect for six buffers tested (with pK_a values in the range of 6 to 9) and resembles the buffer dependence of the catalyzed exchange of ^{18}O between CO_2 and H_2O (Figure 7). Jonsson et al. concluded that this result is consistent with a role for buffer as a proton transfer agent between the enzyme and solution. At buffer concentrations below about 15 m*M* the catalytic rate is slower because protons cannot be transferred away from the active site fast enough; under these conditions the ionization at the active site is not in equilibrium with the proton concentration of the external solution.

In the steady-state experiment any internal proton transfer group is not constrained to remain in its equilibrium ionization state, as was the case for the ^{18}O-exchange conditions, and the unprotonated internal transfer groups would be soon depleted during hydration in the absence of external buffer. However, in the complete absence of external buffer the proton transfer from EH^+ to OH^- would be available. Assuming $10^{10}\ M^{-1}\ sec^{-1}$ for the bimolecular rate constant for proton transfer between EH^+ and OH^-, the expected rate of catalysis in the absence of buffer at pH 7 would be about 0.1% of that measured at maximum buffer in Figure 7. Considering H_2O as a proton acceptor does not increase this figure appreciably. The stopped-flow experiment cannot be meaningfully interpreted in the complete absence of buffer because of large pH changes, so the velocity of catalyzed hydration with OH^- and H_2O as the only proton acceptors cannot be tested. Since these are initial velocity experiments measured before there is an appreciable

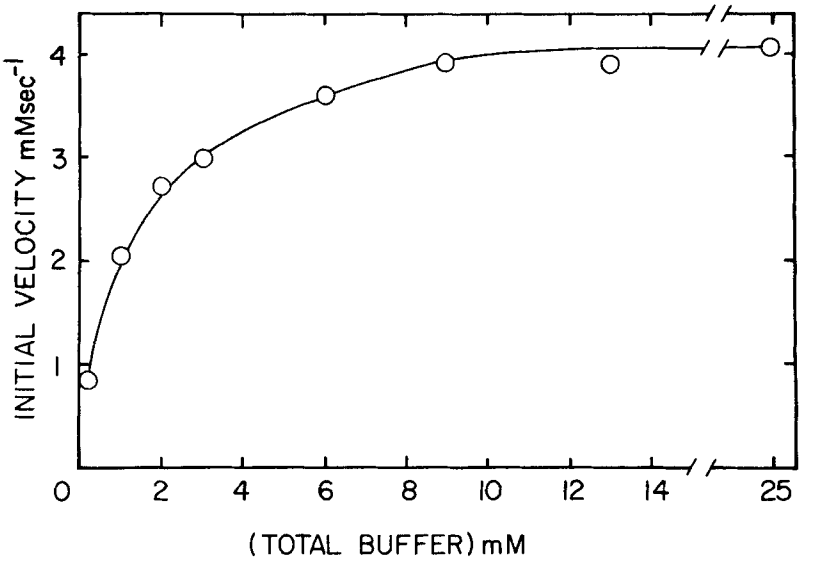
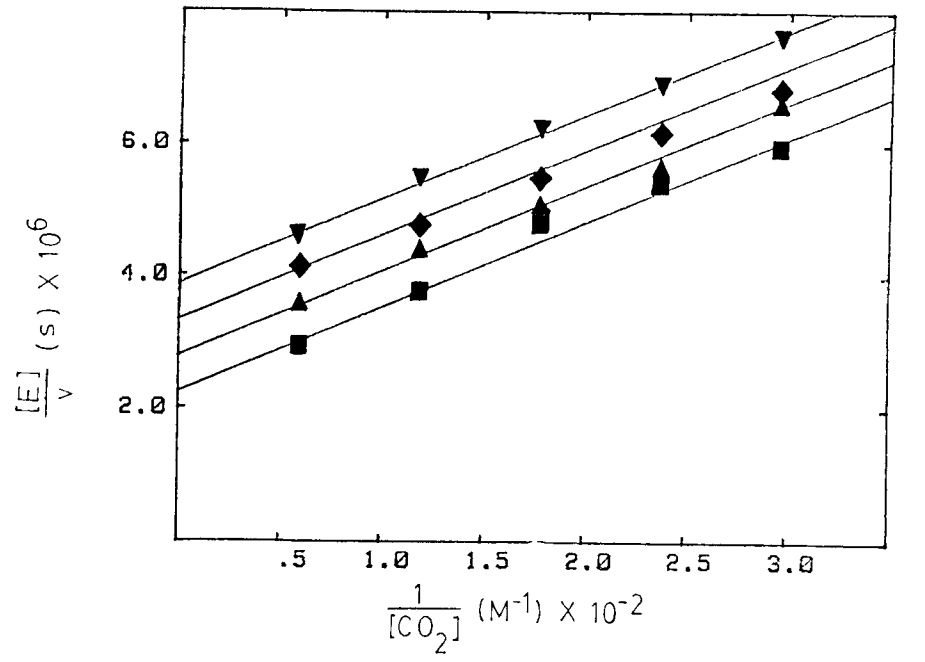


FIGURE 7. The catalyzed component of the initial velocity of CO_2 hydration at 25° C vs. concentration of total buffer. Total buffer is the sum of the concentration of the indicator para-nitrophenol, which was constant at 5×10^{-5} *M*, and the concentration of the buffer imidazole, which was varied. Bovine carbonic anhydrase was present at 2.5×10^{-8} *M* and initial CO_2 was 7.4 m*M*; initial pH was 7.15. Ionic strength was maintained at 0.2 with Na_2SO_4.

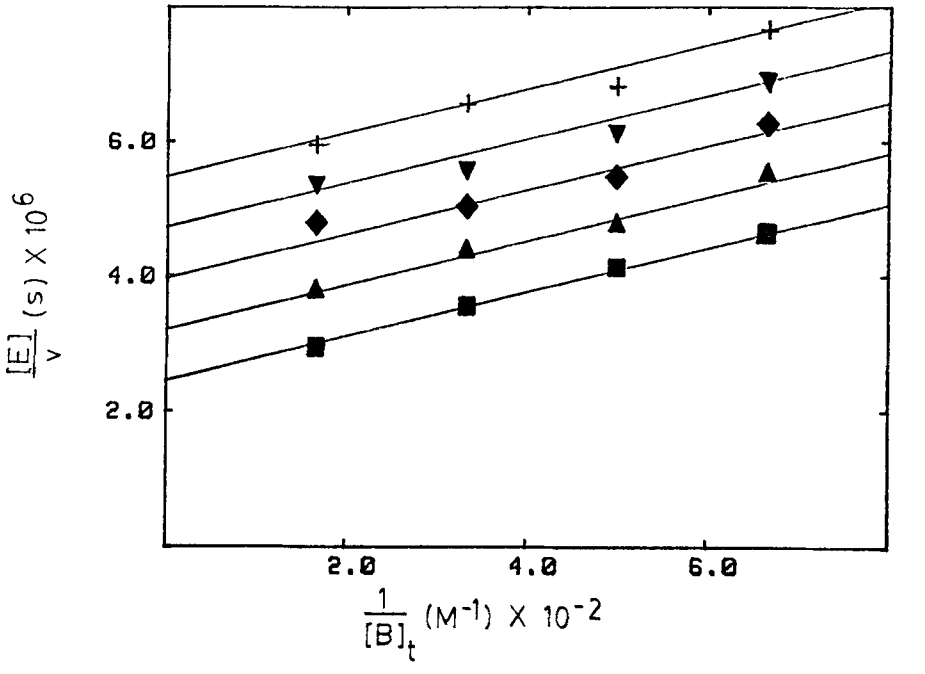
accumulation of product HCO_3^-, there is less of a possibility here than in the equilibrium experiments that substrate acts as buffer. The data in Figure 7 are presented because they are extended to particularly low concentration of total buffer. Here buffers are imidazole, the concentration of which was varied, and *p*-nitrophenol, a pH indicator the concentration of which was held constant at 5×10^{-5} *M*. These data are consistent with and can be fit to a hyperbolic curve passing very near the origin, consistent with our expectations.

The initial velocity measurements of Jonsson et al.[88] using the buffer 2,2-diethylmalonate ($pK_a = 7.3$) at pH 7.8 show an enhancement of the hydration of CO_2 catalyzed by human carbonic anhydrase II and also show that as the concentration of this buffer is varied from 3.3 to 50 m*M* the value of $k_{cat}^{CO_2}/K_m^{CO_2}$ does not change significantly. These first studies of buffer dependence were extended by Rowlett and Silverman[58] to include eight additional buffers (listed in the legend to Figure 9) with values of pK_a between 6 and 9. The ratio $k_{cat}^{CO_2}/K_m^{CO_2}$ was independent of buffer at concentrations of buffer less than 12 m*M*. Low concentrations of buffer were used to minimize the possibility of buffer binding to enzyme. Moreover, considering unprotonated buffer as substrate, $k_{cat}^{CO_2}/K_m^{Buffer}$ or its equivalent k_5 of Equation 4 was independent of CO_2 concentration. Figure 8 shows these patterns for 1,2-dimethylimidazole in which the parallel slopes demonstrate the invariant nature of k_{cat}/K_m.

These results are consistent with the mechanism shown in Equations 2 and 4 which are a special case of the classical ping pong mechanism.[90] We present here arguments that there is no kinetically significant binary enzyme-buffer complex in the mechanism. The presence of buffer does not affect the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium, measured by the exchange-broadened ^{13}C resonances in $H^{13}CO_3^-$,[83] or by ^{18}O exchange.[73] Also, the apparent dissociation constant of the enzyme-HCO_3^- complex is independent of buffer concentration.[83] Furthermore, there are no large deviations from the Brønsted plot in Figure 9 which would be expected for strong binding



A



B

FIGURE 8. Dependence of [E]/v on $1/[CO_2]$ and $1/[B_t]$ for the hydration of CO_2 and protonation of buffer catalyzed by human carbonic anhydrase II, present at 69 n*M*. Ionic strength was maintained at 0.20 with Na_2SO_4, temperature was 25°C and pH 8.5. (A) [1,2-dimethylimidazole] = 6.0 m*M* (■), 3.0 m*M* (▲), 2.0 m*M* (◆), 1.5 m*M* (▼) (these values are total concentraton of buffer.) (B) Replot of same data for $[B]_t$ = 1,2-dimethylimidazole, $[CO_2]$ = 17 m*M* (■), 8.5 m*M* (▲), 5.6 m*M* (◆), 4.2 m*M* (▼), 3.4 m*M* (+). Lines drawn through data points were determined by weighted least-squares analysis and calculated for $k_{cat} = 5.9 \times 10^5$ sec^{-1}, $K_m^{CO_2} = 7.6$ m*M*, $K_m^B = 2.0$ m*M*. (From Rowlett, R. S. and Silverman, D. N., *J. Am. Chem. Soc.*, 104, 6737, 1982. With permission.)

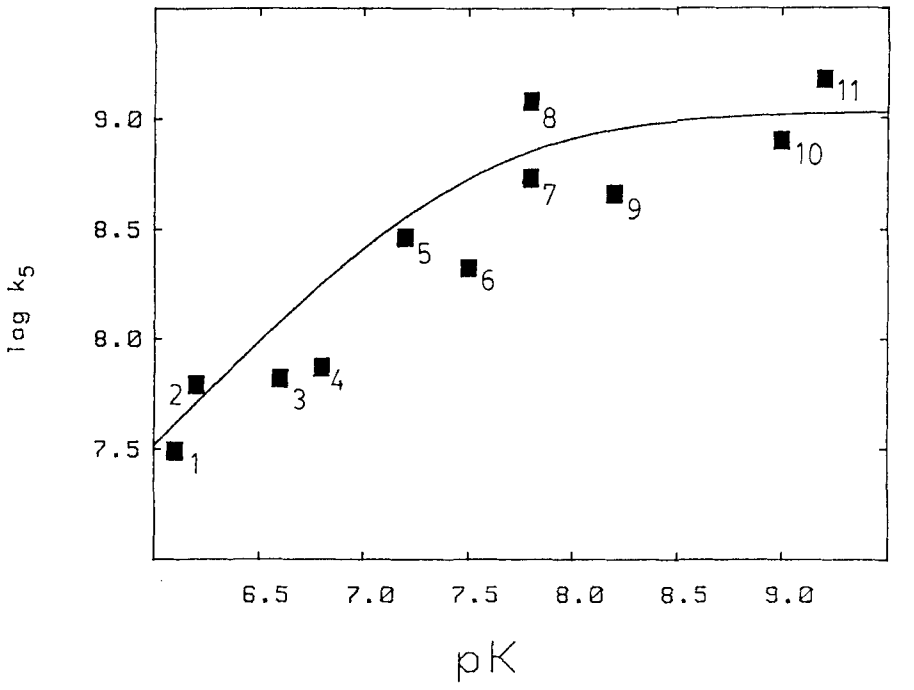


FIGURE 9. Variation of log (k_5) of Equation 4 and Figure 10 with pK_a of external buffers: (1) MES, (2) 3,5-lutidine, (3) 3,4-lutidine, (4) 2,4-lutidine, (5) 1-methylimidazole, (6) Hepes, (7) triethanolamine, (8) 4-methylimidazole, (9) 1,2-dimethylimidazole, (10) Ted, (11) Ches. The curve drawn through the data points was calculated for $k_4 = 1.1 \times 10^9\ M^{-1}\ sec^{-1}$ and $pK_{enz} = 7.6$. (From Rowlett, R. S. and Silverman, D. N., *J. Am. Chem. Soc.*, 104, 6737, 1982. With permission.)

of buffer to the enzyme. Since we are discounting the notion of a strong interaction between buffer and enzyme as well as a role for buffer in the interconversion of CO_2 and HCO_3^-, we also regard as unlikely an ordered bi bi mechanism, which under certain conditions could produce the initial velocity pattern of Figure 8.[90]

Since the catalysis of the initial velocity of CO_2 hydration appears to require proton transfer between buffer and enzyme, it becomes of interest to investigate the extent to which this process conforms to our knowledge of proton transfer between small molecules. Eigen observed that proton transfer between simple acids and bases exhibits a biphasic Brønsted plot.[66] The limiting slope is zero when the pK_a of the proton donor is much less than that of the acceptor, and unity when the pK_a of the acceptor is less than that of the donor, with a transition region in between that spans about 2 pK_a units. The midpoint of the transition region provides an estimate of the point at which the pK_a of the donor equals that of the acceptor. A further characteristic of proton transfer processes between small molecules is that the plateau region of the Brønsted plot corresponds to diffusion-limited proton transfer with a rate constant near 10^9 to $10^{10}\ M^{-1}\ sec^{-1}$.[66]

A Brønsted plot for the transfer of protons between external buffer and human carbonic anhydrase II is shown in Figure 9. The rate constant for the transfer of protons from enzyme to external buffer, k_5 of Equation 4, was determined by Rowlett and Silverman by fitting data such as shown in Figure 8 to the following rate equation which describes catalysis according to Equations 2 and 4:

$$\frac{[E_t]}{v} = \frac{1}{k_5[B]} + \frac{1}{k_{cat}}\left(1 + \frac{K_m^{CO_2}}{[CO_2]}\right) \qquad (9)$$

Here [B] designates the concentration of the basic form of the buffer. The plot of log k_5 against pK_a of buffer in Figure 9 has the appearance expected for a biphasic Eigen curve.[66] The rate constant for proton transfer can be approximated by the expression $k_5 = (k_5)_{max}/(1 + K_{buffer}/K_{enz})$, valid for the case in which the rate constant for the separation from the enzyme of buffer B is equal to that for protonated buffer BH^+.[66] K_{buffer} and K_{enz} are the acid dissociation constants for the buffer and for the donor group on the enzyme. The curve in Figure 9 is a best fit of the data to this equation, which yields these least-squares values and standard errors: $pK_{enz} = 7.6 \pm 0.6$ and $k_5 = (1.1 \pm 0.9) \times 10^9$ $M^{-1}sec^{-1}$. These large errors could have several sources. The possibility of buffer binding to and inhibiting the enzyme has not been taken into account. Moreover, several different chemical classes of buffer are reported in Figure 9, whereas it is best in construction of a Brønsted plot to use buffers of a related group of compounds.

The value of $pK_{enz} = 7.6 \pm 0.6$ estimated from the Brønsted plot is consistent with proton transfer directly between the buffers in solution and the activity-controlling group of pK_a near 7. However, the active site at the metal is at the bottom of a funnel-shaped cleft in the enzyme 12Å deep. That there is adherence to the form of a Brønsted plot with buffers of very different structure is also compatible with the exchange of protons between external buffer and a group of the enzyme nearer to the surface of the protein and more accessible to buffers in solution. His 64 has often been mentioned in such a role, as will be discussed at some length in Section III. The titrations measured by NMR show that His 64 in human carbonic anhydrase II has a pK_a near 7.[91] Certainly the transfer of a proton between external buffer and active site or possible intermediate shuttling groups is very close to the maximal value of bimolecular rate constants found for small molecules. The maximal value of k_5 near 10^9 $M^{-1}sec^{-1}$ obtained from the Brønsted plot in Figure 9 can be compared with the value of 1.2×10^9 $M^{-1}sec^{-1}$ for proton transfer from acetic acid (pK_a 4.8) to imidazole (pK_a 7.1).[68]

D. Critique

The hypothesis of the participation of buffer as a proton-transfer agent in the hydration of CO_2 catalyzed by the rapid type II isozymes of carbonic anhydrase has been the only proposal to account for the very high turnover when HCO_3^- is a product. If H_2CO_3 is the product then there is no need for proton transfer between enzyme and solvent, but the diffusion of H_2CO_3 to the enzyme becomes a problem which to this date has no plausible explanation. The possibility of binding of buffer to enzyme causing rate enhancement without proton transfer would yield hyperbolic activity curves as observed in Figures 5 and 7. However, this possibility is far outweighed by the adherence of rate constants to the Brønsted plot and the failure to observe rate enhancement by buffer analogs which lack proton-transfer capability. Although the magnitude of enhancement of catalytic rate by buffers is modest in both equilibrium and steady-state experiments (Figures 5 and 7), this is accounted for by the nature of the experimental techniques and does not detract from the support of the initial hypothesis.

Whereas the hypothesis of a buffer facilitated catalysis of CO_2 hydration by type II isozymes appears well supported, many critical and interesting details are missing. We do not know the identity of the acceptors on the enzyme of the proton donated by buffer, nor do we know the contribution of H_3O^+ and H_2O to this process. The slower isozymes have not been studied in this regard, although brief reports have appeared that type I isozyme can also be activated by buffers.[88,92] Of interest would be to investigate the possibility of buffer enhancement of the type III isozyme, the activity of which is less than that of type I or II. Preliminary studies indicate no effect of noninhibitory buffers (at concentrations less than 10 mM) on the catalysis by human carbonic anhydrase II of the hydrolysis of *p*-nitrophenyl acetate.[93]

Such a rate-limiting proton transfer between external buffer and enzyme has not been

revealed, to our knowledge, for rapid catalysis caused by enzymes other than carbonic anhydrase, although superoxide dismutase would seem to be a likely candidate for investigation. Silman and Karlin[94] have observed an activation of membrane-bound acetylcholinesterase which is very similar to that which is observed for carbonic anhydrase. Soluble acetylcholinesterase, extracted from its membrane environment, does not show this effect. The hydrolysis of acetylcholine catalyzed by acetylcholinesterase is very efficient with a value of $k_{cat}/K_m = 10^8\ M^{-1}sec^{-1}$, a value as great as that observed for the hydration of CO_2 catalyzed by carbonic anhydrase. Membrane-bound acetylcholinesterase from the electric tissue of an eel shows an increase in hydrolysis activity with increasing buffer concentration consistent with a change in rate-determining step; the activity approximately doubles upon addition of phosphate buffer at pH 7.0 reaching a plateau near 2 m*M* phosphate. Silman and Karlin have interpreted this buffer effect as due to a decrease in the local pH in and around the membrane and unstirred layers caused by the generation of protons accompanying the catalytic hydrolysis of acetylcholine in the absence of buffers. In the presence of buffers this local pH is close to that in bulk solution since the buffers have permitted rapid equilibration with the solution. Another possibility for this buffer dependence is that the enzyme requires buffer facilitated proton transfer to its active site, in a manner similar to carbonic anhydrase. It is our current belief that these two possibilities are closely related. To comment that local pH in the membrane environment is low due to accumulation of protons is just to say that in the membrane environment there are proton shuttle groups. This switches the problem of proton transfer from the active site to some proton shuttle groups nearby which must still rely on external buffers to remove these protons at a rate rapid enough to sustain the catalysis.

III. THE RATE-LIMITING EVENT IN THE CATALYTIC PATHWAY

Here we consider the rate-limiting step or steps in the hydration of CO_2 catalyzed by carbonic anhydrase II, the fast isozyme. We assume that buffer is plentiful to avoid overlap with the previous section; that is, we assume that proton transfer between the enzyme and its solution environment is not a rate-limiting event. This is the situation in vivo since carbonic anhydrase is in contact with cytoplasm where buffers are plentiful. Moreover, carbonic anhydrase is rather indiscriminating, engaging in proton transfer with many different types of buffers probably including the buffering groups of proteins although this has not been shown explicitly. Carbonic anhydrase II catalyzes very rapidly the hydration of CO_2 with $k_{cat}^{CO_2}/K_m^{CO_2} = 10^8\ M^{-1}\ sec^{-1}$ at pH near and above 8. Although this is very large and represents a lower limit for the bimolecular rate constant for encounter of CO_2 and enzyme, it is not evident that this is at the limiting value for a diffusion-controlled reaction of 10^9 to $10^{10}\ M^{-1}\ sec^{-1}$. From the Brønsted plot of Figure 9 we estimate the diffusion-controlled rate constant for encounter of buffer with enzyme to be at least $1 \times 10^9\ M^{-1}\ sec^{-1}$, a factor of ten greater than $k_{cat}^{CO_2}/K_m^{CO_2}$. Other steps which must be considered as possibly rate limiting are the dissociation of a proton from water in the active site to yield hydroxide, the actual catalytic conversion of CO_2 into HCO_3^-, the dissociation of product HCO_3^-, and isomerizations or ionizations of the enzyme itself.

A. Solvent Deuterium Isotope Effects

Since proton transfer is involved in the dissociation of a proton from water bound in the active site and might be involved in the conversion of CO_2 to HCO_3^-, the effect on the catalysis of changing solvent from H_2O to D_2O is of interest. Such studies have been performed by Steiner et al.[95] using human carbonic anhydrase II and by Pocker and Bjorkquist[96] using bovine red cell carbonic anhydrase, also a type II isozyme. These two enzymes are essentially identical in their kinetics and in their response to changing the

Table 3
VALUES OF THE STEADY-STATE PARAMETERS IN H_2O AND D_2O FOR THE HYDRATION OF CO_2 AND DEHYDRATION OF HCO_3^- CATALYZED BY HUMAN CARBONIC ANHYDRASE II

Parameter	Value in H_2O	Value in D_2O		Ratio H_2O/D_2O
$\left(k_{cat}^{CO_2}\right)_{max}$	10	2.6	$\times 10^5$ Sec^{-1}	3.8
$K_m^{CO_2}$	8.3	2.2	mM	3.8
$\left(k_{cat}^{CO_2}/K_m^{CO_2}\right)_{max}$	1.2	1.2	$\times 10^8 M^{-1}$Sec^{-1}	1.0
$\left(k_{cat}^{HCO_3^-}\right)_{max}$	6	1.6	$\times 10^5$ Sec^{-1}	3.8
$K_m^{HCO_3^-}$	32	11	mM	2.9
$\left(k_{cat}^{HCO_3^-}/K_m^{HCO_3^-}\right)_{max}$	1.8	1.4	$\times 10^7 M^{-1}$Sec^{-1}	1.3
K_E[a]	8	2.5	$\times 10^{-8} M$	3.2
K_{eq}[b]	4.8	1.8	$\times 10^{-7} M$	2.7

Note: Measurements were made using 50mM buffers (3,5-lutidine, *N*-methylimidazole, or 1,2-dimethylimidazole) at 25°C and an ionic strength of 0.2 maintained using Na_2SO_4. Estimated errors within 20%.

[a] The ionization constant of the activity controlling group estimated from the pH-dependence of kinetic parameters.

[b] $K_{eq} = (H^+)(HCO_3)/(CO_2)$; determined from titration of $NaHCO_3$ at ionic strength 0.2M, 25°C.

From Steiner, Jonsson, B.H., and Lindskog, S., *Eur. J. Biochem.*, 59, 253, 1975. With permission.

solvent to D_2O. The effect of this change in solvent on k_{cat} and K_m for hydration and dehydration is shown in Figures 3 and 4. The observation which is easiest to interpret is the shift from 6.85 in H_2O to 7.35 in D_2O of the apparent pK_a of the group controlling the enzymatic activity. This apparent pK_a behaves in a manner similar to nearly all acids of pK_a between 3 and 10 in that the pK_a is about 0.5 unit larger in D_2O than in H_2O.[97]

A second observation, shown in Figures 3 and 4 for the bovine and in Table 3 for the human type II carbonic anhydrase, is that k_{cat} and K_m are affected nearly equally by the change from H_2O to D_2O as solvent. This is true for both the hydration and dehydration reactions. The solvent deuterium isotope effect on k_{cat} is the ratio of k_{cat} measured in H_2O to that measured in D_2O, and is in magnitude 3.8 (Table 3) for hydration catalyzed by human type II isozyme. The solvent deuterium isotope effect on $K_m^{CO_2}$ is identical. The isotope effects on $k_{cat}^{HCO_3^-}$ and $K_m^{HCO_3^-}$ are also similar in value. These isotope effects were measured in the plateau regions of Figures 3 and 4 to avoid errors due to the pH dependence of kinetic constants. Solvent deuterium isotope effects of this magnitude are large enough to attribute to primary proton transfer; that is, one or more protons in flight in the transition state. This conclusion assumes, of course, that the change in solvent has not caused a conformational change which affects the enzymatic activity. There are several clues for carbonic anhydrase II that this assumption is correct. First, the enzymatic parameter of kinetic significance k_{cat}/K_m is unaffected by the solvent change. Also, as described later, there is no solvent deuterium isotope effect on the equilibrium rate of interconversion of CO_2 and HCO_3^- as measured by ^{13}C NMR[83] and ^{18}O exchange.[73]

With the result that the solvent deuterium isotope effect on k_{cat}/K_m is unity, but on k_{cat}

and K_m individually is substantial, Steiner et al.[95] proposed a rate-limiting intramolecular proton transfer in a step separate and distinct from the interconversion of CO_2 and HCO_3^-. The ratio k_{cat}/K_m is a composite parameter containing rate constants for steps up to and including the first irreversible step,[98] which in these initial velocity experiments is the release of product HCO_3^- for the hydration direction. Since the isotope effect on $k_{cat}^{CO_2}/K_m^{CO_2}$ is unity, the rate-limiting intramolecular proton transfer must be distinct and separate from the steps beginning with the binding of CO_2 and ending with the dissociation of HCO_3^-. This proposal was supported by studies of the inhibition by HCO_3^- of the hydration of CO_2 catalyzed by human carbonic anhydrase II. Evidence was found for product inhibition consistent with a term containing $[CO_2][HCO_3^-]$ in the denominator of the rate expression. This evidence was readily apparent when product inhibition was measured in D_2O and somewhat less apparent in H_2O.[99] The presence of a term containing $[CO_2][HCO_3^-]$ is expected when an isomerization step such as an intramolecular proton transfer is present in the catalytic pathway, unless that step is very rapid compared to other steps in the pathway.[100] The proposal of a rate-limiting intramolecular proton transfer has profound implications in the analysis of the catalytic mechanism, many of which have been substantiated and are described below. One interesting implication, borne out by ^{13}C NMR, is that the catalytic turnover rate of CO_2 and HCO_3^- measured at chemical equilibrium is even faster, by a factor of 4 or 5, than the turnover rate observed in steady-state experiments.[83] It is our opinion that this proposal by Steiner et al. is the best interpretation of a wide body of data to be discussed in this section including the solvent deuterium isotope effects on kinetic parameters, the lack of pH dependence of $K_m^{HCO_3^-}$, and the product inhibition of CO_2 hydration. An alternative and intriguing second possibility is that the isotope effect is not due to a primary proton transfer, but to a conformational change following the conversion of CO_2 into HCO_3^-, such as a change in coordination number of zinc from 5 to 4 accompanied by solvent reorganization in the active site with concomitant changes in hydrogen bonding and hydrophobic interactions. This alternative possibility does not explain so readily, however, the large solvent deuterium isotope effects observed on k_{cat} and R_{H_2O}. It is probably quite significant, but not yet explained, that there is an inverse isotope effect of about 0.73 on k_{cat}/K_m for the hydrolysis of *p*-nitrophenyl acetate catalyzed by human carbonic anhydrase II,[95] and of about 0.5 on k_{cat} for the hydration of acetaldehyde catalyzed by bovine type II isozyme.[101] These inverse isotope effects can possibly arise from the reactant-state contribution of zinc-bound hydroxide in the rate-limiting step, a step which is different in these cases of slower catalyzed reactions compared with the rapid hydration of CO_2.

Another implication of the isotope effect of unity on k_{cat}/K_m for both directions is that the step which is rate limiting, and involves proton transfer, for k_{cat} may also substantially affect K_m. This is contrary to earlier work which had interpreted the pH-independent value of $K_m^{CO_2}$ to be a dissociation constant.[102,103] Apparent substrate dissociation constants obtained from equilibrium studies using ^{13}C NMR are in fact much larger than K_m,[83] as are the values of the apparent dissociation constants obtained by measurement of the inhibition by equilibrium mixtures of CO_2 and HCO_3^- of the hydrolysis of *p*-nitrophenyl acetate catalyzed by human carbonic anhydrase II.[99]

Steiner et al.[95] interpreted the rate-limiting proton transfer as the step in which water at the active site is split to hydroxide with the proton being transported to the solution. Since buffer concentration in solution is large, it is not the transfer of a proton from the enzyme to external buffer that is rate limiting, but an intramolecular proton transfer between the active site and a proton-transfer group of the enzyme. This hypothesis does not include proton transfer directly between the active site and external buffer. In order to play a role in both the hydration and dehydration directions, the proton shuttle groups must have a pK_a close to 7. This is easiest to accomplish with a histidine group and

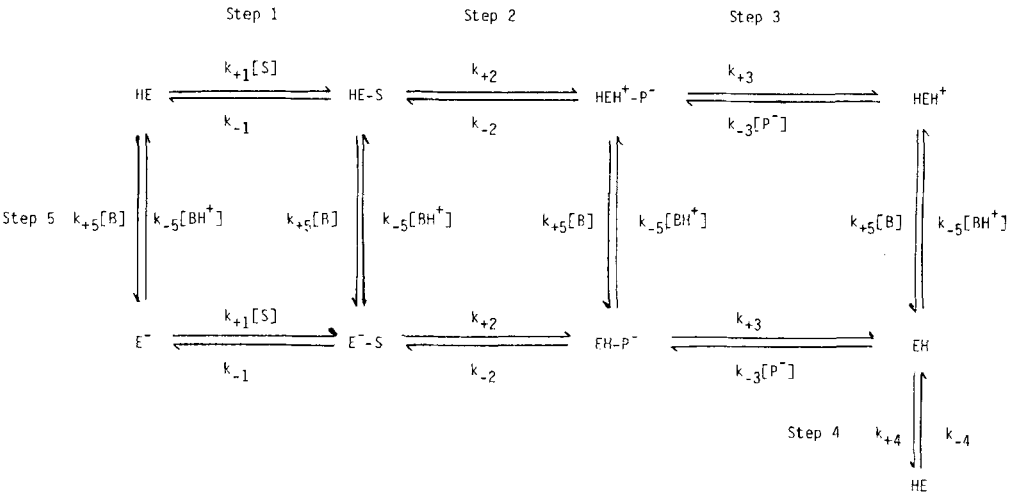


FIGURE 10. A catalytic mechanism for carbonic anhydrase II proposed by Steiner et al.[95] S and P denote CO_2 and HCO_3^-, respectively. The protonated form of the catalytic group is designated EH; the protonated form of the internal proton transfer group is designated HE. The interconversion of CO_2 and HCO_3^- occurs in Steps 1, 2, and 3, which are proposed to be independent of the protonation state of the proton tranfer group. Step 4 is the intramolecular proton transfer proposed to be rate limiting in the presence of an excess concentration of buffers. Steps 5 denote the transfer of protons between the transfer group and buffer in solution.

suggests proton transfer from the active site to His 64 of both the human and bovine type II enzymes. There are few other groups near the active site that could be identified with such a proton transfer function. One possibility suggested by Kannan[44] is Glu 106 hydrogen-bonded to Thr 199, although this requires an anomolously high pK_a for glutamic acid. Ionization with pK_a near 7 of one of the histidine ligands of zinc is not a likely event[104] and is discussed further in Section IV.

Written in more general terms, the proposal of Steiner et al.[95] is shown in schematic form in Figure 10. The rate-limiting event in the catalysis, in the presence of excess external buffer, is the internal proton transfer of Step 4. The intermolecular proton transfer involving external buffer is Step 5. This scheme does not preclude the binding of CO_2 to the form of enzyme with a protonated catalytic group, but does require that such a bound complex EH-CO_2 be kinetically insignificant. There is experimental evidence that imidazole, a competitive inhibitor of CO_2 hydration catalyzed by the type I isozyme, binds equally well to acidic and basic forms of the active site.[106] Moreover, in the scheme of Figure 10, the actual interconversion of CO_2 and HCO_3^- occurs independently of whether the proton transfer group is protonated or unprotonated. In this scheme an isotope effect on the rate-limiting Step 4 affects k_{cat} and K_m equally in both hydration and dehydration directions. Furthermore, when $k_4 = k_{-4}$ the K_m values become independent of pH as is experimentally observed.[95,96]

Venkatasubban and Silverman[107] measured the solvent deuterium isotope affect on V_{max} and K_m as a function of the deuterium content of solvent for hydration of CO_2 catalyzed by bovine red cell carbonic anhydrase in the presence of excess buffer. Plots of V_{max} and K_m against deuterium content of solvent are clearly nonlinear and bulge down, the most straightforward interpretation of which is that more than one hydrogen contributes to the isotope effect. The authors concluded that the intramolecular proton transfer is not consistent with the transfer of a single proton in the transition state of the rate-limiting step, is marginally consistent with the transfer of two protons, but fits best with the transfer of three or more protons. In fact, this is the first case of an enzymic process for which there is a logarithmic relationship between the steady-state rate

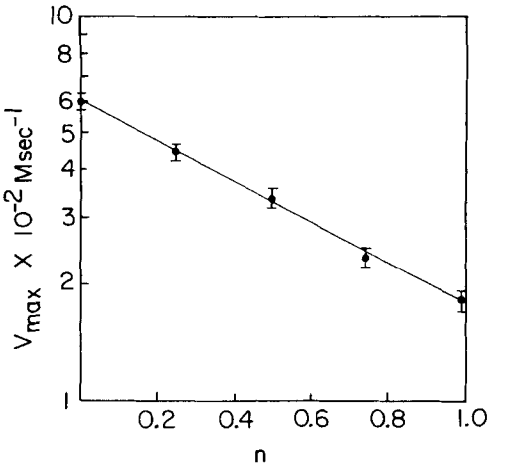


FIGURE 11. Maximum velocity, V_{max}, of the hydration of CO_2 catalyzed by bovine red cell carbonic anhydrase plotted against n, the atom fraction of deuterium in solvent water. The concentration of carbonic anhydrase was 4×10^{-8} *M* in solutions containing 50 m*M* 1,2-dimethylimidazole with *m*-cresol purple as the indicator. The pL was 8.20 and the total ionic strength of solution was maintained at 0.10 using Na_2SO_4. Temperature was 25°C. (From Venkatasubban, K. S. and Silverman, D. N., *Biochemistry*, 19, 4984, 1980. With permission.)

constants and the atom fraction of deuterium to solvent (Figure 11). Such behavior is expected for a multi-proton mechanism in which many protons each contribute a small but approximately equal normal isotope effect.[108] This result is consistent with the possibility that the observed isotope effects are caused entirely or in part by solution changes in the active site which occur in the rate-determining step. However, Venkatasubban and Silverman[107] interpreted this result within the context of the proposal of Steiner et al.,[95] and suggested that the internal proton transfer, Step 4 of Figure 10, involves water in the active site, proceeding through water bridges.

B. Studies of the Catalytic Rates at Chemical Equilibrium

Considerable support for the hypothesis of a rate-determining intramolecular proton-transfer in the enzymic pathway of the hydration of CO_2, as well as considerable evidence for the complexity of the mechanism, comes from studies of the catalyzed rates measured at chemical equilibrium. Since the classic work of Boyer[109] beginning in 1959, isotope exchange measured at equilibrium has been recognized as a useful approach to the study of individual steps in an enzymic pathway. For carbonic anhydrase, the two main approaches have used the exchange-broadening of the ^{13}C resonance of $^{13}CO_2$ and $H^{13}CO_3^-$ which results from the rapid catalytic interconversion of substrate and product, and the exchange of ^{18}O between species of CO_2 and water which also results from the catalytic interconversion of CO_2 and HCO_3^-.

The ^{13}C NMR method was developed for human carbonic anhydrase I by Koenig et al.[110,111] and applied to the type II isozyme by Simonsson et al.[83] It relates the linewidth of the ^{13}C resonances to the equilibrium rate of interconversion of CO_2 and HCO_3^-, which can be expressed in a rate expression of Michaelian form:

$$R_1 = \frac{k_{cat}^{exch}[S][E_t]}{K_{eff}^{S} + [S]} \quad (10)$$

Here R_1 is the equilibrium rate, k_{cat}^{exch} is the maximal rate constant for the interconversion at equilibrium, and K_{eff}^{S} is an apparent dissociation constant for substrate, the value of which depends on the equilibrium between substrate and various forms of the enzyme.[83] This method has contributed to our understanding of the catalytic mechanism in several ways. First, Simonsson et al.[83] observed that k_{cat}^{exch}, the maximal rate constant for catalysis at equilibrium, is larger than either of the maximal turnover numbers from steady-state experiments. The fact that the steady-state values are less indicates that the maximal catalyzed rates under steady-state conditions are limited by steps that are not involved in the interconversion of CO_2 and HCO_3^-. This is in agreement with the interpretation of the solvent deuterium isotope effect of unity observed for the steady-state ratio $k_{cat}^{CO_2}/K_m^{CO_2}$, but of magnitude 3 to 4 observed for $k_{cat}^{CO_2}$. Moreover, Simonsson et al.[83] observed a solvent deuterium isotope effect of unity on R_1. These observations are consistent with a rate-limiting step involving a change in bonding to hydrogen and occurring outside the pathway of interconversion of CO_2 and HCO_3^-. Second, no buffer effect was observed on R_1,[83] indicating that the mechanism by which the ionization of the active site equilibrates with the solvent also occurs outside the pathway of interconversion of CO_2 and HCO_3^-. And third, the values of $K_{eff}^{HCO_3^-}$ obtained differ considerably from the values of the steady-state Michaelis constant $K_m^{HCO_3^-}$. The magnitude and pH-dependencies of $K_{eff}^{HCO_3^-}$ and k_{cat}^{exch} are given in Figure 12 for comparison with the steady-state constants of Figures 3 and 4. Of course, $K_{eff}^{HCO_3^-}$ does not in general equal the true substrate dissociation constant, as discussed by Simonsson et al.,[83] although at acidic pH when $k_2 >> k_{-2}$ in the scheme of Figure 10 then $K_{eff}^{HCO_3^-}$ is a true dissociation constant. However, values of $K_{eff}^{HCO_3^-}$ obtained from ^{13}C NMR are in agreement in both magnitude and pH dependence with the inhibition constants obtained from studies of the inhibition of the hydrolysis of *p*-nitrophenyl acetate by equilibrium mixtures of HCO_3^- and CO_2. The values of $K_{eff}^{HCO_3^-}$ differ in several ways from $K_m^{HCO_3^-}$: (1) $K_{eff}^{HCO_3^-}$ is sharply pH dependent (Figure 12), whereas $K_m^{HCO_3^-}$ is rather independent of pH (Figure 3); (2) $K_{eff}^{HCO_3^-}$ is much larger than $K_m^{HCO_3^-}$; and (3) as anticipated for a binding constant, $K_{eff}^{HCO_3^-}$ is not affected when the solvent is changed to D_2O, whereas $K_m^{HCO_3^-}$ has a solvent deuterium isotope effect between 3 and 4. The conclusion of these observations is that $K_{eff}^{HCO_3^-}$ approximates a substrate dissociation constant and that $K_m^{HCO_3^-}$ is a kinetic constant and not a binding constant. This conclusion serves to eliminate models of the catalysis which, based in part on the pH-independent values of $K_m^{HCO_3^-}$, predict that $K_m^{HCO_3^-}$ is a substrate dissociation constant. This comment can also be extended to $K_{eff}^{CO_2}$. At pH 7.5 the data of Figure 12 give $K_{eff}^{CO_2} = 30$ m*M* which is 3 to 4 times larger than $K_m^{CO_2}$, so that $K_m^{CO_2}$ is probably not a binding constant either.

Since the isotope effects of unity for k_{cat}/K_m and R_1 suggest that there is no rate-limiting proton-transfer in the interconversion of CO_2 and HCO_3^-, it becomes of interest to identify the step or steps of the catalysis in which there is a deuterium isotope effect indicating a rate-limiting proton transfer. The ^{18}O-exchange method has been useful for this purpose since it yields the rates of two steps of the catalysis: R_1 as described in the previous paragraphs; and R_{H_2O}, the rate of release from the enzyme of water bearing substrate oxygen.[73] There seems to be no difficulty with this method in obtaining values of R_1 which agree with the values of R_1 obtained from the ^{13}C NMR experiments[83] and can be related to steady-state constants through the parameters of Equation 10: $k_{cat}^{ex}/K_{eff}^{S} = k_{cat}^{S}/K_m^{S}$ for the substrate S.[83,110,111] On the other hand R_{H_2O} is more difficult to obtain and understand. R_{H_2O} is calculated from a ratio of rate constants for ^{18}O exchange[73] and, for values of R_{H_2O} exceeding 1×10^{-3} Msec^{-1}, can have a large standard deviation from propagated errors. Moreover, R_{H_2O} has not yet been firmly related to any

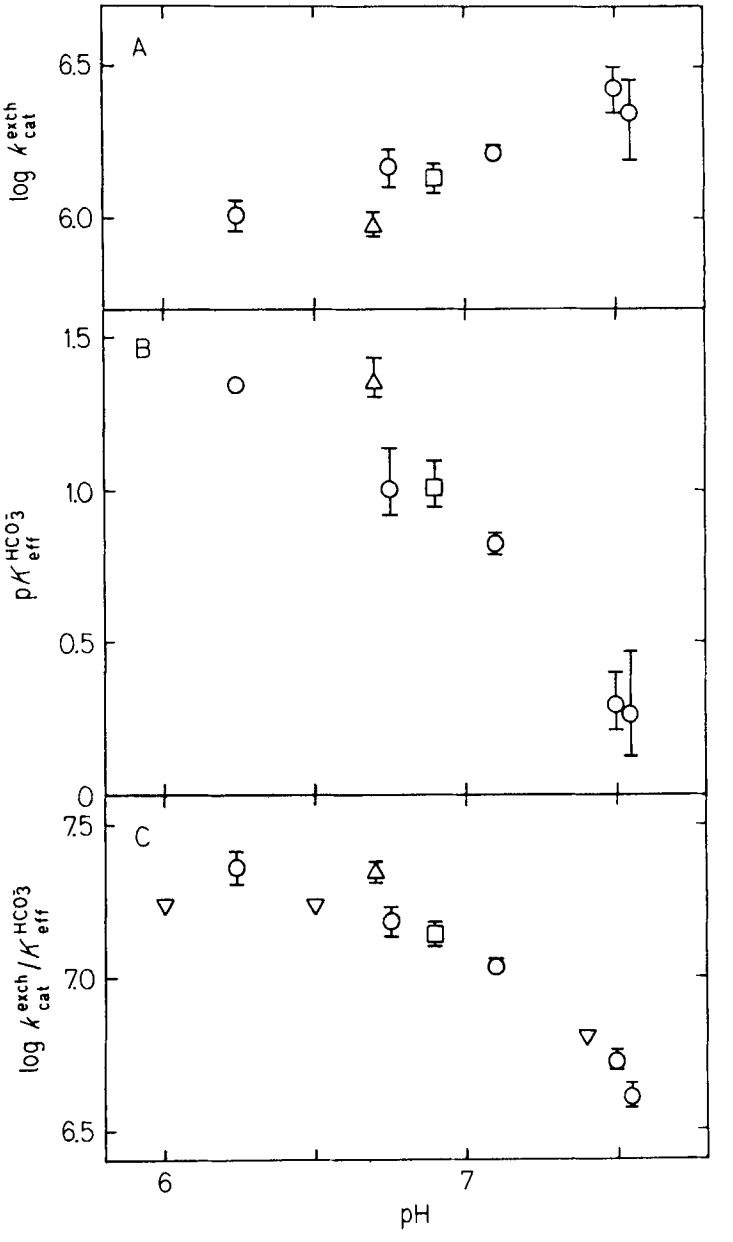


FIGURE 12. pH dependence of the equilibrium exchange parameters of Equation 10. (A) log k_{cat}^{exch} (sec^{-1}); (B) $pK_{eff}^{HCO_3^-} = -\log K_{eff}^{HCO_3^-}$ (M); (C) log $k_{cat}^{exch}/K_{eff}^{HCO_3^-}$ (M^{-1} sec^{-1}). Symbols: (○) no buffer in 94% H_2O and 6% D_2O; (□) no buffer in 99.5% D_2O; (△) 50 m*M* N-methylimidazole in 94% H_2O; (▽) $k_{cat}^{HCO_3^-}/K_m^{HCO_3^-}$ from steady-state studies of Steiner et al.[95] Temperature 25°C with solutions containing human carbonic anhydrase II: ionic strength maintained at 0.2 with Na_2SO_4. (From Simonsson, I., Jonsson, B. H., and Lindskog, S., *Eur. J. Biochem.*, 93, 409, 1979. With permission.)

other observed kinetic or spectroscopic property of carbonic anhydrase. Yet it can be convincingly shown that R_{H_2O} contains the steps which give rise to both the solvent deuterium isotope effects and to the buffer effects in the catalysis.[73]

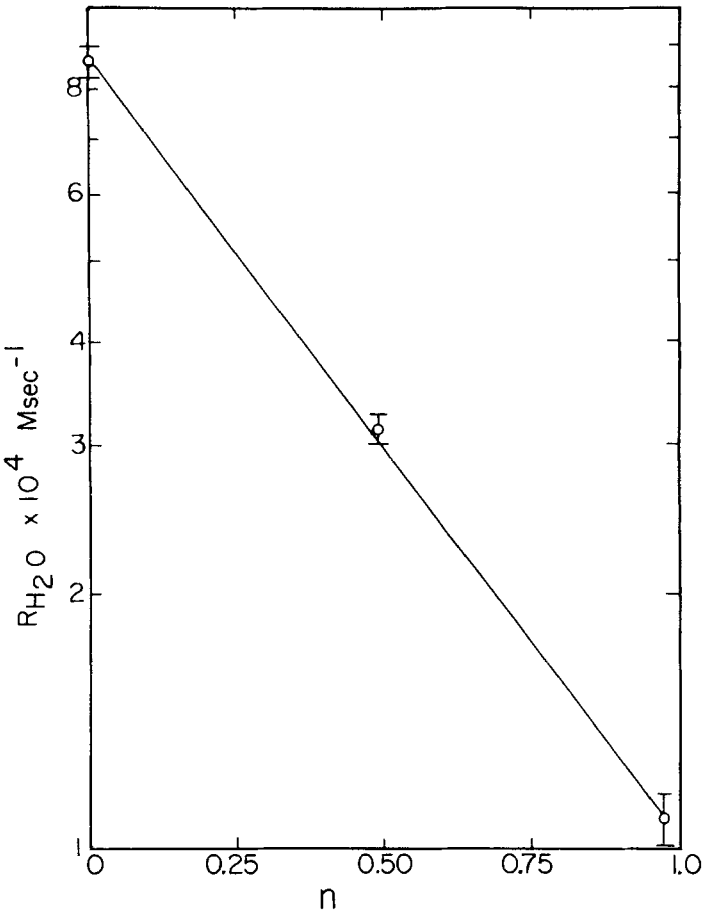


FIGURE 13. R_{H_2O} at three values of n, the atom fraction of deuterium in solvent water. Data are the mean and standard deviation of four experiments. Human carbonic anhydrase II was present at 2.0×10^{-9} *M* and the total concentration of CO_2 species was 15 m*M*. The uncorrected pH-meter reading was 6.6 at 25° C. No buffers were used; ionic strength was maintained at 0.2 with Na_2SO_4. (From Tu, C. K. and Silverman, D. N., *Biochemistry*, 24, 6353, 1982. With permission.)

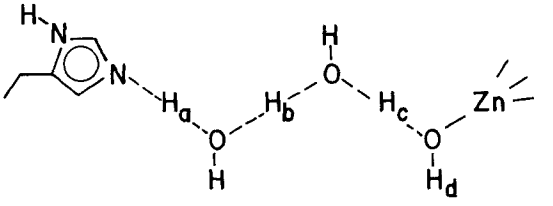
The buffer effect on R_{H_2O}, which was discussed in Section II, and the pH dependence of R_{H_2O} is shown in Figure 6. The bell-shaped pH dependence of R_{H_2O} that was originally reported was obtained using solutions containing Na_2SO_4 to maintain ionic strength.[73] It has since been suggested[79] and shown[55] that sulfate is an inhibitor of carbonic anhydrase II at pH < 7. Figure 6 also shows data taken in the absence of sulfate but in the presence of 30 m*M* MES as buffer (MES is 4-morpholineethanesulfonic acid). These data in the absence of sulfate do not decrease as pH is lowered below 7 and appear to be described, within errors, by a titration curve of pK_a near 7.5. This is the approximate pK_a found from the Brønsted plot of Figure 9, suggesting that the buffer enhancement of R_{H_2O} and buffer enhancement of the initial velocity are functioning through the same group on the enzyme, or at least through groups of similar pK_a. The data of Figure 6 are in agreement with the observations of Simonsson and Lindskog[55] that 50 m*M* Na_2SO_4 inhibits catalysis of the hydrolysis of *p*-nitrophenyl acetate by 56% at pH 6.0.

The ^{18}O method is in agreement with the ^{13}C NMR data that R_1 is not affected by the change of solvent from H_2O to D_2O. However, the solvent deuterium isotope effect on

R_{H_2O} is substantial both in the presence of 50 m*M* imidazole ($R_{H_2O}/R_{D_2O} = 7.2$ at pH(D) 7.4[73]) and in the absence of buffer (Figure 13). The main conclusion that can be drawn from these data is that there is one or more steps involving proton transfer which are rate limiting for R_{H_2O}. Several pertinent facts help us narrow the possibilities for which step this is. First, measuring $1/T_1$ for H_2O. Silverman has shown that the fractionation factor is indistinguishable from unity for exchangeable hydrogen in the inner coordination sphere of cobalt in Co(II)-substituted, bovine carbonic anhydrase.[113] The fractionation factor for the hydrogen of solvent water is also unity. Consequently, it is unlikely that a deuterium isotope effect will be observed for the exchange of water between bulk solvent and its position as a ligand of the metal in carbonic anhydrase. Second, the large deuterium isotope effect on R_{H_2O} was observed in the absence of buffers (Figure 13). Consequently the proton transfer involved in R_{H_2O} is not between external buffer and the enzyme. Substrate itself does not act as a proton transfer agent to affect R_{H_2O}, at least not at pH near 7, since R_{H_2O} is rather independent of total substrate concentration at this pH.[73] Also, the concentrations of H_3O^+ and OH^- are too small near neutral pH to function as effective proton transfer agents, as was shown in the calculations of Section II using the bimolecular rate constants for diffusion-controlled proton transfer. Hence, the proton transfer or transfers influencing the rate-limiting step must be intramolecular and must occur not in the CO_2-HCO_3^- interconversion but in protonation steps that lead to the release to solvent of water bearing substrate oxygen. This proton transfer could very well be that described by Step 4 in Figure 10.

Moreover, the magnitude of the isotope effect on R_{H_2O} has an exponential dependence on n the atom fraction of deuterium in solvent water (Figure 13). This result is similar to that shown in Figure 11 for V_{max} for hydration. The identical observation of exponential dependence of both V_{max} for hydration and R_{H_2O} on n supports further the comment that the same rate-limiting proton transfer is manifested in the isotope effects on V_{max} and R_{H_2O}.

The ^{18}O studies then give a direct confirmation of the interpretation of Steiner et al.[95] that the observed isotope effects originate not in the steps which convert CO_2 to HCO_3^-, but in subsequent steps which also occur in R_{H_2O}. A likely mechanism for a proton transfer is shown in Equation 11, the same as presented by Venkatasubban and Silverman,[107] and involves water bridges in the transfer of a proton between the imidazole side chain of His 64 and the aqueous ligand of zinc. Three hydrogens (H_a, H_b, and H_c) change bonding in the transition state. The fourth (H_d) probably does not, and would be unlikely to affect isotope studies since the fractionation factor for zinc-bound hydroxide is expected to be close to 1.0, the same value expected for zinc-bound water.[113] The remaining hydrogens in Equation 11 do not change bonding in the proton transfer.



The imidazole ring of His 64 is about 6 Å from the zinc[24] in human carbonic anhydrase II, and observation of its magnetic resonance spectrum indicates a pK_a of 7.1.[91] Specific covalent modification of His 64 at the N_τ position by bromopyruvate[114] yields a modified derivative of human carbonic anhydrase II with CO_2-hydration activity decreased by 70 to 85%.[103] The turnover number k_{cat} for the hydration of CO_2 catalyzed by the carboxyketoethylated human II isozyme has a maximal value of 3×10^5 sec^{-1} and has

approximately the same pH dependence as k_{cat} for the native enzyme, which has a maximal value of 1.4×10^6 sec^{-1}. Although this is consistent with the proton shuttle proposal of Steiner et al.,[95] it argues against an essential role for His 64 in the catalytic mechanism since the activity upon modification is not completely abolished. (This conclusion is based on the assumption that the remaining unmodified nitrogen of the imidazole ring of His 64 cannot function in the mechanism.) Khalifah has pointed out the very interesting fact that k_{cat} and K_m for the carboxyketoethylated type II enzyme resemble k_{cat} and K_m for the native type I enzyme.[115]

The rate R_{H_2O} must then include at least one intramolecular proton transfer in a rate-limiting step and a second step which is the release of water from the enzyme to the solvent. The scheme in Equation 11 is then consistent with the observed pH dependence of R_{H_2O} in the absence of sulfate (Figure 6) since only those enzyme molecules with a protonated internal shuttle group (such as His 64) would be able to protonate $\geqslant Zn^{18}OH$ to give the rapidly exchanging $\geqslant Zn^{18}OH_2$. This presumes, as in the original proposal of Steiner et al.,[95] that external buffer in solution does not protonate directly zinc-bound hydroxide at the active site. The proposal then ascribes both the pH dependence of R_{H_2O} (Figure 6) and the Brønsted plot (Figure 9) to the pK_a of one or more shuttle groups of pK_a near 7. Actually, Figures 6 and 9 show evidence of pK_a values somewhat above 7, but experimental scatter and uncertainty are substantial in both sets of data. The rate constant for intramolecular proton transfer can be estimated from the low pH region in Figure 6, in the absence of sulfate, using the microscopic pK_a values obtained by Simonsson and Lindskog:[55] $k_{-4} = 2 \times 10^6$ sec^{-1}. This is to be compared with the value $k_{-4} = 7 \times 10^5$ sec^{-1} estimated by Steiner et al. from stopped-flow studies.[99]

It is necessary to ask at this point how an intramolecular proton transfer between oxygen and nitrogen acids and bases, which is usually a very fast reaction ($k > 10^6$ sec^{-1})[66,116] can exert an isotope effect on R_{H_2O}, which appears to be comparatively slow ($k_{-4} = 10^6$ sec^{-1}). It is possible that an isotope effect is observed on R_{H_2O} because the rate-limiting proton transfer is preceded by an unfavorable equilibrium step in a complex reaction scheme. The slow overall rate would then be a consequence of conformational fluctuations in the enzyme, the proton-bearing residue (such as His 64, for example) and its solution shell being in an orientation favorable for proton transfer only a small fraction of the time. Another possibility, not involving a conformational change, is that the geometry for the intramolecular proton transfer in the active site may not be ideal. That is, the angle or more likely the distance required for the proton transfer between the shuttle group and the active site may be constrained and unfavorable.

It is of interest to end this section on intramolecular proton transfer with one other result. Tu et al.[87] have reported the specific inhibition of R_{H_2O} at pH near 7.3 for human carbonic anhydrase II by cupric and mercuric ions with 50% inhibition of R_{H_2O} at 1×10^{-7} *M* for Cu^{2+} and 1.6×10^{-7} *M* for Hg^{2+}. The inhibition of R_{H_2O} was greatest near pH 7 and was not detectable below pH 6 or above pH 8; the inhibition can be reversed by addition of EDTA. At concentrations of Cu^{2+} and Hg^{2+} less than 10^{-6} *M* the rate of interconversion of CO_2 and HCO_3^- at chemical equilibrium R_1 was not affected. Tu et al. concluded that these metal ions below 10^{-6} *M* are inhibiting either the internal proton transfer or the release of H_2O from the enzyme, or both, without affecting the actual interconversion of CO_2 and HCO_3^-. This suggests the use of these metals as spectroscopic probes of the proposed proton shuttle pathway. It should be pointed out that the inhibition of the initial rate of hydration of CO_2 catalyzed by human carbonic anhydrase II by concentrations of Cu^{2+} less than 10^{-6} *M* was reported by Magid.[117] The relationship between inhibition of initial rate and inhibition of R_{H_2O} at equilibrium has not yet been explored. It should also be noted that anionic inhibitors of carbonic anhydrase such as I^-, N_3^- and SCN^- inhibit both R_1 and R_{H_2O}. Iodide ions at 17 m*M* inhibit both R_{H_2O} and R_1 by

50%. These results are consistent with the inhibition by iodide of the binding of bicarbonate to the active site. Since $HCOO^{18}O^-$ binding is inhibited, so is the labeling by ^{18}O of the active site, as well as the release of $H_2{}^{18}O$ to solvent.

C. Critique

Overall, these studies using D_2O and isotope exchange at equilibrium seem quite firm in demonstrating the separation in the catalysis of two steps. That the first, the interconversion of CO_2 and HCO_3^-, has a solvent deuterium isotope effect of unity and no buffer effect is quite firm. It seems equally firm that the source of the deuterium isotope effect observed on the steady-state parameters V_{max} and K_m is a step occurring after the departure of product. The observation of a rate-limiting intramolecular proton transfer in an enzymic process is not unusual. Such a step is believed to occur, for example, in the trypsin-catalyzed hydrolysis of the oligopeptide analogue *N*-benzoyl-L-phenyl-alanyl-L-valyl-L-arginyl *p*-nitroanilide, which exhibits $(V_{max})_{H_2O}/(V_{max})_{D_2O}$ near 4.[118] The enzyme accelerates C-N bond fission, resulting in the release of nitroaniline, by providing a proton to the nitrogen; the data are consistent with such a proton transfer in the rate-limiting step. What appears to be unusual in the case of carbonic anhydrase is that the rate-limiting, intramolecular proton transfer appears in a step separate and distinct from the steps involved in the conversion of CO_2 to HCO_3^-.

The hypothesis of a rate-limiting, intramolecular proton transfer provides for carbonic anhydrase a qualitative explanation of R_{H_2O}, since only those enzyme molecules bearing protonated shuttle groups would be able to release ^{18}O to solvent. This proposal yields quantitative agreement between the stopped-flow and ^{18}O exchange techniques, which both give a rate constant near 7×10^5 sec^{-1} for this intramolecular proton transfer. Further evidence for the presence of an intramolecular proton transfer are the appreciable values of R_{H_2O} in the absence of external buffer (Figure 6), values that indicate the enzyme has a rapid path for H_2O exchange following dehydration of HCO_3^-. In the absence of buffer the species $E(B')\,{}^{18}OH$ would accumulate, where B′ is the internal proton shuttle group. Introduction of external buffer provides a rapid pathway to the species $E(B'H^+)\,{}^{18}OH$ which can then proceed with intramolecular proton transfer to $E(B')\,{}^{18}OH_2$ and finally exchange $H_2{}^{18}O$ with solvent water.

It is also firm that these ^{18}O exchange and ^{13}C NMR methods are measuring events occurring at the active site. It is for this reason that there should be some comparison with the many studies of the enhancement of the relaxation rate of water protons in the presence of Co(II)-substituted carbonic anhydrase. This enhancement follows a titration curve of pK_a 7, under conditions of physiologic ionic strength, with a maximum at alkaline pH.[65,119,120] This means that at high pH there is a rapidly exchanging proton within the first coordination sphere of the metal with an off-rate near or greater than about 3×10^4 sec^{-1}; and at low pH either no proton exchanging from the first coordination sphere or an exchange which is too slow to measure from magnetic relaxation rates, less than 5×10^3 sec^{-1}. The ^{18}O studies suggest that at the active site, labeled oxygen from substrate does not exchange rapidly with water at high pH, while this substrate oxygen comes out into the solvent water rather rapidly at low pH, with a rate near 10^6 sec^{-1} ($R_{H_2O}/[E_{tot}] = 1.5 \times 10^{-3}$ $Msec^{-1}/1.6 \times 10^{-9}$ *M*, see Figure 6). These values can be compared to the rate of water exchange in $Co(H_2O)_6^{2+}$ which has been measured at 2.4×10^6 sec^{-1} at 25°C by ^{17}O NMR.[121] The rate of exchange of ^{18}O is very dependent on buffer concentration below about 10 m*M* buffer whereas the relaxation rate of water protons in the presence of Co(II)-carbonic anhydrase is not changed by buffer. In comparing these very different experiments, ^{18}O exchange and proton relaxation enhancement, we must remember that $1/T_1$ for water protons is in the fast exchange limit on the NMR time scale. Hence, the proton relaxation rate is not an exchange rate and variations in R_{H_2O} need not be reflected in $1/T_1$ for water protons in the

presence of Co(II)-substituted carbonic anhydrase. Bertini et al.[121a] have measured the frequency dependence of this proton relaxation rate at high pH and have found a correlation time consistent with a tetrahedral metal environment. Thus, the exchange of both oxygen and protons in the high pH form of carbonic anhydrase could be occurring from a tetrahedral metal with hydroxide ion as the fourth ligand. Numerous other possibilities have been suggested.

Lindskog has offered a useful discussion of this problem with some interesting possible solutions.[3] One is that the zinc in carbonic anhydrase is five-coordinated at high pH and bears both a hydroxide and water ligand, only the latter of which exchanges rapidly with solvent water. Koenig and Brown have proposed a catalytic mechanism which is compatible with the NMR result in that it asserts that the only catalytically active form of carbonic anhydrase bears a zinc-bound water and that the pH dependence of catalysis is due to inhibitor binding, especially the inhibitor sulfate.[79] This mechanism is discussed in the literature,[3,80] and it should be noted that the pH-dependence of the catalysis of hydrolysis of *p*-nitrophenylacetate retains a pK_a near 7 in the absence of sulfate and other inhibitory anions.[55]

The ^{113}Cd NMR of Cd(II)-substituted carbonic anhydrases, through the resonance linewidth, is also affected by the rate of exchange of ligands at the metal. The very broad ^{113}Cd resonance at pH values between 5.5 and 10 for human Cd(II)-carbonic anhydrase I indicates a rate of exchange of ligand, most likely water, in the range of 10^3 to 10^4 sec^{-1}.[122] On the other hand, the sharp ^{113}Cd resonance of the type II isozyme, which is rather independent of pH in the range 5.5 to 10, suggests an exchange of the water ligand greater than 10^5 sec^{-1}.[122]

IV. THE MECHANISM OF INTERCONVERSION OF CO_2 AND HCO_3^-

We concentrate finally on the actual interconversion of CO_2 and HCO_3^-. The central issue is whether catalysis of the hydration of CO_2 proceeds by direct nucleophilic attack of zinc-bound hydroxide at the carbon or whether a general base mechanism is involved. This discussion relies on model compounds, which are successful spectroscopic but very poor catalytic mimics of carbonic anhydrase, and relies heavily on kinetic studies, which by two independent equilibrium methods show that the interconversion of CO_2 and HCO_3^- occurs with a solvent deuterium isotope effect of unity.

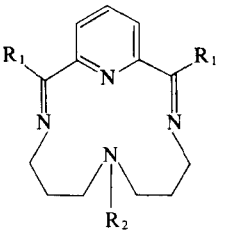
The kinetics of the uncatalyzed reactions betweeen CO_2 and water has been reviewed and discussed.[59,123,124] There may be several clues pertinent to the catalytic mechanism here. Pocker and Bjorkquist[125] have described routes for the uncatalyzed hydration of CO_2 involving nucleophilic attack by water on CO_2 and involving the action of water as a general base to abstract a proton from a second attacking water molecule. The authors found that solvent deuterium isotope effects were not able to distingish firmly between these mechanisms.[125] Dennard and Williams did not observe general base catalysis of the reaction of CO_2 and water in a study over a wide range of pH using many different buffers.[126] *Ab initio* molecular orbital calculations by Jönsson et al.[127] on the reaction of CO_2 and water, pertinent only to conditions of the gas phase, determined an energy barrier for the reaction (219 ± 55 kJ/mol) much greater than that observed in aqueous solution (75 kJ/mol). This, of course, is due to the role of solvent water in stabilizing the charged transition state and the role of solvent water in the transfer of a proton in the formation of bicarbonate. (The solvent deuterium isotope effect for the uncatalyzed hydration is 1.8.[125]) In contrast, calculations show that there is no energy barrier for the reaction between CO_2 and OH^- in the gas phase, whereas in solution the observed activation energy is 55 kJ/mol.[128] Consequently, the energy barrier to the reaction of CO_2 with OH^- can be attributed to solvation effects, a comment which is quite pertinent to the catalysis by carbonic anhydrase. As pointed out by Jönsson et al.,[128] catalytic efficiency

would be promoted by the somewhat hydrophobic environment of the active site cleft for the enzyme containing zinc-bound hydroxide.

A. Model Studies

Model studies have centered on complexes of 3d transition metal ions. These are pertinent to the catalytic mechanism since there is a large body of evidence which indicates that the activity-controlling group in carbonic anhydrase which ionizes with a pK_a of 7 under physiological conditions is zinc-bound water.[3] Campbell et al.[91] showed that none of the titratable histidines near the active site have the properties of the activity-controlling group and Bertini et al.[105] have shown that none of the histidine ligands of the metal ionizes in the range of pH 5.5 to 9.0 ruling out these groups as the activity-controlling group. Of the remaining residues that may be considered, Glu 106 has not been ruled out by experimental evidence; although, if it is responsible for the activity as suggested by Kannan et al.,[44] Glu 106 would have an unusually high pK_a.

There remains then the dissociation of zinc-bound water to form zinc-bound hydroxide, first proposed by Davis[129] to be the form of enzyme active in hydration and formulated more completely in mechanistic terms by Coleman.[130] It is appropriate to ask at this point whether a pK_a of 7 is not also unusual for the dissociation of a proton from zinc-bound water; for the simple aqueous ions of zinc and cobalt, this proton dissociation occurs with values of pK_a near 9 and 10, respectively.[131] A very useful model to help answer this question was devised by Woolley[132] using macrocylcic ligands of the structure shown below:



The metal is a ligand of the four nitrogens plus one or two water molecules. Spectroscopic and crystallographic studies indicate a coordination number of 5 for complexes of Zn^{2+} and Co^{2+}, and coordination number of 6 for Ni^{2+} and Cu^{2+}.[132] Interestingly, the metals Zn^{2+} and Co^{2+} which form complexes with carbonic anhydrase of high catalytic activity also promote the ionization of water in the macrocyclic complex. Values of pK_a as low as 8.1 were found by Woolley for certain of these macrocyclic complexes ($R_1 = R_2 = CH_3$, for Zn^{2+} at low ionic strength.)

Woolley demonstrated that the complex of Zn^{2+} with a macrocyclic ligand ($R_1 = CH_3$, R_2 = H) catalyzes the hydration of acetaldehyde with a pH dependence which is consistent with catalysis by the high pH form, that with a zinc-bound hydroxide. The apparent pK_a obtained from the catalytic efficiency in the hydration of acetaldehyde (9.07, Figure 14) is comparable to the value obtained from the pH titration (9.17). By comparison with the catalysis caused by other bases, Woolley was able to deduce that the mechanism of hydration was by direct nucleophilic attack of zinc-bound hydroxide on the carbonyl carbon of acetaldehyde, not a general-base mechanism.[132,133] As a catalyst for the hydration of CO_2, the zinc-containing macrocyclic complex ($R_1 = CH_3$, $R_2 = H$) was only very slightly active, about as active as copper glycylglycinate.

Another interesting model for catalytic CO_2 hydration has been studied by Dasgupta and Harris:[134] $Co(NH_3)_5\,OH^{2+}$ reacts directly with CO_2 form to $Co(NH_3)_5\,CO_3H^{2+}$. The pK_a for the protonation of $Co(NH_3)_5\,OH^{2+}$ is 7.16.[125] The rate of CO_2 uptake by this hydroxopentamine cobalt(III) complex was measured spectrophotometrically by Pocker and Bjockquist[125] and was found to be $3.1 \times 10^2 M^{-1}sec^{-1}$ at 25°C; moreover, the

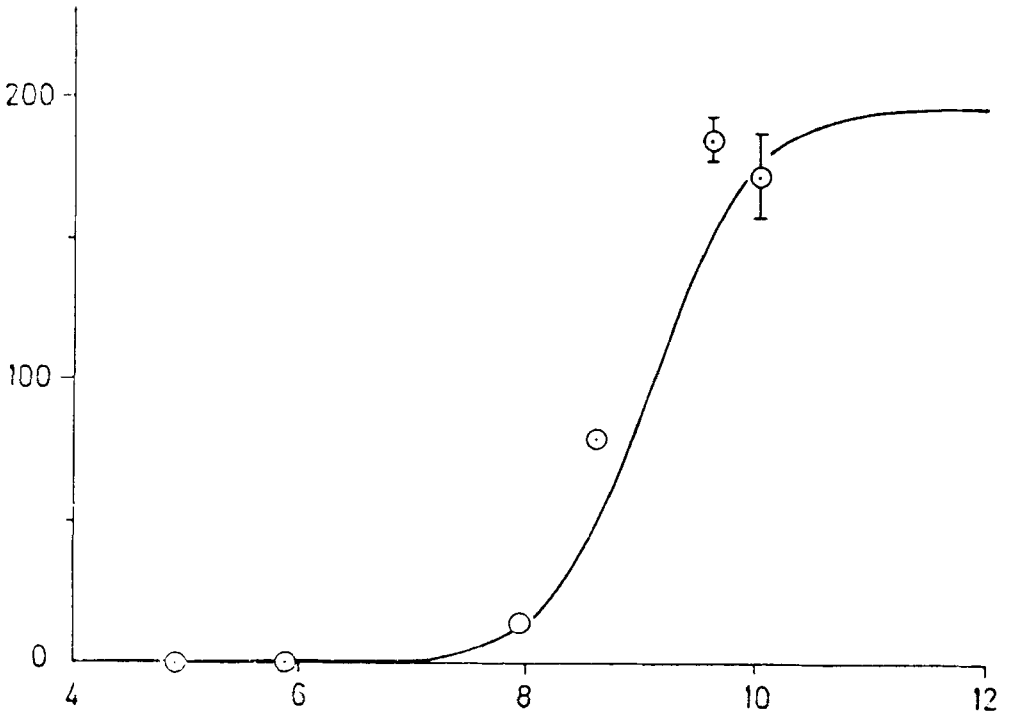
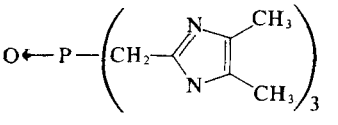


FIGURE 14. The catalytic efficiency in the hydration of acetaldehyde of a macrocyclic ligand of zinc has an apparent pK_a (9.07) close to the pK_a for the ionization of water bound to the zinc in the complex (9.17). The macrocyclic complex is that of Woolley (see text) with $R_1 = CH_3$ and $R_2 = H$. The units of the ordinate are M^{-1} sec^{-1} and the abscissa is pH. (From Woolley, P., *Nature (London)*, 258, 677, 1975. With permission.)

solvent deuterium isotope effect on this rate of CO_2 uptake is unity. This is significant because it indicates that the direct addition of metal-bound hydroxide of CO_2 can occur with a solvent deuterium isotope effect of 1.0. This addition probably occurs by a direct nucleophilic attack since the isotope effect of unity indicates that no proton transfer is likely in the rate-determining step. Later we make analogy to the interconversion of CO_2 and HCO_3^- catalyzed by carbonic anhydrase, the rate of which measured by ^{13}C NMR and ^{18}O exchange also has a solvent deuterium isotope effect near 1.0.

Brown and colleagues[135,136] have prepared a variety of tridentate ligands consisting of three imidazole residues bound via the C-2 carbon to a centeral phosphine which, when bound to Zn^{2+} or Co^{2+}, share spectroscopic and binding properties in common with carbonic anhydrase. The flexible tris imidazole phosphine oxide shown below provides a tridentate ligand for the metal, binding through the imidazole nitrogens. The cobalt(II) complex of this ligand has an ionization of metal-bound water at pK_a near 7.8 in 80% ethanol — 20% water, as deduced from Figure 15. When bound to Zn^{2+} the pK_a is close to 6. Moreover, this spectrophotometric pK_a is shifted to higher values in the presence of



anions, 0.2*M* ClO_4^-, Br^-, and NO_3^- as shown in Figure 15. Regardless of the presence or absence of anions, the same spectrum develops at sufficiently high pH. These properties

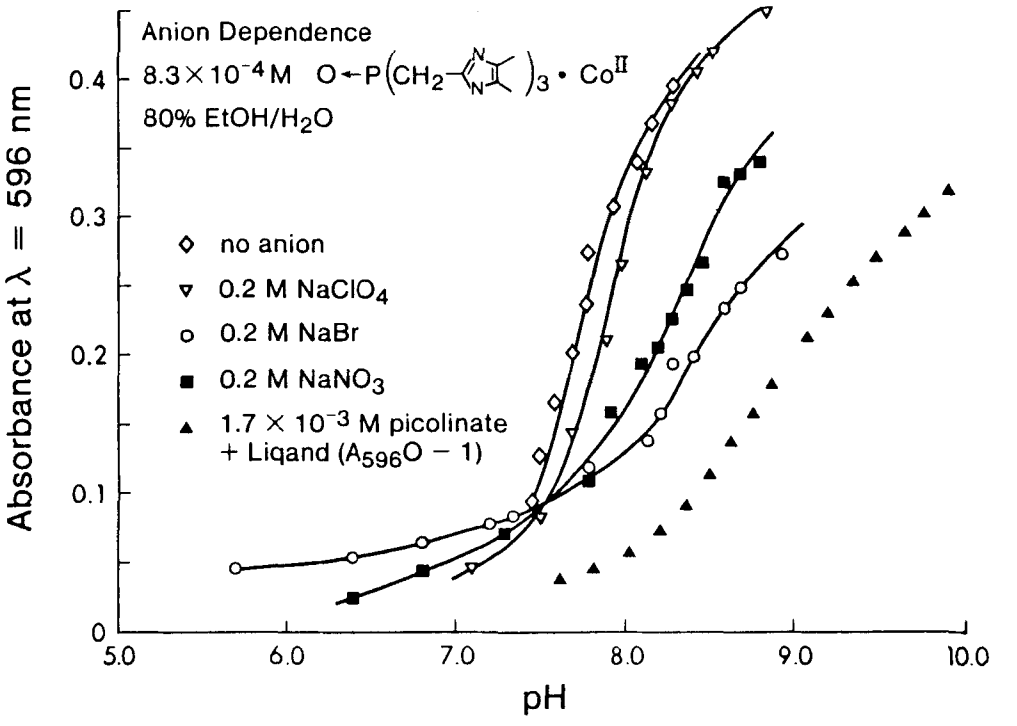
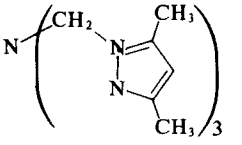


FIGURE 15. The pH-dependence of the absorbance at 596 nm of the tris(imidazole)phosphine oxidide complex with Co(II) of Brown et al. (see text). In the presence of anions, the spectrophometric pK_a shifts to more alkaline values. (From Brown, R. S., Salmon, D., Curtis, N. J., and Kusuma, S., *J. Am. Chem. Soc.*, 104, 3188, 1982. With permission.)

are observed for carbonic anhydrase[49] and are consistent with competition between anion and OH^- for a binding site on the metal. Brown et al.[136] have shown that the deprotonation of cobalt-bound water causes a change in the absorption spectrum of the phosphine oxide complex from that characteristic of a 6-coordinate species to that attributable to a 4 or 5 coordinate cobalt complex. The zinc complex of the phosphine oxide shown above demonstrates a catalysis of CO_2 hydration which is modest, about 200 $M^{-1}sec^{-1}$ compared to 10^8 $M^{-1}sec^{-1}$ for carbonic anhydrase II.

Very similar results have been obtained by Bertini et al.[137] with complexes of cobalt with *tris*[(3,5-dimethyl-1-pyrazolyl)methyl]amine.

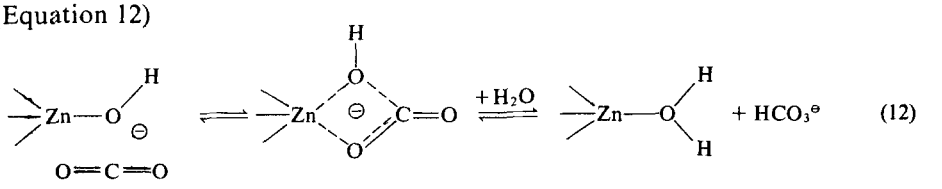


The complex of this ligand with cobalt(II) is water soluble and has been assigned as 5-coordinate with a single water ligand.[137] The electronic spectra of the cobalt complex resembles strongly that of human Co(II)-carbonic anhydrase and its variation with pH has been attributed to a pK_a near 7.5 for the apical nitrogen and near 8.9 for the metal bound water. The anion N_3^- appears to bind predominantly the form with water as a ligand of cobalt. The complex of Bertini et al. exhibits no catalysis of the hydration of CO_2 at pH 8.8.

B. The Catalytic Interconversion of CO_2 and HCO_3^-

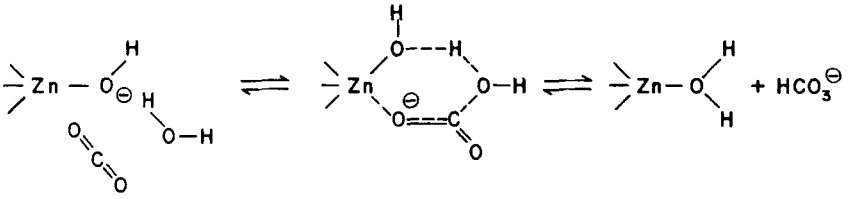
Although direct evidence is lacking for the existence of a zinc-bound hydroxide in

carbonic anhydrase, the model studies mentioned in the previous section as well as a large body of spectroscopic and inhibition data[2,3] support the hypothesis that it is zinc-bound hydroxide which is active in the hydration of CO_2. Of particular importance is the near exact correspondence of catalytic activity with the absorption at 640 nm of Co(II) substituted carbonic anhydrase.[55] Of similar importance is the nearly identical shift in both the apparent pK_a of catalytic activity and of spectroscopic properties when the zinc in the native enzyme is replaced by another metal such as Cd^{2+}.[50] These are some of the strongest criteria, among many, upon which is based the assignment of the pK_a 7 to the ionization of a metal-coordinated water and a role of metal-bound hydroxide in the hydration. Of the numerous possibilities for catalysis by zinc-bound hydroxide, two general schemes are given below, one illustrating direct nucleophilic attack of ZnOH on CO_2 (Equation 12)



(12)

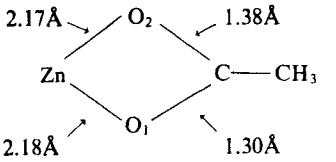
and one a general-base-assisted attack of H_2O on CO_2 (Equation 13)



(13)

Here we rely heavily on certain key observations, some described previously in this review, to support a direct nucleophilic attack of zinc-bound hydroxide on CO_2 without concomitant proton transfer in the actual interconversion (Equation 12). That catalysis of hydration of CO_2 might proceed by direct nucleophilic attack was proposed by Coleman.[130] Carbon dioxide could possibly be a very weak ligand of zinc in this mechanism which would then polarize the molecule and make the carbon more susceptible to nucleophilic attack;[44,138] however, we must add that CO_2 is not known to form coordination complexes with 3d transition metals. Using ^{13}C-labeled substrate and Co(II)-substituted carbonic anhydrase II, Stein et al.[139] measured a weighted ^{13}C-metal distance of 3.6 ± 0.2 Å for bound CO_2 and HCO_3^-. This distance is not compatible with a water bridge between bound substrate and metal-bound hydroxide and supports Equation 12. Using ^{13}C magnetization-transfer NMR for Mn(II)-substituted human carbonic anhydrase I, Led et al.[139a] have determined that substrate HCO_3^- is bonded directly to the metal but that CO_2 associates weakly with the enzyme without a direct bond to the metal. A mechanism such as Equation 12 would permit neutralization by the metal of developing negative charge in the transition state and take advantage of the small activation energy for the reaction of poorly solvated hydroxide with CO_2.[128] The argument that a zinc-bound hydroxide loses much of its nucleophilicity is not borne out by the calculations of Pullman[138] which utilize an *ab initio* SCF molecular orbital method to show that OH^- retains most of its negative charge in binding to zinc in various model complexes containing a zinc-bound hydroxide. The model studies of Woolley[132] and Pocker and Bjorkquist[125] demonstrate that direct attack of a metal bound OH^- on CO_2 does occur in model complexes. Based on the invariance of Glu 106 and Thr 199 in carbonic anhydrases I, II, and III, and on the X-ray structure in which the zinc-bound solvent molecule is hydrogen-bonded to Thr 199 which is hydrogen-bonded to Glu 106, Kannan et al.[44,140] have proposed that this interaction renders the zinc-bound solvent

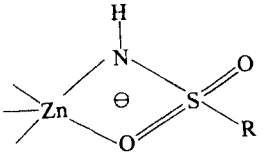
molecule more nucleophilic at the active site. The four-member ring shown in Equation 12 as an intermediate in the catalysis is highly speculative, but the structure is not unprecedented. Van Niekerk et al.[141] reported a bidentate chelation for Zn(II) (acetate)$_2$ involving such four-membered rings. The X-ray structure is a distorted octahedron with two positions occupied by water ligands.



$O_1CO_2 = 111°$

$O_1ZnO_2 = 61°$

The four-membered transition state of Equation 12 bears a similarity to and is supported by a bidentate structure which has been proposed for the binding of sulfonamides to carbonic anhydrase. This class of tight-binding inhibitors has been reviewed;[12,142] the inhibition constant for the well-studied sulfonamide acetazolamide to human type II isozyme is 10^{-8} *M*.[12] Direct evidence for the $-SO_2NH^-$ moiety in the bound complex has been obtained by resonance Raman studies,[143] which are in agreement with earlier spectroscopic investigations of the complex.[144,145] Evelhoch et al.[146] observed the splitting into a doublet of the ^{113}Cd resonance in Cd(II)-carbonic anhydrases by sulfonamides enriched with ^{15}N at the sulfonamide nitrogen. This is firm evidence for the formation of a nitrogen-metal bond in the complex. The X-ray structure has been interpreted to indicate a bidentate complex of the sulfonamide with the metal;[147] evidence for such a structure was also obtained using the method of perturbed angular correlation of gamma rays.[148]



(14)

The binding of sulfonamides to carbonic anhydrase has many complexities not represented in this simple picture of Equation 14: the perturbed angular correlation of gamma rays indicates multiple forms of the acetazolamide complex with Cd(II)-type I enzyme;[148] and Fabry et al.[119] have emphasized the uninhibitable part of the relaxation rate of the protons of water in the presence of a sulfonamide complex with carbonic anhydrase, a result which is difficult to understand if a bidentate sulfonamide diplaces water bound at the metal. A useful discussion of the hydrophobic component of the binding of sulfonamides to carbonic anhydrase has been presented by King and Burgen.[149]

The most pertinent kinetic evidence for a mechanism without proton transfer comes from the equilibrium kinetic methods utilizing ^{13}C NMR and ^{18}O exchange. Both of these methods have been used to determine the rate at chemical equilibrium of the interconversion of CO_2 and HCO_3^-.[73,83] The great advantage of these methods is that they measure only the rate of CO_2-HCO_3^- interconversion and appear to be independent of subsequent steps in the catalysis. Thus, the equilibrium rate of interconversion of CO_2 and HCO_3^-, designated R_1, is independent of concentration of most buffers indicating that the intermolecular proton transfer step which enhances catalysis occurs in steps not directly involved in the CO_2-HCO_3^- interconversion. (The exceptions here are some buffers which inhibit carbonic anhydrase). We can firmly conclude that external buffers play no role in the actual CO_2-HCO_3^- interconversion, although they do participate in a subsequent step, the equilibration of protons between the active site and solvent.

It is also significant that the solvent deuterium isotope effect for R_1 is close to unity as measured by both equilibrium kinetic methods.[73,83] Using the ^{18}O method this has been verified approximately for the range of pH 6 to 8 and accurately at pH(D) 6.6 (uncorrected pH meter reading) for which $(R_1)_{H_2O}/(R_1)_{D_2O} = 1.05 \pm 0.03$.[150] The most straightforward interpretation of these observations is that the catalyzed hydration step proceeds with no change in bonding to hydrogen in the rate-limiting step. As pointed out by Simonsson et al.,[83] this approach eliminates all but one of the mechanisms listed by Pocker and Bjorkquist;[96] the lone possibility being direct nucleophilic attack. All groups reporting these isotope effects in the catalysis by carbonic anhydrase have exercised restraint in interpretation since the isotope effect on R_1 near unity may indicate an asymmetric transition state[151] or that the rate-limiting step is product dissociation, which would not be expected to be different in H_2O and D_2O. This latter possibility is rendered less likely since the off-rate of HCO_3^- from the enzyme can be calculated to be at least 10^7 sec^{-1}, based on a value greater than 30 m*M* for the dissociation constant of the bicarbonate-enzyme complex[83] and assuming diffusion-limited association. There is sufficient information to resolve the possibility of an asymmetric transition state other than to comment that it is rather unlikely for a multi-proton mechanism.

In catalysis, bicarbonate binding at the metal in the manner of anionic inhibitors seems likely.[2,3] However, the binding site of CO_2 in the catalysis is much less certain. Studies using ^{13}C NMR of the binding of CO_2 and HCO_3^- to the active site of Co(II)-substituted bovine red cell carbonic anhydrase indicate that CO_2 binds at least within the second coordination sphere of the metal.[139,152] Much useful information of the CO_2 binding site has been deduced from the discovery of Khalifah that imidazole is a competitive inhibitor of the hydration of CO_2 catalyzed by human carbonic anhydrase I.[54] The neutral form of imidazole appears to inhibit this enzyme independent of other ionizations in the protein, within the range of pH 6 to 9.[106] Alberti et al.[153] extended these studies and found similar properties for the binding of 1,2,3-triazole, 1,2,4-triazole, and tetrazole, each of which also bound to bovine Co(II)-carbonic anhydrase but with an affinity about ten times less than to the type I isozyme. The structure of the enzyme-imidazole complex, determined from X-ray diffraction patterns by Kannen et al.,[44] shows that imidazole binds in a hydrophobic region and may be considered a weak fifth ligand of the metal. That is, this binding apparently does not displace the aqueous species bound to zinc, but binds by expanding the coordination sphere. These results suggest that CO_2, a neutral substrate, also binds equally well to all ionization states of the active site. This, in fact, is the basis of the kinetic scheme of Khalifah and Edsall[103] and has been particularly useful in explaining the pH-independence of $K_m^{CO_2}$. The more recent work of Steiner et al.,[95] however, certainly indicates that a more careful attention must be given to CO_2 binding.

As can be seen, the identity and nature of the CO_2 binding site in carbonic anhydrase is elusive and must be the focus of further work. For this reason model studies and theoretical calculations are particularly valuable. Pullman,[138] using an *ab initio* SCF molecular orbital approach, has calculated the energies of interaction and most favorable positions between a molecule of CO_2 and the two tetraliganded zinc complexes $Zn^{2+}(NH_3)_3 OH_2$ and $Zn^{2+}(NH_3)_3 OH^-$. The most favorable positions are shown in Figure 16 and it is interesting to note that both interactions are energetically favorable, -22 kJ/mol for $Zn^{2+}(NH_3)_3 OH_2$ and -36 kJ/mol for $Zn^{2+}(NH_3) OH^-$. Figure 16b is poised for nucleophilic attack and fits rather comfortably with the scheme in Equation 12. A few of the many relevant points which emerge from these calculations are given here. Apparently the binding of CO_2 near the zinc as shown in Figure 16 does not facilitate the bending of the OCO angle. However, the carbon of the bound CO_2 does lose both σ and π electrons due to redistribution and consequently becomes more susceptible to nucleophilic attack. In harmony with the report of Jönsson et al.[128] on the reaction of CO_2 and OH^-, Pullman finds that the reaction which starts from the geometry of Figure

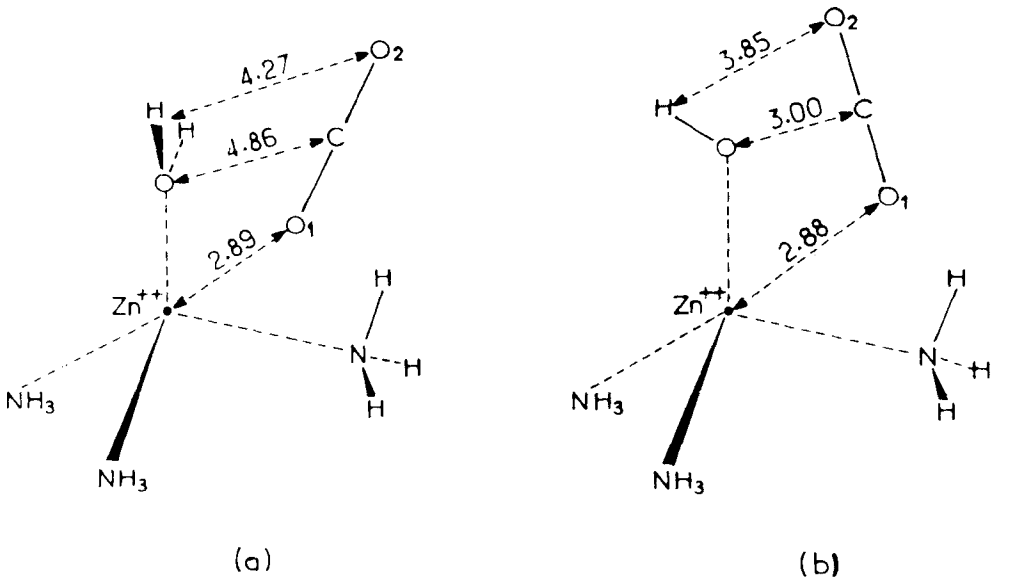


FIGURE 16. The most favorable positions in which CO_2 interacts with (a) $Zn^{2+}(NH_3)_3OH_2$ and (b) $Zn^{2+}(NH_3)_3OH^-$, from the *ab initio* SCF molecular orbital calculations of Pullman. (From Pullman, A., *Ann. N.Y. Acad. Sci.*, 367, 340, 1981. With permission.)

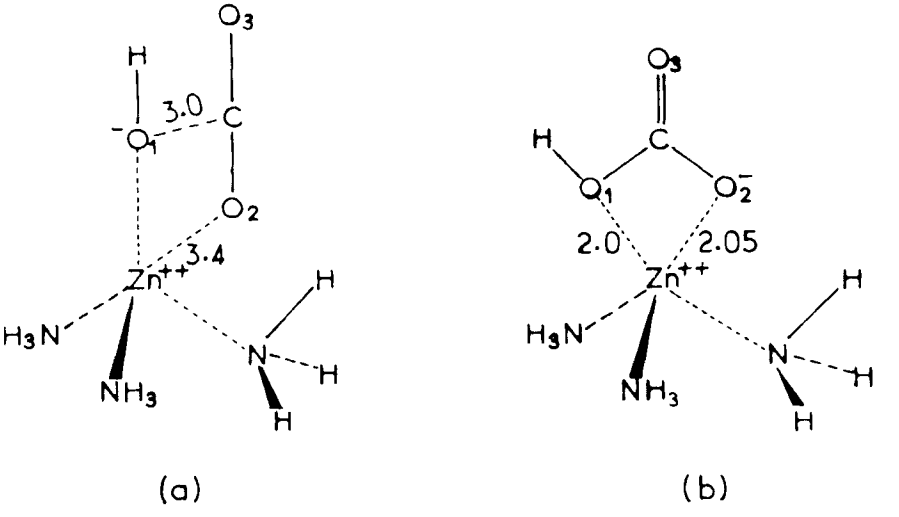


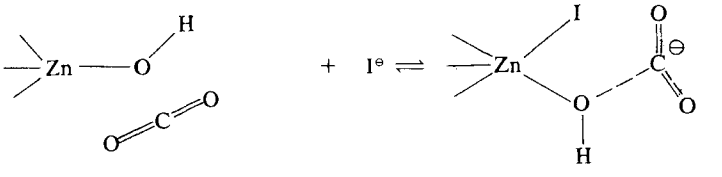
FIGURE 17. Initial (a) and final (b) conformations for the reaction $Zn^{2+}(NH_3)_3OH^- + CO_2 \rightarrow Zn^{2+}(NH_3)_3HCO_3^-$, from the *ab initio* SCF molecular orbital calculations of Pullman. (From Pullman, A., *Ann. N.Y. Acad. Sci.*, 367, 340, 1981. With permission.)

16b to form Zn^{2+} $(NH_3)_3(HCO_3^-)$ requires practically no activation energy; moreover it proceeds without proton transfer and yields the product shown in Figure 17 which is similar in structure to the intermediate in Equation 12.

This intermediate requires that the metal in carbonic anhydrase assume, at least in a catalytic intermediate, a 5-coordinated structure. Evidence is abundant that metals in carbonic anhydrase can become pentacoordinated and that there is room at the active site for two ligands to the metal besides the three histidine ligands from the protein. This topic

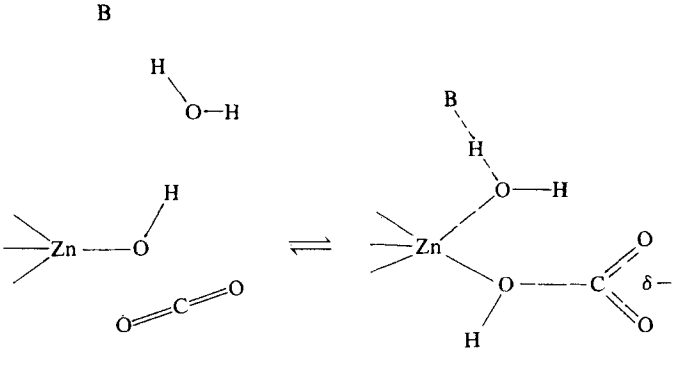
has been reviewed thoroughly[3,4] and just a few pertinent comments are made here. Clear evidence for 5-coordination is provided by Kannan et al.,[44] who have shown from X-ray data that imidazole binds to the metal in carbonic anhydrase I without displacing H_2O which is also a ligand. The metal may also be 5-coordinated in sulfonamide complexes of carbonic anhydrase.[147] The visible absorption spectra of complexes of Co(II)-substituted carbonic anhydrase show a wide variation in band structure and intensity which is related to the coordination geometry of the cobalt. Of these, spectra displaying low intensities ($\epsilon_{max} < 150\ M^{-1}cm^{-1}$) and a weak band ($\epsilon_{max} \approx 10\ M^{-1}cm^{-1}$) between 700 and 800 nm have been attributed to pentacoordinated complexes.[154] The acetate ion is a very interesting example of a ligand which binds in the 5-coordination mode according to this criterion[154] and may form a complex similar to that given in Figure 17. Haffner and Coleman[155] have presented evidence from ESR for a dicyanide complex of Co(II)-carbonic anhydrase which forms a low spin cobalt(II).

It is important to note proposals other than direct nucleophilic attack on CO_2 by zinc-bound hydroxide followed by a rate-limiting intramolecular proton transfer, although this hypothesis is supported in this review as one which we believe is most consistent with the wide body of accumulated data. Pocker and Bjorkquist[96] also measured catalyzed hydration and dehydration in H_2O and D_2O but have not emphasized the solvent deuterium isotope effect of unity on k_{cat}/K_m in both directions. As a consequence, the mechanism developed by Pocker and colleagues does not include the notion of a rate-limiting proton transfer after the departure of product. Pocker and Deits[156] have based a mechanism on the solvent deuterium isotope effect on 3 on k_{cat} and their important findings involving inhibition of catalysis by many monoanions such as SCN^-, I^-, $CH_3CO_2^-$. Although it is known that inhibition by these anions of the hydration of CO_2 catalyzed by type II carbonic anhydrase occurs by a mixed mechanism favoring noncompetitive inhibition near neutral pH,[157] Pocker and Deits discovered that this inhibition is uncompetitive at pH near 9 even though anions bind to the same site at high pH as at neutral pH. This has been interpreted by Pocker and Deits as the binding of two ligands in obligate order: first CO_2 then the anion to yield an inactive ternary complex. CO_2 binding, it is proposed alters the properties of the metal to allow anion



(15)

binding. By analogy in the catalytic mechanism this fifth anionic ligand is an hydroxide ion formed by proton abstraction involving a basic group of the protein or a bound buffer. Pocker and Deits explain that this fifth anionic ligand, by increasing charge density on the zinc, renders the bound hydroxide



(16)

more nucleophilic in its attack on CO_2. One further proton transfer is suggested by Pocker and Deits to allow the departure of product: the transfer of a proton, possibly assisted by Thr 199, from the metal-bound oxygen to the carboxyl group of the product.[156] Thus, such a scheme involves important roles for proton transfer in the actual interconversion of CO_2 and HCO_3^- and is consistent with a solvent deuterium isotope effect near 3 on k_{cat} and also with the result that the transition state of the rate-limiting event in the catalysis involves changes in bonding to two or more hydrogens.[107] In order to reconcile the mechanism of Equation 16 with the lack of a deuterium isotope effect in equilibrium kinetics measured by ^{13}C NMR and ^{18}O exchange, it is necessary to require that substrate encounter or product release be rate limiting or to require an asymmetric transition state for a process involving more than one proton transfer.

Worthy of comment is that uncompetitive inhibition by anions of the hydration of CO_2 catalyzed by type II enzyme is accommodated nicely within the mechanism of Steiner et al.[95] Referring to Figure 10, in the steady-state of CO_2 hydration the rate-limiting intramolecular proton transfer, Step 4, causes an accumulation of the enzyme form EH^+. The binding of anions to this form, consistent with a large body of data[2,3] and not requiring a ternary complex, produces an uncompetitive inhibition with respect to CO_2 in hydration. Moreover, this explanation is consistent with the lack of inhibition by anions at pH near 9 of the hydrolysis of *p*-nitrophenylacetate.[158,159] This is a much slower kinetic process and is not limited by the intramolecular transfer, Step 4 of Figure 10. Consequently, for catalyzed ester hydrolysis there is no accumulation at steady-state of EH^+ to which anions bind. The explanation of Pocker and Deits[156] for lack of anion inhibition of ester hydrolysis at high pH is that the enzyme-ester complex is so short-lived as to preclude concomitant anion binding.

Another proposal for the action of carbonic anhydrase quite different from those described so far has been developed by Koenig and colleagues.[65,79] A major emphasis of their work has been to describe a mechanism consistent not only with the accumulated kinetic and inhibition data but also with the proton relaxation enhancement studies which are not easily explained by the zinc-hydroxide mechanism. The basis of their proposal is that the active form of the enzyme in the hydration of CO_2 is a zinc-bound water and that the pH dependence of the activity of the enzyme and of many spectroscopic properties of the active site, occurring with an apparent pK of 7, are due to anion inhibition of the enzyme.[79] A major basis of this proposal is the study, often repeated,[120,160] of the nuclear magnetic relaxation rate of the protons of water in the presence of cobalt-substituted carbonic anhydrases.[119] A major result, shown in Figure 18, is that the relaxivity, which is directly proportional to $1/T_1$ for the protons of water, follows a titration curve with a pK near 7 at an ionic strength maintained in part by SO_4^{2-} ions. In the high-pH region of this plot, protons are exchanging from the first coordination sphere of the metal at a rate at least as great as 10^5 sec^{-1}. Diffusion limits and our knowledge of the tightness of binding of anions at the active site preclude the exchange of OH^- as the cause of this proton exchange. Consequently, there is a water molecule exchanging from the first coordination sphere at high pH. It is this result that is difficult to reconcile with the zinc-hydroxide mechanism which predicts $\geqslant$Zn-OH as the high-pH form. Recent reports have been favoring a 5-coordinate, high-pH form with both hydroxide and water bound, [$\geqslant$Zn(OH)$H_2$0], although direct evidence for such a complex is lacking (for a useful discussion and review see Reference 3). Several interpretations of Figure 18 suggest that there is no water at the metal of the enzyme at low pH.[65,79] It is likely that this is the case in the presence of anions, including SO_4^{2-} used to maintain ionic strength, when these anions bind to the metal displacing H_2O.[55,79,161] However, measurements of T_1 of the protons of water made in the absence of SO_4^{2-} and inhibitory ions suggest that under these conditions the cobalt-substituted carbonic anhydrase does have a metal-bound water at low pH.[55]

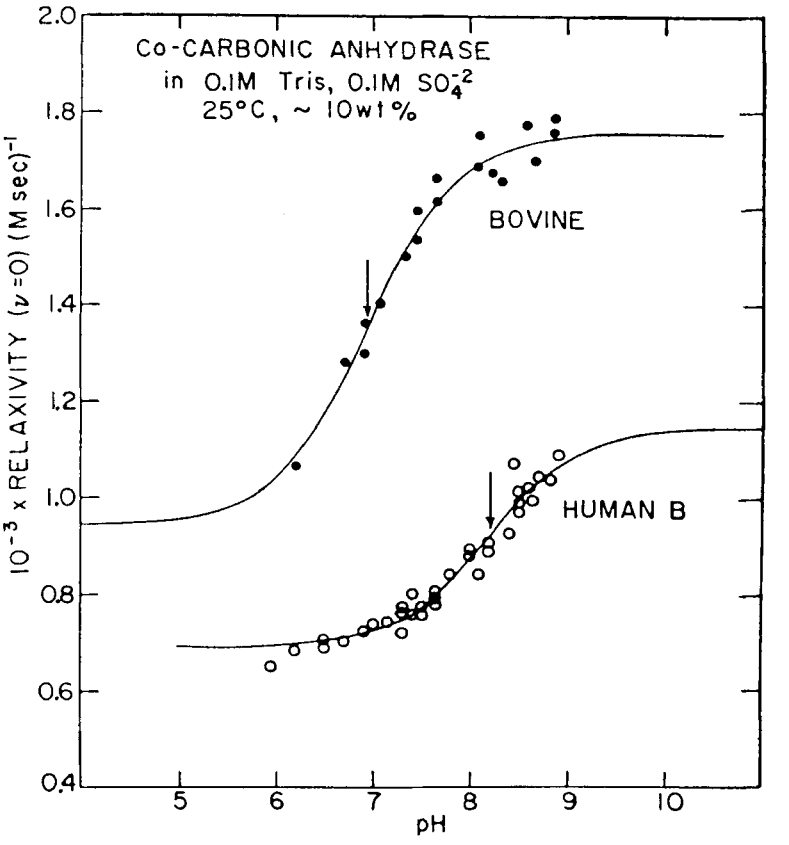


FIGURE 18. The pH dependence of the relaxivity in the limit of low resonance frequency of the proton of water in the presence of Co(II)-substituted bovine red cell and human B carbonic anhydrase. The relaxivity is R: $R = (1\text{-}V)(T_1^{-1} - T_{1w}^{-1})/N$, where T_1^{-1} is the observed relaxation rate of solvent protons in the protein solution, V is the volume fraction occupied by the protein, T_{1w}^{-1} is the relaxation rate in the absence of protein, and N is the enzyme concentration in moles/liter. (From Fabry, M. E., Koenig, S. H., and Schillinger, W. E., *J. Biol. Chem.*, 245, 4256, 1970. With permission.)

On the assumption that this metal-bound H_2O plays a primary role in catalysis, Koenig et al.[79] have proposed this mechanism: In the absence of inhibitors there is just one form of the active site at pH > 5.5, that with zinc-bound water. Koenig et al. have indicated that this water molecule may bind as an anion, OH^-, in lose association with a proton bound to an acceptor group. However, in order to conserve charge in the active site, each anion including OH^- bound at the active site must be accompanied by a proton. This presents the possibility that anion binding to and release from the enzyme would conceivably be dependent on proton transfer rates and could explain the observed buffer dependence of catalysis. This proton acceptor group near the active site is involved in the catalysis also, abstracting a proton from metal bound water to allow attack of OH^- on CO_2. Such a step is required in this proposal since metal-bound H_2O is expected to be a very poor nucleophile. Major differences between this and the zinc-hydroxide mechanism are (1) the source of the pH dependence of the activity, (2) the role of proton transfer in the catalysis, and (3) the role of a zinc-bound water at high pH. Concerning Point 1, there is now a very thorough study of the spectroscopic and kinetic properties of carbonic anhydrase in the absence of inhibitory anions. This work, by Simonsson and Lindskog,[55] shows that the activity of carbonic anhydrase in the hydrolysis of *p*-

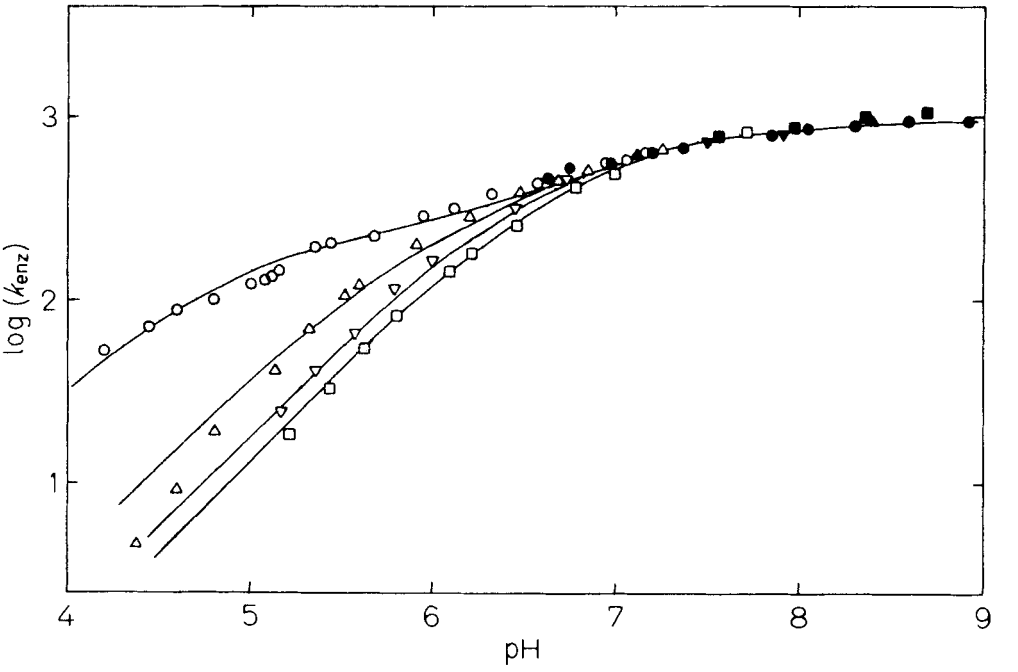


FIGURE 19. The pH dependence of k_{enz}, the apparent second-order rate constant for the hydrolysis of *p*-nitrophenyl acetate catalyzed by bovine red cell carbonic anhydrase at different concentrations of Na_2SO_4. The concentrations of Na_2SO_4 were: (○, ●) 0 m*M*; (△, ▲) 5 m*M*; (▽, ▼) 25 m*M*; (□, ■) 50 m*M*. Open symbols refer to 50 m*M* MES/ NaOH buffers and filled symbols to 50 m*M* Hepes/ NaOH buffers. Temperature, 25° C. (From Simonsson, I. and Lindskog, S., *Eur. J. Biochem.*, 123, 29, 1982. With permission.)

nitrophenylacetate does depend on pH in the absence of sulfate and inhibitory anions and that the pH dependence is dominated by a pK_a of 6.8. Moreover, a similar result was obtained by Chesnovsky and Navon[162] for the hydration of acetaldehyde catalyzed by carbonic anhydrase in the absence of buffers and inhibitory ions. The pH dependence of the *p*-nitrophenylacetate hydrolysis, shown in Figure 19, is perturbed by other ionizing groups in the active site as well. There is evidence of another ionizing group with a pK of 5.1. Addition of sulfate converts this pH-rate profile into a much simpler form, that of a single titration with pK_a near 7 (Figure 19). Changes in the optical spectrum of the cobalt-substituted enzyme parallel those of the activity.[55] Thus it appears that there is strong evidence that the pK 7 component of activity and visible absorption is not due to sulfate or anion inhibition. For Point 2, the lack of both a buffer effect and a solvent deuterium isotope effect on k_{cat}/K_m for hydration of CO_2 and the equilibrium rates of interconversion of CO_2 and HCO_3^- does not favor a scheme in which the departure of product HCO_3^- from the enzyme depends on proton transfer. And finally Point 3, the pH dependence of the rate of release from carbonic anhydrase II of water bearing substrate oxygen in the absence of inhibitory anions, R_{H_2O} in Figure 6, shows a rapid rate of release of H_2O from the active site at pH near 6 and a much smaller rate at pH near 8. This pH dependence is quite different from the lack of pH dependence in the absence of inhibitory anions of $1/T_1$ for water protons in solutions containing cobalt-substituted carbonic anhydrase.[161] Moreover, the buffer dependences of R_{H_2O} and of $1/T_1$ for water protons in the presence of Co(II)-carbonic anhydrase are quite different.[73,120] Hence, these two quantities may be measures of different processes. (However, see the discussion in Section III.C.) Whereas R_{H_2O} is determined from the kinetics of ^{18}O-labeled substrate

and necessarily measures a property of the catalysis, this need not be the case for $1/T_1$ for water protons and it is a possibility that the relaxation of water protons in the presence of Co(II)-carbonic anhydrase measures a process not directly involved in the catalysis.

C. Critique

The hypothesis of a direct nucleophilic attack of zinc-bound hydroxide on CO_2 without proton transfer is the weakest of the major hypotheses we have espoused in this review. This is why we have been obliged to support it with many model studies which although good spectroscopic mimics are poor mimics of the very great catalytic efficiency of the enzyme. It is a fact that the rate-limiting steps in the catalysis up to and including the departure of HCO_3^- exhibit a deuterium isotope effect of unity are not enhanced by the presence of buffer. However, it is certainly possible that there could be a proton transfer in these steps which is not expressed in the isotope effect the departure from the enzyme of CO_2 or HCO_3^- is rate limiting. These are steps which are expected to have deuterium isotope effects near unity. We have argued that this is unlikely based on a diffusion-controlled rate of encounter of substrate with enzyme and a rapid off-rate of product. Another possibility to explain a low isotope effect is an asymmetric transition state involving proton transfer. But with the isotope effects on R_1 and k_{cat}^{CO2}/K_m^{CO2} being so close to unity, this asymmetric transition state would have to resemble the reactants very closely. Hence, the actual mechanism of interconversion of CO_2 and HCO_3^- by carbonic anhydrase needs considerable clarification. One of the most promising prospects to provide clarification is to determine why the three isozymes of carbonic anhydrase have such different catalytic activities.

CONCLUSION

The catalysis of the hydration of CO_2 by carbonic anhydrase II, the most active of the isozymes, can be considered in two parts: (1) the encounter of CO_2 with enzyme, the conversion to HCO_3^-, and the departure of this product, and (2) the transfer of a proton from the active site to solution. A zinc-hydroxide mechanism in the catalysis is so well supported in the literature and previous reviews that it is treated as a peripheral issue here. The rate-limiting steps of the first part exhibit a solvent deuterium isotope effect of unity and are not enhanced by the presence of external buffer. This strongly suggests a direct nucleophilic attack of zinc-bound hydroxide on CO_2 without proton transfer. This hypothesis is supported by many model studies and calculations. However, we are not able to strictly exclude the possibility that these steps are limited by encounter of the enzyme with CO_2 or departure from the enzyme of HCO_3^-, both of which would occur with a deuterium isotope effect near unity. Hence, we cannot rigorously exclude the possibility that proton transfer occurs in a general base mechanism. The transfer of a proton from the active site to solvent is at least as rapid as the catalysis (10^6 sec^{-1}) and requires that the protons be transferred to buffers acting as proton acceptors in solution. At concentrations of buffer below about 5m M at pH near neutral the rate of this proton transfer is small enough that it limits the overall catalytic rate. In the presence of excess buffers so that the proton transfer to solution is not rate limiting, there remains a considerable deuterium isotope effect in the catalysis which by steady-state and equilibrium studies has been assigned to the transfer of protons from the active site to an internal proton transfer group, a residue of the enzyme itself. It is this step which is hypothesized to be rate limiting overall in the catalysis. The proton on this internal transfer group can then be donated to buffers in solution. This hypothesis explains adequately several characteristics which are otherwise difficult to understand: the solvent dueterium isotope effects on $k_{cat}^{CO_2}$ and $k_m^{CO_2}$ which cancel in the ratio $k_{cat}^{CO_2}/k_m^{CO_2}$, the pH

independence of $K_m^{HCO_3^-}$ and $K_m^{CO_2}$, and the nature of the product inhibition of CO_2 hydration which is consistent with the presence of a product term $[CO_2][HCO_3]$ in the rate equation.

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