carried out by Pohl[31,32] have also been shown to be consistent with the analysis of Brandts.[5]

Experimental Section

CT (3× crystallized and lyophilized) obtained from Worthington Biochemical Corporation, Freehold N. J., was used without further purification in the preparation of the derivatives. All other chemicals were of reagent grade.

MMSCT was prepared by oxidation of CT with H_2O_2 in aqueous solution at pH 3.0 and 4°. Enzymic activity of this derivative was virtually identical with that of its precursor CT. Chromatography according to the method of Hirs[33] indicated it to be homogeneous and the preparation was used without further purification. Chemical analysis of the preparation by Dr. H. Schachter showed only one methionine residue oxidized to sulfoxide and from previous work[16] this methionine had been identified as residue 180. We are indebted to Dr. Schachter for this analysis.

DMSCT was prepared by oxidation of CT with H_2O_2 in 8 *M* urea, pH 3.0, 4° according to the method of Schachter, *et al.*[16] After dialysis and lyophilization the disulfoxide preparation showed approximately 40% activity toward *N*-acetyl-L-tyrosine ethyl ester, relative to the activity of CT. Chemical analysis of the product by Dr. Harry Schachter demonstrated that 2.0 methionine were oxidized per molecule. Chromatography indicated the product to be better than 90% homogeneous. Examination of the thermal unfolding characteristics gave no indication that the small amount of heterogeneity, probably due to irreversibly denatured and aggregated protein, interfered with the thermodynamic measurements. Hence the preparation was used without further purification. Unless the impurity contributes significantly to the experimental observable as a result of an unfolding transition of its own which overlaps the one under study, it has no effect on the van't Hoff plot. In addition, so long as the process under study is first order in both forward and backward directions, as proved to be the case in this work, uncertainties in the concentration do not lead to uncertainties in the estimates of the entropy and free-energy changes.

The method for determining solubility in high salt buffer used to establish the fraction of protein in state B was a modified form of the procedure described by Eisenberg and Schwert[34] in which the high salt buffer was maintained at pH 3.6 rather than 3.0. This change was necessary for DMSCT because at pH 3.0 there is a significant fraction of state B protein at all temperatures.

Procedures for preparation of the protein solutions, for the difference spectrum measurements, and for establishing the reversibility of the reaction have been described previously.[9,10]

(31) F. Pohl, *Eur. J. Biochem.*, **4**, 373 (1968).
(32) F. Pohl, *ibid.*, 7, 146 (1968).
(33) C. H. W. Hirs, *J. Amer. Chem. Soc.*, 77, 5743 (1955).
(34) M. A. Eisenberg and G. W. Schwert, *J. Gen. Physiol.*, **34**, 583 (1951).

Bromine Catalysis for Carbon Dioxide Hydration and Dehydration and Some Observations Concerning the Mechanism of Carbonic Anhydrase[1]

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Abstract: The dramatic bromine catalysis of carbon dioxide hydration and dehydration first observed in 1940 has been found to be caused by hypobromous acid. The mechanism for bicarbonate dehydration by HOBr apparently involves a concerted bromination–dehydration reaction and the second-order rate constant for HOBr catalysis is 46 M^{-1} sec^{-1} at 25°; an upper limit equal to 51 M^{-1} sec^{-1} has been set for catalysis of bicarbonate dehydration by elemental bromine. The second-order rate constant for hydronium ion catalyzed dehydration of bicarbonate in 71.5% (v/v) dioxane is increased to 2.5 × 10^6 M^{-1} sec^{-1} at 25°. It is concluded that the apolar character of the active site of carbonic anhydrase contributes relatively little to the catalytic efficacy of the enzyme but probably contributes to the specific facilitation of the dehydration process as compared with the hydration reaction. A mechanism for the carbonic anhydrase reaction has been proposed in which the metal ion associated with the enzyme acts as a general acid catalyst for bicarbonate dehydration.

A comprehensive interpretation has not yet been provided to account for the dramatic halogen catalysis for carbon dioxide hydration and dehydration demonstrated in 1940.[2] We report here evidence which is consistent with a mechanism for bicarbonate dehydration in which hypobromous acid acts as a brominating agent in a concerted decarboxylation-bromination process. These results as well as those obtained in the study of the influence of an apolar solvent on nonenzymic bicarbonate decarboxylation are applied in considerations of the carbonic anhydrase mechanism.

(1) Supported by grants from the National Institutes of Health (GM-11820) and the National Science Foundation (GB-6802). Address reprint requests to the Department of Biochemistry, University of North Carolina, Chapel Hill, N. C. 27514.
(2) M. Kiese and A. B. Hastings, *J. Biol. Chem.*, 132, 267 (1940).

Experimental Section

Materials. Oxygen-18 enriched K_2CO_3 was made by heating an ampoule containing an $H_2{}^{18}O$ solution of an equimolar mixture of K_2CO_3 and $KHCO_3$ for 20 min at 100°. The material was taken to dryness at 80° under vacuum after the $KHCO_3$ had been neutralized with base. Bromine solutions were made up fresh daily and concentrations were determined from a molar extinction coefficient at 400 mμ of 139 cm^{-1}. Dioxane was distilled from sodium. Glass-distilled water was used throughout.

Methods. Rates were determined from the isotopic depletion which is associated with the reversible dehydration–hydration of oxygen-18 enriched bicarbonate in unenriched water. Carbon dioxide gas was released from aliquots of the reaction mixture by injecting the sample through a serum stopper into an evacuated tube containing concentrated sulfuric acid. This tube was cooled in a Dry Ice bath until the gas could be transferred to a vessel for introduction into a mass spectrometer (Consolidated Electrodynamics Model 21-614). The per cent oxygen-18 in carbon dioxide was

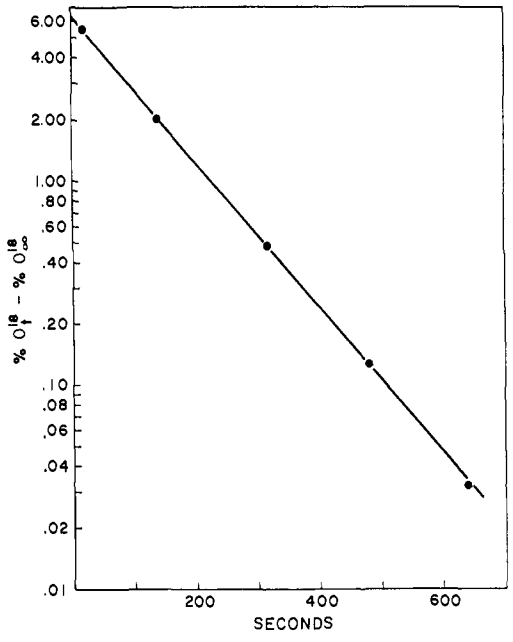


Figure 1. Rate of decarboxylation of bicarbonate at pH 6.65 in 0.5 M phosphate buffer, ionic strength 1.0.

determined from the formula % 18 = [46/44 + 45]100/2 + (46/44 + 45).[3a]

pH's were measured using a Radiometer G202C electrode and a Radiometer TTTlc-PHA 630Ta pH meter. The hydrogen ion concentration in 71.5% (v/v) dioxane was calculated from the measured pH using an experimentally determined relationship: $-\log (H^+)$ = measure pH − 0.51. This was established at ionic strength 2.86 mM, with acid concentrations from 0.286 to 2.86 × 10^{-3} M.[3b]

The pK_a for hypobromous acid was found to be equal to 8.78 at ionic strength 0.05 from spectrophotometric measurements at 330 mμ of freshly prepared 2.7 × 10^{-4} M bromine solutions in borate buffer, pH 8.2–11.2. The results were analyzed by the logistic method of Reed and Berkson.[3d] The pK_a for bicarbonate in 71.5% dioxane was determined to be 12.51 from pH measurements of mixtures containing varying ratios of K_2CO_3 and $KHCO_3$. Since K_w = 3.98 × 10^{-19} in this solvent[4] no correction was made for hydrolysis necessary to generate hydroxide ion.

Rates of bicarbonate decarboxylation in 71.5% dioxane contained 8 × 10^{-3} M bicarbonate as the sole buffer. Very small pH changes were compensated for by addition of 0.1 M HCl with a pH stat.

Results

Isotopic depletion from oxygen-18 enriched bicarbonate requires reaction of normal water with carbon dioxide (eq 1) and the rate of depletion is, therefore,

$$H_2{}^{16}O + C^{18}O_2 \underset{k_r}{\overset{k_f}{\rightleftharpoons}} HC^{16}O_2{}^{18}O^- + H^+ \qquad (1)$$

given by eq 2. Solutions of bicarbonate adjusted to a

$$\text{rate of depletion} = k_f(C^{18}O_2)(H_2{}^{16}O) \qquad (2)$$

(3) (a) This formula is slightly modified from that given by F. S. Klein of the Isotope Department, Weizmann Institute of Science in the Yeda Catalog of Stable Isotopes. Since reactions are first order the method of calculation is without effect on the results. (b) It has previously been found that in 75% dioxane the glass–calomel cell measures hydrogen ion activity, as defined by a hydrogen electrode, and that an empirical calibration of such a system is valid over a wide range of hydrogen ion concentrations if the solvent composition is kept constant.[3c] (c) L. G. Van Uitert and C. G. Haas, *J. Amer. Chem. Soc.*, 75, 451 (1953); (d) W. M. Clark, "Oxidation-Reduction Potentials of Organic Systems," Williams and Wilkins, Baltimore, Md., 1960, p 149.

(4) Interpolated from data in H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold, New York, N. Y., 1950.

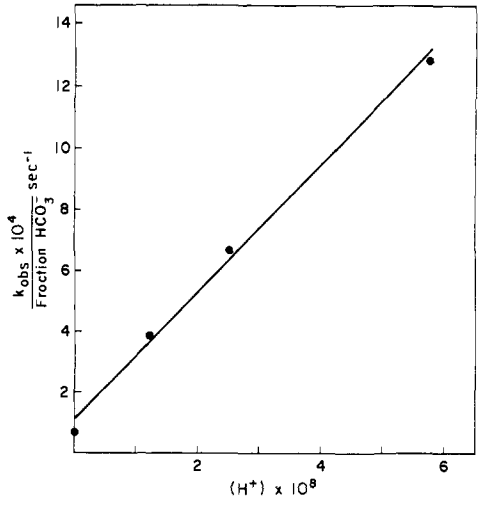


Figure 2. Hydrogen ion dependence of the rate of bicarbonate decarboxylation. Reactions were carried out in 0.08 M N-methylmorpholine buffer, ionic strength 0.05, except for the reaction at the highest pH (9.3) which was in 0.012 M carbonate. The slope is 1.93 × 10^4 M^{-1} sec^{-1}.

fixed pH rapidly attain equilibrium, where

$$k_f[CO_2][H_2O] = k_r[HCO_3^-][H^+] \qquad (3)$$

Solving for the carbon dioxide concentration

$$[CO_2] = \frac{k_r}{k_f}[H^+][HCO_3^-] \qquad (4)$$

and substitution into eq 2 gives

$$\text{rate of depletion} = \frac{k_r}{3}[H^+][HCO_3^-] \qquad (5)$$

where 3 is introduced in the denominator since three cycles are required for full loss of isotope. This method was introduced by Mills and Urey[5] and its advantages are as follows. (1) Rates may be measured in concentrated buffer solutions where spectrophotometric stop-flow assays, which rely upon a pH change, are not feasible. (2) Rates may be made sufficiently slow for easy analysis by pH adjustment. At high pH the rate of the reaction in the thermodynamically unfavorable direction is measured and the dehydration rate may be made progressively slower by increases in pH, at least until a pH independent decarboxylation reaction predominates. At sufficiently low pH eq 2 holds and this reaction is reasonably slow at low temperature. (3) No correction need be made for gas transfer from liquid to gas phase as is the case for rapid reactions measured manometrically.

Results obtained in a typical experiment are given in Figure 1 and the data are summarized in Table I. The hydrogen ion dependence of the rate of decarboxylation is shown in Figure 2. The principal observations are as follows. (1) The second-order rate constant for hydrogen ion catalysis for dehydration is 5.79 × 10^4 M^{-1} sec^{-1} at 25°. This is in good agreement with several values previously reported.[6] (2) Bicarbonate dehydration at constant pH is buffer catalyzed and the second-order rate constant for catalysis by the phosphate mono-

(5) G. A. Mills and H. C. Urey, *J. Amer. Chem. Soc.*, 62, 1019 (1940).

(6) (a) B. H. Gibbons and J. T. Edsall, *J. Biol. Chem.*, 238, 3502 (1963); (b) M. Eigen, K. Kustin, and G. Maass, *Z. Phys. Chem. (Frankfurt am Main)*, 30, 130 (1961).

Table I. Rate of Bicarbonate Dehydration at 25° [a]

pH	Ionic strength	Bromine,[b] $M \times 10^4$	Conditions	$k_{obsd} \times 10^3$, sec^{-1}	$\{[(3k_{obsd}/f) - (k_{H^+}/3)[H^+]]/[HOBr]\}$ [c]
6.65	1.0		0.033 M phosphate	3.24	
6.65	1.0		0.5 M phosphate	8.25	
9.30	0.005		0.012 M bicarbonate	0.062	
10.12	0.14	2.06	0.017 M bicarbonate	0.867	45
8.29	0.15	4.55	0.005 M pyrophosphate 7.9×10^{-4} M bicarbonate	5.41	48
8.30	0.15	3.10	0.005 M pyrophosphate 0.12 M potassium bromide	3.41	44
8.33	0.11	2.40	0.17 M borate 0.1 M potassium bromide	2.77	47
8.32	0.11	2.40	0.17 M borate	2.70	46
7.27	0.15	3.33	0.02 M phosphate	6.59	51
7.27	0.15	12.8	0.02 M phosphate	38.5	89
7.29	0.15	12.5	0.02 M phosphate 0.145 M potassium bromide	11.0	108
7.23	0.15	14.8	0.02 M phosphate	11.6	105

[a] Unless stated otherwise, reactions were carried out with 7.8×10^{-3} M bicarbonate-18, and the ionic strength was contributed by buffer ions and potassium chloride. [b] Sum at the concentration of all species, including Br_2, HOBr, and Br_3^-. [c] Second-order rate constant for hypobromous acid catalyzed decarboxylation. The HOBr concentration was calculated from a pK_a of 8.78, an equilibrium constant equal to 7.2×10^{-9} M^2 for the reaction, $H_2O + Br_2 \rightleftharpoons HOBr + Br^- + H^+$, with water activity set at unity (see F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry," Interscience, New York, N. Y., 1962, p 446) and an equilibrium constant of 16 M^{-1} for the reaction, $Br^- + Br_2 \rightleftharpoons Br_3^-$ (J. W. Mellor, "Comprehensive Treatise on Inorganic Chemistry," Suppl. II, part I, Longmans, Green and Co., New York, N. Y., 1956, p 708). The term f is defined as the fraction of total carbon dioxide existing as bicarbonate at the indicated pH; this has been calculated using a pK_a for bicarbonate of 10.33; k_{H^+} is the second-order rate constant for hydrogen ion catalysis.

anion, calculated from results given in Table I, is 7.7×10^{-2} M^{-1} sec^{-1}.[7a] From results obtained with phosphate concentrations in the range 0.02–0.1 M (ionic strength 0.2) a catalytic constant equal to 5.0×10^{-2} M^{-1} sec^{-1} has been calculated. The catalytic contribution of phosphate is relatively low at these concentrations and aside from the difference in ionic strength for these two studies, the latter constant is probably less accurate since it is calculated from a fairly small difference between large numbers. (3) Phosphate catalysis for carbon dioxide hydration has previously been observed,[6a,7c-e] although it was not detected by Ho and Sturtevant.[8] The observed rate constant at pH 9.3, which is equal to one-third that for the uncatalyzed or water-catalyzed decarboxylation of bicarbonate, is to be compared with a calculated rate constant equal to 1.9×10^{-4} sec^{-1} for this reaction.[6a] (4) Bromine catalysis at pH's 10.1 and 8.3 follows a rate law (eq 6) with

$$\text{rate} = k_{HOBr}[HOBr][HCO_3^-] \quad (6)$$

k_{HOBr} equal to 46 M^{-1} sec^{-1}. There is no evidence for a contribution to the rate from a reaction of carbonate with HOBr or the kinetic equivalent, a reaction of OBr^- with bicarbonate.

At pH 8.3 with 2.3×10^{-4} M bromine the concentration of elemental bromine (*i.e.*, Br_2) is 4.0×10^{-8} M[9] and this concentration is increased 390-fold by the addition of 0.1 M bromide ion. Since bromide ion is without effect on the rate it may be concluded that the catalytic species is hypobromous acid rather than bromine.

At pH 7.3 the calculated rate constant for HOBr catalysis is higher than that observed at more alkaline pH's and this may be associated with catalysis by bromine. With 1.48×10^{-3} M total bromine in 0.145 M potassium bromine the concentration of elemental bromine is 3.56×10^{-4} M (HOBr and Br_3^- are 3.0×10^{-4} M and 8.25×10^{-4} M, respectively)[9] and after correction of the rate for catalysis by HOBr (using a rate constant of 46 M^{-1} sec^{-1} for this reaction) a rate constant equal to 51 M^{-1} sec^{-1} may be calculated for the reaction of bromine with bicarbonate. Because of the paucity of data concerning this point and the fact that the calculation involves a difference between large numbers we consider this constant an upper limit rather than a firm figure.

The observed rate for bicarbonate decarboxylation in 71.5% dioxane has been found to be proportional to pH in the range 9.00–10.28 and the second-order rate constant for hydrogen ion catalysis, with the hydrogen ion concentration calculated as described above, is 2.5×10^6 M^{-1} sec^{-1}; this is only 43 times that observed in pure water. No correction was made in calculating this rate constant for ionization of bicarbonate since the pK of 12.51 precludes ionization in the pH range used in these studies. In a single experiment in 90% dioxane at pH 9.82, the rate for decarboxylation was found to be 4.32 sec^{-1}. There does not appear to be a dramatic increase in the proton-catalyzed reaction in the range 71.5–90% dioxane.

Discussion

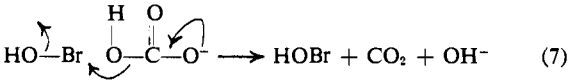
Hypobromous acid is an ambient electrophile since it can donate either a hydronium or bromonium ion. In either case the reaction with bicarbonate regenerates HOBr and is, therefore, catalytic. The remarkable re-

(7) (a) The reactions described in the first two entries in Table I were run with an equimolar mixture of the phosphate mono- and dianion. For calculation of the second-order rate constant for reaction of the phosphate monoanion with bicarbonate the fraction of total carbon dioxide as bicarbonate was calculated from an equilibrium constant for hydration equal to $10^{-5.94}$, which holds at this ionic strength;[7b] (b) H. S. Harned and F. T. Bonner, *J. Amer. Chem. Soc.*, 67, 1026 (1945); (c) F. J. W. Roughton and V. H. Booth, *Biochem. J.*, 32, 2049 (1938); (d) M. M. Sharma and P. V. Danckwerts, *Trans. Faraday Soc.*, 59, 386 (1963); (e) A. E. Dennard and R. J. P. Williams, *J. Chem. Soc. A*, 812 (1966).

(8) C. Ho and J. M. Sturtevant, *J. Biol. Chem.*, 238, 3499 (1963).

(9) See footnote *c* in Table I for method of calculation.

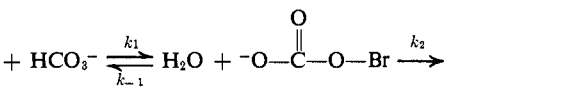
activity of HOBr as a catalyst for decarboxylation, as compared with hydronium ion and the phosphate monoanion, strongly suggests that this compound functions as a brominating agent rather than as a general acid catalyst. From the specific rate constants for hydronium ion and the phosphate monoanion a Brønsted α value equal to 0.7 may be calculated for general acid catalysis of bicarbonate decarboxylation. The reasonableness of this value is supported by a calculated α of 0.6 for the phosphate and hydronium ion catalyzed decarboxylation of *N*-carboxyimidazolidone.[10] Based on the α of 0.7 and a pK of 8.78 the rate for hypobromous acid catalysis is 1.63×10^4 times that predicted for general acid catalysis. The mechanism proposed for HOBr catalysis is



$$\longrightarrow HOBr + CO_2 + OH^- \qquad (7)$$

This mechanism apparently holds despite the fact that the leaving group pK is 15.7, as compared with 8.78 for a mechanism involving general acid catalysis. The high electronegativity of bromine is believed to be responsible here, as it presumably is in the reaction of HOBr with isobutylene where the product is 1-bromo-2-methyl-2-propanol, rather than *tert*-butyl alcohol.[11]

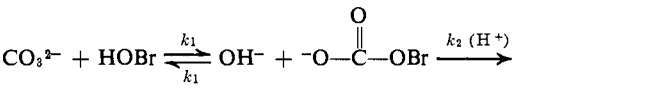
There are good grounds to reject a kinetically equivalent mechanism involving an acylhypobromite intermediate (eq 8). For such a process either $k_{-1} > k_2$, or



$$HOBr + CO_2 + OH^- \qquad (8)$$

$k_{-1} < k_2$. The assignment $k_{-1} > k_2$, which makes the first step a preequilibrium, appears to be ruled out since the position of such an equilibrium would undoubtedly be further to the right, with an accompanying increased rate, were the reaction to occur with bromine. Catalysis by bromine is certainly not nearly that expected if the equilibrium constant for bromine hydration by hydroxide is taken as an indication of the greater driving force expected to be provided by bromine, as compared with HOBr, for the k_1:k_{-1} component of the reaction in eq 8. Also, at pH 8.3 the reaction is first order with respect to bromine in the range $2.4\text{–}4.5 \times 10^{-4}$ M and the upper limit for the acyl hypobromite concentration is, therefore, approximately 2×10^{-4} M at the highest bromine concentration. Since under conditions where $k_{-1} > k_2$ the observed rate equals k_2 (acyl hypobromite), a lower limit for k_2 is 2.7×10^1 sec^{-1}. For $k_{-1} > k_2$, k_{-1} must be at least 1×10^2 sec^{-1}. This rate constant, which is for the reaction of water with an acyl hypobromite is unreasonably high since the rate constant for reaction of water with bromine is only 1×10^2 sec^{-1};[12a] bromine is expected to be more reactive than an anionic acyl hypobromite. The alternate possibility in which $k_{-1} < k_2$ so that the formation of the acyl hypobromite (the k_1 step) is rate limiting may be rejected since it would be expected that the k_1 step would be much faster with carbonate as compared with bicarbonate. No reaction has, however, been detected with carbonate.[12b]

Also consistent with the result is a mechanism in which an acyl hypobromite intermediate is formed in a preequilibrium reaction involving carbonate, with decarboxylation occurring in an acid-catalyzed rate-limiting step (eq 9). Using the same reasoning as

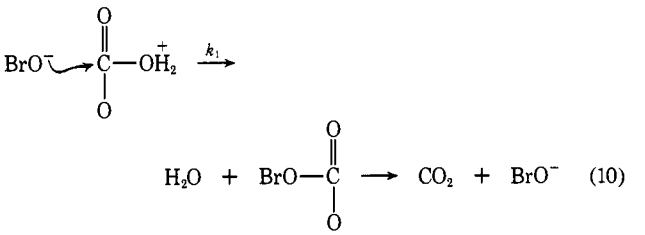


$$CO_2 + HOBr \qquad (9)$$

above, a calculated upper limit for the concentration of acyl hypobromite is 2×10^{-4} M in the presence of 4.5×10^{-4} M bromine, and to account for the observed rate at pH 8.3 and the assignment k_{-1}(OH) (acyl hypobromite) > k_2[H$^+$][acyl hypobromite], values for k_{-1} and k_2 equal to approximately 3×10^8 M^{-1} sec^{-1} and 1.5×10^{10} M^{-1} sec^{-1}, respectively, are required. We consider both of these constants unreasonably large: the former because of the charge repulsion for reaction and the poor leaving tendency of carbonate, and the latter since this constant is about 10^5 times greater than that observed for hydrogen ion catalyzed decarboxylation of bicarbonate. In bicarbonate decarboxylation the leaving group is more basic than with the acyl hypobromite and greater reactivity in an acid-catalyzed reaction is, therefore, expected; in studies of carbamate decarboxylation the rate constant for hydronium catalysis is proportional to the leaving group pK.[13a]

The meager catalysis observed for elemental bromine is inexplicable. Bromine is approximately 1000 times as reactive as HOBr as brominating agent with sodium *p*-anisate.[13b]

Dennard and Williams[7c] have proposed several mechanisms for hypobromite catalysis of carbon dioxide hydration. Since we have studied the reverse reaction, *i.e.*, dehydration, we shall consider their mechanisms in the reverse direction as that previously written. One mechanism proposed is given in eq 10. This reaction



$$H_2O + BrO-\overset{O}{\overset{\|}{C}}-O^- \longrightarrow CO_2 + BrO^- \qquad (10)$$

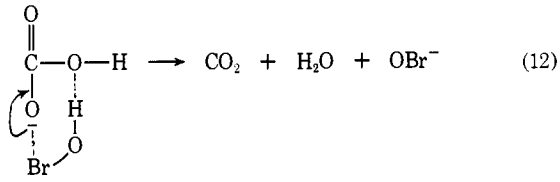
will follow a rate law (eq 11). To account for the rate

$$\text{rate} = k_1[OBr^-][H_2CO_3] \qquad (11)$$

of dehydration at pH 8.29 with 4.55×10^{-4} M bromine the rate constant k_1 in eq 11 must be equal to 4.9×10^6 M^{-1} sec^{-1}.[14] This value appears excessively large for a nucleophilic displacement of a hydroxyl function, even with the internal general acid catalysis provided by the

(10) M. Caplow and M. Yager, *J. Amer. Chem. Soc.*, **89**, 4513 (1967).
(11) J. G. Traynham and O. S. Pascual, *Tetrahedron*, 7, 165 (1959).
(12) (a) M. Eigen and K. Kustin, *J. Amer. Chem. Soc.*, **84**, 1355 (1962); (b) carbonate may be unreactive here because it cannot act as a general acid catalyst for displacement of OH from HOBr coincident with nucleophilic attack on the HOBr molecule. If this is to account for the low reactivity of carbonate it must be assumed that the internal general acid catalysis provided by bicarbonate more than compensates for the $10^{6.7}$ lower basicity (and nucleophilicity) of this substance as compared with carbonate; we consider this unlikely.
(13) (a) M. Caplow, *J. Amer. Chem. Soc.*, **90**, 6795 (1968); (b) D. H. Derbyshire and W. A. Waters, *J. Chem. Soc.*, 569 (1950).
(14) The H_2CO_3 and OBr^- concentrations were calculated using pK's of 3.76 and 8.78, respectively.

neighboring OH group.[15] These workers have also proposed a mechanism, shown in eq 12, in which the abnormal reactivity is ascribed to an association of bi-

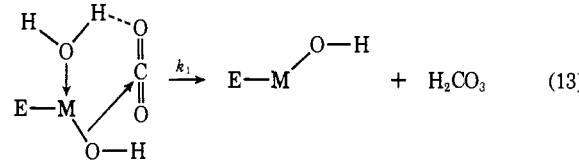


(12)

carbonate and HOBr. However, this interaction of the bicarbonate negative charge would be expected to result in a *decreased* driving force for decarboxylation, in the same way that metal ions prevent decarboxylation of *N*-carboxyimidazolidone.[10]

Carbonic Anhydrase. The remarkable catalytic activity of this enzyme remains unaccounted for, especially the mechanism of the rapid proton transfers which accompany carbon dioxide hydration and dehydration. The active site of the enzyme is evidently highly apolar[16] and since bicarbonate dehydration is accompanied by charge neutralization it might be expected that the reaction in this direction will be facilitated in such an environment. Of course factors which will stabilize the transition state for dehydration will also tend to retard the rate of the hydration process and the Haldane equilibrium relationship may be made to hold despite such unidirectional catalysis if the binding constants for carbon dioxide and bicarbonate have the appropriate values. Such a relationship of the kinetic parameters may be indicative of the physiologic function of the enzyme. The apolar environment of the enzyme may be presumed to play some role in the specific facilitation observed for the dehydration process; at pH 7.05 with carbonic anhydrase B and C the V_{max}'s for dehydration are 1.5 and 0.6 times, respectively, those for hydration,[17] while the nonenzymic rate of dehydration is less than one-seventh that for hydration at this pH. Based upon the fact that the specific rate constant for hydronium ion catalysis for dehydration is only increased 43-fold in 71.5% dioxane it is suggested that the apolar character of the active site contributes relatively little to the catalytic efficacy of the enzyme. The meager rate enhancement observed here suggests that the transition state still posesses substantial charge and probably resembles a zwitterion of carbonic acid.

The rate of carbonic anhydrase catalyzed carbon dioxide hydration at pH 7.05 is 6.2×10^5 sec^{-1} and since the rate is only about half-maximal at this pH the enzyme has a turnover number in the order of 10^6 sec^{-1}. This rate is difficult to account for since one of the groups on the enzyme responsible for catalytic activity, presumably a Zn–OH_2^+ species,[18] has a pK of approximately 7 and the maximum rate of loss of a proton from this group is only about 10^3 sec^{-1}.[19] From studies of carbonic anhydrase catalyzed hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid Kaiser[20a] has proposed a mechanism (eq 13) which obviates the need for proton



(13)

loss to the solvent by having both protons of an attacking water molecule donated to the product of the reaction. This mechanism suffers from a serious defect since microscopic reversibility requires that the reactive species in the dehydration reaction is carbonic acid. It may be calculated that the rate of encounter of enzyme and carbonic acid to form a Michaelis complex is not fast enough to account for the observed rate: at pH 7 with 5.7×10^{-9} M carbonic anhydrase C and 6.8×10^{-2} M bicarbonate (3.9×10^{-5} M carbonic acid) the rate is 3.2×10^{-3} M sec^{-1},[17] while the calculated rate of enzyme–carbonic acid encounter, assuming that the rate constant for such a process is diffusion controlled (10^{10} M^{-1} sec^{-1}), and taking into account the fact that the enzyme is only half-active at this pH, is only 1×10^{-3} M sec^{-1}. Observed rate constants for encounter of enzymes and substrates are in the range 10^6–10^8 M^{-1} sec^{-1} [20b] and the calculated discrepancy is, therefore, a very conservative estimate.[20c]

In addition to this point, the rate law for reaction by eq 13 is

$$\text{rate} = k_1[\text{E–MOH}\cdot\text{H}_2\text{CO}_3] \qquad (14)$$

and to account for the observed rate at pH 7 the rate constant in eq 14 must be equal to 2.1×10^9 sec^{-1}.[21] The requirement for this very high rate constant certainly speaks against such a mechanism.[22]

A metal carbonato complex[18b] is believed to be a key intermediate in carbonic anhydrase catalysis. We would like to point out that based on the low nonenzymic reactivity of the carbonatopentaaminecobalt(III) ion,[24] which decarboxylates with C–O bond cleavage, such an intermediate would be expected to have reac-

(15) An even larger rate constant is required if it is assumed that the reactive species is the very low fraction of carbonic acid existing as the zwitterion, as in eq 10.

(16) (a) R. F. Chen and J. C. Kernohan, *J. Biol. Chem.*, **242**, 5813 (1967); (b) J. E. Coleman, *ibid.*, **243**, 4574 (1968); (c) M. E. Riepe and J. H. Wang, *ibid.*, **243**, 2779 (1968).

(17) B. H. Gibbons and J. T. Edsall, *ibid.*, **239**, 2539 (1964).

(18) (a) J. C. Kernohan, *Biochem. Biophys. Acta*, **96**, 304, 1965; (b) J. E. Coleman, *J. Biol. Chem.*, **242**, 5212 (1967), and references therein.

(19) Rates of proton transfer are calculated from the equilibrium constant and are based on the assumption that the rate is diffusion controlled, and equal to 10^{10} M^{-1} sec^{-1}, in the direction in which the equilibrium is thermodynamically favorable.

(20) (a) E. T. Kaiser and L. W. Lo, *J. Amer. Chem. Soc.*, **91**, 4912 (1969); (b) M. Eigen and G. G. Hammes, *Advan. Enzymol.*, **25**, 1 (1963); (c) a reaction in which a Michaelis complex is formed between enzyme and bicarbonate with protonation of bicarbonate occurring within this complex is ruled out since the rate of protonation of such an intermediate present at a concentration of 5.7×10^{-9} M with 10^{-7} M hydrogen ion is only about 10^{-5} sec^{-1}, assuming the rate constant is that for a diffusion-controlled process. This is slower than the observed enzymic rate (see above).

(21) Based upon pK's equal to 3.76 and 7.0 for carbonic acid and the enzyme aquometal complex, respectively.

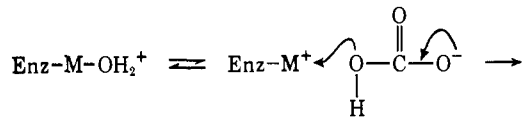
(22) Arguments against carbonic acid as a substrate have previously been developed.[18a,23]

(23) H. DeVoe and G. B. Kistiakowsky, *J. Amer. Chem. Soc.*, **83**, 274 (1961).

(24) The second-order rate constant for hydronium ion catalyzed decarboxylation is about 4×10^6 M^{-1} sec^{-1},[25] which is only 70 times that for bicarbonate breakdown.

(25) (a) D. J. Francis and R. B. Jordan, *J. Amer. Chem. Soc.*, **89**, 5591 (1967); (b) T. P. Dasgupta and G. M. Harris, *ibid.*, **90**, 6340 (1968).

tivity not unlike that of bicarbonate, so that there appears to be little catalytic advantage to this pathway. On the other hand, taking together the indications that catalysis by HOBr proceeds in a concerted mechanism, and evidence—the reasonable calculated Brønsted slope—that catalysis by $H_2PO_4^-$ and H_3O^+ proceeds similarly, it appears that there is some special propensity for a concerted decarboxylation with bicarbonate. We suggest the outline of such a mechanism for carbonic anhydrase in eq 15. This mechanism does not circumvent the dilemma, outlined above, concerning the insufficient rate of proton transfer.



$$\text{Enz-M-OH}_2^+ \rightleftharpoons \text{Enz-M}^+\text{-O(H)-C(=O)-O}^- \rightarrow \text{Enz—M—OH} + \text{CO}_2 \quad (15)$$

Nuclear Magnetic Resonance Study of the Mechanism of Reversible Denaturation of Lysozyme

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Abstract: Reversible denaturation of hen egg white lysozyme was studied by proton magnetic resonance spectroscopy. Temperature, pH, and guanidine concentration were employed as denaturation variables. Denaturation and renaturation appear by this technique to be cooperative transitions between a native state N and a denatured state D. Through the transition region of the denaturant parameter the process is described by the equilibrium $N \rightleftarrows D$; no transition intermediates were detected. In the thermal transition at pH 3.3 between 60 and 75°, ΔH = 73.5 kcal mol^{-1}, ΔS = 215 cal deg^{-1} mol^{-1}, and the $N \rightleftarrows D$ interconversion rate lies between 1.4×10^{-3} and 2×10^2 sec^{-1}. The pmr spectra indicate that D is extended in solution so that all residue side chains are solvated (but does not exclude the possibility of some residual secondary structure) and that N is a unique conformation except for possible mobility of surface residue side chains.

Biological activities of proteins usually are associated with the folded structures that they assume under physiological conditions. The three-dimensional structures of a number of proteins in the solid state now have been determined by X-ray crystallography, including that of hen egg white (HEW) lysozyme,[1] the subject of this study. Many questions remain, however, regarding the structures of proteins in solution, their interactions with the environment, and the processes of protein folding and denaturation.

Several investigations of the denaturation of HEW lysozyme in aqueous solution by optical techniques[2-7] have been reported. The compact folded conformation of the protein converts to a cross-linked (four residual disulfide bonds), random-coil state upon heating, lowering the pH, or addition of guanidine hydrochloride. On reversal of the denaturant parameter, substantial or complete renaturation of the protein to the native state is achieved. The processes of unfolding and folding are rapid for most of the denaturation–renaturation conditions that have been studied so that equilibrium is achieved between the native and denatured states in times from a few seconds to several minutes. If procedures are used during denaturation that break the disulfide bonds, renaturation can occur but at a much slower rate.[8]

Sophianopoulos and Weiss[2] concluded that thermal denaturation of lysozyme proceeded without stable intermediates by a two-state process, where N and D

$$N \rightleftharpoons D$$

are, respectively, the native and denatured (cross-linked random-coil) states. Tanford and coworkers concurred in this conclusion for denaturation induced by guanidine hydrochloride but stated that the final state produced by thermal denaturation retains elements of structure beyond just cross-linking of the random-coil peptide chain by disulfide bonds. They observed changes in the optical rotation of thermally denatured lysozyme upon addition of guanidine hydrochloride that were interpreted in terms of folded structure which was dissipated by the chemical denaturant.[5] The denaturation of lysozyme was represented as

$$N \rightleftharpoons X \rightleftharpoons D$$

where D represents the truly denatured state reached only in concentrated guanidine hydrochloride and X is a distinct but only partially denatured state, structured in undefined fashion, attained by strictly thermal denaturation.

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