measurements are generally done in the temperature interval of 300 to 350°K., E_i should lie between 1600 and 1800 cal./mole.

Introduction of $x = 2.65$ into the heat capacity expression yields

$$(C_p)_{max} \cong 0.75R$$

Thus we may expect an additional negative contribution to $(\Delta C_p^0)_{ij}$ between zero and $0.75R$. It is, of course, highly unlikely that the potential energy minima will be such as to yield the maximum C_p for all hydrogen bonds involved and for all bonds in a given side group. The only rigorous statement that can be made is

$$-1.00\ \nu_{ij}\ R > (\Delta C_p^0)_{ij} > -1.75\ \nu_{ij}\ R$$

We should expect $(\Delta C_p^0)_{ij}$ to lie closer to the less negative value.

The Enzymic Kinetics of Carbonic Anhydrase from Bovine and Human Erythrocytes

By HOWARD DeVOE AND G. B. KISTIAKOWSKY

The rates of the carbonic anhydrase-catalyzed hydration of carbon dioxide and the reverse dehydration of bicarbonate ion were measured as a function of substrate concentration to yield information about the enzymic mechanism. The measurements were made at 0.5° and neutral pH by following, in a "stopped flow" apparatus, optical densities of reaction solutions containing p-nitrophenol indicator and dilute phosphate buffer. Carbonic anhydrases from both bovine and human erythrocytes were studied which contained 0.27 and 0.28% of zinc, respectively. The bovine enzyme requires peptone to stabilize the enzymic activity of dilute solutions, is not affected by p-nitrophenol and is inhibited to varying extents by different neutral salts. Its hydration and dehydration kinetics obey the Michaelis–Menten equation. A simple mechanism which agrees with the pH dependence of the kinetics requires two adjacent sites on the enzyme molecule: one to bind a hydroxide ion and the other to bind the substrates CO_2 and HCO_3^-. The human enzyme differs from the bovine enzyme in not requiring peptone for stabilization, in being activated by p-nitrophenol and in being inhibited less specifically by neutral salts. Its dehydration kinetics obey the Michaelis–Menten equation but its hydration kinetics do not; this may be interpreted as due to activation of the enzyme by CO_2.

Introduction

The physical and chemical properties of carbonic anhydrase, which at neutral pH catalyzes the reaction

$$CO_2(aq.) + H_2O = HCO_3^- + H^+ \qquad (1)$$

have been studied by a number of authors.[1] The kinetics of the hydration reaction with purified horse erythrocyte carbonic anhydrase have been studied by Kiese,[2] the hydration and dehydration reactions with a crude preparation of bovine erythrocyte carbonic anhydrase by Roughton and Booth[3] and the hydration reaction with highly purified human erythrocyte carbonic anhydrase by Davis.[4,5] These workers claim that the dependence of the catalyzed rate on the concentration of substrate (CO_2 or HCO_3^-) follows Michaelis–Menten kinetics and report different values for the hydration Michaelis constants of the three enzyme preparations.

The value of the equilibrium constant of reaction 1 is such that at neutral pH the reaction can be run in either direction with equal convenience. The present work took advantage of this to study the kinetics of the enzymic catalysis in both directions with the same enzyme concentration and comparable solution compositions. Carbonic anhydrases from both bovine and human erythrocytes were studied, using a "stopped flow" apparatus[6] to rapidly mix the reactants and low temperature (0.5°) to minimize the non-enzymic reaction.

Experimental

Method.—The reaction was followed by measuring the rate of pH change of the weakly buffered reaction solution.

An attempt to measure the enzymic reaction with a glass electrode incorporated in the flow apparatus failed, because the apparent rate in a run was greater the longer the electrode had previously contacted an enzyme solution. Experimentation made it clear that enzyme adsorption on the glass membrane of the electrode caused the difficulty, apparently by increasing the local enzyme concentration at the membrane.[7] This finding throws some doubt on the accuracy of Davis'[4,5] carbonic anhydrase rate measurements, since they were made with a glass electrode which was pre-equilibrated with enzyme solution.

The method finally adopted was the photometric measurement of the optical density of p-nitrophenol included as an indicator in the reaction solution. The flow apparatus was thermostated at $0.50 \pm 0.02°$. One of the reactant solutions was the substrate solution, either aqueous CO_2 or aqueous $KHCO_3$. A CO_2 solution was prepared by bubbling a gaseous mixture of CO_2 and N_2 for at least 20 min. through water at the temperature of the flow apparatus. The other reactant solution contained the phosphate buffer, enzyme, indicator and any other materials included in the runs; each batch was used for a series of about eight runs.

About 5 ml. of both reactant solutions were simultaneously discharged from separate glass syringes into a 2 mm. bore glass capillary tube, where turbulent flow mixed the solutions in a 1:1 ratio. The flow was stopped suddenly after about 1 sec., leaving mixed reaction solution at rest in the observation cell of the photometer. During the initial 10 sec. of the run, the fraction of the incident light (in the 400 mμ wave length region) transmitted by the observation cell was measured with a phototube. A photographic record of the signal as a function of time was made from an oscillograph display and was used to determine the initial pH and the initial reaction rate.[8]

The values given for the enzymic reaction rate, v_{enz}, have been corrected for the non-enzymic rate calculated from published values of the hydration[9] and dehydration[10] rate

(1) See F. J. W. Roughton and A. M. Clark, in J. B. Sumner and K. Myrbäck, "The Enzymes," Vol. I, Academic Press, Inc., New York, N. Y., 1951, Ch. 43.

(2) M. Kiese, *Biochem. Z.*, **307**, 400 (1941).

(3) F. J. W. Roughton and V. H. Booth, *Biochem. J.*, **40**, 319 (1946).

(4) R. P. Davis, THIS JOURNAL, **80**, 5209 (1958).

(5) R. P. Davis, *ibid.*, **81**, 5674 (1959).

(6) F. J. W. Roughton and B. Chance, in S. L. Friess and A. Weissberger, "Technique of Organic Chemistry," Vol. VIII, Interscience Publishers, Inc., New York, N. Y., 1953, Ch. 10.

(7) We wish to thank Professor F. J. W. Roughton for alerting us to the possibility that a glass electrode might be incapable of measuring reaction rates accurately in the presence of protein.

(8) Details are given in the Ph.D. thesis of H. DeVoe, Harvard University, 1960.

(9) B. R. W. Pinsent and F. J. W. Roughton, *Trans. Faraday Soc.*, **47**, 263 (1951).

(10) J. A. Sirs, *ibid.*, **54**, 207 (1958).

constants, taking into account the non-enzymic back reaction and catalysis of the reaction by HPO_4^-.[11] Typically, the non-enzymic reaction accounted for from one-tenth to one-half of the total rate (depending on the substrate concentration and other conditions). The random and systematic errors in the values of v_{enz} are believed to amount to less than $\pm$ 5%.

Enzyme Preparations.—The work used two preparations of bovine carbonic anhydrase, designated preps. B-1 and B-2. Prep. B-1 was a dry preparation (Nutritional Biochemicals Corp., lot 6075) which had been isolated from beef erythrocytes using essentially one of the methods of Keilin and Mann.[12] Prep. B-2 consisted of the same lot 6075 enzyme, further purified by the last steps of Keilin and Mann's second procedure (adsorption of impurities with alumina gel followed by fractional precipitation with $(NH_4)_2SO_4$).

Two preparations of human carbonic anhydrase were used, designated preps. H-1 and H-2. Prep. H-1 was isolated from human erythrocytes, following the second procedure of Keilin and Mann[12] at 2°.[13] A portion of prep. H-1 was fractionally adsorbed on aged alumina gel (pH 6.8), eluted with 90 mM phosphate buffer (pH 7.6) and dialyzed against water, resulting in prep. H-2.

The enzyme stock solutions were stored at 1-3°. Aliquots were dried to constant weight at 105-110° to determine the protein concentration in mg. per liter. Solutions of enzyme preps. B-2 and H-1 were analyzed for zinc by colorimetric titration with dithizone[14]; the values reported for $(E)_0$, the total concentration of enzymic sites, are based on these analyses assuming one enzymic site per zinc atom. By calculation from the protein and zinc analyses, the bovine enzyme of prep. B-2 contained 0.27% zinc (within the range of values, 0.22-0.33%, previously reported[12,15,16] for highly purified preparations and corresponding to one zinc atom per enzyme molecule), and the human enzyme of prep. H-1 contained 0.28% zinc.

Other Materials.—The buffer stock solutions were prepared from A. R. grade KH_2PO_4 and Na_2HPO_4. The p-nitrophenol was recrystallized three times. The $KHCO_3$, KCl and KNO_3 were A.R. grade, the NaF was reagent grade and the peptone was Difco Bacto-peptone.

All water was distilled twice, the second time in an all-glass still. Volumetric glassware was cleaned in hot HNO_3-H_2SO_4. Care was taken to avoid contact of solutions with sources of possibly inhibitory heavy metal ions.

Bovine Carbonic Anhydrase.

—Within a series of hydration or dehydration runs performed with the same buffer–enzyme reactant solution (containing 0.1-0.2 mg. per liter of enzyme prep. B—1 or B—2), a steady decrease in v_{enz} from run to run was noted with about half the enzymic activity being lost in 8 hr. Because peptone has been observed to stabilize dilute solutions of bovine carbonic anhydrase,[17,18] the effect of including peptone (0.02%) in the buffer–enzyme reactant solution was investigated. The result was an approximately two-fold increase in the value of v_{enz} and a stabilization of the enzymic activity of the reactant solution. A higher concentration of peptone did not further increase v_{enz}.

When 0.016% Versene was included in a buffer–enzyme reactant solution, the enzymic activity (in dehydration runs) was 10% lower than if peptone instead of Versene had initially been added; and a

(11) F. J. W. Roughton and V. H. Booth, *Biochem. J.*, **32**, 2049 (1938).

(12) D. Keilin and T. Mann, *ibid.*, **34**, 1163 (1940).

(13) The enzyme was purified by Miss Bernice Fingerman three years before the kinetic runs were performed and stored as a concentrated solution at 1-3°.

(14) The zinc analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, New York.

(15) E. Hove, C. A. Elvehjem and E. B. Hart, *J. Biol. Chem.*, **136**, 425 (1940).

(16) D. A. Scott and A. M. Fisher, *ibid.*, **144**, 371 (1942).

(17) D. A. Scott and J. R. Mendive, *ibid.*, **139**, 661 (1941).

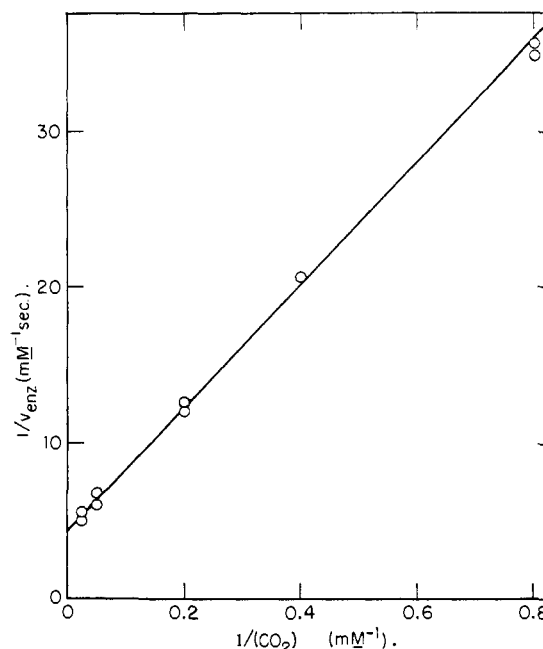(18) A. M. Clark and D. D. Perrin, *Biochem. J.*, **48**, 495 (1951).

Fig. 1.—Typical Lineweaver–Burk plot for a series of hydration runs; enzyme prep. B-2, 2 mM phosphate buffer, pH 7.0-7.1, 0.148 mM indicator.

subsequent addition of 0.02% peptone failed to increase the enzymic activity further. This suggests that the effect of peptone is two-fold: first to chelate trace amounts of inhibitors[1,18] (which Versene can also do), and second, by its surface-active character to protect the enzyme against denaturation at solution interfaces.[19]

All further bovine enzyme work to be described was performed with 0.02% peptone included in the buffer-enzyme reactant solutions.

Hydration Kinetics.—The hydration kinetics of enzyme preps. B-1 and B-2, in the substrate concentration range 1.25-38 mM, accurately obeyed the Michaelis–Menten equation

$$v_{enz} = V_m(CO_2)/[K_M + (CO_2)] \qquad (2)$$

Fitting the data of several series of runs to eq. 2 by weighted least squares analyses[20] yielded values for the standard deviation of v_{enz} of 3-4%. Figure 1 shows a typical Lineweaver-Burk plot of the data for a series of runs. Values of the hydration kinetic parameters V_m and K_M for several reaction conditions, determined either graphically from a Lineweaver-Burk plot or by a least squares analysis, are given in Table I.

For enzyme prep. B-1, the specific activity (V_m divided by the protein concentration) in mole/g. sec. was 2.2 for a protein concentration of 0.80 mg. per liter and was 2.6 for one-ninth of this protein concentration. Enzyme prep. B-2 had a higher specific activity, 3.4, indicating that it contained a smaller percentage of inactive protein.

For enzyme prep. B-2 in 2 mM buffer, V_m increases approximately 15% when the pH is raised from 6.9 to 7.2, while K_M remains constant (9.1 $\pm$

(19) A. M. Clark, Ph.D. Thesis, Cambridge University, 1949.

(20) Each least squares analysis assumed that the substrate concentration was precisely known and that the percentage standard deviation of v_{enz} was a constant.

TABLE I

HYDRATION KINETIC PARAMETERS FOR BOVINE CARBONIC ANHYDRASE[a]

pH[b]	Reaction conditions[c]	V_m (mM/sec.)	K_M (mM)
	Enzyme prep. B-1		
7.0	...	1.8[d]	8.4[d]
7.1	...	0.23[e]	9.3[e]
Enzyme prep. B-2 (0.067 mg. protein/l.; $(E)_0 = 2.8 \times 10^{-9}$ M)			
6.9	...	0.20	9.2
7.1	...	.224 ± 0.007[f]	9.1 ± 0.3[f]
7.1	...	.229 ± 0.007[f]	9.1 ± 0.4[f]
7.2	...	.23	9.1
7.1	0.074 mM indicator	.25	9.1
7.1	0.074 mM indicator	.23	8.5
7.1	10 mM buffer	.26 ± 0.01[f]	13.3 ± 0.6[f]
6.9	50 mM KCl	.11	14
6.9	50 mM KNO₃	.05	30

[a] Each row presents data from a series of runs made with the same buffer-enzyme reactant solution. [b] Median pH value, varied by changing the buffer composition. The pH in a run depended on the CO_2 concentration but deviated by no more than 0.1 pH unit from the value given. [c] 2 mM phosphate buffer, 0.148 mM indicator unless otherwise indicated. [d] 0.80 mg. protein per liter. [e] 0.089 mg. protein per liter. [f] Value and standard deviation from least squares analysis.[20]

0.3 mM) in this pH range (Table I). There is no significant variation of V_m or K_M with the indicator concentration; thus the indicator's presence probably does not affect the enzymic kinetics. When the buffer concentration is changed from 2 mM to 10 mM at pH 7.1, V_m increases 13% and K_M increases from 9 mM to 13 mM; phosphate thus behaves formally as an activator and a competitive inhibitor of the enzyme.
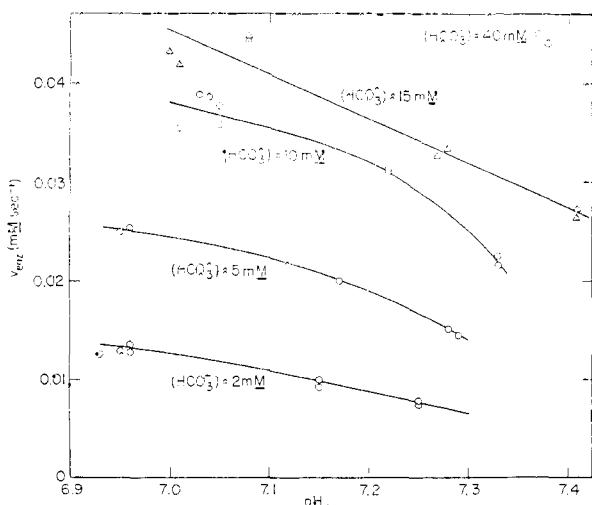

Fig. 2.—Dehydration kinetics of enzyme prep. B-2; $(E)_0 = 2.8 \times 10^{-9}$ M, 2 mM phosphate buffer, 0.148 mM indicator.

The hydration turnover number for enzyme prep. B-2 in 10 mM buffer at pH 7.1 is $V_m/(E)_0 = 9.3 \times 10^4$ sec.$^{-1}$. Roughton and Clark[1] estimate that in pH 7.3 phosphate buffer of unspecified concentration (probably greater than 10 mM) at 0°, the value of $V_m/(E)_0$ for bovine enzyme is 1.6×10^6

sec.$^{-1}$, a value 17 times greater than the present one. Part of the discrepancy can be attributed to the difference in pH and buffer concentration. Roughton and Clark's[1] value of V_m was measured with a crude enzyme preparation which, it can be estimated, contained only one part in 10^4 by weight of active enzyme; and their value of $(E)_0$ was calculated by comparing the activity of this crude preparation (in arbitrary units) with the activity of purified enzyme. The present value of $V_m/(E)_0$, in contrast to Roughton and Clark's higher value, comes from kinetic data and a zinc analysis for the same preparation of highly purified enzyme and is therefore believed to be the more trustworthy value.

Kiese[2] reports for highly purified horse enzyme (at 0.1° in pH 9.0 phosphate buffer) the value $V_m/(E)_0 = 4.5 \times 10^4$ sec.$^{-1}$, close to the present value for bovine enzyme.

Roughton and Booth[3] give $K_M = 9 \pm 1$ mM for bovine enzyme in 24 mM phosphate buffer. The present work predicts a somewhat higher value at this buffer concentration. Kiese[2] found the much lower value $K_M = 1.2$ mM for horse enzyme at pH 7.4.

Both KCl and KNO₃ cause a decrease in V_m and an increase in K_M (Table I), thus acting as inhibitors which are neither purely competitive nor non-competitive. NaF was also found to inhibit, without giving a linear Lineweaver–Burk plot. There is a strong salt inhibition specificity in the order KNO₃ >> KCl >> NaF (for 50 mM salt concentration).

Runs with bovine enzyme from a three year old stock solution (stored at 1–3°) which originally contained 10 mg. protein per liter showed that less than 5% of the enzymic activity remained. Slow thermal denaturation was probably responsible, as the stock solution contained a small amount of white flocculent precipitate.

Dehydration Kinetics.—The dehydration runs were performed with enzyme preparation B-2 at the same concentration as in hydration runs. The KHCO₃ substrate concentration was varied from 2 to 40 mM, and the pH was varied by using three compositions of 2 mM buffer. The rates were strongly dependent on pH, and the pH for each buffer composition depended on the substrate concentration. Therefore, from Fig. 2, a plot of v_{enz} versus pH, values of v_{enz} for the various substrate concentrations were interpolated at each of five pH values and plotted in Lineweaver–Burk plots for each pH (Fig. 3).

The linearity of the plots in Fig. 3 indicates that the kinetics obey the Michaelis–Menten equation for dehydration

$$v_{enz} = V'_m \, (HCO_3^-)/[K'_M + (HCO_3^-)] \qquad (3)$$

Apparent values of the dehydration kinetic parameters V'_m and K'_M were determined graphically from the plots. Because the CO_2 concentration in the runs was not negligible, a correction for the back enzymic reaction was made, based on the expression for the enzymic dehydration rate in the presence of CO_2

$$v_{enz} = \frac{V'_m \, (HCO_3^-)/K'_M - V_m \, (CO_2)/K_M}{1 + (HCO_3^-)/K'_M + (CO_2)/K_M} \qquad (4)^{21}$$
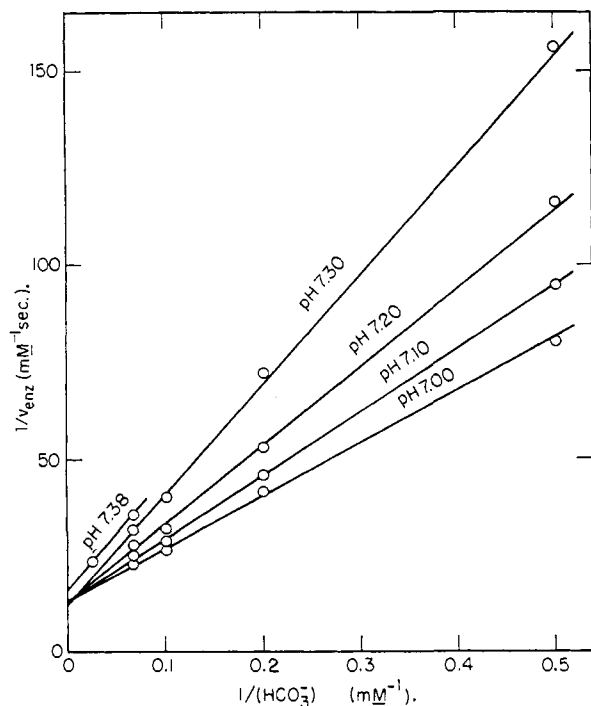
Fig. 3.—Lineweaver–Burk plots for dehydration with enzyme prep. B-2 at several pH values; the points are from the curves of Fig. 2.

Smoothed values of v_{enz}, taken from Fig. 3, were corrected to $(CO_2) = 0$ by the use of eq. 4 (using the apparent values of V'_m and K'_M as first approximations to the true values) and then plotted in new Lineweaver–Burk plots from which the corrected values of V'_m and K'_M were determined.[8]

Table II lists the corrected values of the dehydration kinetic parameters. Included are values from runs with 10 mM buffer; the higher buffer capacity made an interpolation of v_{enz} to constant pH unnecessary. It is unfortunately not possible to tell whether the values are in error from the variations in the ionic strength contributed by the KHCO$_3$ substrate.

TABLE II

DEHYDRATION KINETIC PARAMETERS AND THE HALDANE RELATION FOR BOVINE CARBONIC ANHYDRASE[a]

pH	V'_m (mM/sec.)	K'_M (mM)	$\dfrac{V_m K'_M}{K_M V'_m}$ [b]	$\dfrac{K_{CO_2}}{a_{H^+}\gamma_{HCO_3^-}}$ [c]
7.00[d]	0.074	9.6	3.2	2.9
7.10[d]	.078	12.2	3.9	3.6
7.20[d]	.077	14.7	4.8	4.6
7.30[d]	.085	22.3	6.6	5.7
7.38[d]	.071[f]	20.6[f]	7.3	6.9
7.08[e]	.100	15.6	3.0	3.8

[a] Enzyme prep. B-2; $(E)_0 = 2.80 \times 10^{-9}\ M$; 0.148 m$M$ indicator. [b] Values of V_m and K_M were taken from Table I. [c] $\gamma_{HCO_3^-}$ was calculated from the Debye–Hückel limiting law, taking the ionic strength as that of the buffer alone. [d] 2 mM phosphate buffer. [e] 10 mM phosphate buffer. [f] Based on only two points from Fig. 3 and thus less reliable than the other values.

In 2 mM buffer, V'_m increases 15% between pH 7.0 and 7.3 while K'_M is approximately inversely proportional to the hydrogen ion concentration

(21) See for example R. A. Alberty, *Advances in Enzymol.*, **17**, 1 (1956).
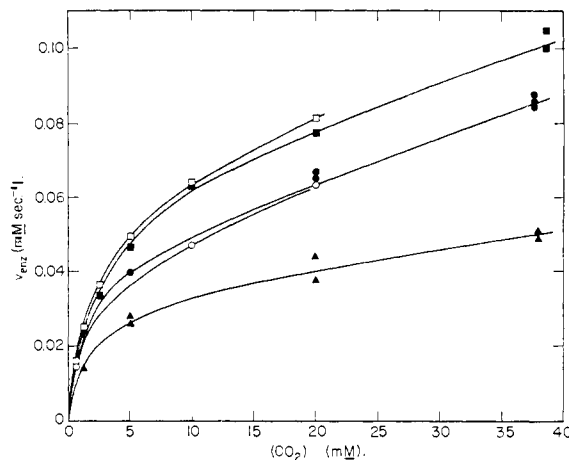

Fig. 4.—Hydration kinetics of enzyme preps. H-1 and H-2; 2 mM phosphate buffer, pH 7.0–7.1; the curve for each series of runs represents eq. 6 with the parameter values of Table V. Reaction conditions: enzyme prep. H-1, 1.06 mg. protein/liter, $(E)_0 = 4.5 \times 10^{-8}\ M$: $O\bullet$, 0.074 mM indicator; $\square\blacksquare$, 0.148 mM indicator. $\blacktriangle$, Enzyme prep. H-2, 0.57 mg. protein/liter, 0.148 mM indicator.

(Table II). When the buffer concentration is increased to 10 mM at pH 7.1, V'_m and K'_M both increase more than 30%; thus phosphate behaves as an activator and a competitive inhibitor for the dehydration reaction as well as for the hydration reaction.

Haldane Relation.—The self-consistency of the hydration and dehydration data for enzyme prep. B-2 can be tested with the Haldane relation,[21] which for reaction 1 takes the form

$$V_m K'_M / K_M V'_m = (HCO_3^-)_{eqm}/(CO_2)_{eqm} =$$
$$K_{CO_2}/a_{H^+}\gamma_{HCO_3^-} \quad (5)$$

where a and γ refer to activity and activity coefficient, respectively. The thermodynamic equilibrium constant of reaction 1, K_{CO_2}, has the value 2.69×10^{-7} at $0.5°$.[22] The last two columns of Table II give calculated values of the right- and the left-hand members of eq. 5; the Haldane relation is seen to be satisfied reasonably well.

Mechanism.—It will be assumed that the observed mild dependence of V_m and V'_m on pH is the result of equilibrium between an inactive form of the enzymic site and an active form produced by dissociation of a proton, with an acid dissociation constant K_a. V_m and V'_m are then proportional to $(E^*)_0$, the concentration of the active enzymic sites, where $(E^*)_0 = (E)_0/[1 + (H^+)/K_a]$. The experimental pH range was too limited for evaluation of K_a. Kiese[2] reports $pK_a = 7.2$ for horse enzyme, whereas Roughton and Booth's[3] data indicate that pK_a is greater than 8 for bovine enzyme.

The correct enzymic mechanism must yield, as the only other pH dependence of the kinetic parameters, an inverse proportionality between K'_M and (H^+). Table III presents three kinetic mechanisms for carbonic anhydrase which are reasonable *a priori*. Mechanism I, the simplest mechanism with CO_2 and HCO_3^- as substrates, fails to satisfy the above criterion for the correct mechanism. Mechanism

(22) H. S. Harned and R. Davis, Jr., THIS JOURNAL, **65**, 2030 (1943).

TABLE III: POSSIBLE KINETIC MECHANISMS FOR CARBONIC ANHYDRASE

Mechanism	V_m	K_M	V'_m	K'_M
I. $E + CO_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}CO_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{-}HCO_3^- \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + HCO_3^-$				
II.^{a} $E\text{-}CO_2 + OH^- \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{-}HCO_3^-$; $E + CO_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}CO_2$; $E + H_2CO_3$; $E\text{-}HCO_3^- \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + HCO_3^-$	$\dfrac{k_3k_5(OH^-)(E^*)_0}{k_3(OH^-) + k_4 + k_5}$	$\dfrac{k_2k_4 + k_2k_5 + k_3k_5(OH^-)}{k_1[k_3(OH^-) + k_4 + k_5]}$	$\dfrac{k_2k_4(E^*)_0}{k_2 + k_3(OH^-) + k_4}$	$\dfrac{k_2k_4 + k_2k_5 + k_3k_5(OH^-)}{k_6[k_2 + k_3(OH^-)] + k_4}$
III.^{b} $CO_2 + {}^-HO\text{-}E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} {}^-HO\text{-}E\text{-}CO_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\text{-}HCO_3^- \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E\text{-}HCO_3^-$; $CO_2 + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-}CO_2$; $E + HCO_3^-$; K'_a	$\dfrac{k_3k_5(E^*)_0}{k_3 + k_4 + k_5}$; $\dfrac{Gk_3k_5(E^*)_0}{k_3 + C(k_4 + k_5)}$	$\dfrac{k_2k_4 + k_2k_5 + k_3k_5}{k_1[k_3 + k_4 + k_5]}$; $\dfrac{Gk_2(k_4 + k_5) + k_3k_5}{k_1[k_3 + C(k_4 + k_5)]}$	$\dfrac{k_2k_4(E^*)_0}{k_2 + k_3 + k_4}$; $\dfrac{Gk_2k_4(E^*)_0}{G(k_2 + k_4) + k_3}$	$\dfrac{K_{H_2CO_3}[k_2k_4 + k_2k_5 + k_3k_5]}{a_{H^+}\gamma_{HCO_3^-}k_6[k_2 + k_3 + k_4]}$; $\dfrac{GK'_a[Gk_2(k_4 + k_5) + k_3k_5]}{(H^+)k_6[G(k_2 + k_4) + k_3]}$

^{a} $K_{H_2CO_3}$ is the dissociation constant of carbonic acid. ^{b} The symbol G is defined by $G \equiv 1 + (H^+)/K'_a$.

II, the simplest for CO_2 and H_2CO_3 as substrates, does satisfy the criterion. Mechanism III assumes that the enzymic site includes a binding site for the substrates, CO_2 and HCO_3^-, and an adjacent site which binds an hydroxide ion in a rapidly established equilibrium (with an acid dissociation constant K'_a). The conversion of one bound substrate to the other occurs by an intramolecular transfer of OH^- between the two sites. Smith[23] has proposed essentially this mechanism, with both binding sites on the zinc atom of the enzyme molecule; whereas Davis[5] has suggested that the zinc atom is an hydroxide site and that the substrate site is elsewhere. Mechanism III satisfies the criterion, provided $K'_a \gg (H^+)$ (in order that $G = 1 + (H^+)/K'_a$ be independent of pH).

While it is not possible to evaluate any of six rate constants of an enzymic mechanism from the experimental values of the four kinetic parameters, it is possible to set a mathematical lower limit on the value of each rate constant. Expressions which can readily be adapted for this purpose have been given.[24] Results for mechanisms II and III are shown in Table IV.

TABLE IV
MINIMUM VALUES OF THE RATE CONSTANTS OF MECHANISMS II AND III FOR BOVINE CARBONIC ANHYDRASE

Rate constant	Mechanism	Expression for minimum value	Min. value^a
k_1	II and III	$\dfrac{V_m + V'_m}{K_M (E^*)_0}$	$1.2 \times 10^7\ (E)_0/(E^*)_0$ M^{-1} sec.$^{-1}$
k_3 and k_5	II and III	$V_m/(E^*)_0$	$8.1 \times 10^4(E)_0/(E^*)_0$ sec.$^{-1}$
k_2 and k_4	II and III	$V'_m/(E^*)_0$	$3.0 \times 10^4(E)_0/(E^*)_0$ sec.$^{-1}$
k_6	II	$\dfrac{(V_m + V'_m)K_{H_2CO_3}}{K'_M(E^*)_0 a_{H^+}\gamma_{HCO_3^-}}$	$2.4 \times 10^{10}(E)_0/(E^*)_0$ M^{-1} sec.$^{-1b}$
k_6	III	$\dfrac{(V_m + V'_m)(K'_a)_{min}}{K'_M (E^*)_0(H^+)}$	$1 \times 10^8(E)_0/(E^*)_0$ M^{-1} sec.$^{-1c}$

^{a} Enzyme prep. B—2, 2 mM phosphate buffer, pH 7.10. ^{b} The value of $K_{H_2CO_3}$, the dissociation constant of carbonic acid, was calculated as 2.1×10^{-4} from the non-enzymic hydration[9] and dehydration[10] rate constants and from K_{CO_2}.[22] ^{c} Taking $(K'_a)_{min} = 10^{-6}$ and $G = 1$.

The value of k_6, the bimolecular rate constant for the binding of H_2CO_3 or HCO_3^- to an enzymic site, must be at least 5×10^{10} M^{-1} sec.$^{-1}$ for mechanism II and at least 2×10^8 M^{-1} sec.$^{-1}$ for mechanism III (from Table IV, assuming that at neutral pH the value of $(E)_0/(E^*)_0$ is at least 2). The difference arises because the concentration of H_2CO_3 is much smaller than that of HCO_3^- at neutral pH. These values for k_6 can be compared with the value for a diffusion-controlled reaction calculated from the model of Alberty and Hammes[25] (in which substrate diffuses into a hemispherical sink without electrostatic interaction). Taking the reaction radius as 5 Å., the diffusion coefficient of the enzyme as 9×10^{-7} cm.^{2} sec.$^{-1}$,[26] and the self diffusion coefficient of the substrate as 6×10^{-6} cm.^{2} sec.$^{-1}$ (from the limiting equivalent conductivity of HCO_3^-), one finds $k_6 = 1 \times 10^9$ M^{-1} sec.$^{-1}$, smaller

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than the minimum value required by mechanism II for H_2CO_3 as substrate. The possibility can be rejected that H_2CO_3 could be supplied at a significant rate to an enzymic site by the protonation of nearby HCO_3^- ions, because the mean residence time of an HCO_3^- ion within a molecular dimension of an enzymic site is much smaller than the lifetime of the ion before it becomes protonated.[8] Thus of the three mechanisms shown in Table III, mechanism III for HCO_3^- as substrate is the most consistent with the experimental data.

Human Carbonic Anhydrase. Hydration Kinetics.—Reactant solutions of the human enzyme did not require peptone for stabilization, nor did peptone if added affect v_{enz} (in contrast to the bovine enzyme's behavior). Therefore, the work described below was done without added peptone.

Plots of v_{enz} versus (CO_2) (Fig. 4), for several series of hydration runs with enzyme preparation H-1 and H-2, do not show an asymptotic approach of v_{enz} to a maximum value as they would if the Michaelis-Menten equation were obeyed. The data of each series were fitted with curves of the form

$$v_{enz} = [a(CO_2) + b(CO_2)^2]/[c + (CO_2)] \qquad (6)$$

equivalent to the Michaelis-Menten expression for v_{enz} (eq. 2) plus a linear term in (CO_2). Table V lists empirical values of the parameters of eq. 6.

TABLE V

VALUES OF THE PARAMETERS OF EQUATION 6

Series symbol (Fig. 4)	a (mM/sec.)	b (sec.$^{-1}$)	c (mM)
O	0.036	0.0015	0.97
●	.043	.0012	1.2
□	.061	.0014	1.9
■	.065	.0011	2.4
▲	.033	.0005	1.6

While most of the human enzyme hydration kinetics measured by Davis[4,5] adhere to the Michaelis-Menten equation, his data for the enzyme stock solution used in his sulfide inhibition work[5] deviate from the Michaelis-Menten equation in the same direction and to about the same extent as do the present data. The remaining portion of this enzyme stock solution was found to exhibit the same deviation in the present flow apparatus.[8] The inconsistency in Davis' data may be due to experimental difficulties or to a kinetic difference between his two supposedly identical[27] enzyme stock solutions.

Enzyme preps. H-1 and H-2 show about the same ratio of v_{enz} to protein concentration (Fig. 4). The fractional adsorption to which enzyme prep. H-2 was subjected thus failed to make it a purer preparation than prep. H-1.

Both KCl and KNO_3 (50 mM) were found to reduce the activity of enzyme prep. H-1 by a factor of ten, the kinetics again deviating from the Michaelis-Menten equation. The KNO_3 was slightly more inhibitory, but this specificity was less pronounced than for the bovine enzyme.

Dehydration Kinetics.—A series of eight dehydration runs was performed with enzyme prep. H-1 under the same conditions as for hydration (2 mM buffer, pH 6.9-7.1, $(E)_0 = 4.5 \times 10^{-8}$ M,

0.148 mM indicator), except that the ionic strength was necessarily increased by the $KHCO_3$ substrate. The data were not interpolated to constant pH or corrected for the enzymic back reaction. In contrast to the hydration kinetics, the dehydration kinetics obeyed the Michaelis-Menten equation (eq. 3). A least squares analysis[20] yielded the values $V'_m = (5.2 \pm 0.3) \times 10^{-2}$ mM sec.$^{-1}$ and $K'_M = 7.1 \pm 0.5$ mM; the standard deviation of v_{enz} was $\pm 4.8\%$.

Mechanism.—A few instances of the type of deviation from Michaelis-Menten kinetics which is shown in Fig. 4 have been reported for other enzymes, among them urease,[28] fumarase,[29] horse liver esterase[30] and α-chymotrypsin.[31]

Activation of the human carbonic anhydrase by CO_2, a special case of a general enzyme-modifier mechanism[32] with CO_2 as the modifier, would account for the observed deviation of the hydration kinetics from Michaelis-Menten kinetics (and the adherence of the dehydration kinetics). Other possible explanations of the deviation include some other component of the reaction solution acting as a modifier,[32] two different kinds of independent enzymic sites[28] and pairs of interacting enzymic sites[28,31]; but these explanations are less reasonable because for each the dehydration kinetics would, in general, also deviate from Michaelis-Menten kinetics.

The relative magnitudes of the parameters of eq. 6 are such that as (CO_2) approaches zero, eq. 6 becomes

$$v_{enz} = a(CO_2)/[c + (CO_2)]$$

which is the Michaelis-Menten equation for hydration (eq. 2). Thus if activation by CO_2 is correct, a and c must have the values of V_m and K_M, respectively, of the non-activated enzyme. The consistency of these values with the experimental values of V'_m and K'_M can be tested with the Haldane relation (eq. 5). For this calculation, average values of a and c are taken from Table V (for enzyme prep. H-1 with 0.148 mM indicator), giving for the left-hand side of eq. 5 the value 3.6. For an average pH of 7.05, the right-hand side of eq. 5 equals 3.3. The agreement is excellent, and the data are, therefore, in accord with activation by CO_2.

It is of interest that the non-activated human enzyme has a smaller hydration turnover number $(a/(E)_0 = 1.4 \times 10^3$ sec.$^{-1})$ than does the bovine enzyme and is thus a less efficient enzyme.

Table V shows that an increase of the p-nitrophenol indicator concentration increases the values of V_m and K_M for the non-activated enzyme. The indicator thus acts formally as an activator and a competitive inhibitor of the enzyme.

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Changes in the Optical Rotation of Proteins after Cleavage of the Disulfide Bonds[1,2]
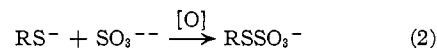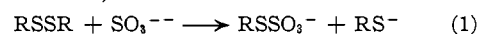
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Increase in dextrorotation of proteins after cleavage of the dithio bonds has been attributed, by other authors, chiefly to changes in conformation, and particularly to the formation of helices from randomly coiled peptide chains. Since the conversion of cystine into cysteic acid or cysteine is accompanied by a large increase in dextrorotation, the influence of the cleavage of dithio bonds in proteins on the optical rotation was investigated. In agreement with earlier observations on the oxidative fission of disulfide bonds in proteins, the present study revealed a similar increase in dextrorotation after reduction with thioglycol and after sulfitolysis, provided that simultaneous changes in the secondary structure were prevented by stabilization of the native state or by complete denaturation prior to the fission of the dithio bonds. It is concluded that the levorotation of cystinyl residues contributes essentially to the levorotation of those proteins which are rich in cystine and that fission of the disulfide bonds is responsible for most of the decrease in levorotation which accompanies reduction or sulfitolysis of these proteins.

It is well known that the levorotation shown by most of the native proteins increases on denaturation. The extent of this change in rotation depends on the protein and on the denaturing agent. An increase in levorotation also occurs when synthetic L-polypeptides are transferred from a non-polar solvent to a more polar solvent.[3] This has been attributed to unfolding of the peptide chains and to the conversion of α-helices into random coils caused by the fission of intramolecular hydrogen bonds. An analogous interpretation has been applied also to the increase in dextrorotation observed after cleavage of the dithio bonds in proteins.[4] Indeed, one could imagine that the disulfide linkages of proteins maintain the peptide chain in a strained configuration and that cleavage of the SS-bonds would allow the peptide chains to assume the α-helix conformation. However, when the dithio groups of serum albumin were oxidized by performic acid to the corresponding sulfonic acids, the increase in dextrorotation was of the order of magnitude calculated for the conversion of cystine into cysteic acid residues. Accordingly, Turner, et al.,[5] attributed the change in optical rotation after oxidation chiefly to the cleavage of the cystinyl residues rather than to configurational changes.

Since a similar increase in dextrorotation is observed when cystine is cleaved by reduction or sulfitolysis, we investigated the changes in rotation of several proteins exposed to these reactions. In contrast to the oxidation, which is usually performed in concentrated formic acid, reduction and sulfitolysis can be carried out in aqueous solutions under mild conditions. Reduction was achieved with thioglycol at pH 7–8 at room temperature. The reaction with sulfite was performed either in the presence or in the absence of an oxidizing agent. Sodium sulfite alone reacts with free cystine and also with peptide-bound cystinyl residues to form one equivalent of cysteine-S-sulfonic acid and one equivalent of cysteine (reaction 1); in the presence of sodium tetrathionate or another oxidant, the cysteine formed in reaction 1 is reoxidized and converted into a second molecule of cysteine-S-sulfonic acid[6] (reaction 2).

$$\text{RSSR} + \text{SO}_3^{--} \longrightarrow \text{RSSO}_3^- + \text{RS}^- \qquad (1)$$

$$\text{RS}^- + \text{SO}_3^{--} \xrightarrow{[O]} \text{RSSO}_3^- \qquad (2)$$

Experimental

Material and Methods.—All the proteins used were in crystalline form. Ovalbumin, bovine serum albumin and pepsin were obtained from the Nutritional Biochemicals Corporation, lysozyme from Sigma Chem. Co., γ-globulin from Armour Laboratories and chymotrypsin from Pentex Incorp. Dr. Marsh of Eli Lilly & Co. kindly supplied the insulin. Urea, sodium sulfite, sodium sulfate and LiBr were reagent grade chemicals, guanidine hydrochloride was recrystallized from methanol–ether. The detergent solutions were prepared from sodium dodecylsulfate (K. & K. Laboratories). Sodium tetrathionate was prepared by the oxidation of sodium thiosulfate with iodine.[7] Glutathione was oxidized according to Mason.[8] Sodium cysteine-S-sulfonate was prepared from cystine and sodium sulfite according to Clarke.[9]

Reduction of Proteins by Thioglycol.—100 or 200 mg. protein were dissolved in a small amount of water in a 10 ml. volumetric flask. Urea, guanidine hydrochloride, LiBr or sodium dodecylsulfate (SDS) was then added to give a final concentration of 8 M urea, 5 M guanidine-HCl, 6 to 12 M LiBr or 0.1 to 0.2 M SDS, respectively. The pH of the solution was maintained between 7 and 8 by the addition of phosphate or tris-(hydroxymethyl)-aminomethane buffer (=tris). When guanidine hydrochloride was used, the sample was kept at 50° for 3 hr. in order to accomplish maximum denaturation. After the addition of 0.1–0.2 ml. of thioglycol, the flask was filled to the mark with distilled

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