[CONTRIBUTION FROM THE GIBBS CHEMICAL LABORATORY, HARVARD UNIVERSITY]

The Kinetics of the Reaction of Human Erythrocyte Carbonic Anhydrase. I. Basic Mechanism and the Effect of Electrolytes on Enzyme Activity

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A rapid recording electrometric method has been developed to observe changes in hydrogen ion activity accompanying the hydration of carbon dioxide in weakly buffered solutions. The kinetics of this reaction were studied at *p*H 7.0 and 1.5° in the absence and in the presence of human erythrocyte carbonic anhydrase. The enzyme-catalyzed reaction follows exactly the Michaelis–Menten mechanism. Both the K_m and V_{max} are functions of electrolyte concentrations, the dependence of the latter being expressed by the relation: $\log V_{max} = -6.2\sqrt{\mu} + \log V^0_{max}$ where μ is the ionic strength of the solution. Changes of K_m are accurately representable by a linear equation: $K_m(\mu) = a + bV_{max}(\mu)$. Such a relation permits an unambiguous identification of the individual rate constants composing $K_m = (k_2 + k_3)/k_1$. The constants k_1 and k_2, which are independent of ionic strength, are found to be $0.46 \times 10^6\ M^{-1}$ sec.$^{-1}$ and 5.5×10^2 sec.$^{-1}$, respectively; k_3 is 18.3×10^2 sec.$^{-1}$ at ionic strength 0.004. These values refer to an enzymatic concentration equivalent to 0.027 micromole of zinc per liter. K_a, the association constant for the enzyme–substrate compound, is $8.3 \times 10^2\ M^{-1}$.

Introduction

Careful physical-chemical investigations of the kinetics of reactions catalyzed by enzymes have demonstrated several enzymes, notably urease,[2,3] fumarase[4] and horse liver esterase,[5] that do not obey the basic mechanism propounded by Michaelis and Menten.[6] It seemed of interest to extend these observations to an enzyme which has a smaller molecular weight and presumably only a single active center. In most enzyme studies, no information is obtained on the magnitude of k_1 and k_2, the forward and reverse rate constants for the formation of the enzyme–substrate complex, and interpretation of the Michaelis constant, by derivation neither a kinetic nor a thermodynamic parameter except in certain limiting cases, is difficult. It has been rarely possible to dissect an enzyme mechanism quantitatively into its component steps. Chance's classical studies[7] of various hemoprotein-catalyzed oxidation–reduction reactions have succeeded in measuring the individual rate constants separately, while Theorell, Nygaard and Bonnichsen[8] determined the specific rate constants, by steady state measurements alone, for the reactions of liver alcohol dehydrogenase and DPN. Alberty and Peirce,[9] by making several reasonable assumptions on the nature of the equilibrium constant for the fumarase reaction and by utilizing the data of inhibition constants, were able to arrive at a range of values for the various rate constants in their enzyme system. A similar analysis of an outwardly "simpler" enzymatic reaction should also provide useful kinetic data.

The hydration of carbon dioxide to form carbonic acid is probably the "simplest" of all enzymatically catalyzed biochemical reactions. The enzyme, carbonic anhydrase, is found primarily in mammalian erythrocytes, gastric mucosa and renal tubular epithelium, where it functions centrally in pulmonary carbon dioxide exchange, hydrochloric acid secretion and sodium ion reabsorption, respectively. Bovine carbonic anhydrase is a relatively small metalloprotein of molecular weight 30,000,[10] with most probably one zinc atom per molecule of protein.[11] Little is known about the physical properties of the enzyme molecule.

The kinetics of the carbonic anhydrase reaction have been previously investigated by gasimetric methods.[12–15] The use of concentrated buffers with profound effects on the catalysis, serious diffusion approximations in the face of the rapidity of the chemical reaction and marked, variable denaturation of the enzyme attendant on the vigorous shaking of the reaction vessel make the interpretation of these investigations difficult. Reports[16,17] of the use of electrometric methods in the study of the carbonic anhydrase reaction have been made, but the observations were not extended. A method based on an electrical measurement of the rate of hydration of carbon dioxide seemed to be the most feasible in being rapid, sensitive and more nearly free of serious approximations. An electrometric method with automatic recording features was developed, and the results of the investigation are reported below.

Experimental Details

The enzyme was obtained from human erythrocytes,[18] freshly provided by normal donors. The sedimented red blood cells were washed several times with 0.15 *M* sodium chloride and lysed with distilled water. The hemoglobin was denatured by chloroform and ethanol and separated by centrifugation. The supernatant aqueous phase was separated from the chloroform–alcohol in a separatory funnel,

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(2) G. B. Kistiakowsky and A. J. Rosenberg, THIS JOURNAL, **74**, 5020 (1952).

(3) G. B. Kistiakowsky and W. E. Thompson, *ibid.*, **78**, 4821 (1956).

(4) R. A. Alberty, V. Massey, C. Frieden and A. R. Fuhlbrigge, *ibid.*, **76**, 2485 (1954).

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(7) B. Chance in "The Enzymes," Vol. II, J. B. Sumner and K. Myrback, eds., Academic Press, Inc., New York, N. Y., 1951, pp. 428–453.

(8) H. Theorell, A. P. Nygaard and R. Bonnichsen, *Acta Chem. Scand.*, **9**, 1148 (1955).

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(11) D. A. Scott and J. R. Mendive, *ibid.*, **140**, 445 (1941).

(12) N. U. Meldrum and F. J. W. Roughton, *J. Physiol.*, **80**, 143 (1933).

(13) F. J. W. Roughton and V. H. Booth, *Biochem. J.*, **40**, 309 (1946).

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

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(16) W. C. Stadie and H. O'Brien, *J. Biol. Chem.*, **103**, 521 (1933).

(17) K. Wilbur and N. G. Anderson, *ibid.*, **176**, 147 (1948).

(18) Kindly given to the author by Dr. James Tullis of the Blood Characterization and Preservation Laboratory, Harvard University.

dialyzed exhaustively against distilled water and fractionated at 0° according to the second procedure of Keilin and Mann.[19] The resultant concentrated enzyme stock solution was stored at 0–3° with only slight change in activity, as will be noted below, over the course of 2.5 years. Each aliquot of enzyme stock solution for the separate investigations to be reported remained constant in activity throughout its use, for periods as long as one year. A progressive increase in concentration of the active form of the enzyme during the fractionation was demonstrated by activity measurements and by determination of zinc concentration.[20] Assuming the reasonable value of a single atom of zinc per molecule of enzyme, as has been determined for bovine carbonic anhydrase,[11] the stock solution contained 2.7×10^{-4} mole of enzyme per liter. No further investigation of the enzyme solution was undertaken apart from the activity studies, because of the paucity of information about the molecular properties of carbonic anhydrase.

The substrate solution of known concentration (calculated from Henry's law) was obtained by bubbling purified carbon dioxide (Liquid Carbonic Corp., Cambridge, Mass.) from a cylinder through a vessel of distilled, ion-exchanged water thermostated at the reaction temperature with a constant $p\mathrm{CO}_2$ maintained above the solution. The carbon dioxide solution flask was connected through a stopcock to a glass syringe which was outside the thermostat. The syringe, through another stopcock and a coil of glass tubing beneath the surface of the bath, communicated with a stainless steel hypodermic needle immersed into the reaction vessel.

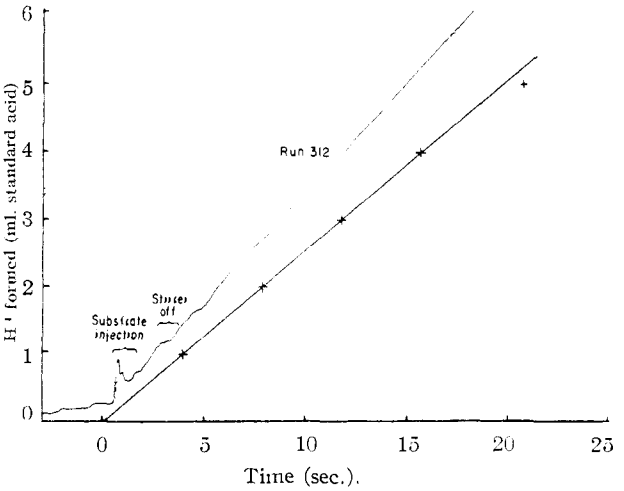

Fig. 1.—A typical experimental run at pH 7.00 and 1.5°: substrate concentration 3.78 mM; enzyme concentration 2.7×10^{-8} M; 0.002 M phosphate buffer; added ionic strength 0.001 NaCl; full scale deflection 20 mv. The experimental curve is reduced by calibration with 0.00986 M HCl to a plot of ml. standard acid formed against time, from which the slope yields the initial rate, in this case 2.52 μM sec.$^{-1}$.

All water was distilled and then passed over a mixed column of Dowex-50 and Amberlite IRA-400 exchange resins to free it of metal ions, ammonium ion and other possible interfering substances. All glassware was cleaned in a hot nitric–sulfuric acid bath, washed ten times with distilled water and three times with distilled, ion-exchanged water.

The inorganic chemicals were Merck Reagent, Mallinckrodt Analytical Reagent, and Baker Analyzed, C.P. grade, employed without further purification. The buffer for all experimental mixtures was 0.002 M potassium phosphate. The pH of the solutions was measured in a Beckman model G pH meter and found to be 7.00 ± 0.02 at the experimental temperature. The experiments were conducted in a well-stirred thermostat at 1.50 ± 0.05°.

The reaction cell was a round-bottomed Pyrex cylinder of about 175-ml. capacity, mounted in the bath so that the solution was well below the surface of the thermostating mixture. The volume of each experimental mixture was 100 ml. Stirring of the solutions was provided by a polyethylene-enclosed permanent magnet driven externally.

The measurement apparatus was essentially a very rapid and sensitive automatic recording pH meter. The changes of hydrogen ion activity of the solutions were measured by glass and calomel electrodes (Beckman Instrument Co., Nos. 1190-80 and 1170, respectively). The impedance of the glass electrode circuit was reduced by an FP-54 electrometer tube in the Du Bridge–Brown circuit, with an attendant voltage amplification factor of about 1/3. To use the Du Bridge–Brown circuit at its balance point, the calomel electrode was connected to one of the e.m.f. terminals of a Type K potentiometer, the other being grounded and the galvanometer terminals being shorted. The potential between the glass electrode and the calomel electrodes could thus be compensated by suitably altering the setting of the Type K potentiometer. The output of the Du Bridge–Brown circuit could be fed either to a box type galvanometer, for preliminary balancing, or to a Model S Speedomax Indicating Recorder Type G (Leeds and Northrup Co.). This instrument is of a constant speed type, requiring two seconds to traverse the chart; it has an input impedance of 0.5 Megohm and a sensitivity corresponding to 20 mv. for full scale (about 25 cm.) deflection. Because of the low voltage amplification factor of the Du Bridge–Brown circuit, 20 mv. correspond to about one unit on the pH scale. The relevant portions of an experiment extended over not more than 0.2 pH unit and the constant speed of the recorder permitted them to be recorded with an effective time constant of about 0.2–0.4 second.

All constituents of the experimental solution except the substrate were mixed in the reaction vessel and allowed to come to the proper temperature with the magnetic stirrer on. In the meantime, the potentiometer and the amplifying circuit were balanced, the appropriate volume of the substrate solution drawn up into the syringe from the generating vessel and the automatic recorder allowed to warm up. After the balancing of the initial signal from the pH 7.0 buffer with the slidewire of the potentiometer, the substrate solution was injected rapidly by the syringe, and, after *ca.* one to two seconds (found empirically to be more than adequate for complete mixing), the magnetic stirrer was stopped. The change in voltage of the glass electrode as the reaction proceeded, a direct function of the activity of the hydrogen ion produced by the reaction sequence

$$\mathrm{CO_2} + \mathrm{H_2O} \longrightarrow \mathrm{H^+} + \mathrm{HCO_3^-} \qquad (1)$$

was recorded directly and graphically on the Speedomax Recorder. At no time was the response of the glass electrode, found by Chance and Love[21] to be about 10 milliseconds, a limiting factor. The 0.002 M phosphate buffer concentration was chosen in order to make conveniently measurable the pH changes in the initial stages of the reaction. Since the change in e.m.f. is not linearly related to the moles of hydrogen ions generated in the reaction, experimental mixtures containing all ingredients except carbon dioxide were titrated with a standard acid, the resulting changes of e.m.f. being recorded by the Speedomax. These calibrations were then used to convert the e.m.f.–time curves of enzymatic and non-enzymatic runs with carbon dioxide into hydrogen ion concentration–time curves. It was found that except for slight disturbances at the start of the run lasting no more than 1 second, linear initial slopes were obtained over a range of at least 0.1 pH unit, after conversion to the hydrogen ion concentration scale. This linear rate was maintained for at least 5 to 10 seconds in enzymatic runs, depending on the concentration of carbon dioxide. The slope of the straight lines was taken as the rate of hydration. Figure 1 shows a typical recorder trace and its conversion to the hydrogen ion concentration scale.

While no comparison studies were made with the gasimetric method, the absence of significant glass electrode effects is strongly suggested by the self-consistency of the data to be reported, the linear relationship between enzyme concentration and activity (Fig. 4), and the maintenance

(19) D. Keilin and T. Mann, *Biochem. J.*, **34**, 1163 (1940).

(20) Kindly performed by Dr. Bert Vallee, Dept. of Biophysics, Peter Bent Brigham Hospital, Boston, Mass., by an ashing procedure followed by dithizonium complexing and spectrophotometric analysis.

(21) B. Chance, personal communication.

of the linear initial rate over long periods in individual rate studies without fall-off (*e.g.*, Fig. 1).

The over-all error of the method is approximately ±5–10%. The errors due to variability of temperature control, pipetting and the interpolation of voltage and time on the recorded plot were less than ±1%. The chief errors were introduced by variations in the delivery of substrate, voltage artifacts in the initial phases of the reaction due both to streaming effects during the delivery of substrate and instability of the calomel electrode fiber and to occasional personal and instrumental inconstancies.

Results

The Non-enzymatic Reaction.—The non-enzymatic hydration of carbon dioxide has been intensively studied by several investigators.[22–25] Faurholt proposes that at *p*H 7, at which all the experiments herein reported were conducted, the reaction sequence is

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \quad (2)$$

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \quad (3)$$

The hydroxyl ion catalyzed reaction does not become significant until above *p*H 8. Reactions 2 and 3 are extremely rapid and must be accounted for in studying the enzymatic rate. It was therefore necessary to measure this rate over the whole experimental range of substrate concentrations. In Fig. 2, the rates of uncatalyzed hydration of carbon dioxide at various carbon dioxide concentrations are presented and, as anticipated, show that the reaction is linear in carbon dioxide concentration. The data have been corrected for a small constant drift inherent in the apparatus. The slope of this plot gives directly the specific rate constant for the uncatalyzed hydration of carbon dioxide. This has the value of 1.87×10^{-3} sec.$^{-1}$, which is very close to the value of 2×10^{-3} sec.$^{-1}$ previously obtained[23,25] at approximately the same temperature and *p*H, by extrapolation from experimental values obtained at higher activating buffer concentrations.

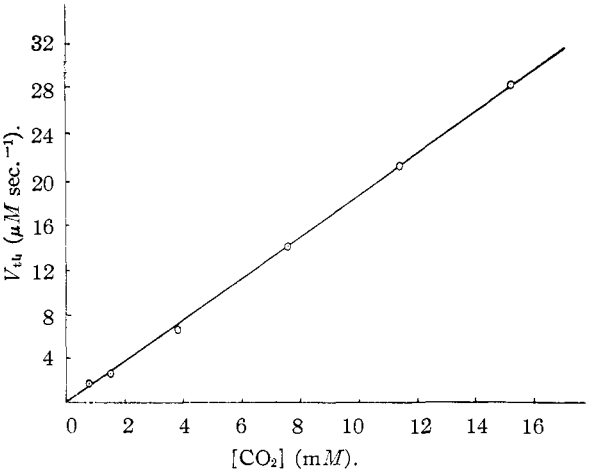

Fig. 2.—The non-enzymatic hydration of carbon dioxide at *p*H 7.00 in 0.002 *M* phosphate buffer at 1.5°, a plot of rate of hydration against CO_2 concentration.

The Enzyme-catalyzed Reaction.—The data for the carbonic anhydrase catalyzed hydration of carbon dioxide, obtained by subtracting the uncatalyzed rate from the total measured rate at each substrate concentration, are presented in Fig. 3 (open circles) plotted according to the method of Lineweaver and Burk.[26] It is evident from the linear relationship between the reciprocals of reaction velocity and substrate concentration that the carbonic anhydrase catalysis precisely follows the classical Michaelis–Menten mechanism

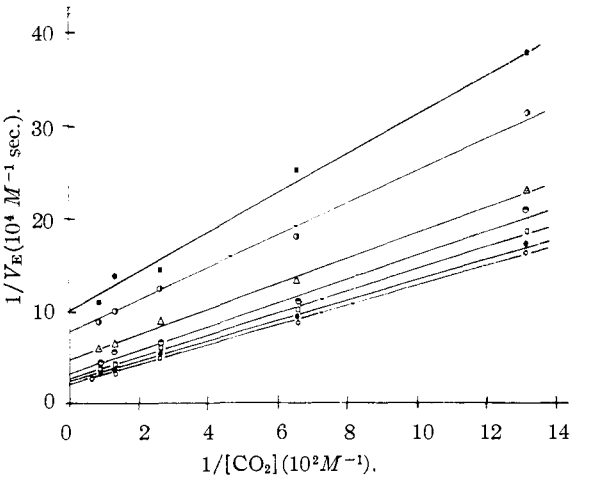

Fig. 3.—The enzymatic hydration of carbon dioxide at *p*H 7.00 in 0.002 *M* phosphate buffer at 1.5°, a plot of inverse rate against inverse CO_2 concentration at various ionic strengths of added salts: ○, no added salt; ●, 0.001 NaCl; □, 0.0015 Na_2SO_4; ◓, 0.002 KNO_3; ▲, 0.01 NaCl; ◑, 0.02 KCl; ■, 0.05 NaCl.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \quad (4)$$

where E is the enzyme; S, the substrate (in this case, carbon dioxide); ES, the enzyme–substrate compound; and P, the products of the reaction (H^+ and HCO_3^-). According to this mechanism, the velocity of the reaction is simply

$$v = -\frac{d(CO_2)}{dt} \cong +\frac{dH^+}{dt} = \frac{k_3(E)(S)}{(S) + (K_m)} \quad (5)$$

where k_3 is the rate constant for the evolution of products from the enzyme–substrate compound, and where the Michaelis constant is

$$K_m = \frac{k_2 + k_3}{k_1} \quad (6)$$

The Michaelis constant of the stored enzyme stock solution was found to decrease about 20% over the course of two years but was constant for each preparation throughout its use. The decrease in K_m was shown to be due to an increase in "specific activity" of the enzyme preparation but was not investigated further, inasmuch as the catalytic behavior of all the preparations was identical. This increase in activity of the enzyme, attended by the slow formation of a precipitate in the stock solution, was tentatively attributed to possible denaturation of a protein impurity, possibly complexed to the

(22) C. Faurholt, *J. chim. phys.*, **21**, 400 (1924).

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

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(25) M. Kiese and A. B. Hastings, *J. Biol. Chem.*, **132**, 267 (1940).

(26) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

TABLE I

RATE OF ENZYMATIC HYDRATION OF CARBON DIOXIDE (μM SEC.$^{-1}$) AT VARIOUS IONIC STRENGTHS AND AT pH 7.00 IN 0.002 M PHOSPHATE BUFFER

Enzyme concentration 2.7×10^{-8} M

CO_2, M	Added salt (μ) None	0.001 NaCl	0.0015 Na_2SO_4	0.002 KNO_3	0.01 NaCl	0.02 KCl	0.05 NaCl
7.57×10^{-4}	6.21	5.86	5.43	4.80	4.42	3.25	2.58
15.1×10^{-4}	11.6	10.9	9.83	9.24	7.65	5.60	4.01
37.8×10^{-4}	20.4	17.9	16.5	15.1	11.3	8.01	6.96
75.7×10^{-4}	28.7	28.0	24.6	17.6	15.6	9.92	7.20
11.4×10^{-3}	...	29.7	27.4	23.8	16.8	11.3	9.06
15.1×10^{-3}	36.8	...	...	...	...	...	..
K_m(mM)	5.22	4.62	4.28	3.90	2.82	2.20	2.08
V_{max} (10^{-5} M sec.$^{-1}$)	4.94	4.26	3.70	3.12	2.10	1.29	1.00

enzyme. The Michaelis constant for enzyme preparation no. 4, which was used for the major portion of the investigations reported herein, was $5.2 \times 10^{-3} M$.

Roughton and Booth[27] found some variability in the dependence of reaction rate on enzyme concentration in their preparations. This relationship proves to be linear for human erythrocyte carbonic anhydrase, as shown in Fig. 4, suggesting a high degree of purity for the enzyme preparation.

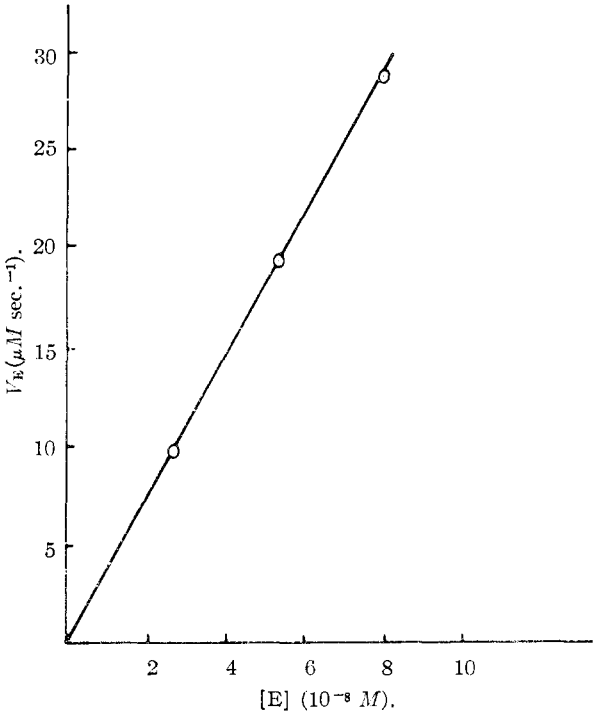

Fig. 4.—The dependence of reaction rate on enzyme concentration at pH 7.00 and 1.5° in 0.002 M phosphate buffer. A plot of reaction rate against enzyme concentration. Substrate concentration 3.78 mM.

The Effect of Neutral Salts on the Enzyme-catalyzed Reaction.—Roughton and Booth[27] have pointed out that neutral salts are profoundly inhibitory to carbonic anhydrase, but knowledge of the mechanism of this inhibition has been totally lacking. Investigation of the inhibition of human erythrocyte carbonic anhydrase by various neutral salts, the data for which are presented in Table I and Fig. 3, demonstrates that salts produce no change in the mechanism of catalysis of carbon dioxide hydration. The mechanism remains that of Michaelis and Menten even at high ionic strengths. There is, indeed, a lack of specificity of this inhibition by NaCl, KCl, KNO_3 and Na_2SO_4, suggesting that the ionic strength effect is due to change in the activity coefficients of ionic participants in the reaction, not due to specific anionic or cationic effects. A test of this hypothesis by the Brønsted-Bjerrum relationship[28,29] (Fig. 5) bears

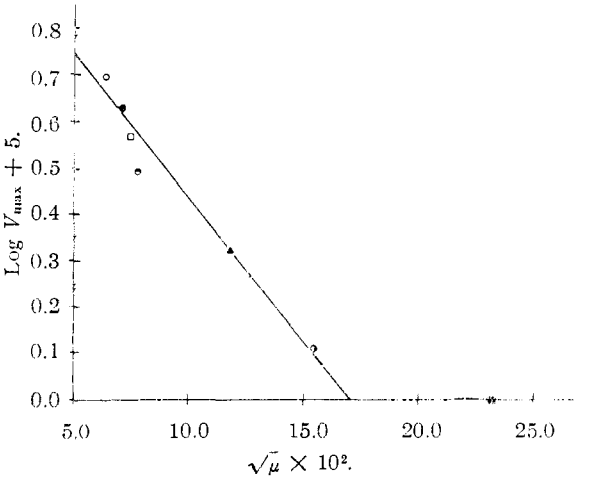

Fig. 5.—The dependence of k_3 of carbonic anhydrase on ionic strength, a plot of the logarithm of the maximum velocity against the square root of the total ionic strength (buffer ionic strength plus added salt). The line conforms to log $V_{max} = -6.2 \sqrt{\mu}$ + constant.

out this hypothesis. Similar behavior has been described previously for urease.[30] The hypothesis is further strengthened by the observation that the apparent K_m and V_{max} for solutions 0.0005 M in Na_2SO_4 are those of a salt solution of ionic strength 0.0015.

Discussion

The direct measurement of the formation of hydrogen ion from the hydration of carbon dioxide by means of an automatic recording apparatus has permitted the study of the uncatalyzed and enzyme-catalyzed reaction in an unambiguous

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

(28) J. N. Brønsted, *Z. physik. Chem.*, **102**, 169 (1922).

(29) N. Bjerrum, *ibid.*, **108**, 82 (1924).

(30) G. B. Kistiakowsky, P. C. Mangelsdorf, Jr., A. J. Rosenberg and W. H. R. Shaw, THIS JOURNAL, **74**, 5015 (1952).

way, free from all the accessory assumptions involved in the gasimetric studies by Roughton[13] and by Kiese.[14] The method, further, enables the study of the hydration reactions at buffer concentrations sufficiently low to free the rate of specific buffer activations, which have been demonstrated[23,25] for uncatalyzed carbon dioxide hydration. The high rate of the thermal (non-enzymatic) reaction necessitates its consideration in the study of the enzymatic reaction. The direct proportionality of the reaction rate to the enzyme concentration, albeit investigated over a relatively narrow range, suggests that the enzyme preparations behaved kinetically in an unambiguous fashion. The failure of Roughton's enzyme preparation to behave in this kinetically "pure" way renders very difficult any kinetic interpretation of his data,

The behavior of carbonic anhydrase in strict Michaelis–Menten kinetics is indeed fortunate for the interpretation of further kinetic studies. Detailed understanding of some aspects of the kinetics of urease[2,3] and of fumarase,[4] for example, has been hampered by the complexity of the kinetic mechanism. Carbonic anhydrase, on the other hand, a small and relatively "simple" protein, behaves kinetically in a "simple" way.

The inhibition of carbonic anhydrase by neutral salts permits a complete analysis of the Michaelis–Menten kinetics. The Michaelis constant K_m and the maximum velocity V_{max} were both found to decrease with increasing ionic strength. K_m, in fact, was found to be a linear function of V_{max} (Fig. 6). This effect cannot be ascribed to an effective decrease in enzyme concentration by "salting out," for then K_m would remain unchanged.

In considering all other possible modes of inhibition

$$E + nI \underset{k'_1}{\overset{k_1}{\rightleftarrows}} EI_n \text{ with an affinity constant } K_i = \frac{k_i}{k_i'}; \quad (7)$$

$$ES + mI \underset{k_{ii}'}{\overset{k_{ii}}{\rightleftarrows}} ESI_m \text{ with an affinity constant } K_{ii} = \frac{k_{ii}}{k_{ii}'} \quad (8)$$

the reaction velocity is

$$v = \frac{k_3(E_0)(S)}{1 + K_{ii}(I)^m} \Big/ \left[\left(\frac{k_2 + k_3}{k_1}\right)\left(\frac{1 + K_i(I)^n}{1 + K_{ii}(I)^m}\right) + (S)\right] \quad (9)$$

and

$$V_{max} = \frac{k_3(E_0)}{1 + K_{ii}(I)^m} \quad (10)$$

and

$$K_m = \frac{k_2 + k_3}{k_1}\left[\frac{1 + K_i(I)^n}{1 + K_{ii}(I)^m}\right] \quad (11)$$

where the k's and K's, but not E_0, may be functions of ionic strength.

By calling to mind that

$$\text{Obsd.: } \log V_{max} = -6.2\sqrt{\mu} + \log V^0_{max} \quad (12)$$

and

$$\text{Obsd.: } K_m = a + bV_{max}, \text{ from (10), (11) and (13)} \quad (13)$$

$$\frac{k_2}{k_1} + \frac{k_3}{k_1} = \frac{a}{\left[\frac{1 + K_i(I)^n}{1 + K_{ii}(I)^m}\right]} + \frac{bk_3(E_0)}{1 + K_i(I)^n} \quad (14)$$

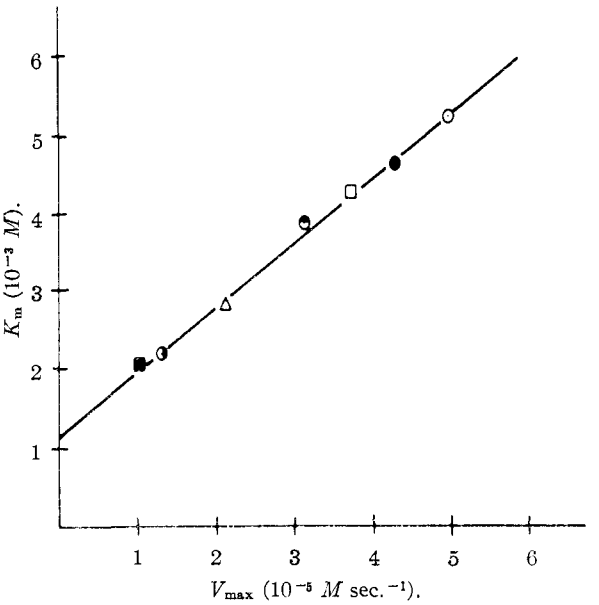

Fig. 6.—The dependence of the Michaelis constant of carbonic anhydrase on k_3 at various ionic strengths and at pH 7.00 in 0.002 M phosphate buffer at 1.5°. A plot of the Michaelis constant against the maximum velocity at each ionic strength; enzyme concentration 2.7×10^{-8} M; added ionic strengths: ⊙, no added salt; ●, 0.001 NaCl; □, 0.0015 Na_2SO_4; ◓, 0.002 KNO_3; ▲, 0.01 NaCl; ◑, 0.02 KCl; ■, 0.05 NaCl.

three alternative solutions are evident: (I) k_2/k_1 may be identified with the first term in (14); k_3/k_1 with the second term. (II) k_2/k_1 may be identified with the second term. k_3/k_1 with the first term. (III) Neither k_2/k_1 nor k_3/k_1 may be identified with either term of (14).

Considering alternative I

$$\frac{k_2}{k_1} = a\left[\frac{1 + K_{ii}(I)^m}{1 + K_i(I)^n}\right] \quad (15)$$

Since k_2/k_1 is the true equilibrium constant for E + S $\rightleftarrows$ ES, it must follow that

$$K_{ii}(I)^m = K_i(I)^n \quad (16)$$

This would lead to a non-competitive inhibitory mechanism, with K_m invariant with μ, unless

$$K_{ii} = K_i = 0 \quad (17)$$

Under these circumstances, which must obtain here

$$\frac{k_2}{k_1} = a \text{ and } \frac{k_3}{k_1} = bk_3(E_0), \text{ or} \quad (18)$$

$$k_1 = \frac{1}{b(E_0)}, \; k_3 = \frac{V_{max}}{(E_0)}, \; k_2 = \frac{a}{b(E_0)} \quad (19)$$

Considering alternative II

$$\frac{k_3}{k_1} = a\left[\frac{1 + K_{ii}(I)^m}{1 + K_i(I)^n}\right] \text{ and } \frac{k_2}{k_1} = \frac{bk_3(E_0)}{1 + K_i(I)^n} \quad (20)$$

Since, again, k_2/k_1 is an equilibrium constant

$$K_i = 0 \text{ and} \quad (21)$$

$$\frac{k_2}{k_1} = bk_3(E_0) \quad (22)$$

Since dependence on ionic strength has been shown not to exist other than

$$\log k_3 = -6.2\sqrt{\mu} + \log k_3^0 \quad (23)$$

the unreasonable situation would now arise that k_2/k_1, a true thermodynamic constant, would be a function of μ and the transition complex of the reaction would also vary with the ionic strength. These are indeed not likely, and alternative II, then, must be rejected.

Considering alternative III, here, neither k_2/k_1 nor k_3/k_1 can be identified with either term of (14). There are an infinite number of solutions, all of which require k_2/k_1, a true equilibrium constant, to be a function of K_i and K_{ii}, and all of which are without physical or *a priori* reasonableness within the constraints (12) and (13) above, unless $K_i = K_{ii} = 0$.

Under these circumstances

$$\frac{k_2}{k_1} + \frac{k_3}{k_1} = a + bk_3(E_0) \quad (24)$$

$$\frac{k_2}{k_1} = F_1 + f_1k_3(E_0) \quad (25)$$

and

$$\frac{k_3}{k_1} = F_2 + f_2k_3(E_0) \quad (26)$$

such that

$$F_1 + F_2 = a \text{ and } f_1 + f_2 = b \quad (27)$$

although F_1, F_2, f_1 and f_2 may individually be functions of ionic strength. Under these circumstances, alternative III like alternative II requires k_2/k_1 to be a function of k_3 and must also be rejected.

We are then left with the very strongly plausible conclusion, not binding mathematically but physically almost so, that alternative I is correct, *viz.*

In eq. 13

$$\frac{k_2}{k_1} = a \quad \frac{1}{k_1(E_0)} = b \quad (28)$$

so that

$$k_1 = \frac{1}{b(E_0)}, \; k_2 = \frac{a}{b(E_0)} \quad (29)$$

Under these circumstances, the ionic strength effect is solely on k_3. This is indeed highly plausible, physically.

The starting reagents for the catalyzed reaction at *p*H 7 are carbon dioxide and water. It is conceivable that the contribution by the charge of the enzyme or water to the partition functions of the transition complex and the starting reagents could cancel in an absolute rate expression. If the products of the reaction were HCO_3^- and H^+, both charged, an ionic strength effect on the rate of breakdown of the enzyme–substrate complex, *i.e.*, k_3, would be quite expected. That the ionic strength effect is indeed solely on k_3 does suggest that the direct product of the enzyme-catalyzed reaction is most probably bicarbonate ion, in contrast to the uncatalyzed reaction, where H_2CO_3 is the chief product at *p*H 7.

Since the enzyme concentration is known from the zinc concentration, utilizing the slope of Fig. 6 and equation 29 above, we can determine k_1 and, from the intercept of Fig. 6, k_2. The analytical separation of the rate constants of the Michaelis Menten mechanism by means of the ionic strength effect yields full quantitative knowledge of k_1, k_2 and k_3, all obtained from rate measurements in the steady state. The Brønsted–Bjerrum relationship, moreover, indicates the participation of 6 units of charge in the ionic strength effect.

The values for the specific rate constants are

$$k_1 = 0.46 \times 10^{-6} M^{-1} \text{ sec.}^{-1}$$
$$k_2 = 5.5 \times 10^2 \text{ sec.}^{-1}$$
$$k_3 = 18.3 \times 10^2 \text{ sec.}^{-1}$$

Also, interestingly, k_2 and k_3 are of the same order of magnitude, under which circumstances the Michaelis constant cannot be the association constant for the enzyme–substrate complex as Kiese[14] has assumed. It is, rather, a hybrid parameter without kinetic or thermodynamic meaning. The association constant for the enzyme–substrate complex is

$$K_a = 8.3 \times 10^2 M^{-1}$$

k_1 is quite large, being of the order of k_1 for catalase and methyl hydrogen peroxide at 25°, as very neatly determined by Chance[7] by direct measurement, and for the liver alcohol dehydrogenase reaction with DPNH, as measured in the steady state by Theorell, *et al.*[8] k_1 for carbonic anhydrase is not as large as estimated by Roughton and Clark,[31] who utilized a calculation based on an approximate enzyme concentration of an admittedly impure and kinetically ambiguous preparation. The Michaelis constant determined by Roughton and Booth[27] for carbonic anhydrase is approximately twice that determined herein. The difference in the two values is easily accountable on the basis of the lack of purification of their preparation and some of the errors of their method.

Kiese's study of highly purified horse erythrocyte carbonic anhydrase by the gasimetric method[14] gave a value for K_m of *ca.* 1.5×10^{-3} *M*, recalculated from his data by the Lineweaver–Burk representation. The ionic strength of Kiese's experimental mixture was approximately 0.08, at which, from Fig. 6, carbonic anhydrase should have an "apparent K_m" roughly that which Kiese's data give. His value for k_3, corrected to *p*H 7, is approximately five times the value reported here, a close approximation considering the possibility of species differences and the errors inherent in enzyme studies.

It has, thus, been possible to identify quantitatively the specific rate constants for carbonic anhydrase by means of the non-specific ionic inhibition which obeys the Brønsted–Bjerrum relationship without the uncertainty inherent in the measurements of inhibition constants necessary for a full evaluation of the kinetics of fumarase.[9] The constants are known from rate measurements in the steady state alone and permit a reconciliation of the previously disparate data on this enzyme.

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