The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. III. Kinetic Studies of the Enzyme-Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate*

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ABSTRACT: Erythrocyte carbonic anhydrase is a powerful catalyst for the reversible hydration of carbon dioxide, as well as of acetaldehyde and related carbonyl compounds. The present investigation demonstrates that erythrocyte carbonic anhydrase is an effective catalyst for the hydrolysis of *p*-nitrophenyl acetate. A detailed pH–rate profile as obtained at 25.0° over the pH range 6.0–10.5 shows a point of inflection at pH 7.5. Lineweaver–Burk reciprocal plots indicate that both apparent K_m and V_m vary with pH. A more detailed treatment of the data shows that the apparent binding, k_1/k_{-1}, is essentially constant over this pH range, while the turnover number, k_2, varies as if full activity is dependent on the basic form of a group having a pK_a of 7.5. Lineweaver–Burk reciprocal plots characterize the salt (anionic) inhibition as noncompetitive. The inhibitory effectiveness, $CN^- \gg SCN^- \simeq N_3^- > I^- > HCO_3^- > NO_3^- > Br^- > AcO^- > Cl^- > F^-$, parallels in essence that observed in hydrase activity. The enzyme forms 1:1 complexes with these anions which are inactive with respect to esterase activity, but still retain their original capacity to bind *p*-nitrophenyl acetate. The various anionic inhibitors act at a site which behaves like an electrophilic center of p$K_a \simeq 7.5$; the loss of a proton from this center reduces but does not abolish the inhibitory effect of these anions. It is suggested that these anions bind at or near a site associated with the hydrolysis process. Both acetonitrile and acetazolamide were also found to be noncompetitive inhibitors. The inhibitory capacity of the former is low, lower even than that of F^-, while that of the latter is higher than that of CN^-. A comparison of these observations with those found in hydrase activity suggests that the hydrating and hydrolyzing sites in the enzyme are similar and that a detailed study of esterase activity should provide an important avenue for the elucidation of the mode of action of carbonic anhydrase.

It was long thought that erythrocyte carbonic anhydrase[1] possessed the remarkable property of absolute specificity with respect to the reversible hydration of carbon dioxide (Davis, 1961; White *et al.*, 1964). Recently the horizons of hydrase activity were extended to include other substrates such as acetaldehyde and related carbonyl systems (Pocker and Meany, 1964, 1965a,b, 1967a,b; Pocker *et al.*, 1965). The similarity between aldehyde hydration (Pocker, 1960) and certain bimolecular mechanisms of ester hydrolysis involving tetrahedral intermediates (Bender, 1951, 1953) led us to explore the possibility that carbonic anhydrase might possess esterase activity (Pocker *et al.*, 1965; Pocker and Stone, 1965a,b). Other investigators have independently tested for this activity (Tashian *et al.*, 1964; Lieflander and Schneider, 1963; J. T. Edsall and J. A. Verpoorte, 1966, personal communication; Malmström *et al.*, 1964; K. W. Lo and E. T. Kaiser, 1966, personal communication). In this paper we present a detailed study of the esterase activity of carbonic anhydrase using *p*-nitrophenyl acetate as substrate. Further detailed studies on the esterase action of bovine carbonic anhydrase using related substrates as well as carbonate esters are under active investigation and will be reported later (Y. Pocker, D. R. Storm, L. J. Guilbert, and J. T. Stone, 1966, unpublished data).

In contrast to the reversible hydration of CO_2 which is generally followed by measuring the rate of pH change of a weakly buffered reaction mixture, the carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl acetate has many distinct advantages. The ester is easily handled, hydrolyzes irreversibly at convenient rates, and allows precise spectrophotometric determinations to be performed at constant pH.

Experimental Section

Materials. *p*-Nitrophenyl acetate (Aldrich) was recrystallized from anhydrous diethyl ether to a constant melting point of 79–80°. *p*-Nitrophenol (Matheson) was recrystallized from water and sublimed,

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[1] Abbreviations used: CA, carbonic anhydrase; BCA, bovine carbonic anhydrase; *p*-NPA, *p*-nitrophenyl acetate.

mp 112°. The molar extinction coefficient of the *p*-nitrophenolate ion at 400 mμ was found to be ε 18,400, a value which accords with previous data (Kézdy and Bender, 1962; Gibbons and Edsall, 1963). Acetonitrile (Baker Analyzed), used to prepare stock solutions of *p*-NPA, was dried by azeotropic distillation with methylene chloride and fractionated.

Buffer components included potassium dihydrogen phosphate, dipotassium hydrogen phosphate, imidazole, and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), and were analytical grade reagents. Buffers containing the mono- and dianions of diethylmalonic acid ($pK_2 = 7.3$) were kindly supplied by Mr. D. G. Dickerson; the synthesis of the corresponding acid was described earlier (Pocker and Meany, 1965b, 1967a,b). We find diethylmalonate buffers to be very suitable in the control of pH since their catalytic efficiency appears to be considerably lower than that of any other buffers of around physiological pH. Sodium chloride and other sodium salts were of reagent grade quality, as was zinc nitrate.

Bovine carbonic anhydrase prepared and purified by the method of Keilin and Mann (1940) from bovine erythrocytes was obtained from Mann Research Laboratories. Two samples of bovine carbonic anhydrase (BCA_I and BCA_{II}) were used. Although similar, the experimental results obtained from each were, nevertheless, kept separate so that each "family" of experiments involved the use of only one of these enzyme samples. These samples were stored dry at −20° and periodic checks of esterase activity revealed no change. Previously (Pocker and Stone, 1965a,b), the specific activity of bovine carbonic anhydrase referred to a known weight of the metalloenzyme. Since the 280-mμ absorbance is a much more reliable measure of the amount of active enzyme, the present results are based on this latter method. Recently, during the preparation of some highly purified bovine carbonic anhydrase, the specific activity of the original Mann preparation was found to be 75% of the purified form as judged by zinc content, by absorption measurements at 280 mμ, as well as by inhibition studies. Zinc ion content was determined using the dithizone method (Malmström, 1957) and flame photometry (λ 214 mμ). In making up solutions we found it easiest to calculate the enzyme concentration from ultraviolet absorption measurements at 280 mμ employing ε 54,000 and a molecular weight of 30,000. A DEAE-cellulose column was used to obtain highly purified bovine carbonic anhydrase similar to that employed earlier (Lindskog, 1960). Three fractions containing isozymes A–C were obtained in this separation. Fractions containing virtually pure A and B forms of bovine carbonic anhydrase were tested at pH 7.5 and found to possess essentially the same esterase activity.

Apparatus. All pH measurements were recorded with a glass electrode on a Beckman research pH meter at 25.0°. The relative accuracy of this pH meter is reported as ±0.001 pH unit. Spectrophotometric determinations were made on a Beckman Model DU 2 equipped with an insulated cell compartment consisting of a specially constructed bath thermostated to 25.00 ± 0.02° by means of a Sargent Model SV (S-82060) thermonitor.

Kinetics and Technique. Prior to dilution with acetonitrile, Tris buffers were 0.005–0.25 M in Tris and 0.01 M in HCl, while phosphate buffers were 0.0006–0.01 M in $H_2PO_4^-$ and 0.001–0.01 M in HPO_4^{2-}. Phosphate and Tris buffers were, in general, prepared according to Perrin (1963). All buffers were brought to an ionic strength, μ, of 0.10 M with added sodium chloride.

A typical procedure for a kinetic run was to initiate the reaction by injecting 0.3 ml of acetonitrile stock solutions of *p*-NPA, by means of a calibrated Yale syringe, into 2.7 ml of appropriate buffer, with or without bovine carbonic anhydrase. The final solution was 10% (v/v) in acetonitrile and ionic strength 0.09. In addition to increasing the ester solubility, the above procedure had the advantage that stable stock solutions of the substrate in dry acetonitrile could be prepared and used for an extensive series of runs.

The pH values for the buffers containing 10% (v/v) acetonitrile were recorded. It was found that identical pH values are obtained when the above buffers are diluted 9:1 with either water or acetonitrile.

The hydrolysis of *p*-NPA was followed spectrophotometrically by monitoring the appearance of *p*-nitrophenolate anion primarily at its peak absorbance at 400 mμ. When high concentrations of *p*-NPA were employed, the appearance of *p*-nitrophenolate ion was monitored at higher wavelengths, primarily at 450 and 465 mμ. It was found that Beer's law was obeyed and that the rate of disappearance of *p*-nitrophenyl acetate was equivalent to the rate of appearance of *p*-nitrophenolate anion at the same pH. Measurements were also carried out at the isosbestic point for *p*-nitrophenol–*p*-nitrophenoxide, λ 348 mμ (Bergmann *et al.*, 1958). Although the molecular extinction and, hence, the sensitivity at this latter wavelength is not as high as at 400 mμ, and because one must also introduce a small correction for the absorption of *p*-NPA itself, such measurements have the advantage that the proportionality factor between the measured absorption and the amount of ester hydrolyzed is independent of pH.

Pseudo-first-order coefficients were obtained by plotting $-\log (A_\infty - A_t)$ *vs.* time, where A_∞ was the experimentally determined absorbance observed after roughly 10 half-lives. When the substrate concentration was high, A_∞ was calculated from the molar absorbance of *p*-nitrophenol previously sublimed and measured at the same pH, ionic strength, and solvent composition as the initial reaction mixture. For low substrate concentrations the plots were linear for at least 2 half-lives and the pH of the buffered medium remained essentially unchanged. For high substrate concentration, initial rates were deduced, because even after 1 half-life time the pH changes accompanying the hydrolysis were significant. The reaction was found to proceed to completion (>99% hydrolysis). The enzymatically catalyzed component

was separated from that accelerated by the various acidic and basic species present in the reaction media using the equation $k_{enzyme} = [k_{obsd} - k_{buffer}](1/[E])$, where $k_{buffer} = k_0 + k_{H_3O} + [H_3O^+] + k_{OH^-}[OH^-] + k_{HA}[HA] + k_B[B]$. One of the attributes of the present reaction is that the enzyme coefficient k_{enzyme} is much larger than that associated with the various buffer components.

For the determination of a pH–rate profile, substrate concentration was held constant at 5.0×10^{-5} M, enzyme concentration was approximately 3×10^{-6} M, and ionic strength and salt concentration were held at 0.09 M. A minimum of three duplicate runs were performed at each pH and the mean value used. In the determination of K_m and V_m at various pH values, 10–15 different substrate concentrations, ranging from 10^{-5} to 5×10^{-3} M, were employed.

Anionic inhibition was studied in Tris buffer of pH 7.55, at an ionic strength $\mu = 0.09$ composed of anion X^- held at a concentration of 0.081 M and of $[Tris \cdot H^+] = [Cl^-]$ held at 0.009 M. The enzyme concentration was held constant at 2.8×10^{-6} M. The following anions were used: fluoride, chloride, bromide, iodide, nitrate, acetate, bicarbonate, cyanide, thiocyanate, and azide. In this series of runs, K_m remained essentially constant so that these inhibitors are noncompetitive with respect to the esterase activity of bovine carbonic anhydrase. The inhibition constant, K_i, for the various anions was evaluated from Lineweaver–Burk plots (Laidler, 1958; Webb, 1963). For chloride ion, such plots were made at seven different pH values: 7.00, 7.55, 7.92, 8.45, 8.92, 9.33, and 10.48. In the pH region 7.00–8.45, K_i values were easily deduced from the above plots. At higher pH values, however, the determination of K_i is difficult because the inhibitory effectiveness of Cl^- is reduced and relatively high salt concentrations are required to induce significant inhibition. By avoiding very high salt concentrations, we were nevertheless able to obtain approximate values for K_i at higher pH values.

Sulfonamides, such as acetazolamide, powerfully inhibit the esterase activity of carbonic anhydrase but have little or no effect on other esterases. This distinguishing feature affords a suitable method for detecting carbonic anhydrase activity. Acetazolamide was used as a specific inhibitor in our studies; its inhibitory effectiveness was determined using 2.8×10^{-6} M bovine carbonic anhydrase and varying acetazolamide concentration from 0 to 40×10^{-6} M at pH 8.45 with μ and $[Cl^-] = 0.09$ M. Lineweaver–Burk plots obtained at seven different acetazolamide concentrations classify this inhibition as noncompetitive. The inhibition constant, K_i, was deduced from the equation $K_i = (E_0 - EI)(I_0 - EI)/(EI)$, where E_0 and I_0 are the total concentrations of enzyme and inhibitor, respectively, and EI is the concentration of the enzyme–inhibitor complex. The assumption that $[EI] = [E_0]((v_0 - v)/v_0)$, where v and v_0 are the enzymatic velocities with and without added inhibitor, was employed in the evaluation of [EI] and hence of K_i.

The implication of both zinc ion and the basic form of a group of pK_a around 7 (such as imidazole) in the active site of carbonic anhydrase prompted us to try to establish the catalytic effectiveness of zinc–imidazole complexes. Zinc nitrate (10^{-3}–10^{-2} M) was introduced into imidazole buffers (0.1 M) while maintaining the pH at 6.95 with added imidazole. The catalytic activity of these solutions was then determined using *p*-nitrophenyl acetate. The stoichiometry of the zinc–imidazole interaction was also studied by following the pH changes associated with the addition of zinc ions to imidazole buffers (Edsall *et al.*, 1954). The catalytic activity of these latter solutions was also determined using *p*-nitrophenyl acetate as substrate.

Results

The spectrophotometric technique employed in determining the rate of *p*-NPA hydrolysis exhibits a reproducibility of $\pm 2\%$. The first-order plot of a typical run derived from such spectrophotometric data is exemplified in Figure 1. The enzymatic component of catalysis was separated from the nonenzymatic catalysis by working in a region where k_{obsd} is a linear function of enzyme concentration, the slope of k_{obsd} *vs.* [E] defining k_{enzyme}. Dilute buffers were used to maintain pH. Under these conditions the only significant contribution at pH>7.0 arose from hydroxide ion, $k_{OH^-} = 888$ min^{-1} M^{-1}. The other buffer components present are, indeed, catalysts (Y. Pocker and J. T. Stone, 1964, unpublished observations); however, their concentration in our experiments coupled with their relatively low catalytic coefficients (Bruice and Lapinski, 1958) was such that they rendered a negligible contribution to the observed rate.

In contrast to results obtained for the chymotrypsin-catalyzed hydrolysis of *p*-NPA (Hartley and Kilby, 1954), all plots of *p*-nitrophenol appearance *vs.* time for the esterase activity of bovine carbonic anhydrase pass through the origin. Thus an important feature of the latter rate plots is the absence of any *detectable* initial "bursts" of *p*-nitrophenol formation. With certain hydrolytic enzymes, a three-step mechanism is minimal for this substrate (eq 1) (Sturtevant, 1960).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P_1 + ES_1 \xrightarrow{k_3} P_2 + E \quad (1)$$

The conversion of ES into the acyl enzyme, ES_1, and *p*-nitrophenol, P_1, is considered to be faster than the hydrolysis of the acyl enzyme to form acetate, P_2, and regenerate enzyme, E. Under certain conditions ($K_{m(app)} > S_0 \simeq E_0$) the rate of appearance of *p*-nitrophenol may be represented by eq 2.

$$\frac{v_{enzyme}}{[S_0] - [P_1]} = (k_2/K_m)[E_0] + (k_2/K_m)[P_1] \quad (2)$$

Here $K_m = (k_{-1} + k_2)/k_1 = ((k_2 + k_3)/k_3)K_{m(app)}$,

where $K_{m(app)}$ represents the experimentally determined Michaelis constant, S_0 and E_0 are the initial concentrations of substrate and enzyme, respectively, v_{enzyme} is the enzymatic rate of *p*-nitrophenol production, and P_1 is the concentration of *p*-nitrophenol produced. For chymotrypsin a plot of $v_{enzyme}/(S_0 - P_1)$ *vs.* P_1 gives a straight line whose intercept divided by the slope is E_0 (Bender *et al.*, 1962). Under similar conditions, the bovine carbonic anhydrase catalyzed hydrolysis of *p*-NPA leads to a $v_{enzyme}/(S_0 - P_1)$ value which does not vary with P_1. The value of $v_{enzyme}/(S_0 - P_1)E_0$, obtained when $K_m > S_0 \simeq E_0$, is numerically the same as that obtained at low bovine carbonic anhydrase concentrations ($K_m > S_0 > E_0$) and equals k_2/K_m. We have also tried to detect a burst in the region where eq 3

$$\frac{P_b}{E_0} = \left(\frac{1}{1 + \frac{k_3}{k_2}}\right)^2 \Big/ \left(1 + \frac{K_{m\,app}}{S_0}\right)^2 \qquad (3)$$

with P_b being a measure of the burst, is applicable (Sturtevant, 1960; M. L. Bender, 1966, private communication). For eq 3 to hold and at the same time for a reasonable burst to be measured, the conditions $S_0 > E_0$, $S_0 > K_m$, and $k_2 > k_3$ must be fulfilled. For certain esterases the first two conditions may be experimentally adjusted; the third is satisfied if a burst does indeed exist. However, with our enzyme only condition $S_0 > E_0$ could be easily satisfied experimentally. The low solubility of the ester in 10% (v/v) acetonitrile imposed certain restrictions so that, under the most favorable conditions, $S_0 \simeq K_m$. Under these conditions no burst is observed. While the latter conditions are far from ideal for quantitatively measuring the size of the burst, they were, nevertheless, sufficient for detecting one, if present.

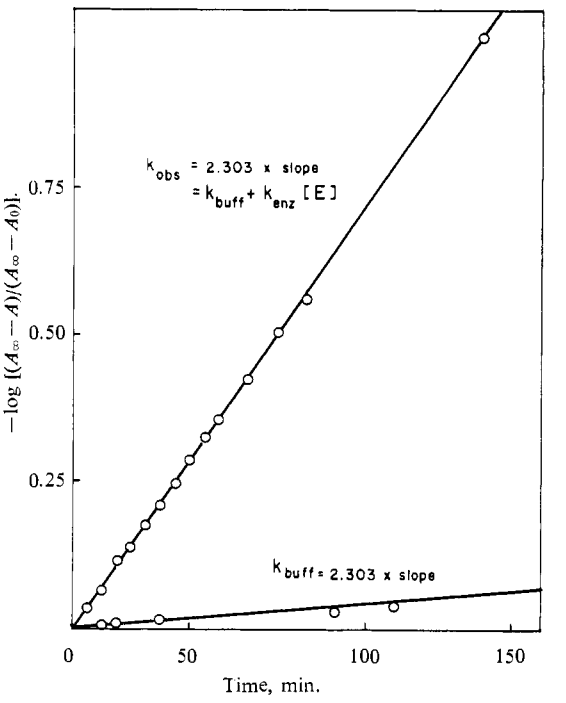


FIGURE 1: Typical first-order rate plots of *p*-NPA hydrolysis with and without BCA at 25.0°: [*p*-NPA] = 5×10^{-5} M, [BCA_T] = 1.4×10^{-6} M, 10% (v/v) acetonitrile, pH 7.55, using Tris buffer at an ionic strength of 0.09 made up of [Tris-H^+Cl^-] = 0.009 M and [NaCl] = 0.081 M. The experimentally determined rate coefficients are: $k_{buffer} = 7.85 \times 10^{-4}$ min^{-1}, $k_{obsd} = 1.65 \times 10^{-2}$ min^{-1}, and $k_{enzyme} = 1.12 \times 10^4$ M^{-1} min^{-1}.

TABLE 1: Hydrolysis of *p*-NPA as Catalyzed by BCA_1.[a]

pH	Buffer[b]	$k_{enzyme} \times 10^{-4}$ (M^{-1} min^{-1})	pH	Buffer[b]	$k_{enzyme} \times 10^{-4}$ (M^{-1} min^{-1})
5.98	P	0.010	7.65	P	1.36
6.25	P	0.038	7.77	P	1.49
6.65	P	0.074	7.83	P	1.55
6.97	P	0.43	7.90	P	1.76
7.00	T	0.43	7.92	P	1.80
7.09	P	0.53	7.92	T	1.84
7.20	P	0.81	8.16	T	1.93
7.29	P	0.91	8.45	T	2.13
7.40	P	1.15	8.72	T	2.29
7.52	P	1.21	8.92	T	2.40
7.56	T	1.23	9.33	T	2.57
7.60	P	1.37	10.48	T	2.64
7.62	P	1.36			

[a] Second-order catalytic constant, k_{enzyme} (M^{-1} min^{-1}), at various pH values in phosphate, P, or Tris, T, buffers at 25.0°. Reaction mixtures contain 10% (v/v) acetonitrile and are kept at an ionic strength of 0.09 with added NaCl. [b] Borate buffers gave lower catalytic constants than either phosphate or Tris buffers; the second-order catalytic constants, $k_{enzyme} \times 10^{-4}$, using borate buffers (0.05 M) are 0.76, 0.96, and 1.47 M^{-1} min^{-1} at pH 7.31, 7.56, and 8.38, respectively.

One of our primary concerns in this study was the determination of the esterase activity of bovine carbonic anhydrase as a function of pH. The catalytic coefficient k_{enzyme} was evaluated at numerous pH values ranging from 6.0 to 10.5 (Table I). Thus a rather detailed pH–rate profile evolved for the hydrolysis reaction at 25° in Tris and phosphate buffers. In order to investigate the enzymatic reaction over a wide pH range it is necessary to use more than one buffer system, and so differential effects arising from the binding of components of the buffer to the enzyme, rather than simply effects of H^+, may be encountered. To minimize these complications we have employed dilute buffers and found Tris buffers to be particularly suited to our pH studies because wide pH ranges may be obtained while maintaining a constant ionic strength and varying only the concentration of uncharged

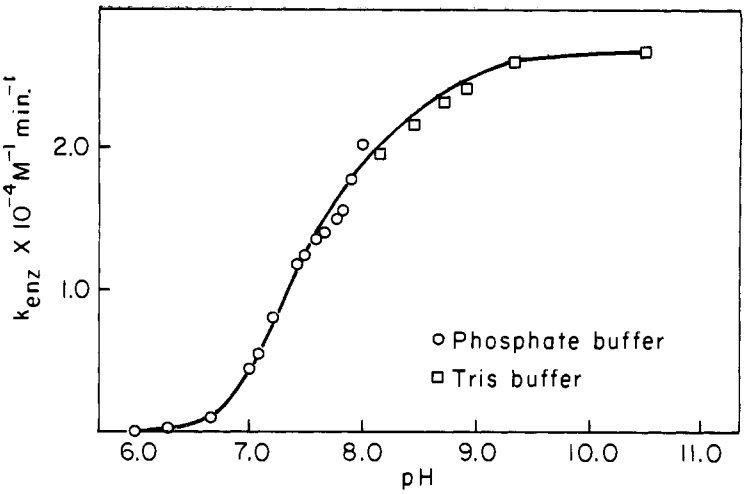


FIGURE 2: The BCA-catalyzed hydrolysis of *p*-NPA as a function of pH in phosphate and Tris buffer at 25.0°; $[BCA_I] \simeq 3 \times 10^{-6}$ M, [*p*-NPA] = 5.0×10^{-5} M, 10% (v/v) acetonitrile, and $[Cl^-] = 0.09$ M, ionic strength 0.09.

buffer component. It was found in regions of overlap that identical values of k_{enzyme} were obtained in Tris and phosphate buffers, whereas borate buffers gave much lower values (Table I). The resulting sigmoid curve when k_{enzyme} is plotted against pH is shown in Figure 2. It will be seen that in acidic media the enzyme was very ineffective, $k_{enzyme} = 110$ min^{-1} M^{-1} at pH 6.0, while in basic media a plateau is reached at pH values >9.5, where $k_{enzyme} = 26{,}400$ min^{-1} M^{-1}. A derivative of this plot, *i.e.*, a plot of $\Delta k_{enzyme}/\Delta$pH *vs.* pH, defines the point of inflection as occurring at pH 7.5.

The dependence of enzymatic rate on substrate concentration was formally analyzed in terms of the Michaelis–Menten scheme.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

It is of course realized that the specific rate constants determined experimentally may be much more complex than those indicated by this simplified scheme. Lineweaver–Burk plots were used to determine formal values of $K_m = [(k_{-1} + k_2)/k_1]$ and $V_m = (k_2[E])$. Rate studies were then undertaken to scrutinize: (1) the variation of K_m and V_m with pH; (2) the effect of the potent and specific sulfonamide inhibitor, acetazolamide; and (3) the effect of various monovalent anionic inhibitors.

It appears that both K_m and V_m vary with pH (Table II), but that in the pH interval 7.0–8.6, K_m is to a first approximation a linear function of the turnover number, k_2. If real,[2] this would imply that the apparent binding constant $K = (k_1/k_{-1})$ is invariant over this pH range, while the inflection point in the pH–rate profile refers to the ionization of a group associated with the hydrolyzing rather than the binding site.

Acetazolamide is a powerful noncompetitive inhibitor of the carbonic anhydrase catalyzed hydration of CO_2 and acetaldehyde. It also noncompetitively inhibits the enzyme-catalyzed hydrolysis of *p*-NPA. A plot of activity *vs.* the ratio of acetazolamide to enzyme concentration (Figure 3) gives a curve in which 75% of the activity is lost at a 1:1 ratio. Extrapolation of the initial slope of inactivation to zero activity indicates that there is one esteratic site per

TABLE II: Results from Lineweaver–Burk Plots for the BCA_{II}-Catalyzed Hydrolysis of *p*-NPA at 25.0°.[a]

pH[b]	$[BCA_{II}] \times 10^6$ (M)	$K_m \times 10^4$ (M)	$V_m \times 10^6$ (M min^{-1})	k_2,[c] (min^{-1})
6.97	3.04	29	35.5	11.7
7.55	3.17	67	258	81.5
7.55[c]	3.15	81	431	137
7.53[d]	3.40	92	500	147
8.01	3.04	79	418	137
8.41	3.48	117	645	185
8.60[e]	3.18	132	662	208
9.20	3.82	90	975	255

[a] Reaction mixtures contain 10% (v/v) acetonitrile, 0.009 M Tris-H$^+$Cl$^-$, and enough Tris to obtain the required pH. [b] Except where indicated, the ionic strength was kept at 0.09 with added NaCl. [c] In the absence of added NaCl; ionic strength in these runs was 0.009. [d] In the presence of 1.8×10^{-3} M $NaHCO_3$. [e] In the presence of 2.0×10^{-2} M $NaHCO_3$.

[2] The difficulty in obtaining quantitative K_m and V_m values from either of the two enzyme preparations used in the present work, coupled with the caution which the long arm of coincidence should always engender, precludes us from taking a more definitive stand on this point.

enzyme molecule. In support of this conclusion is the fact that a plot of $-\log (V_0/V_i - 1)$ *vs.* log (inhibitor) gives a line of slope 1 for the acetazolamide data. Additional results are summarized in Table III. The

TABLE III: Inhibition by Acetazolamide.[a,b]

[Acetazolamide] × 10^7 (M)	[Acetazolamide]/[BCA]	% Act.
0.0	0.0	100.0
0.5	0.0173	95.2
2.5	0.0842	94.9
5.0	0.217	79.8
12.5	0.42	56.2
20.0	0.665	48.0
25.0	0.839	31.9
37.5	1.26	14.5
50.0	1.67	8.4
100.0	3.35	2.8
400.0	13.7	0.0

[a] Reaction mixtures contain 10% (v/v) acetonitrile. Runs carried out in Tris buffer at pH 8.45 using 0.009 M Tris-H^+Cl^- and 0.081 M NaCl to give an ionic strength of 0.09; $[BCA_I] = 2.8 \times 10^{-6}$ M. [b] Acetazolamide (Diamox) was obtained from Lederle Laboratories as the sodium salt.

dissociation constant for the enzyme–acetazolamide complex is estimated as $K_i \simeq 2 \times 10^{-7}$ M at pH 8.45. It should be stated that when an inhibitor as potent as acetazolamide is used, the number ascribed to an inhibitor constant is only an apparent value as the concentration of free inhibitor will be minute, thereby making it difficult to evaluate a true dissociation constant (Webb, 1963). Acetazolamide is a weak acid of $pK_a = 7.2$. At pH 8.45 the basic form of this sulfonamide predominates and inactivates the enzyme. However, we have also shown that the acidic form of this sulfonamide is also a potent inhibitor.

As shown in Figures 4 and 5 the enzyme is also inhibited by a variety of monovalent anions. The kinetic parameters obtained from Lineweaver–Burk plots define this inhibition as noncompetitive, arising from the formation of 1:1 complexes, the order of inhibition being: $CN^- \gg SCN^- \sim N_3^- > I^- > HCO_3^- > NO_3^- > Br^- > AcO^- > Cl^- > F^-$. At the pH under consideration, all these inhibitors are present almost entirely in their anionic form except for HCO_3^- ($pK^{H_2CO_3} = 6.35$) and especially CN^- ($pK_a^{HCN} = 9.1$). The effect of acetonitrile, which was used for substrate solubility purposes, was also determined. It was the weakest of the various inhibitors employed (Table IV).

Salt inhibition appears to be a function of pH. The dissociation constants for the 1:1 complex between

TABLE IV: Binding Constants between BCA and Various Inhibitors.[a]

Inhibitor	pH	K_i Concn for 50% Inhibn (M)	K (M^{-1})
Acetazolamide[b,c]	8.45	$\simeq 2 \times 10^{-7}$	$\simeq 5 \times 10^6$
NaCN[c]	7.55	2.6×10^{-6}	3.8×10^5
NaSCN	7.55	5.9×10^{-4}	1.7×10^3
NaN_3	7.55	5.9×10^{-4}	1.7×10^3
NaI	7.55	8.7×10^{-3}	1.2×10^2
$NaHCO_3$	7.55	2.6×10^{-2}	3.8×10
$NaNO_3$	7.55	4.8×10^{-2}	2.1×10
NaBr	7.55	6.6×10^{-2}	1.5×10
NaOAc	7.55	8.5×10^{-2}	1.2×10
NaCl	7.55	1.9×10^{-1}	5.2
NaF	7.55	1.17	0.85
CH_3CN[d]	8.45	1.24	0.81

[a] The salt inhibition data refer to measurements carried out in 10% (v/v) acetonitrile and an ionic strength of 0.09 made up of 0.081 M inhibiting salt and 0.009 M Tris-H^+Cl^-. The values for 50% inhibition and for enzyme–inhibitor binding constants are calculated neglecting the possible inhibition by 0.009 M Tris-H^+Cl^-. [b] Acetazolamide data refer to measurements carried out on sodium acetazolamide (Lederle Laboratories Division, American Cyanamide Co.) in 10% (v/v) acetonitrile and an ionic strength of 0.09 made up of 0.081 M NaCl and 0.009 M Tris-H^+Cl^-. The 50% inhibition and the inhibitor binding constant are calculated neglecting the inhibition by 0.09 M Cl^-. [c] Considering both conjugate acid and anion to be potential inhibitors; $K_i = [HI + I^-][\Sigma BCA]/[\Sigma BCA \cdot I]$, where ΣBCA is the sum of concentrations of all BCA species not containing inhibitor, and $\Sigma BCA \cdot I$ is the sum of concentrations of all forms of BCA containing bound inhibitor. At the pH under consideration, $[HI + I^-] \simeq [I^-]$, except for CN^-. [d] These data refer to measurements in which varying amounts of CH_3CN were added to an aqueous buffer. The Tris buffer was always adjusted to contain 0.009 M Tris-H^+Cl^-.

enzyme and chloride ion as a function of pH are summarized in Table V. These data show that the anion-sensitive site in the enzyme bears a group with a pK_a in the vicinity of 7.5 and that its transformation to the basic form reduces but does not abolish the inhibitory power of the halide ion. It seems likely that an essential component of the anion-binding site of carbonic anhydrase is electrophilic in nature.

The Michaelis–Menten parameters obtained in the present work permit a formal analysis of the enzymatic process (Figure 6). The best straight line drawn in a plot of K_m *vs.* k_2 has the slope $1/k_1 \cong 0.5 \times 10^{-4}$ M min and an intercept k_{-1}/k_1 of 2×10^{-3} M. Thus the formal binding constant k_1/k_{-1} of 500 M^{-1} arises

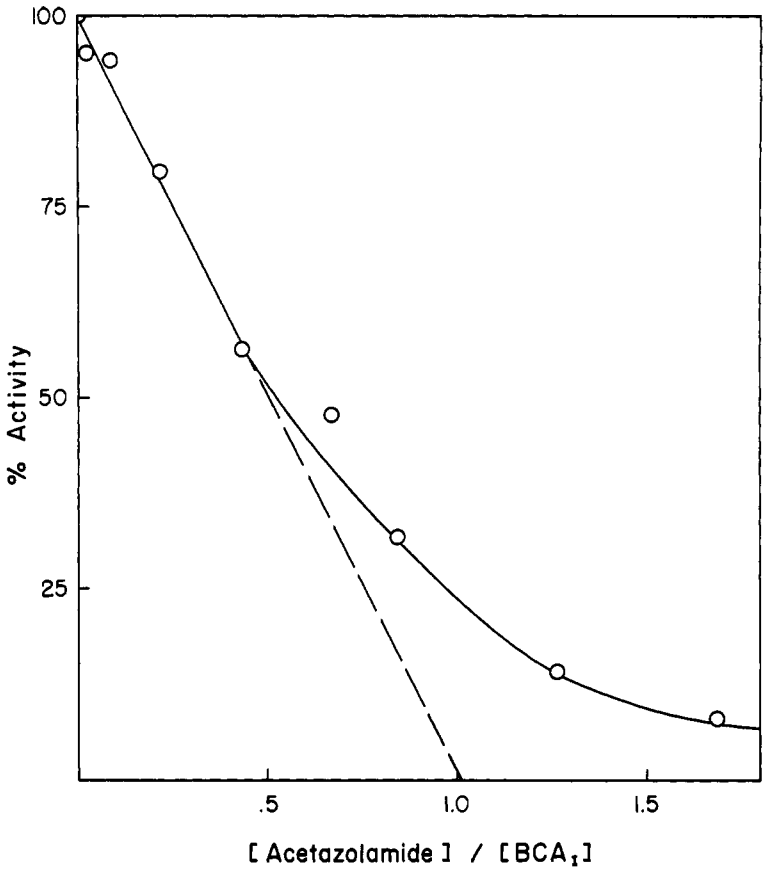


FIGURE 3: Per cent activity as a function of [acetazolamide]/[BCA] ratio at pH 8.45 and 25.0°; $[BCA_I] = 2.8 \times 10^{-6}$ M. 10% (v/v) acetonitrile, Tris buffer, and ionic strength 0.09.

from a k_1 with an apparent minimum value of *ca.* 2×10^4 M^{-1} min^{-1} and a k_{-1} with an apparent minimum value of *ca.* 40 min^{-1}.

It has been demonstrated (Pocker and Meany, 1965a,b) that zinc–imidazole complexes are powerful catalysts for the hydration of acetaldehyde. This should be contrasted to the mild catalytic activity that these complexes exhibit in the hydrolysis of *p*-NPA. Thus, in order to measure the catalytic efficiency of zinc–imidazole complexes in the hydration of acetaldehyde, only small concentrations of zinc ion (10^{-4}–10^{-3} M) need be added to imidazole (0.1 M) buffers. Under such conditions each zinc ion coordinates with four imidazoles, $\bar{\nu} = 4$, whereas ten times larger zinc ion concentrations must be used in studying the hydrolysis of *p*-NPA, making the value of $\bar{\nu}$ uncertain. Furthermore, in hydration the catalytic efficiency of $Zn(Im)_4^{2+}$ or of its stoichiometric equivalent is 33 times larger than that of free imidazole while in the hydrolysis of *p*-NPA imidazole is a very efficient catalyst. The actual catalytic coefficient to be ascribed to these complexes is still in doubt because the amount of free imidazole remaining in solution depends on the assumptions made in evaluating $\bar{\nu}$. Using a value of 4 for $\bar{\nu}$ in the region investigated leads to an upper limit of 40 min^{-1} M^{-1} as the catalytic coefficient of $Zn(Im)_4^{2+}$ or its stoichiometric equivalent. On the other hand, using the assumption that the pH changes accompanying the addition of zinc ions (10^{-3}–10^{-2} M) to imidazole buffers (0.1 M) arise almost entirely from the removal of free imidazole by zinc (Edsall *et al.*, 1954), the average number of imidazole groups bound per zinc ion, $\bar{\nu}$, was calculated as 3.5 for $[Zn^{2+}]_0$

TABLE V: Inhibition of BCA by Cl^- as a Function of pH.[a]

pH	K_i (M)
7.00	0.097
7.55	0.192
7.92	1.40
8.45	1.88
8.92	1.97
9.33	*Ca.* 2.0
10.48	*Ca.* 2.0

[a] Data refer to reaction mixtures containing 10% (v/v) acetonitrile and an ionic strength of 0.09 made up of 0.009 M Tris-H^+Cl^- and 0.081 M NaCl.

$= 10^{-3}$ M and 2.0 for $[Zn^{2+}] = 1.5 \times 10^{-2}$ M. This variable $\bar{\nu}$ leads in turn to a variable catalytic coefficient of 3–5 min^{-1} M^{-1}/zinc ion. The latter, milder catalysis is more in line with the earlier claim of Koltun *et al.* (1956).

Discussion

The most significant feature of the present investigation is that erythrocyte bovine carbonic anhydrase serves not only as a hydrase for CO_2, acetaldehyde, and related carbonyl systems but also as an esterase for *p*-nitrophenyl acetate. This latter activity is especially valuable as it provides additional avenues for studying the relationship between structure and activity. The present investigation shows, *inter alia*, that the carbonic anhydrase catalyzed hydrolysis of *p*-NPA exhibits certain similarities with the enzymatically catalyzed hydration of CO_2 and of acetaldehyde. Thus, the pH–rate profile for carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl acetate has an analogous sigmoid shape to that observed in the reversible hydration of CO_2 (Kernohan, 1964) and of acetaldehyde (Pocker and Meany, 1964, 1965a,b). The point of inflection in the carbonic anhydrase catalyzed hydrolysis occurs at pH 7.5 at 25.0° and should be compared with the corresponding one observed in the hydration of both CO_2 and acetaldehyde which occurs at pH 7.0 at 0.0°.

Erythrocyte carbonic anhydrase is the earliest known zinc–metalloenzyme. The zinc ion associated with the native enzyme has been found to be indispensable for activity (Keilin and Mann, 1940; Lindskog and Malmström 1962). Although the 1:1 zinc–apoenzyme complex (*i.e.*, the native enzyme) possesses a large stability constant, it has proved possible to prepare stable inactive zinc-free apoenzyme to which about 85% activity can be restored by readding the metal ion (Rickli and Edsall, 1962; Lindskog and Malmström, 1962). As a result of the importance of coordinated zinc in the activity of carbonic anhydrase it has been proposed (Davis, 1959) that the function of the protein-bound zinc is to transfer a metal-bound ^-OH directly to the substrate. It was further suggested that the pH dependence of the enzyme may reflect the titration of the protein-bound zinc–aquo complex to a reactive zinc–hydroxo complex, and that such a system would possess a pK around neutrality (Davis, 1958, 1961). Actually, the hydrolytic pK_a of the free (nonchelated) zinc–aquo complex, $Zn(H_2O)_4^{2+}$, at 25° and at low ionic strength is reported to be 9.7 (Hunt, 1963), and the meager data available in the literature on chelated zinc–*aquo* complexes do not contain any examples in which such large shifts in pK_a have been observed. We have earlier contended that the rate-determining step in hydration is not a simple reaction but is coupled with other processes. In particular, a pK around neutrality appeared to us to be also consistent with the participation of an unprotonated base in the protein, such as imidazole, which may promote the interaction between a water molecule

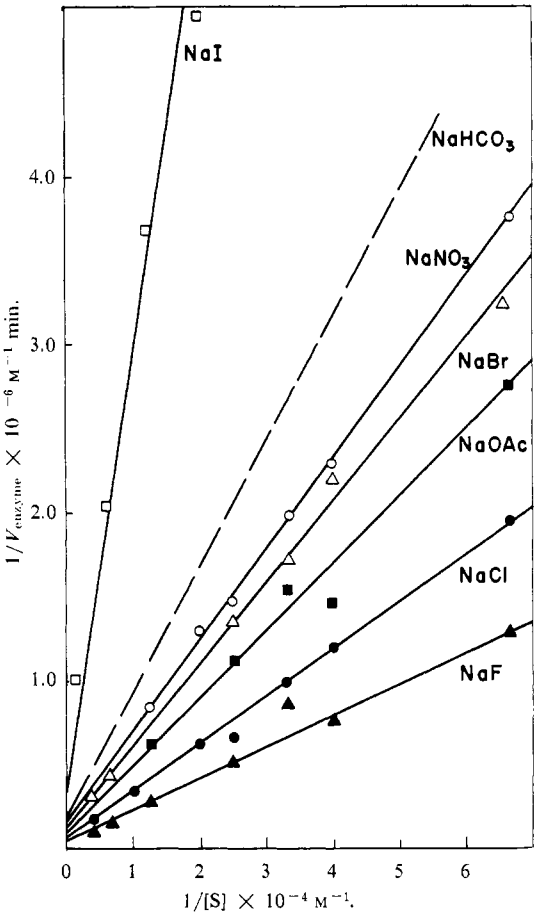


FIGURE 4: Lineweaver–Burk plots for BCA_I-catalyzed hydrolysis of *p*-NPA in the presence of 0.09 M salt made up of 0.009 M Tris-H^+Cl^- and 0.081 M sodium salt.

from the coordination sphere of the ion and the carbonyl carbon of the substrate (Pocker and Meany, 1965a,b; Pocker and Stone, 1965a,b).

For a more detailed understanding of enzymatic ester hydrolysis, it is often advantageous to look for a difference in the rate of production of alcohol or phenol and acid. We have searched for a "burst" in *p*-nitrophenol production using high enzyme concentrations but were unable to detect any in spite of the fact that the BCA-catalyzed hydrolysis of *p*-NPA has an apparent k_2 value which should have allowed its detection. Our inability to detect a "burst"[3] does not invalidate the involvement of an acyl-enzyme intermediate, provided $k_3 > k_2$ (eq 1). This is perhaps not surprising if we follow our working hypothesis to its logical conclusion, namely, that the acyl-enzyme

[3] We are testing the generality of this observation by actively studying the bovine carbonic anhydrase catalyzed hydrolysis of esters for which the condition $S_0 > K_m$ can be easily obtained experimentally.

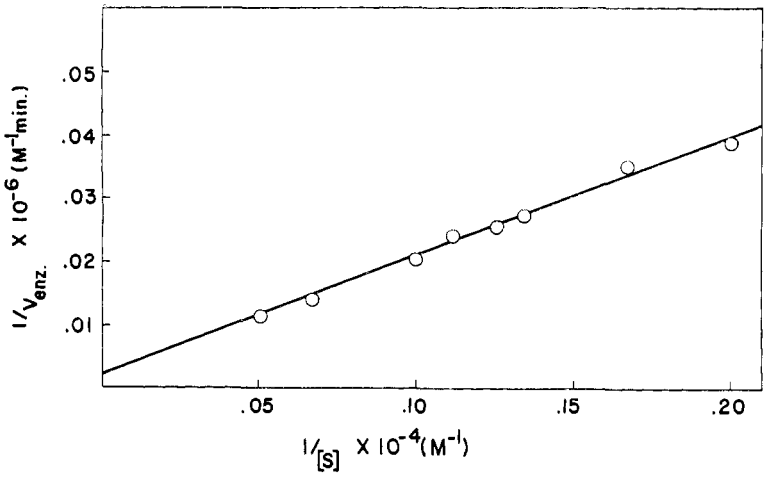
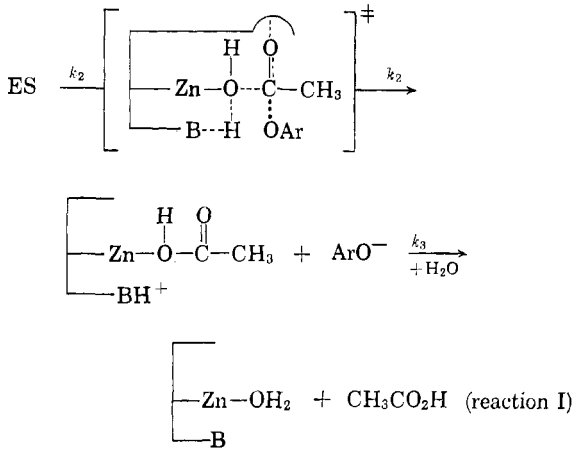
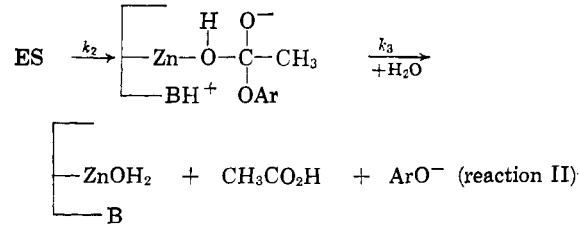


FIGURE 5: Lineweaver–Burk plot for BCA_{II}-catalyzed hydrolysis of *p*-NPA in the presence of $[NaHCO_3] = 18 \times 10^{-2}$ M at pH 7.55 using Tris buffer.

intermediate of carbonic anhydrase is actually a zinc–acetate complex (reaction I).



The acyl-enzyme intermediate in trypsin and chymotrypsin is a relatively stable intermediate but not so in carbonic anhydrase. Step 3 in the latter would involve the displacement of acetate from its coordination to zinc by water. Such a process would be expected to be much faster than k_2. The absence of a burst is of course also consistent with the formation of a tetrahedral intermediate, possibly bound to zinc (reaction II, step k_2), which rapidly decomposes to *p*-nitrophenol and acetic acid, in a concerted or almost concerted step (reaction II, step k_3).



We were able to analyze our kinetic data in terms of three apparent rate coefficients: k_1, k_{-1}, and k_2. Thus, the treatment of K_m as a linear function of k_2 allows a lower limit to be set for the rate coefficients leading to enzyme–substrate association, $k_1 \geq 2 \times 10^4$ M^{-1} min^{-1}, and dissociation, $k_{-1} \geq 40$ min^{-1}. These values are relatively small, implying that the corresponding kinetic parameters may be considerably more complex than indicated by the simple Michaelis–Menten formulation. In particular, the value associated with k_1 is much smaller than that for a diffusion-controlled association and may be indicative of some type of covalent binding.

$$E + S \underset{}{\overset{\text{diffusion}}{\rightleftharpoons}} (E\cdots S) \overset{\text{covalent binding}}{\rightleftharpoons} E\cdot S$$

diffusion complex — covalent complex

It will also be noticed that the apparent binding constant, k_1/k_{-1}, is invariant in the pH region[4] 7–9 while the observed variation of rate with pH is dictated by the turnover number, k_2. The pH–rate profile of the turnover number, k_2, accords with the hypothesis that a group having a pK_a of 7.5 acts in its basic form as a promoter of the direct transfer of water from zinc to the substrate.

The specific effects of various inhibitors have been studied. Both acetazolamide and various common sodium salts were used. The inhibition of bovine carbonic anhydrase catalyzed hydrolysis of *p*-NPA by acetazolamide is similar to that observed for CO_2 and acetaldehyde hydration (Forrest, 1953; Keller

[4] Since the ratio k_1/k_{-1} is essentially invariant over the pH interval 7–9, it is attractive, though perhaps not entirely legitimate (Sturtevant, 1960), to assume that enzyme–substrate binding is controlled by groups whose pK_a lies outside this region. With respect to the experimentally determined K_m (formally $(k_{-1} + k_2)/k_1$) it should be noted that around pH 7.3, $k_{-1} \simeq k_2$; above this pH, $k_2 > k_{-1}$ while below it, $k_{-1} > k_2$.

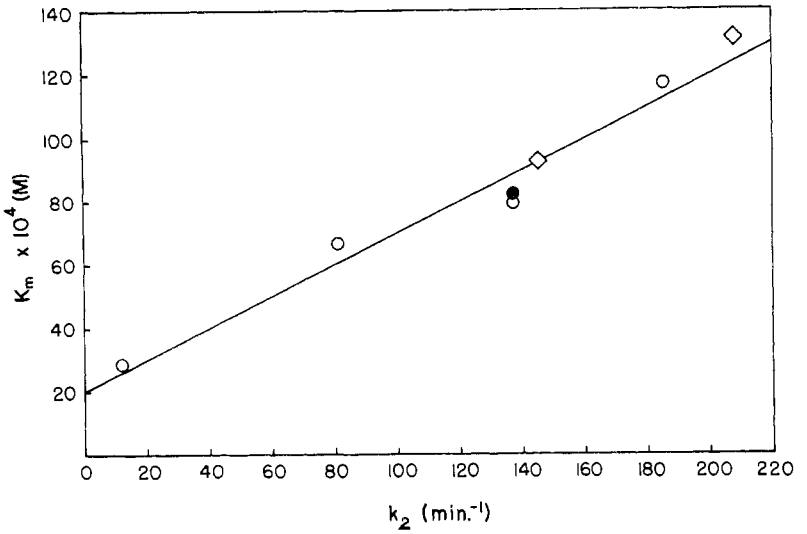


FIGURE 6: The variation of K_m with k_2 for the BCA_{II}-catalyzed hydrolysis of *p*-NPA at 25.0°; $[BCA_{II}] = 3.0–3.8 \times 10^{-6}$ M; (○) with $[Cl^-] = 0.09$ M varying the pH; (●) with $[Cl^-] = 0.009$ M at pH 7.55; (◇) with added $NaHCO_3$.

et al., 1959; Pocker and Meany, 1965b), and is found to be noncompetitive. The extrapolation of the inactivation plot to zero activity coincides with a 1:1 enzyme–inhibitor complex and indicates that there is one esteratic enzyme per molecule. Some preliminary X-ray work on an enzyme–sulfonamide complex indicates that a sulfur (from bound inhibitor) is situated close to the zinc position (Tilander *et al.*, 1965). This may further suggest that acetazolamide noncompetitively inhibits bovine carbonic anhydrase because it binds at or near the zinc site.

Salt inhibitors with the exception of CN^- are much less potent than acetazolamide. This inhibition was found to follow the order: $F^- < Cl^- < AcO^- < Br^- < NO_3^- < HCO_3^- < I < N_3^- \simeq CNS^- \ll CN^-$. A similar order has been obtained for salt inhibition of CO_2 hydration (Roughton and Booth, 1946; De Voe and Kistiakowsky, 1961; Kernohan, 1965). Binding of one anion per sensitive site appears to be sufficient to cause loss of catalytic activity of that site. The Hofmeister lyotropic series appears to be followed in our studies; such a series was obtained for the enzyme, acetoacetic decarboxylase (Fridovitch, 1963). Bovine carbonic anhydrase forms inactive complexes with these anions by binding them at a site which is linked to the ionizing site; furthermore, this site appears to be independent of the substrate-binding site. Loss of a proton from the ionizing group in the enzyme reduces the inhibitory effect of chloride ions. The pK_a of this change is also around 7.5. Similar observations were made in the corresponding hydration of CO_2 (Kernohan, 1964).

The inhibition by HCO_3^- is of particular interest. The hydration of CO_2 is reversibly catalyzed by carbonic anhydrase. The principle of microscopic reversibility would indicate that the binding and hydrating sites for enzyme-catalyzed hydration must be reversed in enzyme-catalyzed dehydration. Thus with respect to *p*-NPA hydrolysis it might be expected that CO_2 would be a competitive inhibitor while HCO_3^- would be a noncompetitive one (reaction III).

$$CO_2 + \underset{\underset{B}{|}}{E{-}Zn{-}OH_2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\underset{B}{|}}{CO_2 \cdot E{-}Zn{-}OH_2} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}$$

$$\underset{\underset{BH^+}{|}}{E{-}Zn{-}OCO_2H} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \underset{\underset{BH^+}{|}}{E{-}Zn{-}OH_2} + HCO_3^-$$

reaction III

The CO_2 test is difficult to apply experimentally because of the low solubility of CO_2 and its rapid hydration. With respect to HCO_3^- we have indeed found the anion is an efficient noncompetitive inhibitor of *p*-NPA hydrolysis with an inhibition constant of $K_i = 0.026$ M at pH 7.55 in 10% (v/v) acetonitrile at 25.0°. Earlier kinetic measurements allow the evaluation of the ratio k_3/k_{-3} for the bovine carbonic anhydrase catalyzed dehydration of HCO_3^- in water at 25.0° (Kernohan, 1964, 1965). Values ranging from 0.012 to 0.09 M are obtained, depending on buffer and ionic strength. The agreement between the value of K_i for noncompetitive inhibition by HCO_3^- and the dissociation constant of $E{\cdot}HCO_3^-$ leads us to believe that the hydrating site for CO_2 must become the binding site for the dehydration of HCO_3^-.

It appears to us that the esterase activity of bovine carbonic anhydrase and its hydrase activity have many features in common. The findings reported herein indicate that these activities are derived from the same part of the enzyme and possibly that the mechanisms are closely related.

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