Stopped-Flow Studies of High pH Activity and Acetazolamide Inhibition of Bovine Carbonic Anhydrase. Enzyme-Catalyzed Hydrolyses of 3-Pyridyl and Nitro-3-pyridyl Acetates†

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ABSTRACT: The activity of bovine carbonic anhydrase after exposure to alkaline pH is carefully delineated and the time-dependent changes leading to activity loss are examined. The esterase activity of bovine carbonic anhydrase is scrutinized with respect to 3-acetoxypyridine, 3-acetoxy-2-nitropyridine, and 3-acetoxy-2,6-dinitropyridine as substrates. For each ester a detailed rate profile is obtained in aqueous media at 25.0° over wide values of pH ranging between 6 and 12. In every case, the pH–activity profiles reveal two inflections, one around pH 7–7.5 and the other around pH 11–11.5. An equation of the form $k_{enz}[E]_0 = k_{EH}[EH] + k_E^-[E^-]$, where $[E]_0 = [EH_2^+] + [EH] + [E^-]$, is fit to the observed data. The second rise in activity is ascribed to a combination of high pH induced conformational changes (substrate dependent) coupled with the formation of a more reactive zinc–hydroxo complex. The ratio of enzymatic activity between the second and the first plateau, k_E^-/k_{EH}, depends upon the acetoxypyridine used and varies between 6 and 13. The pH dependence of the acetazolamide inhibition of the enzymatic hydrolysis of 3-acetoxy-2-nitropyridine is interpreted in terms of a negatively charged species of the inhibitor, $ArSO_2NH^-$, bound to both the "acidic," EH_2^+, and the "neutral," EH, forms of the enzyme. The E^- form of the enzyme does not appear to bind the inhibitor. Alkylation of a specific histidine residue situated in or near the active-site cavity produces a derivative which is inactive around physiological pH. However, the alkylated enzyme is active at high pH and appears to retain the activity associated with the high pH component of the native enzyme, $k_E^-[E^-]$. On the other hand, the apoenzyme is essentially devoid of any esterase activity in the pH range 6–12.

Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) is a zinc-containing metalloenzyme with a mol wt of 30,000. In addition to its known physiological role, the reversible hydration of carbon dioxide, carbonic anhydrase catalyzes the reversible hydration of various aldehydes (Pocker and Meany, 1965a,b; 1967a,b; Pocker and Dickerson, 1968), the dehydration of 2,2-dehydroxy propionate to pyruvate anion (Pocker and Meany, 1970), and the hydrolysis of phenyl esters (Pocker and Stone, 1965, 1967, 1968a–c; Pocker and Storm, 1968; Pocker and Beug, 1972; *cf.* also Tashian *et al.*, 1964; Malmstrom *et al.*, 1964; Armstrong *et al.*, 1966). The esterase function shows many similarities to the hydrase function as both show similar sigmoidal pH–activity profiles around physiological pH and both are subject to the powerful and highly specific inhibitory action of certain sulfonamides. These findings coupled with the fact that the experimental techniques necessary to obtain accurate rate data for many of these substrates are far simpler than those used in the study of the reversible hydration of carbon dioxide have allowed a more comprehensive study of this enzyme.

pH–activity profile studies carried out in these laboratories at pH values above 9 uncovered a second rise in the esterase activity of bovine carbonic anhydrase with a pK_a above 10 (Pocker and Storm, 1968). The nature of the high pH rise in the activity of this enzyme has been under intensive scrutiny in these laboratories (Pocker and Stone, 1968c; Pocker, 1969; Pocker and Guilbert, 1972). Inasmuch as the modification of all the ε-aminolysine and arginine residues and of the one exposed tyrosine residue had no significant effect on enzymatic activity (Nilsson and Lindskog, 1967; Nees *et al.*, 1971) we were led to suggest that the second rise in activity might be a combination of high pH induced conformational changes coupled with the titration of the enzyme-bound zinc–aquo complex to a more reactive zinc–hydroxo complex. The circumstance that pyridyl carbonate esters proved to be excellent substrates for studying the mode of action of bovine carbonic anhydrase over an unusually wide range of pH values, coupled with their capacity to act as sensors of the environment in the vicinity of the active site (Pocker and Guilbert, 1972), has led us to undertake parallel investigations with pyridyl acetates. The pyridyl esters undergo facile hydrolysis and, unlike phenyl esters, are moderately soluble in water so that the need for organic cosolvent is minimal and can be entirely bypassed.

The zinc ion associated with the native enzyme is necessary for the powerful binding of the specific inhibitor, acetazolamide (Lindskog, 1963), and of certain monoanions (Verpoorte *et al.*, 1967; Ward, 1970). In the native enzyme the elements associated with the rapid turnover, *i.e.*, the metal ion in conjunction with the water structure and the amino acid residues at the active site dictate both the pH–activity and pH–inhibition profiles (Pocker and Watamori, 1971). For this reason we have attempted in this paper to study the underlying mechanisms of the enzymatic catalysis by delineating the high pH profile and the inhibition characteristics of bovine carbonic anhydrase with respect to the enzymatic hydrolysis of the three pyridyl esters, AP, ANP, and ADNP.[1]

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: AP, 3-acetoxypyridine; ANP, 3-acetoxy-2-nitropyridine; ADNP, 3-acetoxy-2,6-dinitropyridine.

Experimental Section

Materials. Bovine carbonic anhydrase was obtained as a highly active lyophilized powder from Mann Research Laboratories. For kinetic purposes, further purification was effected by column chromatography on a DEAE-cellulose column (Whatman DE-32). The lyophilized material was dissolved in 0.01 M Tris-HCl buffer, applied to a DEAE-cellulose column preequilibrated with the same buffers, and eluted with 0.08 M Tris-HCl buffer, pH 8.0 (Lindskog, 1960). The leading peak was identified as the B isozyme and the subsequent peak as the A isozyme. The commercial product was shown to consist of a mixture of A and B isozymes in the ratio ~1:3. Based on atomic absorption measurements, these preparations contained 1.0 ± 0.05 atom of zinc per mol assuming a molar absorbancy of 54,000 at 280 nm. Zinc-free bovine carbonic anhydrase B was prepared by dialysis against 0.01 M 1,10-phenanthroline in 0.1 M sodium acetate–acetic acid buffer, pH 5.0. Zinc removal was essentially complete after 10 days. Selective alkylation was first achieved in these laboratories by Dr. J. T. Stone in 1967 (Pocker, 1969). The method employs an excess of iodoacetate or of bromopyruvate. Several lines of evidence indicate that under Dr. J. T. Stone's conditions the major alkylation reaction occurs predominantly in or near the active center: (a) loss of catalytic activity, as assayed around physiological pH, parallels the rate of ^{14}C incorporation into the enzyme; (b) both iodoacetate and bromopyruvate inactivate the enzyme irreversibly only after they first bind as reversible inhibitors; and (c) acetazolamide inhibits the rate of irreversible inactivation. Inasmuch as these alkylation reactions are carried out with a large excess of alkylating agent it proved advantageous to aim for a modification of 1.1 histidine residues in order to ensure the complete 3′-alkylation of a specific histidine residue at or near the active site. The carboxymethylated bovine carbonic anhydrase B was chromatographed on Bio-Rex 70. The hydrase activity of this derivative was at least 100-fold lower than that of the native isozyme. The carboxyketoethylated enzyme, after purification, also showed a highly reduced hydrase activity. Reduction of the carboxy keto function with borohydride produced a 2-carboxy-2-hydroxyethyl derivative of very low activity. There was no indication of a Schiff base formation involving the keto group of the modifier and the amino groups of the native enzyme.

Syntheses of the three pyridyl esters have been previously described (Pocker and Watamori, 1971). Inorganic buffer components were all analytical or reagent grade and used without further purification. 2-Amino-2-hydroxymethylpropane-1,3-diol (Tris) was twice recrystallized from 95% ethanol. Triethylamine and acetic acid were freshly distilled before preparation of triethylammonium acetate buffers. The total buffer concentration in this work was maintained constant at 0.05 M throughout the pH range studied. All buffers were brought to an ionic strength, μ, of 0.15 except for the basic phosphate, HPO_4^{2-}–PO_4^{3-}, buffer in which case the ionic strength, μ, had to be maintained at 0.20 by adding an appropriate amount of Na_2SO_4, KCl, or NaCl. We have confirmed that at high pH the reaction is quite insensitive to changes in ionic strength. Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) was obtained from the Lederle Laboratories Division of American Cyanamide Co. Apparatus, procedure, and general technique for studies around physiological pH have been previously described (Pocker and Watamori, 1971). For the determination of pH–rate profiles in this region, k_{enz} was obtained using the following substrate concentrations: 3.10×10^{-4} M 3-acetoxypyridine; 1.67×10^{-4} M 3-acetoyxy-2-nitropyridine; and 1.0×10^{-4} M 3-acetoxy-2,6-dinitropyridine.

High pH Profile Studies. Rapid hydrolyses of buffer and bovine carbonic anhydrase catalyzed reactions of 3-acetoxypyridine esters at high pH were monitored on a Durrum-Gibson stopped-flow spectrophotometer (Durrum Instrument Corporation, Palo Alto, Calif.) (Pocker and Watamori, 1971). In this apparatus the reaction is initiated by driving the two reactant solutions from syringes by means of plungers actuated by a hydraulic system. The drive–syringe assembly supplied with the instrument was modified in our laboratories to provide mixing ratios varying from 1:1 to 10:1 and at the same time to eliminate leakage. All the buffer solutions for the stopped-flow experiments were made by taking these factors into consideration. The entire fluid handling system was thermostated at 25.0 ± 0.02° by means of an insulated bath compartment consisting of a specially constructed circulating device operating in conjunction with a Sargent thermonitor Model SW (S-82055). Numerous kinetic runs as well as other control experiments covering the changeover from the Beckman Model Kintrac VII (Pocker and Watamori, 1971) to the Durrum-Gibson stopped flow agreed well within 3% indicating a smooth transition. Furthermore, the enzyme and substrate concentrations determined in the mixing chamber of the stopped flow agreed well with values independently deduced from dilution factors. Similarly, pH values in the mixing chamber were shown to be the same as those independently determined by mixing the buffer and the substrate solution in the appropriate ratio. Ester and enzyme were mixed in the stopped-flow instrument and the appearance of the monoanions was monitored at 310, 398, and 387 nm for 3-hydroxypyridine, 3-hydroxy-2-nitropyridine, and 3-hydroxy-2,6-dinitropyridine, respectively. To avoid a significant amount of enzyme deterioration at high pH after mixing in the buffer, the time between mixing and completion of a run in the instrument was maintained within 3 min for the runs above pH 11.5. Also, the absorbance at infinity (A_∞) for the computation of the pseudo-first-order coefficient at these high pH values was calculated according to the method of Swinbourne (1960). The catalytic coefficient of the enzyme (k_{enz}) was determined by dividing the difference between the total rate and the rate due to buffer by the enzyme concentration. The enzymatic rates were proportional to the enzyme concentration over the entire pH range studied (6–12). For the pH–rate profiles, k_{enz} at high pH were obtained using a fixed substrate concentration of 1.8×10^{-4} M for AP, 7.0×10^{-5} M for ANP, and 5.0×10^{-5} M for ADNP. For a given concentration of enzyme, three–five runs were made at each pH and the mean value used.

Very careful scrutiny of the results obtained from over 400 runs allowed a careful delineation of the high pH–activity profiles. However, the quantitative characterization of k_{enz} in terms of k_{cat} ($\equiv k_2$) and K_m, similar to the one carried out in the pH region 6–8 (Pocker and Watamori, 1971) proved considerably more difficult in the high pH region. Nevertheless, with the help of an organic cosolvent such as acetone or acetonitrile, we were able to overcome some of these technical difficulties and to demonstrate that for 3-acetoxy-2-nitropyridine at 25.0° the value of K_m remains effectively constant at *ca.* 0.01 M. Values of k_{cat} are more uncertain than those of k_{enz} because a long extrapolation is involved, but they generally parallel the latter values and show similar inflections; indeed above pH 8, with $[S] < K_m$, $k_{cat} \sim 0.01 k_{enz}$.

Acetazolamide Inhibition. Inhibition of the bovine carbonic anhydrase esterase activity of 3-acetoxy-2-nitropyridine by

acetazolamide was studied as a function of pH. The inhibition constant, K_i, was determined according to eq 1, where $[E]_0$

$$K_i = \frac{([E]_0 - [EI])([I]_0 - [EI])}{[EI]} \quad (1)$$

and $[I]_0$ are the total initial concentrations of enzyme and inhibitor, respectively, and [EI] is the concentration of the enzyme–inhibitor complex. Values for an apparent constant were determined by assuming the relation given in eq 2. The

$$[EI] = [E]_0(1 - k_{enz}{}^I/k_{enz}{}^0) \quad (2)$$

catalytic coefficient, $k_{enz}{}^I$ refers to enzymatic activity at a particular concentration of acetazolamide and $k_{enz}{}^0$ is the enzymatic activity with no acetazolamide present. For less potent pyridol inhibitions the K_i value was determined form the slope of a $1/i$ *vs.* 1/[I] plot. Both pH–activity and pH–inhibition plots are presented as log plots as advocated by Dixon (1953). An additional advantage is that rate data differing in magnitude can be visualized in a single plot.

High pH Stability. Many control experiments attest to the great stability of bovine carbonic anhydrase in the pH region above 5.5 and below 12 (Pocker and Stone, 1967; Nilsson and Lindskog, 1967; Pocker and Storm, 1968; Pocker and Beug, 1972). At pH 12.1, the highest pH used in the present studies, the enzyme was still reasonably stable on the time scale of our stopped-flow experiments; thus, while it showed a tendency to denature on standing, the rate of its inactivation was reasonably slow.

Curve Fitting. Throughout this work all curve fittings were performed by iterating upon the parameters to obtain the optimum fit judged by the minimum sum of the squares of the differences between calculated and experimental points. It is worth emphasizing that, since essentially all the points on the curve contribute to the determination of the parameter in question, the accuracy of the latter is substantially greater than that of the individual points on the curve.

Results

The spectrophotometric technique employed in determining the rate of hydrolysis of 3-acetoxypyridine esters exhibits a reproducibility of ±2% below pH 10 and of ±5% in the stopped-flow region associated with the higher pH. A typical oscilloscope trace produced by the stopped flow instrument is shown in Figure 1. Bovine carbonic anhydrase B is stable above pH 5.5 and below 12. However, above pH 12, the enzyme is unstable and on standing shows a progressive loss in activity as shown in Figure 2. For this experiment, enzyme was dissolved in a sodium hydroxide solution of pH 12.1 and kept at 25°. Aliquots were taken at time intervals varying from 5 min to 4 hr, transferred to a phosphate buffer whose pH value after mixing in the aliquot was 8.0 at 25.0°, and assayed for esterase activity using 3-acetoxy-2-nitropyridine. As seen in Figure 2, bovine carbonic anhydrase at pH 12.1 has a half-life of about 200 min while at pH 8.0 there is no detectable loss of activity even after 3 days.

It was shown previously in these laboratories (Pocker and Storm, 1968; Pocker and Stone, 1968c; Pocker, 1969; Pocker and Guilbert, 1972) that bovine carbonic anhydrase exhibits a second increase in activity above pH 10. However, a quantitative analysis of this high pH region is often limited by the fact that the $k_{OH^-}[OH^-]$ term becomes progressively dominant.

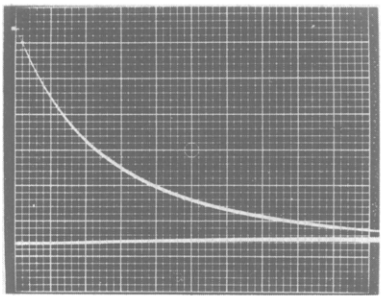

FIGURE 1: A photographic trace derived from a typical run produced by the stopped-flow instrument at 25.0°: [ADNP] = 5.00×10^{-5} M, [bovine carbonic anhydrase] = 2.80×10^{-6} M in dibasic phosphate buffer (0.05 M, μ = 0.20, pH 11.31) followed at 387 nm. The x axis reads 0.2 sec/5 small divisions. The y axis reads 50% transmittance in the middle line and 12.5% transmittance/5 small divisions. The bottom horizontal line is placed after more than 10 half-lives, $k_{obsd} = 1.06\ sec^{-1}$.

Fortunately, the 3-acetoxypyridines, particularly the nitro-substituted esters, are relatively more sensitive to the powerful esterase activity of bovine carbonic anhydrase, and the observed rate of enzymatic hydrolysis is distinctly larger than the buffer rate around the pH associated with the second inflection. In determining k_{enz} values at high pH, great care was taken to achieve maximum accuracy and more than 400 runs were performed using our stopped-flow instrument. These in turn contributed to the successful construction of the high pH–rate profiles presented in Figure 3. Here the respective log k_{enz} values are plotted against pH values ranging from 6 to 12. The rate data pertaining to the low pH portion of the ANP and ADNP profiles are taken from a previous publication (Pocker and Watamori, 1971). Also shown in Figure 3 are theoretical curves based on eq 3–7. Assuming $[E]_0 = [EH_2^+] + [EH] + [E^-]$ we obtain

$$\underset{\text{inactive}}{EH_2^+} \overset{K_1'}{\rightleftharpoons} \underset{\text{active}}{EH} + H^+ \overset{K_2'}{\rightleftharpoons} \underset{\text{very active}}{E^-} + 2H^+ \quad (3)$$

$$\text{rate} = (k_{EH}[EH] + k_{E^-}[E^-] + \Sigma k_i[C_i])[S]$$

Since $k_{buffer} = \Sigma k_i[C_i]$, then $k_{obsd} = k_{EH}[EH] + k_{E^-}[E^-] + k_{buffer}$ and

$$k_{enz} = \frac{k_{obsd} - k_{buffer}}{[E]_0} = \frac{k_{EH}[EH] + k_{E^-}[E^-]}{[E]_0} \quad (4)$$

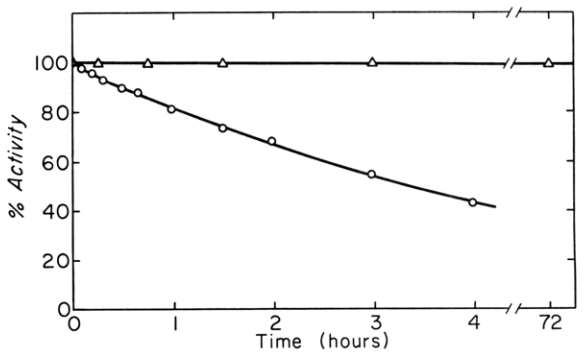

FIGURE 2: The activity of bovine carbonic anhydrase after exposure to alkaline pH at 25.0°. Aliquots were transferred from the alkaline solution to the assay mixture and the activities were all measured at pH 8.0 employing 3-acetoxy-2-nitropyridine as substrate: (○) after exposure to pH 12.1; (△) control experiment at pH 8.0.

TABLE I: Curve Fitting Results for pH–Activity Profiles for Bovine Carbonic Anhydrase Catalyzed Hydrolyses of 3-Acetoxypyridines at 25.0°.[a]

Substrate	pK_1'	$k_{EH} \times 10^{-5}$ (min^{-1} M^{-1})	pK_2'	$k_{E^-} \times 10^{-5}$ (min^{-1} M^{-1})	k_{E^-}/k_{EH}[b]
3-Acetoxypyridine	6.95	0.018	11.4	0.24	13.3
3-Acetoxy-2-nitropyridine	7.50	1.56	11.5	9.7	6.2
3-Acetoxy-2,6-dinitropyridine	7.33	15.3	11.6	134	8.8

[a] Parameters, pK_1', k_{EH}, pK_2', and k_{E^-} are described and obtained using eq 7 in the text. [b] This ratio is unitless and represents the enhanced activity of the high pH form, E^-, over the physiologically active form, EH.

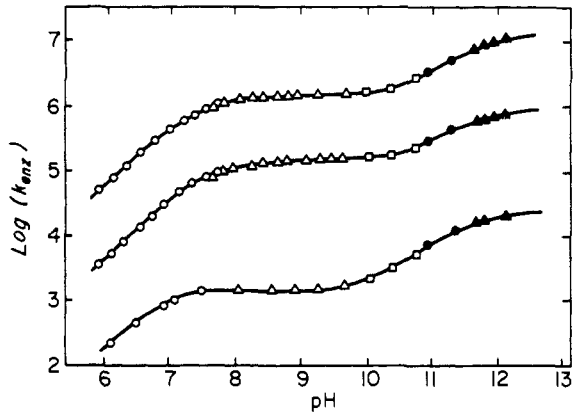

FIGURE 3: The bovine carbonic anhydrase catalyzed hydrolysis of 3-acetoxypyridines as a function of pH at 25.0°: (○) phosphate; (△) Tris; (□) triethylamine; (●) basic phosphate; (▲) hydroxide. Solid lines are the curves calculated from eq 7: upper curve, ADNP; middle curve, ANP; lower curve, AP. Values of k_{enz} in the lower region of pH down to 6.0 for 3-acetoxy-2-nitro- and 3-acetoxy-2,6-dinitropyridines are taken from an earlier publication (Pocker and Watamori, 1971).

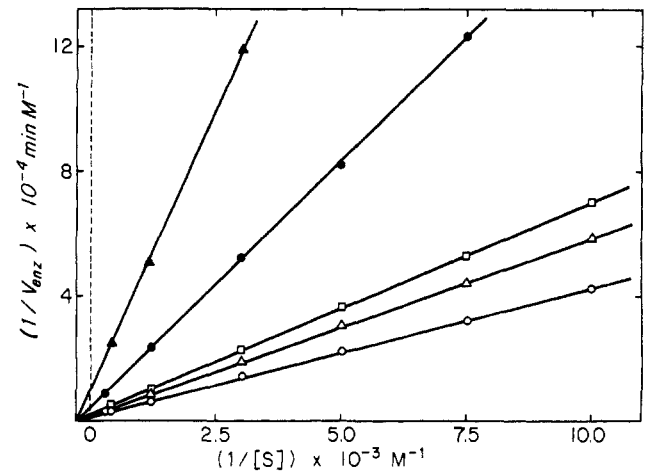

FIGURE 4: Lineweaver–Burk plots at various [acetazolamide]/[bovine carbonic anhydrase] ratios in phosphate buffer (0.05 M, μ = 0.15, pH 7.5) at 25.0° with [enzyme] = 3.3×10^{-6} M: (○) no added acetazolamide; (△) [acetazolamide]/[enzyme] = 0.37; (□) [acetazolamide]/[enzyme] = 0.55; (●) [acetazolamide]/[enzyme] = 0.83; (▲) [acetazolamide]/[enzyme] = 1.20.

Let

$$\alpha_1 = \frac{[EH]}{[E]_0} = \frac{[H^+]K_1'}{[H^+]^2 + [H^+]K_1' + K_1'K_2'} \quad (5)$$

$$\alpha_2 = \frac{[E^-]}{[E]_0} = \frac{K_1'K_2'}{[H^+]^2 + [H^+]K_1' + K_1'K_2'} \quad (6)$$

Substituting eq 5 and 6 into eq 4 we obtain

$$k_{enz} = \alpha_1 k_{EH} + \alpha_2 k_{E^-} \quad (7)$$

Here $[E]_0$, $[EH_2^+]$, [EH], $[E^-]$, [S], and $[C_i]$ are concentration terms pertaining to the total enzyme, its protonated form, its neutral form, its negatively charged form, the substrate, and the buffer component of species i, respectively. The catalytic coefficients, k_{EH}, k_{E^-}, and k_i refer to specific activity of the enzyme species EH, E^-, and the buffer component C_i, respectively. The results of these curve fittings are summarized in Table I. It will be noted that the activity of the E^- form of bovine carbonic anhydrase is significantly greater than that of the EH form, notwithstanding the fact that both depend upon the zinc ion present in the active-site crevice (Pocker, 1969; Pocker and Stone, 1968[2]).

Unlike many enzymes, carbonic anhydrase promotes hydration and hydrolysis reactions which proceed at an appreciable rate in the absence of any catalysts and are furthermore susceptible to general base catalysis (Pocker and Meany, 1965a,b, 1967a,b; Pocker and Stone, 1965, 1967; Pocker and Dickerson, 1968; Pocker and Watamori, 1971[3]). We have taken this opportunity to tabulate the enzymatic efficiency of bovine carbonic anhydrase in the region of the first plateau, because in this region $[E]_0 \simeq [EH]$ and hence, $k_{enz} \simeq k_{EH}$, Table II. The ratios of k_{enz}/k_{OH^-} given in the last column of this table show that the natural substrate, CO_2, is in a class by itself. Thus this ratio is 10^5 times smaller for acetaldehyde, 410 times smaller for *p*-nitrophenyl acetate (in 10% v/v CH_3CN), and *ca.* 60 times smaller for 3-acetoxy-2,6-dinitropyridine.

Acetazolamide has been shown to be a potent specific inhibitor of carbonic anhydrase activity with respect to both hydration (Leibman *et al.*, 1961; Pocker and Meany, 1965b; Pocker and Dickerson, 1968) and hydrolysis (Pocker and Stone, 1965, 1967; Armstrong *et al.*, 1966; Pocker and Storm, 1968). Furthermore, the noncompetitive nature of this inhibition with the binding of one acetazolamide molecule per sensitive site was documented for esterase activity with *p*-nitrophenyl acetate as substrate (Verpoorte *et al.*, 1967; Pocker and Stone, 1967, 1968a). Similar results were obtained with 3-acetoxypyridine esters as substrates (Pocker and Watamori, 1971) and the Lineweaver–Burk plots using various acetazol-

[2] Pocker, Y., and Stone, J. T. (1968), unpublished observations.

[3] Pocker, Y., and Watamori, N. (1971), unpublished observations.

TABLE II: Substrate Specificity of Bovine Carbonic Anhydrase.

Substrate	$pK_{EH_2}{}^{+a}$	pH	Temp (°C)	$k_{enz}{}^b$ (min^{-1} M^{-1})	k_{OH^-} (min^{-1} M^{-1})	k_{enz}/k_{OH^-}
Acetaldehyde[c,d]	7.0	8.0	0	8.4×10^4	7.0×10^5	0.12
Propionaldehyde[e]	6.6	7.8	0	7.1×10^4	1.4×10^5	0.50
2-Pyridinecarboxaldehyde[d]	<6	8.0	0	3.7×10^5	2.6×10^6	0.14
4-Pyridinecarboxaldehyde[d]	<6	8.0	0	1.1×10^6	7.3×10^6	0.15
p-Nitrophenyl acetate[f]	7.5	9.3	25	2.6×10^4	8.9×10^2	2.9×10
p-Nitrophenyl propionate[g]	7.3	9.1	25	6.4×10^3	5.5×10^2	1.2×10
3-Acetoxypyridine[h]	7.0	9.3	25	1.8×10^3	3.9×10^2	4.6
3-Acetoxy-2-nitropyridine[h]	7.5	9.3	25	1.7×10^5	2.1×10^3	8.0×10
3-Acetoxy-2,6-dinitropyridine[h]	7.3	9.3	25	1.7×10^6	1.1×10^4	2.1×10^2
Carbon dioxide[i]	6.9	8.5	25	6×10^9	5×10^5[j]	1.2×10^4

[a] The pK_a value of the group which controls the activity of the enzyme around physiological pH. [b] Value of k_{enz} at the given pH and temperature corresponds to the activity of the enzyme in the first plateau region, *i.e.*, where $k_{enz} \simeq k_{EH}$. [c] Pocker and Meany, 1965b. [d] Pocker and Meany, 1967a. [e] Pocker and Dickerson, 1968. [f] In 10% CH_3CN v/v, Pocker and Stone, 1967. [g] In 1% CH_3CN v/v, Pocker and Storm, 1968. [h] Present work. [i] Kernohan, 1965. [j] Sirs, 1958.

amide concentrations are exemplified in Figure 4.[4] Irrespective of the exact nature of this inhibition, its pH dependency is of much interest (Lindskog, 1963, 1966, 1969; Thorslund and Lindskog, 1967; Lindskog and Thorslund, 1968; Pocker and Storm, 1968; Pocker and Stone, 1968a; Pocker, 1969). Acetazolamide inhibition of bovine carbonic anhydrase catalyzed hydrolysis of ANP was studied as a function of pH. Per cent activities for the enzymatic hydrolysis as a function of the ratio [inhibitor]/[enzyme] at six pH values varying between 8.0 and 11.7 are given in Figure 5. The pH dependency of this inhibition is given in the form of pK_i *vs.* pH plots in Figure 6.

A generalized scheme of this inhibition may be written as follows

$$\begin{array}{ccccc} EH_2^+ + I^- & \overset{K_1'}{\rightleftharpoons} & EH + H^+ + I^- & \overset{K_2'}{\rightleftharpoons} & E^- + 2H^+ + I^- \\ \updownarrow K_A & & \updownarrow K_B & & \updownarrow K_D \\ EH_2I & \overset{K_C'}{\rightleftharpoons} & EHI^- + H^+ & \overset{K_F'}{\rightleftharpoons} & EI^{2-} + 2H^+ \end{array} \quad (8)$$

and

$$HI \overset{K_{HI}}{\rightleftharpoons} I^- + H^+ \quad (9)$$

The several equilibria may be designated thus

$$\begin{aligned} K_A &= [EH_2^+][I^-]/[EH_2I] \\ K_B &= [EH][I^-]/[EHI^-] \\ K_C' &= [EHI^-][H^+]/[EH_2I] \end{aligned} \quad (10)$$

$$\begin{aligned} K_D &= [E^-][I^-]/[EI^{2-}] \\ K_F' &= [EI^{2-}][H^+]/[EHI^-] \end{aligned} \quad (11)$$

$$\begin{aligned} K_1' &= [EH][H^+]/[EH_2^+] \\ K_2' &= [E^-][H^+]/[EH] \\ K_{HI} &= [I^-][H^+]/[HI] \end{aligned} \quad (12)$$

Our experimental observations in the high pH region show that the equilibria designated by the constants K_D and K_F' can for all practical purposes be ignored, leading to the following expressions for K_i (eq 13 and 14).

$$K_i = \frac{[I]_{free}[E]_{free}}{[EI]_{complex}} = \frac{([I^-] + [HI])([EH_2^+] + [EH] + [E^-])}{([EH_2I] + [EHI])} \quad (13)$$

$$K_i = \frac{[I^-](1 + [H^+]/K_{HI})[EH_2^+](1 + K_1'/[H^+] + K_1'K_2'/[H^+]^2)}{[EH_2I](1 + K_C'/[H^+])} = K_A \frac{(1 + K_1'/[H^+] + K_1'K_2'/[H^+]^2)(1 + [H^+]/K_{HI})}{1 + K_C'/[H^+]} \quad (14)$$

Similarly eq 13 can be expressed using K_B in the following form

$$K_i = K_B \frac{(1 + [H^+]/K_1' + K_2'/[H^+])(1 + [H^+]/K_{HI})}{1 + [H^+]/K_C'} \quad (15)$$

Results of the analysis based on this scheme (eq 8–15) are found in the legend to Figure 6.

Inhibition by *o*-nitrophenol was reported to be significantly greater than that by other substituted nitrophenols employing *p*-nitrophenyl acetate as substrate ($K_i = 1.2 \times 10^{-4}$ M, at pH 7.6 and 25.0°). Similar experiments pertaining to the bovine carbonic anhydrase catalyzed hydrolysis of 3-acetoxy-2-nitropyridine but using 3-hydroxy-2-nitropyridine and 3-hydroxy-2,6-dinitropyridine as inhibitors gave K_i values of 1.2×10^{-3} M and 3.0×10^{-3} M, respectively, at pH 7.47.

[4] We must also note here that both Kernohan (1966) and Lindskog (1969) have proposed that inhibition by acetazolamide in carbonic anhydrase reaction with CO_2 may under certain conditions be actually competitive depending upon the rates of formation and dissociation of the enzyme–inhibitor complex. We wish to defer comment on this proposal until our own studies with CO_2 are more complete. In any event, the turnover number for 3-acetoxy-2-nitropyridine hydrolysis at pH 8 is around 1×10^3 min^{-1}, whereas that for CO_2 under the same conditions is 6×10^7 min^{-1}. Thus, the rate constant for the dissociation of the acetazolamide–enzyme complex, k_{diss}, is significantly larger than the turnover number for the enzyme–ANP complex but smaller than the turnover number for the enzyme–CO_2 complex, *i.e.*, $k_{cat}{}^{E-ANP} < k_{diss}{}^{EI} < k_{cat}{}^{E-CO_2}$. It is thus clear that the derivations and quantitative discussions, pertaining to acetazolamide inhibition, presented in this paper are both meaningful and relevant to the problem at hand.

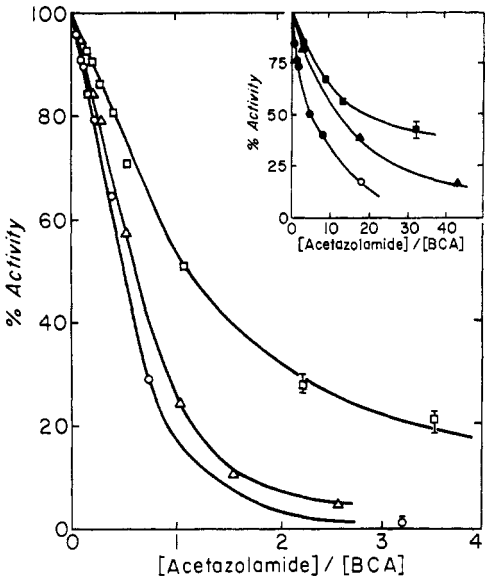

FIGURE 5: Per cent activity for the bovine carbonic anhydrase catalyzed hydrolysis of 3-acetoxy-2-nitropyridine as a function of [acetazolamide]/[enzyme] ratios: (○) Tris buffer (0.05 M, μ = 0.15, pH 8.06); (△) Tris buffer (0.05 M, μ = 0.15, pH 8.90); (□) Tris buffer (0.05 M, μ = 0.15, pH 9.59); (●) triethylamine buffer (0.05 M, μ = 0.15, pH 10.40); (▲) basic phosphate buffer (0.05 M, μ = 0.20, pH 10.90); (■) hydroxide (0.05 M, μ = 0.15, pH 11.66).

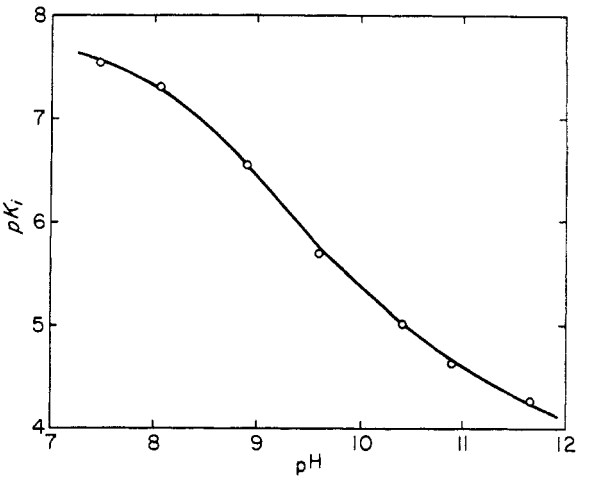

FIGURE 6: pH dependency of the inhibition of bovine carbonic anhydrase catalyzed hydrolysis of 3-acetoxy-2-nitropyridine by acetazolamide at 25.0°. The inhibition constants, K_i, were calculated as described in the text. The solid line is the curve constructed according to eq 14 and 15 using $pK_A = 7.9$, $pK_B = 4.5$, $pK_C' = 10.9$, $pK_1' = 7.1$, $pK_2' = 11.5$, and $pK_{HI} = 7.3$.

Discussion

In the present paper we have attempted to gain further insight into the mode of action of bovine carbonic anhydrase through a study of its esterase activity as a function of pH (Figure 3). A common and prominent feature of the high pH–rate profile of the enzyme-catalyzed hydrolysis of esters is the continual rise in enzymatic activity. We have sought to determine the cause of this "enhanced catalytic power" since its original discovery in these laboratories (Pocker and Storm, 1968). Our working hypothesis was that the esterase activity is promoted by an amino acid residue acting as a general base rather than as a nucleophile (Pocker and Stone, 1967; Pocker and Storm, 1968; Pocker and Watamori, 1971; Pocker and Beug, 1972). The base in question is derived from a conjugate acid which in its special enzymatic environment has a pK_a value near 7. We defer comment on the intrinsic pK_a of the zinc–aquo complex in bovine carbonic anhydrase and wish only to point out that the mathematical model represented by eq 16 best fits the data (Table I, Figure 7). Of course a mathematical model *per se* often characterizes but seldom identifies a group at the active center. However, it is clear from this treatment that the E^- form of the enzyme is produced at the expense of the EH form so that the kinetic term associated with EH decreases above pH 9.5 while the kinetic term associated with E^- increases.

$$k_{enz} = \frac{[H^+]K_1'k_{EH}}{[H^+]^2 + [H^+]K_1' + K_1'K_2'} + \frac{K_1'K_2'k_{E^-}}{[H^+]^2 + [H^+]K_1' + K_1'K_2'} \quad (16)$$

Thus, the overall high pH profile is the sum of two complementary kinetic terms. It might at first appear that a similar pH profile will arise from two concurrent kinetic terms as represented by the mathematical model given in eq 17. According to this model the esterase activity at high pH is explained by the catalytic contribution of one or more basic side chains in the enzyme that are not close to the active center. The latter model fails on three accounts. (a) The data do not converge to give a best fit. (b) Acetazolamide inhibition, although weaker at high pH, is nevertheless capable of essentially eradicating the overall esterase activity of bovine carbonic anhydrase. (c) The catalytic activity of the bovine enzyme is not significantly affected by the chemical modification of *all* lysine and arginine residues and also of the *one* exposed tyrosine residue.

$$k_{enz} = \frac{K_1'k_{EH}}{[H^+] + K_1'} + \frac{K_2'k_{E^-}}{[H^+] + K_2'} \quad (17)$$

Another interesting aspect associated with eq 16 is that when a specific histidine residue is modified with either iodoacetate or bromopyruvate then the alkylated enzyme retains the kinetic term associated with the E^- species, *i.e.*, $K_1'K_2'k_{E^-}/([H^+]^2 + [H^+]K_1' + K_1'K_2')$ (Figure 8), but loses the kinetic term associated with the original EH species, *i.e.*, $[H^+]K_1'k_{EH}/([H^+]^2 + [H^+]K_1' + K_1'K_2')$ (*cf.* also Pocker, 1969; Pocker and Stone, 1968[2]). Thus, at pH ~10 the alkylated enzyme shows a residual activity *ca.* 20% of the overall esterase activity at this pH, a value which is in excellent agreement with the one calculated for the E^- term, eq 16.

In problems of mechanism, illumination often follows from a detailed study of specific inhibitors. Therefore, considerable interest attaches to the binding characteristics of sulfonamides (Lindskog, 1963; Pocker and Meany, 1965a,b, 1967a; Pocker and Stone, 1967, 1968a; Coleman, 1967; Chen and Kernohan, 1967; Galley and Stryer, 1968; Pocker and Dickerson, 1968; Lindskog and Thorslund, 1968; Lindskog, 1969; Taylor *et al.*, 1970; Taylor and Burgen, 1971; Pocker and Watamori, 1971; Pocker and Guilbert, 1972; Pocker and Beug, 1972). The acetazolamide inhibition data of ANP hydrolysis (Figure 6) is in excellent agreement with that obtained using *p*-nitrophenyl acetate as substrate (Pocker and Stone, 1967, 1968a; Thorslund and Lindskog, 1967). In the present paper we have expanded the inhibition scheme of Thorslund and Lindskog (1967) to include the high pH inflection in enzymatic activity as well as the contribution made by the EHI^- species

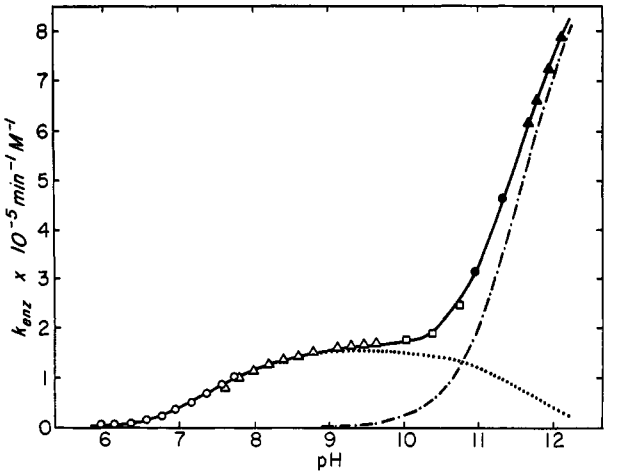

FIGURE 7: pH–activity profile and analysis of the contribution of [EH] and [E⁻] species of the bovine carbonic anhydrase catalyzed hydrolysis of 3-acetoxy-2-nitropyridine constructed according to eq 7 using values listed in Table II: (—) total k_{enz} value; (- - -) $k_{EH}[EH]/[E]_0$; (-·-) $k_E^-[E^-]/[E]_0$.

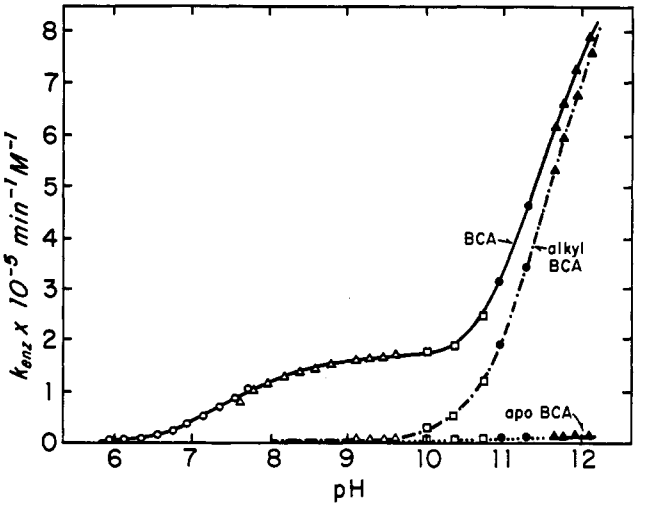

FIGURE 8: The pH variation of k_{enz} for the hydrolysis of 3-acetoxy-2-nitropyridine by the native (—), alkylated (-·-), and apo- (···) bovine carbonic anhydrase.

in the overall inhibition (*cf.* eq 8). The latter species is in equilibrium with both EH and EH_2I species. In the present work we have also noticed that pK_1' shifts to higher pK values if the enzyme is interacting with negatively charged species. Such shifts are also observed when the substrate is modified to include a nitro group (Table I; compare AP with either ANP or ADNP; note that all these results were obtained using identical buffer systems). One striking result which evolved from our present study is that the high pH rise can be eradicated by acetazolamide suggesting that the enzyme-bound zinc ion continues to play an essential role in the enzymatic catalysis at high pH. Furthermore, the pK_i–pH profile (Figure 6) of the acetazolamide inhibition studies reveals that the inhibition mechanism of Lindskog (1969) must be expanded to include new terms in order to accomodate the tailing off phenomenon observed in the high pH region of this profile. We would like to emphasize that the present data (Figures 6–8) are in complete accord with the earlier observations of Dr. J. T. Stone (Pocker, 1969; also Pocker and Stone, 1968[2]) concerning the high pH activity of bovine carbonic anhydrase. The present data are also consistent with our earlier working hypothesis in which we envisioned that the hydrated zinc ion and the ordered solvent structure in the active site are essential in controlling esterase activity and that at physiological pH this activity is further enhanced through hydrogen bonding to the basic form of amino acid residue, probably histidine. The second inflection, *i.e.*, the one observed in the alkaline region, was provisionally ascribed to the formation of a more reactive zinc–hydroxo complex. Especially reactive hydroxo complexes are apparently formed at high pH through the removal of one or more *additional* protons from the histidyl ligands bound to zinc. Thus, it has been recently demonstrated in these laboratories (Pocker and Guilbert, 1971[5]) that in a zinc–histidine chelate the N-3 proton of the imidazole ring titrates lower, $pK_a = 11.5$, than the equivalent proton in free histidine, $pK_a = 14.4$. It should be noted that the activity we claim is lost at physiological pH on alkylation is against pyridyl acetates, substrates which are considerably larger than the physiological substrate, carbon dioxide.

[5] Pocker, Y., and Guilbert, L. J. (1971), unpublished observations.

Meanwhile, important questions have been raised concerning the small residual catalytic activity at physiological pH of the "specifically" alkylated human isozymes B and C with respect to CO_2 hydration (Khalifah and Edsall, 1972). We wish to defer comment on this important paper until our own studies with this much smaller substrate are more complete but would like to draw attention to the following observations: (a) the alkylation of a histidine in or near the active site occurs predominantly but not exclusively at the 3′ position, and (b) the cases which are best documented with respect to residual activity are specifically those in which the alkylation replaces one general base, *i.e.*, the imidazole nitrogen in the native enzyme, by another, *i.e.*, the carboxylate ion of the modifier in the alkylated enzyme.

In recent X-ray studies, the Uppsala group has found evidence for the existence of a hydrogen-bonded network of water molecules in the active site that culminates in the water attached to the zinc atom. Our results indicate that in order to decide whether the modifiable histidine residues act around physiological pH as general bases and thereby promote CO_2 hydration or influence this enzymatic activity in a different, perhaps more subtle (secondary) role, it will be necessary to achieve full alkylation at the 3′ position of a specific histidine residue without at the same time introducing a new general base into the active site. Such alkylations are in progress using halogen derivatives of bifunctional substrates rather than of reversible inhibitors. In concluding this discussion it may be noted that the validity of our kinetic treatment (eq 16) receives strong support from the experimentally determined pH dependence of k_{enz} of the alkylated enzyme (Figure 8).

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