The Esterase Activity of Bovine Carbonic Anhydrase B above pH 9

REVERSIBLE AND COVALENT INHIBITION BY ACETAZOLAMIDE*

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The reversible complex between the metalloenzyme bovine carbonic anhydrase B and the sulfonamide inhibitor acetazolamide can be "frozen" irreversibly by the addition of a covalent bond between the methyl group of the inhibitor and the r-nitrogen of histidine-64. In both cases the inhibited enzyme is inactive as an esterase toward p-nitrophenyl propionate at physiological pH but retains activity controlled by an ionization in the protein exhibiting a pKₐ greater than 10. Similarly, both the covalently and reversibly inhibited enzymes in which the catalytically essential Zn(II) ion has been replaced with Co(II) display the same visible absorption spectrum which is invariant over the pH range from 5 to 12. The evidence therefore indicates that the position of the acetazolamide moiety in the active site is independent of both pH and the presence of the covalent bond to histidine-64. Moreover, when reversibly bound, this inhibitor has been shown to replace the water molecule (or hydroxide ion) known to occupy the fourth coordination position of the metal ion and frequently implicated in the catalytic mechanism of carbonic anhydrases. Thus, the activity exhibited by the inhibited enzymes and consequently the second rise observed in the pH rate profile of the native enzyme above pH 9 cannot reflect the ionization of such a water molecule in contrast to what has been postulated previously (Packer, Y., and Storm, D. R. (1968) Biochemistry 7, 1202-1214). Displacement of the zinc-bound solvent molecule rather than the alkylation of histidine-64 is suggested, however, as the cause of the inactivation of the alkylated enzyme round neutrality. Taken together, the biphasic pH rate profile of native bovine carbonic anhydrase B as well as the activity retained by the alkylated enzyme above pH 9 are best described by a model in which two groups in the enzyme ionize independently, thereby raising the possibility that the high pH activity is controlled by an ionization outside the active site region of the enzyme. Above pH 9.5 the pKₐ for the reversible interaction between native carbonic anhydrase and acetazolamide falls off linearly with increasing pH. The slope of -1.56 suggests that, among other factors, more than one ionization is responsible for the descending limb of the pKₐ-pH profile.

The zinc metalloenzyme carbonic anhydrase (EC 4.2.1.1) exhibits hydratase activity toward a wide variety of substrates. In each case, the activity requires the basic form of a group in the protein ionizing around neutrality. A similar ionization controls the binding of anionic and sulfonamide inhibitors and in each instance the same group is generally believed to be responsible. The identity of this group has become the subject of considerable discussion (1). X-ray crystallography at a resolution of 2 A has shown that in human carbonic anhydrase C the zinc ion is located at the base of a cleft and coordinated tetrahedrally to three imidazole rings (2). The fourth coordination position is occupied by a solvent water molecule and points out into the cleft (2, 3). Additional histidyl side chains protrude into the cavity in the region in front of the metal ion, the number and position depending upon the particular isoenzyme in question (1, 2). Most proposals for the catalytic mechanism of carbonic anhydrase have implicated either the zinc-bound water molecule (4, 5) or a histidyl residue (6-8) as the group ionizing around neutrality. In a series of papers since 1968, Packer et al. (8-14) have demonstrated that with certain substrates above pH 9 bovine carbonic anhydrase B exhibits a second rise in activity corresponding to an ionizing group with a pKₐ above 10.5. These authors have assigned the lower and higher points of inflection in the pH activity profile to a histidyl residue and to the zinc-bound water molecule, respectively.

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Substitution of cobalt for zinc does not appear to perturb grossly the structural or functional properties of the enzyme. Cobaltous bovine carbonic anhydrase retains 100% of the esterase activity of the native enzyme toward p-nitrophenyl acetate (15) and 45% of the carbon dioxide hydration activity (16, 17). The pH dependence of the catalysis (15, 18), the inhibition (19-22), and the metal binding (23) remain largely unchanged. Similarly, neither the ultraviolet optical rotary dispersion curves (23-25) nor the position of the metal ion (3) are affected by the substitution. Although the tendency of cobalt to adopt higher coordination states has been noted (26, 27), studies of magnetic susceptibility (28), magnetic circular dichroism (29), and the visible absorption spectrum (26, 27) indicate that in bovine carbonic anhydrase B the cobaltous ion, like zinc, assumes a tetrahedral or distorted tetrahedral geometry. In view of the above, a covalently modified derivative of bovine carbonic anhydrase and its cobaltous analogue have been used in this study to test for the catalytic involvement of a metal-bound water molecule above pH 9.

Acetazolamide is known to be a powerful, reversible inhibitor of mammalian erythrocyte carbonic anhydrases (30). It has been shown that bromoacetazolamide, an electrophilic derivative, alkylates specifically the active site of bovine carbonic anhydrase B at the \( \gamma \)-nitrogen\(^1\) of histidine-64\(^{*}\) (33-35). The present report compares the effects of reversibly and covalently bound inhibitor on the kinetic and spectral properties of the zinc and cobaltous enzymes, respectively. The data argue against a catalytic mechanism in which a metal-bound water molecule ionizes above pH 9.

**Materials and Methods**

**Enzyme**—A crude mixture of bovine erythrocyte carbonic anhydrase isoenzymes was obtained as a lyophilized powder from Worthington Biochemical Corporation. The B isoenzyme was isolated in highly purified form according to reported procedures (34) and this material was used for all subsequent experiments. All enzyme was stored at \(-20^\circ\)C as a powder lyophilized from distilled water. Azo and cobaltous bovine carbonic anhydrase was prepared according to the method of Lindskog and Malmsröm (18). The preparation of bovine carbonic anhydrase B alkylated at histidine-64 by bromoacetazolamide has been described previously (33). The modified enzyme\(^*\) has been purified further by affinity chromatography using a method originally described by Whitney (36) and reported recently (37). Alkylated cobaltous enzyme was prepared from cobaltous carbonic anhydrase B by the same procedures.

**Inhibitors and Reagents**—Acetazolamide was the gift of Lederle Laboratories and was used without further purification. Bromoacetazolamide (33) and \( p \)-nitrophenyl propionate (38) were prepared as reported. \( p \)-Nitrophenyl acetate was purchased from Eastman Organic Chemicals and was recrystallized four times from anhydrous ether. U.S.P. grade ephedrine sulfate obtained from British Drug Houses was recrystallized once from methanol-acetone (10:1). Organic solvents were reagent grade and redistilled prior to use. All other chemicals were reagent grade or better and were used without further purification.

**Zinc and Cobalt Assays**—Enzyme was analyzed for its zinc or cobalt content by atomic absorption spectrometry using a Perkin-Elmer model 303 spectrometer equipped with a three-slot burner. Protein concentration was approximately 1 and 6 mg/ml for the zinc and cobaltous enzymes, respectively, and solutions were prepared in distilled water. No corrections were applied for sample viscosity (39).

**Electrophoresis**—Electrophoresis on starch gel was performed at pH 3.5 according to reported procedures (40).

**Buffer Preparation**—Several enzyme experiments involving \( p \)-nitrophenyl propionate, buffer solutions were prepared using equivalent amounts of the free base and an aqueous solution of sulfuric acid, except in the case of ephedrine which was commercially available as the sulfate salt. The pH was adjusted with aqueous sodium hydroxide.

In all other cases a solution of the free base was acidified to the desired pH with the appropriate acid, pH was measured using a Radiometer pH meter model PHM 26 equipped with a combination electrode (type GK2301C).

**Determination of Enzyme and Substrate Concentration**—In all enzyme concentration was estimated spectrophotometrically from the absorbance at 280 nm measured in a Beckman DU spectrophotometer. The molar absorptivity of native, apo, and cobaltous bovine carbonic anhydrase B was taken as \( 5.6 \times 10^{4} \text{M}^{-1} \text{cm}^{-1} \) (41). A value of \( 6.08 \times 10^{4} \text{M}^{-1} \text{cm}^{-1} \) was determined for the purified alkylated enzyme by relating the absorbance of the protein solution at 280 nm to the zinc ion concentration of the same solution as determined by atomic absorption spectrometry. The concentration of \( p \)-nitrophenol was estimated at either 548 nm (\( \epsilon_{400} = 5,400 \text{M}^{-1} \text{cm}^{-1} \) (42)) or 400 nm. In the latter case the ratio of phenol (\( \epsilon_{400} = 200 \text{M}^{-1} \text{cm}^{-1} \)) to phenolate anion (\( \epsilon_{400} = 18,100 \text{M}^{-1} \text{cm}^{-1} \)) was calculated from the pH using the value 7.1 reported for the pK, of this ionization (43). The molar absorptivity of \( p \)-nitrophenol acetate at 345 nm was taken as \( 200 \text{M}^{-1} \text{cm}^{-1} \) (42).

**Spectral Measurements**—Visible absorption spectra of the cobaltous enzyme were recorded in a Cary 15 recording spectrophotometer using 5-cm quartz cells (Hellma). To study the effect of pH on their spectra, protein solutions (0.09 mM) were prepared in distilled water containing, when required, a 1.2-fold molar excess of acetazolamide and were centrifuged prior to use to remove suspended material. The pH was adjusted by the addition of aqueous sodium hydroxide or sulfuric acid using a Hamilton microsyringe. Cobaltous bovine carbonic anhydrase B was titrated spectrophotometrically with acetazolamide according to a procedure similar to that of Lindskog and Thorslund (21). Separate solutions of enzyme (0.12 mM) and inhibitor (0.01 mM) were prepared in sodium sulfate (25 mM), adjusting the pH with aqueous sodium hydroxide (1.0 N). The protein solution was centrifuged to remove suspended material and the spectrum recorded at 25°C. Known volumes of the two solutions then were mixed and allowed to equilibrate for at least 15 min. If required, the pH was readjusted while stirring magnetically by the addition of a recorded volume of sodium hydroxide (1.0 N) from a microsyringe. The solution then was transferred to the cuvette using a glass syringe fitted with a Millipore GF15013YP adapter containing a glass fiber filter (RB filters, BF3, 0.45 \( \mu \)m). The process was repeated with additional aliquots of the inhibitor solution until dilution of the enzyme resulted in an absorbance reading at 580 nm of 0.1 or less.

**Enzyme Activity and Inhibition**—All kinetic measurements were carried out at 25°C in one of three buffers used to encompass the pH range from 5 to 10.5: 2.2'-bis(hydroxyethyl)-2,2'-nitrilotriethanol (bis-tris), pH 5 to 7.2, Tris (pH 7.2 to 9.2), and ephedrine (pH 9.2 to 10.5). Above pH 8, considerable attention was paid to the adjustment and maintenance of pH due to the relatively high rate of nonenzymic hydrolysis. In the case of inhibited enzyme this contribution represented as much as 88% of the observed rate. Buffer solutions were therefore readjusted to within 0.01 unit of the appropriate pH following the addition of either enzyme or inhibitor and subsequent manipulation were carried out in this pH range. Buffer solutions of 0.1 or less.

Initial rates of hydrolysis were measured in a Durum Gibson stopped flow spectrophotometer using solutions made up with previously degassed water. Aqueous solutions of \( p \)-nitrophenyl acetate (2 mM) and \( p \)-nitrophenyl propionate (1.6 mM) were prepared by injecting into distilled water with a Hamilton syringe a stock solution of the ester (2.25 mM) in distilled acetone. The pH of the water used was adjusted by injecting either 0.1 N, 2.2'-bis(hydroxyethyl)tetramethyleneamine (bis-tris), or 1.0 N sodium hydroxide (1.0 N) from a microsyringe. The solution then was transferred to the cuvette using a glass syringe fitted with a Millipore GF15013YP adapter containing a glass fiber filter (RB filters, BF3, 0.45 \( \mu \)m). The process was repeated with additional aliquots of the inhibitor solution until dilution of the enzyme resulted in an absorbance reading at 580 nm of 0.1 or less.

\( 1 \) The notation used follows the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature (31).

\( 2 \) Following the suggestion of Andersson et al. (32), the established numbering of residues in human carbonic anhydrase B has been applied to the human C and bovine B enzymes as well.

\( 3 \) When used in connection with bovine carbonic anhydrase B the terms “alkylated” and “modified” refer to the enzyme in which acetazolamide is covalently linked to the \( \gamma \)-nitrogen atom of histidine-64 via the acetylamo group of the inhibitor.
mixing the enzyme and substrate solutions in a ratio of 1:1 in the stopped flow apparatus and the increase in absorbance was followed at either 348 nm (below pH 9) or 400 nm (above pH 9) by means of a storage oscilloscope connected via a log convertor to the photomultiplier. In all cases the total change reflected the hydrolysis of less than 1.6% of the total amount of substrate present. Initial velocities were estimated from the average slope of four oscilloscope traces and corrected for the nonenzymic component of the rate. At higher inhibitor concentrations, this correction included a small but significant contribution due to the presence of acetazolamide.

Second order rate constants for the enzyme-catalyzed hydrolysis of p-nitrophenyl propionate were determined by a method similar to that of Pocker and Storm (8) except that monovalent anions were avoided throughout. In each case the reaction was initiated by injecting 24 μl of a solution of substrate in distilled acetone into 3.0 ml of the enzyme solution contained in a 1-cm cuvette to yield a final ester concentration of 0.05 mM. The course of the hydrolysis was followed by monitoring the increase in optical density for two half-lives on a double beam spectrophotometer. The solution then was stored under argon for a minimum of eight half-lives and subsequently returned to the spectrophotometer for measurement of the optical density at infinite time. Above pH 8 the reaction was followed at 400 nm in a Cary 15 spectrophotometer, whereas around neutrality a Unicam model SP1800 was used at 348 nm. Pseudo-first order rate constants, kobs, were calculated according to the expression \( \ln(A_0 - A) = -k_{obs}t + \ln A_0 \), where \( A_0 \) and \( A \) are the absorbance readings after at least 10 half-lives and at time \( t \), respectively. At each pH the second order rate constant was calculated from the slope of a plot of \( k_{obs} \) versus the total enzyme concentration.

Treatment of Data—Best fit straight lines were obtained by the method of least squares. Least squares estimates of parameters defining nonlinear expressions were obtained by an iterative technique based on the method of Marquardt (44).

RESULTS

Enzymes—Enzyme preparations used in this investigation were essentially identical to those obtained previously in this laboratory (33-35). Purified bovine carbonic anhydrase migrated toward the anode as a single band during electrophoresis on starch gel and catalyzed the hydrolysis of 1 mM p-nitrophenyl acetate at a rate of 55.9 M (M·min)\(^{-1}\) (S.D. 1.4) at 25° and pH 7.6 in 0.075 M Tris-sulfate buffer. The apoenzyme contained less than 0.05 g ion of zinc and the cobaltous derivative 0.96 g ion of cobalt per mol of protein. Migration during starch gel electrophoresis was identical to that of the native zinc enzyme. Following purification by DEAE-cellulose and affinity chromatography, alkylated bovine carbonic anhydrase exhibited less than 0.4% of the native esterase activity toward 1 mM p-nitrophenyl acetate at pH 7.6 in 0.075 M Tris-sulfate. This value compares favourably with that of 0.1% found for the native enzyme in the presence of a 5-fold molar excess of acetazolamide. The modified enzyme migrated toward the anode as a single band ahead of the native enzyme during electrophoresis on starch gel and yielded on acid hydrolysis 1 eq of His (τ CM). The alkylated cobaltous and zinc enzymes were electrophoretically identical.

pH Dependence of p-Nitrophenyl Propionate Esterase Activity In the presence of native or alkylated bovine carbonic anhydrase B and under the conditions used the hydrolysis of p-nitrophenyl propionate exhibits pseudo-first order kinetics over the first two half-lives of the reaction (Fig. 1). The correlation coefficient obtained for the best straight line in plots of \( -\ln (A_0 - A) \) versus time was typically nine to four places of decimal. Given the attainment of a steady state, the observed pseudo-first order rate constant, \( k_{obs} \), is formally equal to \( k_z[E]/K_m + k_f \), where \( k_z \) represents the turnover number, \( K_m \) the Michaelis constant, and \( k_f \) the total contribution of all nonenzymic components of the rate. As illustrated in Fig. 2, \( k_{obs} \) increases linearly with the total enzyme concentration for both native and alkylated carbonic anhydrases. The slope of this relationship represents \( k_m \), the second order rate constant for the enzyme-catalyzed hydrolysis.

Due to the relatively alkaline conditions of these experiments it was necessary to confirm that the kinetic properties of the enzyme were stable over the period of time required for the measurement of \( k_{obs} \). Using the alkylated enzyme, two duplicate series of protein solutions were prepared at pH 10.25 and the value of \( k_{obs} \) was obtained for each solution, allowing a period of approximately 2 hours between the measurements of Series 1 and those of Series 2. When \( k_{obs} \) was plotted versus the enzyme concentration the two values obtained for the second
order rate constant of the enzymic hydrolysis agreed to within 3%.

Small decreases (<0.05 pH unit) in the pH of the sample over the course of the reaction above pH 10 were shown by control experiments to result from the absorption of carbon dioxide. In the alkaline region the effect of such a shift on the measured value of \( A_0 \) is negligible. Similarly, possible effects on the measured pseudo-first order rate constants also were neglected and for the purpose of subsequent comparisons the pH was taken as that at the beginning of the reaction. A small (<0.5%) but consistent increase in the value of \( A_0 \) was observed between the blank solution and that of highest protein concentration at any particular pH. This change is thought to reflect product binding to the protein, but the linearity of the semilogarithmic plots of product formation versus time (Fig. 1) indicates that under the conditions of the experiment the catalysis is not affected to any appreciable degree.

The pH dependence of the second order rate constant, \( k_{\text{enz}} \), for the hydrolysis of p-nitrophenyl propionate in the presence of bovine carbonic anhydrase exhibits two inflection points (Fig. 3, Curve 1) and in good qualitative agreement with that originally reported by Pocker and Storm (8). It has been pointed out (13) that such a biphasic relationship can be described in principle by the models illustrated in Equations 1 and 2, which relate the observed second order rate constant of the catalysis to the hydrogen ion concentration.

\[
k_{\text{enz}} = \frac{[H^+] K_1 k_{\text{EH}} + K_1 K_2 k_E}{[H^+]^2 + [H^+] K_1 + K_1 K_2}
\]

Equation 1

\[
k_{\text{enz}} = \frac{K_1 k_{\text{EH}}}{[H^+] + K_1} + \frac{K_2 k_E}{[H^+] + K_2}
\]

Equation 2

The first model (Equation 1) describes the behavior of a single dibasic group in which the alkaline form is achieved at the expense of the neutral form, which is achieved in turn at the expense of the acid form. The second (Equation 2) reflects two monobasic groups each of which ionizes independently of the other. In either case \( K_1 \) and \( K_2 \) represent dissociation constants of the protons ionizing at lower and higher pH respectively, while \( k_{\text{EH}} \) and \( k_E \) represent the maximum contribution of each ionization of the total activity. Either equation can be shown to be of the form of Equation 3

\[
k_{\text{enz}} = \frac{A[H^+] + B}{[H^+]^2 + C[H^+] + D}
\]

Equation 3

where \( A, B, C, \) and \( D \) are various combinations of the constants \( K_1, K_2, k_{\text{EH}}, \) and \( k_E. \) Consequently, Curve 1 in Fig. 3 represents the best fit of either model to the data points and the appropriate values of the constants obtained by iteration are summarized in Table I. For comparison, the data of Pocker and Storm (8) for the same substrate and enzyme have been fitted in the same manner and these constants have also been included in Table I. The reason for the quantitative difference between the present results and those reported previously is not clear.

The pH rate profile of the alkylated enzyme (Fig. 3, Curve 2) indicates that the activity associated with the group ionizing around neutrality has been lost, while that developing at higher pH has been retained. Equation 4, which describes the titration curve for a single ionization, has been fitted to the experimental data and the appropriate values of \( K_1 \) and \( k_E \) are presented in Table I.

\[
k_{\text{enz}} = \frac{K_2 k_E}{[H^+] + K_2}
\]

Equation 4

The pH dependence of the residual activity of the alkylated enzyme relative to that of the native enzyme is given by the quotient of Equation 4 divided by Equation 2. This is illustrated by the solid line in Fig. 4 which has been calculated according to the appropriate constants in Table I.

Although formally similar, Equations 1 and 2 represent two potentially dissimilar situations. Neglecting the possibility of allosteric effects, the first model (Equation 1) dictates that both ionizations occur within the same region of the protein surface. This is not a requirement in the second model (Equation 2) which describes two independent groups. Curves 3 and 4 in Fig. 3 are theoretical lines drawn to represent the titration of single ionizing groups (Equation 4) characterized by the values of \( k_E \) and \( pK_1 \) given in Table I for the best fit of Equations 1 and 2, respectively, to the data obtained for the native enzyme (Fig. 3, Curve 1). These curves illustrate the contribution made by the group of higher \( pK_1 \) to the total catalysis in each model. The data obtained for the alkylated enzyme (Curve 2), although not superimposable with either of Curves 3 or 4, is in better agreement with that obtained from the "two-site" model of Equation 2 (Curve 4). The extrapolated value for \( k_E \) of 5,280 M\(^{-1}\) min\(^{-1}\) (Table I) obtained for the alkylated enzyme agrees well with that of 5,580 M\(^{-1}\) min\(^{-1}\) calculated for the native enzyme according to Equation 4.

*Equations 1 and 2 are hereinafter referred to as the one-site and two-site models, respectively.*
TABLE I

Parameter values for pH rate profiles of the hydrolysis of p-nitrophenyl propionate by native and alkylated bovine carbonic anhydrase B

By iterating about the values of $K_a$, $K_b$, $k_{EH}$, and $k_E$, Equations 1, 2, and 4 were fitted to the pH rate data obtained as described in the legend to Fig. 3 and under “Materials and Methods” for the enzymic hydrolysis of $p$-nitrophenyl propionate. The deviations represent the 95% confidence limits of the parameters.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Equation</th>
<th>$pK_1$</th>
<th>$pK_2$</th>
<th>$k_{EH} \times 10^{-3}$</th>
<th>$k_E \times 10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine carbonic anhydrase B</td>
<td>1\textsuperscript{a}</td>
<td>6.89 ± 0.06</td>
<td>10.06 ± 0.23</td>
<td>7.97 ± 0.21</td>
<td>13.55 ± 1.18</td>
</tr>
<tr>
<td></td>
<td>2\textsuperscript{b}</td>
<td>6.89 ± 0.06</td>
<td>10.06 ± 0.23</td>
<td>7.97 ± 0.21</td>
<td>5.55 ± 1.07</td>
</tr>
<tr>
<td>Data of Focket and Stern\textsuperscript{c}</td>
<td>1\textsuperscript{a}</td>
<td>7.07 ± 0.11</td>
<td>10.90 ± 0.48</td>
<td>6.06 ± 0.28</td>
<td>27.06 ± 15.03</td>
</tr>
<tr>
<td></td>
<td>2\textsuperscript{b}</td>
<td>7.07 ± 0.11</td>
<td>10.90 ± 0.48</td>
<td>6.06 ± 0.28</td>
<td>21.00 ± 14.70</td>
</tr>
<tr>
<td>Alkylated bovine carbonic anhydrase B</td>
<td>4\textsuperscript{c}</td>
<td>10.22 ± 0.20</td>
<td>5.26 ± 1.47</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} The “one-site” model in which a single group or interrelated groups undergoes two ionizations.

\textsuperscript{b} The “two-site” model in which two groups ionize independently of each other.

\textsuperscript{c} The ionization of a single titrating group.

\textsuperscript{d} Taken from an earlier publication (8) and presented here for the purpose of comparison.

alkylation exerts a secondary influence on the alkaline activity in that the value of $pK_4$ is shifted upward by 0.46 pH unit. It also is noted that the second term in Equation 2 cannot distinguish between a single ionization and several, all of which possess a similar $pK_a$.

Reversible Inhibition of Bovine Carbonic Anhydrase B by Acetazolamide Above pH 9

Above pH 9 the observed initial velocity, $V_i$, of the enzyme-catalyzed hydrolysis of $p$-nitrophenyl propionate can be resolved into two independent components according to Equation 5.

$$V_i = V_O + V_C$$

The reversible binding of 1 eq of inhibitor per molecule of enzyme completely eliminates that portion of the activity represented by $V_O$, whereas the inhibitor can neither bind nor inhibit at the site corresponding to $V_C$. Thus, in the presence of a given concentration of inhibitor, the value of $V_O$ is reduced to $V_i$ such that

$$V_i = V_O + V_C$$

If Equation 6 is divided by Equation 5 and $(1 - V_O/V_C)$ is set equal to the fractional inhibition, $i$, one obtains Equation 7.

$$V_i = (1 - i)(V_O + V_C)$$

Substitution of the expression given by Webb (45) for the fractional inhibition yields Equation 8 which relates the observed initial velocity, $V_i$, to the total concentrations of inhibitor, $[I]$, and enzyme, $[E]$. $K_i$ represents the dissociation constant for the interaction between the enzyme and the inhibitor at the catalytic site susceptible to inhibition. The value of the ratio $[S]/K_a$ representing the free substrate concentration divided by the substrate binding constant has been set equal to zero thereby assuming noncompetitive inhibition. This assumption gave results consistent with our spectral data and eliminated the necessity of measuring $K_i$ or $K_a$. It is pointed out that Equation 8 corresponds to the Zone B mutual depletion system of Straus and Goldstein (46) and Goldstein (47) and as such embodies no approximations regarding the relative amounts of inhibitor and enzyme.
At alkaline pH acetazolamide only partially inhibits the p-nitrophenyl propionate esterase activity of bovine carbonic anhydrase B as illustrated at pH 10.19 in Fig. 5. The solid line represents the best fit of Equation 8 to the data points achieved by iterating about the values of $V_c$ and $K_i$. The value of $V_c$ obtained in this manner represents the activity of the enzyme extrapolated to an infinite concentration of the inhibitor and in this case is equal to 13.7% of the native activity given by $V'_c$. Repetition of this treatment as the pH is raised from 9.51 to 10.38 indicates that a decrease in the binding constant is accompanied by an increase in the proportion of the total activity which cannot be inhibited by saturating amounts of acetazolamide (Table II). The development of acetazolamide resistant activity above pH 9 suggests, in terms of the model described by Equation 8, that an independent, activity linked group in the enzyme ionizes in the alkaline region. As illustrated in Fig. 4, the pH dependence of the per cent residual activity of the reversibly inhibited enzyme (the data points) closely parallels that of the alkylated enzyme (the solid line), suggesting that in either case the same ionization is responsible for the high pH catalysis. The pK, obtained in this manner is presented as a function of pH in Fig. 6 (open circles). It is pointed out that the highest acetazolamide concentration used

![Equation 8]

\[
V_i^t = \left[ \frac{\left( \frac{[I_t] + [E]_t}{K_i} + [S] + 1 \right)}{\left( \frac{[E]_t}{K_S} + 1 \right)^2} \right] \left( \frac{2[E]_t}{K_i} \right)
\]

obtained in this manner represents the activity of the enzyme.

Fig. 5. Effect of acetazolamide on the p-nitrophenyl propionate esterase activity of bovine carbonic anhydrase B. Solutions containing enzyme (27.2 μM) and various concentrations of inhibitor in ephedrine sulfate buffer (0.1 N, pH 10.19) were mixed with equal volumes of a solution of substrate (1.6 mM) in distilled water and acetone (0.64% v/v) at 25° in a stopped flow spectrophotometer. The increase in absorbance was followed at 400 nm and the measured initial velocity was corrected for the nonenzymic component of the rate. The line represents the best fit of Equation 8 to the data points and was obtained by iterating about the values of $K_i$ and $V_c$. Further details are given under “Materials and Methods.”

### Table II

**Parameter values for reversible association of acetazolamide with zinc and cobaltous bovine carbonic anhydrase B**

Values of the parameters were obtained by iteration in fitting Equations 8 and 9 to kinetic (below pH 10.5) and spectral (above pH 10.5) data, respectively. The deviations represent the 95% confidence limits. $V'_t$ and $V_t$ represent the initial rates of enzymic hydrolysis of p-nitrophenyl propionate obtained for the free and acetazolamide-saturated enzymes, respectively. $\epsilon_{580}$ is the observed molar absorptivity at 580 nm of the cobaltous enzyme in the presence of saturating concentrations of acetazolamide. Further details are given under “Materials and Methods.”

<table>
<thead>
<tr>
<th>pH</th>
<th>pK_i</th>
<th>$\epsilon_{580}$</th>
<th>$100 \frac{V'_t}{V_t}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.51</td>
<td>5.72 ± 0.10</td>
<td>5.68 ± 1.57</td>
<td>56.8 ± 1.57</td>
</tr>
<tr>
<td>9.85</td>
<td>5.27 ± 0.12</td>
<td>10.42 ± 2.40</td>
<td>10.42 ± 2.40</td>
</tr>
<tr>
<td>10.19</td>
<td>4.78 ± 0.12</td>
<td>13.68 ± 4.27</td>
<td>13.68 ± 4.27</td>
</tr>
<tr>
<td>10.38</td>
<td>4.49 ± 0.10</td>
<td>20.14 ± 2.95</td>
<td>20.14 ± 2.95</td>
</tr>
<tr>
<td>10.62</td>
<td>3.96 ± 0.20</td>
<td>363 ± 22</td>
<td>363 ± 22</td>
</tr>
<tr>
<td>11.02</td>
<td>3.34 ± 0.05</td>
<td>395 ± 11</td>
<td>395 ± 11</td>
</tr>
<tr>
<td>11.52</td>
<td>2.74 ± 0.11</td>
<td>396 ± 36</td>
<td>396 ± 36</td>
</tr>
</tbody>
</table>

Fig. 6. pH dependence of the affinity of acetazolamide for cobaltous and zinc bovine carbonic anhydrase B. Below pH 10.5 the affinity was estimated kinetically (○) using the zinc enzyme and measuring the initial velocity of p-nitrophenyl propionate hydrolysis in the presence of various concentrations of acetazolamide as described in the legend to Fig. 5. At higher pH, determinations were made spectrophotometrically (□) using mixtures of the cobaltous enzyme and the inhibitor as described in the legend to Fig. 8. Values of $K_i$ were obtained by iteration in fitting Equations 8 and 9 to the kinetic and spectral data, respectively. Confidence limits of 95% are indicated by the bars and the point at pH 9.51 was not included in the linear regression.
The results of this treatment (Table II) indicate that as the pH is raised from 10.6 to 11.5 the molar absorptivity of the enzyme-acetazolamide reversible complex remains constant while the $K_i$ for the interaction increases by a factor of 18. An experimental value obtained from the absorbance of the protein after extrapolation to infinite acetazolamide concentration around neutrality. At lower pH, however, saturation of the enzyme is achieved with equimolar amounts of the inhibitor, whereas much higher concentrations are required in the alkaline region. At any given pH the spectral change can be related to the total inhibitor and enzyme concentrations according to Equation 9 which represents the simple, mass action expression for the interaction between inhibitor and enzyme in the absence of other active site specific components. 

$$\Delta A = \frac{b}{2} (\epsilon_1 - \epsilon_c) \left( \frac{[I]}{[E_i]} + \frac{[E_i]}{[I]} \right)$$

Equation 9

As the pH is raised to 11.8, the spectrum of the enzyme in the presence of reversibly bound acetazolamide reverts to that of the free enzyme at the same pH, indicating that the sulfonamide moiety is displaced from the metal ion by the group displaced is either a water molecule or a hydroxide ion. The pH dependence of the absorbance at 580 nm as a function of the total acetazolamide concentration was fitted using Equation 9 and the residuals have been plotted versus the inhibitor to enzyme ratio. Further details are given under "Materials and Methods."

**Spectral Properties of Cobaltous Enzymes**—At pH 5.4 the same characteristic visible absorption spectrum is exhibited by both modified cobaltous bovine carbonic anhydrase and the native enzyme, having the presence of an equimolar amount of acetazolamide (Fig. 7). The similarity indicates that the alkylating agent displaced occurs in the fourth ligand position of the metal ion, thereby displacing the group normally present in the native enzyme (48). It has been demonstrated by both proton relaxation studies (49) and x-ray crystallography (50) that the group displaced is either a water molecule or a hydroxide ion. As the pH is raised to 11.8, the spectrum of the enzyme in the presence of reversibly bound acetazolamide reverts to that of the free enzyme at the same pH, indicating that the sulfonamide moiety is displaced from the metal ion by the group occupying this position in the native enzyme. Lindskog has shown that the $pK_a$ for this transition exceeds 11.2 (48). In contrast, the spectrum exhibited by the modified enzyme is unperturbed by changes in pH between 7 and 12, indicating that the covalent bond between the acetylamino group of the inhibitor and histidine-64 precludes the displacement observed in the case of the reversible complex. The spectral data therefore demonstrates that in the modified enzyme the sulfonamide group excludes from the fourth coordination position of the metal ion the water molecule or hydroxide ion occupying this position in the native enzyme.

Above pH 10 the addition of sucrose amounts of acetazolamide to a solution of cobaltous bovine carbonic anhydrase results in spectral changes identical with those observed during these measurements was 2.2 mM and thus inhibition exhibiting a $K_i$ greater than about $10^{-2.5}$ would not be detected.
Enzyme-catalyzed hydrolysis of methyl pyridyl carbonates, the cobaltous and zinc enzymes, respectively, produce consistent results. Moreover, in the region around pH 10, similar partial inhibition has been reported (12) for the spectral and kinetic assays for the binding of acetazolamide to a group (or groups) exhibiting a pKₐ, around 10.5. Although a model in which a catalysis developing at higher pH and mammalian erythrocyte carbonic anhydrases (32, 52, 53) and human C enzyme carboxyketomethylated at this position in the catalytic mechanism. Gijthe and Nyman have pointed out below pH 9 where the contribution of the second ionization to the pKₐ of the catalysis around neutrality. The complete inactivation observed in the region of the first point of inflection would appear to reflect the displacement of whatever group occupies the fourth coordination position of the metal ion around neutrality, the alkylated enzyme retains esterase activity corresponding to the basic point of inflection in the biphasic pH rate profile exhibited by the native enzyme. Visible absorption spectra of this derivative in which cobalt(II) replaces zinc suggest that the sulfonamide group of acetazolamide occupies the fourth coordination position of the metal ion at any pH between 5 and 12. The concomitant displacement of the solvent water molecule believed (1) to occupy the same position in the native enzyme rules out the implication of such a group in the catalysis developing above pH 9. It follows that the presence in bovine carbonic anhydrase of an activity-linked histidine-64. Apart from the metal ligands, histidine-64 is the histidyl residue as that controlling the catalysis around neutrality. While the pH rate profile exhibited by the native enzyme can be described by more than one model, a comparison with that of the alkylated enzyme suggests that the two points of inflection reflect at least two independently ionizing groups. Whereas the present evidence is in good accord with the two-site model, the observations of previous investigators have been interpreted in terms of the alternative, one-site model (8–13).

In contrast to an earlier report of complete inhibition (8), the present data indicate that above pH 9 reversibly bound acetazolamide reduces only partially the native esterase activity toward p-nitrophenyl propionate. As in the case of the alkylated enzyme, the kinetic pattern can be described by a model in which a catalysis developing at higher pH and unaffected by acetazolamide is controlled by an independent group (or groups) exhibiting a pKₐ around 10.5. Although a similar partial inhibition has been reported (12) for the enzyme-catalyzed hydrolysis of methyl pyridyl carbonates, the data was interpreted in terms of the scheme of Webb (51) for partial inhibition at a single catalytic site. The latter model requires, however, that the inhibition caused by acetazolamide at the active site vary from complete to partial as the pH is increased from around neutrality to above pH 9. Such a change is not in accord with the observation that the molar absorptivity of the cobaltous enzyme-acetazolamide reversible complex remains constant while the pH is raised to 11.5. The visible absorption spectrum is known to be highly characteristic of the complex reflecting in particular the ligand field of the metal ion. Since the metal ion is closely associated with both the activity and the inhibition, variations in either pattern would appear to be unlikely in the absence of any change in the spectrum. Moreover, the spectral data indicate that even under relatively alkaline conditions neither water nor hydroxide ion is able to compete successfully with acetazolamide at the metal ion when the inhibitor is present in sufficiently high concentration. Any perturbation restoring part or all of the catalytic activity at the active site would be expected to involve such a displacement of the inhibitor.

The identity of the activity-linked group (or groups) exhibiting a pKₐ greater than 10 remains speculative. Although assignment to a residue outside the active site seems attractive, the observation of Pocker et al. (9, 13) that the apoenzyme is completely inactive suggests that a specific interaction is nevertheless involved. Moreover, any assignment based on intrinsic pKₐ alone must take into consideration the reports that the modification of 1 tyrosyl residue as well as all lysyl and arginyl residues in the enzyme has no effect on the activity (8, 9, 12). Kinetic studies above pH 9 using proteins such as serum albumin might contribute to the resolution of this question.

The insensitivity of the acetazolamide moiety in the modified bovine carbonic anhydrase indicates that the activity exhibited above pH 9 by this derivative reflects a retention of the second point of inflection observed with the native enzyme rather than an upward shift in the pKₐ of the catalysis around neutrality. The complete inactivation observed in the region of the first point of inflection would appear to reflect the displacement of whatever group occupies the fourth coordination position of the zinc ion rather than the alkylation of histidine-64. Apart from the metal ligands, histidine-64 is the only active site histidyl residue known to be conserved in all mammalian erythrocyte carbonic anhydrases (32, 52, 53) and therefore might be a reasonable candidate for an essential role in the catalytic mechanism. Göthe and Nyman have pointed out, however, that the presence of residual activity in the human C enzyme carboxyketomethylated at this position weighs against such a role (54).

The continuity of the pKₐ-pH profile (Fig. 6) suggests that the spectral and kinetic assays for the binding of acetazolamide to the cobaltous and zinc enzymes, respectively, produce consistent results. Moreover, in the region around pH 10 noncompetitive kinetics continue to be observed notwithstanding the rapid increase in the value of Kᵢ. This follows from our observation that points calculated assuming competitive inhibition do not appear to lie on the same line as the points obtained with the spectral analysis, the treatment of which involves no such kinetic assumptions. This is in accord with the previous observation that the noncompetitive nature of the inhibition is due to the slow dissociation rate of the enzyme-acetazolamide complex relative to the time scale of kinetic determinations, as well as reports that the association rate and not the dissociation rate accounts for the pH dependence of the Kᵢ (21, 22, 55). Under the conditions used, the value of pKₐ,
appears to be a linear function of pH exhibiting a slope of −1.56 (Fig. 6). A model in which the enzyme-acetazolamide reversible interaction is controlled by two groups ionizing within 0.5 pH unit of neutrality predicts, however, that above pH 9.5 the slope of the pKp-pH profile is −1. Thus, as illustrated in Fig. 6 and pointed out by Lindskog and Thorslund (21), cobaltous bovine carbonic anhydrase deviates from the theoretical pattern in a manner that suggests the involvement of a third ionization such as the amide group of the inhibitor. A minimum slope of −2 is expected, however, when two ionizing groups control the descending limb of the pH affinity curve. The failure to attain such a value could reflect a pH-independent component of the rate of formation of the complex. Indeed, such a leveling off of the pKp at high pH has been observed by Pocker and Watamori (13) using the Zn(II) bovine enzyme and 3-acetoxy-2-nitropyridine as substrate. 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