The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. VI. Kinetic Studies of Noncompetitive Inhibition of Enzyme-Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate*

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ABSTRACT: The esterase activity of bovine carbonic anhydrase has been found to be reversibly inhibited by a large variety of monovalent anions as well as by sulfonamides, amino acids, alcohols, phenol, acetonitrile, and acetone. Anion, sulfonamide, and alcohol inhibitions are noncompetitive with respect to substrate; the former two decrease with pH while alcohol inhibition is not affected by pH. Chloride ions act at a site which behaves like an electrophilic center of pK_a *ca.* 7.5. 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. Likewise sulfonamides produce inactivation by the formation of 1:1 enzyme–inhibitor complexes. Anions such as CNO^-, I^-, and HCO_3^- were found to compete with each other as well as with acetazolamide for the same or for a nearby interacting site which is presumably situated at or near the chelated zinc ion present in the native enzyme and hence near or at a neighboring imidazolium ion. This anion binding site is different from the ester binding site. The sulfonamide binding site actually consists of at least two regions, one a hydrophobic region which attracts the aromatic portion of the sulfonamide, the other a zinc ion containing region which is required for the strong binding of the inhibitor. The dissociation constant of the acetazolamide–enzyme complex rises dramatically at pH >10 as if dependent upon a group in the enzyme of pK_a *ca.* 11. Anion inhibition was found to follow the order $CN^- > HS^- > CNO^-$, SCN^-, $N_3^- > I^-$, $ClO_4^- > HCO_3^-$, $HSO_3^- > NO_3^- > Br^- > AcO^- > Cl^- > F^-$. The order observed with the very strong anionic inhibitors appears to parallel their association constants with zinc, while the order observed with the moderate and weak anionic inhibitors appears to parallel the Hofmeister lyotropic series. The data suggest that the binding site for noncompetitive inhibitors is sensitive to two closely situated electrophilic (cationic) groups, one of pK_a *ca.* 7 and the other of $pK_a > 10$. These findings are shown to parallel the behavior of the esteratic site of carbonic anhydrase.

Studies of specific effects of various inhibitors have often provided important avenues for elucidating the mode of enzyme action. The inhibition of the carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) catalyzed hydration of carbon dioxide has been studied with respect to the effects of anions (Keilin and Mann, 1940; Roughton and Booth, 1946; DeVoe and Kistiakowsky, 1961; Kernohan, 1965) and sulfonamides (Mann and Keilin, 1940; Davis, 1959; Krebs, 1948; Maren *et al.*, 1960; Leibman *et al.*, 1967). The attention given to the former inhibitors is due to their physiological and chemical importance and that given to the latter inhibitors is both due to their importance in molecular pharmacology and to the potent, specific nature of their inhibition. In recent years it has been found in our laboratories that carbonic anhydrase also catalyzes the reversible hydration of a number of aldehydes (Pocker and Meany, 1965a,b, 1967a,b; Pocker and Dickerson, 1968) and that the zinc ion, which is strongly bound to the enzyme, is just as essential for aldehyde as for CO_2 hydration. Moreover the enzymatic hydrations of both acetaldehyde and propionaldehyde are strongly inhibited by sulfonamides and to a lesser degree by anions (Pocker and Meany, 1965b; Pocker and Dickerson, 1968).

The hydrolysis of a number of esters was also found to be catalyzed by carbonic anhydrase (Pocker and Stone, 1965, 1967; Pocker and Storm, 1968; *cf.* also, Tashian *et al.*, 1964; Malmström *et al.*, 1964; Verpoorte *et al.*, 1967; Thorslund and Lindskog, 1967). The pH–rate profile and the effect of zinc removal are similar for both hydrase and esterase activity, probably because the same functional groups in the enzyme are involved in both cases. Furthermore the same compounds which inhibit hydrase activity also inhibit esterase activity (Pocker and Stone, 1965, 1967; Verpoorte *et al.*, 1967; Thorslund and Lindskog, 1967; Pocker and Storm, 1968).

In the subsequent paper in this series, it will be shown that a number of carbonyl-containing substrates as well as substrate analogs competitively inhibit the enzymatic hydrolysis of *p*-nitrophenyl acetate, while bicarbonate ion and aldehyde hydrates, *i.e.*, substrates whose dehydration is enzyme catalyzed, act noncompetitively with respect to ester hydrolysis (Pocker and Stone, 1968). In the present investigation we report on a detailed study of the inhibition of esterase activity by an-

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ions, sulfonamides, and various nucleophilic reagents. This study delineates the number of inhibitor molecules producing inactivation, their inhibitory potency, and the possible sites being employed.

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, mp 112. Buffer components included potassium dihydrogen phosphate, dipotassium hydrogen phosphate, Tris, and ephedrine and were analytical grade reagents. Bovine carbonic anhydrase preparation, storage, and properties have been described (Pocker and Stone, 1967). Common sodium salts, sulfonamides, alcohols, chelating agents, and other organic compounds were commercially available and of excellent purity for the inhibition studies undertaken.

Apparatus, procedure, and technique have been previously described (Pocker and Stone, 1967).

Inhibition Studies. The inhibition constant, K_i, represents the equilibrium between enzyme and inhibitor, $EI \rightleftharpoons E + I$. The magnitude of K_i was generally determined by either Lineweaver–Burk plots or plots of per cent activity, *i.e.*, V_i/V_0 *vs.* (I)/(E), the ratio of inhibitor to enzyme (Laidler, 1958; Webb, 1963). The procedure for determination of the Lineweaver–Burk lines employed in K_i evaluation was identical with that used previously (Pocker and Stone, 1967). In the determination of K_i *via* activity plots, a typical kinetic run procedure was essentially followed. The difference was that varying amounts of stock inhibitor solution were added to the enzyme solution prior to the initiation of the hydrolysis by addition of *p*-NPA.[1] This meant that while the ratio of enzyme to substrate concentration remained constant, the concentration of enzyme varied. Correction to the original enzyme concentration was then made in the calculation of V_i. Other dilution effects such as pH changes and buffer rate variations were also corrected for when necessary. As in previous studies the general reaction conditions of 10% (v/v) acetonitrile, ionic strength of 0.09 maintained with added NaCl, and a temperature of 25.0° were employed throughout the inhibition studies. Enzyme concentrations were in the range of $2–4 \times 10^{-6}$ M. The reaction was followed at either 348, 400, or 465 mμ and correction for absorbance by the inhibitor at these wavelengths was made where necessary. In general, it was possible to prepare stock solutions of inhibitor in water or buffer. Some of the inhibitors such as acetonitrile, acetone, pyridine, or ethylene glycol were added neat. Inhibitors which were water insoluble were prepared in acetonitrile.

Anionic Inhibition. Lineweaver–Burk plots were utilized in the study of anionic inhibition. A Tris buffer of pH 7.55 at an ionic strength of 0.09, composed of the anion held at a concentration of 0.081 M and of Tris–H^+ Cl^- held at 0.009 M, was used to study fluoride, chloride, bromide, iodide, nitrate, and acetate anions. Similar conditions were employed for other anions such as bicarbonate, azide, thiocyanate, and cyanide except that their concentrations were 1×10^{-3}, 9×10^{-4}, 9×10^{-4}, and 9×10^{-6} M, respectively. At the pH under consideration, all of the above anions were present almost entirely in their anionic form except for CN^-, $pK_a = 9.1$, with respect to which both the conjugate acid and anion were considered as potential inhibitors, the K_i then being equal to $[HCN + CN^-][BCA]/[BCA \cdot CN^-]$, where [BCA] is the sum of all BCA species not containing inhibitor, and $[BCA \cdot CN^-]$ is the sum of all forms of BCA containing bound inhibitor.

Activity plots were employed in the evaluation of K_i for cyanate, sulfide, bisulfite, and perchlorate. As with cyanide, H_2S exists as both an anion and conjugate acid, pK_a (H_2S) = 7.0. The data were found to be best analyzed in terms of inhibition by the anion. Several concentrations ranging from 0 to 3×10^{-3} M were used with KCNO and 0 to 3×10^{-5} M with HS^-. Concentrations of bisulfite up to 0.03 M were employed as well as perchlorate up to 0.01 M. Although in sulfite solutions at pH 7.5 the concentrations of sulfur dioxide and sulfurous acid are negligible, $pK_a \sim 1.9$, considerable amounts of both HSO_3^- and SO_3^{2-} are present, $pK_{a_2} \sim 7.3$. Since divalent anions such as SO_4^{2-} and HPO_4^{2-} have long been known to be very weak inhibitors (Roughton and Booth, 1946), the effect of SO_3^{2-} was neglected and inhibition was considered to arise from the equilibrium concentration of HSO_3^-. Both bisulfite and sulfide were found to be efficient catalysts of ester hydrolysis, and suitable corrections for nonenzymatic rates were made. Lineweaver–Burk plots for chloride ion at 0.009 and 0.09 M were made at seven different pH values: 7.00, 7.55, 7.92, 8.45, 9.33, and 10.48.

Sulfonamides. The inhibitory effectiveness of acetazolamide was demonstrated by injecting 0.05 ml of 10^{-4} M into a reaction mixture at pH 7.7 already in progress for 5 min with BCA at 1.6×10^{-6} M. Quantitative data were obtained by premixing acetazolamide ($0–4 \times 10^{-5}$ M) with BCA buffered at pH 8.45; the inhibitor to enzyme ratio ranged from 0 to 13.7. The data were analyzed by plotting % activity, V_i/V_0, against the (acetazolamide)/(BCA) ratio. Similarly, Lineweaver–Burk plots were obtained for seven different acetazolamide concentrations ranging from 0 to 5×10^{-6} M; these plots were used to characterize the inhibition as noncompetitive. Per cent activity as a function of the ratio of inhibitor to BCA was utilized to determine K_i values for acetazolamide at eight pH values ranging from pH 7.0 to 10.0.

In a like manner sulfanilamide ($0–1.6 \times 10^{-4}$ M), sulfaguanidine ($0–1.2 \times 10^{-3}$ M), sulfathiazole ($0–6 \times 10^{-2}$ M), and sulfapyridine ($0–1.6 \times 10^{-3}$ M) were studied at pH 8.2 in Tris buffers.

Miscellaneous Inhibitors. The inhibition of esterase activity by glycine ($0.3–9.1 \times 10^{-2}$ M) was studied at pH 7.4 in Tris buffer, and that by β-alanine ($0.3–9.1 \times 10^{-2}$ M) at pH 7.9 in phosphate buffer. At these pH values the zwitterionic form predominates. Glycine and β-alanine were found to catalyze the production of *p*-

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: *p*-NPA, *p*-nitrophenyl acetate; BCA, bovine carbonic anhydrase.

nitrophenol from *p*-NPA, and a correction had to be applied in the evaluation of enzymatic rates. The chelating agent, 1,10-phenanthroline, used in zinc removal from BCA was tested as an inhibitor at concentrations ranging from 3×10^{-5} to 9.1×10^{-3} M in phosphate buffer at pH 7.4. The enzyme was also incubated for 1 day with 4.5×10^{-3} M 1,10-phenanthroline and tests were made for activity reduction. Acetylacetone and diacetyl were also tested as inhibitors with regard to their metal chelating ability as well as their capacity to interact with both ϵ-amino (lysine) and guanidine (arginine) groups. Concentrations of diacetyl ranging from 4×10^{-3} to 0.7 M produced no detectable inhibition of BCA-catalyzed hydrolysis at pH 7.6. When 0.1 M diacetyl was incubated with BCA for 1 day, precipitation occurred. Acetylacetone concentrations ranging from 0.3 to 9.1×10^{-2} M at pH 7.7 produced no inhibition. Likewise, preincubation of enzyme with acetylacetone for periods varying from 1 hr up to 1 day produced no inhibition

Ethylene glycol (3.3×10^{-4} to 6.3×10^{-2} M), phenol (3.3×10^{-4} to 6.3×10^{-2} M), aniline ($0.3\text{–}6.3 \times 10^{-2}$ M), pyridine (0.04–1.77 M), benzenesulfonate anion (up to 10^{-2} M), and proflavine sulfate (up to 10^{-3} M) were also tested as inhibitors of BCA esterase activity.

Acetone inhibition was studied at pH 7.6 in phosphate buffer using concentrations up to 1.8 M. Stock *p*NPA in acetonitrile was added to give 1% (v/v) acetonitrile, unlike the previous results which were for 10% (v/v) acetonitrile. Likewise ethanol and 1-butanol inhibition was studied using 1% (v/v) acetonitrile.[2] Concentrations of ethanol up to 5.7 M were employed in the determination of esterase activity as a function of ethanol concentration. 1-Butanol concentrations ranging up to 0.7 M were employed in the determination of K_i. Concentrations of alcohol which produced approximately 50% inhibition were employed to characterize their inhibition as noncompetitive by the use of Lineweaver–Burk plots.

The inhibitory ability of acetonitrile was also studied by variation of activity with inhibitor concentration. Acetonitrile concentration was varied from 0 to 3.5 M, *i.e.*, from 0 to 18% (v/v).

Results

Roughton and Booth (1946) were the first to report that anions inhibit the hydrase activity of bovine carbonic anhydrase, and their results were later substantiated by Kernohan (1965). We have earlier reported that these same anions inhibit the esterase activity of this enzyme (Pocker and Stone, 1965, 1967), an observation subsequently shown to hold for the human enzyme (Armstrong *et al.*, 1967), as well as with the bovine cobalt(II) enzyme (Thorslund and Lindskog, 1967). Our results show that the interaction between the enzyme and the various anions is reversible and that the concentration of *p*-NPA does not affect the concentration of the anion-sensitive sites. Thus, for the equilibrium $E + A^- \rightleftharpoons EA^-$ one obtains an enzyme–inhibitor dissociation constant, $K_i = [E][A^-]/[EA^-]$, and a free energy of binding at 25.0° as given in Table I. We find the inhibitory potency of the various anions to follow the order: $CN^- > SH^- > CNO^- > N_3^-$, $CNS^- > I^-$, $ClO_4^- > HCO_3^-$, $HSO_3^- > NO_3^- > Br^- > OAc^- > Cl^- > F^-$. These results are in complete accord with the more limited observations made earlier for the hydration of CO_2 (Roughton and Booth, 1946; DeVoe and Kistiakowsky, 1961; Kernohan, 1965), and for the inhibition of esterase activity of human carbonic anhydrase B (Armstrong *et al.*, 1966; Verpoorte *et al.*, 1967). It will be noticed that a very wide range of inhibitory potency has been observed with these anions. Among the very weak inhibitors were F^-, Cl^-, OAc^-, Br^-, NO_3^-, and HCO_3^-. These results have both physiological significance and great importance to kinetic studies. Chloride, the ion involved in the electroneutrality of blood, is a weak inhibitor and thus does not significantly affect the physiological role of the enzyme in the blood. This is also important as it is commonly employed in maintenance of ionic strength in kinetic studies. The weak inhibitory power of acetate, a product of *p*-nitrophenyl acetate hydrolysis, allows us to neglect product inhibition at substrate concentrations normally employed. Bicarbonate inhibition has a special significance as this anion is the very substrate which embodies the anhy-

TABLE I: Anionic Inhibition of BCA Esterase Activity.[a]

Inhibitor[a]	K_i (M)	$-\Delta F^b$ (kcal/mole)
CN^-[c]	2.6×10^{-6}	7.66
SH^-[d]	1.1×10^{-5}	6.80
CNO^-	1.1×10^{-4}	5.44
SCN^-	5.9×10^{-4}	4.43
N_3^-	5.9×10^{-4}	4.43
I^-	8.7×10^{-3}	2.82
ClO_4^-	1.6×10^{-2}	2.47
HCO_3^-	2.6×10^{-2}	2.18
HSO_3^-[e]	3.0×10^{-2}	2.06
NO_3^-	4.8×10^{-2}	1.81
Br^-	6.6×10^{-2}	1.62
OAc^-	8.5×10^{-2}	1.47
Cl^-	1.9×10^{-1}	0.99
F^-	1.2	−0.09

[a] Reaction mixtures are 10% (v/v) acetonitrile with ionic strength of 0.009 made up of Tris·HCl at pH 7.55. The values of K_i are calculated neglecting the possible inhibition by these substances. [b] Free energy of binding at 25.0°. [c] Considering both conjugate acid and anion as potential inhibitors. [d] Considering HS^- as the inhibitory species. [e] Neglecting the inhibitory effect of SO_3^{2-}.

[2] These experiments are taken from a wider study on alcohol inhibition carried out in our laboratories by Mr. M. Beug. We are indebted to him for permission to include his data on alcohol inhibition.

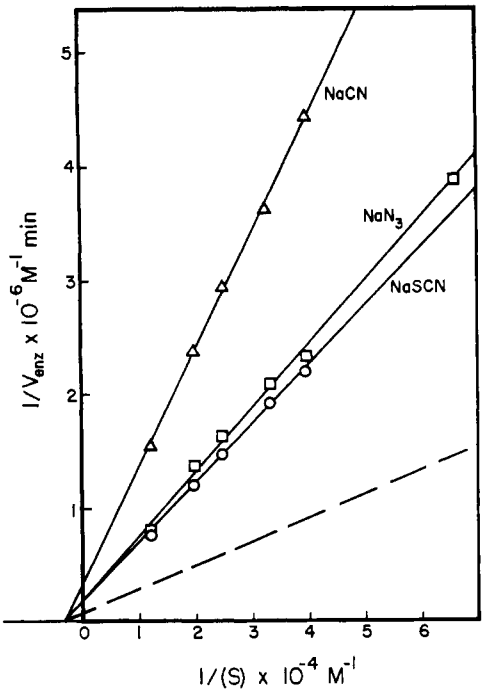


FIGURE 1: Lineweaver–Burk plots for the BCA-catalyzed hydrolyiss of *p*-NPA in the presence and absence of anionic inhibitors, pH 7.55 in Tris buffer with an ionic strength of 0.09 at 25.0°: (---) no added salt; (○) 9×10^{-4} M NaSCN; (□) 9×10^{-4} M NaN_3; (△) 9×10^{-6} M NaCN.

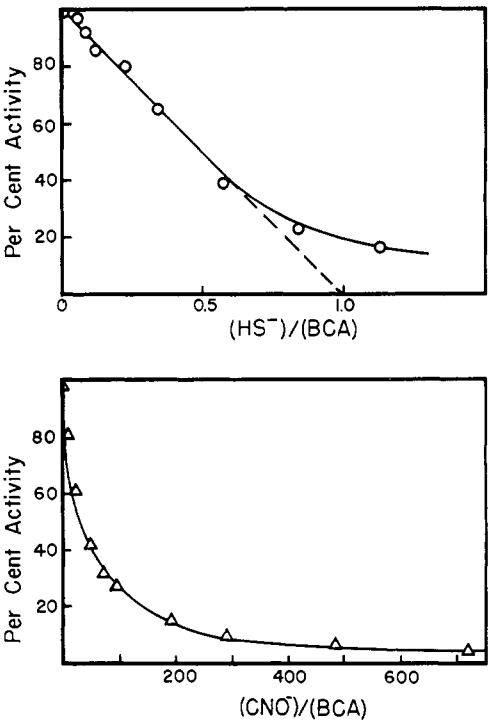


FIGURE 2: Per cent esterase activity. (A) As a function of the $(HS^-)/(BCA)$ ratio at pH 7.6 and 25.0° in phosphate buffer. (B) As a function of the $(CNO^-)/(BCA)$ ratio at pH 7.55 and 25.0° in Tris buffer.

drase activity of the enzyme. Other anions were moderately strong inhibitors with the exception of HS^- and CN^- which were found to be excellent inhibitors of carbonic anhydrase esterase activity.

Anionic inhibition was characterized as noncompetitive by use of Lineweaver–Burk plots (Figure 1). The esterase activity was also studied as a function of anion concentration (Figure 2). It was observed, with the stronger anionic inhibitor HS^-, that binding of one anion per sensitive site was sufficient to cause loss of catalytic activity of that site. When the data are plotted as log $[(V_0/V_i) - 1]$ against log (I), where V_0 and V_i are the velocity with and without inhibitor I, a linear relationship is obtained, the slope of which represents the number of inhibitor molecules producing inactivation of the enzyme (Johnson *et al.*, 1942). With the stronger anions, HS^- and cyanate, this slope was found to be 1.0.

Utilization of either iodide or cyanate with acetazolamide in mutual inhibition experiments revealed that these anions were competing for the same sensitive site (Table II). Parallel experiments in which pairs of inhibitors were employed in mutual inhibition included iodide *vs.* cyanate, acetazolamide *vs.* bicarbonate, and iodide *vs.* bicarbonate. The results indicate that the same sensitive site was employed by all these inhibitors in destroying esterase activity. The carbonic anhydrase site involved in binding these inhibitors might be zinc. However, when one examines the stability constants of simple zinc complexes (Yatsimirskii and Vasil'ev, 1960), one finds that these constants do not exactly parallel anionic inhibitory potency. Indeed, we have reported earlier that the relative inhibitory effects of anions are very similar for carbonic anhydrase and acetoacetic decarboxylase (Fridovich, 1963), and that the order appears to coincide with the Hofmeister lyotropic series. The specificity of this series of anion inhibitors is based on the change in the structure of water which contribute to the stability of the combination of anions with the cationic binding site (Fridovich, 1963). Thus, the anion and solvent themselves determine inhibitory effectiveness. Our preliminary conclusion that zinc in conjunction with a closely situated cationic binding site of $pK_a \sim 7.5$ provides the electrophilic centers of anion binding maintains its validity (Pocker and Dickerson, 1968). The electrostatic interaction factor should be a function not only of pH which to a first approximation controls the equilibrium $BH^+ \rightleftharpoons B + H^+$ and $E \cdot ZnOH_2 \rightleftharpoons E \cdot ZnOH + H^+$ but must also include a hydrogen-bonding factor which in turn affects the pK of these entities.

Loss of a proton from the ionizing group in the enzyme reduces the inhibitory effect of chloride ions (Figure 3A). The apparent pK_a for this effect is also *ca.* 7.5. Specific anion binding seems to be the major effect on the esterase activity of bovine carbonic anhydrase. However, ionic strength and use of different anions produce changes in the pH–rate profile in addition to a reduction in rate due to binding of inhibitor to a sensitive site, *i.e.*, while a similar sigmoidal curve is obtained its pK_a is displaced and the displacement appears to measure the capacity of the anion to form stable hydrogen bonds and thus decrease the tendency of BH^+ to ionize.

Sulfonamides are potent specific inhibitors of car-

TABLE II: Mutual Inhibition by Two Inhibitors.[a]

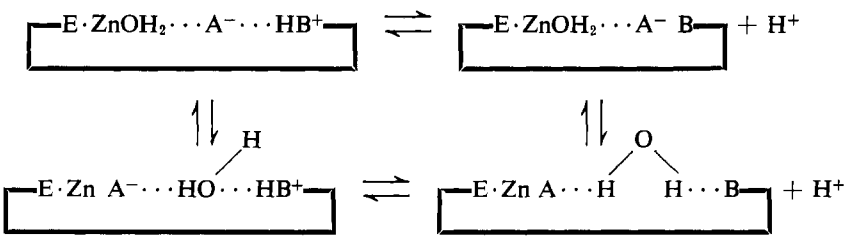
Inhibitors		$V_i/S_0 \times 10^{-2}$			
Acetazolamide (M × 10^7)	I^- (M × 10^2)	Calcd for Identical Sites[b]	Calcd for Independent Sites[c]	Exptl	$V_0/S_0 \times 10^{-2}$
3.29	0.99	2.00	1.95	2.02	2.82
6.49	1.95	1.56	1.44	1.57	
12.5	4.70	1.04	0.82	1.06	
15.4	6.15	0.89	0.65	0.86	
Acetazolamide (M × 10^7)	CNO^- (M × 10^4)				
3.27	1.63	1.63	1.53	1.63	2.82
6.51	1.63	1.42	1.27	1.39	
6.41	3.21	1.16	0.97	1.09	
12.7	3.19	0.97	0.73	0.90	
I^- (M × 10^2)	CNO^- (M × 10^4)				
3.18	1.59	1.52	1.36	1.73	2.95
6.06	3.03	1.05	0.82	1.07	
Acetazolamide (M × 10^7)	HCO_3^- (M × 10^2)				
6.5	1.63	1.51	1.27	1.43	2.95
12.7	3.19	1.42	1.13	1.41	
I^- (M × 10^2)	HCO_3^- (M × 10^2)				
3.18	1.59	2.06	1.93	2.12	2.95
6.06	3.03	1.56	1.32	1.82	
5.97	4.48	1.48	1.20		
Acetazolamide (M × 10^7)	*p*-Nitrophenyl trimethylacetate (M × 10^4)				
6.6	0.66	1.51	1.42	1.44	2.95
13.1	0.66	1.16	1.06	0.98	
13.0	1.31	1.07	0.90	0.80	

[a] At pH 7.8 in phosphate buffer with an ionic strength of 0.09 in 10% (v/v) acetonitrile. [b] Calculated using

$$V_i = \frac{V_0}{1 + \frac{(I)_1}{K_{i_1}} + \frac{(I)_2}{K_{i_2}}}$$

[c] Calculated using

$$V_i = \frac{V_0}{\left(1 + \frac{(I)_1}{K_{i_1}}\right)\left(1 + \frac{(I)_2}{K_{i_2}}\right)}$$



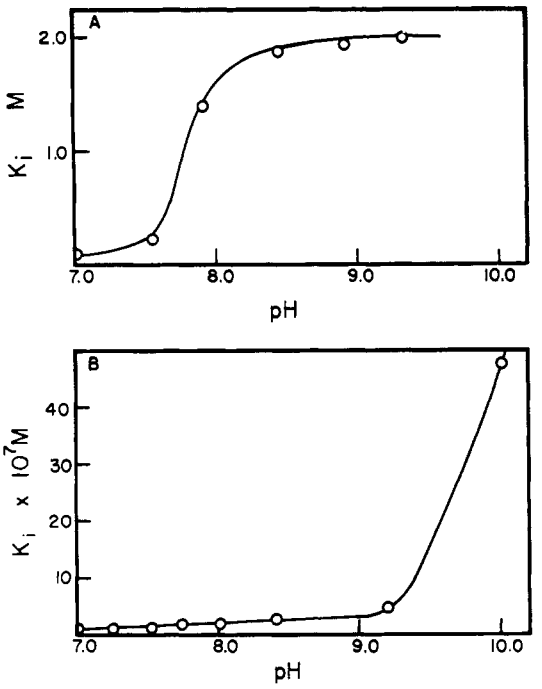


FIGURE 3: Inhibition studies. (A) Chloride inhibition, K_i, as a function of pH at 25.0°. (B) Acetazolamide inhibition, K_i, as a function of pH at 25.0°.

bonic anhydrase. Acetazolamide is one of the most powerful of the sulfonamide inhibitors. Acetazolamide rapidly combines with enzyme to form a complex which is inactive with respect to *p*-NPA hydrolysis (Figure 4).

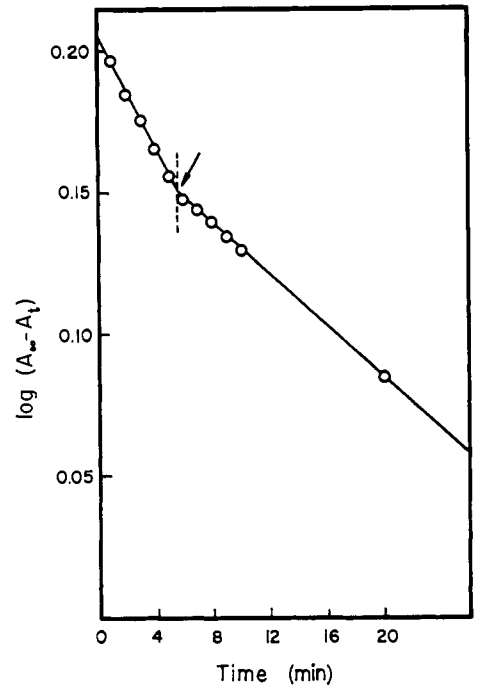


FIGURE 4: Inhibition of the BCA-catalyzed hydrolysis of *p*-NPA by injection of acetazolamide into the reaction mixture 5 min after hydrolysis initiation using pH 7.6 Tris buffer of ionic strength 0.09 with (BCA) = (acetazolamide) = 1.6×10^{-6} M; A_∞ represents the final absorbance and A_t the absorbance at time t.

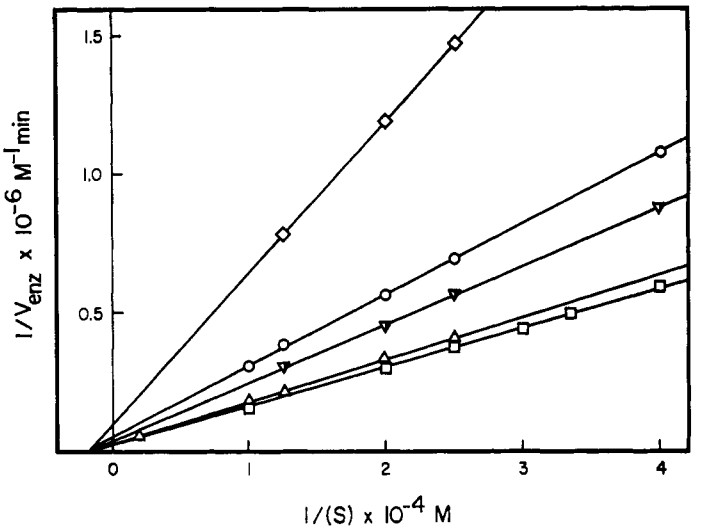


FIGURE 5: Lineweaver–Burk plots at various (acetazolamide)/(BCA) ratios in pH 8.45 Tris buffer of ionic strength 0.09 at 25.0° with (BCA) = 2.8×10^{-6} M. (□) No added acetazolamide; (△) (acetazolamide)/(BCA) = 0.06; (○) (acetazolamide)/(BCA) = 0.16; (▽) (acetazolamide)/(BCA) = 0.50; (◊) (acetazolamide)/(BCA) = 0.63; (◑) (acetazolamide)/(BCA) = 1.26.

No effect on rate is detected when a similar experiment is done with buffer alone. Acetazolamide has been dialyzed from the enzyme to restore full activity (Lindskog, 1963). Thus an equilibrium is rapidly and reversibly established between enzyme and inhibitor, $E + I \rightleftharpoons EI$.

Unlike chloride inhibition which was dependent upon a group in the enzyme with a pK_a of ~7.5, acetazolamide inhibition was found to be dependent on a group with an apparent $pK_a > 10$ (Figure 3B). Inhibition by acetazolamide was much too potent to detect any dependency on a group in the pH region 7–8. One can approximately fit the experimental data to an equation of the form $K_i = K_i(\text{max})/[1 + (H^+/K_a)]$, using a pK_a of ~11 and a $K_i(\text{max})$ of 10^{-5}–10^{-4} M. The latter value corresponds to that which was found for interaction with

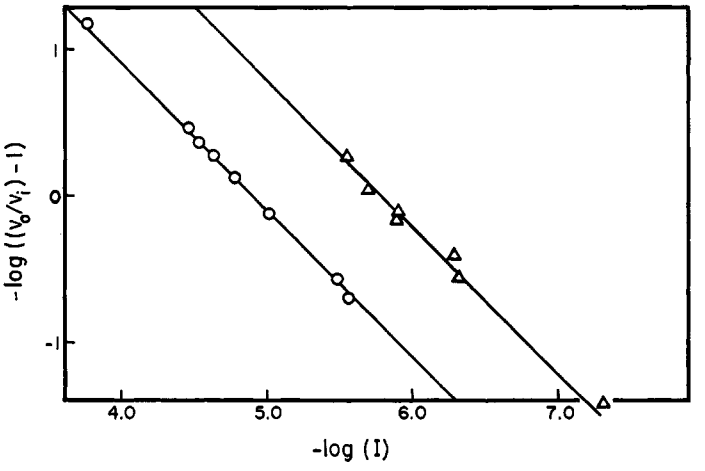


FIGURE 6: Plot of $-\log [(V_0/V_i) - 1]$ *vs.* $-\log$ (I). (○) Sulfanilamide; (△) acetazolamide; (I) is the total inhibitor concentration.

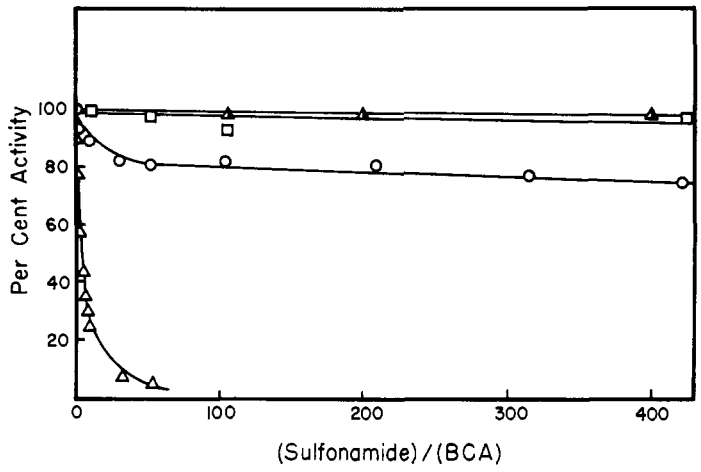


FIGURE 7: Per cent esterase activity as a function of (sulfonamide)/(BCA) ratio in pH 8.45 Tris buffer of ionic strength 0.09 at 25.0° with (BCA) ~3 × 10^{-6} M; (△) sulfanilamide; (○) sulfaguanidine; (□) sulfapyridine; (▲) sulfathiazole.

the apoenzyme (Coleman, 1967) and represents a weaker hydrophobic interaction of the sulfonamide with the enzyme. Chloride ion is much too weak an inhibitor to be used to detect the upper pH dependency; likewise, acetazolamide is much too potent to detect the lower pH dependency. Work is in progress to find a moderate inhibitor which may be employed throughout the entire pH range.

The noncompetitive nature of this inhibition is exemplified in the Lineweaver–Burk plots using various acetazolamide concentrations (Figure 5). A plot of the slope of such Lineweaver–Burk lines against the respective inhibitor concentration may be used to deduce a K_i value. Such a method was in accord with previous results (Pocker and Stone, 1967). Plots of log $[(V_0/V_i) - 1]$ against log (I) revealed that the binding of one acetazolamide molecule per sensitive site was sufficient to eliminate the catalytic activity associated with

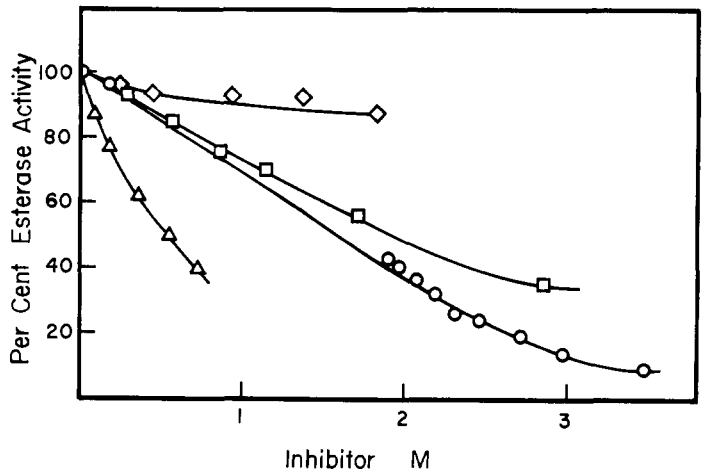


FIGURE 8: Per cent esterase activity as a function of inhibitor concentration at 25.0° in phosphate buffers. (○) Acetonitrile at pH 7.6; (□) ethanol at pH 9.1 with 1% (v/v) acetonitrile; (△) 1-butanol at pH 9.1 with 1% (v/v) acetonitrile; (◊) acetone at pH 7.6 with 1% (v/v) acetonitrile.

TABLE III: Inhibition by Sulfonamides, Cosolvents, and Miscellaneous Compounds.

Inhibitor	K_i[a] (M)	$-\Delta F$[b] (kcal/mole)
Sulfonamides[c]		
Acetazolamide	2×10^{-7}	9.2
Sulfanilamide	1.1×10^{-5}	6.8
Sulfaguanidine	190×10^{-5}	3.7
Sulfapyridine	2100×10^{-5}	2.3
Sulfathiazole	6500×10^{-5}	1.6
Cosolvents[d]		
Acetonitrile[e]	1.7	−0.34
Ethanol[f]	1.9	−0.38
1-Butanol[f]	0.6	0.30
Ethylene glycol[g]	None at saturation	
Acetone[h]	~5	−0.7
Chelating agents[i]		
1,10-Phenanthroline	1.5×10^{-2}	2.5
Acetylacetone	None at 0.1	
Diacetyl	None at 0.7	
Miscellaneous		
Phenol[g]	3×10^{-2}	2.1
Pyridine[c]	5×10^{-1}	0.4
Benzenesulfonic acid[g]	1×10^{-1}	1.4
Aniline[g]	2.8×10^{-2}	2.1
Proflavine sulfate[c]	~1×10^{-2}	2.7
Amino acids[j]		
Glycine	7×10^{-2}	1.6
β-Alanine	10×10^{-2}	1.4

[a] Obtained from plots of per cent esterase activity as a function of inhibitor concentration using K_i = (E)(I)/(EI). [b] Free energy of binding at 25.0°. [c] Reaction mixtures contained 10% (v/v) acetonitrile with ionic strength 0.09 in pH 8.45 Tris buffer. [d] Solvents added neat. [e] In Tris buffer at pH 7.55. [f] Reaction mixtures contain 1% (v/v) acetonitrile at pH 9.1 in Tris buffer. [g] At pH 7.55 in Tris buffer and 10% (v/v) acetonitrile. [h] Using 1% (v/v) acetonitrile at pH 7.6 in phosphate buffer. [i] In pH 7.6 phosphate buffer using 10% (v/v) acetonitrile. [j] In pH 7.8 phosphate buffer where the zwitterion predominates. Compare with K_i = 8.5 × 10^{-2} M for acetate.

that site (Figure 6). The similar results obtained for sulfanilamide indicate the generality of this observation with respect to sulfonamides (Figure 6). The inhibition constants for several other sulfonamides were obtained from plots of activity *vs.* the ratio of inhibitor to enzyme (Figure 7). It can be seen that acetazolamide is by far the most potent of the sulfonamides tested (Table III), being some 50 times more powerful than sulfanilamide. Other sulfonamides such as sulfapyridine, sulfaguanidine, and sulfathiazole were relatively weak.

Several organic solvents were employed as inhibitors. Among them was acetonitrile, the solvent presently employed for ester solubility purposes. It was found to give a mild inhibition of esterase activity (Figure 8) capable of producing a completely inactive EI complex. Under the conditions of 10% (v/v) acetonitrile normally employed, it should be noted that only about 50% esterase activity remains (Figure 8). While this is a serious difficulty, it was, nevertheless, advantageous to employ such conditions. The nature of the inhibition was characterized as noncompetitive.

Alcohols were likewise found to noncompetitively inhibit the esterase activity of carbonic anhydrase (Figure 8). Ethanol and 1-butanol possessed about the same potency as acetonitrile. These compounds apparently compete with anions for the same binding site (Verpoorte *et al.*, 1967). If alcohols replace water at the zinc site, we might anticipate transesterification to occur. Work is presently in progress to analyze for such an effect. Other organic solvents also produce inactivation of esterase activity much as did the alcohols.

Discussion

Sulfonamides are known to be potent, specific inhibitors of hydrase as well as esterase activity of carbonic anhydrase. The results presented in Table III show that for a powerful inhibition of esterase activity, an intact SO_2NH_2 group is necessary, an observation made earlier with respect to hydrase activity (Krebs, 1948; Maren, 1956). Acetazolamide (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide) is one of the most potent sulfonamide inhibitors of hydrase activity (Maren *et al.*, 1954). Its potent inhibition of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate (Pocker and Stone, 1967) is similar to that observed in the enzymatic hydration of CO_2 (Keller *et al.*, 1959; Davis, 1959) as well as of acetaldehyde (Pocker and Meany, 1965a,b). The inhibition has been characterized as noncompetitive in both acetaldehyde hydration and *p*-nitrophenyl acetate hydrolysis. Originally, the enzymatic hydration of CO_2 was also characterized as noncompetitive while the corresponding inhibition of the enzymatic dehydration of bicarbonate was found to be competitive (Leibman and Greene, 1967). We have earlier commented that such behavior might be expected if the hydrating site for the forward reaction becomes the binding site for the reverse dehydration (Pocker and Stone, 1967). Recently, when the sulfonamide inhibition of the enzymatic hydration of CO_2 was studied under special conditions, $(I)_0 \gg (E)_0$, some anomalous behavior was observed (Kernohan, 1966a,b; Lindskog and Thorslund, 1968). From the initial part of the sigmoidal reaction tracings, noncompetitive inhibition was obtained. The rates increased over a short time period, to give an apparent competitive pattern. The proposal was made that this behavior is associated with the relatively slow dissociation of the enzyme–inhibitor complex. We defer comment on this behavior until we have carried out a more detailed analysis of the effect of sulfonamide with and without bicarbonate on the reversible hydration of CO_2. We wish to emphasize, however, that both acetaldehyde hydration (Pocker and Meany, 1965a,b) and *p*-nitrophenyl acetate hydrolysis (Pocker and Stone, 1967) are noncompetitively inhibited by sulfonamides even in the region where inhibitor is fully equilibrated with enzyme.

Sulfonamide inhibition has several important features which are extremely valuable. We have already mentioned that sulfonamides are potent inhibitors of both hydrase and esterase activity. Furthermore, the nature of the site employed by these compounds in binding to the enzyme is partially characterized. Recent X-ray analysis of a sulfonamide–enzyme complex (Tilander *et al.*,1965; Fridborg *et al.*, 1967) reveals that the inhibitor binds at or very near the zinc site. Equilibrium dialysis experiments utilizing both native and apoenzyme as well as spectral studies using the cobalt(II) enzyme (Lindskog, 1963) are consistent with the X-ray analysis. Use of labeled acetazolamide also revealed that the metal was essential for the powerful binding of the inhibitor molecule, although other groups in the protein are involved as well (Coleman, 1967).

The importance of zinc to this enzyme has long been known, and sulfonamide inhibition studies have shed a great deal of light on its role. In the present investigation we have further confirmed that sulfonamides noncompetitively inhibit the esterase activity of bovine carbonic anhydrase throughout the entire pH interval under scrutiny. One sulfonamide inhibitor molecule was found to be sufficient to inhibit esterase activity. Since zinc has been associated with the hydrating or hydrolyzing site and not the carbonyl binding site, these results indicate that only one such site is active. Furthermore, we have observed that activity may be completely destroyed by acetazolamide, *i.e.*, ESI possesses no residual esteratic activity. Increasing the pH was found to reduce but not abolish the inhibitory potency of sulfonamides (Kernohan, 1964; Armstrong *et al.*, 1967; Thorslund and Lindskog, 1967; Pocker and Storm, 1968). The present studies coupled with earlier observations show that the powerful binding of sulfonamide inhibitor is dependent on two groups in the enzyme, one of $pK_a \sim 7.5$ and the other of $pK_a > 10$. The rate of combination of enzyme with benzenesulfonamide was found to increase (*i.e.*, a decrease in K_i) in the pH region 7–8 (Kernohan, 1966a,b). The increase in K_i below pH 8 observed with benzenesulfonamide may be explained by by the fact that the acidic form of the inhibitor and the acidic form of the enzyme interact less efficiently. Likewise, a basic group in the enzyme may promote the ionization of SO_2NH_2 which in turn would increase the inhibitory ability of the sulfonamide. Thus, conversion of BH^+ to B, $pK_a \sim 7.5$, in the enzyme could promote sulfonamide inhibition. Additional interactions between enzyme and sulfonamide inhibitor have been reported (Pocker and Storm, 1968; Pocker and Dickerson, 1968; Coleman, 1967), and are postulated as being essentially hydrophobic. Combination of BCA with 5-dimethylaminonaphthalene-1-sulfonamide reveals that a hydrophobic site situated on the interior of the enzyme is operative in sulfonamide binding (Chen and Kernohan, 1967). Furthermore, ionization of the SO_2NH_2 group promotes binding.

Recently, the esterase activity of BCA with less re-

active substrates has been found to be sensitive to a group of pK_a *ca.* 10.5 (Pocker and Storm, 1968). The question might be raised whether this enhanced activity at high pH is due to a nonspecific catalysis by the ε-amino groups of lysine present in the bovine enzyme. However, Pocker and Storm (1968) have shown that the specific inhibitor acetazolamide is able to eradicate this high pH catalysis and have consequently concluded that it is associated with a specific group which is at or near the active site of carbonic anhydrase. Since the hydrolytic pK_a of the free $Zn(H_2O)_4^{2+}$ complex is reported to be 9.7 (Perrin, 1962; Hunt, 1963), it is possible that the higher inflection corresponds to the titration of a protein-bound zinc–aquo complex. Reduction of the inhibitory potency in this pH region accords with the suggestion that the displacement of a hydroxide ion ligand by acetazolamide would be more difficult than the displacement of a water ligand. An alternative suggestion is that the higher pH dependence is due to a specific ε-aminolysine residue, and that this group is functional in the aminolysis of the ester. Reduced inhibition by acetazolamide could then arise because the negatively charged enzyme interacts more weakly with the negatively charged sulfonamide. However, we have two observations which tend to favor other suggestions over the latter. First, we do not observe a burst of *p*-nitrophenol as might be expected in an aminolysis reaction, and second, control experiments show that the rate of aminolysis of *p*-nitrophenyl propionate by an ε-amino group of lysine, while significant, is far too small (2.5% of the enzymatic rate per lysine residue) to account for the increased enzymatic catalysis observed at high pH (Y. Pocker and J. T. Stone, unpublished observations).

The early suggestion that the metal in native carbonic anhydrase possessed some functional role was based on inhibition studies employing cyanide (Meldrum and Roughton, 1933; Keilin and Mann, 1940). Analogy with simple systems and with other metalloproteins led to the postulation that anions were linked to the metal ion. Spectral studies employing primarily the cobalt(II) enzyme provide direct evidence for such a metal–anion interaction (Lindskog, 1963; Coleman, 1967; Riepe and Wang, 1967). Extensive studies of anionic inhibition (Pocker and Stone, 1965; Armstrong *et al.*, 1967) have led to the observation that a lyotropic series is followed (Pocker and Stone, 1967). Such a series is based on the relationship between the size of the anion and the entropy of binding (Fridovich, 1963). Fridovich's hypothesis is that the specificity of anion inhibition is based on the change in the structure of water coincident to the formation of an enzyme–anion complex. It is possbile that anions interfere with the water structure around zinc, by binding at a cationic site in close proximity to, but other than, the zinc ion, or by binding directly to zinc. Since there is no correlation between association constants with zinc ions and the inhibition constants observed for anions, it is possible that zinc is not the sole binding site of these inhibitors. However, the spectral evidence with the Co(II)–enzyme is overwhelmingly in favor of the suggestion that strongly inhibiting anions bind to the metal ion. Further evidence as to zinc being, at least in part, the anion binding site was obtained through iodoacetamide inhibition studies. Iodoacetamide inactivates the enzyme by reaction at the 3′-imidazole nitrogen of a histidine residue. This initially reversible inhibitor was found to compete with chloride and sulfanilamide for a binding site on the enzyme. In addition, chloride ions do not protect the apoenzyme against inactivation (as measured in terms of activity upon addition of zinc) by iodoacetamide (Whitney *et al.*, 1967a,b; Y. Pocker and J. T. Stone, work in progress). We have contended earlier (Pocker and Dickerson, 1968) that the binding site for noncompetitive inhibitors is sensitive to two cationic groups: the metal ion and the imidazolium cation situated in close proximity. Inhibitors such as iodoacetamide initially react with the inhibitor binding site and subsequently react with the nearby imidazole.

In our work we have noted a striking similarity between sulfonamide inhibition and anionic inhibition. While a lyotropic series is followed for anionic inhibition of the bovine carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl acetate, it appears that the binding of anions is metal ion dependent. Mutual inhibition experiments (Table II) indicate that anions such as cyanate and iodide compete for the same binding site in the enzyme, which is not the locus of attachment of the substrate molecule. Acetazolamide and anions were found to be competitive with respect to each other. Bicarbonate ion appears to bind at a site which is identical with that used by other anions as well as that used for the strong binding of acetazolamide. With the more potent anionic inhibitors such as sulfide or cyanate, we have shown that binding of one anion was sufficient to destroy esterase activity. We have further shown that ESI complexes involving strong anion inhibitors, *e.g.*, CN^-, HS^-, and CNO^-, possessed no residual activity. Consequently, we are tempted to propose an inhibition scheme for anions which is essentially similar to that proposed earlier by Pocker and Dickerson (1968) for explaining anionic inhibition of hydrase activity (*cf.* also Results section of present paper). In the present work we have noted that inhibition by chloride is dependent on a group in the enzyme with an apparent pK_a of 7.5, the acid form of which is more strongly inhibited by this anion. It should also be noted that the inhibitory potency of chloride ion was reduced but not entirely abolished, implying that secondary interactions of a weaker nature still exist at pH >8. We have provisionally ascribed to the transformation of zinc–water to zinc–hydroxyl in the enzyme a pK_a of ~11 (Pocker and Storm, 1968) and have attributed the inflection at pH 7.5 to the ionization of an imidazolium ion, the basic form of which promotes the hydrolysis process by the removal of a proton from the zinc-bound water. It is feasible that at pH values lower than 7.5, the anions interact with two neighboring electrophilic centers in the enzyme, Zn^+OH_2 and BH^+; the neutralization of BH^+ may depend not only on pH but also on the stability of the hydrogen-bonded complex between it and the anionic inhibitor and could be displaced to higher pH values as such interactions become stronger. It is then possible that the more potent inhibitors may dis-

place the zinc-bound water but weak inhibitors such as chloride may not. In other words two distinct enzyme–inhibitor complexes exist: (i) an ion-pair complex and (ii) an inner sphere species of zinc–anion complex.

Anionic inhibitions by bicarbonate and acetate are of particular interest. Acetate anion is the product of hydrolysis of *p*-NPA, while bicarbonate is the product of hydration of CO_2 and is also the substrate of the dehydration process. Bicarbonate ion is apparently produced as an ion pair or an inner sphere complex of zinc when an OH^- group is transferred from EZn^+OH_2 to CO_2 (Pocker and Stone, 1967). In dehydration, the inner sphere complex between zinc and bicarbonate is decomposed to give CO_2. A similar scheme may be envisaged for ester hydrolysis, since acetate is found to bind at or near the zinc ion much like bicarbonate and to produce an ion pair or an inner sphere complex. Thus the idea that water transfer occurs at or near the zinc atom and that the product of hydrolysis is then situated at this same site is indeed in complete accord with the results reported in this work.

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