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The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. VII. Kinetic Studies of Esterase Activity and Competitive Inhibition by Substrate Analogs*

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ABSTRACT: In the present paper evidence is adduced to show (a) that bovine carbonic anhydrase acts as an esterase on a wide variety of phenyl and naphthyl acetates, (b) that various esters as well as aldehydes, aldehyde hydrates, and ketones act as inhibitors of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate, and (c) that hydrase and esterase activity of the enzyme are related. The position of the nitro group, $p > m > o$, and the size of the acyl residue in nitrophenyl esters were found to greatly influence the rate of the enzymatic hydrolysis. With *p*-nitrophenyl acetate as substrate, the inhibitory potency was shown to follow the order: *p*-nitrophenyl trimethylacetate, *p*-nitrophenyl *n*-hexanoate > *o*- and *m*-nitrophenyl acetate > phenyl acetate ≥ naphthyl acetate ≫ methyl, ethyl, *n*-propyl, and *n*-butyl acetates; *p*-nitrophenyl cinnamate and benzoate showed no inhibition at saturation. The aliphatic acetates were weak inhibitors, $K_i \simeq 0.5$ M, whereas the aryl acetates were weak to moderately strong inhibitors, K_i from 10^{-2} to 10^{-4} M. Lineweaver–Burk plots characterize these esters as competitive inhibitors. Furthermore, the binding of one of the above ester molecules to carbonic anhydrase is shown to be sufficient to prevent the binding of *p*-nitrophenyl acetate to the same enzyme molecule. *p*-Nitrophenyl methyl ketone was also found to compete with *p*-nitrophenyl acetate for the same binding site, $K_i \simeq 3 \times 10^{-3}$ M. Propionaldehyde in the presence of its equilibrium amount of hydrate showed a mixed inhibition of esterase activity, $K_i \simeq 1.0$ M, whereas chloral hydrate, which exists almost entirely in the hydrated form, was found to be a noncompetitive inhibitor of esterase activity. Mutual inhibition experiments reveal that bicarbonate and acetazolamide compete with one another for a binding site which is different from that used by the esters, but is the same, or dependent on, the site used by the aldehyde hydrates. Other compounds such as carbon disulfide, acetal, dimethyl carbonate, and triethyl orthoacetate were found to be very weak inhibitors of esterase activity. The totality of these observations is taken as further evidence that significant similarities exist between the hydrase and esterase activities of bovine carbonic anhydrase, not only with respect to the turnover processes, but also with regard to the formation of the corresponding enzyme–substrate complexes. A common enzymatic pathway is therefore proposed for the hydration of carbon dioxide, the hydration of certain aldehydes, and the hydrolysis of esters.

In recent investigations it has been found that besides the reversible hydration of CO_2 (eq 1) carbonic anhydrase catalyzes two general reactions, given by eq 2 and 3. The first of these embodies the physiological significance of the enzyme. The latter two are formally similar to the hydration of CO_2 and their catalytic behavior shows many common features. Versatility with respect to the hydrase activity of carbonic anhydrase was demonstrated with respect to aldehyde hydration (Pocker and Meany, 1965a,b, 1967a,b; Pocker and Dickerson, 1968). The similarity between aldehyde hydration (Pocker, 1960) and certain biomolecular mechanisms of ester hydrolysis involving tetrahedral intermediates (Bender, 1951, 1953) prompted us to explore carbonic anhydrase

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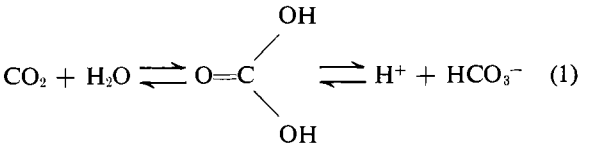

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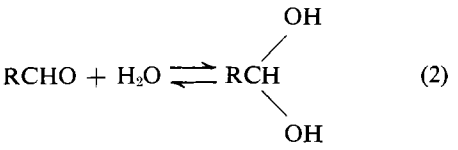

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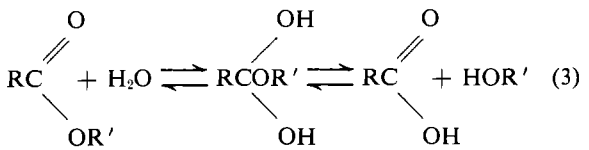

(3)

esterase activity (Pocker and Stone, 1965, 1967). Other investigators have also tested for esterase activity (Tashian *et al.*, 1964; Malmstrom *et al.*, 1964; Armstrong *et al.*, 1966; Duff and Coleman, 1966; Lo and Kaiser, 1966; Thorslund and Lindskog, 1967). The scope of esterase activity has recently been extended in our laboratories to include a homologous series of *p*-nitrophenyl esters (Pocker and Storm, 1968). Apparently, general esterase activity may be ascribed to the same underlying mechanism as is employed in the enzymatic hydrolysis of *p*-nitrophenyl acetate.

In the present paper we have attempted (a) to gain further insight into the mode of action of carbonic anhydrase by examining several related esters as both substrates and inhibitors, and (b) to establish the degree of similarity between esterase and hydrase activity, especially with respect to substrate binding. We have utilized our inhibition studies to demonstrate that esters, aldehydes, and ketones are dependent upon identical or nearly identical sites of the enzyme for their binding, and that these sites are different from those associated with anionic inhibition.

Experimental Section

The purification and properties of *p*-nitrophenol and *p*-nitrophenyl acetate were previously described (Pocker and Stone, 1967). The method of Spasov (1938) was employed in the synthesis of *o*-nitrophenyl acetate, *m*-nitrophenyl acetate, *p*-nitrophenyl cinnamate, and *p*-nitrophenyl benzoate (Pocker and Storm, 1968). The physical constants of these esters agreed well with literature values. Furthermore, spectrophotometric measurements show that upon basic hydrolysis, these esters liberated 99.5–100% of the theoretical amount of the corresponding nitrophenol. Other esters were commercially available and saponification values of the recrystallized products showed them to be analytically pure. The purification of propionaldehyde was previously described (Pocker and Dickerson, 1968). *p*-Nitrophenyl methyl ketone and acetazolamide were of reagent grade purity. The various sodium salts were Baker Analyzed Reagent grade or of similar purity.

Bovine carbonic anhydrase preparation, storage, and properties have been described (Pocker and Stone, 1967). It should be reiterated that the preparation was a mixture of isozymes A and B, but unlike the human enzyme whose isozymes possess markedly different activities (Rickli *et al.*, 1964), the isozymes of bovine carbonic anhydrase were found to exhibit essentially the same esterase activity (Pocker and Stone, 1967). Kinetics on the two isozymes at pH 7.5 in 0.08 M Tris-HCl buffer with ionic strength 0.1 at 25.0° showed k_{enzyme} values of 1.25 and 1.33 $\times$ 10^4 M^{-1} min^{-1} for A and B, respectively.

Buffer components, apparatus, and kinetic procedure were as previously employed with only minor variations. Inhibitory potency was determined by means of BCA[1] activity with respect to *p*-NPA hydrolysis as a function of inhibitor concentration. The inhibition constant may then be deduced from $K_i = ((E_0) - (EI))((I_0) - (EI))/(EI)$, where (E_0) and (I_0) are the total initial concentrations of enzyme and inhibitor, respectively, and (EI) is the concentration of the enzyme–inhibitor complex. The assumption that $(EI) = (E_0)(V_0 - V)/V_0$, where V and V_0 are the enzymatic velocities with an without added inhibitor, respectively, was employed in the evaluation of EI and, hence, K_i. The nature and magnitude of inhibition were characterized by use of Lineweaver–Burk plots (Laidler, 1958; Dixon and Webb, 1964; Webb 1963).

Since the esters used as inhibitors are also potential substrates and since the products of their hydrolysis could be troublesome inhibitors, it proved advantageous to add inhibitor just prior to or simultaneous with the addition of *p*-NPA. In some cases a correction was applied for the accompanying enzyme-catalyzed hydrolysis of the ester used as an inhibitor, but for the most part, conditions of wavelength and concentration could be chosen to avoid this complication.

Aldehyde inhibition of the BCA-catalyzed hydrolysis of *p*-NPA was studied in detail using propionaldehyde in phosphate buffer of pH 7.6. The procedure for a typical run was followed except that propionaldehyde was initially introduced and the mixture allowed to reach equilibrium ($\ll$5 min at 25.0°) before the addition of *p*-NPA. Propionaldehyde was introduced through a microsyringe and the reported inhibitions represent the over-all effect of both aldehyde and its equilibrium amount of hydrate. The relationship between per cent activity and *p*-nitrophenyl methyl ketone concentration (0.3–9.1 $\times$ 10^{-3} M) was examined at pH 7.6 in phosphate buffer.

General esterase activity of bovine carbonic anhydrase was tested for. In each case the buffer rate and the rate with added enzyme were determined and values of k_{enzyme} calculated as with *p*-NPA (Pocker and Stone, 1967). The enzymatic hydrolyses were monitored as follows: β-naphthyl acetate at 330 mμ, phenyl acetate at 270 mμ, *o*-nitrophenyl acetate at 415 mμ, and *m*-nitro-

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

TABLE I: The BCA-Catalyzed Hydrolysis of Various Esters at 25.0°.[a]

Ester	pH	$k_{buffer} \times 10^2$ min^{-1}	$k_{enzyme} \times 10^{-4}$ M^{-1} min^{-1}	$k_{enzyme}(E)/k_{buffer}$
β-Naphthyl acetate	7.75	0.0081	0.0225	8.4
Phenyl acetate	7.50	0.0025	0.0130	15.6
o-Nitrophenyl acetate	7.82	0.065	0.359	16.5
m-Nitrophenyl acetate	7.78	0.047	0.749	47.7
p-Nitrophenyl acetate	7.80	0.090	1.600	53.4
p-Nitrophenyl n-hexanoate	7.80	0.0400	0.090	6.8
p-Nitrophenyl trimethylacetate	7.85	~0	~0	
p-Nitrophenyl cinnamate	10.5[b]	7.44	0	

[a] Reaction mixtures contain 10% (v/v) acetonitrile, μ 0.09, in phosphate or Tris buffer, BCA ~3×10^{-6} M and ester at about 0.1 mM. [b] The higher pH was employed due to the fact that esters such as p-nitrophenyl trimethylacetate show activity at this pH but none at the lower value (Pocker and Storm, 1968).

TABLE II: Ester Inhibition of the BCA-Catalyzed Hydrolysis of p-NPA at 25.0°.[a]

Ester	K_i[b] (M)	pH	Inhibitor Concn Range (M)
o-Nitrophenyl acetate	1.3×10^{-3}	7.8	$0–3.2 \times 10^{-3}$
m-Nitrophenyl acetate	2.5×10^{-3}	7.8	$0–9 \times 10^{-3}$
Phenyl acetate	2×10^{-2}	7.5	$0–2 \times 10^{-2}$
α-Naphthyl acetate	1×10^{-2}	7.8	$0–3 \times 10^{-3}$
β-Naphthyl acetate	2×10^{-3}	7.8	$0–9 \times 10^{-4}$
p-Nitrophenyl cinnamate	None at saturation	10.5	$10^{-5}–10^{-4}$
p-Nitrophenyl benzoate	None at saturation	8.5	~10^{-5}
p-Nitrophenyl trimethylacetate	5×10^{-4}	7.8	$0–1.3 \times 10^{-4}$
p-Nitrophenyl n-hexanoate	3×10^{-4}	7.8	$0–3.4 \times 10^{-4}$
Methyl acetate	0.5	8.2	0–1.1
Ethyl acetate	0.6	8.2	0–0.9
n-Propyl acetate	0.6	8.2	0–0.28
n-Butyl acetate	0.7	8.2	0–0.25
N-Carbobenzoxyglycine p-nitrophenyl ester	5×10^{-4}	7.8	$0–6 \times 10^{-4}$

[a] Reaction mixtures contain 10% (v/v) acetonitrile, μ 0.09, in Tris or phosphate buffers, p-PNA at 0.2 mM, BCA ~3×10^{-6} M. [b] Determined from plots of per cent activity as a function of added inhibitor.

phenyl acetate at 390 mμ. The p-nitrophenyl esters were monitored at both 348 mμ (the isosbestic point) and 400 mμ. The enzymatic hydrolyses of o- and m-nitrophenyl acetates were investigated in some detail. Lineweaver–Burk plots were obtained for each in 10% (v/v) acetonitrile at ionic strength 0.09. Concentrations of o-nitrophenyl acetate ranging from 0.1 to 3.2 mM were used with BCA at 2.6×10^{-6} M, and of m-nitrophenyl acetate from 0.03 to 0.1 mM with 2.7×10^{-6} M BCA. Velocities were calculated in the manner described earlier for p-NPA (Pocker and Stone, 1967).

The enzymatic rates of p-NPA hydrolysis were determined as a function of inhibitor concentration using HS^-, CNO^-, acetazolamide, β-naphthyl acetate, o-nitrophenyl acetate, m-nitrophenyl acetate, p-nitrophenyl n-hexanoate, methyl acetate, propionaldehyde, chloral hydrate, and p-nitrophenyl methyl ketone. These data were then plotted in the form of log $[(V_0/V_i) - 1]$ *vs.* log (I), where V_0 is the velocity with no inhibitor and V_i is the velocity with inhibitor at concentration (I). The slopes of such plots represent the number of inhibitor molecules bound per enzyme molecule (Johnson *et al.*, 1942; Nygaard, 1961).

In the inhibition of the BCA-catalyzed hydrolysis of p-NPA, one may use two inhibitors simultaneously and predict whether they act at the same site or not. If they

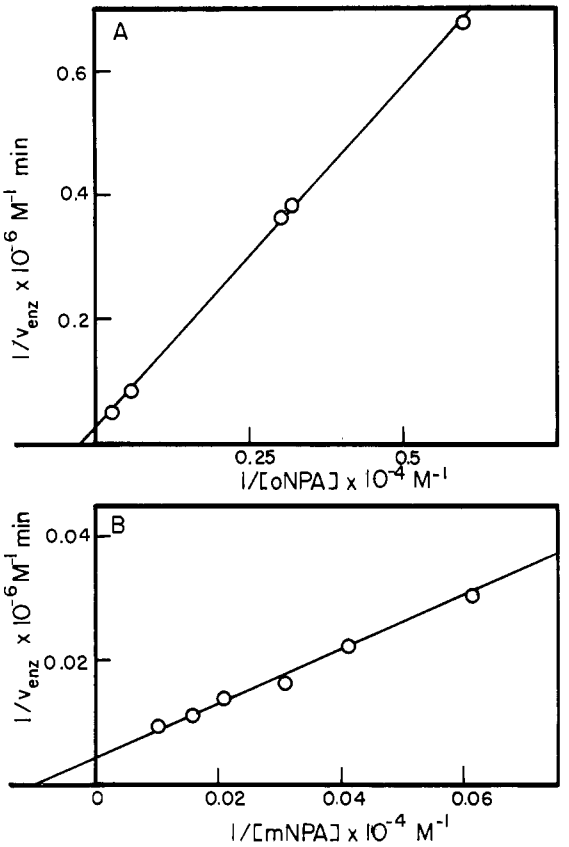

FIGURE 1: Lineweaver–Burk plots. (A) For the BCA-catalyzed hydrolysis of *o*-NPA at pH 7.8 and 25.0° in 10% (v/v) acetonitrile–phosphate buffer with an ionic strength of 0.09. (B) For the BCA-catalyzed hydrolysis of *m*-NPA at pH 7.8 and 25.0° in 10% (v/v) acetonitrile–phosphate buffer with an ionic strength of 0.09.

act at the same site, or on two different but dependent sites, the relationship given by eq 4 holds, while if they act at two different and independent sites, V_i is given by eq 5 (Fridovich, 1963). A typical run at pH 7.8 in phos-

$$V_i = V_0 \frac{1}{\left(1 + \frac{(I_1)}{K_{i_1}}\right)\left(1 + \frac{(I_2)}{K_{i_2}}\right)} \quad (4)$$

$$V_i = V_0 \left(1 + \frac{(I_1)}{K_{i_1}}\right)\left(1 + \frac{(I_2)}{K_{i_2}}\right) \quad (5)$$

phate buffer was used for the evaluation of V_0. The hydrolysis was followed at 348 mμ, and the concentrations of *p*-NPA and BCA were held constant throughout a series of runs. Stock solutions of each inhibitor were prepared and variable amounts added to give the desired concentration. In these mixed inhibition studies, the experimental velocity, V_i, was then compared with the calculated values for identical and for independent sites, using eq 4 and 5, respectively.

Results

A variety of aryl acetate esters were preliminarily tested to examine their ability to serve as substrates for the enzyme-catalyzed hydrolysis (Table I). While the generality of esterase activity was established, a wide range of enzyme efficiency was found. Highest activity was detected with *p*-nitrophenyl acetate (Pocker and Stone, 1965, 1967), but appreciable activity was also found with the *o*- and *m*-nitrophenyl acetates. Similar observations as to the enzymatic hydrolysis of *p*-NPA being faster than that of *o*-nitrophenyl acetate have also been made in other laboratories (Verpoorte *et al.*, 1967; Thorslund and Lindskog, 1967). The introduction of a nitro group accelerates both the chemical and enzymatic hydrolysis of phenyl acetate; however, the enzymatic catalysis is much more sensitive to this substitution. Thus even after correction is made for reduced chemical catalysis, the enzymatic hydrolysis of phenyl acetate appears to be less efficient than that of *o*-nitrophenyl acetate. β-Naphthyl acetate, the substrate used in early genetic work (Tashian *et al.*, 1964), appears to be among the less efficiently catalyzed esters. While the esterase activity of BCA with respect to *p*-nitrophenyl trimethylacetate is significant only above pH 9 (Pocker and Storm, 1968), little or no activity can be detected with respect to *p*-nitrophenyl cinnamate over the entire pH region 7–11. Indeed, the observed rate of hydrolysis was found to be identical with the buffer rate even at pH 10.5: $k_{buffer} = 7.4 \times 10^{-2}$ min^{-1} and $k_{obsd} = 7.2 \times 10^{-2}$ with 10^{-4} M BCA. Similar results were obtained at a pH value of 11.1, a region where BCA shows a dra-

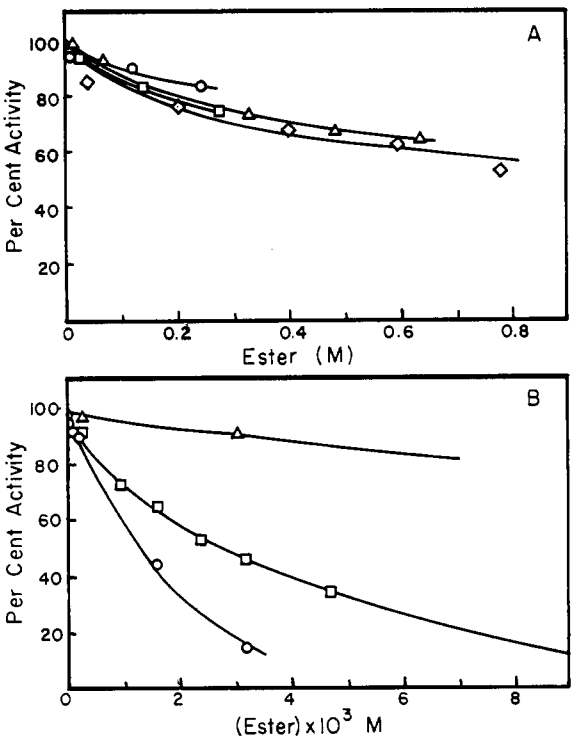

FIGURE 2: Per cent esterase activity as a function of inhibitor concentration. (A) At pH 8.2 and 25.0° in 10% (v/v) acetonitrile–Tris buffer, ionic strength of 0.09, BCA 3.5 × 10^{-6} M; (○) *n*-butyl acetate, (□) *n*-propyl acetate, (△) ethyl acetate, and (◇) methyl acetate. (B) At pH 7.6 and 25.0° in 10% (v/v) acetonitrile–phosphate buffer and an ionic strength of 0.09 with BCA ~2.7 × 10^{-6} M; (△) phenyl acetate, (□) *m*-nitrophenyl acetate, and (○) *o*-nitrophenyl acetate.

matic increase in its esterase activity with respect to most *p*-nitrophenyl esters (Pocker and Storm, 1968). When *p*-nitrophenyl cinnamate was employed as an inhibitor of the enzyme-catalyzed hydrolysis of *p*-NPA, no detectable inhibition was observed at pH 7.0 and 8.5. Likewise, BCA showed little or no esterase activity with regard to *p*-nitrophenyl benzoate as substrate; unfortunately the low solubility of this ester hampered a serious investigation.

Lineweaver–Burk plots were obtained for *o*-nitrophenyl acetate and *m*-nitrophenyl acetate (Figure 1). As with *p*-nitrophenyl acetate, a long extrapolation is necessary in the determination of the kinetic parameters K_m and k_2 and hence it is rather difficult to obtain accurate values (Pocker and Stone, 1967). The values of K_m for the *ortho* and *meta* esters were 42 and 100 $\times$ 10^{-4} M, respectively, while the values of k_2 were 15 min^{-1} for the *ortho* ester and 82 min^{-1} for the *meta* ester at pH 7.8 and 25.0°.

Several aliphatic and aromatic esters were tested for their ability to inhibit the BCA-catalyzed hydrolysis of *p*-nitrophenyl acetate (Table II). The esters display an inhibitory potency ranging from very weak to moderately strong. The aliphatic acetates, *i.e.*, methyl, ethyl, *n*-propyl, and *n*-butyl acetate, are all weak inhibitors (Figure 2A). On the other hand, phenolic esters are moderate inhibitors. Thus, both α- and β-naphthyl acetate as well as phenyl acetate show much greater inhibitory ability than the aliphatic acetates. Introduction of a nitro group into the aromatic function further increases the inhibitory effectiveness (Figure 2B). Both *o*- and *m*-nitrophenyl acetates, as well as *p*-nitrophenyl pivalate and *n*-hexanoate, are moderately strong inhibitors.

Using a group of representative esters the competitive nature of their inhibition of the enzymatic hydrolysis of *p*-NPA was demonstrated by use of Lineweaver–Burk plots (Figure 3). Both aliphatic and aromatic acetates as well as other *p*-nitrophenyl esters were found to bind at the same site as *p*-nitrophenyl acetate. These results imply either a single site or that different but dependent sites are involved in binding. The existence of only one active ester binding site is most essential to a basic understanding of the enzyme-catalyzed hydrolysis.

Various aldehydes were next used as inhibitors of the BCA-catalyzed hydrolysis of *p*-NPA in an effort to correlate hydrase and esterase activity. Numerous aldehydes could not be employed successfully. Acetaldehyde may not be used at 25.0° with any ease because of its low boiling point, 21.0°. Heptaldehyde and benzaldehyde showed no inhibition when saturated solutions of these aldehydes were used. However, success was attained with propionaldehyde (Figure 4). The inhibition was studied by allowing the BCA-catalyzed hydration of propionaldehyde to reach equilibrium prior to ester hydrolysis. Since both the hydration and dehydration processes are enzyme catalyzed one expects that the equilibrated propionaldehyde will inhibit through both the aldehyde and its hydrate (Figure 4A). The Lineweaver–Burk plot (Figure 4B) reveals mixed inhibition by the equilibrated aldehyde. It is attractive to suggest that the unhydrated species acts as a competitive inhibitor, while the corresponding hydrate acts as a noncompetitive inhibitor of esterase activity. Since the equilibrium between aldehyde and hydrate may not be varied except under extreme conditions, it is impossible to test this hypothesis further using propionaldehyde. However, noncompetitive inhibition of esterase activity was obtained using chloral hydrate, an aldehyde which exists entirely in the hydrated form (Figure 5).

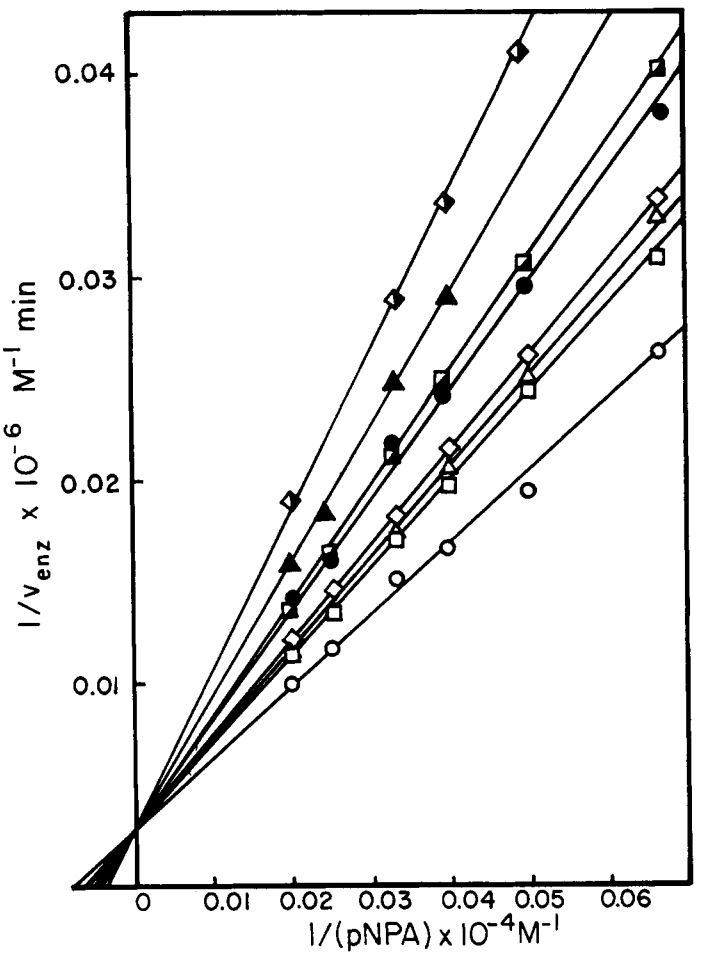

FIGURE 3: Lineweaver–Burk plots of the BCA-catalyzed hydrolysis with added ester inhibitor at pH 7.6 and 25.0°, 10% (v/v) acetonitrile–phosphate buffer and an ionic strength of 0.09, BCA = 2.2 $\times$ 10^{-6} M. (○) No inhibitor added; (□) *p*-nitrophenyl trimethylacetate, 1 $\times$ 10^{-4} M; (△) *m*-nitrophenyl acetate, 3.2 $\times$ 10^{-3} M; (◇) β-naphthyl acetate, 3.2 $\times$ 10^{-4} M; (●) *p*-nitrophenyl *n*-hexanoate, 1.5 $\times$ 10^{-4} M; (◪) ethyl acetate, 0.33 M; (▲) *o*-nitrophenyl acetate, 3.2 $\times$ 10^{-3} M; and (◁▶) phenyl acetate, 1.6 $\times$ 10^{-2} M.

The close structural similarity between *p*-nitrophenyl acetate and *p*-nitrophenyl methyl ketone led us to try the latter as an inhibitor (Figure 6). A moderate inhibition was observed for this compound, $K_i \simeq 2 \times 10^{-3}$ M. Since this ketone exists at equilibrium almost entirely as the unhydrated species, one would anticipate that it would competitively inhibit *p*-NPA hydrolysis, as was actually observed. Other ketones, such as acetone, cyclohexanone, diacetyl, and acetylacetone, were very weak inhibitors of esterase activity.

The close structural similarity between carbon dioxide

TABLE III: Log $[(V_0/V_i) - 1)]$ as a Function of Log (I).[a]

Inhibitor	Concn (M)	Log (Concn)	Log $[(V_0/V_i) - 1]$
HS^-	$\times 10^6$	+6	
	0.278	−0.559	
	0.826	−0.084	
	1.369	+0.135	−1.4908
	2.04	0.307	−1.0605
	2.78	0.441	−0.7670
	5.56	0.741	−0.5969
	8.26	0.916	−0.2725
	13.69	1.135	+0.2122
	20.40	1.307	+0.5363
	27.80	1.441	+0.6953
p-NP-*n*-H[b]	$\times 10^4$	+4	
	0.102	−0.991	−0.932
	0.329	−0.483	0.848
	0.544	−0.264	0.648
	0.679	−0.168	0.693
	0.846	−0.703	0.461
	0.891	−0.050	0.556
	1.184	+0.073	0.491
	1.475	+0.196	0.382
	1.715	+0.234	−0.212
	3.419	+0.534	+0.065
o-NPA[b]	$\times 10^4$	+4	
	0.116	−0.936	−1.714
	0.332	−0.479	−1.443
	1.639	+0.215	−1.060
	3.322	0.521	−1.020
	19.39	1.215	+0.088
	24.39	1.521	+0.773
KCNO	$\times 10^4$	+4	
	0.332	−0.479	−0.600
	0.826	−0.083	−0.192
	1.639	+0.215	+0.145
	2.439	0.387	0.328
	3.322	0.521	0.416
	6.623	0.821	0.733
	9.901	0.996	0.910
	16.39	1.215	1.144
	24.39	1.387	1.277
	32.25	1.521	1.407
β-NA[b]	$\times 10^4$	+4	
	0.322	−0.479	−1.303
	1.639	+0.215	−0.813
	2.439	0.387	−0.666
	3.322	0.521	0.777
	9.901	0.996	−0.548
m-NPA[b]	$\times 10^4$	+4	
	0.332	−0.479	−1.045
	0.990	−0.004	−0.419
	1.639	+0.215	−0.243
	2.439	0.387	−0.055
	3.225	0.521	0.074
	4.762	0.678	0.305
	6.250	0.796	0.793
	9.091	0.996	0.892

TABLE III (*Continued*)

Inhibitor	Concn (M)	Log (Concn)	Log $[(V_0/V_i) - 1]$
Methyl acetate			
	0.205	−0.668	−0.582
	0.404	−0.394	−0.367
	0.597	−0.224	−0.245
	0.783	−0.106	−0.039
	1.139	+0.057	−0.037
p-Nitrophenyl methyl ketone	$\times 10^3$	+3	
	0.99	−0.004	−0.430
	1.64	+0.215	−0.257
	2.44	0.387	−0.086
	3.23	0.509	+0.076
	6.25	0.796	0.412
	0.09	0.959	0.561
Propionaldehyde[c]			
	0.046[c]	1.337	−1.237
	0.137	−0.863	−0.955
	0.226	−0.646	−0.530
	0.445	−0.352	−0.379
	0.657	−0.182	−0.268
	0.862	−0.065	−0.087
Chloral hydrate	$\times 10^2$	+2	
	0.99	−0.044	−0.548
	1.64	+0.215	+0.027
	2.44	0.387	0.221
	4.76	0.678	0.235
	6.25	0.796	0.410

[a] Reaction mixtures in 10% (v/v) acetonitrile, μ 0.09, pH 7.8 phosphate buffer. [b] *p*-NP-*n*-H, *p*-nitrophenyl *n*-hexanoate; *o*-NPA, *o*-nitrophenyl acetate; β-NA, β-naphthyl acetate; *m*-NPA *m*-nitrophenyl acetate. [c] Total concentration, *i.e.*, aldehyde plus hydrate.

and carbon disulfide led us to test the latter as a potential competitive inhibitor of the BCA-catalyzed hydrolysis of *p*-NPA. Carbon disulfide was found to be much too insoluble to be successfully employed as an inhibitor in these studies. The degree of inhibition obtained at saturation (~0.03 M CS_2) is ~3%, indicating an inhibition constant, K_i, of around 1 M. Comparatively, CO_2 binds some 100 times better; this dramatic decrease in the binding capacity of BCA on changing from CO_2 to CS_2 may perhaps be ascribed to the lower basicity of sulfur atoms. Carbonyl sulfide, COS, is presently under investigation as a potential analog to CO_2 and hence a competitive inhibitor of esterase activity. In addition, compounds related to aldehyde hydrates such as triethyl orthoacetate, acetal, and dimethyl carbonate were employed as potential inhibitors of esterase activity. No inhibition was obtained with acetal, up to 0.07 M, or with dimethyl carbonate, up to 0.12 M. Triethyl orthoacetate produced only mild inhibition at saturation, ~10% at ~0.05 M, indicating a K_i of around 1 M. Low solubility hampers serious investigation of any of these inhibitors.

It was found that the esterase activity of BCA is inhibited when the enzyme binds one molecule of ester, aldehyde, aldehyde hydrate, ketone, anion, or sulfonamide (Table III). Thus plots of log $[(V_0/V_i) - 1]$ *vs.* log (I) give rise to slopes which fall within the range 1.0 ± 0.1 (Figure 7 and Table IV).

One vastly simplifies a systematic comparison of the action of a variety of inhibitors on the assumption that they act at the same sensitive site. Mixed-inhibition studies provided the means of substantiating this assumption. Several representative inhibitors were employed (Table V), and it was found that salts and sulfonamides act at the same site whereas sulfonamides and esters bind at two independent sites. These results substantiate our earlier findings which delineate the strong acetazolamide inhibition as noncompetitive (Pocker and Stone, 1965, 1967). Since one acetazolamide molecule produces inhibition, it may be implied that there is only one active esteratic site.

Discussion

Our primary concern in this investigation was to establish that other esters bind to BCA in a manner not un-

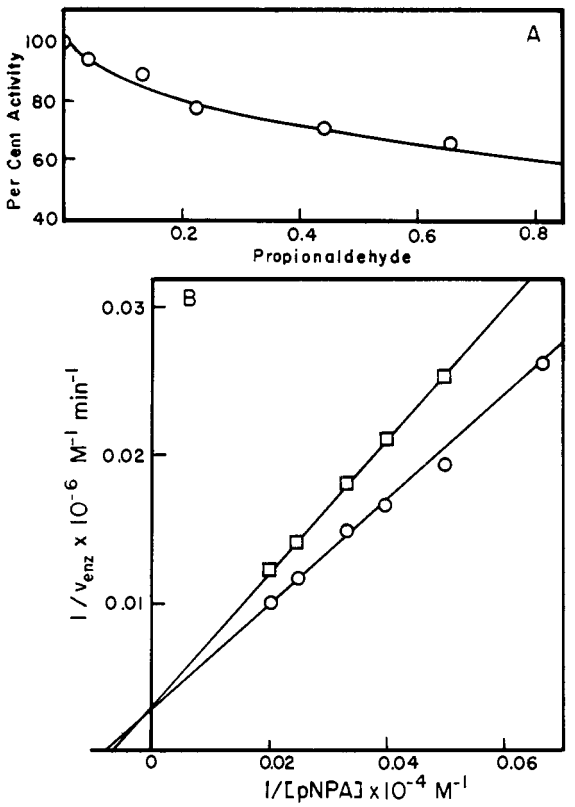

FIGURE 4: Propionaldehyde studies. (A) Per cent esterase activity as function of total propionaldehyde concentration at pH 7.6 and 25.0° in phosphate buffer, 10% (v/v) acetonitrile, and an ionic strength of 0.09. (B) Lineweaver–Burk plots for the BCA-catalyzed hydrolysis of *p*-NPA in the presence and absence of equilibrated propionaldehyde at pH 7.6 and 25.0°, in phosphate buffer, 10% (v/v) acetonitrile, and an ionic strength of 0.09. BCA = 2.3×10^{-6} M; (○) in the absence of propionaldehyde, (□) in the presence of 0.23 M propionaldehyde.

like *p*-nitrophenyl acetate and also to further explore the relationship between BCA esterase and hydrase activity. Consequently related carbonyl compounds such as esters, aldehydes, aldehyde hydrates, and ketones were employed as inhibitors of the BCA-catalyzed hydrolysis of *p*-nitrophenyl acetate.

The esterase activity of the enzyme is sensitive to the chemical nature of both the acyl and the phenolic residue (Pocker and Stone, 1967; Pocker and Storm, 1968; Thorslund and Lindskog, 1967). We have earlier shown that increasing the length of the acyl residue of the ester increases the binding constant but reduces the value of the turnover number (Pocker and Storm, 1968). In the present study we find that BCA shows little if any esterase activity with respect to *p*-nitrophenyl cinnamate as substrate and furthermore that it does not even appear to bind detectable amounts of this ester. Thus, it does appear that there are esters (*e.g.*, cinnamic acid derivatives) which are subject to significant chemical catalysis, as well as catalysis by certain hydrolytic enzymes such as chymotrypsin, but which nevertheless show little or no ability to interact with BCA. On the other hand, we find that the hydrolysis of both α- and β-naphthyl acetates is catalyzed by BCA, and, hence, that the esterase activity of this enzyme, although reduced, is not entirely destroyed even when the phenolic portion of the ester is quite large. Apparently, the formation of active 1:1 complexes between carbonic anhydrase and esters is subject to a higher degree of specificity toward the acyl residue (see Pocker and Storm, 1968) than toward the phenolic group. The importance of a phenolic group should, however, be noted. Thus phenyl acetates are found to bind up to 100 times better than the corresponding aliphatic acetates such as methyl or ethyl acetate. The importance of steric and electronic considerations may be exemplified in the study of *o*-, *m*-, and *p*-nitrophenyl acetate in which an order of activity $p > m > o$ is observed. The lower activity of the *ortho* ester relative to the *para* ester probably arises as a consequence of steric inhibition of the formation of the tetrahedral activated complex in the transition state. Indeed, the pK_a values of the leaving nitrophenolates are essentially the same. Furthermore, on the basis of buffer rates (Table I), one would anticipate a reactivity order, $p > o > m$. While buffer catalysis indicates the importance of both electronic and steric considerations, steric factors appear to be more pronounced in the enzymatic reaction. The trend $p > m > o$ was observed in considering only the enzymatic coefficient and in accounting for the differences in the buffer catalysis, k_{buffer}, by considering the ratio $k_{enzyme}(E)/k_{buffer}$ (Table I). It appears that the esterase activity of BCA is more sensitive to the turnover number, k_2, than to the binding constant of these esters. Indeed, the pseudo-binding constants, $1/K_i$, do not differ markedly on going from *ortho* to *meta* to *para* ester (Table II). Furthermore, the *meta* isomer forms

TABLE IV: Number of Inhibitor Molecules Producing Inactivation.

Inhibitor	No. of Inhibitor Molecules Necessary for Inhibn[a]	Type of Inhibn
m-NPA	1.15	Competitive
o-NPA	0.93	Competitive
β-NA	~0.7	Competitive
p-NP-*n*-H	0.99	Competitive
Methyl acetate	0.90	Competitive
Propionaldehyde	0.95	Competitive[b]
Chloral hydrate	1.15	Noncompetitive
p-Nitrophenyl methyl ketone	1.01	Competitive
KCNO	1.03	Noncompetitive
HS^-	1.07	Noncompetitive
Diamox	1.0	Noncompetitive
Sulfanilamide	1.0	Noncompetitive

[a] Obtained from the slope of a plot of log $[(V_0/V_i) - 1]$ against log (I). [b] Refers to unhydrated aldehyde.

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

Inhibitors		$V_i/(S_0) \times 10^{-2}$			
Acetazolamide M × 10^7	*p*-Nitrophenyl trimethyl acetate M × 10^4	Calcd for Identical Sites	Calcd for Independent Sites	Exptl	$V_0/(S_0) \times 10^{-2}$
6.6	0.66	1.51	1.42	1.44	2.59
13.1	0.66	1.16	1.06	0.98	
13.0	1.31	1.07	0.90	0.80	
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	

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

the least stable ES complex but yet possesses an over-all activity greater than that of the *ortho* ester. Inhibition of *p*-nitrophenyl acetate hydrolysis by various related esters, *o*-nitrophenyl acetate, *m*-nitrophenyl acetate, β-naphthyl acetate, or *p*-nitrophenyl *n*-hexanoate, is competitive (Figure 3), *i.e.*, binding of one of these ester molecules precludes binding of a *p*-nitrophenyl acetate molecule and *vice versa*. The close structural similarity between *p*-nitrophenyl acetate and these related esters leads to the conclusion that only one *p*-nitrophenyl acetate molecule is bound per enzyme molecule in the active ES complex, *i.e.*, we may imply that only one fruitful binding site for esters exists.

Not only have the acyl and alcoholic portions of the ester been varied in this work but also the nature of the carbonyl system itself was changed. When carbon disulfide was used as an analog of CO_2, the binding between enzyme and S=C=S was found to be *ca.* 100 times weaker than the corresponding binding with O=C=O. The stronger attachment of the latter to the enzyme is perhaps due in part to the greater basicity of the carbonyl oxygens and hence a greater capacity to participate in hydrogen bonding. The enzymatic hydrolysis of *p*-nitrophenyl acetate is only mildly inhibited by acetal and triethyl orthoacetate. These inhibitors were employed as unreactive analogs of hydrated carbonyl compounds. The enzymatic hydrolysis of *p*-nitrophenyl acetate is noncompetitively inhibited by bicarbonate anion. It was consequently of interest to evaluate the possible inhibitory power of an analog of the undissociated carbonic acid. In this connection we noted that the inhibition by the relatively stable dimethyl carbonate is so mild that it cannot even be characterized.

Sulfonamide inhibition has been very instructive in the elucidation of the mode of action of BCA. Sulfonamides have been shown to be potent, specific inhibitors of carbonic anhydrase activity (Mann and Keilin, 1940). Acetazolamide is one of the most potent of the sulfonamides (Krebs, 1948; Maren *et al.*, 1954). Its inhibition has been characterized as noncompetitive for CO_2 hydration (Maren *et al.*, 1960; Leibman *et al.*, 1961), acetaldehyde hydration (Pocker and Meany, 1965), and *p*-nitrophenyl ester hydrolysis (Pocker and Stone, 1965, 1967; Pocker and Storm, 1968; Armstrong *et al.*, 1966). In each case one inhibitor molecule binds to the enzyme to produce inactivation, thus implying that one active hydrolytic site exists. Equilibrium dialysis experiments measuring sulfonamide binding to native and apoenzyme indicate that the Zn^{2+} present in the native enzyme is involved in binding these inhibitors (Lindskog,

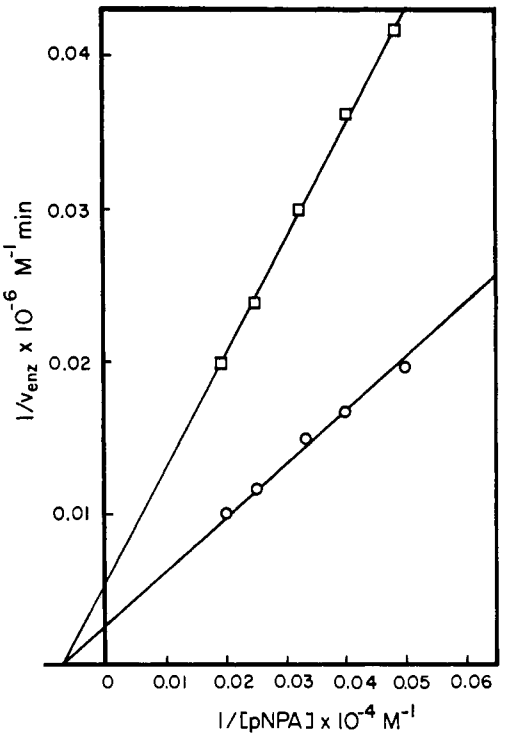

FIGURE 5: Lineweaver–Burk plots for the BCA-catalyzed hydrolysis of *p*-NPA in the presence and absence of chloral hydrate; pH 7.6, 25.0°, phosphate buffer, an ionic strength of 0.09, 10% (v/v) acetonitrile, and BCA = 2.2 × 10^{-6} M; (○) no inhibitor added; (□) 0.016 M chloral hydrate present.

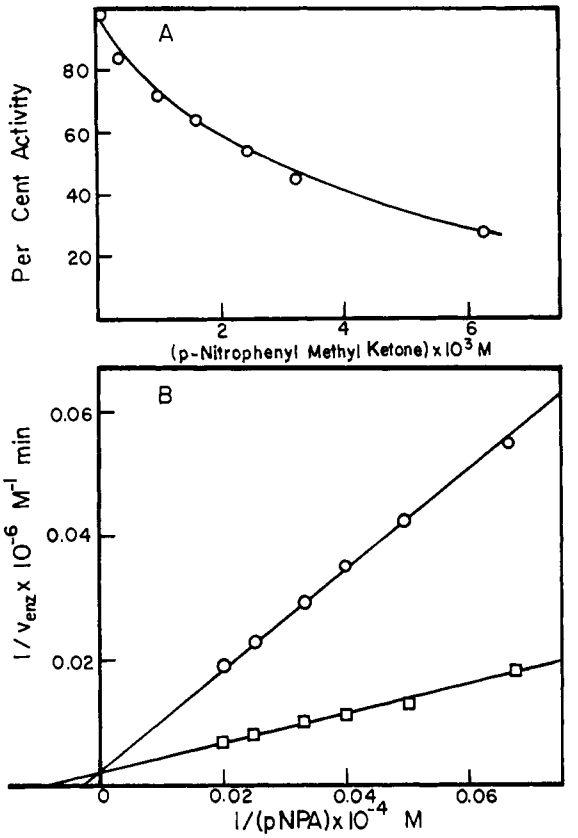

FIGURE 6: *p*-Nitrophenyl methyl ketone studies. (A) Per cent esterase as a function of added *p*-nitrophenyl methyl ketone; pH 7.6, 25.0°; phosphate buffer, 10% (v/v) acetonitrile, an ionic strength of 0.09, BCA = 1.5×10^{-6} M. (B) Lineweaver–Burk plots for the BCA-catalyzed hydrolysis of *p*-NPA in the presence and absence of *p*-nitrophenyl methyl ketone, pH 7.6, 25.0°, phosphate buffer, an ionic strength of 0.09, BCA = 1.5×10^{-6} M; (□) no inhibitor added; (○) *p*-nitrophenyl methyl ketone at 3.2×10^{-3} M.

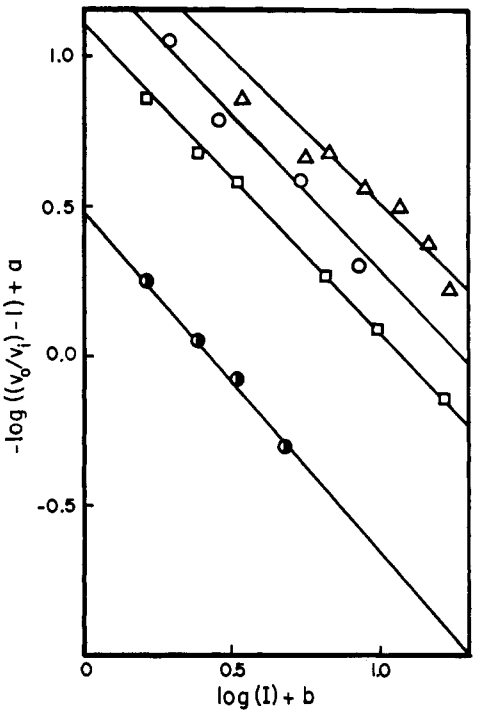

FIGURE 7: Plot of $-\log [(V_0/V_i) - 1]$ *vs.* log (I). (△) *p*-Nitrophenyl *n*-hexanoate, $a = 0$, $b = +5$; (○) HS^-, $a = 0$, $b = +6$; (□) CNO^-, $a = +1$, $b = +4$; (◑) *m*-nitrophenyl acetate, $a = 0$, $b = +4$.

1963). Further evidence that these inhibitors bind at or near zinc has been obtained *via* X-ray analysis (Tilander *et al.*, 1965; Fridborg *et al.*, 1967). Inhibition studies employing acetazolamide are of great value as the point of attachment is reasonably well defined. Likewise, inhibition by anions, especially by bicarbonate, is noncompetitive with respect to ester and competitive with respect to acetazolamide as would accord with a site other than zinc for ester binding. Furthermore, hydrase and esterase activity of BCA were found to be related by the fact that *p*-nitrophenyl methyl ketone, unhydrated aldehydes, and *p*-nitrophenyl acetate all share in part the same binding site. This does not imply that their respective binding sites are identical but only that some of their regions of attachment are close enough to strongly interact with one another. Since hydration is a reversible process, the products themselves may provide information about substrate binding. Bicarbonate is of course the physiological substrate in the dehydration reaction. In the present work we have shown that both bicarbonate and chloral hydrate are noncompetitive with respect to *p*-nitrophenyl acetate hydrolysis and we have further demonstrated that they bind at the same site (or at nearby interacting sites) as acetazolamide and CNO^-.

It therefore becomes apparent that zinc is not directly involved in the binding of CO_2, aliphatic aldehydes, ketones, and phenyl esters, but is involved in the binding of water, anions, acetazolamide, and hydrates.

The nature of the enzyme–substrate interaction has received much attention. We have previously reported that we were unable to detect the formation of stable covalent complexes between substrate and enzyme (Pocker and Stone, 1967). However, for all three reactions catalyzed by carbonic anhydrase, CO_2 hydration (Riepe and Wang, 1967), aldehyde hydration (Pocker and Dickerson, 1968), and ester hydrolysis (Pocker and Storm, 1968), hydrophobic binding between enzyme and substrate has been found to play a dominant role. In an effort to establish a simple model for enzymatic action Bender and his coworkers have found that phenyl ester cleavage was accelerated by cycloamyloses (Van Etten *et al.*, 1967a,b). The interior of these doughnut-shaped molecules is relatively hydrophobic when compared with water. The cycloamyloses appear to form inclusion complexes in which the substrate is inserted into the hydrophobic cavity. If we analyze the model of carbonic anhydrase determined *via* X-ray analysis (Fridborg *et al.*, 1967) we find a pronounced cavity on the surface in which the chelated zinc atom is situated. It is entirely possible that this cavity like the cycloamylose cavity acts as a hydrophobic binding site for the various esters. The chelated zinc is considered to be the hydrolytic site, while the remainder of the cavity may conveniently bind the substrate so that the carboxyl group is placed in the vicinity of the zinc with proper orientation for a hydroxyl group transfer from the en-

zyme to the carbonyl carbon. The competitive nature of inhibition by substrates and substrate analogs presented here implies that their binding sites have many features in common. Furthermore, the simplified reaction scheme previously proposed for the subsequent hydration and hydrolysis (Pocker and Stone, 1967) appears to be generally applicable.

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