Zinc(II) and Cobalt(II) Bovine Carbonic Anhydrases. Comparative Studies and Esterase Activity†

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ABSTRACT: Kinetic studies on the esterase activity of zinc and cobalt bovine carbonic anhydrase with 2,4-dinitrophenyl propionate, acrylate, cyclopropylcarboxylate, and cyclobutylcarboxylate as substrates are reported. For each ester, a detailed pH-rate profile is obtained at 25.0 °C over values of pH that were experimentally feasible (4.7–10.9). The background rate is strongly dependent upon the catalytic effectiveness of hydroxide ions with k_{OH^-} exhibiting the order cyclobutylcarboxylate > acrylate > propionate > cyclopropylcarboxylate, an order which appears to reflect differences in ground-state stability. The enzymatic pH-activity curves reveal an inflection around 7 and a second rise in activity at high pH which seems to be substrate dependent. A comparison of the rate profiles for a common substrate reveals that the pK_a of the group controlling activity around physiological pH for the cobalt carbonic anhydrase is always about 0.1 pK unit lower than that for the native enzyme. The enzyme catalyzed hydrolyses follow Michaelis–Menten kinetics. The variation of the apparent K_m with pH is dictated in every case by the respective turnover number, k_2, while the formal binding constants, k_1/k_{-1}, are nearly independent of pH in the range 7.2 to 9.2. Acetazolamide is a potent inhibitor of the esterase activity. The K_i values at pH 7.5 at 25.0 °C for both the zinc and the cobalt enzymes are in the range of 10^{-8} M with all four substrates. Inhibition studies with the 2,4-dinitrophenyl cyclobutylcarboxylate as substrate suggest that two independent sites are operative at physiological pH. The major site is subject to powerful inhibition by acetazolamide, while the second site contributing ca. 20% to the overall rate of hydrolysis of this "abnormal" ester is unaffected by a 100-fold excess of acetazolamide over enzyme. It is interesting that, with some of these substrates, the second rise in activity at high pH is also observed with the apoenzyme.

In the short history of bioorganic chemistry the use of unnatural substrates has often been valuable in elucidating the mechanism of action of a particular enzyme (Bender et al., 1966; Coleman, 1971). With the exception of the serine proteases, nowhere has this approach been more widely employed than for the enzyme, carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) (CA).[1] The natural function of this enzyme is to catalyze the reversible hydration of carbon dioxide. However, in the past 12 years mammalian CA has also been shown to catalyze the reversible hydration of aliphatic aldehydes (Pocker and Meany, 1965; Pocker and Dickerson, 1968), pyridinecarboxaldehydes (Pocker and Meany, 1967), pyruvic acid (Pocker and Meany, 1970), and ethyl pyruvate (Pocker et al., 1974). Additionally, it has been found to act as an esterase with respect to many monoesters of carboxylic acids (Tashian et al., 1964; Pocker and Stone, 1965, 1967; Verpoorte et al., 1967; Thorslund and Lindskog, 1967; Pocker and Storm, 1968; Pocker and Watamori, 1971, 1973), diesters of carbonic acid (Pocker and Guilbert, 1972, 1974), phosphate esters (Pocker and Sarkanen, 1973, 1975), and a sultone (Lo and Kaiser, 1966, and Kaiser and Lo, 1969). Kinetic investigations with each of these substrates have consistently revealed that the pH dependence of the turnover number is sigmoidal, that the inflection occurs around neutrality, and that the basic form of the enzyme is required for maximum activity.

In addition to these kinetic studies, CA has been explored by a wide variety of other sophisticated techniques and the results of these studies have been thoroughly reviewed (Lindskog et al., 1971; Coleman, 1971, 1973). Briefly, mammalian CA is a zinc metalloprotein of molecular weight 30 000. The high resolution x-ray structure has revealed that the active site consists of a crevice with the zinc(II) ion being coordinated to four ligands at the bottom of the cavity (Kannan et al., 1971; Liljas et al., 1972).[2] Three of the four ligands are apparently histidine residues and the fourth is thought to be a water molecule.[3] Zn(II) can be replaced by a number of divalent transition metals, but only the Co(II) ion restores full activity around physiological pH (Lindskog and Malmstrom, 1962; Coleman, 1967a). Furthermore, the x-ray data also demonstrated that there is an additional histidine (His-63) located in the active site of HCA-C, a result which has been confirmed by sequence work (Henderson et al., 1973; Lin and Deutsch, 1974) as well as by modification studies on HCA-C (Göthe and Nyman, 1972).

Despite the benefit of all this data, neither the mechanism nor the identity of the necessary active base is unambiguously known for CA. It is generally accepted though that a zinc-hydroxo complex either preformed, "fully developed" (Coleman, 1967a,b; Khalifah, 1971), or formed through proton relay to a general base (Pocker and Meany, 1965; Pocker and Storm,

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[1] Abbreviations used are: CA, carbonic anhydrase; BCA, bovine carbonic anhydrase; HCA-C, human carbonic anhydrase isozyme C; DNPP, 2,4-dinitrophenyl propionate; DNPA, 2,4-dinitrophenyl acrylate; DNPCPC, 2,4-dinitrophenyl cyclopropanecarboxylate; DNPCBC, 2,4-dinitrophenyl cyclobutanecarboxylate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

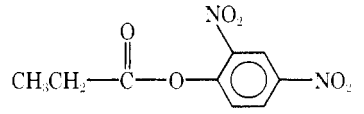
[2] Recent x-ray studies have revealed a somewhat more distant fifth coordination site (Kannan et al., 1977).

[3] Recent ^{1}H NMR studies on CA substituted with a paramagnetic metal have led some researchers to believe that the metal is coordinated to four amino acid residues at pH values below 7 and only at pH values above 7 is one amino acid replaced by a water molecule (Koenig and Brown, 1972; Lanir et al., 1975).

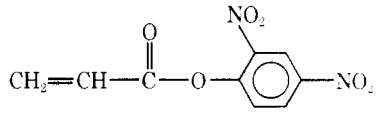
TABLE I: Selected Physical Properties of the 2,4-Dinitrophenyl Carboxylate Esters.

Ester	mp (°C)	Calcd C	Calcd H	Found C	Found H
2,4-Dinitrophenyl propionate	67–68	45.01	3.36	45.21	3.24
2,4-Dinitrophenyl acrylate	46–47	45.39	2.54	45.06	2.48
2,4-Dinitrophenyl cyclopropanecarboxylate	87–87.5	47.63	3.20	47.49	3.11
2,4-Dinitrophenyl cyclobutanecarboxylate	45–46	49.63	3.79	49.72	3.93

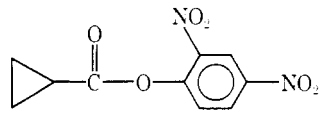
1968) is the nucleophile responsible for enzymatic activity at neutral pH. In an attempt to elucidate the important features of the hydroxo-complex formation and hydroxide-ion transfer in the active site, the following four molecules were synthesized and used in the present study as substrates for both the native and Co(II)-modified BCA. Not only will the hydrolyses of these esters be of interest in their own right, but it is also hoped that a comparative study of the two metallocarbonic anhydrases would help to clarify the role of the metal in the enzyme.



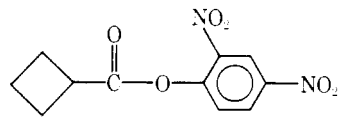
2,4-dinitrophenyl propionate
(DNPP)



2,4-dinitrophenyl acrylate
(DNPA)



2,4-dinitrophenyl
cyclopropanecarboxylate
(DNPCPC)



2,4-dinitrophenyl
cyclobutanecarboxylate
(DNPCBC)

Experimental Section[4]

Materials. The acid chlorides (Aldrich) used in the synthesis of the respective 2,4-dinitrophenyl esters were freshly distilled just prior to use. The 2,4-dinitrophenol (Eastman Kodak) was purified by repeated recrystallization from anhydrous benzene (mp 112.5–113 °C).

2,4-Dinitrophenyl Esters. All of the 2,4-dinitrophenyl esters used in this study were synthesized by the following method. To 0.05 mol of potassium hydroxide dissolved in a minimum amount of hot absolute ethanol was added 0.05 mol of 2,4-dinitrophenol dissolved in 200 mL of absolute ethanol. A bright-orange precipitate was formed and the solvent was removed by filtration. Immediately, the potassium salt of 2,4-dinitrophenol was transferred with extreme care to a pistol drying tube and dried at 101 °C under 2 Torr for 12 h or longer. To a dioxane solution of the dried salt was added dropwise at room temperature 0.045 mol of acid chloride with constant stirring. The mixture was heated to a gentle reflux and stirred for 2 h or more. The solution was then filtered and the dioxane removed under reduced pressure. If the remaining crude product would not recrystallize from ether or ether–petroleum ether mixtures, its purity was enhanced by silica gel chromatography before attempting further recrystallizations. All four esters were obtained in about 50% yield. The results from the elemental analysis along with the melting point of each ester are listed in Table I. Nuclear magnetic resonance spectra of the four compounds all agreed well with their structure. In addition, spectroscopic measurements show that upon base hydrolysis each substrate liberates 1 equiv of 2,4-dinitrophenolate.

Carbonic anhydrase from bovine erythrocytes was prepared and purified as described earlier (Pocker and Guilbert, 1972) and its purity monitored by acetazolamide inhibition of 2,4-dinitrophenyl propionate hydrolysis. Consequently, all enzyme concentrations, deduced from ultraviolet absorbance measurements at 280 nm employing ϵ as 54 000 (Lindskog, 1969), were corrected to reflect the amount of active enzyme actually present. The bovine B isozyme was purchased from Miles-Servac Ltd.

Apo-BCA was prepared by dialyzing a $1\text{–}5 \times 10^{-4}$ M solution of the native enzyme against a 0.1 M sodium acetate buffer, pH 5.0, containing 1×10^{-2} M 1,10-phenanthroline. After dialyzing for 14 days at −5 °C, the 1,10-phenanthroline was removed by dialysis against deionized, distilled water with the small amount of precipitate being removed by centrifugation. The zinc content of the apoenzyme was found to be less than 2% as determined by atomic absorption spectrophotometry.

Co(II) BCA was prepared by dialyzing aliquots of the apoenzyme against frequent changes of a 1×10^{-3} M solution of $CoSO_4$ for 3 h at room temperature. The excess Co(II) was then removed by dialysis against deionized, distilled water and if necessary the enzymatic solution was centrifuged to remove any insoluble precipitate.

Buffer Components and Solutions. All buffers were prepared with deionized, distilled water and the total buffer concentration was maintained at 0.05 M. The ionic strength was brought to 0.15 by addition of sodium sulfate (Baker). The buffer components, sodium acetate (Baker), acetic acid (Baker), sodium dihydrogen phosphate (B&A), dipotassium hydrogen phosphate (Merck), Tris (Matheson, Coleman and Bell), and sulfuric acid (Baker), were all reagent grade or the equivalent and used without further purification. Triethylamine (Eastman Kodak) was refluxed over barium oxide and then distilled (bp 88.5–90.0 °C). Acetazolamide (Lederle Laboratories) was found to be analytically pure (Pocker and Guilbert 1972) and was used as obtained. Reagent-grade acetone (Baker), used as a solvent for the preparation of stock ester solutions, was dried over $CaSO_4$ and then fractionally distilled from P_2O_5 (bp 56.2–56.5 °C).

Instrumentation. Hydrolysis rates were monitored spectrophotometrically on a Varian Techtron Model 635 spectrophotometer equipped with a Forma-Temp Jr. Model 2095

[4] Melting points and boiling points are uncorrected.

circulating bath to maintain the temperature at 25.00 ± 0.05 °C. All pH measurements were recorded at 25 °C with a Beckman Model 101900 research pH meter fitted with a Corning glass electrode (No. 476022) and a Beckman reference electrode (No. 39071). The pH readings were corrected for activity effects using eq 1 and 2, where I and Z stand for the ionic strength and charge, respectively.

$$\text{pH} = -\log([H_3O^+]f_\pm) \quad (1)$$

$$\log f_\pm = \frac{-0.51Z^2I^{0.5}}{1 + 1.5I^{0.5}} \quad (2)$$

Nuclear magnetic resonance spectra for structure verification were obtained with a Varian Associates T-60 instrument. The zinc content of apo-BAC was determined using a Perkin-Elmer Model 303 atomic absorption spectrophotometer at 213.9 nm.[5]

Kinetics and Technique. The hydrolysis of the 2,4-dinitrophenyl carboxylate esters was followed spectrophotometrically by monitoring the appearance of the 2,4-dinitrophenolate anion at its peak absorbance (360 nm, ϵ 1.40 × 10^4 cm^{-1} M^{-1} in aqueous 10% (v/v) acetone). When a high concentration of the ester was used, the appearance of the anion was monitored at longer wavelengths. A typical procedure for a kinetic run was to initiate the reaction by injecting 10 μL of an acetone stock solution containing the desired ester by means of a Hamilton microliter syringe into the spectrophotometric cell containing 3 mL of the appropriate buffer, with or without enzyme.

At low ester concentration, $[S]_0 \ll K_m$, pseudo-first-order rate coefficients were evaluated using a Fortran IV computer program executed on a CDC 6400 digital computer. The program was written by Dr. N. Watamori to calculate the best slope for first-order rate plots by means of a least-squares method. The program analyzed the data as plots of log $(A_\infty - A_t)$ vs. time. Only those rates with a correlation coefficient for the slope better than 0.9990 were used. All rates were done in triplicate.

The observed first-order rate constant for the hydrolysis of the 2,4-dinitrophenyl carboxylate esters in buffered aqueous media is best described by eq 3

$$k_{buff} = k_0 + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + k_{HB^+}[HB^+] + k_B[B] \quad (3)$$

where k_0 is the catalytic coefficient for the water-catalyzed reaction, and k_{HB^+} and k_B are the respective catalytic coefficients for the acidic and basic components of the buffer. A 4 × 4 Tris buffer matrix was used in order to evaluate the coefficients k_0, k_{OH^-}, k_{TrisH^+}, and k_{Tris} (Bell and Darwent, 1950). When enzyme is added to the buffer the expression for the observed rate constant is now given by eq 4.

$$k_{obsd} = k_{buff} + k_{enz}[enz] \quad (4)$$

The catalytic coefficient for the enzyme was determined by eq 5.

$$k_{enz} = [k_{obsd} - k_{buff}](1/[enz]) \quad (5)$$

When it was desired to separate k_{enz} into values for K_m and k_{cat}, the method of Lineweaver-Burk was employed. In order to achieve greater initial ester concentrations, these rates were monitored in 10% acetone.

Inhibition of BCA activity by acetazolamide was studied as a function of inhibitor concentration. The inhibition constant, K_i, represents the equilibrium between enzyme and inhibitor, EI ⇌ E + I. K_i is defined by

$$K_i = \frac{([E]_0 - [EI])([I]_0 - [EI])}{[EI]} \quad (6)$$

where $[E]_0$ and $[I]_0$ are the total concentrations of BCA and acetazolamide, respectively, and [EI] is the concentration of the enzyme-inhibitor complex. A value for [EI] was calculated from the relationship

$$[EI] = [E]_0\left(1 - \frac{k_{enz}^I}{k_{enz}^0}\right) \quad (7)$$

where k_{enz}^I is the rate constant at a particular concentration of acetazolamide and k_{enz}^0 is the rate constant with no inhibitor present. This relation is valid provided the enzyme-inhibitor complex is completely inactive with respect to substrate hydrolysis.

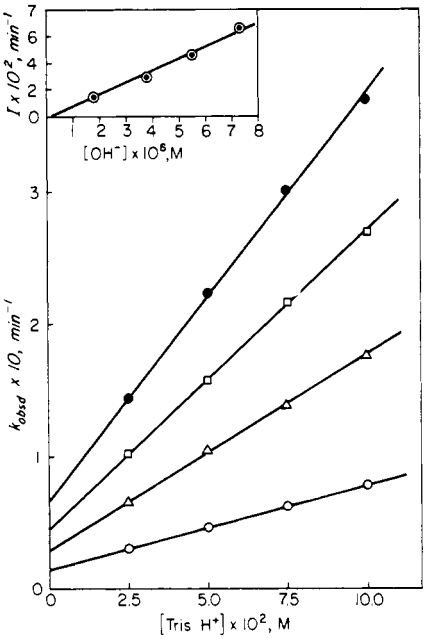


FIGURE 1: Bell-Darwent plots for Tris-catalyzed hydrolysis of 2,4-dinitrophenyl cyclobutanecarboxylate; k_{obsd} vs. [TrisH⁺] at 25.0 °C; μ = 0.15; (○) pH 8.24; (△) pH 8.57; (□) pH 8.74; (●) pH 8.86. Insert: Intercept vs. [OH⁻].

Results

Before analyzing the enzyme-catalyzed hydrolysis of the four esters, we felt it would be advantageous to study their chemical hydrolysis. The individual rate coefficients in eq 3 were separated by the method of Bell and Darwent (see Pocker et al., 1975 for details). Figure 1 illustrates the dependence of the rate constant, k_{obsd}, on the Tris buffer concentration at four different pH values for the hydrolysis of 2,4-dinitrophenyl *cyclobutanecarboxylate*. By plotting the intercept of each of these lines against the corresponding hydroxide ion concentration it is possible to deduce k_{OH^-}, Figure 1 (insert). Similarly, by plotting the slope of the lines in Figure 1 against the corresponding buffer ratio, [Tris]/[TrisH⁺], it is possible to deduce k_{Tris} and k_{TrisH^+}. A summary of the values for k_{OH^-} and k_{Tris} determined by this method is listed in Table II.

The individual pH-rate profiles for enzymatic activity (Figure 2) were determined under the condition $[S] \ll K_m$ with the temperature and ionic strength held constant at 25.0 °C and 0.15, respectively. At most pH values the buffer rate contributed only 5 to 10% of the enzymatic rate and at no time did it exceed 50%. As can be seen from Figure 2, the enzyme-

[5] The experiment was kindly performed by Mr. Dick Huntamer of the Laboratory of Radiation Ecology in the College of Fisheries.

TABLE II: Catalytic Coefficients for the Hydrolysis of 2,4-Dinitrophenyl Carboxylate Esters.[a]

Ester	$k_{OH^-} \times 10^{-3}$ (min^{-1} M^{-1})	k_{Tris} (min^{-1} M^{-1})
2,4-Dinitrophenyl propionate	2.57	0.625
2,4-Dinitrophenyl acrylate	5.59	3.01
2,4-Dinitrophenyl cyclopropanecarboxylate	1.02	0.163
2,4-Dinitrophenyl cyclobutanecarboxylate	10.8	0.747

[a] Respective values for k_{TrisH^+} and k_0 were too small to be accurately determined.

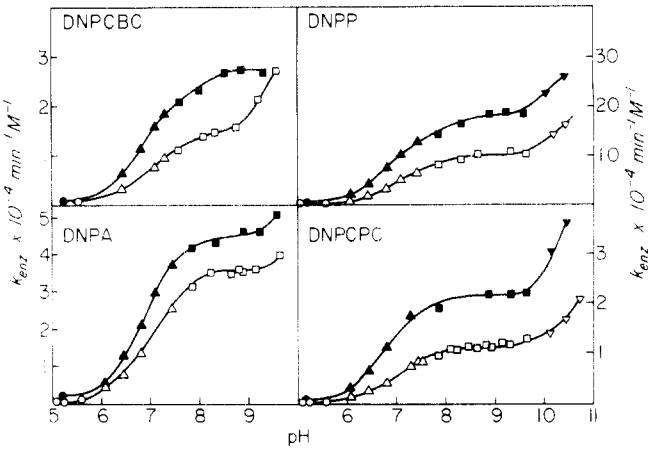


FIGURE 2: The BCA-catalyzed hydrolysis of 2,4-dinitrophenyl carboxylate esters as a function of pH at 25.0 °C. (○) acetate; (△) phosphate; (□) Tris; (▽) triethylamine. k_{enz} vs. pH plot. (Open symbols refer to the Zn BCA catalyzed hydrolysis and filled symbols refer to the Co BCA catalyzed reaction.)

catalyzed hydrolysis of all four esters increases with pH and seems to exhibit a first inflection between pH 7 and 7.5. To obtain a more exact value for the point of inflection and to determine the number of protons involved in the ionization of the active group(s), plots of log k_{enz} against pH were made. Table III summarizes the results.

Since a rapid release ("burst") of 2,4-dinitrophenolate was not observed during the hydrolysis, the dependence of rate on substrate concentration was formally analyzed in terms of the limiting Michaelis–Menten scheme, eq 8.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (8)$$

Lineweaver–Burk plots were used to determine formal values of $K_m \equiv (k_{-1} + k_2)/k_1$, and $V_m = k_2[E]$ at various pH values between 7.2 and 9.2 for all four esters (Table IV). Plots of K_m vs. k_2 were linear in each case which indicates that the binding term, $K_s \equiv (k_1/k_{-1})$, remains constant and that K_m is to a first approximation a linear function of the turnover number, k_2. Consequently, the sigmoidal dependence around neutral pH of k_{enz} must be due solely to changes in the turnover number. The plots of K_m vs. k_2 also allow k_1 and k_{-1} to be assessed. A value for k_1 is obtained from the reciprocal of the slope and that for k_{-1} can be deduced from the intercept after dividing by k_1. Table V compares the values obtained for k_1, k_{-1}, K_s and the $\Delta G°$ for binding with those obtained from a series of *p*-nitrophenyl carboxylate esters (Pocker and Storm, 1968).

A common and predominant feature of the pH–rate profiles for the BCA-catalyzed hydrolysis of carboxylic acid esters is a second rise in activity at high pH (Pocker and Storm, 1968; Pocker and Watamori, 1973). As seen from Figure 2, a second increase in activity for each of the four esters is easily detected at pH values around 10. However, the exact pH which marks

TABLE III: Selective Parameters Resulting from log k_{enz} vs. pH Plots.

2,4-Dinitrophenyl Ester	Zn BCA		Co BCA	
	Slope	pK_a	Slope	pK_a
Propionate	0.97	7.18	1.0	7.15
Acrylate	0.97	7.10	1.0	7.05
Cyclopropanecarboxylate	0.94	7.05	1.02	6.90
Cyclobutanecarboxylate	0.64	7.35	0.75	7.27

TABLE IV: Results from Lineweaver–Burk Plots for Zn BCA Catalyzed Hydrolysis of Esters as a Function of pH at 25.0 °C.[a,b]

Substrate[c,d]	pH	$K_m \times 10^3$ (M)	$V_m \times 10^3$ (min^{-1} M)	$k_2 \times 10^{-2}$ (min^{-1})
DNPP	7.46	8.33	7.41	7.91
	8.26	25.0	8.00	31.9
	9.16	50.0	33.3	68.7
DNPA	7.39	5.55	1.25	1.07
	8.00	20.0	6.90	8.31
	8.71	33.3	8.00	14.8
DNPCPC	7.94	3.57	0.235	0.321
	8.24	5.56	0.833	0.837
	8.39	7.69	0.909	1.36
DNPCBC	7.44	2.50	0.204	0.219
	7.81	2.86	0.286	0.354
	9.16	6.66	1.33	1.49

[a] The concentration of Zn BCA ranged from 2.51×10^{-6} to 9.95×10^{-6} M. [b] Determined in 0.05 M Tris buffer; ionic strength maintained at 0.15 with Na_2SO_4 in 10% (v/v) acetone. [c] DNPP, 2,4-dinitrophenyl propionate; DNPA, 2,4-dinitrophenyl acrylate; DNPCPC, 2,4-dinitrophenyl cyclopropanecarboxylate; DNPCBC, 2,4-dinitrophenyl cyclobutanecarboxylate. [d] Lineweaver–Burk plots were used to determine the formal values of K_m and V_m. For each ester and given pH the line of best fit was obtained from five data points. In most cases when these data points were plotted in reciprocal form they fell on a straight line which coincided with the best fitting line calculated by the method of least squares (Bjorkquist, 1975).

TABLE V: Comparison of the Binding Constants of BCA with 2,4-Dinitrophenyl Carboxylate Esters and *p*-Nitrophenyl Carboxylate Esters at 25.0 °C.

Ester	$k_1 \times 10^{-4}$ (min^{-1} M^{-1})	$k_{-1} \times 10^{-2}$ (min^{-1})	$K_s = (k_1/k_{-1})$ $\times 10^{-2}$ M^{-1}	$\Delta G°$ (cal)
p-Nitrophenyl acetate[a]	2.00	0.400	5.00	3680
p-Nitrophenyl propionate[b]	1.23	0.066	18.50	4460
p-Nitrophenyl isobutyrate[b]	0.216	0.004	56.30	5120
p-Nitrophenyl *n*-caproate[b]	0.146	0.0003	500.0	6410
2,4-Dinitrophenyl propionate[c]	14.5	4.36	3.33	3450
2,4-Dinitrophenyl acrylate[c]	5.0	1.75	2.86	3370
2,4-Dinitrophenyl cyclopropanecarboxylate[c]	2.34	0.502	4.67	3660
2,4-Dinitrophenyl cyclobutanecarboxylate[c]	3.06	0.44	5.56	3760

[a] Values determined (Pocker and Stone, 1967) in 10% (v/v) acetonitrile. [b] Values determined (Pocker and Storm, 1968) in 1% (v/v) acetonitrile. [c] Values determined in 10% (v/v) acetone.

TABLE VI: Residual Activity of Apo-BCA[a] at 25.0 °C.

2,4-Dinitrophenyl Ester	pH	$k_{enz} \times 10^{-4}$ M^{-1} min^{-1} k_{enz}^{apo}	k_{enz}^{native}
Propionate	5.53	0.027	0.10
	6.42	0.23	1.86
	7.09	0.26	5.25
	7.57	0.45	7.20
	8.06	0.45	8.72
	10.2	0.90	14.3
	10.49	2.7	17.0
Cyclobutanecarboxylate	5.58	0.05	0.11
	6.42	0.08	0.31
	7.09	0.13	0.76
	7.50	0.25[b]	1.10
	8.06	0.26	1.39
	8.75	0.34	1.60
	9.14	1.20	2.13
	9.57	2.83	2.73
Cyclopropanecarboxylate	7.50	0.024	1.23
	8.88	0.048	1.63
	10.49	1.1	1.73
	11.12	2.1	

[a] Values are uncorrected for activity due to residual Zn BCA (less than 2%). To remove traces of metal ions present in analytical reagents, all buffer solutions were shaken with successive portions of a 0.001% solution of dithizone in CCl_4 until the organic phase remained pure green (J. E. Stein, unpublished observations, 1976). In the absence of these precautions, somewhat higher values of k_{enz}^{apo} are obtained (Figure 3). [b] At pH 7.5, in the presence of a 26-fold excess of acetazolamide, $k_{enz}^{apo} = 0.23 \times 10^4$ M^{-1} min^{-1}.

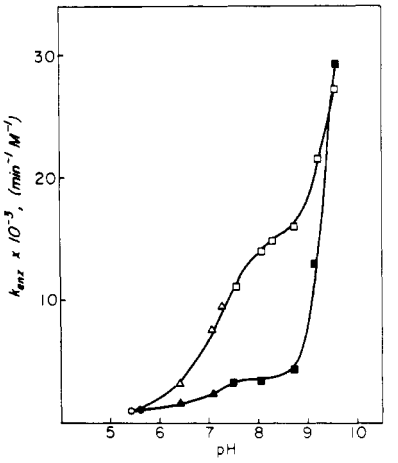


FIGURE 3: The BCA-catalyzed hydrolysis of 2,4-dinitrophenyl cyclobutanecarboxylate as a function of pH at 25.0 °C. Plots of k_{enz} vs. pH: open symbols, Zn BCA; filled symbols, apo-BCA; (○ and ●) acetate; (△ and ▲) phosphate; (□ and ■) Tris.

the beginning of the second rise in activity seems to be dependent to a large degree on the size of the substrate. To determine whether the zinc(II) ion is an essential component for the high pH activity, a pH profile of the hydrolysis of DNPCBC with apo-BCA was performed (Figure 3). Two striking observations resulted from this study. First, in contrast to DNPP, DNPA, and DNPCPC, the apo-BAC catalyzed hydrolysis of DNPCBC possesses 20% of the activity of the native enzyme at neutral pH values (Table VI). Secondly, as illustrated in Table VI the activity of the apoenzyme adequately accounts for the additional high pH activity of the native enzyme for the substrates tested.

To determine whether the 20% residual activity for the hydrolysis of DNPCBC with the apoenzyme could be caused by turnover in a second esteratic binding site independent of zinc, the hydrolysis was monitored in the presence of acetazolamide. It is well documented that acetazolamide can abolish carbonic anhydrase activity with respect to both hydration (Kernohan, 1965; Pocker and Meany, 1965; Pocker and Dickerson, 1968) and hydrolysis (Pocker and Stone, 1965; Armstrong et al., 1966; Pocker and Watamori, 1973). Moreover, there is ample evidence that this particular inhibitor binds near enough to the zinc to be coordinated to it (Tilander et al., 1965; Fridborg et al., 1967; Liljas et al., 1969; Coleman, 1965). Plots of enzymatic activity vs. the ratio of acetazolamide to enzyme concentration are given in Figure 4. Values of the dissociation constants for the enzyme-acetazolamide complex in the presence of DNPP, DNPA, DNPCPC, and DNPCBC are 7.9×10^{-9}, 1.4×10^{-8}, 2.7×10^{-8}, and 3.7×10^{-8} M, respectively, at pH 7.5. It should be noted that for all the kinetics with acetazolamide its concentration is very low and free [I] values are not accurately known. Therefore, these inhibition constants are only apparent values.

Finally to check that the 20% residual activity is not caused by an impurity, the hydrolysis of DNPCBC was followed by a Tris buffer of pH 7.57 containing pure BCA-B. Even when an acetazolamide to BCA-B concentration ratio of 36 was reduced, the 20% residual activity remained.

Discussion

The study of the chemical hydrolysis of these four esters has uncovered interesting information about their structure–reactivity relationship. Their hydrolysis was primarily catalyzed by hydroxide ions, but the basic component of a Tris buffer also made a small contribution to the observed rate (Table II). The observed order for the value of k_{OH^-} is DNPCBC > DNPA > DNPP > DNPCPC. In fact, the value of k_{OH^-} for the cy-

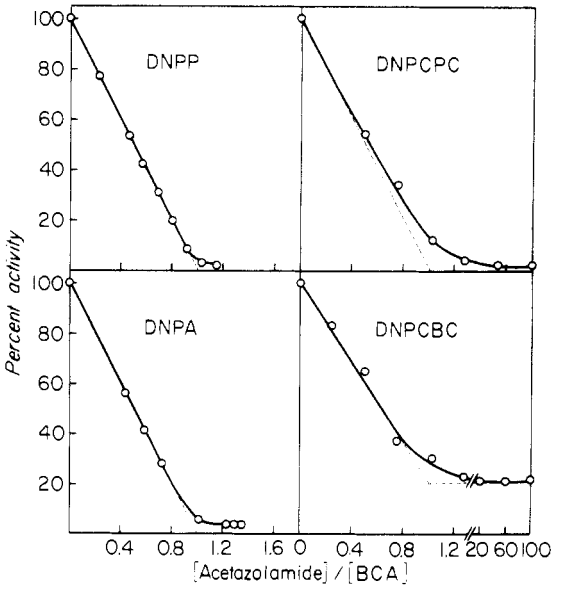


FIGURE 4: Plots of esterase activity as a function of [acetazolamide]/[BCA] ratio for the Zn BCA catalyzed hydrolyses of 2,4-dinitrophenyl propionate (DNPP), acrylate (DNPA), cyclopropanecarboxylate (DNPCPC), and cyclobutanecarboxylate (DNPCBC) at 25.0 °C.

clobutyl carboxylate ester is about ten times larger than that for the cyclopropyl carboxylate ester. This surprising result is just the opposite of what would be predicted on steric grounds. Therefore, it is necessary to search for an electronic effect in order to explain the observed trend. Recent molecular orbital calculations have shown that the cyclopropyl ring structure can stabilize an adjacent positive center better than a vinyl group, which in turn is better than a cyclobutyl ring system. If it is assumed that the difference in reactivity of these four esters depends on the partial positive charge developed on the carbonyl carbon in the ground state, then an adjacent cyclopropyl ring can donate electrons to the unsaturated center and render the molecule less susceptible to nucleophilic attack.

The ionization of either the zinc-aquo complex or the imidazolium ion of a histidine residue has been suggested to account for the increase in carbonic anhydrase activity at neutral pH. If the zinc ion is involved in the process, then an exchange of this metal by cobalt might affect the ionization of the metal-bound water and consequently shift the inflection of the pH-rate profile. On the other hand, if the ionization of an imidazolium ion is important, then to a first approximation the inflection in the pH-rate profile should be independent of the metal present in the active site. The results from Table III indicate that the inflection is consistently shifted to a lower value by about 0.1 pK unit in going from the zinc to the cobalt enzyme. However, this variation is too small to unambiguously distinguish which of the two acids is responsible for catalysis.

It should be noted that there is some experimental evidence suggesting that the pK_a of Co(II) and Zn(II) carbonic anhydrase might coincidentally be the same. Coates et al. have published pK_a values for $[Zn(H_2O)_6]^{2+}$, $[ZntrenH_2O]^{2+}$, and $[ZnMe_6trenH_2O]^{2+}$ of 9.5, 10.26, and 9.00, respectively. The corresponding values for the cobalt complexes are 9.8, 10.22, and 8.8 (Coates et al., 1974). Certainly the trend in these data suggests that the ionization of the metal-bound water of a zinc and cobalt complex will be about the same as long as both metals are coordinated by an identical set of ligands. Preliminary x-ray data have revealed that zinc and cobalt do indeed bind at the same location in the active site of carbonic anhydrase and that they presumably are both coordinated by three histidines and one water molecule (Liljas et al., 1972; Kannan et al., 1975). Therefore, a shift in acidity by an equal amount for both zinc and cobalt would be expected in going from aqueous solution to the microenvironment in the active site of the enzyme, since in both instances one set of identical ligands is being replaced by another. In this regard it is interesting to speculate that the shift in acidity from $[Mn(H_2O)_6]^{2+}$ to Mn-BCA will also be by the same amount as for cobalt and zinc. Assuming that the ionization of the metal-bound water is controlling the activity at neutral pH and using 10.6 as the pK_a of $[Mn(H_2O)_6]^{2+}$, then a rough estimate for the pK_a of Mn BCA would be 8.1, a value actually found experimentally for the Mn BCA catalyzed hydrolysis of *p*-nitrophenyl acetate (Lanir et al., 1975). It has been recently demonstrated that the Cd enzyme also exhibits esterase activity but the pH-activity profile is shifted to even higher pH values (pK 9 ± 0.2) as compared to the Zn(II) and Co(II) enzyme (Bauer et al., 1976; Y. Pocker and J. T. Stone, unpublished observations).

An integral part of the esterase profile of carbonic anhydrase is the second rise in activity at high pH values. In general it appears that this enhanced activity results from the attack of an enzyme-affiliated nucleophile or general base whose conjugate acid is being titrated at pH > 9. It has been suggested that the increase in enzymatic activity at high pH might in certain cases be due to the formation of a more active zinc-hydroxo complex (Pocker and Storm, 1968). However, this complex is clearly not responsible for the activity of apo-BCA-B with respect to the hydrolysis of DNPCBC (Figure 3). The zinc ion has been removed in this instance, but the high pH activity is still observed. In fact, the apoenzyme seems to adequately account for most if not the entire high pH increase in activity for DNPP and DNPCPC as well (Table V). Furthermore, preliminary inhibition data with acetazolamide indicate that two concurrent kinetic terms account for the high pH activity (Y. Pocker, L. Bjorkquist, and D. Bjorkquist, unpublished observations). One of the terms must be associated with the titration of an amino acid whose pK_a is nine or above, and the other term is a constant value associated with the contribution by the conjugate base of the acid controlling the activity at neutral pH.

The inhibition studies with acetazolamide have also been useful in identifying the existence of a secondary esteratic binding site for BCA. Figure 4 illustrates that, at high acetazolamide to enzyme concentration ratios, differing percentages of BCA activity can be abolished. With respect to the hydrolysis of DNPP, DNPA, and DNPCPC, acetazolamide is capable of eradicating nearly all of the activity at pH 7.5. However, at the same pH and with a concentration of acetazolamide 100 times that of BCA only 80% of the enzymatic activity can be abrogated with respect to the hydrolysis of DNPCBC. This result clearly suggests that there is a second binding site at neutral pH which is independent of zinc and accounts for 20% of the activity.

Further confirmation for the idea of a second esteratic binding site at neutral pH comes from three independent experiments with DNPCBC. (1) When the rate of hydrolysis was monitored at pH 7.5 with apo-BCA, approximately 20% residual activity remained. This result clearly was expected, since the removal of the zinc should not affect the catalysis occurring at a secondary binding site independent of zinc. Furthermore, as would be predicted, the addition of acetazolamide to apo-BCA could not reduce the activity significantly. (2) Plots of $1/v$ against $1/[S]_0$ demonstrated marked upward curvature at high substrate concentrations (Bjorkquist, 1975). This be-

havior is in good agreement with the expected kinetics for an enzyme with two binding sites, providing that the binding of substrate to one site will affect the properties of the other (Webb, 1963). (3) The pH–rate profiles also assist in revealing that the BCA-catalyzed hydrolysis of DNPCBC is different from the other three esters studied. By plotting log k_{enz} vs. pH, the number of protons involved in the ionization process can be determined from the slope of the curve in the acidic region. Results from such a plot (Table III) indicate that there is one proton involved in the hydrolysis of DNPP, DNPA, and DNPCPC near neutral pH. However, the data resulting from the study with DNPCBC indicates otherwise. Since the slope of the line is only 0.68, it is quite possible that the binding of the bulky DNPCBC substrate in the "normal" ester binding site results in a conformational change that not only reduces the enzymatic activity, but causes irreversible changes in protein conformation as well which lead to the creation of a new secondary binding site. Additional changes in conformation at high pH may further alter this secondary site and bring the substrate into position to be decomposed by an amino acid residue whose conjugate acid ionizes above pH 9.

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