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The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. IV. Kinetic Studies of Enzyme-Catalyzed Hydrolyses of *p*-Nitrophenyl Esters*

Y. Pocker† and D. R. Storm

ABSTRACT: Kinetic studies on the esterase activity of bovine carbonic anhydrase with *p*-nitrophenyl propionate, *n*-butyrate, isobutyrate, *n*-valerate, isovalerate, *n*-caproate, and trimethyl acetate as substrates are reported. Esterase activity rises continuously but non-uniformly with increasing pH between 4.0 and 10.9. Below pH 9.0 the profiles for propionate, *n*-butyrate, isobutyrate, and isovalerate are quite similar to that previously obtained for *p*-nitrophenyl acetate: they are sigmoidal with an inflection occurring around pH 7.3. In contrast, the two longer substrates, *p*-nitrophenyl *n*-valerate and *p*-nitrophenyl *n*-caproate, exhibit an "abnormal" behavior. (i) Their rate profiles show inflections at pH 6.2 and 5.7, respectively, rather than at 7.3, and (ii) their K_i values for the specific inhibitor acetazolamide are 10^3 times larger than those found with *p*-nitrophenyl acetate and propionate as substrates. The sterically hindered trimethyl acetate ester is subject to little or no enzymatic catalysis below pH 8.5. The enzymatic hydrolyses of all the esters reported in this paper exhibit a second inflection, of much greater magnitude around pH 10.5; this dramatic increase in enzymatic activity at high pH has not been reported in any previous kinetic study pertaining to carbonic anhydrase.

A formal Michaelis–Menten treatment of these enzyme-catalyzed hydrolyses shows that both k_2 and K_m vary with pH. The variation of K_m with pH is shown to be dictated by the pH dependency of the turnover number (k_2) while the apparent binding constant (k_1/k_{-1}) appears to be pH independent below 9. A comparison of the various esters reveals that binding increases with the size of the ester. The relative increase in free energy of binding associated with the larger esters is shown to be well accounted for by a parallel increase in the free energy of hydrophobic interactions. D_2O studies indicate that k_2 in D_2O is twice as large as it is in H_2O.

Recent investigations conducted in these laboratories have demonstrated that carbonic anhydrase catalyzes the reversible hydration of various aldehydes (Pocker and Meany, 1965a,b, 1967a,b; Pocker *et al.*, 1965) and the hydrolysis of *p*-nitrophenyl acetate (Pocker and Stone, 1965, 1967). Kinetic studies on propionaldehyde, isobutyraldehyde, and pivalaldehyde hydrations revealed that the catalytic efficiency of bovine carbonic

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TABLE I: Physical Properties of *p*-Nitrophenyl Esters.

Substrate	Physical Appearance	Mp (°C)	Bp (°C)	$\epsilon_{400} \times 10^{-3}$ (M^{-1} cm^{-1})[a]
Propionate	White solid	62.5–63		17.9
Isobutyrate	White solid	39.5–40		17.9
Trimethylacetate	White solid	95.5–96		18.0
n-Butyrate	Pale yellow liquid		50[b]	17.9
n-Valerate	Yellow liquid		67[b]	17.9
n-Caproate	Yellow liquid		89[b]	18.0
Isovalerate	Yellow liquid		80[b]	17.8

[a] On complete hydrolysis by sodium hydroxide in 1% (v/v) acetonitrile. [b] At 0.1 mm.

anhydrase[1] with respect to these substrates was inversely proportional to the size of the aldehyde (Pocker *et al.*, 1965). In the present paper we have attempted to gain further insight into the mode of action of carbonic anhydrase by delineating its substrate specificity, with respect to the enzymatic hydrolysis of a homologous series of *p*-nitrophenyl esters. These hydrolyses can be studied over a much wider pH range than the hydration of CO_2 (Davis, 1961) or acetaldehyde (Pocker and Meany, 1965a,b). This is particularly true for the *p*-nitrophenyl esters of the larger aliphatic acids because their hydrolyses can be easily monitored even at high pH. The low solubility of *p*-nitrophenyl esters has been and still remains a major difficulty in any direct observation of the zero-order region in carbonic anhydrase. Nevertheless, valuable information can be obtained by varying substrate concentration up to $[S] \simeq K_m$. The present study has allowed us to define the structural limits of the *p*-nitrophenyl esters upon which carbonic anhydrase will act catalytically, and to gain some understanding of the nature of the forces and groups shaping its esterase activity.

Experimental Section

Materials

p-Nitrophenyl Esters. All of the *p*-nitrophenyl esters used in this study were synthesized by refluxing nitrophenol (0.20 mole) with freshly distilled acid chloride (0.24 mole) in the presence of powdered magnesium metal (0.20 mole) in 200 ml of benzene (Spasov, 1938; Huggins and Lapides, 1947). Reaction times for esterification varied from 1 to 3 hr depending upon the particular acid chloride used. Refluxing was continued until the evolution of HCl ceased. The resulting reaction mixture was washed with distilled water, then with saturated aqueous bicarbonate, and was finally dried over anhydrous sodium sulfate. Esters were collected by removing the benzene under reduced pressure. The solid esters were repeatedly recrystallized from diethyl ether and the melting points given in Table I compare favorably with values previously reported (Spasov, 1938; Huggins and Lapides, 1947; McDonald and Balls, 1957). The liquid esters were distilled at 0.1 mm with a molecular distillation apparatus. Prior to distillation the liquid esters were brown liquids; after distillation they were pale yellow. The infrared and nuclear magnetic resonance spectra agreed with the designated structures. In addition, weighed amounts of each ester were compared with the spectrophotometric concentration of *p*-nitrophenol produced on complete hydrolysis. Purity was further checked by determining k_{enzyme} values for each substrate during progressive stages of the purification scheme.

p-Nitrophenol obtained from the Matheson Co. was recrystallized from diethyl ether and then twice sublimed to a constant melting point of 114–114.5°. A value of 18,100 was obtained for the molar extinction coefficient of *p*-nitrophenolate ion at 400 mμ in 1% (v/v) acetonitrile and this value compares favorably with values previously reported (Kézdy and Bender, 1962; Gibbons and Edsall, 1963; Pocker and Stone, 1967).

Acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, obtained from American Cyanamid (Lederle Laboratory Division) was used without further purification. Reagent grade acetonitrile (Baker Analyzed) was used as a solvent for preparation of stock solutions of the various *p*-nitrophenyl esters. Analysis by vapor phase chromatography showed that this acetonitrile contained less than 0.1% water. Stock solutions of the various esters underwent no detectable decomposition on prolonged standing.

Bovine carbonic anhydrase was supplied by Mann Research Laboratories where it is isolated from bovine erythrocytes and purified by the method of Keilin and Mann (1940). The enzyme showed no loss of esterase activity when stored at −20° under anhydrous conditions. Activity was periodically checked by determining the enzymatic catalytic constant for the hydrolysis of *p*-nitrophenyl propionate at pH 9.0 in 0.05 M Tris buffer brought to a final ionic strength of 0.09. Two different samples of enzyme were used and the results from these are kept separate and identified as BCA_I and BCA_{II}. There were, however, no detectable

[1] Abbreviation used: BCA, bovine carbonic anhydrase.

TABLE II: Buffer System Employed for Kinetics.

Buffer	pK_a (25.0°)[d]	pH Range Used
Acetate[a]	4.75	4–5
Phosphate[a]	7.21	6–7.5
2-Amino-2-hydroxylmethyl-1,3-propanediol (Tris)[b]	8.08	7.5–9.5
Ephedrine[b]	9.55	9.5–10.5
Triethylamine[c]	10.67	10.5–10.9

[a] Only sodium salts were used. [b] Used as Tris–Tris·H^+,Cl^- and ephedrine–ephedrine·H^+,Cl^-. [c] Used as Et_3N–Et_3N^+H,O^-Ac. [d] Values of pK_a taken from Perrin (1964).

kinetic differences between the two samples. The enzyme preparations used were a mixture of the two isozymes A and B. It has been previously demonstrated (Pocker and Stone, 1967) that the two isozymes exhibit identical esterase activity at pH 7.0 with *p*-nitrophenyl acetate as substrate.

Buffer Components and Solutions. Erythrocyte carbonic anhydrase exhibits activity which is quite buffer dependent and it is therefore necessary to employ a set of buffers whose inhibitory effect is either negligibly small or at least fairly similar. Furthermore, it is advantageous fo choose buffer components which do not excessively promote the hydrolysis or aminolysis of the *p*-nitrophenyl esters. The family of buffers listed in Table II meets the above criteria for the bovine carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl esters. All buffer components with the exception of Tris and triethylamine were analytical grade reagents. Tris was purified by sublimation and triethylamine was purified by fractional distillation.

The total buffer concentration used in this work was maintained constant at 0.05 M throughout the pH range studied. Ephedrine and Tris buffers were prepared by acidifying solutions of the respective amines to the desired pH with HCl. The phosphate buffers were prepared by accurately weighing samples of NaH_2PO_4 and Na_2HPO_4 to the desired buffer ratio. Triethylamine hydrochloride was found to exhibit inadequate buffering capacity at pH values greater than 10.5 but the corresponding acetate proved satisfactory. Triethylammonium acetate buffers were made by acidifying a solution of triethylamine to the desired pH with acetic acid. All buffer solutions were brought to an ionic strength of 0.09 by adding an appropriate amount of NaCl.

Instrumentation

Ester hydrolysis was followed with a Beckman DU-2 spectrophotometer equipped with an insulated cell compartment consisting of a specially constructed bath thermostated to 25.0 ± 0.02° by means of a Sargent Model SV (S-82060) thermonitor. All pH and pD determinations were made with a Beckman 101900 research pH meter equipped with a Beckman Calomel Internal 39071 frit junction reference electrode and a Beckman glass electrode 41263. The pD values reported are pD = pH reading + 0.41 (Glasoe and Long, 1960; Li *et al.*, 1961). This pH meter has a relative accuracy of ±0.001 pH unit and was calibrated against standard Beckman buffers. The absorption of CO_2 by buffer solutions at pH $\geqslant$ 10 necessitated the use of a nitrogen atmosphere over these solutions. pH and pD determinations were made on reaction mixtures immersed in a 25.0° thermostat. Nuclear magnetic resonance spectra for structure verification were obtained with a Varian Associates A-60 instrument.

Kinetics and Technique. Kinetic runs were initiated by the following method. Acetonitrile (0.03 ml) containing the substrate was injected with a calibrated Hamilton syringe into 3 ml of the appropriate buffer solution giving a reaction mixture which was 1% (v/v) acetonitrile. This method had several important advantages; stock solutions of esters could be prepared and stored for prolonged periods of time with no decomposition. The acetonitrile enhanced the solubility of these esters which proved particularly important in deducing Michaelis–Menten parameters. In addition, acetonitrile is both transparent above wavelengths of 280 mμ and is only a weak inhibitor of bovine carbonic anhydrase activity (Pocker and Stone, 1965, 1967).

Above pH 6.5, the hydrolyses of the *p*-nitrophenyl esters were studied spectrophotometrically by following the appearance of *p*-nitrophenolate anion at 400 mμ. These reactions are pseudo-first order and proceed to completion (>99% hydrolysis). Plots of $-\log (A_\infty - A_t)$ *vs.* time were linear with substrate concentrations below 5×10^{-5} M for at least 2 half-lives. First-order rate constants were obtained from the slopes of these plots. A FORTRAN IV computer program was written for the evaluation of first-order rate constants. All computer calculations were executed by an IBM 7094 digital computer. This program was written to calculate the best slope for first-order plots by means of the least-square method. The output of the program included the standard deviation and confidence limits for each kinetic run as well as the first-order rate constant for each half-life followed.

pH values reported in this study are for the initial reaction mixture which included 1% (v/v) acetonitrile, enzyme, substrate, and the buffer components. At low ester concentrations the pH remained constant (±0.01 pH unit) throughout a given run, but at high substrate concentrations we were able to detect small pH changes. When these changes occurred, k_{obsd} was calculated from first-order plots of $-\log (A_\infty - A_t)$ *vs.* time, where A_t were absorbance readings for the initial part of the reaction, and A_∞ was the infinity absorbance corrected to correspond to the initial pH. The correct infinity absorbance at each pH was deduced using a conversion factor relating absorbances at 400 and 348 mμ. The isosbestic point for the *p*-nitrophenol–*p*-nitrophenolate ion is located at 348 mμ (ϵ_{348} 5200) (Bergmann *et al.*, 1958), while at 400 mμ (ϵ 18,100)

for *p*-nitrophenolate ion and 200 M^{-1} cm^{-1} for *p*-nitrophenol (Gibbons and Edsall, 1963). For additional confirmation, the entire reaction was monitored at 348 mμ, this proved particularly useful at pH <6.5 where absorptivity is actually greater than at 400 mμ.

The observed first-order rate constant for the bovine carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl esters in buffered aqueous media can be represented by eq 1. Here k_0 is the catalytic constant for the

$$k_{obsd} = k_0 + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + k_{HB}[HB] + k_{B^-}[B^-] + k_{enzyme}[E] \quad (1)$$

water-catalyzed reaction and the various k's are catalytic constants for each species present in the system. HB and B^- are the acidic and basic forms of the buffer. The catalytic coefficient (k_{enzyme}) was determined by varying enzyme while keeping the concentrations of all other components constant. Plots of k_{obsd} *vs.* [E] gave straight lines whose slopes defined k_{enzyme}. At each pH, an average of six different concentrations of bovine carbonic anhydrase varying from 1×10^{-6} to 1.5×10^{-5} M were used for the determination of k_{enzyme}. The enzyme concentration was determined spectrophotometrically from its absorbance at 280 mμ. The molar extinction coefficient at this wavelength for bovine carbonic anhydrase has been previously determined as 54,000 based upon a molecular weight of 30,000 (Lindskog, 1960). With all substrates, concentrations of bovine carbonic anhydrase greater than 10^{-5} M had to be employed at pH >9.5, because hydroxide ion catalysis becomes significant relative to enzymatic catalysis. For the determination of pH–rate profiles, k_{enzyme} was obtained using 5×10^{-5} M substrate. For the determination of K_m and V_{max}, substrate concentrations were varied from 10^{-5} to 4×10^{-4} M. These concentrations were deduced from the *p*-nitrophenol–*p*-nitrophenolate absorbance at 400 and 348 mμ.

Inhibition of bovine carbonic anhydrase esterase activity by acetazolamide was studied as a function of inhibitor concentration. Percentage activity was determined from the $k_{enzyme}^{I}/k_{enzyme}^{0}$ ratios at various concentrations of acetazolamide. The catalytic coefficient, k_{enzyme}^{I}, refers to enzymatic activity at a particular concentration of acetazolamide and k_{enzyme}^{0} is the enzymatic catalytic coefficient with no acetazolamide present. The inhibition constant, K_i, is defined by

$$K_i = \frac{([E_0] - [EI])([I_0] - [EI])}{[EI]} \quad (2)$$

where $[E_0]$ and $[I_0]$ are the total concentrations of bovine carbonic anhydrase and acetazolamide, respectively, and [EI] is the concentration of the enzyme–inhibitor complex. Values for an apparent inhibition constant were determined by assuming the relation given in eq 3.

$$[EI] = [E_0]\left(1 - \frac{k_{enzyme}^{I}}{k_{enzyme}^{0}}\right) \quad (3)$$

TABLE III: The Catalytic Coefficients for *p*-Nitrophenyl Ester Hydrolysis.[a]

Substrate	$k_0 \times 10^4$[b] min^{-1}	k_T[c] (M^{-1} min^{-1})	k_{OH^-}[d] (M^{-1} min^{-1})
Propionate	4.0	0.0610	550
n-Butyrate	3.5	0.0383	440
n-Valerate	2.0	0.0371	390
n-Caproate	1.5	0.0310	370
Isobutyrate	3.0	0.0370	400
Isovalerate	~1.0	0.0220	190
Trimethyl-acetate	~1.0	0.0150	87

[a] Ionic strength 0.06. [b] The intercepts for deducing k_0 give in principle, $k_0 + k_{H_3O^+}[H_3O^+]$, but the latter term is small and can be neglected. [c] The contribution of the catalytic term $k_{TH^+}[TH^+]$ was negligibly small. [d] The coefficients k_{OH^-}'s were determined on the basis of a_{OH^-} rather than $[OH^-]$.

This relation is valid provided the enzyme–inhibitor complex is completely inactive with respect to substrate hydrolysis.

Results

As a preliminary to our kinetic studies of carbonic anhydrase catalysis we have determined the catalytic rate coefficients associated with H_2O, OH^-, and Tris using the method of Bell and Darwent (1950). The results are given in Table III. The reaction with the amino alcohol, Tris, is in part an aminolysis and in part a transesterification (Bruice and York, 1961). The values of k_T reported in Table III were obtained over the pH interval 8.0–8.9.

Enzymatic Activity as a Function of pH. The titration of enzymatic activity of bovine carbonic anhydrase is not only important in terms of its physiological significance but it also aids in the identification of titratable groups contributing to its activity. The method for determining k_{enzyme} has been described in the Experimental Section. Values of k_{enzyme} as a function of pH are listed for *p*-nitrophenyl propionate, *n*-butyrate, *n*-valerate, *n*-caproate, isobutyrate, isovalerate, and trimethylacetate in Table IV. It should be noted that trimethylacetate hydrolysis was apparently not catalyzed by bovine carbonic anhydrase at pH less than 8.5. The following general conclusions can be drawn about the pH–rate profiles depicting the bovine carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl esters (Figures 1–3).

(1) Values of k_{enzyme} obtained in different buffers, *e.g.*, Tris and phosphtae at pH 7.6 or Tris and ephedrine at pH 9.5, are in excellent agreement. (2) In the acidic region, the esterase activity of bovine carbonic anhydrase is greatly diminished for all substrates (Figures

TABLE IV: pH Dependency of k_{enzyme} for the Hydrolysis of *p*-Nitrophenyl Esters at 25.0°.

Substrate	pH, Buffer[a]	$k_{enzyme} \times 10^{-3}$[b]	pH, Buffer	$k_{enzyme} \times 10^{-3}$	pH, Buffer	$k_{enzyme} \times 10^{-3}$
Propionate	6.15, P	0.58	8.02, T	5.73	9.50, T	6.40
	6.79, P	1.83	8.62, T	6.00	9.55, E	6.40
	8.62, P	1.83	8.70, T	6.20	10.02, E	8.80
	7.39, P	4.01	8.81, T	6.25	10.26, E	10.00
	7.43, T	4.17	9.00, T	6.25	10.64, TA	13.42
	7.50, T	4.70	9.10, T	6.40		
n-Butyrate	6.12, P	0.42	8.02, T	0.71	9.96, E	1.62
	6.82, P	0.45	8.81, T	0.81	10.03, E	1.88
	7.39, T	0.57	9.55, E	1.46	10.76, TA	4.41
n-Valerate	3.99, A	0.16	7.50, T	1.24	9.96, E	1.39
	5.00, P	0.40	8.02, T	1.29	10.41, E	1.60
	6.12, P	0.77	9.00, T	1.30	10.81, TA	5.38
	6.81, P	1.16	9.56, E	1.30		
n-Caproate	4.00, A	0.07	7.49, T	0.85	9.93, E	1.65
	5.00, P	0.20	8.02, T	0.95	10.35, E	3.80
	6.12, P	0.57	9.00, T	1.02		
	6.84, P	0.73	9.54, E	1.25		
Isobutyrate	6.11, P	0.09	8.01, T	0.18	9.95, E	0.93
	6.79, P	0.09	8.81, T	0.30	10.37, E	1.18
	7.36, T	0.14	9.05, T	0.41	10.42, E	1.65
	7.53, T	0.17	9.11, T	0.45	10.65, TA	2.54
	7.96, T	0.18	9.51, E	0.60		
Isovalerate	6.11, P	0.10	8.01, T	0.17	10.00, E	0.36
	6.82, P	0.10	9.00, T	0.17	10.40, E	1.11
	7.36, P	0.14	9.55, E	0.26	10.84, TA	3.53
Trimethylacetate	6.00, P	0.00	9.00, T	0.046	10.65, TA	0.791
	7.00, P	0.00	9.57, E	0.200	10.90, TA	1.971
	8.00, T	0.00	10.40, E	0.350		

[a] 1% (v/v) acetonitrile, buffer concentrations (0.05 M) A (acetate), P (phosphate), T (Tris), E (ephedrine), and TA (triethylamine); ionic strength 0.09. [b] Units of k_{enzyme} in M^{-1} min^{-1}.

1–3). (3) Below pH 9.0 the profiles are all sigmoidal with propionate, *n*-butyrate, isobutyrate, and isovalerate exhibiting an inflection around pH 7.3. This latter inflection is apparently displaced to pH ~6.3 for *n*-valerate and ~5.7 for *n*-caproate. Each rate profile exhibits a plateau between pH 8.50 and 9.0 where enzymatic activity is apparently pH independent. (4) With all substrates, esterase activity rises very steeply above pH 10. Unfortunately, the relative magnitude of the hydroxide ion catalysis precluded accurate k_{enzyme} determinations above pH 11. Nevertheless, it is quite apparent that the slopes in the various pH–rate profiles are extremely steep around 10.5, indicating that a catalytically active group of pK_a around 10.5 participates in its basic form in the hydrolysis process. This second inflection dominates the appearance of the pH profiles of the larger, more sterically hindered esters. As an extreme example, *p*-nitrophenyl trimethylacetate has a k_{enzyme} = 46 min^{-1} M^{-1} at pH 9.0 and a k_{enzyme} = 1970 min^{-1} M^{-1} at pH 10.9.

Michaelis–Menten Kinetics. The value of k_{enzyme} actually expresses the combined ability of the enzyme to bind and hydrolyze a particular substrate (eq 4).

$$k_{enzyme} = \frac{v}{[S][E]} = \frac{k_2}{K_m + [S]} \quad (4)$$

Although we were unable to obtain substrate concentrations in excess of $[S_0] \simeq K_m$, the results at each pH gave good linear plots of v^{-1} against $[S]^{-1}$ or of S/v against S, which in spite of the long extrapolation, allowed the determination of the Michaelis parameters, K_m and V_{max}.

One of our first concerns in the investigation of the esterase activity of bovine carbonic anhydrase was the

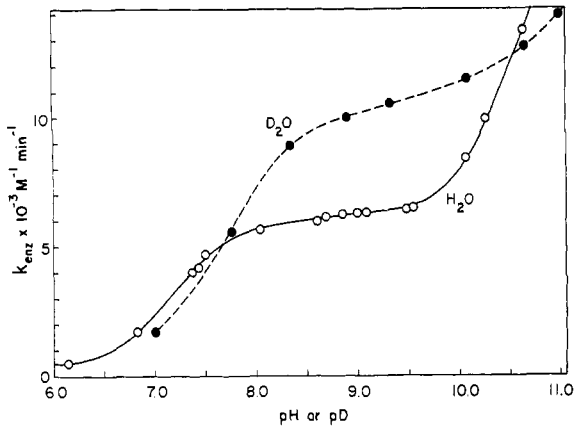

FIGURE 1: The bovine carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl propionate as a function of pH and pD at 25.0°. 1% (v/v) acetonitrile; buffer concentration, 0.05 M; ester concentration, 5×10^{-5} M; $[BCA]_I = 1\text{–}15 \times 10^{-6}$ M; ionic strength, 0.09. (○) Hydrolysis in H_2O. (●) Hydrolysis in D_2O.

detection of an initial "burst" of *p*-nitrophenol formation. This phenomenon is observable under presteady-state conditions (Sturtevant, 1960), *i.e.*, if $k_2 > k_3$ in the three-step mechanism (eq 5). For many enzymes,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P_1 + ES_1 \xrightarrow{k_3} P_2 + E \quad (5)$$

the rate of formation of the acyl-enzyme intermediate (ES_1) and *p*-nitrophenol (P_1) is significantly faster than the rate of hydrolysis of acyl-enzyme intermediate to give carboxylate anion (P_2) and enzyme. With carbonic anhydrase under a variety of conditions including $K_m \simeq [S_0] \gg [E_0]$, all plots of *p*-nitrophenol formation passed through the origin and led to predictable k_{enzyme} values. In other words, no "burst" was detected under the most favorable conditions that were experimentally attainable with our substrates. It should be noted that the accurate measurement of the size of the "burst" is best made when $[S_0] > [E_0]$ and $[S_0] > K_m$. (Bender *et al.*, 1966). The first inequality was easily satisfied experimentally but the latter was not. However, the condition $[E_0] \leq [S_0] \simeq K_m$ is sufficient for the detection of a burst, provided $k_2 > k_3$ (Dixon and Neurath, 1957). Our failure to detect a burst leads us to the conclusion that k_3 is actually larger than k_2.

Values for K_m and k_2 were determined for all substrates at pH 9.5 in a 0.05 M Tris buffer and ionic strength 0.09. A comparison of enzymatic catalysis for *p*-nitrophenyl ester hydrolysis with hydroxide catalysis is made in Table V. The following conclusions can be made about the specificity of the enzyme for the various *p*-nitrophenyl esters.

(1) The hydrolyses of the larger substrates, which are most unlike the natural substrate CO_2, are catalyzed the least. The greatest decrease in k_{enzyme} occurs between the propionate and *n*-butyrate. A similar discontinuity occurs between the esters derived from straight-chain acids and their branched isomers. (2) Qualitatively, the trend in k_2 parallels the one in k_{enzyme}. This is understandable because $k_{enzyme} = k_2/(K_m + [S])$. Qualitatively, k_2 exhibits a much greater substrate specificity than k_{enzyme}. The comparatively smaller substrate specificity exhibited by k_{enzyme} is reflected in the respective values of K_m. The value of $1/K_m$, increases with the size of these esters but k_2 favors the smaller ones. The net result of these two opposing tendencies is a decreased substrate specificity for the total enzymatic process. For example, the ratio of k_2 for propionate compared to *n*-butyrate is 12 to 1 but the corresponding k_{enzyme} ratio is only 4.4 to 1. This difference is due to a counterbalancing effect as expressed by K_m values of 16.2×10^{-4} M for propionate and 6.39×10^{-4} M for *n*-butyrate. (3) Both trimethylacetate and isovalerate have significantly smaller values of k_2 compared to *n*-valerate. However, the K_m values are greater for isovalerate and trimethylacetate than for *n*-valerate. This indicates that the enzyme binds *n*-valerate somewhat better than isovalerate or trimethylacetate. (4) The most meaningful comparison between hydroxide ion and enzyme catalysis is given by the ratio $k_2/k_{OH^-}[OH^-]$. The efficiency of the enzyme as expressed by this ratio follows a trend qualitatively similar to that demonstrated by k_2 and k_{enzyme}.

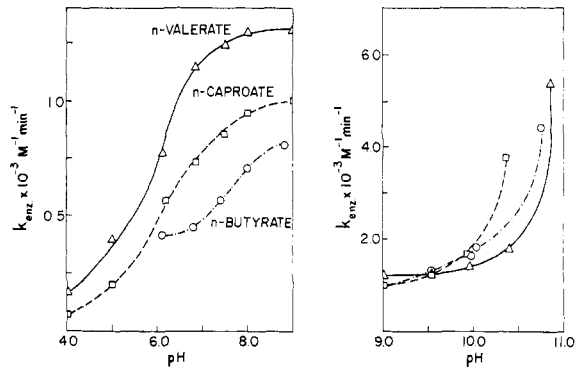

FIGURE 2: The bovine carbonic anhydrase catalyzed hydrolyses of *p*-nitrophenyl esters as a function of pH at 25.0°. 1% acetonitrile; buffer concentrations, 0.05 M; ester concentration, 5×10^{-5} M; $[BCA]_I = 1\text{–}15 \times 10^{-6}$ M; ionic strength, 0.09. (○) *n*-Butyrate, (△) *n*-valerate, and (□) *n*-caproate.

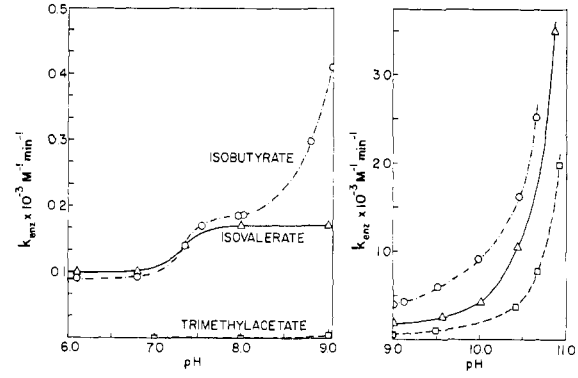

FIGURE 3: The bovine carbonic anhydrase catalyzed hydrolyses of *p*-nitrophenyl esters as a function of pH at 25.0°. 1% (v/v) acetonitrile; buffer concentrations, 0.05 M; ester concentration, 5×10^{-5} M; $[BCA]_I = 1\text{–}15 \times 10^{-6}$ M; ionic strength, 0.09. (○) Isobutyrate, (□) isovalerate, and (△) trimethylacetate.

TABLE V: Hydrolysis of *p*-Nitrophenyl Esters. Comparison of Enzymatic Catalysis with Hydroxide Ion Catalysis at pH 9.50 and 25.0°.

Substrate	k_{enzyme} (M^{-1} min^{-1})	$K_m \times 10^4$ (M)	k_2 (min^{-1})	k_{OH^-} (M^{-1} min^{-1})	k_{enzyme}/k_{OH^-}[a]	$k_2/k_{OH^-}[OH^-]$[a]
Propionate	6400	16.2	10.7	550	11.6	615
n-Butyrate	1461	6.39	0.89	440	3.32	63.6
n-Valerate	1300	3.85	0.70	390	3.33	56.5
n-Caproate	1250	2.84	0.49	370	3.38	41.9
Isobutyrate	600	2.86	0.19	400	1.50	15.0
Isovalerate	260	5.35	0.18	190	1.37	30.0
Trimethylacetate	200	4.56	0.10	87	2.30	35.7

[a] These ratios, k_{enzyme}/k_{OH^-} and $k_2/k_{OH^-}[OH^-]$, are unitless.

pH Dependency of Michaelis-Menten Parameters. Detailed rate studies were also undertaken to determine the pH dependency of the Michaelis-Menten parameters. These are summarized in Table VI and the following conclusions can be drawn. (1) Values of k_2 are more uncertain than those of k_{enzyme} since a long extrapolation is involved; but they generally parallel the latter values for *p*-nitrophenyl propionate and show similar inflections, one around pH 7.3, the other above pH 10.0, probably around pH 10.5. (2) Both k_2 and K_m increase with pH, but the dependency of K_m on pH is reflected solely in the turnover number k_2. Figure 4 verifies that K_m is a linear function of k_2 over a wide pH range for *p*-nitrophenyl propionate, isobutyrate, and *n*-caproate. It should be noted, however, that this linear dependency cannot be documented at pH greater than 9.5 using propionate or *n*-caproate. (3) Formally speaking, the slopes in each of the plots depicted in Figure 4 are $1/k_1$ and the intercepts are k_{-1}/k_1 for the respective esters. Consequently, it was possible to determine formal values of k_1 and k_{-1} for the three esters studied in detail. The binding constants for the formation of the respective substrate-enzyme complexes as expressed by the ratio k_1/k_{-1}, for acetate, propionate, caproate, and isobutyrate are compared in Table VII. Formal values for k_1 and k_{-1} for acetate were previously determined (Pocker and Stone, 1967) in 10% (v/v) acetonitrile. Examination of this data reveals that the larger esters bind much better to the enzyme than the smaller esters. Both k_1 and k_{-1} decrease from acetate to caproate but k_{-1} varies by a factor of 10^3 and k_1 varies by a factor of 10. The values of $\Delta F°$ for binding reveal a significant trend; they increase by a relatively constant increment of 725 ± 50 cal for each methyl or methylene group added. The values of $\Delta F°$ for the respective binding constants were calculated and included in Table VII.

TABLE VI: Results from Lineweaver-Burk Plots for Bovine Carbonic Anhydrase Catalyzed Hydrolysis of *p*-Nitrophenyl Esters as a Function of pH at 25.0°.

Substrate	pH	$k_{enzyme} \times 10^{-3}$ (M^{-1} min^{-1})	$K_m \times 10^4$ (M)	k_2 (min^{-1})
Propionate	6.15	0.58	5.7	0.36
	6.82	1.70	7.0	1.3
	7.50	5.00	8.0	4.1
	7.98	5.40	11.3	6.4
	8.62	6.40	12.2	8.2
	9.11	6.40	14.5	9.9
	9.12	6.40	15.0	10.6
	9.13	6.40	15.7	11.2
	9.52	6.40	16.2	10.7
	9.94	8.40	~17[b]	~14[b]
	10.26	10.00	~18[b]	~18[b]
Isobutyrate	7.53	0.17	1.97	0.05
	8.01	0.18	2.10	0.07
	9.11	0.45	2.12	0.12
	9.51	0.60	2.7	0.19
	9.95	0.81	3.3	0.31
	10.37	1.13	~3.8[b]	~0.49[b]
n-Caproate	6.84	0.74	0.60	0.07
	8.67	1.00	1.10	0.17
	9.00	1.02	1.30	0.19
	9.54	1.25	2.84	0.49
	10.37	3.80	~2.7[b]	~1.23[b]

[a] Values of k_{enzyme} refer to a substrate concentration of 5×10^{-5} M and ionic strength 0.09. [b] These values are uncertain and were obtained by a long extrapolation.

Inhibition by Acetazolamide. Acetazolamide has been shown to be a potent specific inhibitor of carbonic anhydrase activity with respect to both hydration and hydrolysis. This inhibition has been characterized as noncompetitive for CO_2 (Maren *et al.*, 1960; Liebman *et al.*, 1961), acetaldehyde (Pocker and Meany, 1965b), and *p*-nitrophenyl acetate (Pocker and Stone, 1965, 1967; Armstrong *et al.*, 1966). The apparent dissociation constant for the enzyme-acetazolamide complex was determined from plots of activity *vs.* the ratio of acetazolamide to enzyme concentration. An extrapolation

TABLE VII: Comparison of the Binding Constants of Bovine Carbonic Anhydrase with *p*-Nitrophenyl Esters at 25.0°.

Substrate	$k_1 \times 10^{-4}$ (M^{-1} min^{-1})	k_{-1} (min^{-1})	k_1/k_{-1} (M^{-1})	$\Delta F°$ (cal)	$(\Delta(\Delta F°)/n)^b$ (cal)
Acetate[a]	2.00	40.0	500	3,680	0
Propionate	1.23	6.64	1,850	4,460	775
Isobutyrate	0.216	0.385	5,630	5,120	717
n-Caproate	0.146	0.029	50,000	6,410	682

[a] Values determined (Pocker and Stone, 1967) in 10% (v/v) acetonitrile. [b] The denominator (n) is the difference in the number of carbon atoms between each subsequent ester and the acetate ester.

of the initial slope for *p*-nitrophenyl propionate hydrolysis at pH 8.55 to zero activity (Figure 5) indicates that there is one esteratic site per enzyme molecule as observed earlier with *p*-nitrophenyl acetate (Pocker and Stone, 1965, 1967). Values of the dissociation constants for the enzyme–acetazolamide complex in the presence of various *p*-nitrophenyl esters are given in Table VIII. The value of K_i determined from the enzyme-catalyzed hydrolyses of *p*-nitrophenyl acetate (Pocker and Stone, 1967), propionate, and isobutyrate is 2×10^{-7} M. Although K_i should be substrate independent much larger values of K_i were observed with *n*-butyrate, *n*-valerate, and *n*-caproate with respect to which acetazolamide exhibited mixed inhibition. The greatest discontinuity for K_i values occurred between *n*-butyrate and *n*-valerate; K_i from *n*-valerate and *n*-caproate hydrolysis was approximately 1000 times greater than that determined from acetate and propionate hydrolysis. It is interesting to note that the pH profiles of the two substrates possessing longer chains exhibited inflection points at pH 6.2 and 5.7, respectively, rather than at 7.3. These observations imply that the conformation of the enzyme may be significantly perturbed when it binds larger substrates.

Acetazolamide has been shown (Tilander *et al.*,

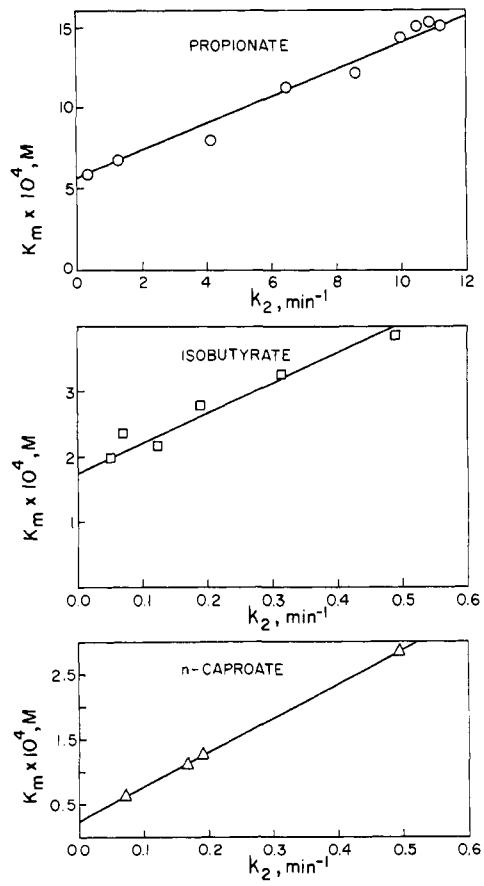

FIGURE 4: Plots of K_m *vs.* k_2 for the esterase activity of bovine carbonic anhydrase with *p*-nitrophenyl esters at 25.0°. 1% (v/v) acetonitrile; buffer concentrations 0.05 M; ionic strength, 0.09. (○) Propionate, (□) isobutyrate, and (Δ) *n*-caproate.

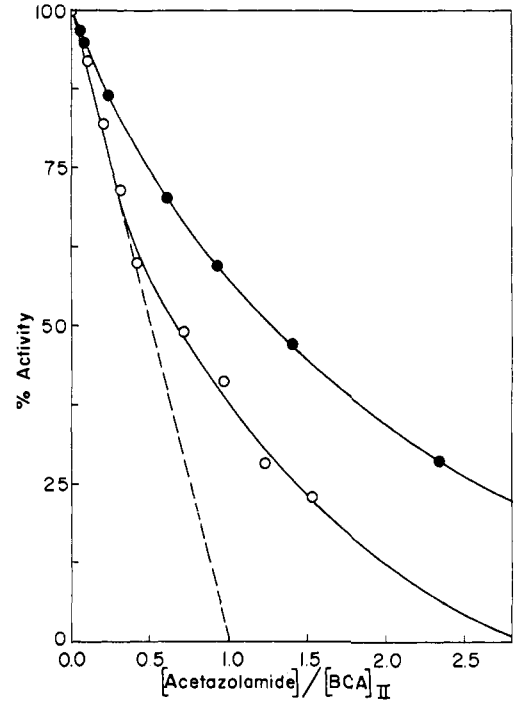

FIGURE 5: Per cent activity for the BCA-catalyzed hydrolysis of *p*-nitrophenyl propionate as a function of [acetazolamide]/[BCA] at pH 8.55 (lower curve) and pH 9.90 (upper curve). 1% (v/v) acetonitrile; 0.05 M Tris (pH 8.55) and ephedrine (pH 9.9) buffers; $[BCA]_{II} = 3.27 \times 10^{-6}$ M (pH 8.55) and $[BCA]_{II} = 8.48 \times 10^{-6}$ M (pH 9.90); ionic strength, 0.09.

TABLE VIII: Dissociation Constants for the Enzyme-Acetazolamide Complex in the Presence of *p*-Nitrophenyl Esters at pH 8.55 and 25.0°.

Substrate	K_i (M)[b]	Substrate	K_i (M)[b]
Acetate[a]	2×10^{-7}	*n*-Butyrate[c]	9×10^{-6}
Propionate	2×10^{-7}	*n*-Valerate[c]	2×10^{-4}
Isobutyrate	2×10^{-7}	*n*-Caproate[c]	4×10^{-4}

[a] Determined in 10% acetonitrile (Pocker and Stone, 1967). [b] Determined in 1% (v/v) acetonitrile, 0.05 M Tris buffer, ionic strength 0.09. [c] Mixed inhibition as defined by Webb (1963).

1965; Fridborg *et al.*, 1967) to bind with carbonic anhydrase at or near the zinc atom. The effect of this inhibitor on the enyzme-catalyzed hydrolysis of *p*-nitrophenyl propionate was to reduce k_2. Although K_m also varied, it did so only in proportion to k_2, thus characterizing this inhibition as noncompetitive. Evidently, *p*-nitrophenyl propionate like *p*-nitrophenyl acetate is not bound to the zinc atom. It was also found in our laboratories (Y. Pocker and J. T. Stone, unpublished results) that *p*-nitrophenyl caproate and trimethylacetate are competitive inhibitors of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate, thus indicating that all these esters apparently bind at the same site.

By studying the *p*-nitrophenyl propionate hydrolysis at high pH, we were able to show that the dramatic rise in esterase activity can be entirely inhibited by the highly specific reagent acetazolamide (Figure 5). We found that K_i changed from 2×10^{-7} at pH 8.55 to 5×10^{-6} M at pH 9.9; yet the value of K_i at each pH was essentially independent of inhibitor concentration and the extent of inhibition at a given ratio I_0/E_0 was very nearly independent of *p*-nitrophenyl propionate concentration. The strength of binding between acetazolamide and enzyme appears to be highly sensitive to pH changes particularly in the high pH region where esterase activity rises dramatically with increase in pH.

Comparison of the Esterase Activity in D_2O and H_2O. The pD dependency of k_{enzyme} for the bovine carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl propionate was determined by the same procedure as that employed for the pH profiles. The values of k_{enzyme} as a function of pD are listed in Table IX and plotted in Figure 1 along with the pH-activity profile. The lower inflection point shifts from 7.25 in H_2O to 7.8 in D_2O. This is consistent with the observation that an ionizable group of pK around 7 is generally displaced toward higher pH by approximately 0.55 pH unit in D_2O (Glasoe and Long, 1960; Li *et al.*, 1961). Consequently, the entire lower portion of the pH profile is shifted about 0.55 pH unit toward higher pH in D_2O.

We deduced k_{enzyme} in D_2O and H_2O at respective values of pD and pH where the ratio of the basic form of the enzyme to its conjugate acid is equal. In general

TABLE IX: Relative Effectiveness of Bovine Carbonic Anhydrase Catalysis of *p*-Nitrophenyl Propionate Hydrolysis in D_2O and H_2O.[a]

pD	$k_{enzyme} \times 10^{-3}$ (D_2O)[b]	pH	$k_{enzyme} \times 10^{-3}$ (H_2O)[b]	k_D/k_H
7.00	1.7	6.54	1.00	1.7
7.76	5.52	7.21	3.31	1.67
8.35	8.90	7.80	5.30	1.68
8.88	10.06	8.33	6.00	1.68
9.33	10.61	8.78	6.20	1.71
10.09	11.59	9.54	6.60	1.76
10.63	12.80	9.79[c]	8.60	1.85[c]
11.00	13.90	10.26[c]	11.50	1.67[c]

[a] In 1% (v/v) acetonitrile, 0.05 M buffer, and ionic strength 0.09. [b] Units of k_{enzyme} in M^{-1} min^{-1}. [c] Values of k_{enzyme} in D_2O are compared to those in H_2O taking into consideration the difference in the dissociation constant of an acid of $pK_a = 10.5$ in H_2O and D_2O, respectively, $pK = pK_a^{D_2O} - pK_a^{H_2O} = 0.84$.

we have used the relationship, pD = pH + 0.55, and values of k_{enzyme} determined in D_2O and H_2O at corresponding values of pD and pH are compared in Table IX.

It is apparent from an inspection of the general shape of the pD and pH profiles that the second inflection is displaced by 0.8–0.9 pH unit in D_2O while the first inflection was displaced by 0.55 pH unit in D_2O. If the second inflection corresponds to the titration of a catalytically active group of pK_a around 10.5, it would be expected that its pK would change by approximately 0.84 pH unit in D_2O (Glasoe and Long, 1960). Therefore k_D and k_H values corresponding to the second inflection (high pH region) should be obtained at pD = pH + 0.84. The last two points in Table IX were treated in this fashion and the values deduced for the k_D/k_H ratio compare favorably with those obtained at lower values of pH.

For the bovine carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl propionate values of k_2 and K_m were also determined in D_2O at pD 8.39; $k_2 = 12.6$ min^{-1} and $K_m = 16.0 \times 10^{-4}$ M. The corresponding values in H_2O at pH 7.84 are $k_2 = 6.20$ min^{-1} and $K_M = 10.0 \times 10^{-4}$ M. The ratio of $k_2^{D_2O}/k_2^{H_2O}$ is 2.0, indicating that the actual hydrolysis step is favored in D_2O, while $K_m^{D_2O}/K_m^{H_2O}$ is 1.6 indicating that the enzymatic affinity at this pH is higher in H_2O than in D_2O. These results accord with earlier observations pertaining to the hydrase activity of the enzyme with acetaldehyde as substrate (Pocker and Meany, 1965a, 1967c).

Discussion

Previous investigations conducted in these laboratories on the catalytic activity of carbonic anhydrase have demonstrated the versatility of this enzyme with

respect to aldehyde hydration (Pocker and Meany, 1965a,b, 1967a,b; Pocker *et al.*, 1965) and *p*-nitrophenyl acetate hydrolysis (Pocker and Stone, 1965, 1967; *cf.* also Tashian *et al.*, 1964; Malmstrom *et al.*, 1964; Armstrong *et al.*, 1966; Duff and Coleman, 1966). The esterase activity of bovine carbonic anhydrase was shown to exhibit a number of important similarities with its hydrase activity, and it is believed that the underlying mechanisms for the enzyme-catalyzed hydrations and hydrolyses are similar (Pocker and Stone, 1967). The present study demonstrates that carbonic anhydrase also exhibits significant esterase activity with respect to other *p*-nitrophenyl esters derived from related carboxylic acids. The activity of the enzyme with respect to the hydrolysis of various esters reflects a marked substrate specificity. Since all attempts to observe a presteady-state release of *p*-nitrophenol proved negative, the enzyme-catalyzed process was formally analyzed in terms of the classic two-step Michaelis–Menten scheme. It is of course realized that the mechanism of the enzymatic process is much more complex but partly for simplicity and partly in order to make clear the reasons for elaborations, it appeared to us useful to develop our interpretation in stages. A number of observations confirm the suggested binding between enzyme and the various substrates studied.

(1) At sufficiently low concentrations the rate was found to be proportional to the first power of the substrate concentration, but at higher concentrations the rate increase was less than linear in substrate. Low ester solubility precluded the attainment of the region at which the rate is actually independent of substrate concentration (zero-order kinetics); nevertheless, for all the esters reported in this paper, it was possible to deduce both K_m and V_{max} from Lineweaver–Burk plots. (2) *p*-Nitrophenyl *n*-caproate and trimethylacetate *competitively* inhibited the bovine carbonic anhydrase catalyzed hydrolysis of *p*-nitrophenyl acetate by forming enzyme–substrate (1:1) complexes (Y. Pocker and J. T. Stone, unpublished data.) (3) The association constant (K_i) for the specific inhibitor, acetazolamide, with bovine carbonic anhydrase was greatly increased in the presence of *p*-nitrophenyl *n*-valerate and caproate as substrates. Ideally, K_i for acetazolamide inhibition should be independent of the nature of the various esters; unless, of course, the final conformational state depends on the bound substrate. Control experiments showed that *p*-nitrophenyl *n*-valerate and *n*-caproate did not react with acetazolamide so that the profound effects that these esters have no acetazolamide inhibition as well as on the pH–rate profile can be best understood in terms of conformational changes induced by the binding substrates. It has been suggested (Fridborg *et al.*, 1967; Coleman, 1967) that acetazolamide may possibly bind to the enzyme through two concurrent interactions; a coordination to zinc coupled with hydrophobic interactions at other sites near the metal ion. The strength of this binding would be highly sensitive to perturbations inasmuch as the conformational adaptability of the enzyme must be differently affected by the larger substrates.

As was reported in the Results section, no "burst" of *p*-nitrophenol was observed for any of the *p*-nitrophenyl esters studied. Pocker and Stone (1967) have previously pointed out that the absence of a "burst" is consistent with a three-step mechanism (eq 5) in which $k_3 > k_2$. One such mechanism visualizes a double displacement, with step k_2 leading to the formation of a labile zinc–carboxylate intermediate (scheme a, Figure 6). According to this scheme the displacement of a zinc-bound carboxylate ion by water occurs much faster than the formation of an acyl-enzyme intermediate leading to $k_3 > k_2$. Another mechanism consistent with the absence of a burst visualizes the formation of a zinc-coordinated tetrahedral intermediate which subsequently decomposes simultaneously to give *p*-nitrophenol and a carboxylic acid (scheme b, Figure 6). A common feature of both schemes is the participation of an unprotonated base (B) in the transfer of H_2O from zinc to the carboxyl carbon of the ester. In fact there are several possible explanations for the first inflection in the pH–rate profile observed with the "more natural" substrates

$$\underset{\text{inactive}}{EH_2^+} \overset{pK_1 \simeq 7.0}{\rightleftharpoons} \underset{\text{active}}{EH} + H^+$$

Davis (1958, 1959) has suggested that the pK_a of the zinc–aquo complex in the native enzyme might be around 7.0. Actually the pK_a of $Zn(H_2O)_4^{2+}$ is 9.7 (Hunt, 1963); and while in the native enzyme three amino acid residues and a water molecule appear to be coordinated to the zinc ion (Fridborg *et al.*, 1967), the data in the literature does not contain any examples where a pK_a of around 7.0 has been observed for a zinc–aquo complex. We have earlier contended (Pocker and Meany, 1965a,b, 1967a,b; Pocker and Stone, 1965, 1967) that round physiological pH the transfer of H_2O from zinc may be promoted in the enzyme by a general base (B) having a pK_{BH^+} of *ca.* 7.0. Two amino acid residues exhibit pK_{BH^+} in this region, a terminal α-amino group whose pK_a in the protein matrix varies from 7.6 to 8.4 and a histidine residue (imidazole) whose pK_a in the protein matrix varies from 5.6 to 7.0 (Dixon and Webb, 1964). It is attractive to implicate the imidazole residue of histidine, for the known dependence of its apparent pK_a on the protein matrix would still accord with the range of pH–rate profile inflections (5.7–7.5) which we observed in the enzymatic hydrolysis of the various *p*-nitrophenyl esters. Furthermore, preliminary studies have shown that carbonic anhydrase reacts with iodoacetamide and iodoacetate to give a modified enzyme in which a histidine residue is alkylated at the 3 position of the imidazole ring. The modified enzyme shows an exceedingly low activity and no inflection whatsoever in the pH region 6–8.5. On the other hand the dramatic rise in activity at higher pH which is associated with the "second inflection" is but little affected, except that in the modified enzyme it starts from a very low level of activity.

The most significant feature of our present investigation is the detection of a hitherto unknown second rise in the pH–rate profile of carbonic anhydrase. This

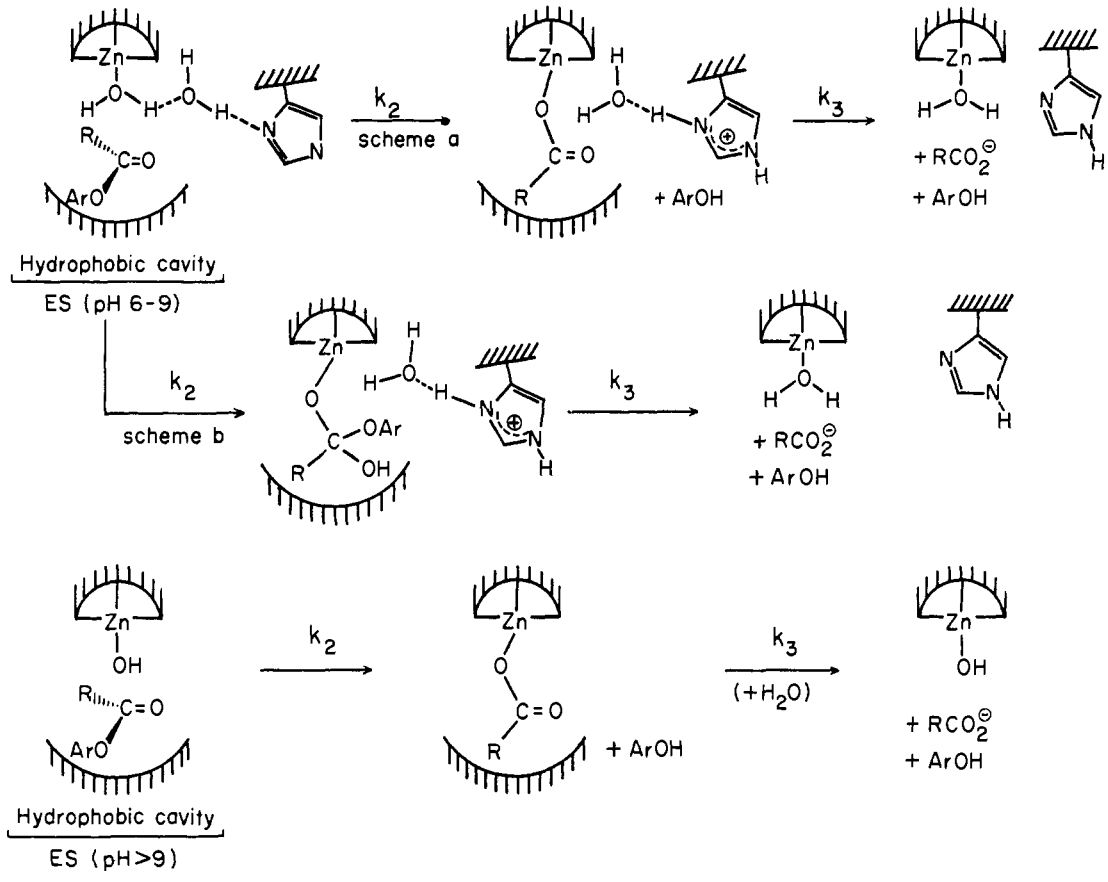

FIGURE 6: Hypothetical mechanisms for ester hydrolysis by bovine carbonic anhydrase in the pH region 6–9 (schemes a and b) and above pH 9.5.

dramatic rise in rate accords with

$$\underset{\text{active}}{EH} \overset{pK_2 \simeq 10.5}{\rightleftharpoons} \underset{\text{very active}}{E^-} + H^+$$

and should be examined in the light of several alternative explanations. It is possible that this second inflection could be due to the participation of other basic groups in the enzyme having a pK_a in this region. The following functional groups exhibit pK_a's in the region of interest: (1) phenolic hydroxyl (tyrosine), pK_a = 9.8–10.4; (ii) ϵ-amino (lysine), pK_a = 9.4–10.6; (iii) guanidinium (arginine), pK_a = 11.6–12.4. (The absence of cysteine in bovine carbonic anhydrase eliminates the requirement for considering sulfhydryl groups.) In this connection as inspection of the hydrogen ion equilibria around pH 10.5 of human carbonic anhydrases B and C (Riddiford, 1964, 1965; Riddiford *et al.*, 1965) and of bovine carbonic anhydrase B (Nilsson and Lindskog, 1967) as well as the chemical modifications of lysine and tyrosine residues in the bovine enzyme (Y. Pocker and D. G. Dickerson, unpublished observations; Nilsson and Lindskog, 1967) are particularly instructive. Thus most of the tyrosine residues appear to be masked in native bovine carbonic anhydrase and only one titrates freely ($pK_{int} \simeq 10.8$); however, the modified enzyme in which one tyrosine residue has been acetylated or nitrated retained full catalytic activity. Similarly, amidination of all lysine residues, including the carboxyl-terminal lysine, caused no loss of activity. On the other hand, since the hydrolytic pK_a of the free zinc-aquo complex, $Zn(H_2O)_4^{2+}$, is reported to be 9.7 (Perrin, 1962; Hunt, 1963), it is possible that the higher inflection might correspond to the titration of a protein-bound zinc-aquo complex whose apparent pK_a may lie around pH 10.5. This latter suggestion, although strictly hypothetical, appears to be consistent with the experimental evidence presented in this work.

(1) Acetazolamide, a specific inhibitor of carbonic anhydrase activity, was found to powerfully and *noncompetitively* inhibit the *entire* enzyme-catalyzed hydrolysis of *p*-nitrophenyl propionate in the region of the second inflection (pH >10). Equilibrium dialysis experiments measuring sulfonamide binding to native and apocarbonic anhydrase indicate that the Zn^{2+} present in the native enzyme is involved in binding these inhibitors (Lindskog, 1963). The K_i for acetazolamide inhibition in the region of the second inflection is somewhat higher than that at pH 8.5; this observation accords with the suggestion that the displacement of a hydroxide ion ligand by acetazolamide anion would be more difficult than the displacement of an H_2O ligand. (2) Cyanide ions, powerful inhibitors of carbonic anhydrase activity, act noncompetitively probably by coordinating to Zn^{2+} and displacing ligand water. The enzyme-catalyzed hydrolysis of *p*-nitrophenyl

propionate can be entirely inhibited by CN^- in both the plateau region and in the region of the second inflection. Here again, the value of K_i is somewhat higher in the region of the second inflection than at pH 8.5; an observation which accords with the suggestion that the displacement of ligand H_2O by CN^- is more facile than that of ligand ^-OH (Y. Pocker and D. R. Storm, unpublished data). (3) The high rates of displacement of zinc-bound acetate by H_2O as reported by Eigen and Wilkins (1965) support our earlier contention (Pocker and Stone, 1967) that the acetate release from the zinc ion (k_3 in scheme a, Figure 6) may be significantly faster than the liberation of *p*-nitrophenol (k_2 in scheme a). (4) The faster turnover in D_2O accords with studies involving model systems which show that metal ion assisted transfer of OD^- is *ca.* 1.5–2.0 times faster than the respective transfer of ^-OH (Pocker and Meany, 1965a, 1967c; also unpublished data).

Provisionally, we follow our earlier speculative model and regard hydration and hydrolysis in the region of the first inflection ($pK_1 \simeq 7.0$) as a general base (imidazole) assisted transfer of ^-OH from $E \cdot ZnOH_2$; and further suggest that at high pH ($pK_2 \simeq 10.5$) the formation of the zinc–hydroxo complex, $E \cdot ZnOH$ would no longer require the cooperation of an internal base to promote this transfer.

Another significant feature of the present investigation is that we were able to determine values for the *apparent* rate coefficients k_1 and k_{-1} as well as k_2. This allowed us to gain further insight into the binding of esters with enzyme. The relatively small values of k_1 and k_{-1} suggest that the formation of an enzyme–substrate complex may involve either an undetected covalent binding or slow conformational changes. Since the relaxation times associated with single noncovalent interactions such as hydrogen bonding, hydrophobic interactions, etc., are considerably shorter, the slow conformational changes associated with the formation of the enzyme–substrate complex must involve highly cooperative interactions.

Tanford (1962) has shown that the addition of a methylene group to an amino acid residue or a hydrocarbon increases the magnitude of its hydrophobic interaction by 745 cal. Our values of $\Delta F°$ for ester binding reveal a similar trend; they also increase by a relatively constant increment of 725 ± 50 cal for each methyl or methylene group added (Table VII). Apparently, the stronger binding of the larger esters is due to increased hydrophobic interactions with the enzyme. It is realized, of course, that other interactions such as hydrogen bonding probably contribute to the over-all binding, but their magnitude should not vary significantly from ester to ester. Inasmuch as binding appears to be largely hydrophobic in nature, it is interesting to examine the X-ray model described by Fridborg *et al.* (1967). In their model they show the presence of a pronounced cavity on the enzyme surface in the vicinity of the zinc atom. It is possible that this structural feature may be the binding site for the various *p*-nitrophenyl esters. Thus, if the carboxyl end of the ester, in the enzyme–substrate complex, were situated near the zinc atom, which we consider to be the esteratic site, the remainder of the acid chain could conveniently fit in the hydrophobic cavity on the enzyme surface.

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Amine Specificity in Transpeptidation. Inhibition of Fibrin Cross-Linking*

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ABSTRACT: A systematic survey was made of some 25 amines, including several newly synthesized ones, to assess their inhibitory effect in the enzymatic cross-linking of fibrin. Compounds were selected from the point of view of different basicities and side-chain substitutions.

Best inhibitors contained a pentamine or hexamine residue attached to an apolar group, toluene, or naphthalene. *N*-(5-Aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide, the most potent inhibitor so far, was shown to act as a substrate for the cross-linking enzyme. It became incorporated into fibrin, serving as a fluorescent label for the acceptor cross-linking sites of this protein. Incorporation of the fluorescent amine showed saturation kinetics with an apparent K_M of 1.6×10^{-4} M (at a fixed initial fibrin concentration of 5.45×10^{-6} M). This may be compared with a value of 3×10^{-3} M for the incorporation of [^{14}C]glycine ethyl ester (Lorand, L., and Ong, H. H. (1966b), *Biochemistry* 5, 1747). The ratio of Michaelis constants for these two amines is in good agreement with their relative potencies in inhibiting the cross-linking of fibrin.

The concept that cross-linking of fibrin[1] occurred through an enzyme-catalyzed transpeptidation (Lorand *et al.*, 1962) between selected amino groups (now shown to be those of ε-lysine; Lorand *et al.*, 1966a) of one protein molecule and carbonyl group (γ-glutamyl; Lorand and Ong, 1966a) of another (see Added in Proof), led us to examine the influence of synthetic amines ($H_2N \cdot R$) on the reaction. Many of the compounds proved to be powerful inhibitors of cross-linking which is illustrated here (eq 1) by the formation of a dimer

$$H_2N \cdot F \cdot CO \cdot Y + H_2N \cdot F' \cdot CO \cdot Y \xrightarrow[H_2N \cdot R]{e\backslash} H_2N \cdot F \cdot CO \cdot NH \cdot F' \cdot CO \cdot Y + HY \quad (1)$$

through a single amide bond. It is important to point out that amines do not seem to interfere at all with the reversible "self-assembly" of fibrin, that is, clot formation; they specifically inhibit only the subsequent cross-linking reaction between fibrin units which, in the absence of amines, would yield structures insoluble in

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[1] Abbreviations used: F, fibrin; e or e·SH, the cross-linking enzyme, *i.e.*, the thrombin-activated fibrin-stabilizing factor (FSF*); Y, the leaving group in fibrin cross-linking, presumed to be NH_2; dansyl, 1-dimethylamino-5-naphthalenesulfonyl; Cbz, benzyloxycarbonyl; Ts or tosyl, *p*-toluenesulfonyl; TAME, N^{α}-tosyl-L-arginine methyl ester.