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Catalytic Versatility of Erythrocyte Carbonic Anhydrase. IX. Kinetic Studies of the Enzyme-Catalyzed Hydrolysis of 3-Pyridyl and Nitro-3-pyridyl Acetates*

Y. Pocker† and N. Watamori

ABSTRACT: The present investigation demonstrates that bovine carbonic anhydrase powerfully catalyzes the hydrolysis of 3-acetoxypyridine, 3-acetoxy-2-nitropyridine, and 3-acetoxy-2,6-dinitropyridine. The reactions follow Michaelis–Menten kinetics over the whole range of substrate concentrations studied, including the region [S] > K_m. The pH–activity profiles are approximately sigmoid; the esterase activity is very small below pH 6 and rises to an intermediate plateau above pH 8. The inflection point at 25.0° lies at pH 7.56 for 3-acetoxy-2-nitropyridine and pH 7.4 for 3-acetoxy-2,6-dinitropyridine. A more detailed treatment of the data shows that for all these substrates the mild variation of K_m with pH is dictated by the respective turnover number (k_2) while the formal binding constants (k_1/k_{-1}) are nearly independent of pH in the range 6.0–8.0. A comparison of these kinetic parameters suggests that when the first nitro group is incorporated into the ester it leads to an increase in esterase activity through both a larger turnover number and a better binding; of these, the former factor predominates. However, the incorporation of the second nitro group into the ester leads only to an increase in the turnover number but leaves the formal binding constant, k_1/k_{-1}, essentially unchanged. The relatively high solubility of these esters makes them ideal substrates for the kinetic identification of transient acyl-enzyme intermediates preceding a slower turnover reaction. However, throughout this study, no evidence could be obtained of an initial "burst" of release of either 3-hydroxy-2-nitropyridine or of 3-hydroxy-2,6-dinitropyridine even under highly favorable conditions. Acetazolamide is a powerful inhibitor of esterase activity. The K_i values at pH 7.47 and 25.0° are: 2.8×10^{-7} M with 3-acetoxypyridine, 2.9×10^{-8} M with 3-acetoxy-2-nitropyridine, and 2.0×10^{-8} M with 3-acetoxy-2,6-dinitropyridine. The inhibitions appear to be noncompetitive.

It has been demonstrated in these laboratories that erythrocyte carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) (CA)[1] is not, as has been previously thought, an absolutely specific catalyst for the reversible hydration of carbon dioxide, a catalysis that does indeed embody its physiological function, but the enzyme also powerfully catalyzes the reversible hydration of various aldehydes (Pocker and Meany, 1965a,b, 1967a,b; Pocker and Dickerson, 1968), and the hydrolysis of nitrophenyl esters (Pocker and Stone, 1965, 1967; Pocker and Storm, 1968; *cf.* also Tashian *et al.*, 1964; Malmström *et al.*, 1964; Armstrong *et al.*, 1966). This versatility was later shown to include also the hydrolysis of sulfonate esters (Lo and Kaiser, 1966; Y. Pocker and S. Sarkanen, unpublished observations) and of 1-fluoro-2,4-dinitrobenzene (Henkart *et al.*, 1968). More recently it has been shown that the enzyme also catalyzes the dehydration of the 2,2-dihydroxypropionate anion to pyruvate ion (Pocker and Meany, 1970). These findings, coupled with the fact that the experimental techniques necessary to obtain accurate rate data for many of these substrates are far simpler than those used in the study of the reversible hydration of carbon dioxide, have allowed a more comprehensive study of this enzyme and a fuller appraisal of its mode of action.

Kinetics studies on propionaldehyde, isobutyraldehyde, and pivaldehyde hydrations revealed that the catalytic efficiency of bovine carbonic anhydrase (BCA) with respect to these substrates was inversely proportional to the size of the aldehyde (Pocker *et al.*, 1965). Furthermore, a comparison of the various homologous series of *p*-nitrophenyl esters re-

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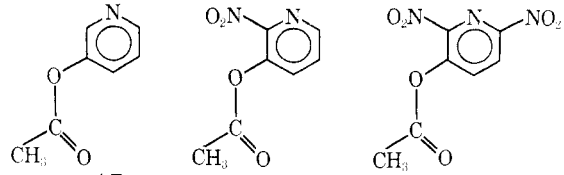
[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: CA, carbonic anhydrase; BCA, bovine carbonic anhydrase; *p*-NPA, *p*-nitrophenyl acetate; AP, 3-acetoxypyridine; ANP, 3-acetoxy-2-nitropyridine; ADNP, 3-acetoxy-2,6-dinitropyridine.

vealed that binding increased with the size of the ester chain, and this was well accounted for by a parallel increase in the free energy of hydrophobic interactions (Pocker and Storm, 1968). A logical outgrowth of our previous work on esterase activity was to seek compounds which, in addition to their ability to undergo facile hydrolysis, also contain the elements necessary for significantly stronger binding and to evaluate their capacity as substrates for carbonic anhydrase. A number of pyridyl and pyridoyl esters seemed to us to satisfy these requirements and a series of kinetic studies were commenced. The circumstance that pyridyl carbonate esters proved to be excellent substrates for delineating the mode of action of BCA over an unusually wide range of pH coupled with their capacity to act as sensors of the environment in the vicinity of the active site (Y. Pocker and L. J. Guilbert, to be submitted for publication as Part X in this series) has led us to undertake parallel studies with pyridyl acetates. There are many distinct advantages in studying the enzymatic hydrolysis of these latter compounds. Unlike phenyl esters, the pyridyl esters are moderately soluble in water and the need for organic cosolvent is minimal and can be entirely bypassed.

In addition, the possibilities of isomerism among pyridyl esters are greater than with phenyl esters, since not only does the relative position of the constituents to one another enter into the question, but also their position with regard to the ring nitrogen. Accordingly, pyridyl esters have an advantage in acting as more sensitive reporter substrates. Most significant of all, the esterase activity can be easily monitored even under rather extreme conditions, such as those required for the kinetic detection of covalent enzyme–substrate intermediates. In the present study we have attempted to gain further insight into the mode of action of carbonic anhydrase by delineating its specificity with respect to the enzymatic hydrolysis of 3-acetoxypyridine esters, AP, ANP, ADNP.



Experimental Section[2]

Materials. The nitropyridols used in the syntheses of the respective nitro-3-pyridyl esters were prepared by a step-by-step nitration of 3-hydroxypyridine:[3] 3-hydroxy-2-nitropyridine, mp 66–67° (lit. (De Selms, 1968) mp 67–69°); 3-hydroxy-2,6-dinitropyridine, mp 133–133.5° (lit. (Czuba and Plazek, 1958) mp 132°).

Pyridyl Esters. All of the 3-acetoxypyridine esters used in this study were synthesized by the following method. To a solution of 0.04 mole of 3-hydroxypyridine and 0.04 mole of pyridine in 100 ml of benzene was added dropwise 0.04 mole of acetyl chloride. The mixture was stirred an additional hour, filtered, and vacuum distilled and/or recrystallized from $CHCl_3$–CCl_4 (De Selms, 1968): 3-acetoxypyridine (AP), bp 64–64.5° (1) (lit. (Ueno *et al.*, 1964) bp 92° (9)); 3-acetoxy-2-nitropyridine (ANP), mp 50–51° (lit. (De Selms, 1968) mp 50–51°); 3-acetoxy-2,6-dinitropyridine (ADNP), mp 97.5–98.5°; nmr $\delta_{Me_4Si}^{CDCl_3}$ 2.43 (s, 3, CH_3), 8.43 (ABQ, J_{AB} = 8.5 Hz, 2, H_4, and H_5). *Anal.* Calcd for $C_7H_5N_3O_6$: C, 37.02; H, 2.22; N, 18.50. Found: C, 37.06; H, 2.21; N, 18.35.

Nuclear magnetic resonance (nmr) spectra of all the substituted 3-hydroxy- and 3-acetoxypyridines agreed well with their structures. Furthermore, spectroscopic measurements show that upon basic hydrolysis AP, ANP, and ADNP liberated 99.5–100% of theoretical amount of the corresponding hydroxypyridine.

Acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, obtained from American Cyanamid (Lederle Laboratory Division) was used without further purification. Several inhibitor stock solutions were prepared in water for convenient concentration range and the concentration was determined spectrophotometrically ($\epsilon_{292\,nm}$ 1.215×10^4). Reagent grade acetonitrile (Baker Analyzed), used as a solvent for preparation of stock solutions of AP, ANP, and ADNP, was freshly distilled. The buffer solutions employed in these experiments were usually prepared from the commercially available compounds, analytical or reagent grade, without further purification. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) was twice recrystallized from 95% ethanol. The total buffer concentration used in this work was maintained constant at 0.05 M throughout the pH range studied. All buffers were brought to an ionic strength, μ, of 0.15 by adding an appropriate amount of Na_2SO_4, KCl, or NaCl.

Bovine carbonic anhydrase, BCA (carbonate hydrolyase, EC 4.2.1.1), was a product of Mann Research Laboratories prepared and purified from bovine erythrocytes by the method of Keilin and Mann (1940). The specific activity of this preparation was found to be 74% of the highly purified form as judged by its zinc content (measured at 214 nm) as well as by inhibition with acetazolamide. For control purposes, chromatography of the commercial sample was carried out following the procedure of Lindskog (1960) except that DEAE-Sephadex was substituted for DEAE-cellulose. The purified product was shown to consist of a mixture of A and B isomers in the ratio 1:2. Unlike the human enzyme whose isozymes possess markedly different activities (Rickli *et al.*, 1964; Verpoorte *et al.*, 1967), the two resolved isozymes of bovine carbonic anhydrase were found to exhibit essentially identical catalytic properties. The various enzyme preparations were stored dry at −20°, and periodic checks of esterase activity revealed no change during the course of the experiments. Enzyme concentrations, deduced from ultraviolet absorbance measurement at 280 nm employing ϵ 54,000 and a molecular weight of 30,000 (Lindskog, 1960), were corrected for the per cent zinc actually present in the enzyme preparation.

Apparatus. All pH measurements were recorded with a glass electrode on a Beckman research pH meter at 25.0°. The relative accuracy of this pH meter is reported as ±0.001 pH unit. Spectrophotometric determinations of steady-state kinetics were made on a Beckman Model Kintrac VII equipped with a 0.1–10-in./min recording capability and with an insulated cell compartment thermostatted to 25.0 ± 0.02° by means of a Forma-Temp Jr. refrigerating and heating device Model No. 2095-1 which contained, in addition to the thermostatting mechanism, a pump to circulate the coolant.

Rapid hydrolyses were monitored on a Durrum-Gibson stopped-flow spectrophotometer (Durrum Instrument Corp., Palo Alto, Calif.) which, under favorable conditions, permitted accurate measurements down to periods of 5 msec. The drive-syringe assembly supplied with the instrument was replaced by one designed in our laboratories by Mr. Ron Reaugh and constructed by the departmental workshop. This

[2] Melting points and boiling points are uncorrected.

[3] We wish to thank Mr. R. Anderson for his assistance in the preparation of these 3-hydroxynitropyridines.

modified assembly eliminated leakage and made it possible, through the use of two syringes of different bore to injection into the mixing chamber amounts of solution in a ratio approaching 10:1. The bath was also modified, and thermostatting was achieved by means of an insulated bath compartment consisting of a specially constructed circulating device thermostatted to 25.0 ± 0.02° by means of a Sargent thermonitor Model SW (S-82055). Nmr spectra for structure verification were measured in $CDCl_3$ with tetramethylsilane as internal standard on a Varian Associates A-60 instrument. Ultraviolet spectra were recorded on a Cary 14 spectrophotometer.

Kinetics and Technique. A typical procedure for a kinetic run was to initiate the reaction by injecting 15 ~ 25 μl of acetonitrile stock solution of a substrate ester by means of a calibrated Hamilton microliter syringe into the spectrophotometer cells containing 3 ml of appropriate buffer, with or without BCA. The hydrolysis of the 3-acetoxypyridine esters was followed spectrophotometrically by monitoring the appearance of anions of 3-hydroxypyridines primarily at their peak absorbances. The values of ϵ at the wavelengths employed for each of the 3-pyridinols are 3-hydroxypyridine: λ 314 nm, ϵ 2.82 × 10^3 (pH 7.0); 3-hydroxy-2-nitropyridine: λ 398 nm, ϵ 5.89 × 10^3 (0.1 N NaOH); and 3-hydroxy-2,6-dinitropyridine: λ 387 nm, ϵ 9.88 × 10^3 (0.1 N NaOH). All work employed deionized distilled water.

At low ester concentration, $[S]_0 \ll K_m$, pseudo-first-order coefficients were obtained by plotting log $(A_\infty - A_t)$ *vs.* time, where A_∞ was the exerimentally determined absorbance observed after roughly 10 half-lives. A FORTRAN IV computer program was written for the evaluation of first-order rate constants. All computer calculations were executed by a CDC 6400 digital computer. The program was written to calculate the best slope for first-order plots by means of the least-squares method. Correlation coefficient of the slope was better than 0.995 in most runs (>90%). For ester concentrations greater than 10^{-3} M, initial velocities were obtained by plotting the first part of the reaction progress against time and deducing the tangent at the origin.

pH values reported in this study are for the initial reaction mixture, and except at very high substrate concentrations, they remained essentially unchanged throughout a given run. For high substrate concentration, initial rates were deduced using the infinity absorbance corrected to correspond to the initial pH. The reaction was found to proceed to completion (>99% hydrolysis). The observed first-order rate constant for the BCA-catalyzed hydrolysis of 3-acetoxypyridine esters in buffered aqueous media can be represented by eq 1.

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

Here k_0 is the catalytic constant for the 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 of the enzyme (k_{enz}) was determined by varying enzyme concentration while keeping the concentration of all other components constant. Plots of k_{obsd} *vs.* [E] gave straight lines whose slopes defined k_{enz}. For the determination of pH–rate profiles, k_{enz} was obtained using a fixed substrate concentration of 1.67 × 10^{-4} M for ANP and 1.00 × 10^{-4} M for ADNP. A minimum of three duplicate runs were performed at each pH and the mean value used. In the determination of K_m and k_2 at various pH values, average of seven different concentrations ranging from 1.0 × 10^{-4} to 4.5 × 10^{-3} M (AP), 1.0 × 10^{-4} to 1.0 × 10^{-2} M (ANP), and 6.7 × 10^{-5} to 3.0 × 10^{-3} M (ADNP), were employed.

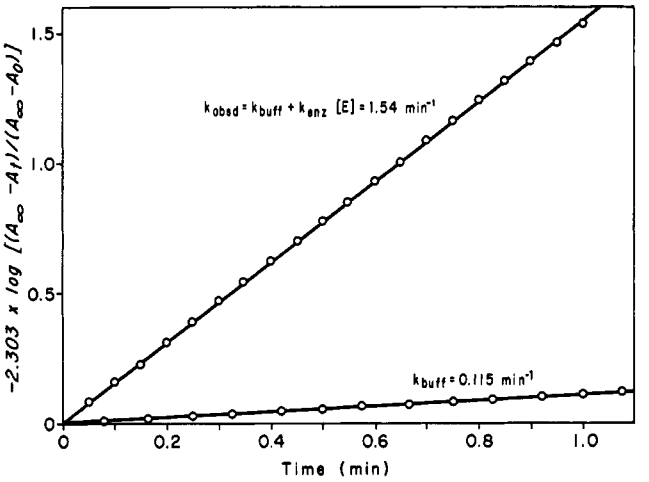


FIGURE 1: Typical first-order rate plots of 3-acetoxy-2,6-dinitropyridine hydrolysis with and without BCA at 25.0°: [ADNP] = 1.00 × 10^{-4} M, [BCA] = 1.86 × 10^{-6} M in phosphate buffer (0.05 M, μ = 0.15, pH 7.36) followed at 387 nm. Plots furnish k_{enz} = 7.63 × 10^5 M^{-1} min^{-1}.

Inhibition of BCA esterase activity by acetazolamide was studied as a function of inhibitor concentration. Percentage activity was determined from the $k_{enz}^I : k_{enz}^0$ ratios at various concentrations of acetazolamide. The catalytic coefficient, k_{enz}^I, refers to enzymatic activity at a particular concentration of acetazolamide and k_{enz}^0 is the enzymatic activity 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 BCA 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

$$[EI] = [E]_0\left(1 - \frac{k_{enz}^I}{k_{enz}^0}\right) \quad (3)$$

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

Throughout this work all curve fittings were performed by iterating upon the parameters to obtain the optimum fit judged by the minimum sum of the squares of the differences between calculated and experimental points. It is worth emphasizing that, since essentially all the points on the curve contribute to the determination of the parameter in question, the accuracy of the latter is substantially greater than that of the individual points on the curve.

Results

The spectrophotometric technique employed in determining the rate of hydrolysis of 3-acetoxypyridine esters exhibits a reproducibility of ±2%. A first-order plot derived from spectrophotometric readings observed in a typical run is exemplified in Figure 1. As a preliminary to our kinetic studies of carbonic anhydrase catalysis we have determined the cata-

TABLE I: pH Dependence of k_{enz} for the Hydrolysis of 3-Acetoxy-2-nitropyridine and 3-Acetoxy-2,6-dinitropyridine at 25.0°.

Substrate	pH, Buffer[a]	$k_{enz} \times 10^{-4}$ [b]	pH, Buffer[a]	$k_{enz} \times 10^{-4}$ [b]	pH, Buffer[a]	$k_{enz} \times 10^{-4}$ [b]
3-Acetoxy-2-nitropyridine	5.94, P	0.342	7.35, P	7.06	8.38, T	13.6
	6.13, P	0.535	7.56, P	8.94	8.67, T	14.6
	6.35, P	0.828	7.68, T	8.44	8.78, T	15.5
	6.55, P	1.50	7.75, P	10.2	9.11, T	16.5
	6.76, P	2.38	7.80, T	10.2	9.29, T	16.7
	6.94, P	3.73	7.99, T	11.5	9.48, T	16.9
	7.15, P	5.25	8.29, T	12.6	9.63, T	17.0
3-Acetoxy-2,6-dinitropyridine	5.94, P	4.50	7.20, P	65.6	8.06, T	128.0
	6.15, P	7.40	7.37, P	77.2	8.25, T	135.0
	6.35, P	12.2	7.57, P	101.0	8.43, T	146.0
	6.55, P	19.8	7.68, T	103.0	8.63, T	155.0
	6.75, P	32.0	7.77, P	115.0	8.82, T	162.0
	6.99, P	46.4	7.85, T	115.0		

[a] Buffer concentration (0.05 M): P (phosphate), and T (Tris); ionic strength 0.15. [b] Units of k_{enz} in M^{-1} min^{-1}.

lytic rate coefficients associated with buffer components using the method of Bell and Darwent (1950) (Y. Pocker and N. Watamori, details to be submitted for publication). In the presence of enzyme the overall rate coefficients determined in a buffered solution consist of a sum of catalytic terms (eq 1). The enzymatic component of catalysis was separated from the nonenzymatic catalysis by working in a region where k_{obsd} is a linear function of enzyme concentration. Values of k_{enz} as a function of pH are listed for 3-acetoxy-2-nitropyridine and 3-acetoxy-2,6-dinitropyridine in Table I. The resulting sigmoid curves when k_{enz} is plotted against pH are shown in Figures 2 and 3. Also shown in these figures are theoretical curves based on eq 4–7, where $[E]_0$, EH_2^+, EH, S, and C_i are

$$\underset{\text{inactive}}{EH_2^+} \overset{K_a'}{\rightleftharpoons} \underset{\text{active}}{EH} + H^+; \quad [E]_0 = [EH_2^+] + [EH] \tag{4}$$

$$\text{rate} = (k_{EH}[EH] + \Sigma k_i[C_i])[S] \tag{5}$$

$$k_{obsd} = k_{EH}[EH] + \Sigma k_i[C_i] \tag{6}$$

$$k_{buffer} = k_i[C_i]$$

$$k_{enz} = \frac{(k_{obsd} - k_{buffer})}{[E]_0} = k_{EH}\frac{[EH]}{[E]_0} = k_{EH}\frac{K_a'}{K_a' + [H^+]} \tag{7}$$

the total enzyme concentration, the protonated form of the enzyme, the neutral form of the enzyme, the substrate, and the buffer component of species i, respectively. k_{EH} and k_i are the catalytic coefficients of the active enzyme species EH, and buffer component C_i. Since it was shown previously in these laboratories (Pocker and Storm, 1968; Pocker and Stone, 1968c) that BCA exhibited a second inflection of much greater magnitude above pH 10, the above treatment is an approximate analysis of the first inflection of this enzyme. The BCA-catalyzed hydrolysis rates for 3-acetoxy-2-nitropyridine and 3-acetoxy-2,6-dinitropyridine above pH 9 require special ex-

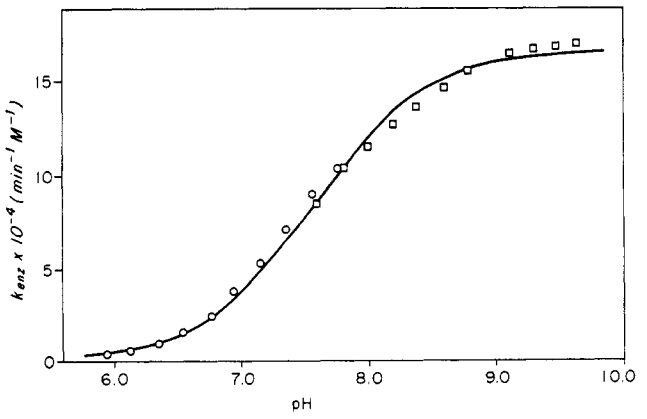


FIGURE 2: The BCA-catalyzed hydrolysis of 3-acetoxy-2-nitropyridine as a function of pH in phosphate (○) and Tris (□) buffer at 25.0°; [BCA] = 1–25 × 10^{-6} M, [ANP] = 1.67 × 10^{-4} M, ionic strength, 0.15. Solid line is the curve calculated from the experimental points.

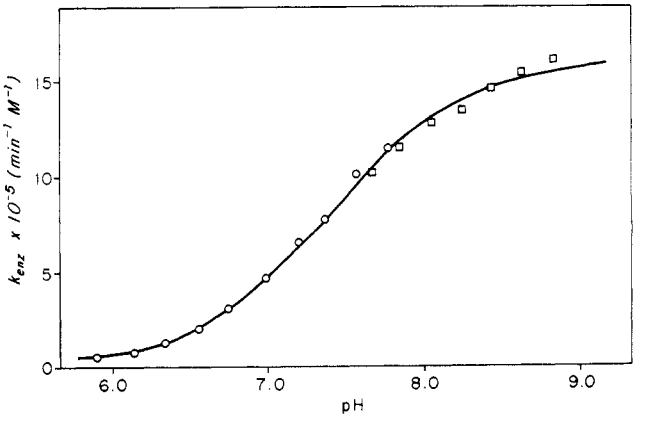


FIGURE 3: The BCA-catalyzed hydrolysis of 3-acetoxy-2,6-dinitropyridine as a function of pH in phosphate (○) and Tris (□) buffer at 25.0°; [BCA] = 4–120 × 10^{-7} M, [ADNP] = 1.00 × 10^{-4} M, ionic strength, 0.15. Solid line is the curve calculated from the experimental points.

TABLE II: Results from Lineweaver–Burk Plots for BCA-Catalyzed Hydrolysis of 3-Acetoxypyridine Esters at 25.0°.[a]

Substrate	pH	No. of Runs	[BCA] × 10^6 (M)	K_m × 10^3 (M)	V_m × 10^4 (min^{-1} M)	k_2 × 10^{-1} (min^{-1})	γ[b]
3-Acetoxypyridine	6.45	18	12.7	5.4	0.17	0.13	0.9993
	6.95	20	10.2	5.6	0.39	0.38	0.9996
	7.45	18	10.3	6.3	0.75	0.73	0.9996
	7.90	20	10.1	7.1	1.1	1.1	0.9978
3-Acetoxy-2-nitropyridine	6.03	18	3.23	1.65	0.227	0.703	0.9956
	6.45	18	3.22	1.82	0.554	1.72	0.9995
	6.94	20	3.48	2.21	2.94	8.45	0.9992
	7.47	21	3.33	4.02	9.52	28.6	0.9990
	7.86	15	3.15	9.04	26.9	85.4	0.9998
3-Acetoxy-2,6-dinitropyridine	6.03	18	0.633	1.58	0.530	8.37	0.9990
	6.45	21	0.688	1.66	1.73	25.1	0.9970
	6.97	18	0.586	1.83	4.06	69.3	0.9991
	7.47	21	0.656	2.19	13.4	204.0	0.9983
	7.90	19	0.644	2.48	20.5	318.0	0.9971

[a] Determined in 0.05 M phosphate buffer; ionic strength, μ, 0.15. [b] Correlation coefficient.

perimental techniques and would be reported fully in a subsequent publication together with a complete documentation of the second inflection. Theoretical curves give, respectively, pK_a' and k_{EH} values of 7.56 and 1.65 × 10^5 min^{-1} M^{-1} for 3-acetoxy-2-nitropyridine and of 7.40 and 1.61 × 10^6 min^{-1} M^{-1} for 3-acetoxy-2,6-dinitropyridine.

Although ANP and ADNP are considerably better substrates with respect to the esterase activity of BCA than *p*-NPA (Pocker and Stone, 1967), we were still unable to detect any initial "burst" of release of the respective nitropyridol even under practically the most advantageous conditions so far encountered with our enzyme. This suggests that an acetyl-enzyme intermediate either does not form or hydrolyzes very rapidly, *i.e.*, $k_3 > k_2$ in eq 8. For many hydrolytic enzymes,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P_1 + ES_1 \xrightarrow{k_3} P_2 + E \qquad (8)$$

the rate of formation of the acyl-enzyme intermediate (ES_1) and the product phenol (P_1) is significantly faster than the rate of hydrolysis of the covalent acyl-enzyme intermediate to give carboxylate anion (P_2) and enzyme (E) (*i.e.*, $k_2 > k_3$) (Sturtevant, 1960; Bender *et al.*, 1962; Bender and Kezdy, 1965). However, it is not unreasonable to suppose that with carbonic anhydrase k_3 would be much faster than k_2, for if we follow our working hypothesis to its logical conclusion, the acyl-enzyme intermediate of carbonic anhydrase is actually the highly labile zinc–acetate complex. Consequently, the dependence of enzymatic rate on substrate concentration was formally analyzed in terms of the Michaelis–Menten scheme (eq 9). It is of course

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (9)$$

realized that the specific rate constants determined experimentally may be considerably more complex than those indicated by this simplified scheme. Lineweaver–Burk plots were used to determine formal values of $K_m = [(k_{-1} + k_2)]/k_1$ and $V_m = k_2[E]$ for the enzymatic hydrolyses of 3-acetoxypyridine, 3-acetoxy-2-nitropyridine, and 3-acetoxy-2,6-dinitropyridine at various pH values. Since such plots are sensibly linear, K_m and V_m values were therefore calculated by a linear least-squares procedure. These results are given in Table II. It appears that both K_m and V_m vary with pH, but that in the pH interval 6.0–8.0, K_m is to a first approximation a linear function of the turnover number, k_2. This would imply that the apparent binding constant $K_s = (k_1/k_{-1})$ is invariant over this pH range, while the inflection point in the pH–rate profile refers to the ionization of a group associated with the hydrolyzing rather than the binding site. Formally speaking, the slopes in K_m *vs.* k_2 plots 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. These results, including the apparent binding constants k_1/k_{-1} and the $\Delta F°$ values for binding, are compared in Table III together with those of *p*-NPA previously determined in 10% (v/v) acetonitrile (Pocker and Stone, 1967).

Acetazolamide has been shown to be a potent specific inhibitor of carbonic anhydrase activity with respect to both hydration (Leibman *et al.*, 1967; Pocker and Meany, 1965b; Pocker and Dickerson, 1968) and hydrolysis (Pocker and Stone, 1965; Armstrong *et al.*, 1966; Pocker and Stone, 1967; Pocker and Storm, 1968). Plots of enzymic activity *vs.* the ratio of acetazolamide to enzyme concentration are given in Figure 4. Extrapolations of the initial slopes for the hydrolysis of 3-acetoxypyridine esters to zero activity indicate that there is one esteratic site per enzyme molecule. Values of the dissociation constants for the enzyme–acetazolamide complex in the presence of 3-acetoxypyridine, 3-acetoxy-2-nitropyridine, and 3-acetoxy-2,6-dinitropyridine are 2.8 × 10^{-7}, 2.9 × 10^{-8}, and 2.0 × 10^{-8} M, respectively, at pH 7.47. It should be stated that when an inhibitor as potent as acetazolamide is used, the number ascribed to an inhibition constant is only an apparent value as the concentration of free inhibitor

TABLE III: Comparison of the Binding Constants of BCA with 3-Acetoxypyridine Esters and *p*-Nitrophenyl Acetate at 25.0°.

Substrate	$k_1 \times 10^{-4}$ (M^{-1} min^{-1})	$k_{-1} \times 10^{-2}$ (min^{-1})	$k_1/k_{-1} \times 10^{-2}$ (M^{-1})	$\Delta F°$ (cal)
3-Acetoxypyridine	0.55	0.28	2.0	3100
3-Acetoxy-2-nitropyridine	11.5	1.80	6.37	3820
3-Acetoxy-2,6-dinotropyridine	351.0	55.8	6.28	3810
p-Nitrophenyl acetate[a]	2.00	0.400	5.00	3680

[a] Value determined (Pocker and Stone, 1967) in 10% (v/v) acetonitrile.

TABLE IV: Acetazolamide Inhibition of the BCA-Catalyzed Hydrolysis of 3-Acetoxy-2-nitropyridine at 25.0°.[a]

[Acetazolamide] $\times 10^6$ (M)	No. of Runs	$K_m \times 10^3$ (M)	$V_m \times 10^2$ (min^{-1} M)	γ[b]
0.0	21	4.0	9.5	0.9990
1.21	10	4.2	7.1	0.9994
1.82	12	3.2	4.8	0.9998
2.74	12	4.0	2.6	0.9999
3.97	10	4.0	1.1	0.9999
4.57	6	4.8	0.23	0.9997

[a] Determined in 0.05 M phosphate buffer, pH 7.5; ionic strength, μ, 0.15; [BCA] = 3.3×10^{-6} M. [b] Correlation coefficient.

will be minute, thereby making it difficult to evaluate a true dissociation constant (Webb, 1963). Acetazolamide has been shown to bind with carbonic anhydrase at or near the zinc atom (Tilander *et al.*, 1965; Fridborg *e al.*, 1967; Liljas *et al.*, 1969). The effect of this inhibitor on the enzyme-catalyzed hydrolyses of AP, ANP, and ADNP is to reduce V_m without significantly affecting the value of K_m, thus characterizing this inhibition as noncompetitive[4] (Table IV). Since enzymatic catalysis depends essentially on the binding between enzyme and substrate, and on the rate of breakdown of the active enzyme–substrate complex, it is particularly instructive to compare the magnitudes of k_2 to k_{OH^-} [OH^-] for the hydrolyses of AP, ANP, ADNP, and *p*-NPA (Table V). It will be noted that the efficiency of the enzyme as expressed by the ratio k_2:k_{OH^-} [OH^-] is between 6×10^4 and 6×10^5 times better than the hydroxide ion component around pH 7.5.

Discussion

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 hydration and hydrolysis are similar (*cf.* Pocker and Stone, 1967). All available evidence is compatible with the view that carbonic anhydrases from erythrocytes obey simple Michaelis–Menten kinetics. They have none of the characteristic properties of "allosteric" enzymes.[5] The zinc ion associated with the native enzyme is essential both for its hydrase and esterase activity. Both activities show sigmoid pH–rate profiles from which it can be inferred that in each case a basic group with an apparent pK_a value close to 7 is involved. Also, there are strong indications that this group is associated with the turnover process but plays little or no role in the binding of CO_2, acetaldehyde, or *p*-NPA. Accordingly, k_2 values dictate the pH–rate profile whereas the formal binding constants, k_1/k_{-1}, are pH independent in the region 6–8.5. (Pocker and Meany, 1965a,b;

[4] The statistical analysis of the Lineweaver–Burk plots for all three esters points to the noncompetitive nature of these inhibitions. This observation is in accord with earlier studies of *p*-NPA hydrolysis (Pocker and Stone, 1965, 1967), and contrasts with the enzymic hydrations of 2- and 4-pyridinecarboxaldehydes which are competitively inhibited by acetazolamide (Pocker and Meany, 1967a,b). This difference could arise if the ring nitrogen of the pyridinecarboxaldehydes were positioned closer to the zinc atom than that of the 3-acetoxypyridines. Clearly the inhibition of the enzymatic catalysis of pyridyl and pyridoyl systems needs further investigation.

We must also note here that both Kernohan (1966) and Lindskog (1969) have proposed that inhibition by acetazolamide in carbonic anhydrase reaction with CO_2 may under certain conditions be actually competitive depending upon the rates of formation and dissociation of the enzyme–inhibitor complex. We wish to defer comment on this proposal until our own studies contrasting CO_2 with other highly reactive substrates are more complete.

[5] All the mammalian carbonic anhydrases are single polypeptide chains of molecular weight approximately 30,000 and contain 1 zinc atom/molecule. On the other hand, the plant enzyme has a molecular weight of 180,000 and is made up of 6, possibly identical, subunits whose zinc analysis indicates the presence of 1 zinc atom/29,000 molecular weight subunit (CA from parsley leaves, Tobin, 1970; CA from spinach leaves, Y. Pocker and Mrs. J. Ng, unpublished results; *cf.* also Pocker *et al.*, 1971). Although a powerful catalyst for the reversible hydration of carbon dioxide, the enzyme from spinach leaves does not show the catalytic versatility associated with the mammalian enzymes and is furthermore subject to regulatory control (Y. Pocker and Mrs. J. Ng, unpublished results). It is interesting to speculate about the regulatory control of CO_2 by plant carbonic anhydrase and its relevance to the photosynthetic cycle in plants.

TABLE V: Comparison of Turnover Number to Hydroxide Ion Catalysis at 25.0°.

Substrate	k_{OH^-} (M^{-1} min^{-1})	K_m (M)	k_2 (min^{-1})	k_2/k_{OH^-} $[OH^-]$[a,b]
3-Acetoxy-pyridine	3.87×10^2	6.3×10^{-3}	pH 7.45 7.3	6.7×10^4
3-Acetoxy-2-nitropyridine	2.11×10^3	4.02×10^{-3}	pH 7.47 2.86×10^2	4.60×10^5
3-Acetoxy-2,6-dinitropyridine	1.14×10^4	2.19×10^{-3}	pH 7.47 2.04×10^3	6.07×10^5
p-Nitrophenyl acetate[c]	8.9×10^2	4.6×10^{-3}	pH 7.51 5.4×10	1.9×10^5

[a] This ratio is unitless. [b] In calculating the concentration of hydroxide ions it was assumed that $[OH^-] = a_{OH^-}$. [c] In 10% (v/v) acetonitrile (Pocker and Stone, 1968c). In 1% acetonitrile the value k_2 is higher, leading to a k_2/k_{OH^-} $[OH^-]$ ratio which is much closer to the one deduced for 3-acetoxy-2,6-dinitropyridine.

Kernohan, 1965; Pocker and Stone, 1967; Pocker and Storm, 1968; Pocker and Dickerson, 1968; Khalifah, 1971). Whereas an *acidic* group of pK_a around 7 facilitates anion binding including that of anionic substrates undergoing dehydration such as HCO_3^- (Kernohan, 1965) and $CH_3C(OH)_2CO_2^-$ (Pocker and Meany, 1970), no essential acidic groups have as yet been detected as promoters of the hydrase or esterase activity. The present study demonstrates that BCA exhibits powerful esterase activity with respect to 3-acetoxypyridine esters (Table V). The pH–rate profiles for 3-acetoxy-2-nitro (Figure 2) and -2,6-dinitropyridines (Figure 3) reveal sigmoidal curves analogous to those observed in the reversible hydration of CO_2 (Kernohan, 1964), of aldehydes (Pocker and Meany, 1964, 1965a,b; Pocker and Dickerson, 1968) and of *p*-nitrophenyl esters (Pocker and Stone, 1967; Pocker and Storm, 1968). Because of their pronounced solubility in water, Michaelis–Menten parameters could be easily and accurately determined. Thus, using maximum velocities and Michaelis constants at various pH values of the forward hydrolysis we were able to determine for each of our substrates three formal rate constants k_1, k_{-1}, and k_2 (eq 9) (Tables II and III). It would appear that the binding constants, $K_s = k_1/k_{-1}$, are roughly similar for ANP, ADNP, and *p*-NPA and that AP binds only *ca.* three times poorer than the other three esters. On the other hand, the formal rate constants of ES formation, k_1, are much slower than diffusion-controlled rates, and furthermore show a significant degree of specificity, ADNP:ANP:*p*-NPA:AP = 640:21:4:1. Similar substrate specificity is also encountered in the formal rate constants, k_{-1}, for the dissociation of the enzyme–substrate complex, ADNP:ANP:*p*-NPA:AP = 200:6.4:1.4:1. Since K_s is practically independent of pH between 6 and 8, the neutral esters AP, ANP, and ADNP seem to bind with equal strength to both the acidic and the basic form of the enzyme. Kernohan (1965) found the K_m value for the hydration of CO_2 by bovine carbonic anhydrase to be nearly independent of pH, between 5.5 and 9; all his values lay in the range 14 ± 3 mM. Assuming that in this hydration, $K_m = 1/K_s$, one obtains a binding constant for CO_2 of 71 ± 12 M^{-1} and it would thus appear that the bovine enzyme binds 3-pyridyl esters significantly better than its natural substrate. The following conclusions may be drawn. (1) The binding nature of BCA with AP, ANP, and ADNP as substrates is essentially hydrophobic. (2) Other interactions such as hydrogen bonding and possibly charge transfer complexation probably contribute to the more efficient overall binding of these esters into the reaction center. (3) The esterase activity of BCA with respect to these substrates is more sensitive to the turnover number, k_2, than to their respective binding constants.

Here we wish to point out that there is increasing evidence which suggests that CO_2 (Riepe and Wang, 1968; Khalifah, 1971), aldehydes (Pocker and Dickerson, 1968), and esters (Pocker and Stones, 1968a,b) may also be bound to the enzyme in a nonproductive mode. The possibility for nonproductive substrate binding arises for two reasons. Firstly these substrates are small enough to fit into the relatively deep crevice of the active site in more than one orientation. Secondly, like with the binding of anions there are additional sites away

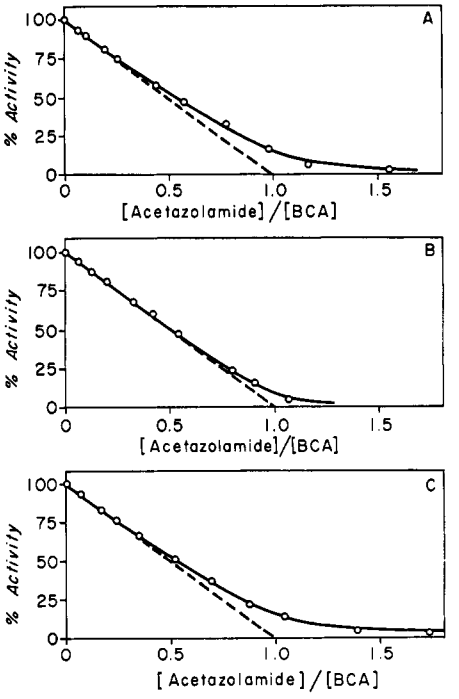


FIGURE 4: Per cent esterase activity as a function of [acetazolamide]:[BCA] ratio at 25.0° in phosphate buffer (0.05 M, μ = 0.15, pH 7.47). (A) 3-Acetoxypyridine, [BCA] = 1.03×10^{-5} M; (B) 3-acetoxy-2-nitropyridine, [BCA] = 3.41×10^{-6} M; (C) 3-acetoxy-2,6-dinitropyridine, [BCA] = 6.55×10^{-7} M. Solid lines are the curves calculated from the experimental points.

TABLE VI: Comparison of Enzymatic to Hydroxide Ion Catalysis in Aldehyde Hydration and Carboxylic Ester Hydrolysis.

Substrate	pH	Buffer[a]	Temp (°C)	k_{enz} (min^{-1} M^{-1})	k_{OH^-} (min^{-1} M^{-1})	$k_{enz}:k_{OH^-}$[b]
Acetaldehyde[c,d]	8.01	P	0	8.38×10^4	7.0×10^5	0.12
Propionaldehyde[e]	7.84	D	0	7.10×10^4	1.41×10^5	0.50
Isobutyraldehyde[e]	7.99	D	0	1.53×10^4	1.06×10^5	0.14
2-Pyridinecarboxaldehyde[d]	8.00	D	0	3.73×10^5	2.6×10^6	0.14
4-Pyridinecarboxaldehyde[d]	8.03	D	0	1.12×10^6	7.3×10^6	0.15
p-Nitrophenyl acetate[f]	7.92	T	25	1.84×10^4	8.90×10^2	20.7
p-Nitrophenyl propionate[g]	8.02	T	25	5.73×10^3	5.50×10^2	10.4
p-Nitrophenyl isobutyrate[g]	8.01	T	25	1.8×10^2	4.00×10^2	0.45
3-Acetoxypyridine[h]	8.06	T	25	1.74×10^3	3.87×10^2	4.50
3-Acetoxy-2-nitropyridine[h]	7.99	T	25	1.15×10^5	2.11×10^3	54.5
3-Acetoxy-2,6-dinitropyridine[h]	8.06	T	25	1.28×10^6	1.14×10^4	112.0

[a] T (Tris), P (phosphate), and D (diethyl malonate). [b] This ratio is unitless. [c] Pocker and Meany (1965b). [d] Pocker and Meany (1967a). [e] Pocker and Dickerson (1968). [f] In 10% (v/v) acetonitrile (Pocker and Stone, 1967). [g] In 1% (v/v) acetonitrile (Pocker and Storm, 1968). [h] Present work.

TABLE VII: Reactions of Nitropyridyl Acetates with High Enzyme Concentrations.

Substrate	pH	[S], M	[E], M	Condition	k_{enz} (min^{-1} M^{-1})
3-Acetoxy-2-nitropyridine	6.43			Steady state	1.15×10^4[a]
		1.0×10^{-2}	3.7×10^{-5}	$[E] < K_m < [S]$	1.16×10^4
		7.0×10^{-5}	3.7×10^{-5}	Initial 5 sec	1.17×10^4
	6.37			Steady state	1.01×10^4[a]
		2.0×10^{-4}	1.5×10^{-4}	$[E] \simeq [S] < K_m$	1.06×10^4
3-Acetoxy-2,6-dinitropyridine	6.43			Steady state	1.59×10^5[b]
		4.0×10^{-3}	3.7×10^{-5}	$[E] < K_m < [S]$	1.62×10^5
		5.0×10^{-5}	3.7×10^{-5}	Initial 5 sec	1.50×10^5
	6.39			Steady state	1.46×10^5[b]
		4.0×10^{-3}	1.1×10^{-4}	$[E] < K_m \leq [S]$	1.38×10^5

[a] Interpolated from the pH–activity profile given in Figure 2. [b] Interpolated from the pH–activity profile given in Figure 3.

from the active site which are capable of binding small substrates. We believe that changes in conformation and internal prototropic isomerizations probably precede and almost certainly follow the formation of the ES complex. We defer comment on the rates of these processes until more complete evidence using temperature-jump relaxation studies is available.

One meaningful comparison between enzymatic and hydroxide ion catalysis is given by the ratio $k_2:k_{OH^-}$ $[OH^-]$ (Table V). It will be noted that the efficiency of the enzyme as expressed by this ratio is higher with nitro-substituted esters ($2–6 \times 10^5$ at pH 7.5) but does not vary appreciably between *p*-NPA, ANP, and ADNP. Another meaningful comparison is given by the ratio $k_{enz}:k_{OH^-}$ (Table VI). It will be noted here that although k_{enz} values for ester hydrolysis are lower than the respective values for aldehyde hydration, the enzymatic catalysis as measured by the $k_{enz}:k_{OH^-}$ ratio is actually more effective. An inspection of the data indicates that with respect to all three substrates (AP, ANP, and ADNP), carbonic anhydrase activity is quite impressive. With certain enzymes a covalent intermediate is formed during the turnover of ES (eq 8) (Bender and Kezdy, 1965; Koshland and Neet, 1968; Jencks, 1969). We have searched earlier for such intermediates but were unable to detect them in the BCA-catalyzed hydrolysis of *p*-NPA (Pocker and Stone, 1967). The present substrates are much more water soluble and the opportunity presented itself to expand the search for a covalent acetyl–BCA intermediate using highly favorable conditions. However, in the present work we were again unable to detect acetylation of BCA by an extensive kinetic study (Table VII), although in these experiments high enzyme concentrations were used at pH values around 6.4 where the turnover is low and nitropyridol production is easily monitored. In contrast to α-chymotrypsin, bovine carbonic anhydrase showed no "initial burst" of 2-nitropyridol or 2,6-dinitropyridol even under highly favorable reaction conditions for its detection

by stopped-flow kinetics. Strandberg and his collaborators in Uppsala (Liljas *et al.*, 1969; also private communication, 1970) have collected X-ray diffraction data on human carbonic anhydrase C to a 2-Å resolution and the corresponding three-dimensional electron density map has been computed. The zinc is situated at the bottom of a deep crevice and its ligands have been tentatively identified as three imidazole rings. There appear to be one or more imidazole residues in the active-site region at a distance of three or more water molecules from the zinc. The kinetic evidence requires the presence in the active site of a basic group which is either linked to or acts in cooperation with the metal ion. It has been suggested that the pH–rate profile reflects the dissociation around pH 7 of a proton from a metal-bound water molecule (Davis, 1959). The assumption of a pK_a' value near 7 for this ionization is open to question. The corresponding pK_a value for similar imidazole–zinc–aquo complexes is about 10 (Y. Pocker and J. T. Stone, unpublished observations). Of course the possibility exists that the environment of zinc in the deep crevice could conceivably shift the pK to much lower values (Coleman, 1969). On the other hand, Pocker and Meany (1965a,b, 1967a,b) as well as Pocker and Stone (1965, 1967, 1968a–c) have suggested that around physiological pH, the basic form of an imidazole group in a histidine residue may play a key role as a proton acceptor from a water molecule in the hydration sphere of the zinc–aquo complex and at the same time may be involved in the regulation of the water structure in the active site. Indeed, the value of the temperature coefficient associated with the pK_a' of the BCA-catalyzed hydrolysis, $\Delta pK_a'/\Delta T \sim -0.02$ unit/deg (Pocker and Stone, 1968c), is in excellent agreement with the respective coefficient for imidazole (Perrin, 1964). Furthermore, there is strong evidence for the presence of an imidazole group in the active-site region of bovine carbonic anhydrase B (Pocker, 1969; Y. Pocker and J. T. Stone, unpublished observations; Kandel *et al.*, 1970). Specific modifications of histidine residues leading to major activity losses have also been achieved with human carbonic anhydrase B (Whitney *et al.*, 1967; Bradbury, 1969). Wang has proposed a somewhat different role for the active-site imidazole from that originally proposed by Pocker and his coworkers. Wang uses the imidazole to promote a proton transfer from zinc-bound OH^- to the oxygen atom of CO_2. The assumed proton transfer is rather unusual for it involves a base-assisted proton removal from a zinc–hydroxo complex which is itself a powerful base. Furthermore, the localization of the negative charge in the developing HCO_3^- on the oxygen atom closest to the positively charged zinc does not appear to be a necessity in view of the fact that HCO_3^- may actually be formed as a solvent separated rather than tight ion pair (Pocker and Dickerson, 1968; Liljas *et al.*, 1969). Of course, the mere fact that imidazole is present near the metal ion suggests that it should have some function, but the data so far obtained do not allow a unique conclusion about its role in this catalysis.

Acetazolamide inhibits the enzymatically catalyzed hydrolysis of AP, ANP, and ADNP. The extrapolation of the inactivation plot to zero activity coincides with a 1:1 enzyme–inhibitor complex and indicates that there is one esteratic site per enzyme molecule (Figure 4). The noncompetitive inhibition observed in the present work (Table IV) implies that the protein-bound zinc is not the binding site for pyridyl esters in spite of the fact that these esters are bifunctional in character in that they not only have a carboxylic ester group which hydrolyzes with ease, but also possess, in principle, the capacity to interact with enzyme bound zinc *via* the ring nitrogen. Perhaps the most striking result which evolved from our studies with sulfonamides is that the inhibitory effect caused by acetazolamide in pyridyl ester hydrolysis and pyridinecarboxaldehyde hydration (Pocker and Meany, 1967a) differs not only in magnitude ($K_i = 2 \times 10^{-8}$ M with ADNP as opposed to $K_i = 4.8 \times 10^{-5}$ M with 4-pyridinecarboxaldehyde) but also in type (noncompetitive as opposed to competitive). It would appear that pyridinecarboxaldehyde bind closer to zinc than the pyridyl esters. Thus it is apparent that radical changes are not necessarily required in order to explain the observed differences between pyridyl and pyridoyl systems; rather these differences can be rationalized in terms of a flexible active site whereby conformational changes are induced by substrate (Koshland, 1963). This is a concept that we shall certaintly try to test further. The present study in turn prepares the way for a discussion of the special features associated with the enzymatic hydrolysis of pyridyl carbonates (Y. Pocker and L. J. Guilbert, to be submitted for publication as Part X in this series).

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Purification and Properties of Carbonic Anhydrase from Sheep Erythrocytes*

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ABSTRACT: The single major component and three minor components of sheep red cell carbonic anhydrase were purified using a combination of column chromatography and isoelectrofocusing. The major form behaved as a single component in sedimentation velocity experiments ($s^0_{20,w}$ = 3.0 S; observed mol wt 30,000) and during starch gel electrophoresis. Hydrase and esterase activities in conjunction with amino acid composition data suggest that the single major component is homologous with the high-activity form known to occur in other mammals. Comparison of the amino acid composition data of the sheep major and minor forms indicates these structures are also apparently conformational isomers of the same protein. Cyanogen bromide treatment of sheep enzyme releases two soluble fragments. Composition and sequence data indicate the C-terminal fragment is identical with the corresponding fragment seen in bovine carbonic anhydrase except for a Gly–Val substitution four residues from the C terminus. We conclude that sheep red cells contain a single high-activity carbonic anhydrase in contrast to most other mammalian red cell systems where both high- and low-activity forms are known to occur.

The erythrocytes of most mammalian species studied to date (*cf.*, *e.g.*, Tashian *et al.*, 1972) contain two isozymes of carbonic anhydrase which differ markedly in specific activity. In addition, genetic studies (*cf.* Tashian *et al.*, 1971) and comparative sequence analyses of the two human isozymes (Henderson *et al.*, 1971) clearly indicate that the two enzymes are the products of two different autosomal genes. Additional isozymes, seen at low levels in many mammalian carbonic anhydrase systems, appear not to be products of additional genetic loci, but rather represent secondarily altered forms of the major isozymes (*cf.* Funakoshi and

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