Catalytic Versatility of Erythrocyte Carbonic Anhydrase. Kinetic Studies of the Enzyme-Catalyzed Hydrolysis of Methyl Pyridyl Carbonates*

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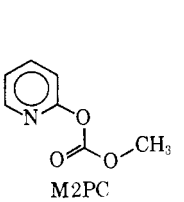
ABSTRACT: The present investigation demonstrates that bovine carbonic anhydrase is an effective catalyst for the hydrolysis of diesters of carbonic acid. The enzymatic hydrolyses of methyl 2-, 3-, and 4-pyridyl carbonate were studied between pH 6 and 10.5. Micromolar bovine carbonic anhydrase concentrations increase the hydrolysis rate of the 2- and 3-esters more than tenfold over their respective buffer rate but do not appear to change the rate of hydrolysis of the 4 isomer beyond the experimental error. The pH–rate profiles of the bovine carbonic anhydrase catalyzed hydrolyses of the methyl 2- and 3-pyridyl carbonates are similar and show two inflections, one at pH 7.03 and another beyond pH 10. An equation of the form $k_{enz} = k_{EH}[EH]/[E]_0 + k_E^-[E^-]/[E]_0$ is fit to the observed pH–rate data. The specific inhibitor acetazolamide completely represses the enzymatic activity below pH 9 but only partially inhibits above pH 10. Acetazolamide is found to be a noncompetitive inhibitor in regard to methyl 3-pyridyl carbonate hydrolysis but it competes with methyl 2-pyridyl carbonate for a binding site. On the other hand, iodide ion acts as a noncompetitive inhibitor in regard to the hydrolysis of the 2-ester. The K_m values for methyl 2- and 3-pyridyl carbonate are large, 0.13 and 0.32 M, respectively. Turnover numbers indicate that the 3-ester is more effectively hydrolyzed by the enzyme than is the 2-ester, although free zinc ions catalyze only the hydrolysis of the latter. The data are analyzed in terms of binding sites which do not call for direct coordination to the enzyme-bound zinc for either of the carbonate esters; the lower turnover number of the 2-ester is interpreted as a reflection of a less productive binding site.

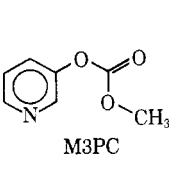
In addition to its known physiological role, the reversible hydration of carbon dioxide, carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) from erythrocytes has been found to catalyze the reversible hydration of aliphatic aldehydes (Pocker and Meany, 1965; Pocker and Dickerson, 1968), pyridinecarboxaldehydes (Pocker and Meany, 1967), and pyruvic acid (Pocker and Meany, 1970). Mammalian carbonic anhydrases also act as esterases for the hydrolysis of a number of nitrophenyl esters of carboxylic acids (Tashian *et al.*, 1964; Pocker and Stone, 1965, 1967; Verpoorte *et al.*, 1967; Thorslund and Lindskog, 1967; Pocker and Storm, 1968). The esterase function appears to be identical with the hydrase function as both show similar pH–rate profiles around physiological pH and both are subject to the powerful and highly specific inhibitory action of certain sulfonamides.

Detecting carbonic anhydrase activity through its esterase function is direct and inherently more accurate than studies with the enzyme's natural substrates and can be employed over a wide pH range. Studies in these laboratories carried out at pH >9 uncovered a second rise in the esterase activity of bovine carbonic anhydrase (BCA)[1] with a pK_a = 10–11 (Pocker and Storm, 1968). The value of the second pK_a suggests that this rise in activity might be the consequence of a zinc–aquo complex being titrated to a more active zinc–hydroxo species, especially if the zinc ion was also coordinated to electron-rich amino acid nitrogen atoms. The nature of this high pH rise in enzyme activity has been extensively investigated in these laboratories (Pocker, 1969; Y. Pocker, J. T. Stone, and N. Watamori, unpublished results).

The high pH rise is here examined as part of a study of a new class of carbonic anhydrase substrates, the organic diesters of carbonic acid. Unsubstituted pyridine carbonates are capable of interacting with divalent metal ions, just as pyridinecarboxaldehydes have been found to (Pocker and Meany, 1968). This property makes them useful substrates for a broad pH study of the zinc–metalloenzyme carbonic anhydrase.

The zinc atom bound to carbonic anhydrase is necessary for the powerful binding of sulfonamides of the type $ArSO_2NH_2$ (Lindskog, 1963) and is also an important component in the binding of anionic inhibitors (Verpoorte *et al.*, 1967; Ward, 1970). The nature of these inhibitors makes them useful in the study of the character of pyridine carbonate binding. Because the aromatic sulfonamide acetazolamide powerfully, specifically, and completely inhibits carbonic anhydrase activity at neutral pH, we have further used it to study the high pH–activity of the enzyme.


M2PC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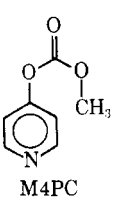
M4PC

We have in general examined organic carbonates as carbonic anhydrase substrates and have further demonstrated the catalytic versatility of this enzyme. We have found that the

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: BCA, bovine carbonic anhydrase; M2PC, methyl 2-pyridyl carbonate; M3PC, methyl 3-pyridyl carbonate, M4PC, methyl 4-pyridyl carbonate.

special properties of pyridine carbonates, M2PC, M3PC, and M4PC, have enabled us to look at what appears to be a directionally specific hydrogen-bonding site near the zinc atom associated with the native enzyme.

Experimental Section

Materials

The methyl carbonates were prepared from methyl chlorocarbonate (Matheson Coleman & Bell) and the corresponding pyridol or pyridolate ion under anhydrous reaction conditions. Traces of HCl, which interfere with the reaction by protonating the pyridol or pyridolate anion, were removed from methyl chlorocarbonate by shaking it with calcium carbonate, filtering, and distilling it immediately before use. The physical properties of the methyl pyridine carbonates are given in Table I.

Methyl 3-Pyridyl Carbonate (M3PC). Methyl chlorocarbonate (1.5 equiv) was slowly added to 1 equiv of 3-pyridol (Aldrich) dissolved in acetonitrile. Calcium carbonate, about 5 equiv, was added to neutralize the accumulating HCl. The crude product, which remains with the solvent, was isolated by stripping away the acetontrile from the filtered solution on a Büchi Rotavap thermostated at 35°. The resulting brown oil was vacuum distilled through a 5-cm microcolumn, bp 55–56° (0.1 mm). The clear liquid solidified at room temperature, mp 33°. Hydrolysis of the product in glycine buffers at pH 10 yielded 1.02 equiv of 3-hydroxypyridol determined spectrophotometrically at 298 nm (ϵ 4.06×10^3). *Anal.* Calcd $C_7H_7NO_3$: C, 54.8; H, 4.61; N, 9.16; O, 31.3. Found:[2] C, 54.8; H, 4.72; N, 9.24; O, 31.5.

Methyl 2-Pyridyl Carbonate (M2PC). This ester is prepared by reacting the anion of 2-pyridone with dry methyl chlorocarbonate. The sodium salt of 2-pyridone precipitates from a concentrated aqueous solution of 2-pyridone (Aldrich) with a 2 molar equiv of sodium hydroxide dissolved in the minimum amount of water. The precipitate was collected and dried. Methyl chlorocarbonate (1.5 equiv) was slowly added to 1 equiv of a 2-pyridone anion slurry in diethyl ether. The reaction mixture was rapidly stirred for 1 hr at 0°, filtered, and the excess methyl chlorocarbonate and diethyl ether was removed from the filtrate in a Büchi Rotavap at room temperature. The residue was vacuum distilled on a 1-cm molecular distillation apparatus, bp 30° (0.01 mm). This initially distilled ester preparation contained small quantities of the isomeric 1-carbomethoxy-2-pyridone which apparently has a boiling point very close to that of M2PC. Increased separation by distillation over larger columns necessitated higher pot temperatures and produced a decarboxylated, rearranged product, *N*-methyl-2-pyridone, which also boils at a temperature near the boiling point of M2PC and cannot be separated by distillation. Pure M2PC was obtained by extracting an ether solution with sodium chloride saturated water until an aliquot of the ether fraction indicated a minimum and constant absorbancy at 300 nm. The ether fraction was immediately dried to prevent excessive product hydrolysis by passing it through a 4×15 cm sodium sulfate column, cooling the solution to −100°, and filtering out any ice crystals onto cold diatomaceous earth in a dry box. The diethyl ether was immediately stripped away on the Rotavap at 20°. Molecular distillation of the residue gave a product with less than 0.2% impurities. The hydrolysis of 1 equiv of M2PC in glycine buffers at pH 10 gave 0.996 equiv of 2-pyridone, determined spectrophotometrically. The elemental analysis is in agreement with that calculated for $C_7H_7NO_3$. Since a major impurity is an isomer which has the same hydrolysis product and the same empirical formula, one additional criterion for purity is the absence of an absorbancy peak in the uv at 309 nm, the λ_{max} of 1-carbomethoxy-2-pyridone. *Anal.* Calcd for $C_7H_7NO_3$: C, 54.8; H, 4.61; N, 9.16; O, 31.3. Found: C, 55.0; H, 4.74; N, 9.23; O, 31.4.

TABLE I: Physical Properties of the Methyl Pyridine Carbonates.

Property	M2PC	M3PC	M4PC
Melting point (°C)	<−100[a]	33	80 (dec)
Boiling point (°C)	63–64[b]	55–56[c]	
Ultraviolet spectra			
λ_{max} (nm)	257[d]	285, 223[d]	283, 275, 268[d]
Log ϵ	3.78	3.91, 3.43	4.28, 4.41, 4.33
Infrared spectra			
λ (μ)	5.64	5.64	5.64[e]

[a] Amorphous liquid below −20°. [b] 0.2 mm. [c] 0.1 mm. [d] In acetonitrile. [e] Mineral oil suspension.

Methyl 4-Pyridyl Carbonate (M4PC). The 4-ester was obtained in a manner analogous to the preparation of M2PC. Methyl chlorocarbonate was slowly added to a cold, dry diethyl ether slurry of sodium pyridolate. The reaction mixture was stirred for 2 hr at 0° under dry nitrogen and filtered. The product was isolated from the filtrate by stripping away 90% of the diethyl ether solution on a Rotavap thermostated at 25°. The crude product was recrystallized by dissolving it in the minimum amount of dry acetonitrile at room temperature and cooling the solution to −45° with an acetonitrile–Dry Ice bath. The crystals were collected in the dry box and thoroughly dried in a refrigerated vacuum desiccator. The ir spectrum shows a strong carbonyl band at 5.64 μ which is characteristic of the pyridine carbonates. One equivalent of the product produces 1.02 equiv of 4-pyridone as determined spectrophotometrically at pH 10 in glycine buffers.

Extinction coefficients for 2- and 4-pyridone and 3-pyridol were determined for the Gilford 2000 spectrophotometer using highly purified compounds. 2-Pyridone was twice recrystallized from benzene–Norit and then sublimed. 3-Pyridol was twice recrystallized from acetonitrile–Norit before it was sublimed. The extinction coefficients for 4-pyridone were determined with material thrice recrystallized from ethanol–Norit. The organic cosolvent acetonitrile (Baker analyzed) was dried by azeotropic distillation with methylene chloride and fractionated through a 14-in. bubble-cap column. The buffer components potassium dihydrogen phosphate (Mallinckrodt), disodium hydrogen phosphate (Mallinckrodt), and sodium acetate (Baker) were analytical grade and used without further purification. Tris (Aldrich) was recrystallized from 95% ethanol and sublimed. Imidazole (Aldrich) was recrystallized twice from benzene. *N,N*-Dimethylglycine was prepared from *N,N*-dimethylglycine·HCl (Nutritional Biochemicals Corp.) by titrating a concentrated aqueous solution of the hydrochloride with sodium hydroxide to its first end point, near pH 6. The solution was evaporated to dryness and the neutral *N,N*-dimethylglycine sublimed from the mixture. Potassium hydroxide, sodium hydroxide, cobalt

[2] Elemental analysis of M2PC and M3PC were carried out at the Alfred Bernhardt Microanalytical Laboratory in West Germany.

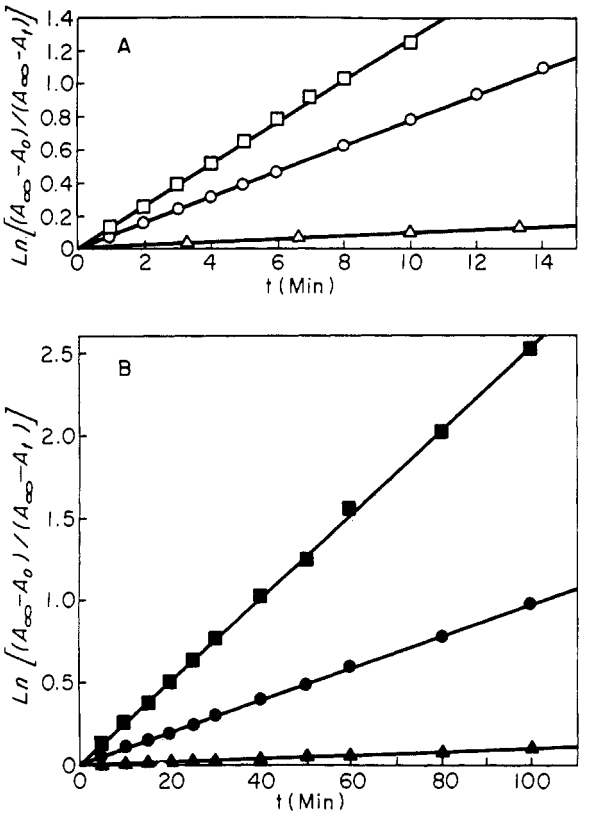


FIGURE 1: Metal ion and bovine carbonic anhydrase catalyzed hydrolysis of pyridine carbonates at 25°, I = 0.10. (A) M2PC in pH 8.91, Tris buffers. (△) Buffer only, $k = 0.17 \times 10^{-3}$ sec^{-1}. (○) [Zn^{2+}] = 3.5×10^{-6} M, $k = 1.30 \times 10^{-3}$ sec^{-1}. (□) [Co^{2+}] = 3.63×10^{-5} M, $k = 2.16 \times 10^{-3}$ sec^{-1}. (B) M3PC in pH 7.70, phosphate buffers. (▲) Buffer only. (●) [BCA] = 2.82×10^{-6} M. (■) [BCA] = 7.05×10^{-6} M.

(II) nitrate, zinc acetate, and sodium chloride were Baker analytical grade reagents. Acetazolamide (Lederle Laboratories) was titrated with 0.1 N sodium hydroxide to its second end point and found to analytically pure (100 ± 1%); consequently it was used without further purification. Ethoxzolamide, generously donated by the Upjohn Co., and sodium iodide (Merck) were also used without further purification. Carbonic anhydrase from bovine erythrocytes was prepared and purified as described earlier (Pocker and Stone, 1967; Pocker *et al.*, 1971). To eliminate relative error, all enzyme kinetics experiments involved the use of a single purified batch. For this batch the enzyme concentration in solution, as calculated from its uv absorbance at 280 nm (ε 54,000; mol wt 30,000) is equal to the zinc concentration of this solution as determined by flame photometry (λ 214 nm).

Apparatus. All pH measurements were recorded at 25° with an Orion Model 801 digital pH meter fitted with a Corning glass electrode (476022) and a Beckman reference electrode. The relative accuracy of this apparatus is reported to be 0.001 pH unit. Hydrolysis rates were monitored spectrophotometrically on a Gilford 2000 multiple-sample recording spectrophotometer. The cell compartment, a water bath of our own design, was thermostated to 25.0 ± 0.05° by means of a Sargent Model SV (S-82060) thermonitor unit. Ultraviolet and visible absorption spectra traces were obtained on a Cary 14 recording spectrophotometer. Infrared absorption traces were recorded on a Perkin-Elmer 137 sodium chloride spectrophotometer.

TABLE II: Hydrolysis of Pyridine Carbonates. Wavelengths and Extinction Coefficients for Spectrophotometric Rate Determinations.

Ester	λ (nm)	Log ε	Conditions
M2PC	256	3.38	Above pH 10, M2PC absorbancy decrease
M2PC	294	3.74[a]	Below pH 10, 2-pyridone absorbancy increase
M2PC	302.5	3.732	pH 6.9 (method B), 2-pyridone absorbancy increase
M3PC	298	3.61[a]	Above pH 8.0, 3-pyridolate absorbancy increase
M3PC	314	3.48[b]	Below pH 8.0, 3-pyridol absorbancy increase
M4PC	275	4.41[c]	pH 6 to 10, M4PC absorbancy decrease

[a] ε determined at pH 10.2 in glycine buffers. [b] ε determined at pH 8.2 in Tris buffers. [c] ε determined at pH 6.0 in citrate buffers.

Kinetics and Technique. Buffers were uniformly prepared at constant ionic strength, I = 0.10, with *no added* sodium chloride. For examples of this method, see Perrin (1963). Dilute sodium or potassium hydroxide solutions for use in kinetics were prepared using a Radiometer automatic titrator and stored in a CO_2-free box. The ionic strength of the hydroxide solutions was kept constant at 0.1 by addition of solid sodium or potassium chloride. These solutions were transferred to spectrophotometer cuvet inside the CO_2-free box.

Two procedures for kinetics runs were used. In the first and most commonly employed method, the reaction was initiated by injecting 10 μl of a substrate stock solution (in acetonitrile) into 3.0 ml of a buffered solution in a 1-cm cuvet. The alternate method, used only with very high substrate concentrations, calls for weighing out the carbonate ester into the cuvet, and starting the reaction by injecting 3.0 ml of buffer solution into a 1-cm cuvet or 1.25 ml into a 5-mm cuvet. The acetonitrile concentration was kept the same in both methods at 0.33% by volume. The hydrolyses of the three pyridine carbonate esters were followed by spectrophotometrically monitoring either the disappearance of the carbonate ester or the appearance of the respective pyridol. The spectrophotometric parameters for the rate studies are summarized in Table II.

The spectrophotometric rate data were analyzed by two methods. (A) At low substrate concentrations, [S] ≪ K_m, the first-order rate coefficient, k_{obsd}, was obtained from plots of ln $[(A_\infty - A_0)/(A_\infty - A_t)]$ *vs.* time. Such plots were linear past two half-lives (Figure 1 is typical) and the coefficients of ester hydrolysis, k_{obsd}, were reproducible to ±3%. The BCA-catalyzed hydrolysis of M2PC and M3PC is first order in both enzyme and substrate as plots of the first-order hydrolysis rate constants, k_{obsd}, against their respective enzyme concentrations are linear over the range 5×10^{-7} to 5×10^{-5} M. The slope obtained from such a plot is the second-order catalytic rate constant, k_{enz}, and the intercept at [E] = 0 is the buffer rate (see Figure 2). One of the attributes of the bovine carbonic anhydrase catalyzed hydrolysis of M2PC and M3PC

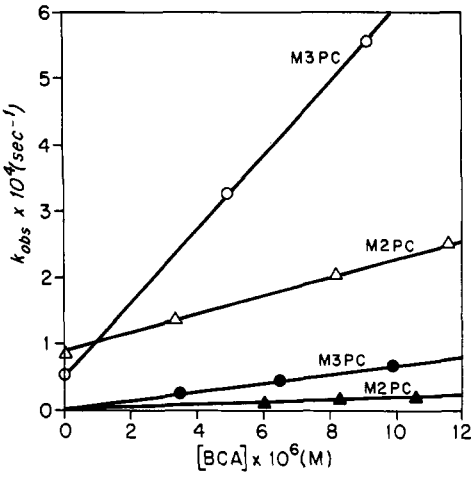


FIGURE 2: Enzymatic hydrolyses of pyridine carbonates. Plot of k_{obsd} *vs.* [BCA] at 25°, I = 0.10. (○) M3PC in Tris, pH 8.54. (●) M3PC in phosphate pH 6.3. (△) M2PC in Tris (pH 8.54). (▲) M2PC in phosphate (pH 8.54).

is that at micromolar levels of enzyme, the enzymatic catalysis is significantly larger than that associated with the various buffers. The reverse, however, is true in regard to M4PC. (B) For ester concentrations greater than 10^{-2} M straight-line plots of the optical density of the respective appearing pyridol or pyridone *vs.* time, starting at $t = 0$, produced accurate determinations of initial velocities. The extinction coefficient for the appearing species, separately determined for the specific reaction conditions, converts optical density velocities into molar velocities, V_{obsd}, which corrected for the buffer component, V_{buffer}, leads to the enzymatic component, V_{enz} (eq 1).

$$V_{obsd} = V_{enz} + V_{buffer} \equiv dx/dt = (dA/dt)/\epsilon_x; \quad A \equiv \text{optical density} \quad (1)$$

$K_{m,app}$ and V_m values were calculated from Lineweaver–Burk plots, the ester concentration varying between 1.4×10^{-2} and 1.3×10^{-1} M. Since the highest attainable $[S]_0$ values were not far below the calculated $K_{m,app}$ values, the determination of the kinetic constants involved only a short extrapolation. The exact concentrations of the esters were known from weighing them directly into the cuvet. The high concentrations of M2PC and M3PC needed to saturate the enzyme made it imperative that the ester preparations be very pure. As mentioned above, 1-carboxymethyl-2-pyridone may be present in less than 0.2% in M2PC. Because this carbamate ester is also powerfully hydrolyzed by carbonic anhydrase (Y. Pocker and L. J. Guilbert, unpublished observations), and because it absorbs near 300 nm, we resorted both to the laborious purification mentioned above and to monitoring the appearance of 2-pyridone at 302.5 nm, the isosbestic point of the hydrolysis of the carbamate into 2-pyridone. The M3PC preparations were quite pure, having an initial optical density of less than 0.05 at 314 nm when the calculated optical density at the completion of hydrolysis was near 200.

Certain aromatic sulfonamides, such as acetazolamide, powerfully inhibit the esterase activity of carbonic anhydrase but have little or no effect on other esterases. This distinguishing features affords a suitable method for detecting carbonic anhydrase activity. The character of the inhibition was determined from inverse rate plots (Laidler, 1958). A similar procedure was followed in determining the character and the constant, K_i, for iodide ion inhibition. At physiological pH, aromatic sulfonamides show such a high degree of affinity for mammalian carbonic anhydrase that there is relatively little unbound sulfonamide left. This condition, which has been labeled "a zone C mutual depletion inhibition," produces only an apparent value of the inhibition constant, but allows the active enzyme concentration to be accurately determined (Webb, 1963a). The value of the inhibition constant was obtained from the relationship $K_i = [(1 - i)([I]_0 - i[E]_0)]/i$, where i = (fractional inhibition) = 1 − (fractional activity) in the region where the fractional inhibition was no longer directly proportional to the sulfonamide concentration.

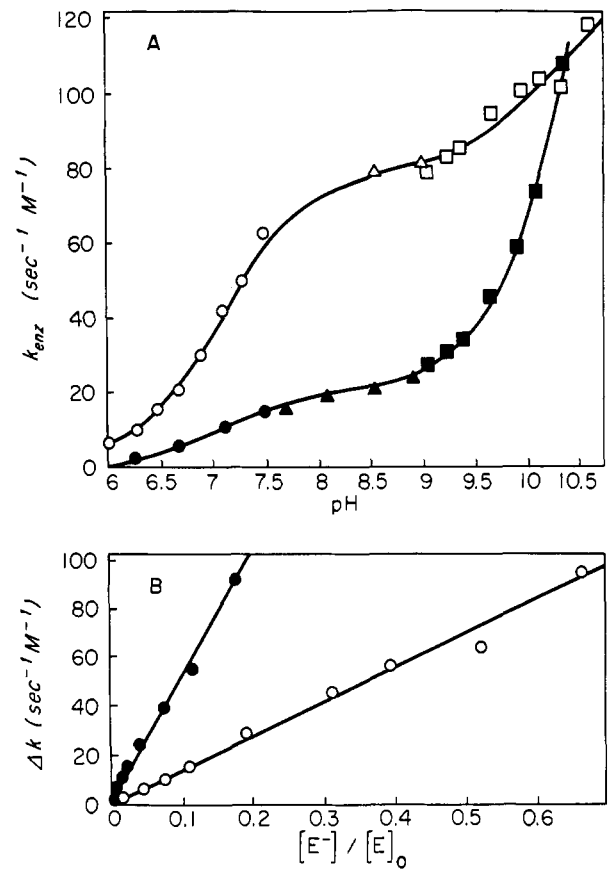


FIGURE 3: Bovine carbonic anhydrase catalyzed hydrolysis of pyridine carbonates as a function of pH at 25.0° and I = 0.10. (A) pH–rate profile. (○) M3PC, phosphate buffers. (△) M3PC, Tris buffers. (□) M3PC, dimethylglycine buffers. (●) M2PC, phosphate buffers. (▲) M2PC, Tris buffers. (■) M2PC, dimethylglycine buffers. Parameters to eq 5 for the drawn curves are

Substrate	k_{EH}	pK_{EH}	k_{E^-}	pK_{E^-}
M2PC	21.4	7.03	503	10.9
M3PC	79.6	7.03	137	10.2

(B) High pH-activity rise as a function of $[E^-]/[E]_0$ from eq 5. (●) M2PC, pK_{E^-} = 10.9. (○) M3PC, pK_{E^-} = 10.2.

Results

The individual pH–rate profiles for enzymatic activity (Figure 3) were determined at a low substrate concentration, ([S] $\ll K_m$), [M2PC] = 2.5×10^{-4} M, [M3PC] = 3.2×10^{-4} M, with the ionic strength and temperature held constant throughout at 0.10 and 25.0°, respectively. Between pH 6 and 9 the

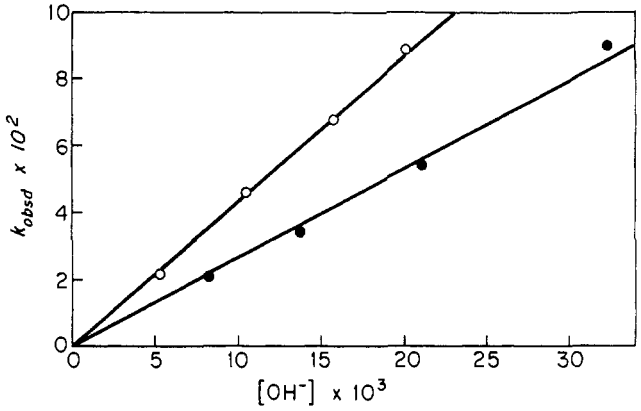


FIGURE 4: Hydroxide ion catalyzed hydrolysis of methyl 3-pyridyl carbonate and methyl 2-pyridyl carbonate at 25.0° and $I = 0.10$. (○) Methyl 3-pyridyl carbonate, NaOH–NaCl. (●) Methyl 2-pyridyl carbonate, KOH–KCl.

buffer rate for M2PC and M3PC hydrolyses remains a small fraction of the enzymatic rate. Buffer rates at pH >9 are predominantly due to hydroxide ion catalysis, a process slow enough with M2PC and M3PC to be measured independently between pH 11.5 and 12.5 in hydroxide solutions (Figure 4). Enzyme concentrations were maintained high enough so that even around pH 10 the buffer rates for M2PC and M3PC hydrolyses never exceeded 40% of the observed rate. On the other hand, even with 10^{-5} M bovine carbonic anhydrase there is no significant enzyme-catalyzed hydrolysis of M4PC around neutral pH. The values of k_{enz} for M4PC hydrolysis may in fact be the same or somewhat larger than the corresponding values associated with M2PC hydrolysis at pH >8, but the 4 isomer has a much larger buffer rate which masks the enzymatic rate. The condition $k_{buffer} \gg k_{enz}[E]$ for M4PC hydrolysis can be seen at pH 8.1 in Tris buffers (Table III) where the enzymatic portion of the M4PC hydrolysis rate with $[E] = 8 \times 10^{-6}$ M amounts to only 5%. As the enzymatic process is determined by monitoring the rate of appearance of the respective pyridol, the fate of the remaining two hydrolysis products is of interest. In a study of the symmetrically substituted carbonate ester, bis(4-nitrophenyl) carbonate, it was found that the enzymatic hydrolysis proceeds as a smooth first-order decomposition producing two equivalents of 4-nitrophenol (Y. Pocker and L. J. Guilbert, unpublished observations). The rate of hydrolysis of a carbonate alkyl monester around neutral pH is much faster than the respective rate for a homologous alkyl aryl carbonate diester (Faurholt, 1927; Dittert and Higuchi, 1963). At higher pH values, $k_{enz}[E]$, the first-order rate constant for monoester formation approaches the estimated first-order rate constant for the decomposition of the monoester intermediate (Faurholt, 1927). However, enzymatic rates were first order over the entire pH range, thus monocarbonate intermediate buildup apparently does not affect carbonic anhydrase catalysis of the first step. It is noteworthy, however, that the enzymatic reaction does not proceed in two distinct steps as does the nucleophilic hydrolysis of bis(4-nitrophenyl) carbonate in imidazole buffers (Y. Pocker and L. J. Guilbert, unpublished data).

The simplest enzymatic process for carbonate diester hydrolysis is a three-step mechanism (eq 2). The last step may

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} P_2 + E \qquad (2)$$
$$\searrow P_1$$

TABLE III: Hydrolysis of Pyridine Carbonates. Enzymatic Component of Hydrolysis Rate Relative to Buffer Component in Tris Buffers at pH 8.1.

Ester	[BCA] × 10^6 (M)	$k_b \times 10^5$ (sec^{-1})	$(k_{enz}[BCA]/k_b) \times 100$ (%)
M2PC		6.1	
M2PC	8.0	21.8	72.1
M3PC		3.5	
M3PC	8.0	62.2	94.5
M4PC		109	
M4PC	8.0	114	4.6

merely involve the release of a monocarbonate ester intermediate, or it may involve the hydrolysis of this intermediate while it is still attached to the enzyme. The presence of a carbonate intermediate of a α-chymotrypsin has been suggested (Shah and Connors, 1968). In contrast to results obtained for the chymotrypsin-catalyzed hydrolysis of carbonate esters (Hartley and Kilby, 1952), all plots of pyridol appearance *vs.* time for the esterase activity of carbonic anhydrase pass through the origin. Buildup of an ES′ complex as manifested by a decrease in v_{enz}, thereby producing an "initial burst," can most conveniently be measured under the conditions $[S]_0 > [E]_0$, $[S]_0 > K_{m,app}$ providing $k_2 \gg k_3$ (Sturtevant, 1960). Under these conditions a measure of the burst is P_b in eq 3.

$$P_b/[E]_0 = (1/(1 + k_3/k_2))^2/(1 + K_{m,app}/[S]_0)^2 \qquad (3)$$

Here $K_m = (k_{-1} + k_2)/k_1 = ((k_2 + k_3)/k_3)K_{m,app}$, where $K_{m,app}$ is the experimentally determined Michaelis constant with $[S]_0$ and $[E]_0$ representing the initial concentrations of substrate and enzyme, respectively. The first condition can be met, but the second cannot in the case of pyridine carbonates. The values of $K_{m,app}$ for pyridine carbonate hydrolysis by BCA are near or slightly larger than the solubility limits of these esters in water at 25°, although no burst could be detected at $[S]_{max} \cong K_m$.

$$v_{enz} = dx/dt = (k_2/K_m)([E]_0 - x)([S]_0 - x) \qquad (4)$$

Here $x = P_1$ = concentration of pyridol produced and v_{enz} is the enzymatic rate of pyridinol production. For the chymotrypsin-catalyzed hydrolysis of 4-nitrophenyl acetate, a plot of $v_{enz}/([S]_0 - x)$ *vs.* x gives a straight line whose intercept divided by the slope is $[E]_0$ (Bender *et al.*, 1966). Equation 4 is valid providing $[S]_0 \cong [E]_0 \gg K_{m,app}$. In accord with the above observations concerning "burst" detection, this condition could not be met as first-order and not second-order kinetics with pyridine carbonates were observed. Thus within the limitations of both these experiments, *i.e.*, $[S]_{max} \simeq K_{m,app}$, no conclusion can be reached as to the relative magnitude of k_3 *vs.* k_2.

Carbonic anhydrase activity in regard to carbonate esters as a function of pH follows eq 5 which derives from the hydrogen ion equilibrium represented by eq 6. The term $[E]_0$ repre-

$$k_{enz} = k_{EH}[EH]/[E]_0 + k_E\text{-}[E]/[E]_0$$

$$[EH]/[E]_0 = (K_{EH}/a_{H^+})(a_{H^+} + K_{EH}(1 + K_E^-/a_{H^+})) \qquad (5)$$

$$[E^-]/[E]_0 = ([EH]/[E]_0)K_E^-/a_{H^+}$$

TABLE IV: pH Dependency of the Bovine Carbonic Anhydrase Catalyzed Hydrolysis of Pyridine Carbonates.[a]

M2PC pH	Buffer[b]	k_{enz} (M^{-1} sec^{-1})	M3PC pH	Buffer[b]	k_{enz} (M^{-1} sec^{-1})
6.30	P	2.7	6.06	P	6.3
6.68	P	5.9	6.30	P	9.9
7.10	P	11.0	6.48	P	15.6
7.50	P	15.0	6.68	P	20.3
7.70	T	15.1	6.88	P	30.0
8.13	T	18.6	7.10	P	40.9
8.54	T	20.4[c]	7.29	P	49.3
8.92	T	23.3	7.50	P	62.1
9.05	DMG	28.3	8.54	T	79.0[c]
9.23	DMG	31.8	9.00	T	81.0
9.38	DMG	35.4	9.04	DMG	78.5
9.65	DMG	45.8	9.23	DMG	83.1
9.90	DMG	58.7	9.38	DMG	85.4
10.10	DMG	74.1	9.65	DMG	93.3
10.32	DMG	108[c]	9.94	DMG	100.0
			10.10	DMG	103.4
			10.32	DMG	101.2[c]
			10.58	DMG	118.5

[a] At 25.0°; ionic strength, I = 0.10. [b] Abbreviations are: P = phosphate, T = Tris, and DMG = dimethylglycine. [c] k_{enz} values determined with and without 10^{-5} M EDTA were identical.

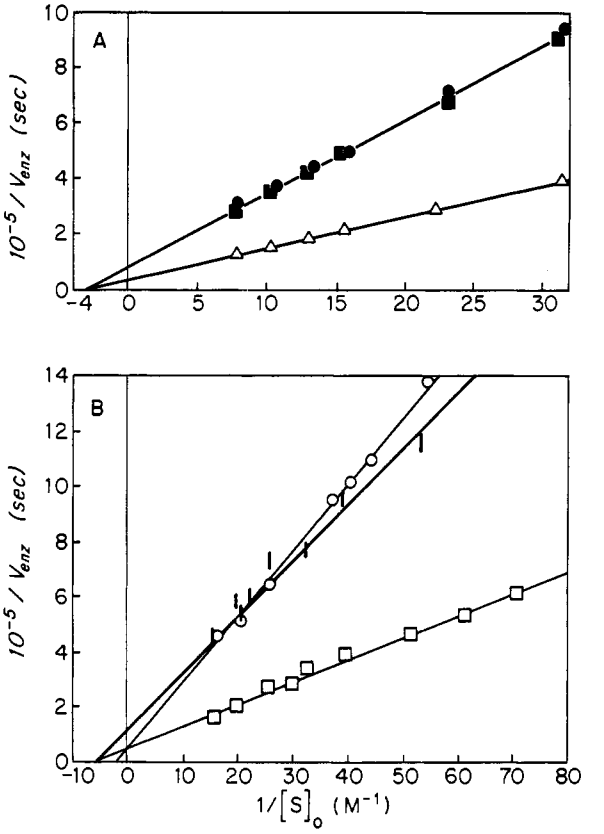


FIGURE 5: Michaelis–Menten kinetics for the inhibited and uninhibited enzymatic hydrolyses of pyridine carbonates. All runs were made in phosphate buffers, I = 0.10, pH 6.90 at 25°. (A) Bovine carbonic anhydrase catalyzed hydrolysis of M3PC, [BCA] = 2.82×10^{-6} M. (△) Uninhibited rates. (■) Inhibited by 1.5×10^{-6} M ethoxzolamide. (●) Inhibited by 1.5×10^{-6} M acetazolamide. (B) Bovine carbonic anhydrase catalyzed hydrolysis of M2PC, [BCA] = 2.65×10^{-6} M. (□) Uninhibited rates. (○) Inhibited by 1.5×10^{-6} M acetazolamide. (◧) Inhibited by 9.2×10^{-3} M I^-.

sents total concentration of enzyme. The protonated species EH_2^+ in eq 6 does not lead to hydrolysis. No enzymatic hydrolysis of M3PC beyond that expected for the equilibrium concentrations of EH can be detected in acetate buffers at pH 5.2. A least-squares fit of eq 5 to the enzymatic rate data

$$EH_2^+ \overset{K_{EH}}{\rightleftharpoons} EH \overset{K_{E^-}}{\rightleftharpoons} E^- \qquad (6)$$

$$K_{EH} = a_H{}^+[EH]/[EH_2^+],\ K_{E^-} = a_H{}^+[E^-]/[EH]$$

(Table IV) was performed, iterating upon the values of k_{EH}, k_{E^-}, K_{EH}, and K_{E^-} to obtain the optimum fit. The theoretical curves giving the best fit in each instance are drawn in Figure 3 along with the experimental points taken from Table IV. Values of the parameters k_{EH}, k_{E^-}, pK_{EH}, and pK_{E^-} giving the optimum fit to the observed data are listed in the legend to Figure 3.

In order to investigate the enzymatic reaction over a wide pH range, it is necessary to use more than one buffer system, and so differential effects arising from the binding of components of the buffer to the enzyme, rather than simply effects of the proton, may be encountered. Differential effects were noted only in Tris buffers and these were resolved by allowing for chloride ion inhibition (Pocker and Stone, 1967).

Since no "initial burst" could be detected, the dependence of rate on substrate concentration was formally analyzed in terms of the limiting Michaelis–Menten scheme (eq 7). The rate constants determined experimentally may be much more complex than those indicated by this simplified scheme. Line-

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E \qquad (7)$$

weaver–Burk plots are used to graphically determine values of $K_{m,app}$ and $V_m = k_2[E]_0$ (Laidler, 1958). Enzymatic rates, obtained under the optimum conditions,[3] were used to determine apparent values of K_m and k_2 and to delineate the effect of acetazolamide, ethoxzolamide, and iodide ion on the Michaelis parameters (Figure 5). The Michaelis constants, K_m, for the two pyridine carbonates (Table V) are much larger than those for pyridinecarboxaldehydes (Pocker and Meany, 1967) or for 3-acetoxypyridine (Y. Pocker and N. Watamori, submitted for publication as Part IX in this series). The turnover numbers, k_2, for pyridine carbonate and 4-nitrophenyl acetate (Pocker and Stone, 1967) hydrolyses are similar; both are larger than the observed turnover number for the BCA-catalyzed hydrolysis of 3-acetoxypyridine. For a number of substrates, the ratio $k_2/k_{OH^-}[OH^-]$ provides a means of comparing their enzymatic efficacy as normalized by their respective susceptibility to hydroxide ion attack. Apposition of the ratios listed in Table V shows that 2- and 3-pyridine carbonates were indeed efficiently hydrolyzed by carbonic anhydrase.

The aromatic sulfonamides, acetazolamide and ethoxzolamide, specifically and powerfully inhibit carbonic anhydrase

[3] Optimum conditions are pH 6.9 in phosphate buffers. In spite of the very high ester concentrations necessary to saturate the enzyme, by following initial rates we were able to keep the pH constant and the bicarbonate ion concentration low.

TABLE V: Chemical and Enzymatic Reactions of Carbonic Anhydrase Substrates. Comparison of Enzymic Catalysis with Hydroxide Ion Catalysis at pH 6.9.

Substrate[a]	k_{enz} (M^{-1} sec^{-1})	K_m (M)	k_2 (sec^{-1})	k_{OH^-} (M^{-1} sec^{-1})	k_{enz}/k_{OH^-}[g]	$k_2/k_{OH^-}[OH^-]$[g] $\times 10^6$
M2PC	13.3	0.13	1.7	2.59	5.27	6.4
M3PC	42.9	0.32	14	4.24	10.2	32
2PA[b]	4,530	0.015	68	4.33×10^4	0.105	0.022
3PA[b]	4,140	0.007	29			
4PA[b]	15,000	0.011	165	12.2×10^4	0.123	0.020
2NPC[c]	837	0.0029	2.4	16.6	50.4	1.4
PNPA[d]	71.6	0.0029	0.195	14.1	5.08	0.132
CO_2	3.52×10^7	0.012[e]	4.22×10^5	8.5×10^3[f]	4.15×10^3	473

[a] Abbreviations are: 2PA = 2-pyridinecarboxaldehyde, 2NPC = methyl 2-nitro-3-pyridyl carbonate, and PNPA = 4-nitrophenyl acetate. [b] Pocker and Meany (1967) (0°). [c] Y. Pocker and L. J. Guilbert, unpublished data (25°). [d] Pocker and Stone (1967) (10% CH_3CN, 25°). [e] Kernohan (1965) (BCA, 25°). [f] Sirs (1958). [g] Ratios are unitless.

activity. As regards ester hydrolysis their inhibition has been consistently found to be noncompetitive in nature (Pocker and Stone, 1967; Verpoorte *et al.*, 1967). The enzymatic hydrolysis of the pyridine carbonate ester, M3PC, is in this respect normal as the extrapolated lines representing the sulfonamide-inhibited and uninhibited rates intercept on the inverse concentration axis of a Lineweaver–Burk plot (Figure 5). The inverse rate data with [acetazolamide] = [ethoxzolamide] fall on the same line although ethoxzolamide is thought to be a more effective inhibitor in nanomolar enzyme concentrations (Wistrand, 1964). In micromolar enzyme solutions the respective inhibitor–enzyme dissociation constants are both too low to allow an accurate determination of the concentration of unbound inhibitor. This exemplifies a zone C, mutual exclusion inhibition, as denoted by Webb (1963a). Whereas in bovine carbonic anhydrase catalyzed hydrolysis of 4-nitrophenyl acetate, iodide ion and acetazolamide are found to bind at overlapping or nearby interacting sites (Pocker and Stone, 1968), the enzyme-catalyzed hydrolysis of M2PC shows that the iodide ion does not increase the apparent Michaelis constant, K_m, while acetazolamide does without affecting V_m. M2PC then is atypical in that acetazolamide competes with it for a binding site in the bovine carbonic anhydrase cavity.

Values for the inhibitor constant, K_i = [I][E]/[EI], cannot be obtained from the Lineweaver–Burk plots in Figure 5 unless the concentration of unbound inhibitor is accurately known. In all the sulfonamide runs this concentration is very low and its exact value is unknown. Thus only an apparent inhibitor constant can be calculated from these data. In the case of iodide ion inhibition of enzyme-catalyzed M2PC hydrolysis, a true K_i value can be evaluated because $[I]_0 \gg [E]_0$ and hence $[I]_{free}$ is very nearly identical with $[I]_0$ (Table VI). Apparent values of K_i for sulfonamide inhibition are found by fitting the equation $K_i = (1 - i)([I]_0 - i[E]_0)/i$ to the appropriate data. Theoretical curves for the calculated K_i values are drawn through the observed data points in Figures 6A (M2PC, pH 8.54) and 7A (M3PC, pH 9). These values of K_i are interpreted as upper limits to the actual K_i. The most accurate value for the inhibition constant in regard to the potent sulfonamide inhibitors is obtained where $[I]_{free} \geq [E]_{free} \simeq [EI]$, that is, where $K_i \simeq [I]_0 \geq [E]_0$. In the present communication these conditions are met only at pH values greater than 10. The inhibition constants K_i at pH <10 are in agreement with those previously obtained for acetazolamide at analogous pH values (Pocker and Stone, 1968; Verpoorte *et al.*, 1967).

Inhibition of carbonic anhydrase activity at pH values greater than 9 becomes difficult to measure because of several converging problems. (a) The inhibitory potency of sulfonamides falls off markedly above pH 9. (b) The enzymatic fraction of the first-order hydrolysis rate constant, $(k_{obsd} - k_{buffer})/k_{obsd}$, starts to decrease at pH >9 because of the increasing contribution of hydroxide ion catalysis, $k_{OH^-}[OH^-]$, to the buffer rate constant k_{buffer}. However, carbonic anhydrase activity in the hydrolysis of pyridine carbonates also rises with pH allowing inhibition experiments to be carried out even around pH 10. Above pH 10 carbonic anhydrase activity in regard to M2PC and M3PC hydrolyses can only be partially inhibited by acetazolamide (Figures 6B and 7B).

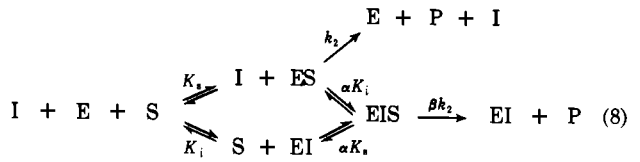
Although a number of multiparameter equations can be

TABLE VI: Inhibition of the Bovine Carbonic Anhydrase Catalyzed Hydrolyses of Pyridine Carbonates.

Substrate	Inhibitor	pH	K_i (M)	β
M2PC	Acetazolamide	8.54	7.5×10^{-8}	*a*
M2PC	Acetazolamide	10.0	1×10^{-5}	0.45[b]
M2PC	Iodide	6.90	1.66×10^{-2}	*c*
M3PC	Acetazolamide	9.0	8×10^{-7}	*d*
M3PC	Ethoxzolamide	9.0	5×10^{-7}	*d*
M3PC	Acetazolamide	10.58	4×10^{-5}	0.15[b]

[a] Complete and probably competitive inhibition. [b] Without further proof the inhibition above pH 9 may be either partial competitive or partial noncompetitive, as the inhibition curves in Figures 6 and 7 do not differentiate between the two behaviors. Partial inhibition is here expressed in terms of β from eq 9 keeping $\alpha = 1$. This limits eq 9 to an expression for partial noncompetitive inhibition (Webb, 1963b). The parameters to eq 9 for partial competitive inhibition are evaluated by fixing $\beta = 1$ and letting $\alpha = 1/\beta_{PNC}$, where β_{PNC} is the β value listed in the above table. [c] Complete noncompetitive inhibition. [d] Complete and probably noncompetitive inhibition.

used to account for partial inhibition, we have found it convenient (Pocker and Dickerson, 1968) to analyze the data in terms of eq 8, from Webb (1963b). Equation 9 is derived by



Webb from eq 8 and does not involve possible states of enzyme protonation but only comments on the partial nature of the inhibition as observed at any pH. Here $K_s = [E][S]/[ES] \cong$

$$i = \frac{[I]([S](1 - \beta) + K_s(\alpha - \beta))}{[I]([S] + \alpha K_s) + K_i\alpha([S] + K_s)} \quad (9)$$

$K_{m,app}$ and i = fractional inhibition. The parameters for the best fit of eq 9 to the inhibition data are summarized in Table VI. Theoretical curves based on the parameters are drawn through the appropriate set of data points in Figures 6B and 7B. The high values of K_i necessitate high concentrations of acetazolamide to observe inhibition. The effect of acetazolamide upon the nonenzymatic rate was determined and corrected for. The K_i values reported here are at least twofold higher than the value determined in these laboratories in regard to 4-nitrophenyl acetate hydrolysis at pH 10 (Pocker and Stone, 1968). These observations will be analyzed in more detail in the Discussion.

The effect which zinc ions have on the rate of hydrolysis of the pyridine carbonates was determined at pH 8.92 in Tris buffers (Figure 1). It was found that Zn^{2+} ions are more effective catalysts for the hydrolysis of M2PC than the enzyme, $k_{enz}/k_{Zn^{2+}} = 23.3/34.5 = 0.68$. On the other hand, the enzyme is much more effective than are Zn^{2+} ions at catalyzing the hydrolysis of M3PC, $k_{enz}/k_{Zn^{2+}} = 80/0.0965 = 830$. In contrast to Zn^{2+} catalysis of M2PC where the metal ion can bind with the pyridine nitrogen and at the same time remain close to the carbonyl oxygen, the enzyme appears to overcome these restrictions on substrate and configuration and is an efficient catalyst for the hydrolysis of both M2PC and M3PC. This tendency has been previously observed in a study of 2- and 4-pyridinecarboxaldehyde hydration (Pocker and Meany, 1967). It is seen in Figure 1 that Co^{2+} is a better catalyst than Zn^{2+} in regard to M2PC hydrolysis, a tendency also found for the metal ion catalysis of 2-pyridinecarboxaldehyde hydration.

It is clear from the above data that zinc alone is an effective catalyst for the hydrolysis of M2PC. Inasmuch as metalloenzymes often carry extra metal ions (Fabry *et al.*, 1970), any contamination by these ions has to be carefully controlled. Our preparation was free of any such contamination. Furthermore addition of 10^{-5} M EDTA effected no significant variation in k_{enz} in regard to the enzyme-catalyzed hydrolyses of *both* M2PC and M3PC (see Table IV).

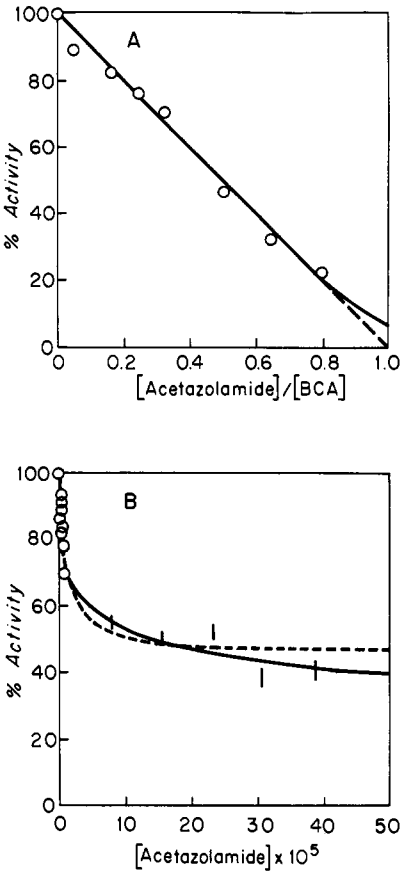


FIGURE 6: Acetazolamide inhibition of the enzymatic hydrolysis of methyl 2-pyridyl carbonate. (A) Tris buffers, pH 8.54, $I = 0.10$, [BCA] = 1.33×10^{-5} M. (B) Dimethylglycine buffers, pH 10, $I = 0.10$. (○) [BCA] = 8.46×10^{-6} M. (●) [BCA] = 9.87×10^{-6} M. ——, best fit to points. ---, theoretical curve from eq 9 with parameters given in Table VI.

Discussion

The zinc ion associated with the native enzyme is known to be an essential component for the powerful binding of sulfonamides (Lindskog, 1963; Coleman, 1967) and anions (Verpoorte *et al.*, 1967; Ward, 1970). The 5.5-Å X-ray resolution of the crystal structure of the human carbonic anhydrase C–acetoxymercurisulfanilamide complex places the sulfonamido group within the volume of the inner coordination sphere of the enzyme-bound zinc, and indicates that sulfonamides are bound through an $ArSO_2NH^-$–Zn^{2+} linkage (Fridborg *et al.*, 1967). It was further reported that an iodide ion bound within the active volume of the enzyme was situated 4.44 Å from the zinc atom. This is about 1.5 Å greater than the calculated maximal touching distance.[4] The anion is pointing from the zinc ion into the deeper part of the active-site crevice and can be considered to lie within the second coordination sphere of the zinc ion as originally visualized by Pocker and Dickerson (1968). The solvent separated ion-pair concept is also in accord with the observations of Pocker and Stone (1968) that the inhibitory power of a series of weak anionic inhibitors, including iodide ion, follows not the anions' ability to coordinate to zinc, but rather follows the Hoffmeister Lyotropic series, which is a measure of their capacity to break and enter water structure (Fridovich, 1963).

The positions taken by the iodide ion and acetazolamide relative to the central zinc atom may be reflected in their in-

[4] The extra 1.5 Å is probably occupied by a molecule of water. X-Ray studies of $CoI_2 \cdot 6H_2O$ crystals place six water molecules about the cobalt ion with the iodide ion placed outside 4.55 Å from the central cation (Shchukarev *et al.*, 1963). Combination of crystal radii gives the touching distance of cobalt to iodide as 2.9 Å, which subtracted from 4.55 Å, the observed cobalt to iodide distance, gives 1.65 Å as the crystal diameter of H_2O when sandwiched between a divalent metal ion and the iodide ion.

hibitory behavior in regard to the BCA-catalyzed hydrolysis of M2PC (Figure 5). The data would appear to allow a qualitative decision to be made as to the exact mode of inhibition by the iodide ion. In fact, least-squares treatments of the data place the x-axis intercept either as shown in Figure 5 or slightly to the left of it. Although a more exact consideration concerning the binding site of M2PC awaits further studies with anionic inhibitors, this substrate appears to bind close enough to the zinc atom to reflect the apparent competitive nature of the sulfonamide inhibition.[5]

Whatever its exact binding site, M2PC binds differently from M3PC. Acetazolamide inhibits the bovine carbonic anhydrase catalyzed hydrolysis of M3PC in a noncompetitive fashion; one which is normal in regard to carboxylic acid esters (Pocker and Stone, 1967). The inhibition results discussed above suggest that neither carbonate ester is coordinated to the zinc ion. In fact basicity alone does not dictate the ability of these substrates to bind to carbonic anhydrase, Table VII, and it appears then that the free energy of binding pyridine substrates to BCA derives predominantly from hydrophobic interactions just as in the case of 4-nitrophenylcarboxylic acid esters (Pocker and Storm, 1968) and aliphatic aldehydes (Pocker and Dickerson, 1968). In the case of pyridine carbonates, specific binding sites within the crevice may be determined by the overall environment of the site and not just by its hydrophobic forces. The physical properties of M2PC and M3PC are not so much different that they should cause the esters to bind at different sites. The two esters have identical carbonate side chains (see carbonyl absorbancies in the infrared region, Table I), and similar pyridine rings. Although the basicity of the pyridine nitrogen differs (Table VII), M3PC having the more basic nitrogen, this particular physicochemical property does not increase its binding as compared to M2PC and is thus unlikely to influence the choice of binding sites. The relative water solubility of these esters can be taken as a measure, an inverse one, of the extent to which these isomeric substrates will bind to a uniform hydrophobic environment (Hansch *et al.*, 1966). Their observed maximum solubilities in water at 25° are close: $[M2PC]_{max} = 8 \times 10^{-2}$ M, $[M3PC]_{max} = 15 \times 10^{-2}$ M. It is not likely that this reflects enough lipophilic difference to cause the two esters to seek different sites. More likely, the esters interact with what must be considered a nonuniform hydrophobic environment which is sensitive to the substitution position on their pyridine ring. The nature of their structural variant, juxtaposition between pyridine nitrogen and the carbonate side chain, suggests that this additional interaction with the protein which places the esters in different sites may also involve hydrogen bonding. M2PC, because of its structure, can form two hydrogen bonds even when both come from the same general direction. This structural characteristic may allow M2PC, after it has been transferred into the cavity by hydrophobic forces, to hydrogen bond weakly to the protein-bound water structure in the vicinity of the zinc ion. M3PC, since it does not bind in a position perturbed by a simultaneously bound sulfonamide molecule, may not be oriented by hydrogen bonding to the degree which M2PC appears to be. At any rate, M3PC binding is affected in the same way by acetazolamide as is the binding of molecules such at 4-nitrophenyl acetate which have fewer sites basic enough to be as efficiently stabilized by hydrogen bonding. The complex M2PC–BCA is not as effectively turned over into products as the corresponding M3PC–BCA complex. One measure of effectiveness is determined by comparing the first-order rate coefficient for the decay of the enzyme–substrate complex, k_2, to the respective first-order rate coefficient for hydroxide ion catalyzed hydrolysis, $k_{OH^-} \cdot [OH^-]$. In general, it is difficult to make a quantitative estimate of the amount of rate acceleration brought about by bovine carbonic anhydrase compared with the nonenzymatic reaction with OH^-. The former follows a rate law altogether different from that of latter. In fact, the enzymatic reaction is likely to proceed by a mechanism which is entirely different from that induced by OH^-. Nevertheless, the concept of "catalytic power" can be treated in terms of the unitless parameter $k_2/k_{OH^-}[OH^-]$. Such a comparison (Table V) shows enzyme-bound M2PC to hydrolyze about one-fifth as effectively as does the M3PC–BCA complex. By this same measure of enzyme efficiency, even M2PC is more effectively catalyzed than are the pyridinecarboxaldehydes or 4-nitrophenyl acetate (Table V).

A common and prominent feature of the high pH–rate profile of the bovine carbonic anhydrase catalyzed hydrolysis of esters is the continual rise in enzymatic activity. We have sought to determine the cause of this "enhanced catalytic power" since its discovery in our laboratories in 1966 (Pocker and Storm, 1968). In general it appears that the rise in enzymatic activity at high pH results from the attack of an enzyme-affiliated nucleophile or general base whose conjugate acid is being titrated between pH 9 and 12. After extensive modification studies involving a tyrosyl residue having an intrinsic pK_a of 10.8 and all the lysyl residues,[6] we were led to suggest that the acid being titrated is actually the zinc–aquo complex and that the nucleophile or the general base formed in this titration is a zinc–hydroxo complex (Pocker and Storm, 1968). Indeed, the pH-dependency profiles for enzymatic activity in regard to pyridine carbonates can be analyzed in terms of this model (see Results and Figure 3).

Whatever the nature of the active nucleophile causing the dramatic rise in activity above pH 9, the pK_a of its conjugate acid appears to also be a function of the substrate. The high pH (second) inflection appears at different values of pH (10–12) and with different intensities for different substrates. Pyridine carbonates provide an interesting study of this relationship. The pH profiles for BCA-catalyzed hydrolysis of three such carbonates, M2PC, M3PC, and methyl 2-nitro-3-pyridyl carbonate show, respectively, a rapid increase in activity above pH 9, a more gradual increase above pH 9, and no additional increase in activity from pH 9 to 11, where the activity starts rising again. Since none of these esters have labile protons which titrate between pH 6 and 12, it is the ES complex that is being titrated above pH 9. The enzyme-bound substrate then affects the pK_a of a particular group, the basic form of which

[5] Time-dependent sulfonamide binding has been reported for the carbonic anhydrase catalyzed hydration of carbon dioxide (Kernohan, 1966). We were unable to detect this phenomenon, possibly because the enzyme-catalyzed hydrolysis of the pyridine carbonates occurs at a much slower rate relative to the enzymatically catalyzed hydration of carbon dioxide. However, it is interesting in this respect that the enzymatic hydrolysis of M3PC is noncompetitively inhibited by acetazolamide.

[6] Spectrophotometric titrations at alkaline pH show that most of the tyrosines are masked in the native enzyme. Only one tyrosine seems to titrate freely but with a rather high value of the intrinsic pK (10.8) The remaining seven tyrosines ionize above pH 12 during a time-dependent change leading to activity loss. Tetranitromethane reacts with only one tyrosine in the native (as well as the metal free) enzyme. The catalytic activity is not affected by this modification. Methyl and phenyl *N*-methylacetimidates react with all the lysyl residues without loss of enzymatic activity (Y. Pocker, M. W. Beug, and D. G. Dickerson, unpublished observations; Nilsson and Lindskog, 1967).

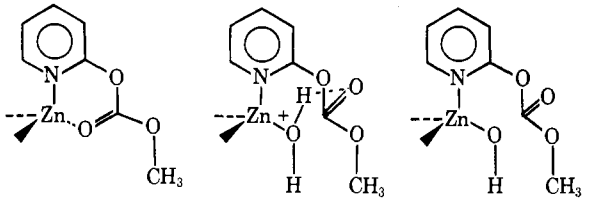
TABLE VII: pK_a Values of Some Pyridine Compounds and Binding to Bovine Carbonic Anhydrase.

Compound	pK_a	K_i or K_m (M)
Pyridine	5.23	0.5[a]
M2PC	3.28[b]	0.15
M3PC	4.88[c]	0.33
2-Pyridinecarboxaldehyde	4.1[d]	0.015[e]
4-Pyridinecarboxaldehyde	5.2[d]	0.011[f]

[a] Pocker and Stone (1968) (pH 8.45, 10% CH_3CN). [b] pK_a of model compound (2-methoxypyridine) (Albert and Phillips, 1956). [c] pK_a of model compound (3-methoxypyridine) (Albert and Phillips, 1956). [d] The fractions of hydration of 2- and 4-pyridinecarboxaldehydes as functions of pH show inflections at pH 4.1 and 5.2, respectively (Pocker *et al.*, 1967). A similar inflection in the pH dependency of the fraction of hydration of pyruvic acid corresponds well with the known pK_a of that acid (Pocker *et al.*, 1969). [e] Pocker and Meany (1967) (pH 6.8). [f] Pocker and Meany (1967) (pH 6.89).

is involved in enzyme action. Inasmuch as acetazolamide, a specific inhibitor of carbonic anhydrase activity, is reasonably effective even at high pH, it is attractive to suggest that the basic group in question is situated near the zinc, possibly even within the coordination sphere of the metal ion.

In the crystal structure of human carbonic anhydrase, the zinc ion appears to be tetrahedral, being firmly held to the protein through three histidine residues, and leaving only one site available for catalytic action (A. Liljas, private communication, 1970). The metal-chelating agent EDTA at 10^{-5} M levels does not enter the enzyme to inhibit M2PC hydrolysis at pH 8.54. Preliminary results using nonionic bidentate inhibitors similar to M2PC such as di(2-pyridyl)ketone and 2-pyridone indicate that even at pH around 10 only one coordination site is readily available on enzyme-bound zinc. The zinc ion catalysis of pyridine carbonate hydrolysis, although it is not an ideal model for the enzymatic hydrolysis of pyridine carbonates, even at physiological pH, is instructive.



The above zinc–M2PC complexes which could lead to accelerated hydrolysis are structures which cannot exist for M3PC–Zn(II) complexes. All such structures with M2PC require at least two coordination sites to be available on zinc. Thus catalytic mechanisms involving bovine carbonic anhydrase bound zinc would appear to be limited to the use of a single coordination site whereas zinc ions in solution are catalysts only when at least two sites are available. The comparison of second-order catalytic rate coefficients below shows that the catalytic process associated with zinc ions is much more

$$k_{Zn^{2+}}^{M2PC}/k_{Zn^{2+}}^{M3PC} = 34.5/0.0965 = 358 \text{ and } k_{enz}^{M2PC}/k_{enz}^{M3PC} = 23.3/80.0 = 0.29$$

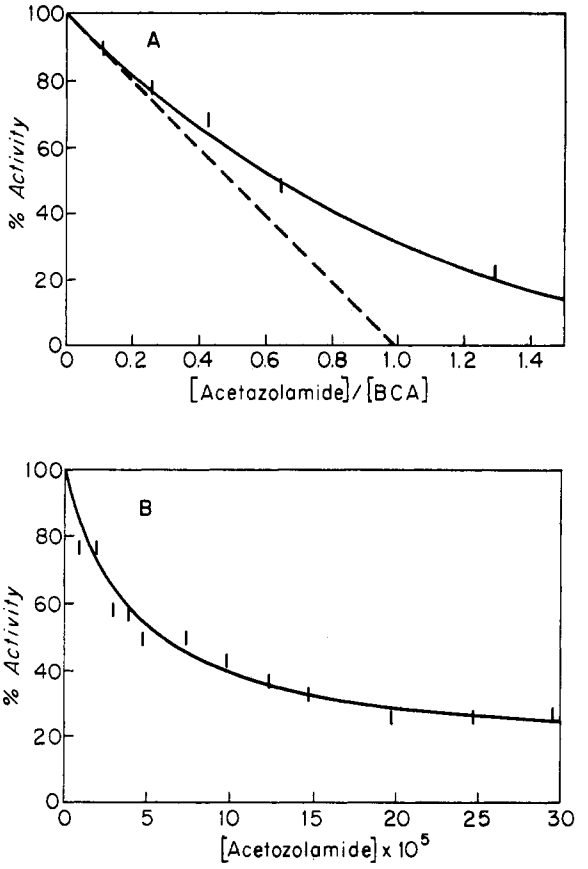


FIGURE 7: Acetazolamide inhibition of the enzymatic hydrolysis of methyl 3-pyridyl carbonate. (A) Tris buffers, I = 0.10, pH 9.0, $[BCA]_0 = 5.72 \times 10^{-6}$ M. (B) Dimethylglycine buffers, I = 0.10, pH 10.58, $[BCA]_0 = 1.61 \times 10^{-5}$ M. The curve follows eq 9 with parameters from Table VI.

sensitive to substrate structure than is the enzymatic process and that the two processes are oppositely sensitive to substrate geometry. In this respect it is interesting that zinc ions in solution are more efficient, per mole of zinc, than is this zinc-metalloenzyme at hydrolyzing M2PC at pH 8.91 (see Results).

The sulfonamide, acetazolamide, because of its efficiency of action and ubiquity in usage, has become the inhibitor, which when effective, is an accepted criterion for carbonic anhydrase activity. Since at physiological pH aromatic sulfonamides bind to carbonic anhydrase within the inner coordination sphere of its zinc atom (Fridborg *et al.*, 1967), acetazolamide inhibition can also be considered an indicator of zinc involvement in the enzymatic process. It is of interest then that around pH 10 acetazolamide only partially inhibits the bovine carbonic anhydrase catalyzed hydrolysis of M2PC and M3PC (Figures 6B and 7B). Partial inhibition at pH values around 10 would be expected if the process associated with k_{EH} (eq 5) is completely inhibited by sulfonamides and the process associated with k_{E^-} is not subject to sulfonamide inhibition. The predicted fractional inhibition at a given pH is then $(k_{EH}[EH]/[E]_0)/k_{enz}$. However, an inspection of Figures 6B and 7B would indicate that the fraction of inhibition is much larger than that predicted by eq 5. The predicted fractional inhibition can be increased by considering an alternate explanation for the pH dependency of enzymatic activity. The shape of the pH–rate profiles at high pH can arise from an extra and developing enzymatic activity being superim-

posed upon a plateau of activity which developed at physiological pH. This case, expressed mathematically in eq 10, can be derived from eq 5 and would result if the extra activity at

$$k_{enz} = k_I + k_{II}[E^-]/[E]_0 \quad (10)$$

high pH is a consequence of an enzymatic mechanism unrelated to the physiological mode of action. This scheme predicts the fractional inhibition, here k_I/k_{enz}, for M2PC hydrolysis at pH 10 to be 0.28, yet 55% of the enzyme rate is inhibited by acetazolamide. Likewise, at pH 10.58 for M3PC, $k_I/k_{enz} = 0.66$, yet 85% of the rate can be inhibited by acetazolamide. This latter scheme seems doubly improbable when the observation that both the processes associated with k_I and k_{II} depend upon the presence of the Zn^{2+} ion is considered (Pocker, 1969; Y. Pocker and J. T. Stone, unpublished results).

The inhibition data at high pH is only incompatible with the pH–rate profile data as interpreted by eq 5 if acetazolamide binds to the zinc in the high pH region as reported for acetoxymercurisulfanilamide in the crystal state (Fridborg *et al.*, 1967). However, there is evidence that sulfonamide binding at pH >11 may not involve direct attachment to the metal ion associated with the native enzyme. Formation of the acetazolamide complex at neutral pH is accompanied by changes in the energy of the d–d transitions of the cobalt enzyme. As pH is increased, the difference between the cobalt–BCA and the acetazolamide–cobalt–BCA spectra vanishes with a pK_a value near 11.2 (Lindskog, 1963). This change in relationship between acetazolamide and metal ion with increasing pH is manifested by the tremendous increase in K_i observed above pH 9 in the present work (see also Pocker and Stone, 1968; Lindskog, 1969). An acetazolamide binding site at pH >10 near but not at zinc could lead to K_i values around 10^{-5} M and could also account for the partial nature of the acetazolamide inhibition at these pH values. Indeed, such weak binding of acetazolamide does not require a zinc coordination site (Coleman, 1967; see also, equilibrium dialysis data for the binding of a second sulfonamide molecule, Lindskog, 1963). Most likely another ligand (*e.g.*, OH^-) becomes strongly attached to the metal ion, causing acetazolamide to lose its primary attachment. It should be noted that around pH 10 acetazolamide is predominantly in the form of the dianion. The loss in stability of the BCA–acetazolamide complex with increasing pH may also be the consequence of a substrate-induced deprotonation of an amino acid residue which formed a part of the primary inhibitor binding site. The latter interpretation is similar to the one invoked for the rise in K_m at pH >9 in the α_1-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester (Valenzuela and Bender, 1971).

The experiments reported in this paper indicate that the enzymatic process at high pH operates within the same general cavity as does the enzymatic process which is operative around neutral pH. While not proving it, the results are not incompatible with a mechanistic model which assigns the high pH rise in BCA esterase activity to the appearance of a zinc–hydroxo complex. Carbonic anhydrase also undergoes configurational changes above pH 10 as evidenced by major changes in optical rotation (Coleman, 1965) and hence the high pH–activity increase may in part arise from suitable conformational changes.

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