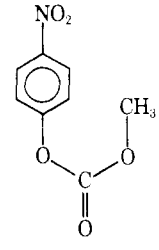
Carbonic Anhydrase Catalyzed Hydrolysis and Decarboxylation. Kinetic Studies of Enzyme-Catalyzed Decomposition of Mono- and Disubstituted Derivatives of Carbonic Acid†

Y. Pocker* and L. J. Guilbert‡

ABSTRACT: The effect of bovine carbonic anhydrase on the stepwise hydrolysis of carbonate diesters is examined. While biphasic enzyme kinetics cannot be detected in the case of bis(4-nitrophenyl) carbonate, it is demonstrated that at pH 10.45 methyl 4-nitrophenyl carbonate is enzymatically hydrolyzed to produce an intermediate monoester, methyl carbonate, which does not undergo enzyme-catalyzed decarboxylation. However, it is shown that at pH 7.27 methyl carbonate decarboxylation is accelerated by carbonic anhydrase. The enzymatic pH–rate profile for methyl 4-nitrophenyl carbonate release of 4-nitrophenol rises with increasing pH, while the pH dependency for the enzyme-catalyzed decarboxylation of methyl carbonate is such that it decreases with increasing pH. In this respect, the kinetic behavior of bovine carbonic anhydrase in regard to methyl 4-nitrophenyl carbonate hydrolysis appears to be similar to that observed in CO_2 hydration; in both the rate varies as though dependent on the ionization of a group in the enzyme with pK near 7, only the basic form being active. On the other hand the enzyme-catalyzed decarboxylation of methyl carbonate appears to be formally similar to that of bicarbonate; with these two anions the rate varies as though dependent on the ionization of a group in the enzyme of essentially the same pK (~7) with only the acid form being active. Although at pH 7.27 the first-order rate coefficient, k_{buf}, for methyl carbonate decomposition is 1.8 times larger than that for bicarbonate dehydration, the second-order enzymatic rate coefficient, k_{enz}, for bicarbonate is three orders of magnitude greater than that for methyl carbonate. The large differences in these k_{enz} values are discussed in terms of the role of the labile bicarbonate proton in the mechanism of carbonic anhydrase catalysis.

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) from erythrocytes is a remarkably versatile enzyme, catalyzing, in addition to the reversible hydration of carbon dioxide, that 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 with respect to certain activated monoesters of carboxylic acids (Tashian *et al.*, 1964; Pocker and Stone, 1965, 1967; Verpoorte *et al.*, 1967; Thorslund and Lindskog, 1967; Pocker and Watamori, 1971) as well as diesters of carbonic acid (Pocker and Guilbert, 1972). In the latter case the hydrolyses of water-soluble pyridine carbonates were examined. These carbonate diesters were very efficiently turned over by carbonic anhydrase; however, the Michaelis constants were found to be very large (K_m > 0.1 M at pH 6.9). In an attempt to lower K_m while retaining the high turnover numbers of carbonate diesters, the more lipophilic carbonate substrates, methyl 4-nitrophenyl carbonate (I) and bis(4-nitrophenyl) carbonate (II), were chosen for the present study.

The hydrolysis of aryl alkyl carbonate diesters has been observed to proceed in stepwise fashion, with the decarboxylation of the monoalkyl carbonate intermediate generally thought to be more rapid than the rate of its formation



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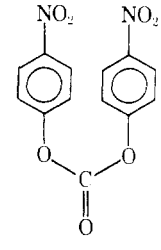
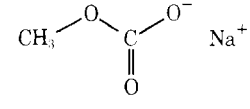


II

(Dittert and Higuchi, 1963). Because carbonic anhydrase greatly accelerates the hydrolysis of the diester methyl 4-nitrophenyl carbonate, especially at pH values above 9, intermediate methyl carbonate (III) accumulation becomes



III

probable. Consequently, the present study also attempts to answer the following questions. (a) Is intermediate III formed during the enzymatic hydrolysis of I? (b) Does the presence of III interfere with the carbonic anhydrase catalyzed hydrolysis of the diester I? (c) Is the decarboxylation of the intermediate ester subject to carbonic anhydrase catalysis? In the course of this inquiry, it was discovered that methyl carbonate is indeed an excellent analog of bicarbonate.

There is decisive evidence that under physiological conditions significant amounts of blood carbon dioxide are present as carbamates of hemoglobin and plasma proteins (Edsall and

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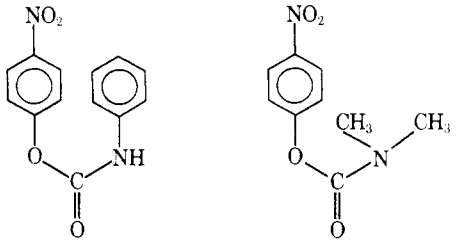
Wyman, 1958). However, less is known about carbamate formation between CO_2 and the ϵ-amino lysyl residues of carbonic anhydrase. Clearly, carbonic anhydrase presents itself as a likely promoter of carbamate formation and decomposition. Three carbamate esters, the rates of which can be spectrophotometrically monitored, were for the above reasons studied as potential carbonic anhydrase substrates. The esters are 4-nitrophenyl *N*-phenylcarbamate (IV), 4-nitrophenyl *N,N*-dimethylcarbamate (V), and neostigmine bromide (VI).



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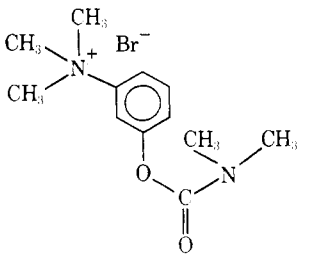


VI

Experimental Section

Materials

Bis(4-nitrophenyl) carbonate was prepared by treating 2.5 equiv of dry sodium 4-nitrophenolate with 1 equiv of phosgene in dry toluene (Glatthard and Matter, 1963). The product recovered from the toluene was twice recrystallized from benzene–Norit and once from chloroform, mp 141–142°.

Methyl 4-nitrophenyl carbonate, obtained from methyl chlorocarbonate and sodium 4-nitrophenolate (for method see Pocker and Guilbert, 1972), was recrystallized three times from methanol, mp 111–112°.

Methyl carbonate, sodium salt, precipitates from a methanol solution of sodium methoxide through which very pure CO_2 is bubbled. It cannot be overemphasized that extreme precautions are to be taken as to the purity and dryness of the starting materials since the above-mentioned precipitate must be filtered, dried, and used with no further purification. The equivalent weight of this precipitate, determined titrimetrically, was 98.4 ± 0.6 (theoretical, 98).

4-Nitrophenyl N,N-dimethylcarbamate was prepared as described by Lellmann and Benz (1891). The product was twice recrystallized from benzene–Norit. Residual benzene was removed in a pistol dryer (78° (0.1 mm) for 5 hr), yielding crystals mp 108–109°.

4-Nitrophenyl N-phenylcarbamate was prepared from 4-nitrophenol and phenyl isocyanate in toluene and recrystallized twice from chloroform, mp 158–159°.

Neostigmine bromide, the *N,N*-dimethylcarbamoyl ester of 3-hydroxy-*N,N,N*-trimethylanilinium bromide, was obtained from Mann research (Pharmaceutical grade) and was used without further purification.

The preparation or purification of the organic co-solvent (acetonitrile), the buffer components (potassium dihydrogen phosphate, disodium hydrogen phosphate, Tris, imidazole, and *N,N*-dimethylglycine), the erythrocyte enzyme (bovine carbonic anhydrase), and the inhibitor (acetazolamide) has been previously described (Pocker and Guilbert, 1972).

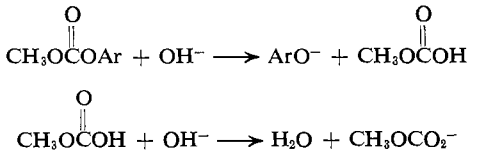
Apparatus. All pH measurements were recorded at 25° with an Orion Model 801 digital pH meter fitted with a Corning glass electrode (no. 476022) and a Beckman reference electrode. The relative accuracy of this apparatus is reported to be 0.001 pH unit. Hydrolysis rates were monitored spectrophotometrically 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. The rapid kinetic studies were carried out on a Durrum-Gibson stopped flow spectrophotometer with electronics, drive syringes, and temperature control systems modified in our laboratory by Mr. Ronald Reaugh. For pH-Stat kinetics a Radiometer automatic titrator with an ABU-1b 2.5-ml Auto-Burette was used. This assembly was set up in two ways. The electrode head and reaction vessel were modified as described by Magid (1968) to allow for rapid removal of CO_2 from the solution by nitrogen scrubbing. Alternatively, the electrode head was modified to make it more nearly air tight and to allow initiation of the rate by syringe delivery. Mineral oil layered over the reaction solution further prevented gas exchange. A jacketed reaction vessel with a precision bath-pump maintained the contents at constant temperature. An IBM 1094-1090 computer pair and later a teletype terminal leading to a CDC 6400 computer were used as aids in the analysis of the kinetic data.

Methods

In general, buffer solutions were prepared as previously described (Pocker and Guilbert, 1972). Those for bis(4-nitrophenyl) carbonate hydrolysis studies with the enzyme were of ionic strength 0.02 before dilution with organic cosolvent. All others were ionic strength 0.10 before dilution.

The hydrolysis rate of the aryl carbonate and carbamate esters was followed by monitoring the appearing ultraviolet (uv) or visible absorbancy band of their respective phenolic moiety. The appearance of 4-nitrophenol was monitored at 400 nm (phenoxide), ϵ 1.8×10^4, or at 320 nm (phenol), ϵ 9.2×10^3, and that of 3-hydroxy-*N,N,N*-trimethylanilinium bromide at 294 nm, ϵ 3.4×10^3.

The pH-Stat titrant at pH 10.45 was 5×10^{-4} M NaOH, while at pH 7.27 it was 5×10^{-3} M HCl. All reaction solutions were brought to ionic strength 0.10 with sodium sulfate. No buffer was used at pH 10.45; at pH 7.27, 8×10^{-4} M phosphate was used. At pH 10.45, the total decomposition of methyl 4-nitrophenyl carbonate formally takes up 2.73 hydroxide ions, two of which are used up in the initial diester cleavage and disappear concomitantly with 4-nitrophenoxide appearance (eq 1). Finally, the fraction of hydroxide ion is taken up by



(1)

the subsequent decomposition of methyl carbonate to the carbonate–bicarbonate equilibrium system which has an

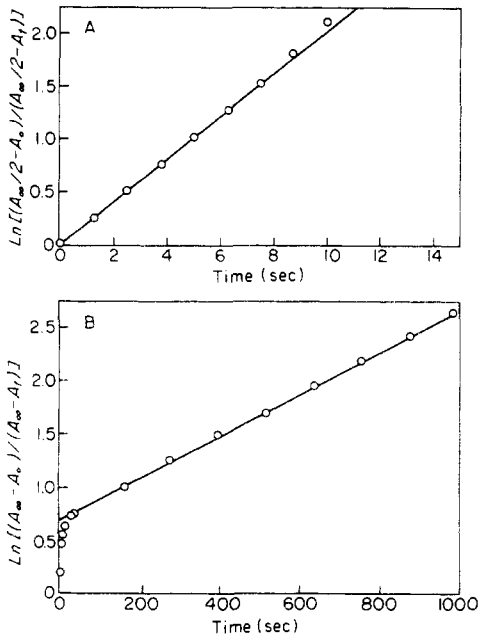


FIGURE 1: The imidazole-catalyzed hydrolysis of bis(4-nitrophenyl) carbonate: [imidazole] = 0.085 M, 15% acetonitrile (v/v$_t$), 25°, ionic strength = 0.085. Release of (A) first and (B) second 4-nitrophenol (determined spectrophotometrically).

apparent $K_{HCO_3^-}$ of 1.14×10^{-10} M at ionic strength 0.10 and 25° (Harned and Owen, 1958) (eq 2).

$$\begin{aligned} CH_3OCO_2^- &\rightleftharpoons CH_3O^- + CO_2 \\ CH_3O^- + CO_2 + H_2O &\rightleftharpoons CH_3OH + HCO_3^- \\ HCO_3^- + OH^- &\rightleftharpoons CO_3^{2-} + H_2O \end{aligned} \qquad (2)$$

On the other hand, the decomposition of methyl carbonate around physiological pH was followed by removing CO_2 from solution with nitrogen gas as rapidly as it is formed and maintaining constant acidity with the pH-Stat (eq 3). Such a

$$\left[\begin{array}{c} CH_3OCO_2^- + H_3O^+ \\ \Updownarrow \\ CH_3OCO_2H + H_2O \end{array}\right] \longrightarrow CH_3OH + H_2O + CO_2 \qquad (3)$$

procedure has been utilized with a high degree of precision in regard to the dehydration kinetics of the bicarbonate ion (Magid, 1968; Magid and Turbeck, 1968). We have, with the above-mentioned methodology, determined the rate of dehydration of bicarbonate at pH 7.27 which is entirely in agreement with Magid's results.

The first-order rate coefficient, k_{obsd}, was obtained from the spectrophotometric rate data as the slope of the plot ln $[(A_\infty - A_0)/(A_\infty - A_t)]$ *vs.* time. The relationship for pH-Stat kinetics was ln $[V_\infty - V_0)/(V_\infty - V_t)]$ where V is the volume of titrant in milliliters. Such plots were typically linear past 2 half-lives; however, the reaction of bis(4-nitrophenyl) carbonate with imidazole and the hydrolysis of methyl 4-nitrophenyl carbonate at pH 10.45 as determined with the pH-Stat are special cases of limiting first-order plots (see Results). The k_{obsd} determined in the absence of carbonic anhydrase is referred to as k_b and is a summation of the buffer and water species catalyses; $k_b = k_0 + k_{OH^-}[OH^-] + k_{H_3O^+} \cdot [H_3O^+] + k_B[B] + k_{HB}[HB]$. The buffer rate, k_b, was separated into its constituent parts by the method of Bell and Darwent (1950). The enzymatic second-order rate coefficient, k_{enz}, was obtained from the slope of plots of k_{obsd} *vs.* the respective enzyme concentration with the intercept at [E] = 0 being k_b. The data from enzyme inhibition studies, net first-order rate coefficients, $k_{obsd} - k_b$, for enzymatic hydrolysis taken at constant [E] with the inhibitor concentration varying, were analyzed in two ways. (1) Where $[E] \gg K_i = [I][E]/[EI]$, the fractional inhibition, i, is proportional to $[I]_0$ until most of the enzyme has been titrated. A plot of per cent activity = $100(1 - i)$ *vs.* $[I]_0$ at the lower $[I]_0$ forms a straight line, the zero activity intercept of which is equal to the active enzyme concentration, $[E]_0$. Where i is no longer proportional to $[I]_0$, at the higher values of $[I]_0$, a value of K_i was determined by the method of Easson and Stedman (1936). The intercept of this plot (where $1/(1 - i) = 0$) also equals $[E]_0$. (2) Where $[E]_0 \ll K_i$, the slope of the straight line plot of $1/i$ *vs.* $1/[I]_0$ is K_i (Webb, 1963). The inhibition is complete if the intercept at $1/[I]_0 = 0$ is unity.

Results

Hydrolysis of Nitrophenyl Carbonates. Before turning to the enzymatic catalysis of methyl 4-nitrophenyl carbonate and bis(4-nitrophenyl) carbonate hydrolysis, it is instructive to consider briefly the nature of hydrolytic reactions as catalyzed by weak bases and the hydroxide ion. In phosphate and Tris buffers bis(4-nitrophenyl) carbonate hydrolyzes without accumulation of intermediate with the release of both 4-nitrophenols in the course of a first-order rate process. This hydrolysis is catalyzed by the phosphate dianion, but very little by the phosphate monoanion. The hydrolysis of bis(4-nitrophenyl) carbonate is powerfully catalyzed by imidazole, the release of the 4-nitrophenols being here stepwise (Figure 1). The value of k_{OH^-}, the second-order catalytic rate coefficient for the hydroxide ion, is sensitive to the solvent. When measured in 15% dioxane solutions, k_{OH^-} is some 50 times larger than the value found with 15% acetonitrile solutions (Table I).

The spontaneous rate coefficient, k_0, is approximately the same in both solvents. Similar solvent effects on k_{OH^-} can be found in the literature pertaining to 4-nitrophenyl acetate hydrolysis (Bender and Turnquest, 1957; Bruice and Lapinski, 1958). Bis(4-nitrophenyl) carbonate is far more readily hydrolyzed than is methyl 4-nitrophenyl carbonate with the ratio $k_{OH^-,bis}/k_{OH^-,methyl}$ being 13.5 The hydrolysis of this latter mentioned, unsymmetrical carbonate diester is stepwise provided $k_I[I] \gg k_{III}[CH_3OCOO^-]$ in eq 4. Both

$$\underset{\text{I}}{4\text{-}O_2NC_6H_4OCOCH_3} \xrightarrow{k_I} \underset{\text{4-nitrophenol}}{CH_3OCO^-} \xrightarrow{k_{III}} \underset{\text{III}}{CH_3OH + HCO_3^-} \qquad (4)$$

k_I and k_{III} are first-order rate constants; both are complex, being equal to $k_0 + \Sigma_i k_{c_i}[\text{catalyst}]_i$. The first-order rate constant k_I can be measured for any situation, with no interference from the second step, by spectrophotometric methods, *i.e.*, by following the rate of appearance of 4-nitrophenol. Above pH 9, the remaining first-order constant, k_{III}, is reported to be independent of $[OH^-]$ (Faurholt, 1927); using a pH-Stat we have determined k_{III} at pH 10.45 (see Table I). Equation 5 from Frost and Pearson (1961) predicts the maxi-

$$[CH_3OCO_2^-]_{max}/[I]_0 = \kappa^{\kappa/(1-\kappa)} \qquad (5)$$

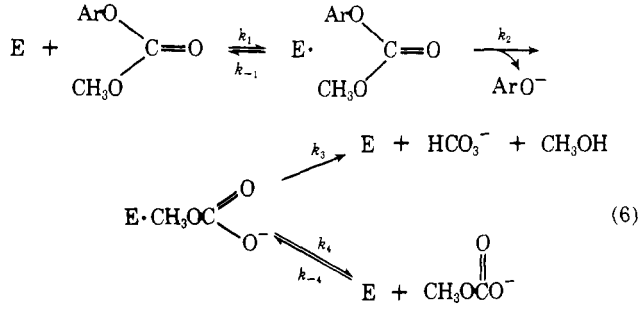
$$\kappa = k_{III}/k_I$$

TABLE I: Rate Coefficients Pertaining to the Hydrolysis of Carbonate Esters.

Substrate	Conditions	Catalyst	k_{cat} (M^{-1} sec^{-1})
(A)			
Bis(4-nitrophenyl) carbonate	*a*	HPO_4^{2-} [b]	1.9×10^{-2}
	a	$H_2PO_4^-$ [b]	$<10^{-4}$
	a	HPO_4^{2-} [c]	7.1×10^{-2}
	a	$H_2PO_4^-$ [c]	$<10^{-3}$
	a	OH^- [b,d]	1.2×10^{2}
	a	OH^- [b,e]	1.17×10^{2}
	a	OH^- [c,d]	6.19×10^{3}
	a	H_2O [b,d]	4.0×10^{-6}
	a	H_2O [c,d]	4.5×10^{-6}
	f	Imidazole[b]	23.5
	g	HPO_4^{2-} [h]	1.89×10^{-1}
	g	Imidazole[i]	23.2
Methyl 4-nitrophenyl carbonate	*j*	OH^-	8.75
(B)		pH	$k_{obsd} \times 10^3$ (sec^{-1})
Methyl carbonate, sodium salt	*k*	7.27	6.4 ± 0.1
	k	10.45	2.0 ± 0.04

[a] Present work; 25°, ionic strength 0.017. [b] 15% (v/v$_t$) acetonitrile. [c] 15% (v/v$_t$) dioxane. [d] Determined in phosphate buffers. [e] Determined in Tris buffers. [f] Present work; 25°, ionic strength 0.085. [g] Fife and McMahon (1970); 1.63% acetonitrile. [h] Determined at 50°. [i] Determined at 30°. [j] Present work; 0.67% acetonitrile, 25°, ionic strength 0.10. [k] Present work; 25°, ionic strength 0.10.

mum fraction of intermediate, monocarbonate anion, which would appear in eq 4. The ratio $[CH_3OCOO^-]_{max}/[I]_0$ reaches 0.10 as k_I reaches a value of 2.75×10^{-4} sec^{-1}. In the absence of any other catalyst than the hydroxide ion, this situation exists at pH 9.40. However, the first step of the hydrolysis can be greatly accelerated by bovine carbonic anhydrase. At pH 10.45 the second-order catalytic rate coefficient, k_{enz}, for this first step is about 1200 M^{-1} sec^{-1} so that $k_{I,enz} = k_{enz}[E]_0 = 1.2 \times 10^{-2}$ sec^{-1} when $[E]_0 = 10^{-5}$ M. Equation 5 predicts $[CH_3OCOO^-]_{max}/[I]$ to be 0.70 for the enzymatic portion of the rate in this instance. The predicted intermediate buildup due to enzymatic action at pH 10.45 thus allows experiments pertaining to the fate of the enzyme–methyl carbonate complex to be carried out. As the decomposition of carbonate diesters by carbonic anhydrase must involve at least three steps (eq 6), it is of interest to determine whether regeneration



(6)

of free enzyme is concomitant with the destruction of molecular methyl carbonate (the upper, k_3, route as opposed to the lower, k_4, route in eq 6).

Figure 2 shows that a measurable concentration of intermediate is formed by the enzymatic hydrolysis of methyl 4-nitrophenyl carbonate at pH 10.45. The initial, curved portion of curve A in Figure 2 is the first step of eq 4, a hydrolysis which takes up two hydroxide ions. The upper, linear portion

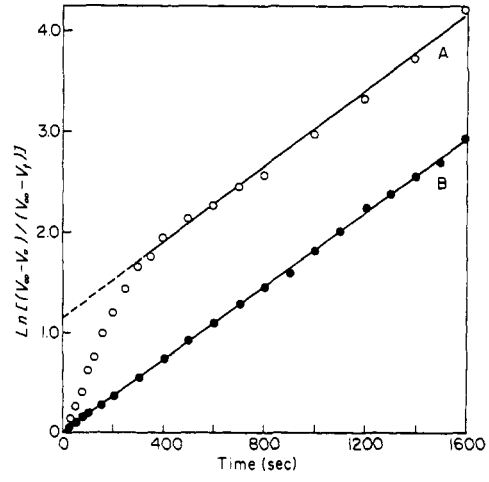


FIGURE 2: The rate of methyl carbonate decomposition (B) as compared to the rate of decomposition of the intermediate generated during the enzyme-catalyzed hydrolysis of methyl 4-nitrophenyl carbonate (A). Titrimetric measurements: $[E]_0 = 7.0 \times 10^{-6}$ M in both A and B, pH 10.45, 25°, ionic strength = 0.10.

of curve A is very nearly parallel to the slope of line B, the first-order plot for the decomposition of methyl carbonate, sodium salt, at pH 10.45 in the presence of the same concentration of bovine carbonic anhydrase as was held for curve A, 7×10^{-6} M. The first-order rate constants for the linear portion of curve A and for line B are, respectively, $2.0 \pm 0.3 \times 10^{-3}$ and $2.1 \pm 0.2 \times 10^{-3}$ sec^{-1}.[1] The methyl carbonate which is seen to decompose in the upper portion of curve A must have been dissociated intact from the active site; thus, the route

[1] The presence of carbonic anhydrase appeared to increase CO_2 absorption at pH 10.45 despite the already mentioned precautions, thus increasing the error of these kinetic determinations as compared to the excellent (2%) reproducibility in the nonenzymatic rate constant (see Table I).

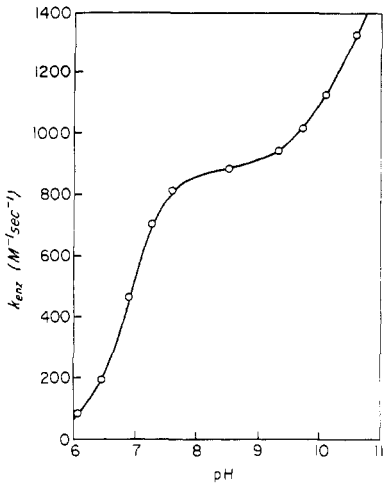


FIGURE 3: Bovine carbonic anhydrase catalyzed hydrolysis of methyl 4-nitrophenyl carbonate as a function of pH: ionic strength = 0.10, 25°, 0.6% (v/v_t) acetonitrile.

involving k_4 in eq 6 is operative. Because of the error limits involved, it is questionable whether the enzyme actually catalyzes the decomposition of the monoester at pH 10.45.

A major difficulty in regard to interpretation of methyl carbonate decomposition data is whether one is looking at hydrolysis or decarboxylation. Clearly the proton release data gathered under conditions which suppress CO_2 evolution do not differentiate between hydrolysis and decarboxylation. At neutral pH the decarboxylation of methyl carbonate produces methanol and CO_2 as primary products while hydrolysis leads to methanol and bicarbonate (eq 7). At pH

$$H_2O + CH_3CO_3^- + BH^+ \rightleftharpoons \begin{matrix} CH_3OH + CO_2 + H_2O + B \\ \text{(steps } k_3, k_{-4}, k_4\text{)} \\ CH_3OH + HCO_3^- + BH^+ \end{matrix} \quad (7)$$

7.27, the removal of CO_2 from the reaction mixture with an inert carrier gas prevents bicarbonate formation from CO_2 and allows the determination of the rate of decomposition in terms of the rate of proton uptake. Proton uptake under these conditions can arise from direct decarboxylation (step 6, eq 7), or from the dehydration of the intermediate bicarbonate produced by hydrolysis (steps 7 and 9 in eq 7). However, we have shown that the rate of proton uptake by methyl carbonate is faster than that by bicarbonate; thus bicarbonate cannot be an intermediate and decarboxylation appears to be the major mechanism of methyl carbonate decomposition at physiological pH. As in the case of bicarbonate dehydration, first-order plots of methyl carbonate decarboxylation are linear to 2 half-lives provided CO_2 is efficiently removed. First-order rate constants for methyl carbonate decarboxylation are given in Table I. Carbonic anhydrase is a catalyst for the decarboxylation of methyl carbonate at pH 7.27, with a k_{enz} value of 3.2×10^3 M^{-1} sec^{-1}. The mode of complete hydrolysis of alkyl aryl carbonate diesters by bovine carbonic anhydrase therefore involves both reversible dissociation of intact alkyl carbonate anion (k_4, k_{-4} step in eq 6) and enzymatic cleavage of the bound intermediate (k_3 step).

The pH–rate dependencies for the enzymatic hydrolysis yielding 4-nitrophenol from methyl 4-nitrophenyl carbonate and both 4-nitrophenols from bis(4-nitrophenyl) carbonate are given respectively in Figure 3 and in Table II. That pertaining to the methyl ester is typical of the pH dependency of the

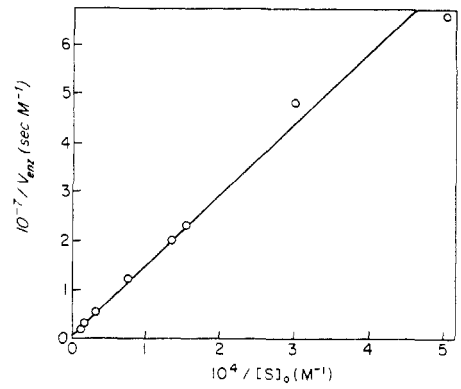


FIGURE 4: Bovine carbonic anhydrase catalyzed hydrolysis of methyl 4-nitrophenyl carbonate. A double reciprocal plot at pH 7.65: $[E]_0 = 10^{-6}$ M, 25°, 15% (v/v_t) acetone.

TABLE II: pH Dependence of k_{enz} for the Hydrolysis of Bis(4-nitrophenyl) Carbonate.[a]

pH	k_{enz} (M^{-1} sec^{-1})	pH	k_{enz} (M^{-1} sec^{-1})
7.48	45.4	8.20	85.7
7.58	49.9	8.28	97.5
7.70	56.0	8.35	114.0
7.87	61.9	8.40	138.0
8.00	69.1	8.45	155.0
8.12	76.4	8.50	162.0

[a] Ionic strength 0.017, Tris buffers, 10% acetonitrile (v/v_t), 25°.

esterase function of bovine carbonic anhydrase (see Pocker and Stone, 1967; Pocker and Guilbert, 1972) in having a sharp inflection near pH 7 and a continuing rise in activity above pH 9. However, enzymatic activity in regard to bis(4-nitrophenyl) carbonate hydrolysis smoothly rises from pH 7.5 to 9 and is much lower[2] than that for the methyl ester.

Both nitrophenyl carbonate diesters are sparingly soluble in water. Even with 15% organic cosolvent (acetonitrile, dioxane, or acetone) the bisester precipitates from solution when concentrations rise above 8×10^{-5} M at 25°. Although the methyl ester is considerably more water soluble, the Lineweaver–Burk plot depicted in Figure 4, carried out in 15% acetone solutions, shows K_m to be much larger than the maximum solubility, $[S]_{max} = 8 \times 10^{-4}$ M.

The aromatic sulfonamide–acetazolamide, a specific and powerful carbonic anhydrase inhibitor, completely suppresses enzymatic activity in regard to methyl 4-nitrophenyl carbonate hydrolysis at pH 7.27 (Figure 5A). Where the fractional inhibition, i, is no longer proportional to $[I]_0$, the inhibitor concentration, a value for $K_i = 1.4 \times 10^{-8}$ M was obtained from an Easson–Stedman plot (see Experimental Section). The vertical axis intercept in this latter mentioned plot is equal to $[E]_t$, the total concentration of active sites being inhibited, and is identical with the horizontal line intercept of the dashed line

[2] The hydrolysis of bis(4-nitrophenyl) carbonate by carbonic anhydrase exhibits monophasic kinetics, whereas that by α-chymotrypsin exhibits biphasic kinetics (Bender and Wedler, 1972). Apparently under our reaction conditions the release of the first 4-nitrophenol is slow enough to be rate determining.

extrapolated from the linear portion of Figure 5A. The ratio of $[E]_t$ to $[E]_0$, the enzyme concentration, is very nearly unity. At pH 7.27, the enzymatic acceleration of methyl carbonate anion hydrolysis, the second step of the hydrolysis of the above-mentioned carbonate diester, is also inhibited by acetazolamide.

Aromatic sulfonamides characteristically lose inhibitory power at pH values above 9 (Pocker and Stone, 1968a; Lindskog, 1969). In addition, the inhibition by acetazolamide at high pH pertaining to the enzymatic hydrolysis of pyridine carbonates was found not to be complete (Pocker and Guilbert, 1972). However, the acetazolamide inhibition of the carbonic anhydrase catalyzed hydrolysis of methyl 4-nitrophenyl carbonate at pH 10.58 is essentially complete as the intercept of a plot of $1/i$ *vs.* $1/[I]_0$ is 1 (Figure 5B).

Carbonic Anhydrase and the Hydrolysis of Carbamate Esters. N,N-Disubstituted carbamate esters are used, in part, as pharmaceutical agents because of their stability in aqueous solution. At pH 10.6 the first-order hydrolysis rate constants, $k_{obsd} = k_{OH^-}[OH^-]$, are 1.5×10^{-7} sec^{-1} for neostigmine bromide (Christenson, 1964) and 1.3×10^{-7} sec^{-1} for 4-nitrophenyl *N,N*-dimethylcarbamate (Dittert and Higuchi, 1963). At this pH in 10 μM enzyme solutions, the hydrolysis of neither ester was accelerated beyond this very slow base rate. Additional experiments, where $[S]_0 \gg [E]_0$, were carried out such that released 4-nitrophenol or 3-hydroxy-*N,N,N*-trimethylanilinium bromide of concentrations equal to the enzyme concentration could be accurately monitored on the Gilford 2000 spectrophotometer. Under these conditions no product "burst" could be detected; thus, a deactivating modification of the active site such as happens with acetylcholinesterase when treated with neostigmine bromide (Wilson, 1960) is not likely.

The enzymatic dissociation constant, $K_s = k_r/k_f$ (eq 8), in

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \qquad K_s = k_r/k_f = K_i \tag{8}$$

regard to unreactive substrates can easily be determined by using them as inhibitors for the enzyme-catalyzed hydrolysis of an "active" substrate (Pocker and Stone, 1968b). The active substrate used in this case was methyl 4-nitrophenyl carbonate. At pH 10.58 4-nitrophenyl *N,N*-dimethylcarbamate concentrations up to 7.1×10^{-3} M and neostigmine bromide concentrations as high as 2.4×10^{-2} M did not inhibit carbonic anhydrase activity. Thus at this pH minimum limits as to the value of K_s can be placed at about 0.05 M for the 4-nitrophenyl ester and 0.3 M for neostigmine bromide. At pH 6.9 neostigmine bromide does inhibit the enzyme, with an apparent dissociation constant, K_s, of 0.06 ± 0.01. Since a K_i value of 0.066 has been reported for sodium bromide at pH 7.55 (Pocker and Stone, 1968a) it would appear that the neostigmine cation binds no better to bovine carbonic anhydrase than does the sodium ion.

The monosubstituted carbamate ester 4-nitrophenyl *N*-phenylcarbamate is readily hydrolyzed at neutral pH, $k_b = 3.2 \times 10^{-3}$ sec^{-1} at pH 7.2. However, 10^{-5} M enzyme does not accelerate this rate. No attempt was made to determine a binding constant for this ester because of its rapid nonenzymatic hydrolysis. It appears likely that the hydrolysis of N-monosubstituted carbamates involves abstraction by a base (OH^-) of the carbamine proton to form an intermediate

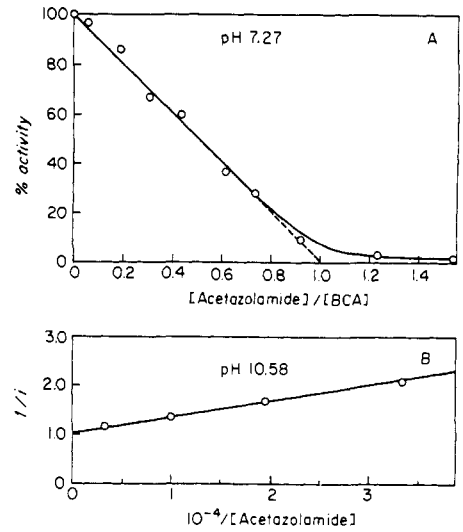
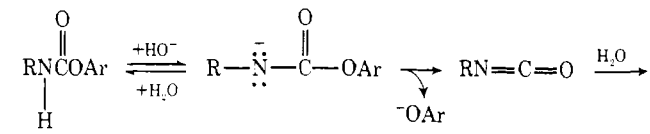


FIGURE 5: Acetazolamide inhibition of the bovine carbonic anhydrase catalyzed hydrolysis of methyl 4-nitrophenyl carbonate, 15% acetonitrile (v/v$_t$), 25°: (A) pH 7.27, $[E]_0 = 1.48 \times 10^{-5}$ M; $K_i = 3.25 \times 10^{-5}$ M; (B) at pH 10.58; reciprocal fractional inhibition, $1/i$, as a function of reciprocal inhibitor concentration, $1/[I]$.

isocyanate (Dittert and Higuchi, 1963; Hutchins and Fife, 1973; see eq 9). None of the known carbonic anhydrase



$$RNH_2 + CO_2 \quad (9)$$

substrates hydrolyze *via* a reaction path involving such a proton removal.

Discussion

Carbonate Diesters. The pH–rate profiles pertaining to carbonic anhydrase catalytic activity for a wide variety of ester substrates under conditions such that $[S]_0 \ll K_m$ reflect apparent titrations of a family of diprotic acids characterized by a pK_1 near 7 and a pK_2 greater than 10 (Pocker and Stone, 1967; Pocker and Storm, 1968; Pocker and Watamori, 1971, 1973; Pocker and Guilbert, 1972; Pocker and Beug, 1972). Despite the close similarities in the points of inflection in the sigmoid curves, there are significant dissimilarities in overall activity. Neutral pH associated activity relative to that activity appearing at high pH values, approximately measured by the ratio of k_{enz} values at pH 10.5 and 8.5,[3] appears to be a function of substrate. Limiting our observations to carbonate diester substrates only, we see these ratios vary from 1.03 to 7.3 (Table III). In addition to these obvious pH profile dif-

[3] The pH profiles described above have been analyzed in terms of the titration of a diprotic acid (Pocker and Guilbert, 1972) with $k_{enz} = \alpha k_\alpha + \beta k_\beta$ where α and β are the fractions of dissociation of the conjugate acids of the functional bases at the enzyme active site respectively at neutral and high pH. Thus k_α represents the total enzymatic activity attributable to that form of the enzyme made active by the titration of an acid having a pK_a near seven. The quantity k_α can approximately be determined as the horizontal inflection of these pH–rate profiles which occurs at an average pH of 8.5 for carbonate diesters. In general pH 10.5 was the highest pH value at which enzyme kinetics were carried out in regard to carbonate diester hydrolysis.

TABLE III: Bovine Carbonic Anhydrase Catalyzed Hydrolysis of Carbonate Diesters. A Comparison of Chemical and Enzymatic Second-Order Rate Coefficients.

Carbonate Ester	$k_{enz,8.5}$ (M^{-1} sec^{-1})	$k_{enz,10.5}/k_{enz,8.5}$	k_{OH^-} (M^{-1} sec^{-1})	$k_{enz,8.5}/k_{OH^-}$
Bis(4-nitrophenyl)[a]	162.0		120	1.35
Methyl 2-pyridyl[b]	20.4	7.3	2.59	7.88
Methyl 3-pyridyl[b]	79.0	1.6	4.24	18.7
Methyl 2-nitro-3-pyridyl[c]	1680.0	1.03	16.6	101
Methyl 4-nitrophenyl[d]	888.0	1.5	8.75	101

[a] Present work; 10% (v/v_t) acetonitrile, 25°. [b] 0.33% acetonitrile, 25°; Pocker and Guilbert (1972). [c] 0.67% acetonitrile, 25°; Guilbert (1971). [d] Present work; 0.67% acetonitrile, 25°.

ferences among carbonate substrates, there is wide variation in the ability of bovine carbonic anhydrase to catalyze the hydrolysis of these esters. A useful measure of "catalytic power" when comparing different substrates is the unitless ratio k_{enz}/k_{OH^-} even though the enzymatic reaction is likely to proceed by a mechanism which is entirely different from that induced by OH^-. This ratio for carbonate diesters varies by almost two orders of magnitude with the extremes represented by the 4-nitrophenyl esters metioned in the present report (Table III). It is tempting to ascribe the 100-fold ratio difference between the 4-nitrophenyl carbonate esters to the different steric requirements of the two esters, with bis(4-nitrophenyl) carbonate being much bulkier than methyl 4-nitrophenyl carbonate. However, the term k_{enz} equals k_{cat}/K_m (eq 10), and because of the already mentioned solubility

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} \text{product} + E \qquad K_m = (k_{cat} + k_r)/k_f \tag{10}$$

problems with these substrates, it cannot be determined whether k_{cat} or K_m or both cause this interesting ratio variation.[4] A comparative examination of acetazolamide inhibition of the enzyme-catalyzed hydrolysis of organic carbonates at high pH is also informative. In a previous report, the authors reported partial acetazolamide inhibition in regard to pyridine carbonate hydrolysis near pH 10, noting that the enzyme activity inhibited was always greater than that ascribed to the neutral pH rise in catalysis, k_α (see footnote 3). At pH 10.58 the enzyme-catalyzed hydrolysis of methyl 4-nitrophenyl carbonate is totally inhibited (see Figure 5B); thus it appears that the observed high pH activity is substrate sensitive, probably with the various carbonate substrates binding in overlapping sites, as acetazolamide at a given pH will bind in the same site. The pH 7 inflection in the rate profiles and the acetazolamide inhibition at neutral and higher pH values for the carbonic anhydrase catalyzed hydrolysis of organic carbonates point to binding sites and enzymatic modes of action which are similar to CO_2 hydration. However, the above-mentioned dissimilarities in catalytic behavior demonstrate that carbonic anhydrase does not accommodate even these carbonate esters equally in all details. The acetazolamide inhibition behavior at high pH, partial with the more water-soluble pyridine carbonates and complete with the more lipophilic 4-nitrophenyl substrates, underscores the role of hydrophobic interactions in the formation of the enzyme–substrate intermediate.

Aryl N,N-Dimethylcarbamates. The inability of carbonic anhydrase to significantly catalyze the hydrolysis of either of the two *N,N*-dimethylcarbamate esters mentioned in this report can be better understood in terms of the information contained in Table III. The spectrophotometric methodology employed to detect catalysis (see Results) allows an upper limit as to a value of k_{enz} for both carbamate esters to be placed at about 10^{-4} M^{-1} sec^{-1} at pH 10.6. Both esters have k_{OH^-} values of about 2.5×10^{-4} M^{-1} sec^{-1} (Dittert and Higuchi, 1963; Christenson, 1964); thus, the upper limit of the ratio k_{enz}/k_{OH^-} is about 0.4 at this pH. This limiting value is not so much lower than the ratios listed in Table III so as to allow a conclusion that carbamate esters are unreactive with respect to carbonic anhydrase catalysis. Studies on more reactive N,N-disubstituted carbamate esters, those which have k_{OH^-} values near 1 M^{-1} sec^{-1}, would seem to be more promising. In this regard, the carbamate ester 1-carbomethoxy-2-pyridone appears to be catalytically hydrolyzed by carbonic anhydrase, having a $k_{enz} \simeq 20$ M^{-1} sec^{-1} at pH 6.9 (Guilbert, 1971). It should also be noted that neostigmine bromide is a salt and would not be expected to bind well in a hydrophobic cavity unless a properly placed negative charge exists within the cavity as is observed for acetylcholinesterase (Wilson, 1960). The apparent high value of K_s for the neostigmine bromide–carbonic anhydrase complex dissociation indicates that this enzyme at pH 10.58 does not develop a site which even approximates the hydrophobic–anionic system in acetylcholinesterase.

Methyl Carbonate. The pH dependency of k_{enz} in regard to the carbonate diester, methyl 4-nitrophenyl carbonate, is such that k_{enz} increases with increasing pH (Figure 3). In contrast, the value of k_{enz} for the decomposition of methyl carbonate, an intermediate in the hydrolysis of the diester, decreases with increasing pH. It is likely that the lack of carbonic anhydrase catalyzed decomposition of methyl carbonate at pH values near 10 is due, at least in part, to the marked decrease in the ability of the enzyme to bind anions beyond pH 8 (Pocker and Stone, 1968a; Ward, 1970). A direct consequence of the above-mentioned enzymatic property is that the enzyme-catalyzed hydrolysis of the carbonate diester at high pH is unaffected by the hydrolysis intermediate, the methyl carbonate anion.

While the esters previously studied show an analogous behavior to CO_2 because of enzyme-associated catalytic properties such as pH–rate profiles (Pocker and Stone, 1967; Edsall, 1968; Lindskog *et al.*, 1971), it is significant that the ester, methyl carbonate, appears to resemble bicarbonate in its behavior. Indeed, methyl carbonate and bicarbonate are

[4] The Michaelis constant, K_m, for methyl 4-nitrophenyl carbonate is probably within an order of magnitude of the ester's maximum solubility in water. The above statement reflects a rule of thumb developed in our laboratories concerning the esterase function of carbonic anhydrase. The rule is simply that the ratio of the substrate's maximum solubility, $[S]_{max}$, to the respective Michaelis constant, K_m, is, within an order of magnitude, constant. The ratio $[S]_{max}/K_m$ is usually less than unity (Pocker and Stone, 1967; Guilbert, 1971; Pocker and Guilbert, 1972); thus, synthesis of very water-soluble substrates may not always aid in the separation of K_m and k_{cat}. This "rule of thumb" is in accord with the proposed hydrophobic nature of substrate-carbonic anhydrase interactions (Pocker and Storm, 1968; Chen and Kernohan, 1967). In addition, there are important instances where the ratio $[S]_{max}/K_m > 1$ (Pocker and Watamori, 1971; Guilbert, 1971).

structurally quite similar, the methoxy group of the former replacing the hydroxyl group of the latter. This structural change, however, has a marked effect on enzymatic catalysis. Table IV compares enzyme kinetic coefficients at pH 7.27.

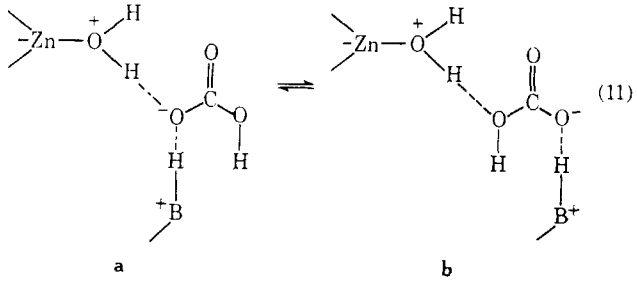
TABLE IV: Spontaneous and Carbonic Anhydrase Catalyzed Decarboxylations of Bicarbonate and Methyl Carbonate at pH 7.27.[a]

Substrate	k_{enz} (M^{-1} sec^{-1})	k_b (sec^{-1})	$k_{enz}[E]/k_b$
$HOCO_2^-$	2.5×10^6 [b]	3.5×10^{-3} [c]	7×10^2 [d]
$CH_3OCO_2^-$	3.2×10^3	6.4×10^{-3}	0.5[d]

[a] At 25.0°, ionic strength 0.1. [b] Calculated from the data of Kernohan (1964, 1965). [c] Obtained by the method of Magid and Turbeck (1968). [d] [E] = 10^{-6} M bovine carbonic anhydrase.

If the enzymatic reaction at pH 7.27 paralleled the nonenzymatic rate, one would expect k_{enz} ($=k_{cat}/K_m$) for the two carbonates to be about the same. The nonenzymatic first-order decarboxylation rate associated with methyl carbonate is about 1.8 times faster than that for bicarbonate; however, the k_{enz} value for bicarbonate exceeds that for methyl carbonate by three orders of magnitude. It is obvious from the data in Table IV that k_{enz} for bicarbonate reflects the very special relationship between carbonic anhydrase and its natural substrate. It behooves us then to examine the structural differences between natural and methylated carbonate substrates in terms of the mechanism of the breakdown of the probable enzyme–substrate complexes into products.

The affinity of carbonic anhydrase toward bicarbonate ion follows the order $I^- > HCO_3^- > NO_3^- > Br^-$, an order governed by the Hofmeister lyotropic series (Pocker and Stone, 1968a). If the binding characteristics toward methyl carbonate are similar then the three orders of magnitude difference in the respective k_{enz} values is probably attributable to a much larger k_{cat} in the case of bicarbonate. One of the more interesting implications of this line of reasoning is that the conversion of enzyme–bicarbonate complex to enzyme–carbon dioxide complex appears to require the labile bicarbonate proton. Although the CH_3–O and H–O bond energies are roughly the same, the activation energy difference involved in splitting them is very large (Frost and Pearson, 1961). It is thus probable that the presence of a kinetically unmovable methyl group is significant in the very large differences in ES breakdown rates. The presence of methyl on the enzyme-bound carbonate substrate may prevent an intramolecular substituent shift as depicted in eq 11. The enzyme-bound acid which transfers the proton in eq 11 may be water hydrogen bonded to protein, or it may be a protonated amino acid residue near zinc. Clearly structure b allows a rapid collapse to CO_2 plus water with facilitated electron flow toward zinc, a logical electron sink. Similarly if the methyl carbonate were placed as in b with the methyl group on the left, this structure would be ideal for decarboxylation. However, both bicarbonate and methyl carbonate would be expected to bind as in a, but only bicarbonate would be able to assume without rotation structure b. A consideration of detailed mechanisms concerning enzyme–methyl carbonate breakdown indeed includes such possibilities as molecular rotation to b prior to breakdown, or an intermolecular proton transfer to the ES complex in a, coupled with an electron flow away from zinc.[5] All such possibilities are relatively high-energy processes when compared to the intramolecular proton transfer available to HCO_3^-. It is then of interest as to how the enzyme decarboxylates methyl carbonate at all. Presently under way in our laboratories is a more extensive examination of the enzymatic and spontaneous decarboxylation of methyl carbonate. A parallel study involving bicarbonate is also being carried out. It is our hope that a quantitative comparison of chemical and enzymatic rate parameters in regard to bicarbonate and methyl carbonate will result in a greater understanding of the mechanistic constraints which enable carbonic anhydrase to rapidly turn over its natural substrates.



a b (11)

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References

Bell, R. P., and Darwent, B. (1950), *Trans. Faraday Soc. 46*, 34.
Bender, M. L., and Turnquest, B. W. (1957), *J. Amer. Chem. Soc. 79*, 1656.
Bender, M. L., and Wedler, F. C. (1972), *J. Amer. Chem. Soc. 94*, 2101.
Bruice, T. C., and Lapinski, R. (1958), *J. Amer. Chem. Soc. 80*, 2265.
Chen, R. F., and Kernohan, J. C. (1967), *J. Biol. Chem. 242*, 5813.
Christenson, I. (1964), *Acta Chem. Scand. 18*, 904.
Dittert, L. W., and Higuchi, T. (1963), *J. Pharm. Sci. 52*, 852.
Easson, L. H., and Stedman, E. (1936), *Proc. Roy. Soc., Ser. B 121*, 142.
Edsall, J. T. (1968), *Harvey Lect. 62*, 191.
Edsall, J. T., and Wyman, J. (1958), Biophysical Chemistry, Vol. I, New York, N. Y., Academic Press, p 571.
Faurholt, C. (1927), *Z. Phys. Chem., Stoechiom. Verwandschaftslehre 126*, 211.
Fife, T. H., and McMahon, D. M. (1970), *J. Org. Chem. 35*, 3699.
Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, New York, N. Y., Wiley.
Glatthard, R., and Matter, J. (1963), *Helv. Chim. Acta 46*, 795.
Guilbert, L. J. (1971), Ph.D. Dissertation, University of Washington.
Harned, H. S., and Owen, B. B. (1958), The Physical Chemistry of Electrolytic Solutions, 3rd ed, New York, N. Y., Reinhold, p 580.
Hutchins, J. E. C., and Fife, T. H. (1973), *J. Amer. Chem. Soc. 95*, 3786.

[5] The same considerations apply if in eq 11a and 11b the bicarbonate anion, or the methyl carbonate anion, is coordinated directly to the zinc ion of carbonic anhydrase.

Kernohan, J. C. (1964), *Biochim. Biophys. Acta 81*, 346.
Kernohan, J. C. (1965), *Biochim. Biophys. Acta 96*, 304.
Lellmann, E., and Benz, E. (1891), *Ber. Deut. Chem. Ges. 24*, 2108.
Lindskog, S. (1969), *NASA Spec. Publ. NASA* SP-188, 1570.
Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O., and Strandberg, B. (1971), *Enzymes, 3rd Ed., 5*, 587.
Magid, E. (1968), *Biochim. Biophys. Acta 151*, 236.
Magid, E., and Turbeck, B. O. (1968), *Biochim. Biophys. Acta 165*, 515.
Pocker, Y., and Beug, M. (1972), *Biochemistry 11*, 698.
Pocker, Y., and Dickerson, D. G. (1968), *Biochemistry 7*, 1995.
Pocker, Y., and Guilbert, L. J. (1972), *Biochemistry 11*, 180.
Pocker, Y., and Meany, J. E. (1965), *Biochemistry 4*, 2535.
Pocker, Y., and Meany, J. E. (1967), *Biochemistry 6*, 239.
Pocker, Y., and Meany, J. E. (1970), *J. Phys. Chem. 74*, 1486.
Pocker, Y., and Stone, J. T. (1965), *J. Amer. Chem. Soc. 87*, 5497.
Pocker, Y., and Stone, J. T. (1967), *Biochemistry 6*, 668.
Pocker, Y., and Stone, J. T. (1968a), *Biochemistry 7*, 2936.
Pocker, Y., and Stone, J. T. (1968b), *Biochemistry 7*, 3021.
Pocker, Y., and Storm, D. R. (1968), *Biochemistry 7*, 1202.
Pocker, Y., and Watamori, N. (1971), *Biochemistry 10*, 4843.
Pocker, Y., and Watamori, N. (1973), *Biochemistry 12*, 2475.
Tashian, R. E., Douglas, D. P., and Yu, T. L. (1964), *Biochem. Biophys. Res. Commun. 14*, 256.
Thorslund, A., and Lindskog, S. (1967), *Eur. J. Biochem. 3*, 117.
Verpoorte, J. A., Mehta, S., and Edsall, J. T. (1967), *J. Biol. Chem. 242*, 4221.
Ward, R. L. (1970), *Biochemistry 9*, 2447.
Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, Vol. 1, New York, N. Y., Academic Press, p 71.
Wilson, I. B. (1960), *Enzymes, 2nd Ed., 4*, 501.

Kidney Phenylalanine Hydroxylase from Man and Rat. Comparison with the Liver Enzyme†

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ABSTRACT: The recent observation that phenylalanine hydroxylase is present in kidney, as well as in liver, poses the question of whether the enzymes from the two organs are the same, or whether they are tissue-specific isozymes. A comparison of liver and kidney enzymes from man and rat was made on the basis of kinetic parameters and immunological reactivity. The apparent Michaelis constant for tetrahydrobiopterin and the relative rates with tetrahydrobiopterin and tetrahydro-6,7-dimethylpterin were different for the enzymes from different species, but were identical for kidney and liver enzymes of the same species, as also were the apparent Michaelis constants for phenylalanine and tetrahydro-6,7-dimethylpterin. Antibodies to rat liver phenylalanine hydroxylase reacted identically with rat liver and rat kidney enzymes. Both human kidney and human liver phenylalanine hydroxylase showed only 4% of the reactivity of the rat enzymes indicating that 4% of the antibody binding sites of the rat enzymes are present on the human enzymes. Rat liver and kidney enzymes were also compared by their elution patterns and equilibrium distribution on Bio-Gel A-5, and by the effect of phenylalanine on their sedimentation characteristics in sucrose density gradient centrifugation. Neither of these criteria, which are reflections of the molecular size, density, and shape, could differentiate between the kidney and liver enzymes. From these results a tentative conclusion can be made that phenylalanine hydroxylase in kidney and liver is the same enzyme. If tissue-specific isozymes do exist they could not be detected by any of the above criteria.

Until recently phenylalanine hydroxylase (EC 1.14.3.1) had been detected in mammals only in the liver. It was first shown to be present in kidney in the mouse in 1969 (Tourian *et al.*, 1969), and since then has also been reported in the kidney of rat and guinea pig (McGee *et al.*, 1972; Berry *et al.*, 1972).

We have found that phenylalanine hydroxylase is also present in human kidney cortex, at a level of about 20% of that found in human liver (Ayling *et al.*, 1973c, 1974). We wished to establish whether or not the liver and kidney enzymes are identical, or whether they are tissue-specific isozymes. This is of particular interest since it has been shown that in phenylketonuria, the part of the phenylalanine hydroxylating system which is defective or missing (Jervis, 1953) is the hydroxylase itself, and not the cofactor, or cofactor regenerating system (Justice *et al.*, 1967).

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