The Catalytic Versatility of Erythrocyte Carbonic Anhydrase. II. Kinetic Studies of the Enzyme-Catalyzed Hydration of Pyridine Aldehydes*

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ABSTRACT: The present investigation establishes that carbonic anhydrase from bovine erythrocytes very powerfully and reversibly catalyzes the hydrations of 2-, 3-, and 4-pyridine aldehydes. These reactions were studied spectrophotometrically in diethylmalonate buffers. Since such hydrations are subject to general acid–general base catalysis, the major advantage in using diethylmalonate buffers is that at physiological pH the threshold of catalysis associated with the mono- and dianions of diethylmalonic acid is low in comparison to the enzymatic rate. The dependence of the enzymatic rate on substrate concentration formally obeys the Michaelis–Menten relationship. The increase in over-all enzymatic catalysis, k_2/K_m, for the reversible hydration of 2- and 4-pyridine aldehydes in the pH region 6–8 is only about one-half that observed for the corresponding enzymatic hydration of acetaldehyde. Indeed, while the point of inflection in the pH–rate profile for the enzymatic hydration of acetaldehyde occurs at pH 7.0, that for the corresponding pyridine aldehyde hydrations appears to fall below pH 6. Whereas the pyridine aldehydes seem to form more stable complexes with bovine carbonic anhydrase than acetaldehyde, the turnover numbers are significantly larger in the enzymatic hydration of the latter. The hydrations of 2- and 4-pyridine aldehydes are competitively inhibited by acetazolamide. This is contrasted with the noncompetitive inhibition observed in the enzymatic hydration of both CO_2 and acetaldehyde by acetazolamide. The modification in binding and turnover number induced by the use of these "abnormal" substrates containing a ring nitrogen is discussed in terms of their capacity to coordinate with the zinc ion in bovine carbonic anhydrase which with "normal" substrates is assumed to be associated only with the hydration step.

Earlier claims pertaining to the absolute specificity of erythrocyte carbonic anhydrase (carbonate hydrolyase EC 4.2.1.1) (CA)[1] with respect to the reversible hydration of carbon dioxide have been generally accepted (Davis, 1961; White *et al.*, 1964). However, this enzyme has been shown in our laboratories to be a powerful catalyst for the reversible hydration of acetaldehyde (Pocker and Meany, 1964, 1965a,b), and related aliphatic aldehydes (Y. Pocker and D. G. Dickerson, 1965, unpublished data) as well as for the hydrolysis of *p*-nitrophenyl acetate (Pocker and Stone, 1965) and related esters (Y. Pocker, L. J. Guilbert, and D. R. Storm, 1966, unpublished data). All these substrates formally obey the classical Michaelis–Menten relationship and in general show that their binding to the enzyme is independent of hydronium ion concentration around physiological pH values. The apparent turnover number, k_2, dictates the observed increase in enzymatic rate with pH. In all these cases the basic form of an ionizable group of pK_R around 7 is implicated. A scrutiny of the steric requirements of the active site shows that the catalytic efficiency of BCA with respect to aldehyde hydration follows the order $CH_3CHO > CH_3CH_2CHO > (CH_3)_2CHCHO >> (CH_3)_3CCHO$, an order dictated by the turnover number rather than by binding which actually follows the reverse order (Pocker *et al.*, 1965).

The catalytic versatility of the zinc ion firmly incorporated into the structure of the native enzyme should be contrasted with the negligible activity possessed by the organic matrix of the protein molecule. Different avenues of approach are thus offered for the delineation of the active site in erythrocyte CA. In the present paper we examine the versatility of BCA with respect to substrates which contain the elements necessary for both coordination to the metal and hydration.

Experimental Section

Materials. 2-, 3-, and 4-pyridine aldehydes were purchased from Aldrich Chemical Co., Inc., and were twice distilled under nitrogen at 12 mm of pressure

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[1] Abbreviations used in this work: CA, carbonic anhydrase; BCA, bovine carbonic anhydrase; PA, pyridine aldehyde.

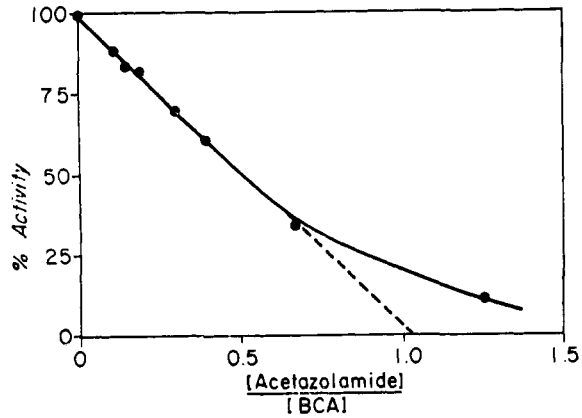

FIGURE 1: Acetazolamide inhibition of the enzymic hydration of acetaldehyde in 0.01 M diethylmalonate buffer (pH 7.2) at 0.0°. [BCA] = 1.05×10^{-5} M.

at the following temperatures: compounds 2-PA, 3-PA, and 4-PA at T_B 60–61, 82–83, and 76–77°, respectively. The distillations were always carried out directly before use to avoid the possibility of oxidation of these substrates. The dianion of diethylmalonic acid was prepared by refluxing the ethyl ester in strong alkali. Neutralization of the dianion with HCl produced diethylmalonic acid, $K_1 = 6.15 \times 10^{-3}$, $K_2 = 5.1 \times 10^{-8}$ (Gane and Ingold, 1931), which was purified by recrystallization from benzene (mp 128–128.5°).

Baker Analyzed reagent grade acetonitrile was distilled in the presence of CH_2Cl_2 used to remove trace amounts of H_2O so that the compound could be employed as a suitable solvent for the pyridine aldehydes when extremely small amounts of substrate were desired.

Bovine carbonic anhydrase (BCA) was a product of Mann Research Laboratories prepared and purified from bovine erythrocytes by the method of Keilin and Mann (1940). The enzyme was stored dry at −20° and its activity was periodically determined by observing its catalytic effect on acetaldehyde hydration in 0.002 M phosphate buffer at pH 7.12. During the course of the experiments described in this paper, this method of enzyme assay showed that no variation in activity occurred.

Since BCA contains one zinc ion per molecule, we were able to standardize several enzyme solutions by assay of zinc *via* the dithizone method described by Malmstrom (1953). Zinc ion content was further confirmed by atomic absorption spectrophotometry. Protein content was then calibrated at 280 mμ and found to have an ϵ of 54,000 based on a molecular weight of 30,000 which is in perfect agreement with that obtained by Lindskog (1960). Thus, ϵ was employed in deducing enzyme concentrations used in our kinetic studies.

Our enzyme assay was further documented by testing acetazolamide inhibition of BCA with respect to acetaldehyde hydration. Acetazolamide concentration alone was varied throughout a series of runs. A plot of enzyme activity *vs.* the ratio of inhibitor/enzyme concentration shows that an extrapolation to zero activity occurs at a 1/1 molar ratio of inhibitor/enzyme (Figure 1). Since there is evidence that acetazolamide inhibits specifically by coordination at or near the zinc ion in CA (Malmstrom *et al.*, 1964; Tilander *et al.*, 1965), this experiment confirms our BCA assay and implies that the enzyme preparations employed in this investigation contain no other active catalyst.

Control experiments involving the denaturation of BCA substantiate in part the above findings by indicating the absence of catalytic contaminants of nonproteinic nature. Thus, a solution of 2.0×10^{-5} M BCA in 0.01 M diethylmalonate buffer (pH 7.20) was heated to 70° until the precipitation of the enzyme occurred. After centrifugation, no catalysis with respect to both 2- and 4-pyridine aldehyde hydration was observed. The rates of hydration in the above solution were identical with those observed in pure 0.01 M diethylmalonate buffer at this pH.

Apparatus. The reactions were followed on a Gilford high-speed recording spectrophotometer, Model 2000. An insulated cell compartment in place of the conventional chamber was attached to the Gilford. The compartment consisted of a bath containing a mixture of water and methanol. An internal coil through which coolant (methanol) flowed lowered the temperature of the bath as required. The temperature of the circulating coolant was kept at about −5° allowing the cell compartment to be thermostated to 0.0 ± 0.02° by means of a Sargent Model SV (S-82060) thermonitor unit.

The phototube housing of the Gilford instrument is neither enclosed in an air-tight fashion nor equipped with desiccant. Consequently, for runs performed at 0.0°, it was necessary to allow a constant stream of dry nitrogen gas to flow into this compartment so that shorting of the phototube by condensation could be avoided. Nitrogen gas was also used to eliminate water vapor condensing on the outside of the windows at the entrance to and the exit from the cell compartment.

Method. The hydration kinetics considered in this paper are similar to those described for acetaldehyde hydration in an earlier publication (Pocker and Meany, 1965b) and the pseudo-first-order rate constants are deduced in the same fashion. However, the hydration of the pyridine aldehydes proceeds much more rapidly.

All values of k_{obsd} in this paper refer to the sum of first-order rate coefficients for the forward, k_f, and for the reverse, k_r, processes, $k_{obsd} = k_f + k_r$, at 0.0°. Such data is converted to first-order rate coefficients for the forward reaction, k_f, by multiplying by the fraction of hydration, χ, at 0.0°: substrates 2-PA, 3-PA, and 4-PA had χ 0.48, 0.28, and 0.66, respectively. The apparent fractions of hydration, $\chi = (A_0 - A_\infty)/A_0$, were obtained from kinetic runs in which the initial absorbancies of the pyridine aldehydes, A_0, were determined by extrapolation to zero reaction, and the final absorbancies, A_∞, were those observed from the equilibrated reaction solutions.[2]

Since one must be cautious in using the spectrophotometric method for the determination of fractions of hydration of heteroaromatic aldehydes, we have further confirmed the validity of these observations by comparing values of χ obtained spectrophotometrically in D_2O both at 25 and 40° to those obtained from the nuclear magnetic resonance (nmr) spectra associated with the hydrated and unhydrated forms of the pyridine aldehydes under the same conditions.

The spectrophotometric determinations of χ for each of the pyridine aldehydes were carried out at 278, 305, and 320 mμ and were found to be independent of wavelength in this region. Furthermore, the presence of BCA or inhibitors in the concentrations employed in this work had no detectable effect on these values. A typical run involving the hydration of 4-PA is shown in Figure 2 in which a plot of log $(A_t - A_\infty)$ *vs.* time gives a straight line with $k_{obsd} = -(2.3)$(slope), and $k_f = -(2.3)(\chi)$(slope).

For the data described in this paper, the aldehydes were injected neat into the reaction solution using calibrated Hamilton syringes. Most of this work involves the determination of enzymatic catalysis as a function of substrate concentration. Since relatively high substrate concentrations had to be employed these runs were followed not at λ_{max} but at wavelengths where the molar extinction coefficient of each pyridine aldehyde allowed accurate spectrophotometric measurements. The values of ϵ at the wavelengths employed for each of the pyridine aldehydes are: substrates 2-PA, 3-PA, and 4-PA had λ 305, 305, and 320 and ϵ 50, 48, and 33, respectively, were ϵ values refer to the molar absorbancies of the pyridine aldehydes in equilibrium with their respective hydrates.

It should be noted that the enzymatic catalysis for each run was evaluated by subtracting the buffer-catalyzed portion of the reaction from the over-all catalysis. In general, enzyme concentrations were of such magnitude that the enzymatically catalyzed reaction accounted for about 90% of the over-all rate.

Results

The measurement of the hydration of the pyridine aldehydes using the equipment described in the Experimental Section affords results which are generally reproducible within ±2%. The enzyme concentrations employed were such that the first-order rate coefficients

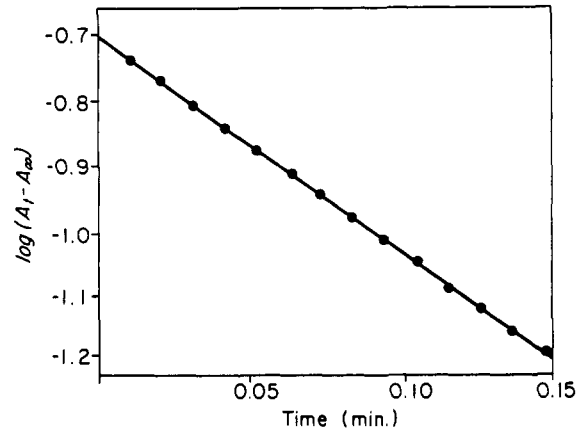

FIGURE 2: A typical 4-pyridine aldehyde hydration run with 4.3×10^{-6} M BCA in 0.01 M diethylmalonate buffer (pH 7.22) at 0.0°. [4-PA] = 8.09×10^{-5} M. $k_f = (\chi)(k_{obsd}) = -(2.3)(0.66)$(slope) = 5.15 min^{-1}.

obtained, k_{obsd}, usually ranged from 1 to 10 min^{-1}.

Our kinetic studies of the reversible hydrations of 2-, 3-, and 4-pyridine aldehydes show them to be general acid–general base catalyzed processes (Y. Pocker and J. E. Meany, unpublished data, 1966). In the presence of enzyme, the over-all rate coefficients for the forward process, k_f, determined in a buffered solution consist of a sum of catalytic terms as given in eq 1

$$k_f = k_0 + K_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + k_{HA}[HA] + k_{A^-}[A^-] + k_{enzyme}[E] \quad (1)$$

We have chosen low substrate concentrations of both 2-PA and 4-PA (where [PA] $< < K_m$) in order to show that under such conditions the enzymatically catalyzed reaction is indeed first order in enzyme. These experiments were carried out at pH 7.20 in 0.01 M diethylmalonate buffer.

For reactions following eq 1 it is necessary to isolate the enzymatically catalyzed portions of these hydrations from the contributions of other catalytically active molecules. Consequently, in the pH range 6–8, we searched for a buffer system whose threshold of catalysis is low in comparison to the enzymatic rate. The use of diethylmalonate buffers was found to be advantageous for studying these hydrations in that the components of catalysis associated with the mono- and dianions of diethylmalonic acid are sufficiently low so that with 0.01 M buffer the forward catalytic rate constant, k_f, is essentially given by eq 2

$$k_f = k_0 + k_{OH}[OH^-] + k_{enzyme}[E] \quad (2)$$

pH–rate profiles for the nonenzymatic hydration of 2- and 4-pyridine aldehydes in 0.01 M diethylmalonate buffers appear in Figure 3. The rate coefficient k_{OH^-} for 2- and 4-PA can be deduced from plots of k_f *vs.*

[2] In previous papers (Pocker and Meany, 1965a,b) we have followed the general procedure of presenting the kinetic data for strictly reversible reactions in terms of the experimental rate coefficient for equilibration, k_{obsd}, which is actually the sum of forward and reverse rate coefficients, $k_{obsd} = k_f + k_r$. Hence, k_{enzyme} referred to the sum of rate coefficients for the enzymatic hydration and dehydration. In this paper, catalytic rate coefficients for several substrates are compared. Thus, it is more meaningful to represent the corresponding values for the forward processes alone since their respective fractions of hydration differ.

TABLE I: Results from Lineweaver–Burk Plots for BCA-Catalyzed Hydration of 2- and 4-Pyridine Aldehydes in 0.01 M Diethylmalonate Buffers at 0.0°.

			2-Pyridine Aldehyde			
pH	6.15	6.80	7.15	7.53	8.00	7.15[a]
K_m (mole l.$^{-1}$)	0.014	0.015	0.014	0.013	0.011	0.033
k_2 (min^{-1})	3,100	4,100	4,600	4,300	4,100	4,400
			4-Pyridine Aldehyde			
pH	6.35	6.89	7.18	7.54	8.03	7.18[b]
K_m (mole l.$^{-1}$)	0.012	0.011	0.011	0.012	0.012	0.033
k_2 (min^{-1})	9,200	9,900	11,400	12,300	13,400	11,500

[a] In the presence of 9.54×10^{-5} M acetazolamide. [b] In the presence of 9.90×10^{-5} M acetazolamide.

hydroxide ion concentration.

The pH dependencies for the enzymatic hydrations of 2- and 4-pyridine aldehydes were studied as a function of substrate concentration. Lineweaver–Burk plots were used to determine K_m and k_2 for the enzymatic hydrations of 2- and 4-pyridine aldehydes at various pH values.[3] These results are given in Table I.

[3] The constants k_1, k_{-1}, and k_2 are the specific rate constants in the simplified mechanism

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

When S represents 2-, 3-, or 4-pyridine aldehyde, the reaction velocity may be expressed as

$$-\frac{d[S]}{dt} = \frac{d[P]}{dt} = \frac{(V_m/K_m)[S]}{1 + \frac{[S]}{K_m}} \qquad \text{(ii)}$$

It is realized that the kinetic parameters determined experimentally may be considerably more complex than indicated by the above formulation. Indeed, the simplest mechanism by which a strictly reversible enzyme-catalyzed process might be expected to proceed is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EX \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} E + P \qquad \text{(iii)}$$

where EX is the enzyme–substrate complex common to both aldehyde and hydrate. The concentration of water which is formally also a substrate might be included in k_1 of k_2. The steady state treatment of this mechanism yields the following equation for the reaction rate (Haldane, 1930)

$$-\frac{d[S]}{dt} = \frac{d[P]}{dt} = \frac{(V_m^f/K_m^f)[S] - (V_m^r/K_m^r)[P]}{1 + \frac{[S]}{K_m^f} + \frac{[P]}{K_m^r}} \qquad \text{(iv)}$$

This rate equation is also in agreement with the experimental findings when account is taken of the fact that when reciprocal initial velocities are plotted *vs.* reciprocal initial concentrations of substrates, we are dealing with a case where [P] = 0 and eq iv becomes equivalent to eq ii.

Because hydroxide ion catalysis is dominant in the region pH >8, determination of enzymatic catalysis in these basic media becomes inaccurate. Determinations of K_m and k_2 values are also problematical at pH <6 because it is difficult to maintain a constant pH while at the same time using low buffer concentrations necessary to minimize enzyme inhibition by the anionic components of the buffer. The change in pH arises because the conjugate acids of the pyridine aldehyde–pyridine aldehyde hydrate systems are such as to induce pH increases with increasing substrate concentration. Nevertheless, employing acetate buffers, approximate values of the ratio k_2/K_m were obtained for both 2- and 4-pyridine aldehydes at pH 5.1. These were found to be only about 15% of those observed at the pH values at which maximal enzymatic activity occurs for each of the pyridine aldehydes.

The maximum values of k_2/K_m, as defined by $k_{enzyme-(max)}$, for the enzymatic hydrations of 2- and 4-pyridine aldehydes are compared to the corresponding catalyses

It is mechanistically difficult to visualize a common complex being formed between enzyme–aldehyde and enzyme–hydrate. Consequently, it would appear more reasonable to anticipate two or more complexes where k_2 would be the actual hydration step and k_{-2} the actual dehydration step

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} EP \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E + P \qquad \text{(v)}$$

where now the experimental Michaelis constant for the forward process becomes

$$K_m^f = \frac{k_{-1}k_3 + k_{-1}k_{-2} + k_2k_3}{(k_2 + k_{-2} + k_3)k_1} \qquad \text{(vi)}$$

and the forward maximum velocity is represented by

$$V_m^f = \frac{k_2k_3[E]_0}{k_2 + k_{-2} + k_3} \qquad \text{(vii)}$$

Since all these mechanisms reduce to the same over-all equation at [P] = 0, they are kinetically indistinguishable when steady-state conditions are operative.

TABLE II: Comparison of Enzymatic Catalysis to Hydroxide Ion Catalysis at 0.0°.

Substrate	k_{OH^-} (l. mole^{-1} min^{-1})	$k_{enzyme\ (max)}$[a] (l. mole^{-1} min^{-1})	$k_{enzyme(max)}/k_{OH^-}[OH^-]$[a] (l. mole^{-1})	k_2 (min^{-1})	$k_2/k_{OH^-}[OH^-]$[a]
2-PA	2.6×10^6	3.7×10^5	1.2×10^6	4,100	13,000
4-PA	7.3×10^6	1.1×10^6	1.3×10^6	13,400	16,100
Acetaldehyde	7.0×10^5	8.4×10^4	1.1×10^6	54,000	676,000

[a] Catalytic rate coefficients for the forward processes taken at pH 8.0.

by hydroxide ions at pH 8.0 in Table II. Similar data has been included for the hydration of acetaldehyde. Interestingly enough, the ratio of BCA catalysis to that by hydroxide ions for acetaldehyde hydration is of the same order of magnitude as that found for the hydration of 2- and 4-pyridine aldehydes.

The enzymatically catalyzed hydration of 3-pyridine aldehyde was also studied as a function of substrate concentration at pH 7.2 and allows a comparison of the relative values of K_m and k_2 for the reversible hydration of the aldehydes which have thus far been investigated. Table III summarizes this data together

TABLE III: Comparison of the Kinetic Parameters, k_2 and K_M, for the BCA-Catalyzed Hydrations of Acetaldehyde, 2-, 3-, and 4-Pyridine Aldehydes at pH 7.2.

Substrate	2-PA[a]	3-PA	4-PA[a]	Acetaldehyde
k_2 (min^{-1})	4,600	2,000	11,400	48,000
K_m (mole l.$^{-1}$)	0.014	0.007	0.011	0.65

[a] The values given for K_m and k_2 for 2-PA and 4-PA are interpolated from values at pH 7.15 and 7.18, respectively.

with that obtained for acetaldehyde using the same enzyme preparation. It will be noticed that the turnover numbers, k_2, follow the order (CH_3CHO) > (4-PA) > (2-PA) > (3-PA), while K_m varies in the order (3-PA) < (4-PA) $\leqslant$ (2-PA) < (CH_3CHO).

Inhibition studies were carried out on the enzymatic hydration of 2- and 4-pyridine aldehydes. The activity of BCA approaches zero with increasing acetazolamide concentration.

Additionally, these enzymatic processes were studied as a function of substrate concentration in the presence of acetazolamide. The inhibited reactions also followed Michaelis–Menten kinetics and were analyzed *via* Lineweaver–Burk plots. The constancy in K_m and variation in k_2 categorizes these inhibited processes as

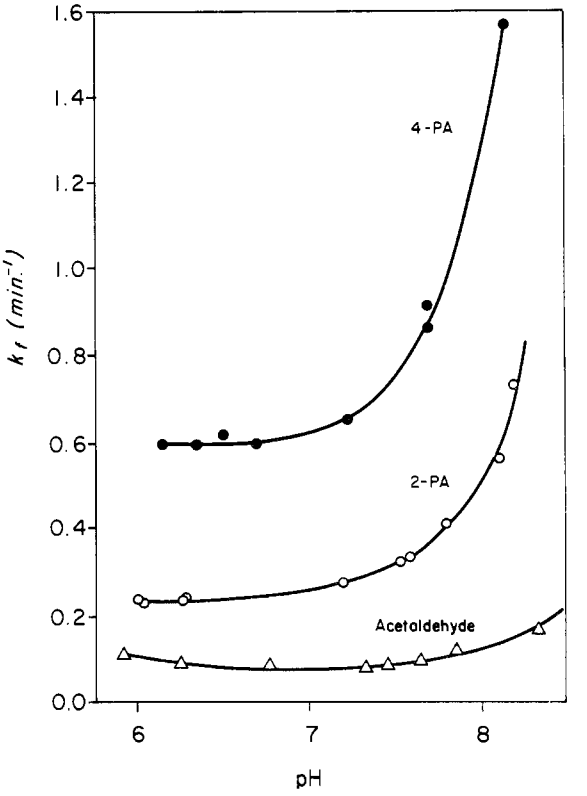

FIGURE 3: Catalysis of the 2- and 4-pyridine aldehydes' hydration by 0.01 M diethylmalonate buffers as a function of pH at 0.0°. Catalysis of acetaldehyde hydration added for comparison.

competitive. The apparent dissociation constant, K_i, of the complex formed between BCA and acetazolamide was calculated from these reciprocal plots and is given in Figures 4 and 5.

Discussion

The noncompetitive inhibition observed with specific reagents which generally coordinate to zinc ions, implies that protein-bound zinc is not the binding site in either CO_2 or acetaldehyde hydration. Consequently, we were interested to explore the possibility of enzymatic

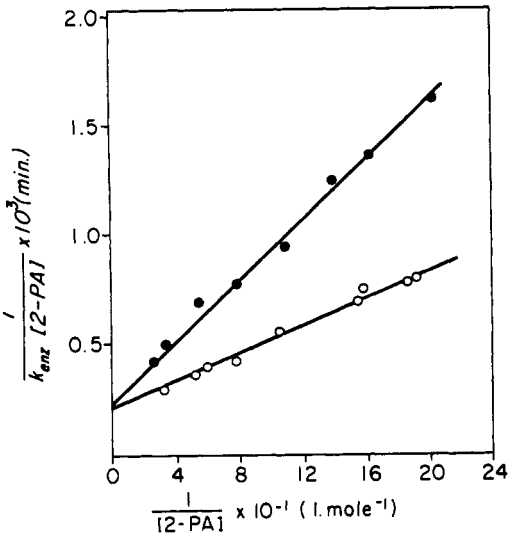

FIGURE 4: Michaelis–Menten kinetics for the inhibited and uninhibited enzymatic hydrations of 2-pyridine aldehyde. All runs were made in 0.01 M diethylmalonate buffer (pH 7.15) at 0.0°. ○, BCA-catalyzed hydrations. K_m = 0.014 mole l.$^{-1}$. ●, BCA-catalyzed hydrations inhibited by 9.54 × 10^{-5} M acetazolamide. K_m = 0.033 mole l.$^{-1}$. k_2 = 4400 min.$^{-1}$. From the Michaelis–Menten parameters associated with the inhibited and uninhibited processes, K_i = 8.2 × 10^{-5} M.

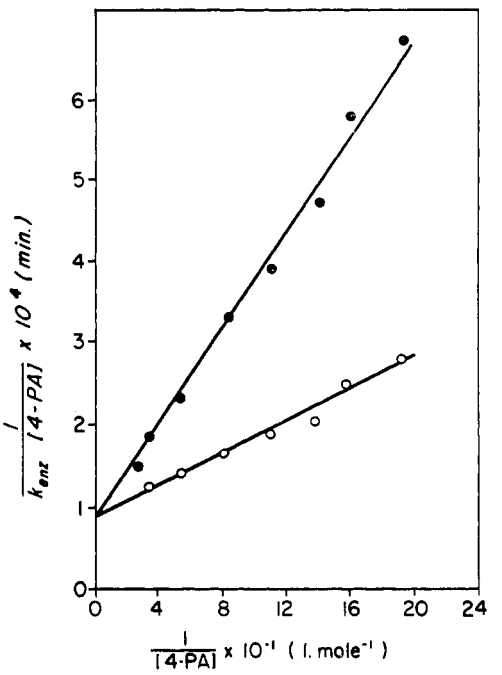

FIGURE 5: Michaelis–Menten kinetics for the inhibited and uninhibited enzymatic hydrations of 4-pyridine aldehyde. All runs were made in 0.01 M diethylmalonate buffer (pH 7.18) at 0.0°. ○, BCA-catalyzed hydrations. K_m = 0.011 mole l.$^{-1}$. k_2 = 11,400 min^{-1}. ●, BCA-catalyzed hydrations inhibited by 9.9 × 10^{-5} M acetazolamide. K_m = 0.033 mole l.$^{-1}$. k_2 = 11,500 min^{-1}. From the Michaelis–Menten parameters associated with the inhibited and uninhibited processes, K_i = 4.8 × 10^{-5} M.

catalysis involving "abnormal" substrates which are bifunctional in character. The unprotonated pyridine aldehydes satisfy this requirement in that they not only possess a carbonyl group which hydrates with ease, but also have the capacity to interact with enzyme bound zinc *via* the ring nitrogen.

BCA proved to be an efficient catalyst for the hydrations of the three pyridine aldehydes. It was evident from the linear relationship between the reciprocals of reaction velocity and substrate concentration that the carbonic anhydrase catalysis can be formally analyzed in terms of the Michaelis–Menten treatment.

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

When S represents 2-, 3-, or 4-pyridine aldehyde, the reaction velocity may be expressed as

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

Consequently, it was possible to analyze the enzymatic catalysis in terms of the experimentally determined Michaelis constant, $K_m = (k_{-1} + k_2)/k_1$, and the turnover number, k_2.

Earlier work has shown that the enzymatic hydration of acetaldehyde can also be analyzed in terms of the Michaelis–Menten treatment (Pocker and Meany 1964, 1965b). Account being taken of the data presented in this paper, it will be noted that the apparent binding between BCA and the heterocyclic aldehydes is much stronger than that with acetaldehyde. There may be additional opportunities for enzyme–substrate interaction with the pyridine aldehydes due to the presence of the ring nitrogen. These additional modes of interaction may involve either hydrogen bonding with suitable acidic groups in the enzyme, or chelation of the pyridine aldehydes to protein-bound zinc. *A priori*, 2-pyridine aldehyde, because it can theoretically act as a bidentate ligand, might be expected to form a more stable enzyme–substrate complex. However, the apparent binding between these substrates and BCA follows the order: 3-PA > 4-PA $\geqslant$ 2-PA. Perhaps this is not surprising since *o*-phenanthroline, a powerful bidentate ligand having a high capacity for complexing with divalent zinc, does not inhibit BCA except by removal of the zinc ion on prolonged incubation (Lindskog and Malmstrom, 1960). This could suggest that the zinc ion in the native enzyme is associated with the proteinic part in such a way as to leave only one additional site available for chelation with an external ligand (*e.g.*, H_2O, certain BCA inhibitors, or perhaps even the pyridine aldehydes). Whereas the

pyridine aldehydes appear to form more stable complexes with BCA than does acetaldehyde, the turnover numbers are significantly larger in the enzymatic hydration of the latter (see Table III).

Since the enzymatic catalysis depends essentially on the binding between the enzyme and substrate and the rate of breakdown of the active enzyme–substrate complex, the "over-all" catalytic effect may be defined as the ratio, $k_2/K_m = k_{enzyme(max)}$. Our experimental rate coefficient, k_{enzyme}, indeed becomes equivalent to k_2/K_m when substrate concentrations are minute compared to K_m (see eq B).

It is interesting to compare the magnitudes of $k_{enzyme(max)}$ to the catalysis of these hydrations by hydroxide ions for the aldehydes investigated. Such a comparison indicates that at pH 8.0, the ratios of $k_{enzyme(max)}/k_{OH^-}$ $[OH^-]$ for the 2-pyridine aldehyde, 4-pyridine aldehyde, and acetaldehyde, vary by only about $\pm 10\%$ from the average value of 1.2×10^6.

We envisage the turnover number, k_2, as governing the hydration step, so that a more meaningful comparison may be given by the ratio $k_2/k_{OH^-}[OH^-]$ at pH 8.0 (see Table II). Such a comparison shows that the efficiency of the turnover number relative to hydroxide ion catalysis is about 40 times greater for acetaldehyde than for either 2- or 4-pyridine aldehyde.

Acetazolamide inhibits the enzymatically catalyzed hydration of 2- and 4-pyridine aldehydes; the inhibition becoming complete with increasing quantities of the inhibitor. The inhibited reactions also formally obey Michaelis–Menten kinetics as indicated by the linearity of the reciprocal plots used to deduce the Michaelis–Menten parameters for the inhibited processes. The equality in turnover numbers observed for the inhibited and uninhibited processes indicate that the breakdown of the enzyme–substrate complex, k_2, is unaffected by acetazolamide. These investigations suggest that the 2- and 4-pyridine aldehydes interact with a site at or near the zinc ion in BCA.

It should also be noted that acetazolamide does not inhibit the enzymatic hydrations of the pyridine aldehydes (see Figures 4 and 5) as potently as the corresponding acetaldehyde hydration where $K_i = 6.1 \times 10^{-7}$ mole $l.^{-1}$ (Pocker and Meany, 1965b). We first considered the possibility of interaction between the pyridine aldehydes and the inhibitor since such combination could provide a rational for this phenomenon. We were able to find no evidence to confirm this. Ultraviolet spectra of the inhibitor and substrates, separately and together, in the range 220–320 mμ showed no new peaks resulting from the addition of the inhibitor to either substrate. Also, the absorbancies of all peaks for the mixture could be accounted for by a superposition of the corresponding spectra of inhibitor and substrates taken individually. Further, the presence of relatively high acetazolamide concentrations (0.00337 M) did not alter the apparent fraction of hydration of 0.005 M 4-pyridine aldehyde, whereas one may have expected a change in this constant if such interaction had occurred.

Finally, the rate of hydration of acetaldehyde in the presence of a mixture of 2×10^{-5} M BCA, 1.78×10^{-4} M acetazolamide, and 0.005 M 4-pyridine aldehyde was determined. It was found that the inhibition of the enzymatic process was not reduced by the presence of 4-pyridine aldehyde, this implying that there is little or no interaction between acetazolamide and the heterocyclic aldehyde which might diminish the availability of the inhibitor.

Ideally, one would expect that the magnitude of inhibition constants with respect to a particular enzyme and inhibitor would be independent of the substrate, provided inhibition reaches completion with increasing inhibitor concentration. However, Klein (1960) observed similar anomalies in the inhibition of pig kidney D-amino acid oxidase by benzoate where determinations of K_i from Lineweaver–Burk plots led to different values for various substrates.

Perhaps the most striking result which evolved from our inhibition studies is that the inhibitory effect caused by acetazolamide with respect to the enzymatic hydration of acetaldehyde and the pyridine aldehydes differs in type (noncompetitive as opposed to competitive), not only in magnitude. Consequently, it may be suggested that the binding site of BCA is at least modified to accommodate the pyridine aldehydes. If interactions were to occur between substrate and zinc, enzymatic hydration might have been completely eradicated. Since enzymatic activity is still operative in spite of such interactions, one may expect that the mode of enzyme action has become significantly distorted. Such distortions could induce competitive inhibition by inhibitors which normally act noncompetitively in the hydration of CO_2 and acetaldehyde. At present, one cannot exclude the possibility that the hydrating site is also altered when the enzyme is complexed to the various pyridine aldehydes. It now appears that the enzyme is not only capable of promoting the hydration of a number of substrates, but possesses versatility with respect to its mode of action also.

Earlier investigations (Lindskog and Malmstrom, 1962) indicate that the one zinc ion in each molecule of erythrocyte BCA is an obligatory component for the enzymatic hydration of CO_2. The complete inhibition of the BCA-catalyzed hydration of acetaldehyde and the pyridine aldehydes by acetazolamide also suggests the necessity of the zinc ion for these processes as well. Evidence pointing to the generality of the effectiveness of zinc ions as a hydrating catalyst was furnished by our investigations involving the use of aqueous solutions of divalent zinc in various buffers as catalytic media. From these studies, it was observed that zinc ions in the presence of imidazole buffers coordinate to four imidazole molecules and provide an efficient catalyst for the hydration of acetaldehyde (Pocker and Meany, 1964, 1965a,b). As a logical extension of these studies, we have investigated divalent metal ion catalysis of a compound which could act simultaneously both as substrate and complexing agent. 2-Pyridine aldehyde seemed to satisfy both of these requirements. The relative catalytic effectiveness of divalent cobalt and zinc for the hydration of this compound could be

conveniently compared to that of one of its isomers, 4-pyridine aldehyde (Y. Pocker and J. E. Meany, 1966, unpublished data). Thus, we find divalent cobalt and zinc to be extremely potent catalysts for the hydration of 2-pyridine aldehyde ($k_{Zn^{2+}}$ = 27,900 l. mole^{-1} min^{-1}; $k_{Co^{2+}}$ = 32,500 l. mole^{-1} min^{-1}). The catalysis of the reactions of enzyme substrates or their analogues by metal ion is of particular interest since such reactions may reveal mechanistic features characteristic of catalysis by metallo enzymes.

It should be recalled that the pH–rate dependency of the enzymatic hydration of acetaldehyde showed sharp changes in the catalytic capacity of the enzyme in the pH region 6–8. The enzyme exhibits a fourfold increase in its catalysis from pH 6 to 8 and reaches a limiting value at pH 8. The constancy of K_m and the variation of k_2 suggests that while the apparent enzyme-to-substrate binding is unaffected, the actual hydration step depends on the basic form of an ionizable group in the enzyme having a pK_a of 7.0. Essentially the same enzymatic behavior has been observed through similar studies involving the hydration of CO_2, where the pH dependence appears to be the titration of a weak base with a pK_a of 7.2 (Kiese, 1941). For this process also, the hydration step rather than the enzyme-to-substrate binding appears to reflect the variation of enzymatic activity with pH.

In contrast to these processes, the enzymatic hydrations of 2- and 4-pyridine aldehydes showed only mild pH dependency in the pH range 6–8. K_m is essentially independent while k_2 varies by about 30% for these substrates. Indeed, because only 15% of the enzymatic activity remains at pH 5.1 and since relatively small variations occur from pH 6 to 8, the inflection point in the pH–rate profile appears to be shifted to *ca.* pH 5.5. It should be noted however, that if the variation in enzymatic catalysis with pH is due to changes in the magnitude of k_2 the experimentally observed pK_a of the ionizable group in the enzyme must depend on ES rather than free enzyme (Dixon and Webb, 1964). Indeed, the ionization constants of groups on the enzyme may be perturbed by the presence of various substrate molecules. The exact nature of such perturbations is not fully understood and work is in progress in an attempt to distinguish whether protein-bound zinc, imidazole, or a third unspecified group is intimately associated with the 2- and 4-pyridine aldehyde ES complexes.

The main physiological role of carbonic anhydrase appears to be the catalysis of the reversible hydration of CO_2. The behavior of the enzyme in its catalysis of acetaldehyde hydration seems to parallel that involved in the CO_2 process in many ways described in earlier publications (Pocker and Meany, 1965a,b). Consequently, it would appear that the enzymatic hydration of acetaldehyde may be used as a model in the study of the physiological action of carbonic anhydrase as well as an accurate method for assaying the enzyme.

Certain apparent differences in enzymatic behavior occur when substrates having "abnormal" characteristics (*e.g.*, a capacity to coordinate with zinc) are imposed upon the enzyme. If substrates such as the pyridine aldehydes can *bind* at or near the hydrating site, the mode of action becomes sufficiently distorted to affect the pH–rate profile with respect to both binding and the breakdown of the active enzyme–substrate complex. Additionally such distortion may affect the type of inhibition by specific inhibitors.

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