A Comparative Study of Enzymatic and Metal Ion Catalyzed Hydration of Pyridine Aldehydes[1a]

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Abstract: The present investigation represents a comparative kinetic study of the catalysis of the reversible hydration of 2- and 4-pyridine aldehyde by the metalloenzyme carbonic anhydrase, by divalent zinc and cobalt ions, as well as by water and hydroxide ion. The catalytic efficiency of bovine carbonic anhydrase is *ca.* 10^8 times larger than that of water for both 2- and 4-pyridine aldehyde hydrations. Divalent zinc and cobalt are *ca.* 10^7 times more effective than water for the 2-pyridine aldehyde hydration while much less effective in the 4-pyridine aldehyde hydration. The proximity and juxtaposition of the carbonyl group and the pyridine nitrogen in 2-pyridine aldehyde affords a unique environment for these divalent metal ions to efficiently promote the reversible hydration. In contrast to the efficiency of the metal ion catalysis of 2-pyridine aldehyde hydration where the metal ion can bind to the nitrogen and at the same time remain close to the aldehydic group, the enzyme appears to overcome these restrictions on substrate configuration and is an efficient catalyst for both 2- and 4-pyridine aldehyde hydration.

In our earlier publications we have established that the catalytic effect of the metalloenzyme carbonic anhydrase is not limited to CO_2 hydration, but that this enzyme very powerfully and reversibly catalyzes the hydration of acetaldehyde and many other carbonyl compounds.[2-6]

It has become increasingly obvious that the mode of action of the metalloenzyme erythrocyte carbonic anhydrase cannot be explained in terms of the chelated zinc ion alone or in terms of the protein alone and that consequently a delineation of the role of the metal will benefit from a comparative study of the catalytic effectiveness of both enzyme and suitably chelated metal ion.

The catalysis of the reactions of enzyme substrates or their analogs by metal ion is of particular interest since such reactions may reveal mechanistic features characteristic of catalysis by metalloenzymes.[7-12]

In carboxypeptidase the zinc ion participates both in the formation of certain enzyme substrate complexes and also in their subsequent hydrolyses.[13] On the other hand it is generally believed that the zinc ion in erythrocyte carbonic anhydrase is not the binding site for CO_2 or acetaldehyde but appears to function only as part of the hydrating site.[4,14] A logical outgrowth of our previous work involving the enzymatic hydration of aliphatic aldehydes[2-4] was to seek compounds which in addition to their ability to hydrate also contain the elements necessary for coordination to the metal and to evaluate their capacity as substrates for carbonic anhydrase. The 2- and 4-pyridine aldehydes seemed to satisfy both of these requirements since they not only hydrate with ease but might also be expected to coordinate with protein-bound zinc.

Experimental Section

Bovine carbonic anhydrase (BCA) was a product of Mann Research Laboratories prepared and purified from bovine erythrocytes by the method of Keilin and Mann.[15] 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 each molecule of native carbonic anhydrase from bovine erythrocytes 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[16] and further confirmed these assays by atomic absorption spectrophotometry. The extinction coefficient, ϵ, of this enzyme was spectrophotometrically determined at 280 mμ and found to be 54,000 based on a molecular weight of 30,000. This is in perfect agreement with the corresponding value obtained earlier by Lindskog.[17] Consequently, enzyme concentrations used in kinetic studies were determined spectrophotometrically.

These assays were further substantiated by inhibiting the enzyme-catalyzed hydration of acetaldehyde with the specific and powerful inhibitor, acetazolamide. By keeping the enzyme concentration constant throughout a series of runs, while varying only the acetazolamide concentration, plots of enzyme activity *vs.* the ratio of inhibitor to enzyme concentration show by extrapolation that complete inactivation occurs at essentially a 1:1 molar ratio of inhibitor to enzyme. Since there is evidence that acetazolamide specifically inhibits carbonic anhydrase by coordination at or near the zinc ion,[18,19] this experiment confirms our assay and implies that the

(1) (a) Support for this work by the National Institutes of Health of the Public Health Service is gratefully acknowledged. Data given in this paper were taken in part from a dissertation presented by John E. Meany to the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Aug 11, 1966. (b) Author to whom correspondence should be addressed. (c) NASA Predoctoral Fellow, 1963–1966.

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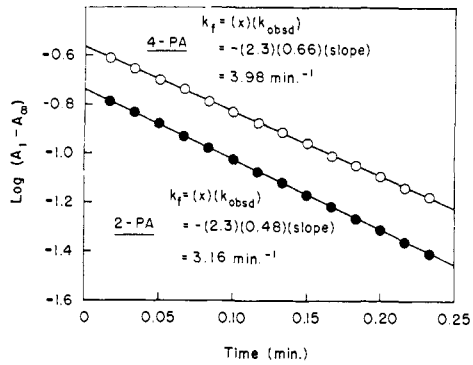

Figure 1. Typical 2- and 4-pyridine aldehyde hydration runs in 0.01 M diethylmalonate buffer (pH 7.2) at 0.0°. Kinetic zero is actually 3 sec after mixing. At completion (equilibrium) 2-pyridine aldehyde is hydrated to the extent of 48% and 4-pyridine aldehyde to the extent of 66%: O, 4-pyridine aldehyde hydration catalyzed by 3.02×10^{-6} M bovine carbonic anhydrase; ●, 2-pyridine aldehyde hydration catalyzed by 6.39×10^{-6} M bovine carbonic anhydrase.

enzyme preparations employed in this investigation contain no other active catalyst.

Control experiments involving the denaturation of bovine carbonic anhydrase 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 bovine carbonic anhydrase 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 either 2- or 4-pyridine aldehyde hydration was observed. The hydration rates in the above solution were identical with those observed in pure 0.01 M diethylmalonate buffer at this pH.

Cobalt and zinc ion solutions were made up in the form of their nitrates which were obtained from Baker and Adamson in reagent grade. The nitrate was chosen as the anion for the solution since the association of nitrate with these metallic ions is small compared with that of halogens and other common anions.[20]

The cobalt nitrate concentrations were checked spectrally (ϵ 4.60 at λ 520 mμ)[21] and those of zinc nitrate by dithizone determinations.[16]

The 2- and 4-pyridine aldehydes were purchased from Aldrich Chemical Co., Inc., and were twice distilled under reduced pressure in a nitrogen atmosphere: bp (2-pyridine aldehyde) 60–61° (12 mm); bp (4-pyridine aldehyde) 76–77° (12 mm).

The distillations were always carried out directly before use to minimize the oxidation of the 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, which was purified by recrystallization from benzene and dried *in vacuo* (mp 128–128.5°). The first and second dissociation constants were determined by Gane and Ingold[22] (K_1 = 6.15×10^{-3}; K_2 = 5.1×10^{8}). To remove trace amounts of water, Baker Analyzed reagent grade acetonitrile was fractionated in the presence of CH_2Cl_2. The drying of acetonitrile allows it to be used in the preparation of stock solutions of the respective pyridine aldehydes. Such dilution was necessary so that extremely small amounts of the aldehydes could be introduced accurately into the reaction mixtures.

The hydrations 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-83060) 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 the condensation of water vapor on the outside of the windows at the entrance to and the exit from the cell compartment.

The kinetics for the reversible hydration of 2- and 4-pyridine aldehyde are similar to those described for acetaldehyde hydration in an earlier publication[2,4] and the pseudo-first-order rate constants are deduced in the same fashion. Although the hydration of these pyridine aldehydes proceeds much more rapidly, the measurement of the first-order rate coefficients using the equipment described above affords results which are generally reproducible within ±2%. The catalyst concentrations employed were such that the rate constants obtained usually ranged from 1 to 10 min^{-1}.

The experimental rate constant, k_{obsd}, is actually 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 are converted to first-order rate coefficients for the forward reaction, k_f, by multiplying k_{obsd} by the fraction of hydration, χ, at 0.0°: χ(2-pyridine aldehyde) = 0.48; χ(4-pyridine aldehyde) = 0.66.

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.[23] It is observed that the fractions of hydration were unchanged by the presence of catalytic amounts of enzyme, Co^{2+}, or Zn^{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 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.

The reactions were initiated by injecting constant amounts of dilute acetonitrile solutions of 2- or 4-pyridine aldehyde through a Hamilton microliter syringe into the spectrophotometer cells containing 3 ml of the reaction mixture. Small amounts of the pyridine aldehydes could in this way be conveniently introduced into the cuvettes. The reaction rates were followed by observing the rate of diminution of the absorbancy of the respective pyridine aldehydes at 278 mμ. Typical runs involving the hydrations of 2- and 4-pyridine aldehyde are shown in Figure 1 in which plots of log $(A_t - A_\infty)$ *vs.* time are linear with $k_{obsd} = -(2.3)$(slope), and $k_f = -(2.3)(\chi)$(slope).

The catalysis of the hydrations of both 2- and 4-pyridine aldehyde by bovine carbonic anhydrase is very powerful so that only small concentrations of the enzyme need be used in the determination of the respective rate coefficients. Similar considerations apply for the catalysis of 2-pyridine aldehyde hydration by divalent zinc and cobalt. Thus again only small concentrations of these catalysts were required for the accurate determination of the respective rate coefficients. These concentrations were always below 2×10^{-4} M and had a negligible effect on the pH of the 0.01 M diethylmalonate buffer employed. On the other hand, it was necessary to employ higher concentrations of these divalent cations in the determination of their relatively low catalytic coefficients operative in the 4-pyridine aldehyde hydration. Both divalent zinc and cobalt appear to have a tendency to bind diethylmalonate dianion in a 1:1 ratio.[24] Consequently, if the concentrations of these ions are similar to that of the buffer components, their addition causes an

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(21) K. Jorgensen, *Acta Chem. Scand.*, **8**, 175 (1954).
(22) R. Gane and C. K. Ingold, *J. Chem. Soc.*, 2153 (1931).

(23) In previous papers[2b,4] 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_{enz} 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.

(24) The pH changes accompanying the addition of these metal ions to diethylmalonate buffers allow us a preliminary determination of the stoichiometry of the complexes formed. Our observations accord with those found earlier for the interaction of heavy metal ions with malonate dianions.[25,26]

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alteration of the buffer ratio resulting in a pH decrease. These pH changes, when encountered, were counteracted by the addition of appropriate quantities of 0.01 *M* diethylmalonate dianion in order to restore the pH to its original value of 7.2.

Results

Our kinetic studies of the reversible hydrations of 2- and 4-pyridine aldehydes show them to be general acid, general base catalyzed processes.[6]

Consequently, it is necessary to isolate the enzymatically and metal ion catalyzed portions of these hydrations from the contributions of other catalytically active molecules. We have searched for a buffer system whose threshold of catalysis is relatively low in the pH range 6–8. 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 (HA^- and A^{2-}, respectively) are low in comparison to both the enzymatic and the metal ion catalyzed rates.

Because each acidic and basic species present in the reaction solution catalyzes the hydration processes independently, the forward rate constant, k_f, in diethylmalonate buffers may be represented by eq 1. Around

$$k_f = k_0 + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + k_{HA^-}[HA^-] + k_{A^{2-}}[A^{2-}] \quad (1)$$

neutral pH the term $k_{H_3O^+}[H_3O^+]$ is negligible so that the main contributors to k_f are: k_0, $k_{OH^-}[OH^-]$, and the catalytic terms arising from the buffer components, $k_{HA^-}[HA^-] + k_{A^{2-}}[A^{2-}]$. We find that the catalytic coefficients for diethylmalonate buffer, k_{HA^-} and $k_{A^{2-}}$, are relatively small so that in 0.01 *M* buffer, essentially the only remaining contributors are k_0 and $k_{OH^-}[OH^-]$.

Consequently, the catalytic coefficients k_0 and k_{OH^-} were evaluated by carrying out a series of runs in 0.01 *M* diethylmalonate buffer at various pH's.[6] We find that the spontaneous hydration coefficient, k_0, for the forward reaction of 2-pyridine aldehyde is 0.24 min^{-1} while that for 4-pyridine aldehyde is 0.51 min^{-1}. For hydroxide ions we find for the forward reaction of 2-pyridine aldehyde $k_{OH^-} = 2.6 \times 10^6$ l. $mole^{-1}$ min^{-1} while for 4-pyridine aldehyde $k_{OH^-} = 7.3 \times 10^6$ l. $mole^{-1}$ min^{-1}.

In the presence of bovine carbonic anhydrase (BCA) the over-all rate coefficients for the forward process, k_f, determined in diethylmalonate buffers around neutral pH consist of a sum of catalytic terms given by eq 2.

$$k_f = k_0 + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + k_{AH^-}[HA^-] + k_{A^{2-}}[A^{2-}] + k_{enz}[BCA] \quad (2)$$

The corresponding treatment of the metal ion catalyzed hydration in buffered solution is given by eq 3.

$$k_f = k_0 + k_{H_3O^+}[H_3O^+] + k_{OH^-}[OH^-] + k_{HA^-}[HA^-] + k_{A^{2-}}[A^{2-}] + k_{M^{2+}}[M^{2+}] \quad (3)$$

The catalytic coefficients, k_{enz}, were evaluated for both 2- and 4-pyridine aldehydes from a series of runs in which only the enzyme concentration was varied (Table I). Since throughout such a series of runs, all other catalytic components in eq 2 remain constant,[27]

(27) Actually for experiments at pH 7.2 in 0.01 *M* diethylmalonate buffer the catalytic components $k_{H_3O^+}[H_3O^+]$, $k_{HA^-}[HA^-]$, and $k_{A^{2-}}[A^{2-}]$ are negligible.

Table I. Catalysis of 2- and 4-Pyridine Aldehyde Hydration by Bovine Carbonic Anhydrase, Zn^{2+}, and Co^{2+} at pH 7.2[a]

(i) [2-PA][b] = 3.8×10^{-5} mole $l.^{-1}$

$[Zn^{2+}] \times 10^5$, mole $l.^{-1}$	k_f, min^{-1}	$[Co^{2+}] \times 10^5$, mole $l.^{-1}$	k_f, min^{-1}	[BCA] $\times 10^5$, mole $l.^{-1}$	k_f, min^{-1}
0	0.77	0	0.77	0	0.77
1.82	1.55	3.33	1.85	0.160	1.21
3.64	2.02	6.67	3.06	0.320	1.91
5.46	2.34	10.0	4.12	0.480	2.54
7.28	2.98	13.3	5.14	0.639	3.16
9.09	3.46	16.7	6.09	0.798	3.33
13.1	4.29			1.46	6.00
19.6	6.00			2.19	8.76

(ii) [4-PA][b] = 8.1×10^{-5} mole $l.^{-1}$

$[Zn^{2+}] \times 10^3$, mole $l.^{-1}$	k_f, min^{-1}	$[Co^{2+}] \times 10^3$, mole $l.^{-1}$	k_f, min^{-1}	[BCA] $\times 10^6$, mole $l.^{-1}$	k_f, min^{-1}
0	0.66	0	0.66	0	0.66
0.40	0.71	3.17	0.70	1.51	2.09
0.72	0.73	4.55	0.74	3.02	3.98
1.50	0.76	6.34	0.79	4.54	5.23
2.20	0.83	9.31	0.87	6.05	6.94
4.32	1.07	13.7	1.00	7.56	8.46
6.50	1.29				

[a] All runs were carried out in 0.01 *M* diethylmalonate buffer at 0.0 ± 0.02°. The concentration of bovine carbonic anhydrase was deduced as indicated in the Experimental Section. Both Zn^{2+} and Co^{2+} were introduced in the form of their nitrates. [b] Acetonitrile stock solutions of both 2- and 4-pyridine aldehydes were introduced into the reaction mixture using a microsyringe.

k_{enz} was deduced from plots of k_f *vs.* the concentration of bovine carbonic anhydrase. The slope of the resulting straight line is defined as k_{enz} (Figures 2 and 3). We have independently shown[6] that the enzymatically catalyzed hydrations of 2- and 4-pyridine aldehyde formally obey the Michaelis–Menten relationship. For the results reported in this paper, the second-order rate coefficients, k_{enz}, are numerically equivalent[28] to K_2/K_m since the concentrations of the substrates were very small ([2-pyridine aldehyde] = 3.77×10^{-5} *M*, [4-pyridine aldehyde] = 8.09×10^{-5} *M*) compared to their respective K_m values (K_m = 0.014 *M* for 2-pyridine aldehyde; K_m = 0.011 *M* for 4-pyridine aldehyde).

(28) The simplest mechanism by which a strictly reversible enzyme-catalyzed hydration might be expected to proceed is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EX \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P \quad (i)$$

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 or k_2. The steady-state treatment of this scheme yields the following equation for the reaction rate[29]

$$v = -\frac{d(S)}{dt} = \frac{d(P)}{dt} = \frac{(V_m^f/K_m^f)(S) - (V_m^r/K_m^r)(P)}{1 + \frac{(S)}{K_m^f} + \frac{(P)}{K_m^r}} \quad (ii)$$

where S represents 2- or 4-pyridine aldehyde and P the corresponding hydrate. When reciprocal initial velocities are plotted *vs.* reciprocal initial concentrations of substrates we are dealing with a case where (P) = 0, and eq ii reduces to eq iii

$$v = -\frac{d(S)}{dt} = \frac{d(P)}{dt} = \frac{(V_m/K_m)(S)}{1 + \frac{(S)}{K_m}} \quad (iii)$$

which may in turn be simplified to eq iv

$$v = k_{enz}(E)(S) = \frac{k_2(E)(S)}{K_m + (S)} \quad (iv)$$

(29) J. B. S. Haldane, "Enzymes," Longmans Green and Co., London, 1930, p 81; also the Massachusetts Institute of Technology Press, Cambridge, Mass., 1965, p 81.

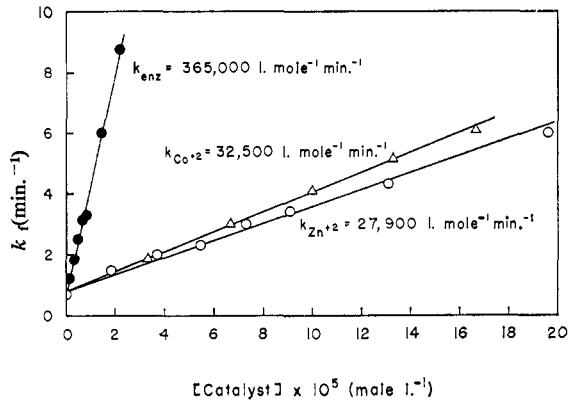


Figure 2. Determination of second-order catalytic rate coefficients. The hydration of 2-pyridine aldehyde in 0.01 *M* diethylmalonate buffer (pH 7.2) at 0.0° as catalyzed by: ●, bovine carbonic anhydrase; △, divalent cobalt; ○, divalent zinc.

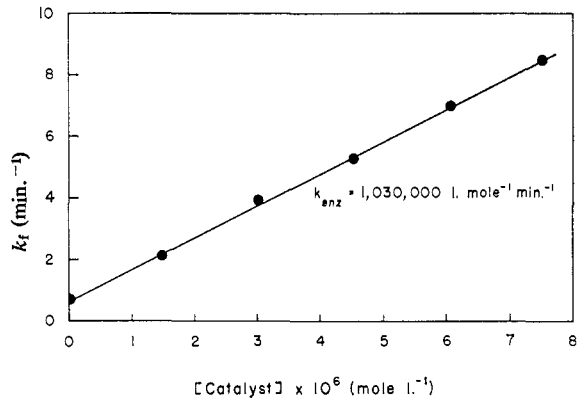


Figure 3. Determination of second-order catalytic rate coefficients. The hydration of 4-pyridine aldehyde in 0.01 *M* diethylmalonate buffer (pH 7.2) at 0.0° as catalyzed by bovine carbonic anhydrase.

Values of k_{enz} at pH 7.2 in 0.01 *M* diethylmalonate buffer for both 2- and 4-pyridine aldehyde hydration appear in Table II.

Table II. Catalytic Rate Coefficients Involved in the Reversible Hydration of 2- and 4-Pyridine Aldehydes[a]

	2-PA		4-PA	
Catalyst	k_c, l. mole^{-1} min^{-1}	$\frac{k_c}{k_{H_2O}}$	k_c, l. mole^{-1} min^{-1}	$\frac{k_c}{k_{H_2O}}$
H_2O[b]	0.0043	1	0.0091	1
Zn^{2+}	27,900	6.5×10^6	100	1.1×10^4
Co^{2+}	32,500	7.6×10^6	25	2.7×10^3
BCA	365,000	8.5×10^7	1,030,000	1.1×10^8
OH^-	2,600,000	6.0×10^8	7,300,000	8.0×10^8

[a] All catalytic rate coefficients refer to the forward processes. [b] The respective spontaneous rate coefficients for hydration, k_0 (in min^{-1}), were divided by 55.5 mole l.$^{-1}$ to obtain k_{H_2O} in l. mole^{-1} min^{-1}.

Corresponding runs for these substrates were carried out using varying concentrations of divalent zinc and colbalt. These runs also were performed at pH 7.2 in 0.01 *M* diethylmalonate buffers (Table I).

Within these series of runs plots of k_f *vs.* metal ion concentration were used to evaluate the respective catalytic rate coefficient associated with each divalent metal ion for 2- and 4-pyridine aldehyde hydration.

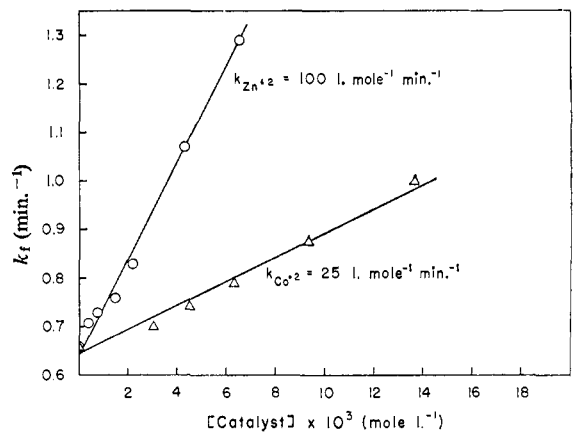


Figure 4. Determination of second-order catalytic rate coefficients. The hydration of 4-pyridine aldehyde in 0.01 *M* diethylmalonate buffer (pH 7.2) at 0.0° as catalyzed by: △, divalent cobalt; ○, divalent zinc.

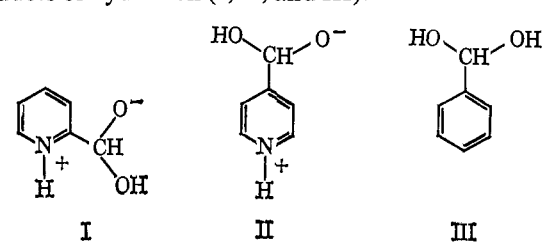
The variation of the metal ion concentrations over a rather broad range showed that the specific rates of hydration are linear in metal ion for both zinc and cobalt (Figures 2 and 4). It will be noted that both metallic ions are extremely effective catalysts for 2-pyridine aldehyde hydration and have similar catalytic coefficients ($k_{Co^{2+}}$ = 32,500 l. mole^{-1} min^{-1} and $k_{Zn^{2+}}$ = 27,900 l. mole^{-1} min^{-1}).

The catalysis of the hydration of 2-pyridine aldehyde by 1.66×10^{-4} *M* zinc ion was also evaluated in 0.01 *M* acetate buffer, at pH 6.07, where $k_{Zn^{2+}}$ = 26,400 l. mole^{-1} min^{-1}. This result suggests that the zinc ion catalysis is not very sensitive to the buffer employed in the pH region 6–7.2.

In contrast to the very efficient catalysis of 2-pyridine aldehyde hydration, the promotion of the 4-pyridine aldehyde reaction by these ions is exceedingly mild as is apparent from Figure 4 where $k_{Co^{2+}}$ = 25 l. mole^{-1} min^{-1} and $k_{Zn^{2+}}$ = 100 l. mole^{-1} min^{-1}.

Discussion

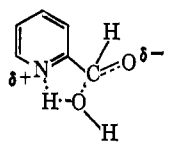
Assuming the ratio of activity coefficients f_{hydr}/f_{ald} for dilute solutions to equal unity, the equilibrium constant, K = [aldehyde hydrate]/[aldehyde], for 2- and 4-pyridine aldehyde hydration at 0.0° is 1.0 and 1.9, respectively. At first sight these constants may appear to be somewhat large in view of the significant loss in resonance energy accompanying the formation of the above hydrates. Indeed, a related loss in resonance energy might be the prohibiting factor for the presence of detectable amounts of benzaldehyde hydrate in equilibrium with benzaldehyde. The above-mentioned equilibrium constants may however be rationalized by contrasting the probable products of hydration (I, II, and III).



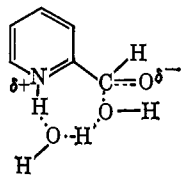
Thus one might expect that owing to the charged nature of the zwitterionic hydrates (I and II) the dif-

ference in solvation energy between the hydrates and their respective reactants may counterbalance the loss in resonance energy caused by these transformations.

It is instructive to compare the relative rate constants for the spontaneous hydration of 2- and 4-pyridine aldehyde. For 2-pyridine aldehyde one might have expected that the ring nitrogen would assist the spontaneous hydration by participating as a conveniently located general base. Such assistance is explicitly considered in transition states IV and V which differ in the number of water molecules bridging between the ring nitrogen and the nearby carbonyl carbon. The

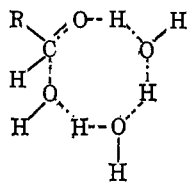


IV



V

corresponding transition states for the hydration of 4-pyridine aldehyde are prohibited owing to the distance separating the ring nitrogen and the aldehydic group. Because the water-catalyzed hydrations of 2- and 4-pyridine aldehyde are of comparable magnitude (actually $k_0^{4\text{-PA}}$ is 2.1 times larger than $k_0^{2\text{-PA}}$) one cannot ascribe to transition states IV and V a significant role in these spontaneous hydrations. To account for the similarity in the values of k_0 for these hydrations, one may follow Eigen[30] in writing cyclic transition states applicable to the hydration of aldehydes in



VI

general (VI). Other alternatives would include multistep hydration processes or concerted mechanisms not involving cyclic transition states.[31a,b]

Further comparison can be made between the spontaneous rates of hydration of these pyridine aldehydes with that of acetaldehyde. The spontaneous rate coefficients for the former reactions are larger ($k_0^{4\text{-PA}} \simeq 8k_0^{CH_3CHO}$; $k_0^{2\text{-PA}} \simeq 4\,k_0^{CH_3CHO}$), despite the larger loss in resonance energy which accompanies the formation of the corresponding transition states for the 2- and 4-pyridine aldehydes. The loss in resonance energy on going from ground state to transition state may be partially offset by the significant gains in solvation energy.

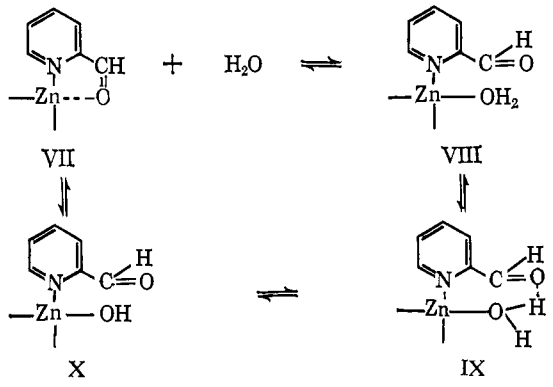
In an earlier publication[2b] we have shown that zinc ions in the presence of imidazole buffers provide an efficient catalyst for the hydration of acetaldehyde. In the present paper we discuss the results of a comparative study involving the catalysis of 2- and 4-pyridine aldehyde hydrations by bovine carbonic anhydrase and by the two divalent metal ions known to restore activity to apocarbonic anhydrase, *i.e.*, divalent zinc and cobalt.[32]

Complexes between the oximes of 2-pyridine aldehyde and divalent cations are well documented.[33] Although there is not as much evidence regarding complexes between these dications and 2-pyridine aldehyde, their existence, at least in equilibrium concentrations, has often been invoked.[34] Indeed, a green complex composed of 1 mole each of $CuCl_2$, 2-pyridine aldehyde, and ethanol was isolated from these components in absolute ethanol.[35]

By employing divalent zinc and cobalt as catalysts for the hydration of 2-pyridine aldehyde, we have shown that these hydrations are first order in both 2-pyridine aldehyde and in divalent metal ion. These kinetic studies indicate that the stoichiometry of the activated complex is 1:1 and further suggest that a complex between 2-pyridine aldehyde and the divalent metal is an intermediate in the hydration process. Indeed, the actual species undergoing hydration could be a divalent metal–2-pyridine aldehyde complex, and the metal ion catalysis could be derived from a proportional increase in the concentration of this complex.

The rate coefficient for spontaneous hydration, k_0, of 4-pyridine aldehyde exceeds that for the 2-pyridine aldehyde process by a factor of 2.1. The corresponding ratio for hydroxide ion catalysis, $k_{OH}^{4\text{-PA}}/k_{OH}^{2\text{-PA}}$, is similar and has a value of 2.8. If this is a fairly general trend then one might have expected, *a priori*, that in the absence of special effects, the catalytic effectiveness of divalent zinc and cobalt would parallel this trend. Actually the trend is reversed in that the catalysis afforded by these metal ions is much greater for the 2-pyridine aldehyde hydration. The respective ratio for zinc is $k_{Zn^{2+}}^{2\text{-PA}}/k_{Zn^{2+}}^{4\text{-PA}} \simeq 280$ and that for cobalt ion is $k_{Co^{2+}}^{2\text{-PA}}/k_{Co^{2+}}^{4\text{-PA}} \simeq 1300$. We ascribe this reversal together with the magnitude of the respective catalytic terms to the special arrangement of the ring nitrogen and aldehydic group in 2-pyridine aldehyde which is absent in 4-pyridine aldehyde.

One can envisage four possible structures (VII–X) for coordination compounds involving divalent metal ions and 2-pyridine aldehyde.[36]



VII VIII X IX

(30) M. Eigen, *Angew. Chem.*, 75, 489 (1963); *Discussions Faraday Soc.*, 39, 7 (1965).

(31) (a) R. P. Bell, "The Proton in Chemistry," Cornell University Press, Ithaca, N. Y., 1959, p 147; (b) Y. Pocker, *Proc. Chem. Soc.*, 17 (1960).

(32) S. Lindskog and B. G. Malmstrom, *J. Biol. Chem.*, 237, 1129 (1962).

(33) S. Bolton and R. I. Ellin, *J. Pharm. Sci.*, 51, 533 (1962).

(34) B. Kirson and S. Yariv, *Bull. Soc. Chim. France*, 2969 (1964).

(35) D. Kutscher and E. Hoyer, *Z. Chem.*, 3, 68 (1963).

(36) (a) In diethylmalonate buffers, two of the remaining ligand sites may be occupied by a diethylmalonate dianion.[23] (b) We have considered several structures because the position of the metal in its most stable coordination compound with 2-pyridine aldehyde tells little about its position in the transition state and the chelation in the ground state cannot be used *a priori* as a compelling argument for a mechanism involving a cyclic intermediate such as VII in the transition state.

In promoting this hydration, the divalent metal ion is conveniently held in place by the ring nitrogen and could function in either of two limiting capacities: (a) as a general acid polarizing the carbonyl oxygen directly (VII), or indirectly, through the bridging of water (IX); (b) by participating in the direct transfer of water (VIII) or OH^- (X) to the aldehydic group.

(a) It is evident that the chelation of 2-pyridine aldehyde through pyridine nitrogen and carbonyl oxygen to the divalent metal would increase its reactivity toward H_2O (or OH^-) and other nucleophilic reagents. Thus the divalent metal ion may assist the hydration by polarizing the carbonyl group and making the carbonyl carbon more susceptible to nucleophilic attack by external H_2O (VII and IX). Although the metal ion[37] is a weak Lewis acid it has some properties not possessed by a proton. By virtue of its coordination number, size, and the steric requirements of its coordinate bonds, it would be expected to interact with several atoms. For the reaction under consideration the metal ion may act as a general acid with respect to the carbonyl oxygen (either directly or through the bridging of water) while at the same time being conveniently held in place by coordination to the nitrogen. In case a, hydration is achieved *via* an attack by external water on the carbonyl carbon in VII or IX.

(b) An alternate mechanism may involve the *intramolecular* transfer of metal-bound H_2O (or OH^-) to the carbonyl carbon in VIII or X. The metal ion may coordinate with both substrate and nucleophilic agent and serve as a "collection point" for both components of a bimolecular reaction presumably by making the entropy of activation more positive. Other things being equal, the inherent activity of a metal-bound hydroxide should be less than that of a free hydroxide ion. The virtue of a metal ion complex lies however in its ability to carry appreciable amounts of potential OH^- at a pH where very little free OH^- could exist. The metal ion complex containing water or hydroxide ion could be considered to be a bifunctional catalyst, the metal ion serving as a general acid and the hydroxide ion acting as a nucleophile.

At present, we cannot differentiate between a hydration arising from reaction schemes involving an attack by external water on the metal–2-pyridine aldehyde complex and one in which the attacking water itself comes from the metal hydrate because of the labile nature of these complexes. The observations reported in this paper are taken from a wider study, now in progress, attempting to distinguish between and scrutinize these possibilities.

With respect to the enzymatic catalysis the following points are of interest.

(37) Two of the ligand sites on the divalent metal may be occupied by diethylmalonate dianion.

(1) Bovine carbonic anhydrase is an excellent catalyst for both 2- and 4-pyridine aldehyde hydrations. It is important to note that the ratio of the enzymatic second-order rate coefficients to the water rate coefficient for both 2- and 4-pyridine aldehyde hydrations is very similar (Table II).

$$\frac{k_{enz}^{2\text{-PA}}}{k_{H_2O}^{2\text{-PA}}} \simeq \frac{k_{enz}^{4\text{-PA}}}{k_{H_2O}^{4\text{-PA}}} \simeq 10^8$$

A similar comparison can be made with hydroxide ion catalysis (Table II).

$$\frac{k_{enz}^{2\text{-PA}}}{k_{OH^-}^{2\text{-PA}}} \simeq \frac{k_{enz}^{4\text{-PA}}}{k_{OH^-}^{4\text{-PA}}} \simeq 0.14$$

Because of the similarity in the Michaelis constant, K_m, for the enzymatic hydrations of 2- and 4-pyridine aldehyde ($K_m^{2\text{-PA}} = 0.014$ mole $l.^{-1}$; $K_m^{4\text{-PA}} = 0.011$ mole $l.^{-1}$) parallel comparisons can be made using k_2 rather than k_{enz}. The parallelism between k_{H_2O}, k_2 ($=k_{enz}K_m$), and k_{OH^-} is in accord with our earlier view[4] that the turnover number governs the actual rate of hydration and that the enzyme can be regarded as an efficient donor of OH^- under conditions (pH 7.2) such that the free concentration of OH^- is negligibly small.

(2) In contrast to the metal ion catalysis of 2-pyridine aldehyde where the metal ion can bind with the pyridine nitrogen and at the same time remain close to the aldehydric group, the enzyme appears to overcome these restrictions on substrate configuration and is an efficient catalyst for both 2- and 4-pyridine aldehyde hydrations.

(3) Our earlier investigations[6] show that acetazolamide is a competitive inhibitor in the hydration of both 2- and 4-pyridine aldehydes, thus implying: (a) that in contrast to acetaldehyde[2-4] and CO_2[14a] these substrates appear to bind at or near the protein-bound zinc, and (b) that the zinc ion associated with the enzyme may not be the water donor, at least in the case of "abnormal" substrates such as 2- and 4-pyridine aldehyde.

(4) Because of the similarity in K_m for both 2- and 4-pyridine aldehyde hydration it would appear that the enzyme–substrate binding is similar for both. Consequently, 2-pyridine aldehyde probably does not act as a bidentate ligand with respect to the protein-bound metal.

(5) The efficiency of enzymatic hydration, in spite of the distortions of the active site caused by these "abnormal" substrates, would require the proximity of a well-oriented water molecule. This water molecule could be associated through hydrogen bonding with any number of basic groups in the protein in the vicinity of the aldehydic group undergoing hydration and need not be transferred by the zinc ion.