Kinetic Study of the Hydration Mechanism of Vitamin B_6 and Related Compounds

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Abstract: In the present investigation, a detailed study of the proton and hydroxide catalysis of the formyl hydration of vitamin B_6 and related compounds as followed by temperature-jump relaxation technique is reported. It is found that in N-heterocyclic formyl compounds intramolecular catalysis predominates over that by protons and hydroxide ions in solution. The k^0 values for intramolecular catalysis are higher for these substances by several orders of magnitude than those of aliphatic aldehydes. The absolute values of the rate constants of proton catalysis are also reduced compared to those of aliphatic aldehydes and benzaldehyde by more than one order of magnitude. Both of these differences are interpreted in terms of the increase of polarization of the carbonyl π system due to electron withdrawal by the ring nitrogen.

Vitamin B_6 compounds are interesting because they are known to be important cofactors of a number of enzymes. A considerable amount of literature already exists concerning the absorption spectra of these substances.[1-7] Rapid reaction techniques provide a new approach to the elucidation of the reaction pathways of these substances.

This paper is concerned with temperature-jump relaxation studies of the acid dependence of the formyl hydration of several vitamin B_6 compounds and of those closely related to them such as pyridinecarboxaldehydes and 5′-deoxypyridoxal. The protolytic reactions of these compounds will be dealt with in a following paper.

In addition to kinetic measurements some absorption and nmr spectra have been obtained.

Experimental Section

Uv spectral measurements were made with a Cary-14 spectrophotometer fitted with cell holders thermostated at 25°. For pH measurements a Radiometer Model 22 pH meter was used at 25°. The nmr equipment was the recently developed Varian Model XL-100 and was used by kind permission of Varian AG, Darmstadt; the nmr spectra were taken at 25°. (We wish to thank Dr. Bremser of Varian AG for experimental advice and introduction to handling of the spectrometer.)

Compounds were of analytical grade and were purchased from Fluka AG and Ega Chemie. The pyridinecarboxaldehydes were redistilled under reduced pressure and kept under dry nitrogen. 5′-Deoxypyridoxal was generously provided by Professor H. H. Inhoffen. The N-methylated pyridoxal 5′-phosphate and 3-methoxypyridoxal 5′-phosphate were kindly donated by Professor E. H. Fischer.

Kinetic experiments were performed using a temperature-jump relaxation apparatus (T-jump).[8] The wavelengths for observation were chosen from λ 295 to 400 nm, depending on the absorption maximum shifting with pH. The initial temperature was 18°, and a final temperature of 25° was reached after a T-jump of $\Delta T = 7°$. The concentration of reactants was usually 2×10^{-4} M. Solutions were brought to the appropriate pH by addition of hydrochloric acid and sodium hydroxide, and the ionic strength was adjusted to 0.1 M by addition of sodium chloride. The following substances have been studied: pyridine-3- and 4-carboxaldehyde, pyridoxal, pyridoxal 5′-phosphate, 5′-deoxypyridoxal, *N*-methylpyridoxal 5′-phosphate, 3-methoxypyridoxal 5′-phosphate, pyridoxal 5′-phosphonate, and the sodium salt of 2-sulfobenzaldehyde.

Results

Spectra. Addition reactions to the carbonyl double bond of a formyl compound and/or alteration of the neighboring groups by protonation give rise to marked changes in the uv and nmr spectra of the carbonyl compound.

Uv absorption spectra of vitamin B_6 and related heterocyclic formyl compounds have been reported previously.[1-7] In most cases spectral changes were observed as a function of pH and were discussed in terms of hydration and protolytic dissociation equilibria. Thus, for a considerable number of vitamin B_6 compounds and their analogs, pK values have been determined by optical titration. Spectra obtained by us were in good agreement with those previously reported, and this was taken as a criterion for the purity of the compounds used. Morozov, *et al.*, have extensively studied[3-5] the hydration equilibria of the various states of protonation of pyridoxal and pyridoxal 5′-phosphate by analysis of the uv spectra.

Nmr spectra of pyridoxal and pyridoxal 5-phosphate were recorded in D_2O solution at different pD values. The chemical shift data and the coupling constants obtained from the spectra are summarized in Table I.

The pmr spectra of pyridoxal 5′-phosphate clearly reflect the hydration at the formyl group. Upon addition of water to the >C=O group the magnetic environment of the C-4′ proton is altered drastically, causing an upfield shift of the pmr signal of about 400 cps with respect to the position of the formyl resonance. From the integrated signals, the relative equilibrium concentrations of both the *gem*-diol and the free aldehydic forms of pyridoxal 5′-phosphate are evaluated (see Table II). The two signals collapse at higher pD values (pD >13), indicating the onset of a rapid reaction between both the hydrated and the unhydrated

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Table I. Chemical Shift Data and Coupling Constants from Nmr Spectra of Pyridoxal and Pyridoxal 5'-Phosphate

Compound	pD	Chemical shifts, δ_{TMS}, ppm: CH_3–	–CH_2–	H (aromatic)	H–C(–O–)$_2$–		H–C(=O)–
Pyridoxal	2.4	2.76	5.24, 5.43, J_{HH} = 14 cps	8.26	6.80		
	8.4	2.47	5.04, 5.26, J_{HH} = 13 cps	7.57	6.56		
	9.7	2.40	4.89[b]	7.39[a]	6.49[a]		10.2[a]
	12.6	2.40	4.90	7.42		8.04[a]	
	13.7	2.41	4.96	7.41		7.47	
Pyridoxal 5'-phosphate	1.1	2.73	5.27, J_{HP} = 7 cps	8.26	6.53		10.51
	7.3	2.47	5.09, J_{HP} = 7 cps	7.71	6.27		10.40
	13.6	2.42	4.97 J_{HP} = 5 cps	7.62		8.89	

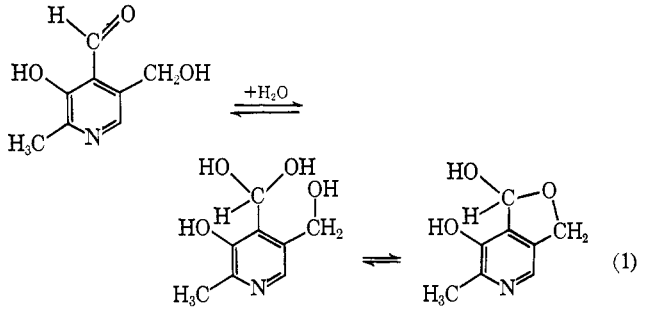
[a] Signal strongly broadened. [b] Signal partially hidden by the HDO absorption peak.

Table II. Relative Intensities of Formyl and *gem*-Diol Proton Resonances

Compound	pD	Intensities, %: H–C(–O–)$_2$–	H–C(=O)–
Pyridoxal	2.4	100	
	8.4	100	
	9.7	78	22
Pyridoxal 5'-phosphate	1.1	75	25
	7.3	17	83

species. A rough estimation from line broadening leads to a reaction rate in the millisecond range.

An analysis of the pyridoxal pmr spectrum is complicated by hemiacetal formation between the formyl group in the 4 position and the hydroxymethylene group in the neighboring 5 position. Therefore, the free aldehyde is present only in very low concentrations at the pD values at which reaction 1 can proceed.



(1)

Around pD ~9, the formyl group proton resonance becomes detectable (see Table II), though the signal is greatly broadened; at higher pD's the signal merges into the resonance of the C-4' proton because of rapid interconversion of both species (Figure 1). Nevertheless, at pD ~9, the ratio of integrals is in agreement with Morozov's data for the relative amounts of hydrated and unhydrated forms of pyridoxal. Therefore, one can conclude that under these conditions hydration alone determines the pmr spectrum. The strong line-broadening effect and the relatively small pD range over which the signal can be observed make detection difficult and result in considerable uncertainty in the calculation of equilibrium constants.[9]

Temperature-Jump Studies. All vitamin B_6 compounds exhibit two relaxation processes. The first process, which could be resolved only when the pH lay between ~5.5 and ~7.5, only occurs with those substances which possess at least two donor–acceptor functions. This proton-transfer process will be discussed in more detail in a forthcoming paper.

The second process was found to be independent of the concentration of reactants, but varied within the range of seconds and 10^{-4} seconds, depending on

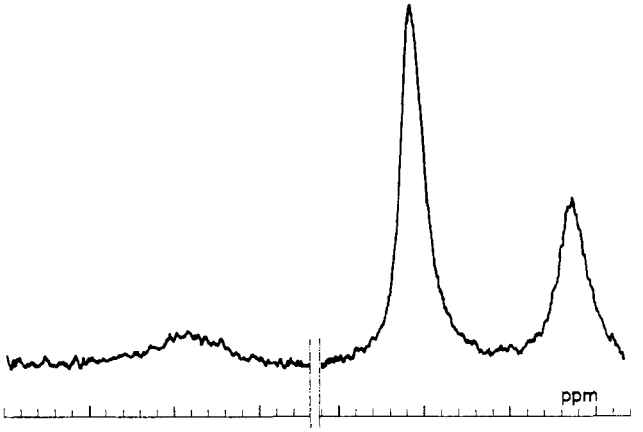
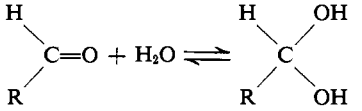


Figure 1. Formyl and *gem*-diol proton magnetic resonance signals in pyridoxal at pH 9.2. In addition, the resonance signal of the aromatic proton is shown (*cf.* Table I).

pH. It was only observed with those molecules bearing a formyl group, and therefore it was attributed to the hydration of this group. Support of this interpretation is given by the change of nmr spectra with pH, because by observing the collapse of the pmr signals of the formyl proton and the *gem*-diol proton a reaction rate is evaluated which is identical with that obtained by T-jump relaxation spectrometry at corresponding pH values.

Reaction Mechanisms. The hydration of a formyl group in aqueous solution generally occurs according to a pseudo-first-order reaction scheme



(2)

Kinetic studies on aliphatic aldehydes[10] revealed the hydration reaction to be catalyzed by general acids and bases, resulting in a pronounced pH dependence of the rates. The rate law common to all substances of this class studied so far is pseudo first order with respect to water, and also includes second-order terms for proton and hydroxide catalysis and for catalysis by general acids and bases.

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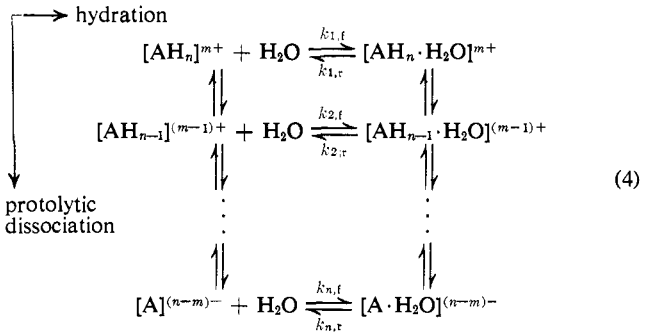
(10) R. P. Bell, *Advan. Org. Chem.*, **4**, 1 (1966).

In the case of a first-order reaction, the reciprocal relaxation time represents the sum of the forward and the reverse rate constants. Thus the reciprocal relaxation time $1/\tau$ of the hydration reaction (2) is given by

$$1/\tau = (k_f + k_r) = (K_H + 1)k_r =$$
$$(K_H + 1)\{k_r^0 + k_r^H c_H + k_r^{OH} c_{OH} + \Sigma k_r^j c_j\} \quad (3)$$

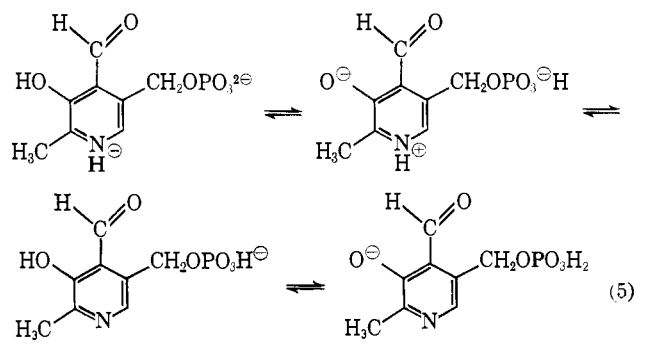
where $K_H = c_{hydrate}/c_{aldehyde} = k_f/k_r$ is the hydration equilibrium constant and k_f and k_r are the forward and reverse pseudo-first-order rate constants for the hydration reaction. k_r^0 indicates intramolecular and solvent catalysis, whereas k_r^H, k_r^{OH}, and k_r^j refer to catalysis by H_3O^+, OH^-, and general acids and bases, respectively. c_j represents the concentrations of the respective catalysts.

In the case of vitamin B_6 compounds and those related to them, the substituent R to which the formyl group is attached possesses at least one proton donor–acceptor function, so the state of protonation of R will be altered by changing pH. Because the extent of hydration on the formyl group depends on the molecular environment, each state of protonation of the molecule is associated with a characteristic constant of hydration ($K_{H,i}$). Therefore, the complete reaction scheme for the formyl hydration of a compound with n dissociable protons consists of $n + 1$ parallel hydration reactions coupled to one another by protolytic dissociation steps of the hydrated and the unhydrated species.



(4)

In addition, the pyridoxal derivatives, if not totally protonated or deprotonated, exist in several zwitterionic forms. For instance, in the dianion of pyridoxal 5′-phosphate, the two remaining protons are shared among four acceptor centers. Therefore, four isomeric forms of pyridoxal 5′-phosphate are present at pH values between pK_2 and pK_3



(5)

This gives rise to another three hydration equilibria occurring at that particular state of protonation. However, for the derivation of the relaxation time from mechanism 4 these species need not be taken into account separately, since the isomerization equilibria 5 will always be established rapidly compared to the rate of the hydration reactions. Thus all equilibrated isomers can be treated as one compound. A similar argument holds for the dissociation equilibria. The protolytic rates in mechanism 4 are faster than those of the hydration reactions by several orders of magnitude. Therefore, the protolytic equilibria are established immediately compared to the hydration reaction. Thus, the only rate constants entering into the expression for $1/\tau$ are those concerning hydration, whereas protolysis will be only represented by equilibrium constants.

The complete expression for $1/\tau$ describing the hydration kinetics according to mechanism 4 is essentially the same as that for a simple one-step hydration as shown in reaction 2. However, in the case of the complex mechanism, the complete expression is given by the sum of forward and reverse pseudo-first-order rate constants $k_{i,f}$ and $k_{i,r}$ for any of the hydration reactions, and in addition each individual term must be multiplied by a factor in order to account for the contributions from the protolytic reactions.

$$\frac{1}{\tau} = \frac{k_{1,f}}{1 + \frac{K_1}{c_H} + \frac{K_1K_2}{c_H^2} + \frac{K_1K_2K_3}{c_H^3}} + \frac{k_{1,r}}{1 + \frac{K_{-1}}{c_H} + \frac{K_{-1}K_{-2}}{c_H^2} + \frac{K_{-1}K_{-2}K_{-3}}{c_H^3}} + \frac{k_{2,f}}{\frac{c_H}{K_1} + 1 + \frac{K_2}{c_H} + \frac{K_2K_3}{c_H^2}} + \frac{k_{2,r}}{\frac{c_H}{K_{-1}} + 1 + \frac{K_{-2}}{c_H} + \frac{K_{-2}K_{-3}}{c_H^2}} + \frac{k_{3,f}}{\frac{c_H^2}{K_1K_2} + \frac{c_H}{K_2} + 1 + \frac{K_3}{c_H}} + \frac{k_{3,r}}{\frac{c_H^2}{K_{-1}K_{-2}} + \frac{c_H}{K_{-2}} + 1 + \frac{K_{-3}}{c_H}} + \frac{k_{4,f}}{\frac{c_H^3}{K_1K_2K_3} + \frac{c_H^2}{K_2K_3} + \frac{c_H}{K_3} + 1} + \frac{k_{4,r}}{\frac{c_H^3}{K_{-1}K_{-2}K_{-3}} + \frac{c_H^2}{K_{-2}K_{-3}} + \frac{c_H}{K_{-3}} + 1} \quad (6)$$

In eq 6 $1/\tau$ is represented for a reaction scheme possessing three dissociation constants. K_i and K_{-i} given above are the dissociation constants of the unhydrated and of the hydrated species, respectively.

$$K_i = [AH_{n-1}]^{(m-1)+} c_H / [AH_n]^{m+}$$

$$K_{-i} = [AH_{n-1} \cdot H_2O]^{(m-1)+} c_H / [AH_n \cdot H_2O]^{m+}$$

$k_{i,f}$ and $k_{i,r}$ are the rate constants of the hydration reaction in the ith state of protonation (*cf.* mechanism 4). $k_{i,f}$ and $k_{i,r}$ may be split into catalytic contributions as was shown for the simple one-step hydration in (2).

Since the relaxation measurements on pyridoxal compounds were done in unbuffered solutions, no

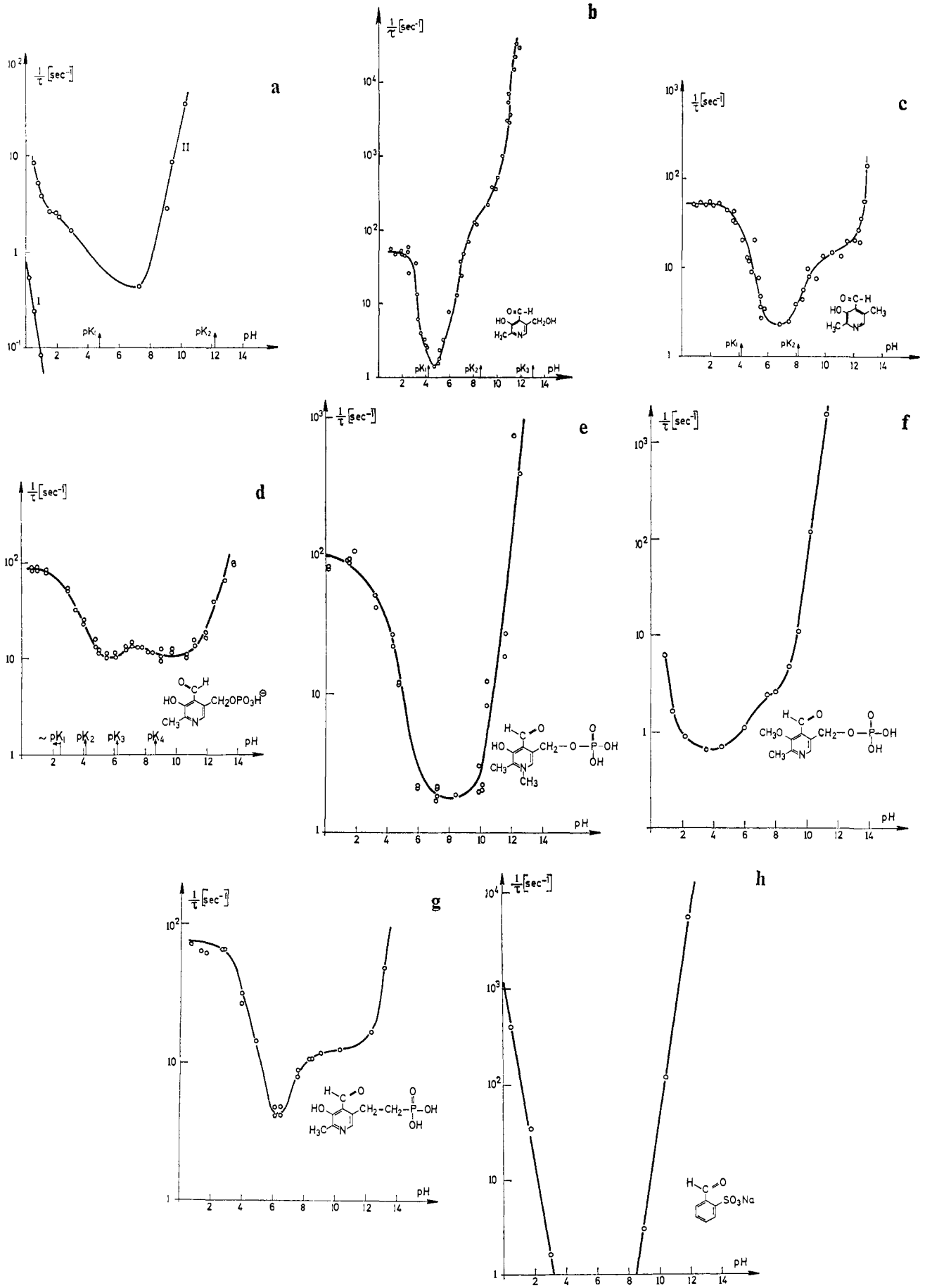


Figure 2. Acidity dependence of $1/\tau$ for the hydration reaction of N-heterocyclic formyl compounds. Measured values of log $1/\tau$ *vs.* pH: (a) pyridine-3-carboxaldehyde (I) and pyridine-4-carboxaldehyde (II), (b) pyridoxal, (c) 5-deoxypyridoxal, (d) pyridoxal 5′-phosphate, (e) *N*-methylpyridoxal 5′-phosphate, (f) 3-methoxypyridoxal 5′-phosphate, (g) pyridoxal 5′-phosphonate, (h) 2-sulfobenzaldehyde.

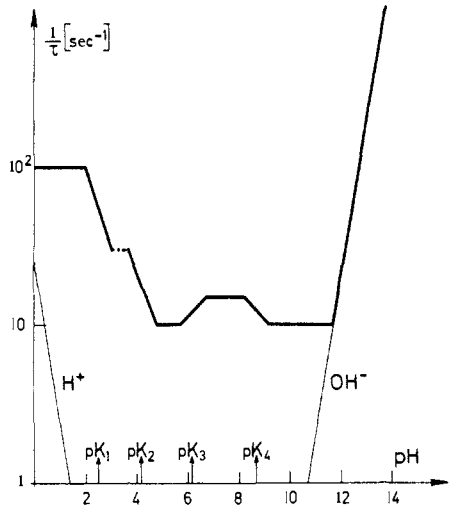


Figure 3. Schematic diagram of log $1/\tau$ *vs.* pH as typical for the hydration of a heterocyclic formyl compound associated with four pK values. H^+ and OH^- denote acid and base catalysis.

general acids or bases were present, and therefore the term concerning these catalysts in eq 3 can be omitted.

$$k_{i,f} = k_{i,f}^0 + k_{i,f}^H c_H + k_{i,f}^{OH} c_{OH}$$
$$k_{i,r} = k_{i,r}^0 + k_{i,r}^H c_H + k_{i,r}^{OH} c_{OH} \quad (7)$$

If hydration is studied at extreme pH values, essentially all of the substance will be in one state of protonation, either completely protonated at pH $\ll$ pK_1 or totally deprotonated at pH $\gg$ pK_n. Therefore, for these pH's $1/\tau$ is reduced to

$$1/\tau = (1 + K_{H,1})(k_{1,r}^0 + k_{1,r}^H c_H) \quad (7a)$$

at pH $\ll$ pK_1 and

$$1/\tau = (1 + K_{H,n+1})(k_{n+1,r}^0 + k_{n+1,r}^{OH} c_{OH}) \quad (7b)$$

at pH $\gg$ pK_n. Most of the substances under investigation are characterized by a number of pK values. For instance, with pyridoxal 5′-phosphate four pK values can be distinguished, each of them differing from the preceding one by 2–3 pK units. Therefore, around each pK value the substance will be present in at least two states of protonation. Hence, in eq 6 at pK_i < pH < pK_{i+1}, *a priori* the kinetic contributions from the adjacent states of protonation have to be taken into account. However, by a rough estimation of the contributions, it can be shown that within each range of pH limited by adjacent pK values the kinetic contributions from the neighboring states become vanishingly small. Thus, at pK_i < pH < pK_{i+1} the substance can be treated like a single carbonyl compound (*cf.* mechanism 2). Then, the rate becomes, to a good approximation

$$1/\tau = k_{i,f} + k_{i,r}$$

for pK_i < pH < pK_{i+1}. Figures 2a–h illustrate the dependence upon pH of log $1/\tau$ for the formyl hydration of several vitamin B_6 compounds, pyridine-3- and -4-carboxaldehydes, and 2-sulfobenzaldehyde. A nonlinear pH dependence of log $1/\tau$ is obvious from these plots except for that of 2-sulfobenzaldehyde. With the exception of the latter case, $1/\tau$ remains essentially constant or at least levels off over the pH range bounded by two adjacent pK values for most of the substances. This implies that the compounds undergo predominantly intramolecular catalysis, and therefore the forward and reverse rates adopt the following forms

$$k_{i,f} = k_{i,f}^0 \qquad k_{i,r} = k_{i,r}^0$$

The acid dependence of log $1/\tau$ for the hydration of a carbonyl compound associated with four pK values is shown schematically in Figure 3. The limiting values for catalysis by H_3O^+ and OH^- are taken from the measurements on heterocyclic formyl compounds. Even if one allows the constants for proton and hydroxide catalysis to vary over several orders of magnitude, it becomes obvious from the diagram (Figure 3) that the hydration kinetics of a formyl compound which is restricted to a limited pH range are not affected by H_3O^+ or OH^- catalysis unless the intramolecular catalysis would decrease by about five or six orders of magnitude. The complete curve obtained for the pH range between pH 0 and 14 only approximates the curve for the hydration of a simple compound according to mechanism 2. The slopes of the ascending and descending branches in the log $1/\tau$ *vs.* pH plot need no longer be ± 1, and even intermediate reversal of the sign of slopes can occur. When pH = pK_i a point of inflection must occur, because of the change of protonation. Furthermore, the schematic illustration demonstrates that a true constant will not be achieved if two pK values are separated by only about one unit; in this case the curve will only level off slightly.

Discussion

All the catalytic rate constants which can be obtained from the measurements taking into account the above considerations are listed in Table III.

Each of the hydration curves shown in Figure 2 exhibits individual properties.

At very low pH values the hydration curve of pyridine-4-carboxaldehyde (Figure 3) starts with a steep branch of slope -1, which levels off only when the pH approaches pK_1. At pH >8 the log $1/\tau$ *vs.* pH points fall on a straight line again, now of slope $+1$. Pyridine-3-carboxaldehyde should behave similarly. However, the hydration reaction becomes too slow at pH >1 to be measured by the T-jump method, and so points are given only for pH <1.

This behavior indicates that with the pyridinecarboxaldehydes at extreme pH values, proton or hydroxide ion catalysis predominates, resulting in the direct proportionality between log $1/\tau$ and pH, *i.e.*, $k_{1,f}^H \gg k_{1,f}^0$ and $k_{2,f}^{OH} \gg k_{2,f}^0$, and the equivalent for the respective dehydration reactions. The catalytic constants for proton and hydroxide catalysis of the hydration of pyridine-4-carboxaldehyde and that for proton catalysis of the hydration of pyridine-3-carboxaldehyde are summarized in Table III. Intramolecular catalysis is markedly faster with the pyridinecarboxaldehydes than with the aliphatic aldehydes.

The curve obtained for pyridoxal 5′-phosphate comes nearest to the schematic diagram. Since its pK values are almost equally distributed over the pH axis, and since the k^0's for the pH range between 4 and 10 do not differ very much, the total curve for pyridoxal 5′-phosphate becomes more or less symmetrical.

Table III. Rate Constants for Intramolecular and H_3O^+ and OH^- Catalysis in the Formyl Hydration of N-Heterocyclic Formyl Compounds

	Compound	pK values[a]				Catalytic rate constants[b]		
		pK_1	pK_2	pK_3	pK_4	$(k_{i,f}^{H} + k_{i,r}^{H})$, M^{-1} sec^{-1}	$(k_{i,f}^{0} + k_{i,r}^{0})$, sec^{-1}	$(k_{i,f}^{OH} + k_{i,r}^{OH})$, M^{-1} sec^{-1}
a	Pyridine-4-carboxaldehyde	4.77				25	~2.5, <0.45	2.5×10^5
a	Pyridine-3-carboxaldehyde	3.80				8		
b	Pyridoxal	4.20	8.64			<55	55, 1.5	5×10^6
c	5′-Deoxypyridoxal	4.17	8.14			<50	50, 2.0, ~15	1.4×10^3
d	Pyridoxal 5′-phosphate	2.5	4.14	6.20	8.69	<85	85, 10, 13, 10	$\geq 1.3 \times 10^2$
e	*N*-Methylpyridoxal 5′-phosphate					<100	100, 1.7	1.6×10^4
f	3-Methoxypyridoxal-5′-phosphate					30	0.65	5.5×10^5
g	Pyridoxal phosphonate					<75	75, 4.5, 12	3.5×10^2
h	2-Sulfobenzaldehyde					1.3×10^3		4×10^5
i	Acetaldehyde					9×10^2	$\sim 7 \times 10^{-3}$	8×10^4

[a] pK values of the hydrated formyl group are not tabulated. [b] Intramolecular rate constants $(k_{i,f}^{0} + k_{i,r}^{0})$ are listed according to the order of pK values given in the second column.

Deoxypyridoxal and pyridoxal are characterized by only two pK values, the acid dissociation of the hydrated formyl groups being ignored for the purposes of this study. Because of these two pK values, the hydration curves for these two compounds resemble that of the phosphate but are more unsymmetrical. These compounds exhibit strong intramolecular catalysis which predominates over proton catalysis even at pH 0. It is likely that proton catalysis occurs at a similar rate to that of pyridinecarboxaldehydes and that this effect is small compared to the contribution from the intramolecular catalysis.

For comparison, some derivatives of pyridoxal 5′-phosphate have been studied (Figure 2e, f, g). In general, pyridoxal 5′-phosphonate and the N-methylated pyridoxal 5′-phosphate behave as their parent compounds do. However, with 3-methoxypyridoxal 5′-phosphate there is no indication of intramolecular catalysis in the low-pH range, at least at pH 2. From the very low rate at pH 2 one can conclude that in highly acidic media this substance might behave rather more like the pyridinecarboxaldehydes than like the other pyridoxal compounds. The last compound listed in Table III is 2-sulfobenzaldehyde. In this case no marked difference is observed compared with aliphatic aldehydes.

The most remarkable feature of the hydration of the N-heterocyclic formyl compounds is the pronounced effect of intramolecular catalysis. However, the absolute values of the rate constants of proton catalysis are reduced, compared to those of aliphatic aldehydes and benzaldehyde, by more than one order of magnitude.

Both of these effects are attributed to the influence of the ring nitrogen. Owing to its high electronegativity, the nitrogen atom withdraws a lone electron pair from the π electrons of the ring system. Because of the coupling between the ring electrons and the carbonyl group, withdrawal of electron density from the ring increases the polarization of the carbonyl π system. This turns the formyl group into a more reactive conformation. On the other hand, attack by a proton from the solution on the carbonyl π system becomes more difficult. This effect is absent with 2-sulfobenzaldehyde, and therefore the hydration kinetics of this compound are similar to those of the aliphatic aldehydes.

In addition, substituents which are capable of forming hydrogen bridges to the solvent in the vicinity of the formyl group favor solvation and therefore facilitate the hydration. This latter effect can only result in an increase in the intramolecular catalysis but cannot account for a reduction in the proton catalysis. Thus, it may be argued that this is responsible for the lack of a plateau at low pH values in the hydration curve of 3-methoxypyridoxal 5′-phosphate.

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