# THE KINETIC BEHAVIOR OF VITAMIN-$B_6$ COMPOUNDS HYDRATION AND PROTON TRANSFER

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## 1. Introduction

Most physico-chemical studies of vitamin-$B_6$ and its derivatives have been concerned with the equilibrium properties of the compounds [1–4]. Few studies exist of their kinetic behavior [5–8]. The present work is concerned with the kinetics of hydration of the formyl group, and with the inter- and intramolecular proton transfers involved in the ionization of the compounds between their uncharged and zwitterionic forms present in the pH range between 5.5 and 7.5 [9–11]. It was found that in compounds lacking the phosphate group, protons are transferred by an intermolecular process, whereas, in compounds possessing the phosphate group intramolecular reaction is facilitated by that group acting as a proton mediator. This fact may be significant in the behavior of pyridoxal-5′-phosphate as a co-factor in enzymatic catalysis.

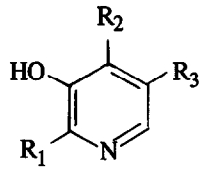
## 2. Materials and methods

The compounds are presented in table 1. The substances were of analytical grade and were purchased from Fluka AG and Ega-Chemie. The pyridinealdehydes were redistilled at reduced pressure and kept under dry nitrogen. 5-Desoxypyridoxal was generously provided by Prof. H.H.Inhoffen.

Kinetic experiments were performed at 25°C using a temperature-jump relaxation apparatus. In the hydration experiments the concentration of the reactants was usually $2 \times 10^{-4}$M, and in the proton transfer experiments it was varied within the range $2 \times 10^{-5}$ M – $4 \times 10^{-4}$M. Solutions were brought to the appropriate pH by addition of hydrochloric acid and sodium hydroxide, and the ionic strength in all experiments was adjusted to 0.1 M by addition of sodium chloride.

Table 1

| Compound | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Pyridoxal | $CH_3$ | CHO | $CH_2OH$ |
| Pyridoxal-5′-phosphate | $CH_3$ | CHO | $CH_2OPO_3H_2$ |
| Pyridoxamine-5′-phosphate | $CH_3$ | $CH_2NH_2$ | $CH_2OPO_3H_2$ |
| 5-Desoxypyridoxal | $CH_3$ | CHO | $CH_3$ |
| 3-Hydroxypyridine | H | H | H |
| Pyridine-3-aldehyde | H | H | CHO |
| Pyridine-4-aldehyde | H | CHO | H |



## 3. Results and discussion

All compounds except the hydroxypyridines exhibit two relaxation processes. One ($\tau_I$) is attributed to the hydration of the formyl group. $\tau_I$ was found to be independent of the concentration of reactants, but varied within the range between seconds and microseconds depending on pH. The second process ($\tau_{II}$), which could be resolved only when the pH of the solution lay between 5.5 and 7.5, occurs only with vitamin-$B_6$ compounds and the hydroxypyridines, which possess at least two proton donor-acceptor functions. $\tau_{II}$ was found to depend on pH, and also, except with pyridoxalphosphate and pyridoxaminephosphate on the concentration of reactants.

### 3.1. *Hydration reactions*

Fig. 1 illustrates the dependence upon pH of log $(1/\tau_I)$ for pyridoxal-5′-phosphate; the other vitamin-$B_6$ compounds behave similarly.

Pyridoxal-5′-phosphate has four $p$K-values ($pK_1 < 2.5$; $pK_2 = 4.1$; $pK_3 = 6.2$; $pK_4 = 8.7$). The $p$K-values are sufficiently separated so that at a pH lying between two adjacent $p$K's the compound is present in a particular state of protonation. Because the extent of hydration depends upon the molecular environment, each state of protonation of the molecule is associated with a characteristic constant of hydration

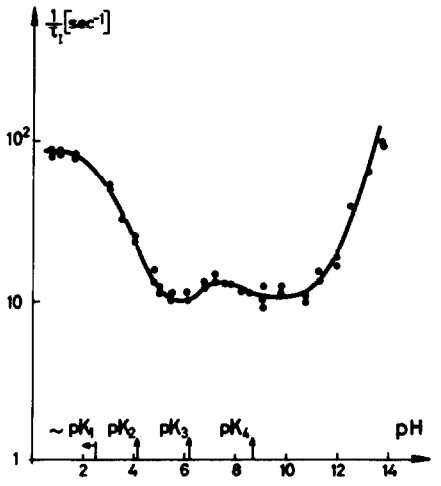
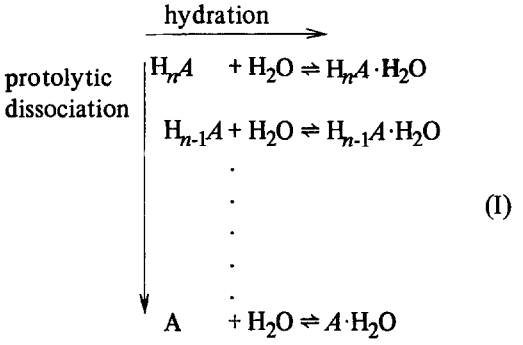



Fig. 1. Acidity dependence of $1/\tau_I$ for the hydration reaction of pyridoxal-5′-phosphate. Measured values of log $(1/\tau_I)$ versus pH.

($K_{H,i}$). The complete reaction scheme for the hydration is given below:




The various hydration equilibria are coupled via the protolytic reactions which are fast compared with the hydration steps. A rigorous treatment of this mechanism yields a complex expression for $1/\tau_I$, but it can be shown that within each range of pH limited by adjacent $p$K-values, the following expression is a good approximation:

$$1/\tau_{I,i} = \vec{k}_{I,i} + \overleftarrow{k}_{I,i} = \overleftarrow{k}_{I,i}\ (1 + k_{H,i}). \qquad (1)$$

$\vec{k}_{I,i}$ and $\overleftarrow{k}_{I,i}$ are pseudo-monomolecular rate constants for the forward and backward step of the hydration, respectively. As in the case of the aliphatic aldehydes, $\alpha$-ketoacids, and benzaldehyde [12–14], it must be assumed that the hydration of the vitamin-$B_6$ compounds and their analogs are catalyzed by $H^+$- and $OH^-$-ions. In addition, contributions to the rate of hydration resulting from intramolecular catalysis must be taken into account. The following equations express the rates of the forward and backward steps of the hydration, respectively:

$$\vec{k}_{I,i} = \vec{k}^{O}_{I,i} + \vec{k}^{H}_{I,i} c_H + \vec{k}^{OH}_{I,i} c_{OH};$$
$$\overleftarrow{k}_{I,i} = \overleftarrow{k}^{O}_{I,i} + \overleftarrow{k}^{H}_{I,i} c_H + \overleftarrow{k}^{OH}_{I,i} c_{OH}. \qquad (2)$$

Now, the third term of eqs. (2) becomes negligible under strongly acidic conditions, and the second term under strongly alkaline conditions. Thus, provided that intramolecular catalysis is slow, a plot of log $(1/\tau_I)$ versus pH should yield a straight line of slope -1 or +1 depending on whether the reaction is acid or base catalyzed. But, as the data (fig. 1) show, $1/\tau_I$ remains approximately constant over the pH range

bounded by two adjacent $p$K-values. This implies that the compounds studied here undergo predominantly intramolecular catalysis, and therefore eqns. (2) adopt the following forms:

$$\vec{k}_{I,i} = \vec{k}^{\circ}_{I,i} \quad \text{and} \quad \overleftarrow{k}_{I,i} = \overleftarrow{k}^{\circ}_{I,i}.$$

The acidity dependence of log $(1/\tau_I)$ for the hydration of a compound associated with four $p$K-values is shown schematically in fig. 2.

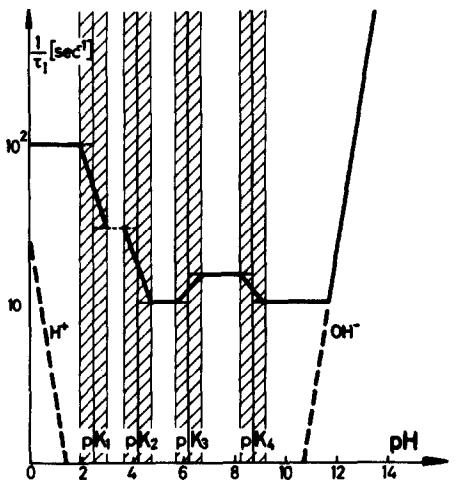


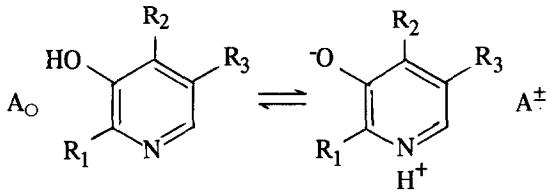

Fig. 2. Schematic diagram of log $(1/\tau_I)$ versus pH as typical for the hydration of a heterocyclic formyl compound associated with four $p$K-values. $H^+$ and $OH^-$ denote acid and base catalysis.

The limiting values for catalysis by $H^+$ and $OH^-$, and the values of the several intermediate $k^{\mathbb{Q}}$'s illustrate typically the behavior of heterocyclic formyl compounds. The data for pyridoxal-5'-phosphate (fig. 1) show that intramolecular reaction is dominant even under strongly acidic conditions; however, other compounds examined in this work were observed to undergo intermolecular acid catalysis.

The data in table 2 show that, in contrast with the situation observed in other aldehydes, hydration of the formyl group of pyridine derivatives proceeds overwhelmingly by intramolecular catalysis. However under conditions in which intermolecular catalysis was observed, the hydration appeared slower than that observed with other aldehydes. This kind of behavior towards the addition of water is ascribed to the very high number of protons available in the reactant molecules. A more detailed discussion will be presented in a later paper.

### 3.2. *Proton transfer reactions*

Pyridine hydroxy derivatives at medium pH can be present in solution in two different electrically neutral forms with an equilibrium between the uncharged species $A_o$ and the zwitterionic form $A^{\pm}$:




This isomerization implies that proton transfer may proceed both by an intermolecular and by an intramolecular path. The experimental data show that in all compounds without a phosphate group the proton transfer between the ring nitrogen and the oxygen in position 3 occurs predominantly by an intermolecular reaction. Upon introduction of the phosphate group, as in pyridoxal-5'-phosphate, the type of reaction changes from a two- to three-center reaction. Depending on pH, one or two protons are shared amongst three functional groups. Direct proton transfer between the ring nitrogen and the oxygen in position 3 as described above is possible, but in the presence of another group with proton donor-acceptor functions an additional mode of intramolecular proton transfer occurs. In this respect the phosphate group is unique because its $p$K-value lies between the $p$K's of the other two functional groups. Such a transfer in a three center process involves a contribution to the relaxation time independent of the partner concentration, as is found experimentally.

This may be relevant to the function of phosphorylases for it has been shown [15,16] that pyridoxal cofactors not possessing the phosphate group are enzymatically inactive. In addition, the importance of the phosphate group cannot be due solely to the electrostatic interaction between this group and positively charged side chains on the enzyme, for the enzyme becomes inactive on substitution of the phosphate

Table 2

Rate constants for intramolecular and $H^+$- and $OH^-$-catalysis in the formyl hydration of pyridinealdehydes and vitamin-$B_6$ compounds. Intramolecular rate constants ($k^{\circ}_{I,i} + k^{\circ}_{I,i}$) are listed according to the order of $pK$-values given in the first column.

| Compound | $pK$-values * | | | | ref. | Catalytic rate constants | | |
|---|---|---|---|---|---|---|---|---|
| | $pK_1$ | $pK_2$ | $pK_3$ | $pK_4$ | | $(k^{H}_{I,i} + k^{H}_{I,i})$ [$M^{-1}$ $sec^{-1}$] | $(k^{\circ}_{I,i} + k^{\circ}_{I,i})$ [$sec^{-1}$] | $(k^{OH}_{I,i} + k^{OH}_{I,i})$ [$M^{-1}$ $sec^{-1}$] |
| Pyridine-4-aldehyde | 4.77 | | | | [10] | 25 | $\sim 2.5; \leqslant 0.45$ | $2.5 \times 10^5$ |
| Pyridine-3-aldehyde | 3.80 | | | | [10] | 8 | | |
| Pyridoxal | 4.20 | 8.66 | | | [1] | $<55$ | 55; 1.5; | $5 \times 10^6$ |
| 5-Desoxypyridoxal | 4.17 | 8.14 | | | [1] | $<50$ | 50; 2.0; $\sim 15$ | $1.4 \times 10^3$ |
| Pyridoxal-5'-phosph. | 2.5 | 4.14 | 6.20 | 8.69 | [14] | $<85$ | 85; 10; 13; 10 | $\geqslant 1.3 \times 10^2$ |
| Acetaldehyde | | | | | | $9 \times 10^2$ | $\sim 7 \times 10^{-3}$ | $8 \times 10^4$ |

* $pK$-values of the hydrated formyl groups not tabulated.

group [15]. A decision about the detailed mechanism of the proton transfer must await further experiments, but it appears possible that the acid-base function of the phosphate group plays an important role in the enzymatic action of phosphorylases.

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## References

[1] D.E.Metzler and E.E.Snell, J. Am. Chem. Soc. 77 (1955) 2431.
[2] Y.Matsushima and A.E.Martell, J. Am. Chem. Soc. 89 (1967) 1322.
[3] O.A.Gansow and R.H.Holm, Tetrahedron 24 (1968) 4477.
[4] Yu.V.Morozov, N.P.Bazhulina, L.P.Cherkashina and M. Ya.Karpeiskii, Biofizika 12 (1967) 397.
[5] Y.Pocker and J.E.Meany, J. Am. Chem. Soc. 89 (1967) 631.
[6] Y.Pocker and J.E.Meany, Biochemistry 6 (1967) 239.
[7] Y.Pocker and J.E.Meany, J. Phys. Chem. 72 (1968) 655.
[8] F.Bergel and K.R.Harrap, J. Chem. Soc. (1961) 4051.
[9] S.F.Mason, J. Chem. Soc. (1958) 674.
[10] K.Nakamoto and A.E.Martell, J. Am. Chem. Soc. 81 (1959) 5857.
[11] K.Nakamoto and A.E.Martell, J. Am. Chem. Soc. 81 (1959) 5863.
[12] R.P.Bell, in: Advances in physical and organic chemistry, Vol. 4 ed. V.Gold (Academic Press, London and New York 1966) p. 1.
[13] M.-L.Ahrens, Ber. Bunsenges, Phys. Chem. 72 (1968) 692.
[14] M.-L.Ahrens, G.Maass, P.Schuster and H.Winkler, to be published.
[15] S.Shaltiel, J.L.Hedrick, A.Pocker and E.H.Fischer, personal communication.
[16] A.Pocker and E.H.Fischer, personal communication.