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Catalytic Reactions Involving Azomethines. VI. The Mechanism of the Transamination of 3-Hydroxypyridine-4-aldehyde by Glutamic Acid*

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ABSTRACT: Under the conditions of L-glutamic acid >> 3-hydroxypyridine-4-aldehyde (30°, μ = 0.5 M with KCl), the disappearance of aldehyde has been found to be first order in amino acid and first order in aldehyde and not to require metal ion catalysis (no inhibition by added EDTA). From a knowledge of (a) the pK_a' values of the reactants, (b) the *p*H dependence of the equilibrium constant for imine formation from reactants, and (c) the *p*H dependence of the pseudo-first-order rate of the overall disappearance of aldehyde, it is possible to calculate the concentration of reactant and intermediate species in solution and identify the species undergoing the rate-limiting step (*N*-[3-hydroxy-4-pyridinium methylene]glutamate; $S^{\oplus}$ of Chart I).

By isotope-dilution analyses, the fraction of product arising via a transamination reaction has been found to be only *ca.* 0.4 at *p*H 4.5 and *ca.* 0.1 at *p*H 5.7. Therefore the acid dependency of the transamination reaction is somewhat greater than that for the overall disappearance of aldehyde. The rate-limiting spontaneous prototropy of the transamination reaction is suggested to occur through species $S^{\oplus}$ by way of intramolecular general acid catalysis by the 3-hydroxyl group in its undissociated form. The prototropic shift converting $S^{\oplus}$ to the corresponding ketimine is catalyzed by imidazole-free base. For the imidazole-catalyzed reaction, isotope-dilution experiments indicate that the disappearance of aldehyde can be quantitatively accounted for by transamination (*p*H 7.12). The mechanism of the catalyzed reaction is suggested to be a concerted bimolecular general base (by imidazole) and an intramolecular general acid-catalyzed process (by 3-hydroxyl group).

Pyridoxal phosphate and related members of the vitamin B_6 group play a central role in intermediary metabolism. The various enzymatic reactions for which this group of compounds serve as cofactors have been recently reviewed (Snell, 1958; Braunstein, 1960; Snell *et al.*, 1963). We have undertaken the study of model systems in an attempt to elucidate, in detail, the mechanism of the transamination reaction between amino acids and pyridoxal and its analogs. Earlier investigations, which form the basis for our approach to the problem, have been reviewed in paper I (Bruice and Topping, 1963a).

At any constant acidity the transamination reaction may be written as:

$$AH + PCHO_T \underset{\text{fast}}{\overset{K_{p\text{H}}}{\rightleftharpoons}} S_T' \overset{k_2'}{\underset{k_{-2}'}{\rightleftharpoons}} S_T'' \overset{K_3'}{\underset{\text{fast}}{\rightleftharpoons}} K_T + PCH_2NH_{2T} \quad (1)$$

where AH = total amino acid, $PCHO_T$ = total aldehyde, S_T' = total aldimine [PCH=N—CH(R)COOH], (i.e., aldimine in all its ionic forms), S_T'' = total ketimine [PCH_2—N=C(R)COOH], K_T = total keto acid, and

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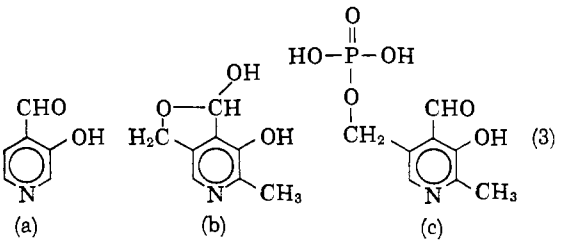
PCH_2NH_2 = total amine.[1] In equation (1) the slow step must be the prototropic shift (k_2'/k_{-2}'), and this has been established by Bruice and Topping (1963a,b,c) to be subject to concerted general acid, general base catalysis by imidazole-imidazolium ion in the transamination of pyridoxal by α-aminophenylacetic acid. In an important study, Blake *et al.* (1963) found that α-deuterio-L-alanine transaminates with pyridoxal at approximately one-third the rate observed with α-protio-L-alanine. This result supports the suggestion (Bruice and Topping, 1963a) that the rate-limiting step in (1) involves proton abstraction from S_T'. Banks *et al.* (1961) attempted a detailed kinetic investigation of the transamination reactions between pyridoxal and alanine and between pyridoxamine and pyruvic acid. Owing to the small rate constants, competing side reactions, and an unexplained stoichiometry, few deductions could be made. In experiments where the reactions were followed to a maximum of 6% completion, evidence was offered to support general acid catalysis in (1).

In (1) the reactants, intermediates, and products exist in various ionic forms in acid-base equilibria. The concentration of these species is dependent upon the *p*H sensitivity of the apparent constants K_{pH}, k_2'/k_{-2}', and K_3'. Investigation of the *p*H dependence of the equilibrium and rate constants should then allow a detailed description of the species undergoing the rate-determining step and a meaningful study of its catalysis. The simplest system, from a kinetic point of view, is one in which AH >> $PCHO_T$, so that the overall reaction proceeds to completion via pseudo-first-order kinetics:

$$AH + PCHO_T \underset{\text{fast}}{\overset{K_{pH}}{\rightleftharpoons}} S_T' \xrightarrow{k_2'} \text{products} \qquad (2)$$

providing that the rate of establishment of equilibrium K_{pH} is faster than k_2'. Determination of the *p*H dependence of the pseudo-first-order rate constant as well as the independent determination of the *p*H dependence of K_{pH} would then allow, when combined with the pK_a' values, a complete description of the system. The low solubility of α-aminophenylacetic acid precluded this approach in the studies by Bruice and Topping (1963a,b,c). In the present study, we have employed the more soluble, and biologically important, amino acid, L-glutamic acid. One of the major objectives of these experiments has been to ascertain whether or not imidazole can act as a general base catalyst in the transamination of a naturally occurring amino acid and to determine which species of imine is sensitive to the catalytic action of imidazole.

For the aldehyde (PCHO) we have used 3-hydroxypyridine-4-aldehyde (equation 3a) which possesses the essential functional groups for transamination (Braunstein, 1960; Snell, 1958) but lacks the complicating features associated with pyridoxal (3b) and pyridoxal-5'-phosphate (3c).



(3)

In aqueous system, (3a) would appear to be more closely analogous to enzyme-bound (3c) than either (3b) or (3c). For example, it is known that pyridoxal (3b) exists, as indicated, primarily as an internal hemiacetal in aqueous solutions (Metzler and Snell, 1955). Since the enzymatically active cofactor (3c) is phosphorylated at the 5' position, such internal hemiacetal formation as in (3b) is impossible. (At the active site of a pyridoxal phosphate enzyme, the 4-aldehyde group may be in imine linkage with the ε-amino group of a lysine residue [Fischer *et al.*, 1958; Jenkins, 1961].) The biologically active form (3c) is not a good model compound because the phosphate group greatly and unnecessarily complicates *p*H-equilibrium and *p*H-rate profile studies in water. For these reasons then, (3a) represents at least as satisfactory a model for the enzymatic reactions as do (3b) and (3c).

The metal ion catalysis of the disappearance of (3a) by glutamic acid is also described herein. In earlier studies (Metzler and Snell, 1952; Metzler *et al.*, 1954b; Longenecker and Snell, 1957a,b; Matsuo, 1957), the effectiveness of various metal ions as a function of their concentration and *p*H on (1) were determined. The usual procedure was to incubate aqueous solutions of reactants at 100° for various periods of time and analyze the resultant reaction solutions. No attempts at the determination of rate constants were performed in these highly valuable semiquantitative studies. The purpose of our examination of the metal ion catalysis of the disappearance of (3a) in the presence of excess glutamic acid has been principally to determine if metal ion promotion at ambient temperatures is significant and to determine if reproducible pseudo-first-order kinetics could be obtained. A number of metal ions have been examined and the dependence of the pseudo-first-order constants on metal ion concentration and *p*H has been examined in a few select cases. Rate constants for metal ion and non(metal ion) reactions have been compared.

Experimental

Materials. 3-Hydroxypyridine-4-aldehyde was prepared by the method of Heinert and Martell (1959) and stored in the dark *in vacuo* until used.

Anal. Calcd for $C_6H_5O_2N$: C, 58.53; H, 4.09; N, 11.28. Found: C, 58.31; H, 4.16; N, 11.32.

[1] Abbreviations used in this work: PCHO, 3-hydroxypyridine-4-aldehyde; PCH_2NH_2, 3-hydroxy-4-aminomethylpyridine; K, α-ketoglutaric acid; AH, L-glutamic acid; S', the aldimine formed between 3-hydroxypyridine-4-aldehyde and L-glutamic acid; S'', the ketimine formed between 3-hydroxy-4-aminomethylpyridine and α-ketoglutaric acid; Im_T, total imidazole (where $Im_T = Im_F + ImH^{\oplus}$); Im_F, imidazole-free base; $ImH^{\oplus}$, imidazolium ion; a_H, hydrogen-ion activity as measured by the glass electrode at 30°.

Stock solutions of 3-hydroxypyridine-4-aldehyde were usually prepared just prior to use. If not immediately used, the stock solutions were refrigerated but kept no longer than 2 days. α-Ketoglutaric acid (A grade) was purchased from Calbiochem. L Glutamic acid was obtained from Fisher Scientific Co. and was reagent grade. *o*-Phenylenediamine was Eastman Practical grade and was recrystallized from chloroform before use. Imidazole was Eastman White Label grade and was recrystallized prior to use from acetone–petroleum ether and stored over P_2O_5 *in vacuo*. L-[C^{14}]Glutamic acid was obtained from Schwartz Bioresearch, 125–168 mc/mmole. All other chemicals were of reagent-grade quality. Water used in these experiments was distilled, deionized, and then redistilled from an all-glass apparatus under N_2 and stored under N_2.

Apparatus. Spectrophotometric measurements were made with either a Perkin-Elmer Model 350 double-beam recording spectrophotometer equipped with a thermostated cylindrical cell housing, or with a single-beam Zeiss PMQ II spectrophotometer fitted with a hollow brass cell holder. Constant temperature (30 ± 0.1°) was maintained by Haake circulating water baths. Standard taper stoppered cuvet and cylindrical cells of 1 cm path length were used in all kinetic experiments. These were filled so as to leave no air space. Measurements of *p*H carried out with a Model 22 *p*H meter at constant temperature (30 ± 0.1°) employed a combined-type GK Radiometer electrode and a Radiometer Model PHA 630 Pa scale expander.

Kinetics. All kinetic experiments reported in this paper were carried out at 30 ± 0.1° at a calculated ionic strength of 0.5 M (with KCl). In the initiation of a kinetic experiment, an aliquot of a stock solution of 3-hydroxypyridine-4-aldehyde (0.025 M) was mixed with an aliquot of 0.2 M L-glutamic acid solution at the desired *p*H to give a final aldehyde concentration of 2.5–5.0 × 10^{-4} M. The time of mixing was taken as the zero time of the run. The reaction mixture was transferred to a cylindrical cell or cuvet and gassed for several minutes with a stream of nitrogen, and the cells were then stoppered and sealed with a strip of Parafilm. The change in absorbance (read against a solution containing no aldehyde) was recorded at convenient time intervals between 260 and 400 mμ on the Perkin-Elmer Model 350 recording spectrophotometer or on the Zeiss PMQ II spectrophotometer. In the latter case, absorbance measurements were made at 390 and 320 mμ (± 5mμ depending on the *p*H of the reaction mixture). Reaction solutions were thermostated in the dark except when absorbance measurements were being made.

The value of the experimentally obtained rate constant, k_{obs}, was initially calculated by the method of Guggenheim (1926) at 390 mμ. From the values of $t_{1/2}$ so determined, the change in absorbances, ΔOD, at t_∞ could be calculated. Employing these values of ΔOD_∞, the values of k_{obs} were then recalculated using the conventional integrated form of the first-order rate law, $k_{obs}t = 2.3 \log \Delta OD_\infty/(\Delta OD_\infty - \Delta OD_t)$. External buffers used were acetate (*p*H 5.76, 0.1–0.3 M) and imidazole (*p*H 6.12, 0.1–0.4 M; *p*H 6.65, 0.1–0.6 M; *p*H 7.14, 0.2 and 1.0 M).

For the metal ion catalyzed runs, a convenient salt of the metal was dissolved in the reaction mixture to give a metal ion concentration of 0.01 M. This generally caused a fairly large change in the *p*H but the *p*H did not change further in the course of the reaction. Metal ion addition also caused spectral shifts and the reactions were followed at wavelengths corresponding to the peaks for aldehyde and amine. Most of the rate constants were obtained using the Zeiss spectrophotometer, but with Mn^{2+} and Al^{3+} as the metal ion catalysts, several runs were followed on the Perkin-Elmer Model 350. In these cases the isosbestic points were found to hold until fairly near the end of the reaction.

Product Analysis. A 10-ml reaction mixture was prepared which was 0.2 M in L-glutamic acid at *p*H 4.45 (μ = 0.50 M with KCl), 5 × 10^{-4} M in 3-hydroxypyridine-4-aldehyde, and contained 50 μc of L-[C^{14}]glutamic acid. An identical reaction mixture containing no aldehyde was carried through all subsequent procedures as a radioactivity control.

The reaction was allowed to proceed to *ca.* 50% completion as judged by the absorbancy at 390 mμ. α-Ketoglutaric acid (41 mg, 0.28 mmole was then added as carrier followed by the addition of 270 mg (2.5 mmoles) *o*-phenylenediamine in 2.5 ml of 1 N HCl. After 15 minutes, the contents was strongly acidified with concentrated HCl. After several hours, the quinoxaline derivative was collected and washed with 1 N HCl, water, ethanol, and ether. The compound was recrystallized to constant specific activity from glacial acetic acid–water.

The quinoxaline derivatives (0.875 mg in 0.5 ml of 95% ethanol) were counted in 10 ml of Bray's solution (1960), employing a Packard Tri-Carb Model 314 DC scintillation counter or a Nuclear Chicago Model 723 scintillation counter. The counts found in the control run (no aldehyde) were subtracted from those found in the complete run. The specific activity of the L-glutamic acid in the reaction medium was determined by diluting 10 μl of the reaction solution to 10 ml with 95% ethanol and then counting a 0.5-ml aliquot of this in 10 ml of Bray's solution. In experiments similar to the one described above, external buffers used were acetate (0.2 M, *p*H 5.71) and imidazole (1.0 M, *p*H 7.12).

Results

The equilibrium constants for imine formation between L-glutamate ($NH_2CHRCOO^{\ominus}$) and the three ionic species of 3-hydroxypyridine-4-aldehyde have been reported in a previous communication from this laboratory (French *et al.*, 1965). These constants are related to equilibrium constants K_1 and K_2 of Chart I in the present study by the acid dissociation constants of glutamic acid (amino group) and 3-hydroxypyridine-4-aldehyde. Values for equilibrium constants K_1 and K_2 in Chart I can therefore be calculated to be 0.86 and 21.6 M^{-1}, respectively.

Over time periods greater than those required to determine equilibrium constants, disappearance of al-

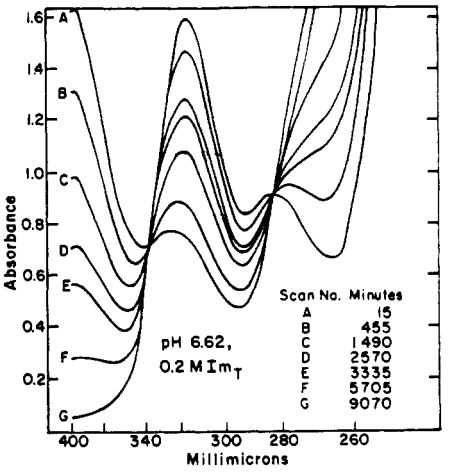


FIGURE 1: Spectral time study for the reaction of 3-hydroxypyridine-4-aldehyde (4×10^{-4} M) and L-glutamic acid (0.2 M). Temperature, 30°; μ = 0.50 M.

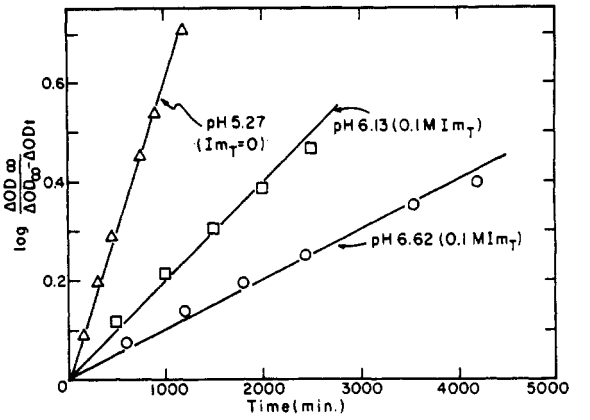
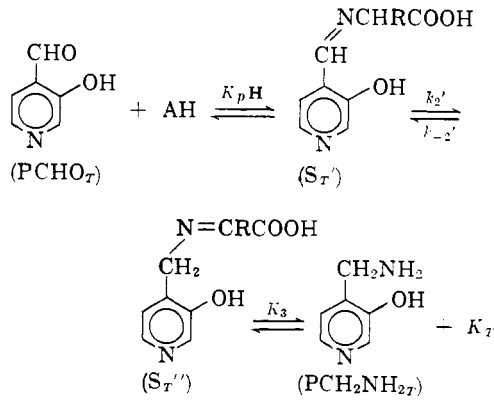


FIGURE 2: First-order plots for the disappearance of 3-hydroxypyridine-4-aldehyde at 395 mμ at several *p*H values (L-glutamic acid = 0.2 M, μ = 0.50 M, 30°).

dehyde plus aldimine occurred as measured by the decrease in absorbance at 390 mμ. A typical spectral time study is shown in Figure 1. The maintenance of tight isosbestic points during the course of the reaction indicates that aldimine is in rapid equilibrium with amino acid and aldehyde and that there is no significant accumulation of other intermediates. A linear relationship exists between the decrease in absorbance at 390 mμ (absorption maximum of aldehyde and aldimine) and the increase in absorbance at 320 mμ (products). These conditions held at all *p*H values investigated. Addition of EDTA (5.4×10^{-3} M) to a reaction mixture at *p*H 4.75 caused no decrease in rate, indicating that trace metal impurities in the reagents cannot be responsible for the disappearance of aldehyde. From the overall equilibrium constants (K_{pH}; see French *et al.*, 1965) it can be calculated that 21% of the aldehyde is in imine form at *p*H 4.5 and 83% of the aldehyde is in imine form at *p*H 7.14 when total glutamic acid and aldehyde equal 0.2 M and 4×10^{-4} M, respectively. Since the ratio of aldehyde to imine will always be constant in the presence of a large excess of glutamic acid, the decrease in absorbance at 390 mμ is a direct measure of the rate of disapperance of total aldimine (S_T') and aldehyde; the increase in absorbance in the 320-mμ region must be due to the formation of pyridine derivatives having no conjugated exocyclic double bond. Pyridine derivatives not possessing exocyclic unsaturation would be S_T'' and 3-hydroxy-4-aminomethylpyridine arising from transamination (4) or from products arising via other routes, e.g., aldol condensations of the type producing β-pyridoxylserine (Snell, 1958).

Regardless of the paths leading to products, the rate-limiting step follows formation of S_T'. In the presence of a large excess of L-glutamic acid, the kinetic scheme for transamination (4) reduces to the pseudo-first-order one as shown in equation (2), since the rate-limiting prototropic shift (k_2') is considerably slower (*vide infra*) than



(4)

the time required for establishment of equilibrium in aldimine (S_T') formation (Metzler, 1957).

The decrease in absorbance at 390 mμ was found to follow fairly good first-order kinetics at all *p*H values studied (Figure 2). The experimental values of k_{obs} are plotted as a function of *p*H in Figure 3, the points being experimental and the line drawn from a theoretical equation (10). In those *p*H regions where a buffer other than glutamic acid was required, rates were measured at several buffer concentrations (μ = 0.50 M) and the values of k_{obs} were determined by extrapolation to zero buffer concentration. These extrapolated values are employed in Figure 3. Control reaction mixtures consisting solely of aldehyde and the external buffers listed (at the highest concentrations used in kinetic experiments; see experimental) showed no significant change with time in the absorbancies at 390 and 320 mμ.

A detailed mechanistic interpretation consistent with the experimental observations is shown in Chart I. From this scheme the rate of disappearance of aldehyde and aldimine species is defined as

$$v = k_2 S^{\oplus} = k_2 \left[\frac{a_H}{a_H + K_S^{\oplus}} \right] S_T \qquad (5)$$

where $S_T = S^{\oplus} + S = K_1(PCHO)(AH) + K_2(PCHO^{\ominus})\cdot(AH)$.

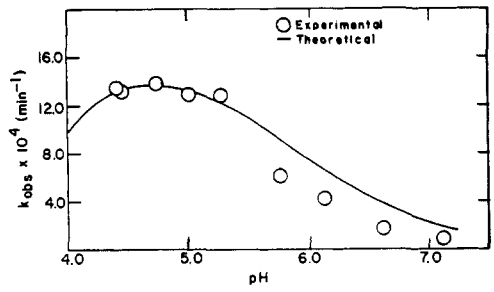


FIGURE 3: Plot of the observed first-order rate constants, k_{obs} (see text), versus *p*H. The theoretical curve is constructed from equation (10).

Therefore, from equation (5)

$$v = k_2\left[\frac{a_H}{a_H + K_S^{\oplus}}\right] \times [K_1(PCHO) + K_2(PCHO^{\ominus})](AH) \quad (6)$$

Defining $(PCHO_T) = (PCHO^{\oplus}) + (PCHO) + (PCHO^{\ominus})$ and solving for $(PCHO_T)$ in terms of (PCHO), one obtains:

$$(PCHO) = (PCHO_T) \times \left[\frac{a_H K_{PCHO}^{\oplus}}{a_{H^2} + K_{PCHO}^{\oplus} a_H + K_{PCHO}K_{PCHO}^{\oplus}}\right] \quad (7)$$

and $(PCHO^{\ominus}) = (PCHO_T)$

$$\times \left[\frac{K_{PCHO}K_{PCHO}^{\oplus}}{a_{H^2} + K_{PCHO}^{\oplus} a_H + K_{PCHO}K_{PCHO}^{\oplus}}\right] \quad (8)$$

Combination of (6), (7), and (8) supplies (9):

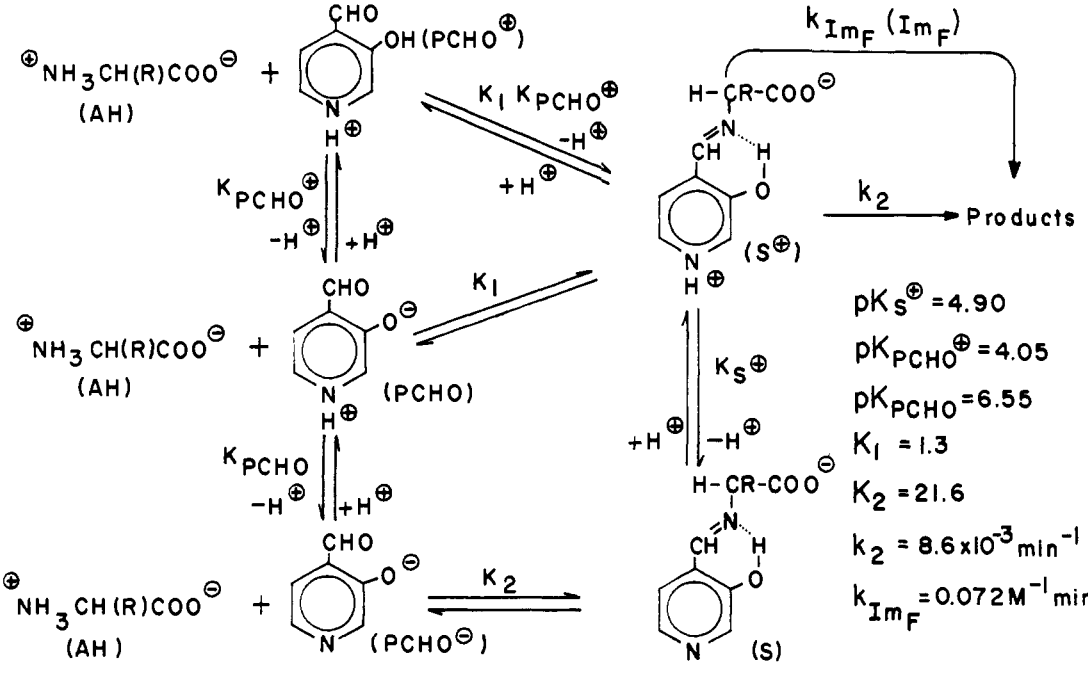
$$v = k_2\left[\frac{a_H}{a_H + K_S^{\oplus}}\right] \times \underbrace{\left[\frac{K_1 a_H + K_2 K_{PCHO}}{a_{H^2}/K_{PCHO}^{\oplus} + a_H + K_{PCHO}}\right]}_{(B)}(PCHO_T)(AH) \quad (9)$$

Experimentally, $v = k_{obs}(PCHO)_T$. Therefore, from (9),

$$k_{obs} = k_2\left[\frac{a_H}{a_H + K_S^{\oplus}}\right](B)(AH) \quad (10)$$

The limited solubility of glutamic acid below *p*H 4.5 prevented determination of the rate of the reaction at these more acid *pH* values. However, experiments in this laboratory (T. C. Bruice and D. S. Auld, unpublished observations) indicate that the disappearance of 3-hydroxypyridine-4-aldehyde in the presence of a large excess of glycine does in fact decrease with decreasing *p*H. Similar observations have been reported by Blake *et al.* (1963) for the transamination reaction between L-alanine and pyridoxal. At a higher *p*H value (*p*H 8.48), aldehyde disappearance in the presence of 0.2

CHART I



$$k_{obs} = k_2\left[\frac{K_1 aH + K_2 K_{PCHO}}{K_{PCHO} + aH + aH^2/K_{PCHO}^{\oplus}}\right]\left[\frac{aH}{aH + K_S^{\oplus}}\right](AH)$$

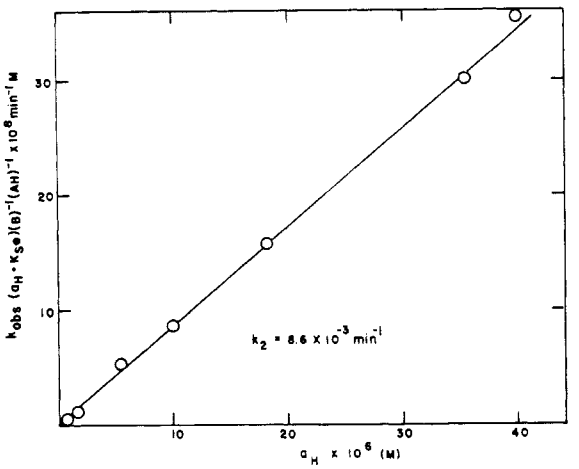


FIGURE 4: Plot of equation (11). The slope provides the value of k_2.

M L-glutamic acid was exceedingly slow and no attempt was made to follow the reaction.

The solid line in Figure 3 has been calculated from equation (10) employing values of 1.26×10^{-5} M and 1.3 M^{-1} for the equilibrium constants $K_S^{\oplus}$ and K_1, respectively. The constants given in Chart I meet the requirements of dynamic equilibrium within a factor of 2.5, which is considered satisfactory in view of the multitude of experimental constants employed in the derivation.

Rearrangement of (10) provides (11)

$$k_{obs}(a_H + K_S^{\oplus})(B)^{-1}(AH)^{-1} = k_2 a_H \qquad (11)$$

A plot of equation (11) is shown in Figure 4; the slope provides the true first-order rate constant for the conversion of imine $S^{\oplus}$ to products and is calculated to be 8.6×10^{-3} min^{-1}.

Isotope-dilution procedures were employed to determine what per cent of aldehyde disappearance could be attributed to a transamination reaction. Theoretically, if the reaction is all transamination one would expect the α-ketoglutarate formed to have the same specific activity as the glutamic acid after correction for the amount of carrier added. At *p*H 4.5 it was found that transamination was responsible for *ca.* 40% of the aldehyde disappearance. The stability of the α-keto acid at *p*H 4.5 was examined to determine if a reaction subsequent to the formation of the α-ketoglutarate might be responsible for recovery of only 40% of this compound. To this end, 0.03 M α-ketoglutarate was incubated at 30° with 0.2 M glutamate buffer (*p*H 4.52). Gravimetric analysis (as the quinoxaline) of 10 ml of the reaction mixture over a 24-hour period showed no decrease in the amount of α-ketoglutaric acid under these conditions. Addition of 0.03 M pyridoxamine to this incubation mixture caused no decrease in the amount of recoverable quinoxaline (at least 90% of theory). Therefore the α-ketoglutarate is stable under the experimental conditions employed.

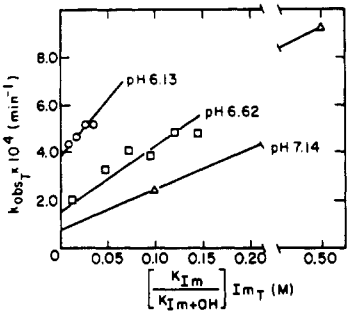


FIGURE 5: Plot of k_{obs_T} versus imidazole free base.

Isotope-dilution experiments at *p*H 5.71 (0.2 M acetate buffer) indicated that less than 10% of the aldehyde disappearance is due to a transamination reaction. In reaction solutions containing imidazole buffers, it was found that k_{obs_T} increased in a linear fashion with increasing concentrations of imidazole free base (Figure 5); k_{obs_T} is the experimentally observed rate constant at a given imidazole concentration, *i.e.*,

$$k_{obs_T} = k_{obs} + k_{cat} \qquad (12)$$

where k_{obs} is the value obtained upon extrapolation to zero imidazole and k_{cat} is equal to the difference between k_{obs_T} and k_{obs} and has been found to fit the relation:

$$k_{cat} = k_{Im_F}\left[\frac{a_H}{a_H + K_S^{\oplus}}\right] \times \left[\frac{K_1 a_H + K_2 K_{PCHO}}{K_{PCHO} + a_H + a_H^2/K_{PCHO}^{\oplus}}\right]\left[\frac{K_{Im}}{K_{Im} + a_H}\right] \times (Im_T)(AH) \qquad (13)$$

This equation predicts a linear relation when $k_{cat}\left[\frac{a_H + K_S^{\oplus}}{a_H}\right]\left[\frac{a_H^2/K_{PCHO}^{\oplus} + a_H + K_{PCHO}}{K_1 a_H + K_2 K_{PCHO}}\right]$ is plotted against $\left[\frac{K_{Im}}{K_{Im} + a_H}\right](Im_T)$ (Figure 6). The slope of the line is equal to the total concentration of amino acid times the true second-order rate constant, k_{Im_F}, for the reaction of Im_F and the protonated imine $S^{\oplus}$, in the rate-limiting prototropic shift. The value of k_{Im_F} calculated from Figure 6 is 0.072 M^{-1} $min.^{-1}$.

Isotope-dilution experiments at *p*H 7.12 in the presence of 1.0 M imidazole indicate that aldehyde disappearance can be quantitatively attributed to transamination (100% recovery of the calculated amount of radioactive α-ketoglutaric acid as the quinoxaline). Obviously then, the general base, Im_F, directs the reaction pathway, and this has important consequences in enzymatic reactions.

The metal ion catalyzed reactions showed, in most cases, good kinetics and were first order over the first

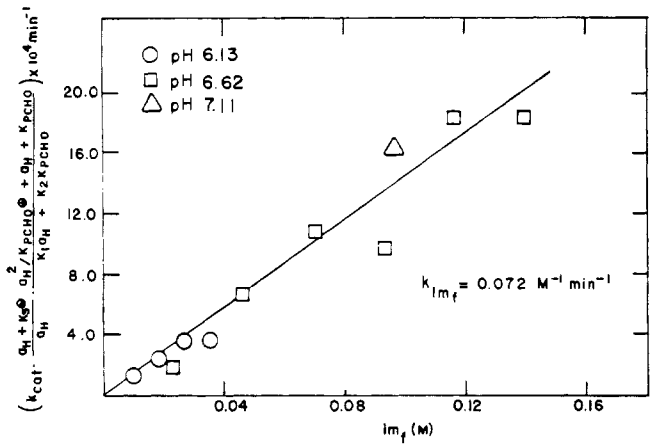


FIGURE 6: Plot of equation (13). The slope provides the value of k_{Im_F} (AH), where k_{Im_F} is calculated to be 0.072 M^{-1} min^{-1}.

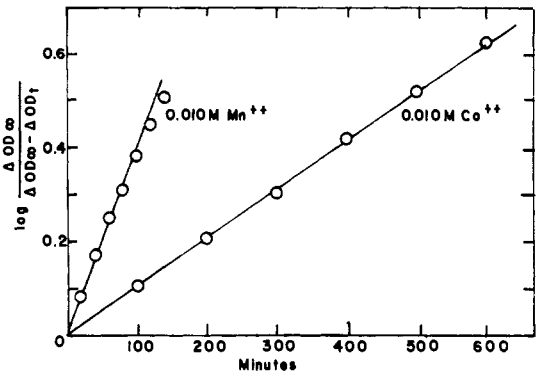


FIGURE 7: Typical pseudo-first-order plots for the transamination between 3-hydroxypyridine-4-aldehyde and L-glutamic acid in the presence of Mn^{2+} and Co^{2+} (0.01 M, *p*H = 4.7, μ = 0.5 M).

two half-lives. In all cases the disappearance of aldehyde and appearance of products were followed; the rates of these two reactions were, in general, about the same although the addition of metal tended to accelerate the disappearance of aldehyde more than the appearance of products at the lower wavelength. The rate constants quoted here are those calculated for the disappearance of aldehyde. Typical first-order plots for two metals are shown in Figure 7.

TABLE I: Metal Ion Catalysis (0.01 M) in the Reaction of 3-Hydroxypyridine-4-aldehyde with Glutamic Acid.

Metal	*p*H	10^3k (min^{-1})
Mn^{2+}	4.67	13.2
Fe^{2+}	4.71	9.3
In^{3+}	4.60	6.0
Al^{3+}	4.68	5.5[a]
Ni^{2+}	4.65	3.3
Co^{2+}	4.72	2.4
Mg^{2+}	4.74	1.6
Pb^{2+}	4.80	1.5
Ba^{2+}	4.84	1.4
None	4.74	1.4
Hg^{2+}	4.69	1.3
Zn^{2+}	4.64	1.2

[a] Extrapolated from lower metal-ion concentrations.

Table I summarizes the results obtained in this study, the metals being listed in the order of their effectiveness. Detailed comparisons of the number in Table I are not particularly meaningful owing to the nonlinear relationship between reaction rate and metal ion concentration (*vide infra*). However, keeping the metal ion constant at 0.01 M does provide some basis for comparison. Certain metals (Sc^{3+}, Cr^{3+}) did not give first-order kinetics; others (Cu^{2+}, Fe^{3+}) formed highly colored solutions upon addition to the reaction mixture (presumably by complexing with the amino acid), and no attempt was made to follow the course of the reaction.

The effect of metal ion concentration upon the rate of reaction was studied using manganese and aluminum as catalysts. In the former case extensive investigation at *p*H 4.67 did not give a consistent picture. In the region of 0.004 M Mn^{2+} the observed rate constant jumped suddenly from about 1.3 × 10^{-3} min^{-1} to *ca.* 8 × 10^{-3} min^{-1}, reaching a maximum of 13.2 × 10^{-3} min^{-1} at 0.01 M Mn^{2+} and then falling off to *ca.* 7 × 10^{-3} min^{-3} at 0.03 M Mn^{2+}. However, there was considerable lack of reproducibility in the rate constants. A much clearer picture is obtained with the aluminum-catalyzed reaction. This follows the pattern described by Longenecker and Snell (1957a,b). Initially, addition of Al^{3+} increases the rate linearly up to a certain concentration but above this concentration the catalytic effect of the metal falls off (see Figure 8).

According to Metzler and Snell (1952) the effectiveness of a metal as a catalyst depends critically upon the *p*H of the reaction mixture. This was investigated over the *p*H range 4.36–6.94 using indium as the catalyst. At *p*H values where buffers other than glutamic acid were required, no attempt was made to extrapolate to zero buffer concentration as the effect of the external buffer is so much less than that of the metal. The concentration of indium was maintained constant at 10^{-2} M in all the runs. The results are shown in Table II. Similar experiments with aluminum were not possible as a precipitate was evident at *p*H values above 4.7. This was also true for indium at low buffer concentrations, but on using 0.4 M imidazole and 0.3 M acetate, no precipitation occurred.

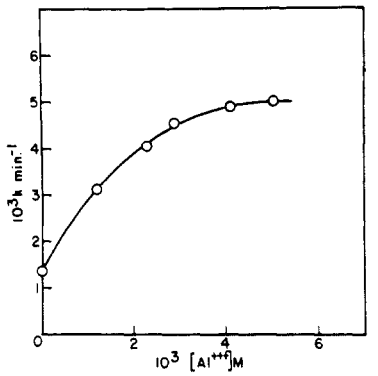


FIGURE 8: Effect of Al^{3+} concentration on k_{obs} (pH = 4.6, μ = 0.5 M).

TABLE II: Effect of pH on the Indium-catalyzed Reaction.

pH	$10^3 k_{obs}$ (min^{-1})[a]	$10^3 k_o$ (min^{-1})[b]	$10^3 k_{In}$ (min^{-1})[c]	k_{In}/k_o
4.36	13.7	1.29	12.4	9.6
4.60	6.0	1.36	4.6	3.4
5.50	5.2	0.98	4.2	4.3
6.32	7.5	0.53	7.0	13.2
6.94	7.5	0.24	7.3	30.4

[a] Observed rate in the presence of 0.01 M In^{3+}. [b] Observed rate in the absence of added metal taken from the theoretical line of Figure 3. [c] $k_{In} = k_{obs} - k_o$.

Discussion

In an earlier study in this laboratory a kinetic investigation was made of the transamination reaction between pyridoxal and the activated (i.e., α-substituted to stabilize an incipient carbanion) and unnatural amino acid, α-aminophenylacetic acid (Bruice and Topping, 1963a,b,-c). Because of the rather low solubility of α-aminophenylacetic acid in water the reaction was examined under the second-order conditions of (amino acid) $\cong$ (pyridoxal). The reaction occurred in two distinct steps (a and b of [14]).

$$\text{(a) } C_6H_5CH(NH_3^{\oplus})COO^{\ominus} + \text{pyridoxal} \overset{K_1}{\rightleftharpoons} \text{aldimine} \overset{k_r}{\rightleftharpoons} \underset{\text{(steady state)}}{\text{ketimine}} \overset{k_{-r}}{} \text{(fast)} \quad (14)$$

$$\text{(b) ketimine} \overset{K_3}{\rightleftharpoons} C_6H_5COCOO^- + \text{pyridoxamine (slow)}$$

In the present study, the kinetics of the reaction between 3-hydroxypyridine-4-aldehyde and L-glutamic acid have been investigated at 30° in aqueous solutions between pH 4.5 and 7.1 at a constant ionic strength of 0.50 M. Under conditions where the amino acid concentration greatly exceeds that of the aldehyde, the kinetic problem at any single pH is simplified from one of form

$$A + B \overset{K}{\rightleftharpoons} C \underset{k_{-rate}}{\overset{k_{rate}}{\rightleftharpoons}} D$$

to a pseudo-first-order form

$$E \overset{k_{obs}}{\rightarrow} D$$

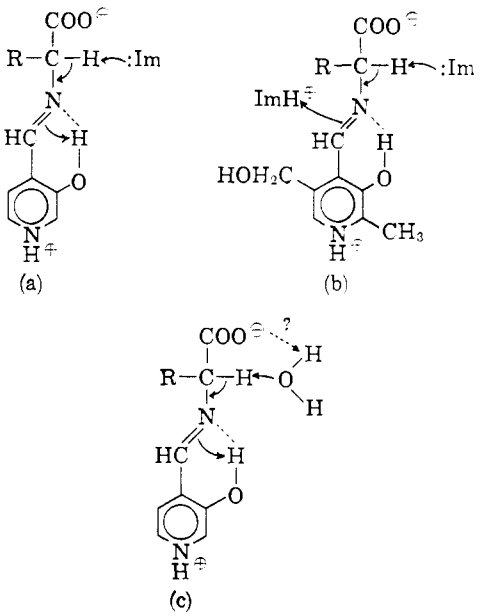
(Figure 2), where E represents an equilibrium mixture of 3-hydroxypyridine-4-aldehyde and its aldimine with glutamic acid. From the known pK_a' values of reactants and from the separately determined pH dependence of the apparent equilibrium constants for formation of aldimine (French *et al.*, 1965) the concentration of the various species of imine in acid-base equilibrium at any pH may be determined. Over the pH range investigated the principal ionic forms of the aldimine have been determined to be S and $S^{\oplus}$ (Chart I). The experimental points in Figure 3 reveal that total aldehyde disappearance in this system increases with increasing acidity even though total aldimine decreases with increasing acidity (French *et al.*, 1965) The acid dependency of aldehyde disappearance as a result of the transamination reaction is even greater as indicated by the isotope-dilution experiments (*ca.* 40% of the total at pH 4.5 and *ca.* 10% at pH 5.7). With this information, plausible mechanisms for the transamination reaction may be postulated. The experimental results are in accord with either of two kinetically indistinguishable pathways; *viz.*, $S^{\oplus}$ undergoes a spontaneous prototropic shift (Chart I) or S is subjected to a specific acid-catalyzed prototropic shift ($k_r = 6.8 \times 10^2$ M^{-1} min^{-1}).

The transamination of 3-hydroxypyridine-4-aldehyde by glutamic acid at constant pH has been found to be a linear function of the concentration of imidazole buffer. The apparent second-order rate constant ($k_{Im_F} = 0.072$ M^{-1} min^{-1}) is found to be constant (Figure 6) at pH values of 6.13, 6.62, and 7.11 if it is assumed that the catalytic species is imidazole-free base and the aldimine undergoing prototropy is $S^{\oplus}$ (Chart I); alternatively the catalytic species is imidazolium ion and the aldimine undergoing prototropy is S ($k_{ImH} = 4.65 \times 10^{-4}$ M^{-1} min^{-1}). *A priori*, one might expect general acid and/or general base catalysis by reactant glutamic acid. It was found that at pH 4.50 the apparent second-order rate constants for the disappearance of 3-hydroxypyridine-4-aldehyde at 390 mμ were 0.69, 0.68, and 0.67 $\times 10^{-2}$ M^{-1} min^{-1} when glutamic acid was at 0.20, 0.15, and 0.10 M, respectively, all other conditions being dentical (μ = 0.50 M, T = 30°). Therefore the reaction is first order in amino acid and there does not appear to be general catalysis by the amino acid itself.

As previously mentioned, isotope-dilution analysis for α-ketoglutaric acid indicates that aldehyde disappearance in the presence of imidazole can be quantitatively accounted for by transamination, whereas this is not the case under other conditions employed in these ex-

periments. Obviously, there is a multiplicity of mechanism by which aldehyde can disappear in the presence of a large excess of glutamic acid, and it is considered significant with respect to enzyme-catalyzed reactions that imidazole directs the reaction toward transamination to the exclusion of other pathways. Conceivably, the imidazole-containing side chain of a histidine residue located in the catalytic region of a transaminase may function in directing the course of the reaction. It is well known that reactions of pyridoxal in model systems are complex and multiple (Snell, 1958). The high specificity of pyridoxal phosphate–requiring enzymes, in contrast, is very likely due to functional groups on the enzyme which act as general acids or bases as required.

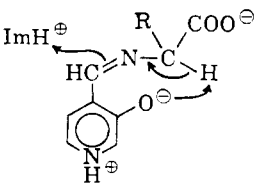
On the basis that meaningful roles may be assigned to the pyridine nitrogen (Metzler *et al.*, 1954a) and hydroxyl group, the favored mechanism of the imidazole-catalyzed prototropic shift in the present study is suggested to be (15a). In the imidazole-catalyzed transamination of pyridoxal by α-aminophenylacetic acid the reaction was found to be second order in buffer. The mechanism of the latter reaction in the *p*H range investigated was suggested to be (15b), occurring with imine species possessing either $-O^-$ or $-OH$ at the 3 position. (Bruice and Topping, 1963b).



(15)

In both (15a) and (15b) imidazole is suggested to act as a general base in the abstraction of a proton from the α position in the rate-determining step. The proton abstraction is suggested to be concerted with intramolecular general acid catalysis by the hydrogen-bonded 3-hydroxyl group in (15a) and with intermolecular general acid catalysis by imidazolium ion in (15b). Kinetically (15a) is indistinguishable from (16). However the rate constant associated with (16) would be prohibitively large owing to the low concentration of the imine species.

The termolecular nature of reaction (15b) was ascribed to a complex formed between the imidazole and imidazolium species and total aldimine (S_T'). This suggestion was in accord with the observation that the reaction followed Michaelis-Menten type kinetics (i.e., first order in each of imidazole, imidazolium ion, and S_T' at low total buffer concentration and zero order in catalyst at high buffer concentration).



(16)

In the spontaneous transamination of $S^{\oplus}$ (Chart I) the mechanism is suggested to be one of intramolecular general acid catalysis by the 3-hydroxyl group, with water acting as a proton acceptor (15c), possibly assisted by the α-carboxyl group. Both the 3-hydroxyl group and the α-carboxyl group are required for reaction. Thus, in an experiment where pyridine-4-aldehyde was substituted for 3-hydroxypyridine-4-aldehyde, no reaction occurred at *p*H 4.74 and γ-aminobutyric acid could not replace glutamic acid in the *p*H range 4.5–9.0. It has been found previously by Metzler *et al.* (1954a) that the 3-hydroxyl group is absolutely essential to obtain a transamination reaction in their system. The necessity of the α-carboxyl group is undoubtedly related to its stabilizing influence on the transition state for proton abstraction.

The phenolic hydroxyl group serves the important function of allowing for considerable imine formation at *p*H values near neutrality (Metzler, 1957). For example, the pK_a' value for the hydroxyl group in free 3-hydroxypyridine-4-aldehyde is 4.05, whereas the corresponding pK_a' in imines of 3-hydroxypyridine-4-aldehyde is around 9.3 (French *et al.*, 1965). The mechanistic implications of this are seen in Chart I where an amino acid reacts with neutral (PCHO) or negatively charged aldehyde ($PCHO^{\ominus}$) to give the corresponding imines ($S^{\oplus}$ and S) via acid-insensitive equilibrium constants. In the case of pyridine-4-aldehyde the formation of imine is dependent on the concentration of unprotonated amino acid and, owing to the acidity dependence of the equilibrium constant for imine formation, the concentration of imines formed with this aldehyde and various amino acids at neutral *p*H values is vanishingly small (French and Bruice, 1964). The increase in the pK_a' of the 3-hydroxyl group on imine formation has been suggested to be due to internal hydrogen bonding to the imine linkage (Metzler, 1957). It is therefore reasonable to suppose that the already hydrogen-bonded hydroxyl group should donate its proton at the time of proton abstraction from the α-carbon of the amino acid in the transamination reaction. Thus the role of the 3-hydroxyl group is to increase the concentration of aldimine species ($S^{\oplus}$) at neutral *p*H and possibly to act as an internal general acid catalyst in the ensuing prototropic shift. The implication of this group in (15a) and (15c) is in accord with the proposed mechanisms of Metzler *et al.* (1954a) for metal-ion catalysis of the transamination reaction, where the metal ion is

suggested to act as an electrophilic catalyst in the abstraction of the α-hydrogen.

The metal ion facilitation of the disappearance of 3-hydroxypyridine-4-aldehyde in the presence of excess glutamic acid has been found to afford first-order kinetics at 30°. The order of metal ion activation was found to be similar to that noted by Longenecker and Snell (1957a,b) at 100°. However Mn^{2+} appears to be an effective catalyst at 30°, whereas it was reported to be ineffective at 100° (1957a,b). A series of metal ions was investigated at *p*H 4.7 (Table I); in one instance (In^{3+}) the reaction was studied over the same *p*H range as the noncatalyzed reaction (Table II). In this case the reaction was found to be relatively insensitive to *p*H whereas the spontaneous reaction exhibits *p*H sensitivity. As a result, In^{3+} (0.01 M) at a *p*H of 4.60 increases the rate of reaction only 3-fold but at *p*H 6.94 a 30-fold increase is obtained.

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