Catalytic Reactions Involving Azomethines. VIII.[1] Water and Alanine Catalysis of the Transamination of 3-Hydroxypyridine-4-Aldehyde by Alanine

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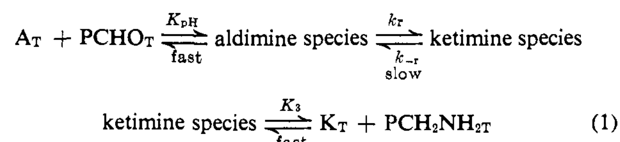
Abstract: The transamination of 3-hydroxypyridine-4-aldehyde by DL-alanine has been studied in detail over the pH range 2.7–7.0 in an aqueous medium in the absence of metal ions at 30° (μ = 1.0 with KCl). The keto acid product of the transamination reaction, pyruvic acid, was determined quantitatively by polarographic and condensation methods. If the pseudo-first-order rate constants (k_{obsd}) are divided by the total amino acid concentration ([A_T]) and plotted *vs.* [A_T] at a constant pH, the kinetics of the transamination reaction can be resolved into a term associated with the alanine-catalyzed isomerization (k_S) and a term associated with spontaneous isomerization (k_I). From a prior knowledge of the pK_a's of the aldimine and aldehyde species and one equilibrium constant of aldimine formation (part VII), the rate constants for the aldimine species undergoing both spontaneous and alanine-catalyzed transamination can be calculated. Both mechanisms associated with the prototropic rearrangement appear to involve a slow proton transfer from aldimine species since a large deuterium isotope effect is obtained in both the alanine catalysis term (k_S) and the spontaneous or water catalysis term (k_I) when DL-alanine-d_4 is substituted for DL-alanine. Since there is no observable transamination of pyridine-4-aldehyde by amino acids even in the pH region above 8 where the apparent equilibrium constant of aldimine formation for pyridine-4-aldehyde is greater than that for the 3-hydroxypyridine-4-aldehyde, the 3-hydroxyl group must play a critical role in the conversion of aldimine species into ketimine species. Therefore, the mechanism of the rate-determining prototropic shift is suggested to be a bimolecular general base catalysis by water or the zwitterion form of DL-alanine coupled with intramolecular assistance by the 3-hydroxyl group which established a partial positive charge on the azomethine nitrogen, thereby stabilizing the transition state.

Several reviews of the enzymatic transamination reaction have appeared.[4–8] The nonenzymatic transamination reaction has also recently been reviewed.[6,9] In our series of papers, "Catalytic Reactions of Azomethines," we have attempted to elucidate, in detail, the mechanism of the nonenzymatic transamination reaction between pyridoxal or its analog 3-hydroxypyridine-4-aldehyde and amino acids in aqueous media at 30°.[1] Since metal ions appear to play no role in the enzymatic reaction,[9] the studies of the model systems were carried out in their absence.

In an aqueous medium the rate-determining step in the transamination reaction is the transformation of aldimine into ketimine (eq 1) since aldehyde and amino acid rapidly equilibrate with aldimine at all pH's from pH 4 to 12.[1e,10]



$$A_T + PCHO_T \underset{\text{fast}}{\overset{K_{pH}}{\rightleftharpoons}} \text{aldimine species} \underset{k_{-r}\ \text{slow}}{\overset{k_r}{\rightleftharpoons}} \text{ketimine species}$$

$$\text{ketimine species} \underset{\text{fast}}{\overset{K_3}{\rightleftharpoons}} K_T + PCH_2NH_{2T} \qquad (1)$$

where A_T = total amino acid, $PCHO_T$ = total aldehyde species, PCH_2NH_{2T} = total amine species, K_T = keto acid species, and K_{pH} = observed pH-dependent equilibrium constant of aldimine formation. Evidence for general base and general acid catalysis of the prototropic shift of aldimines of pyridoxal has been provided independently by Banks, Diamantis, and Vernon[11] and by Bruice and Topping.[1a] Banks, *et al.*, studied the reaction between pyridoxal and alanine and the reverse of the reaction at 25° in an aqueous medium. Because of the slowness of the reaction, competing side reactions prevented the following of the reaction to greater than 6% of completion. The evidence offered to support general acid catalysis was not compelling.

Bruice and Topping[1a] studied the transamination of pyridoxal by α-aminophenylacetic acid in an aqueous medium at 30° in the absence of metal ions. The reaction displayed Michaelis–Menten type kinetics and the prototropic shift was found to be subject to catalysis by imidazole–imidazolium ion. General acid and general base catalysis was offered as the mechanism for the imidazole–imidazolium ion catalysis. The low solubility of α-aminophenylacetic prevented the study of the reaction under the pseudo-first-order conditions of [A_T] ≫ [$PCHO_T$]. This condition was met in the study of Thanassi, Butler, and Bruice[1f] in the quantitative transamination of 3-hydroxypyridine-4-aldehyde by

(1) For parts I, II, and III of this study see: (a) T. C. Bruice and R. M. Topping, *J. Am. Chem. Soc.*, **85**, 1480 (1963); (b) *ibid.*, **85**, 1488 (1963); (c) *ibid.*, **85**, 1493 (1963); for part IV: (d) T. C. French and T. C. Bruice, *Biochemistry*, **3**, 1589 (1964); part V: (e) T. C. French, D. S. Auld, and T. C. Bruice, *ibid.*, **4**, 77 (1965); part VI: (f) J. W. Thanassi, A. R. Butler, and T. C. Bruice, *ibid.*, **4**, 1463 (1965); part VII: (g) D. S. Auld and T. C. Bruice, *J. Am. Chem. Soc.*, **89**, 2083 (1967).

(2) National Institutes of Health Predoctoral Fellow, 1963–1966. Part of the work to be submitted by D. S. Auld in partial fulfillment for the Ph.D. Degree, Cornell University.

(3) To whom inquiries should be addressed.

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(6) E. E. Snell, P. M. Fasella, A. E. Braunstein, and A. Rossi-Fanelli, Ed., "Chemical and Biological Aspects of Pyridoxal Catalysis," in "Proceedings of the Symposium of the International Union of Biochemistry, Rome, 1962, Macmillan and Co., New York, N. Y., 1963.

(7) A. Meister, *Enzymes*, **6**, 193 (1962).

(8) B. M. Guirard and E. E. Snell in "Comprehensive Biochemistry," Vol. 15, M. Florkin and E. H. Stolz, Ed., Ellsevier Publishing Co., Inc., New York, N. Y., 1964, Chapter V.

(9) T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. II, W. A. Benjamin, Inc., New York, N. Y., 1966, Chapter 8.

(10) D. E. Metzler, *J. Am. Chem. Soc.*, **79**, 485 (1957).

(11) B. E. Banks, A. A. Diamantis, and C. A. Vernon, *J. Chem. Soc.*, 4235 (1961).

L-glutamate in imidazole buffers. The prototropic shift was suggested to be imidazole and water general base catalyzed and intramolecularly general acid catalyzed by the 3-hydroxyl group. The low solubility of L-glutamate below pH 4.5 and the extreme slowness of the reaction above pH 6 prevented the extension of this study to a wider pH range.

Since glutamate underwent a transamination reaction with 3-hydroxypyridine-4-aldehyde, an investigation of the reaction of other naturally occurring amino acids with 3-hydroxypyridine-4-aldehyde was undertaken. Of the amino acids studied in a cursory fashion DL-alanine was chosen because of its high solubility over the pH range 2–12 (at least 1.6 *M*) and the number of methods of analysis existing for pyruvic acid, the product of its transamination.

Paper VII[1g] in this series reported the pH profiles at 30° for equilibria and for rate constants of formation and hydrolysis of the aldimines formed from DL-alanine and 3-hydroxypyridine-4-aldehyde. This paper will deal with the mechanisms of the transformation of aldimine into ketimine as elucidated by dependence of the rate and product yield on pH and amino acid concentration and the deuterium isotope effects obtained on substituting DL-alanine-d_4 for DL-alanine.

Experimental Section

Materials. For purity of 3-hydroxypyridine-4-aldehyde and alanine see paper VII in this series. The purity of deuterated alanine (Merck Sharp and Dohme of Canada) was checked by nmr. The fully deuterated alanine is believed to be at least 95% α deuterated. Potassium chloride and potassium hydroxide were reagent grade chemicals. The disodium salt of ethylenediaminetetraacetic acid was Fischer Certified Reagent. Salicylaldehyde (Eastman White Label), α-methylindole (Aldrich White Label), and the potassium salt of pyruvic acid (Calbiochem A grade) were used without further purification. All solutions were prepared in triply distilled, deionized water.

Apparatus. The spectrophotometer employed for rate studies was either a Zeiss M4Q III monochromator or a Beckman DU monochromator combined with a Gilford multiple-sample absorbance recorder. Temperature was kept constant at 30 ± 0.1° by circulation of water through Beckman double thermospacers. The combination Beckman–Gilford spectrophotometer was equipped with a dual wavelength control enabling the recording of the decreasing absorbance at 390 mμ and the increasing absorbance at 320 mμ at alternate times. The time interval between readings was normally about 15 min, during which time the light passed through the blank solution only. The time interval for reading a sample's optical density was 5 sec. Some preliminary kinetic studies and scans of the absorbing product were made with a Perkin-Elmer Model 350 double-beam recording spectrophotometer equipped with a thermostated, cylindrical cell housing. Standard taper stoppered cuvette or ₮ stoppered cylindrical cells of 1-cm path length were used in all kinetic experiments. All pH measurements were made with a Radiometer Model 22 pH meter equipped with a Radiometer Model PHA 630 Pa scale expander. The combined glass calomel electrode (Radiometer GK 2021C) and electrode cell compartment were thermostated at 30 ± 0.1°. The polarograph used for product analysis was a Sargent 1600. A water-jacketed cell was designed for use with the polarograph.

Kinetics. All kinetic measurements in this paper were carried out at 30 ± 0.1° at a calculated ionic strength of 1.0 (with KCl) under pseudo-first-order conditions of a great excess of DL-alanine. The ratio of molar concentrations of DL-alanine to 3-hydroxypyridine-4-aldehyde was at least 1000:1. Stock solutions of the aldehyde were normally 0.025 *M* and were kept frozen when not in use. No solution was kept longer than 1 week. Normally 0.10 ml of the stock aldehyde solution was added to the alanine buffer at the appropriate concentration, ionic strength, and pH, and a dilution to 10 ml was made. The concentration of EDTA was 5.0 × 10^{-3} *M* in all kinetic experiments. The pH was then recorded and the solution transferred to a 2-ml standard taper stoppered cuvette or cylindrical cell. Nitrogen was bubbled in for *ca.* 2 min; the ₮ cuvettes were then stoppered, sealed with parafilm, and equilibrated at 30° in the cell housing of the spectrophotometer for *ca.* 10 min. Optical density was then read alternatingly at 390 and 320 mμ at a fixed time interval. Comparison of the pH's before and after a reaction indicated the drift in pH was never greater than 0.05 pH unit and normally was within 0.02 unit. Pseudo-first-order rate constants were obtained from plots of log $[(OD_0 - OD_\infty)/(OD_t - OD_\infty)]$ *vs.* time for 390 mμ and plots of log $[(OD_\infty - OD_0)/(OD_\infty - OD_t)]$ *vs.* time for 320 mμ. The final optical density at 390 mμ was assumed to be zero since the presumed aromatic product, 3-hydroxy-4-aminomethylpyridine, should not absorb at this wavelength. Experimentally, zero absorbance at 390 mμ was found at t_∞ for pH's below neutrality. Above *ca.* pH 6 a small residual absorbance was obtained (normally less than 10% of the initial optical density). In the acid region, good first-order kinetics were obtained to at least 70% completion of reaction. In the vicinity of pH 6, deviations from first-order kinetics began to be serious but the reactions were still first order to at least 40% completion at pH 7. Above pH 8 the kinetics were not first order, the logarithmic plots exhibiting a decided downward curvature. The values of the intercept (k_I) and slope (k_S) for plots of the pseudo-first-order rate constants (k_{obsd}) divided by total amino acid concentration ($[A_T]$) *vs.* $[A_T]$ were calculated from a least-squares computer program. The errors in the intercept and slope were calculated from the standard error of estimate, standard deviations, and the Student t distribution at the 70% confidence level.

Product Analyses. The keto acid formed in the transamination of 3-hydroxypyridine-4-aldehyde by alanine is pyruvic acid. This product was determined quantitatively by three different methods. The main method used was a polarographic one. Solutions 0.5 *M* in DL-alanine, 2.5 × 10^{-3} *M* in EDTA, and 2.0 × 10^{-4} *M* to 1.0 × 10^{-3} *M* in pyruvic acid were prepared at pH 2.80 ± 0.01 and ionic strength 0.67 (with KCl). These solutions were analyzed on the polarograph at 0.06 μa/mm, t = 2.52 ± 0.02 sec^{-1}, and T = 30.0 ± 0.1°. A linear relationship between concentration and diffusion current was obtained at the half-wave potential of −0.75 v. Product analysis reaction mixtures were prepared from pH 2.7 to 9.1 at intervals of approximately one pH unit. These reaction mixtures were 2.0 × 10^{-3} *M* in 3-hydroxypyridine-4-aldehyde. The solutions were placed in black-taped, screw-cap vials, N_2 was bubbled in for 2 or 3 min, and the vials were capped, wrapped with parafilm, and placed in a constant temperature bath at 30.0 ± 0.1°. After 90–95% reaction, a 5-ml sample was withdrawn and added to a 10-ml volumetric flask. The appropriate amounts of 3 *N* HCl and 4 *M* KCl to assure a constant pH and μ were added, and a dilution was made to 10 ml. The final pH was 2.78 ± 0.01 and the ionic strength 0.67. The solutions were then analyzed on the polarograph under the previously stated conditions for the standards. The other two methods used as a check on the polarographic method were the spectrophotometric determination of the condensation products of pyruvate with salicyaldehyde in base[12] or with α-methylindole in acid.[13]

Results

When DL-alanine and 3-hydroxypyridine-4-aldehyde are allowed to react there is a rapid formation of aldimine, which equilibrates with the aldehyde and alanine in a matter of seconds. A second reaction follows having a half-life of hours, which characteristically shows a decreasing absorbance in the region of 390 mμ and an increasing absorbance in the region of 320 mμ. A linear relationship was obtained between the decreasing absorbance in the 390–365-mμ region and the increasing absorbance in the 320–304-mμ region throughout the entire pH range. The decrease in absorbance at 390 mμ (characteristic of exocyclic conjugation to the pyridine ring) is due to the disappearance of aldehyde and aldimine which are always present in a constant ratio. The increasing absorbance at 320 mμ must be due to pyridine derivatives not possessing exocyclic conjugation to the pyridine ring. At pH's where the yield of pyruvic acid is high the ab-

(12) S. Berntsson, *Anal. Chem.*, 27, 1659 (1955).
(13) Z. Dische, R. Weil, and E. Landsberg, *J. Biol. Chem.*, 208, 23 (1954).

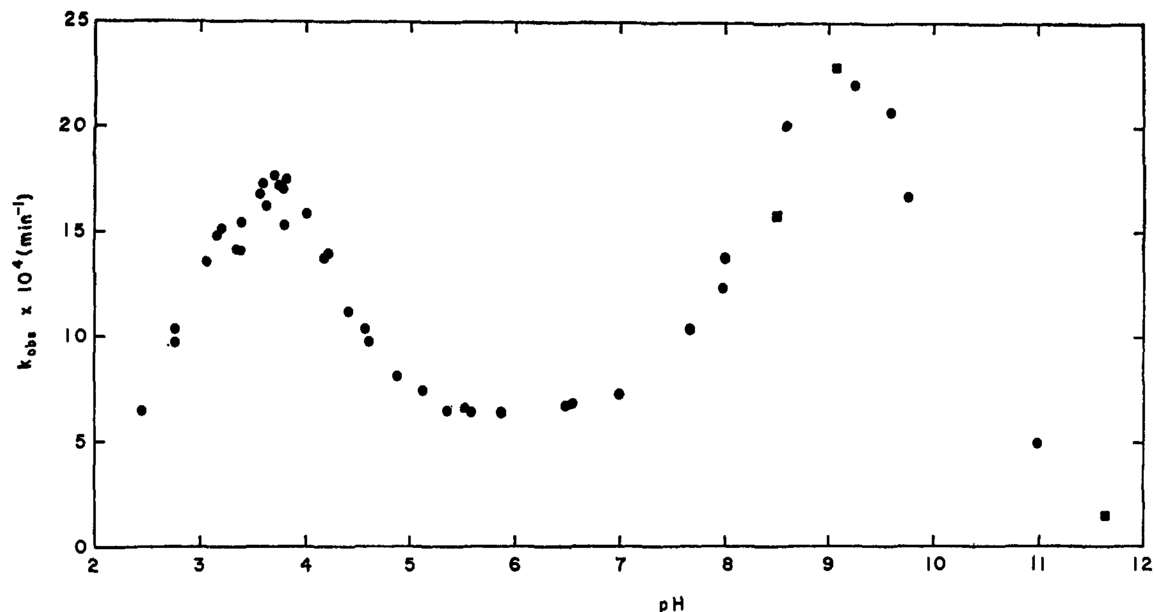


Figure 1. pH-pseudo-first-order rate constant profile at 1.0 M DL-alanine for the transamination of 3-hydroxypyridine-4-aldehyde by DL-alanine. All rate constants calculated at 390 mμ except ■ (305 mμ); μ = 1.0 M; temperature, 30°; [EDTA] = 5 × 10^{-3} M.

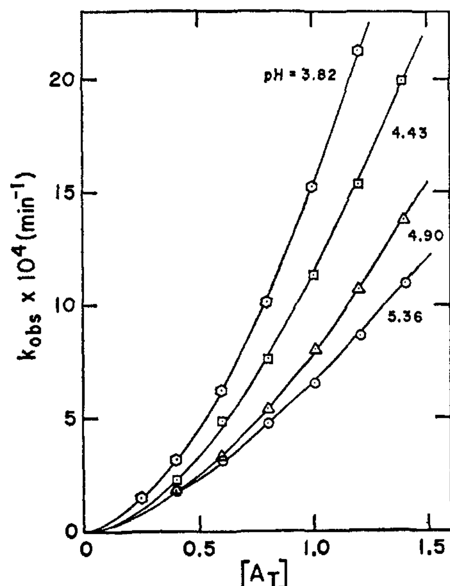


Figure 2. Plots of the pseudo-first-order rate constants (k_{obsd}) for the transamination of 3-hydroxypyridine-4-aldehyde by DL-alanine *vs.* concentration of DL-alanine ($[A_T]$); μ = 1.0; temperature, 30°; [EDTA] = 5 × 10^{-3} M.

sorbance at 320 mμ must be largely due to the pyridoxamine analog, 3-hydroxy-4-aminomethylpyridine.

In all kinetic experiments reported $[A_T] >> [PCHO_T]$ so that pseudo-first-order kinetics were obtained and all reactions proceeded to completion. Comparison of the pH's before and after a reaction indicated that essentially no pH drift occurred during the reaction.

As a safeguard against possible interference from trace metal ions all kinetic studies were carried out at 5.0 × 10^{-3} M in EDTA. The amount of EDTA present did not appear to affect the rate in either the acid or base region in reactions which were only buffered with DL-alanine. Thus, the pseudo-first-order rate constants at pH 3.76 in the absence and presence of 5.0 × 10^{-3} M EDTA were 17.5 and 17.3 × 10^{-4} min^{-1}. The ionic strength was kept constant at 1.0 M with KCl, but increasing or decreasing the ionic strength by 50% caused essentially no change in rate. Above pH 8 first-order kinetics cannot be achieved beyond 15 to 25% of reaction. A buffer dilution in DL-alanine at 9.77 yields a linear relationship between the pseudo-first-order rate constants and DL-alanine concentration but intersects the rate constant axis well above zero at zero alanine concentration. There is, however, only very slight spectral changes for an aldehyde solution in the absence of DL-alanine in either the acid or base region. The entire pH profile of pseudo-first-order rate constants at 1.0 M DL-alanine is shown in Figure 1. We have not attempted at this time to treat in detail the reaction above pH 7.0 because good pseudo-first-order kinetics are not followed when $[A_T] >> [PCHO_T]$, and the dependence of initial rate on $[A_T]$ is quite complex.

Figure 2 shows some typical examples of the dependence of k_{obsd} on the value of $[A_T]$ below pH 7. The upward curvature in the plot of k_{obsd} *vs.* $[A_T]$ suggests that the rate of reaction is dependent on $[A_T]$ to more than the first power. This finding suggests that the rate-determining prototropic shift (Chart I) is both spontaneous and DL-alanine catalyzed.

$$v = d[PCHO_T]/dt = k_{H_2O}[S_T][H_2O] + k_{AH}[S_T][A_T] \quad (2)$$

and since $K_{pH} = [S_T]/[PCHO_T][A_T]$

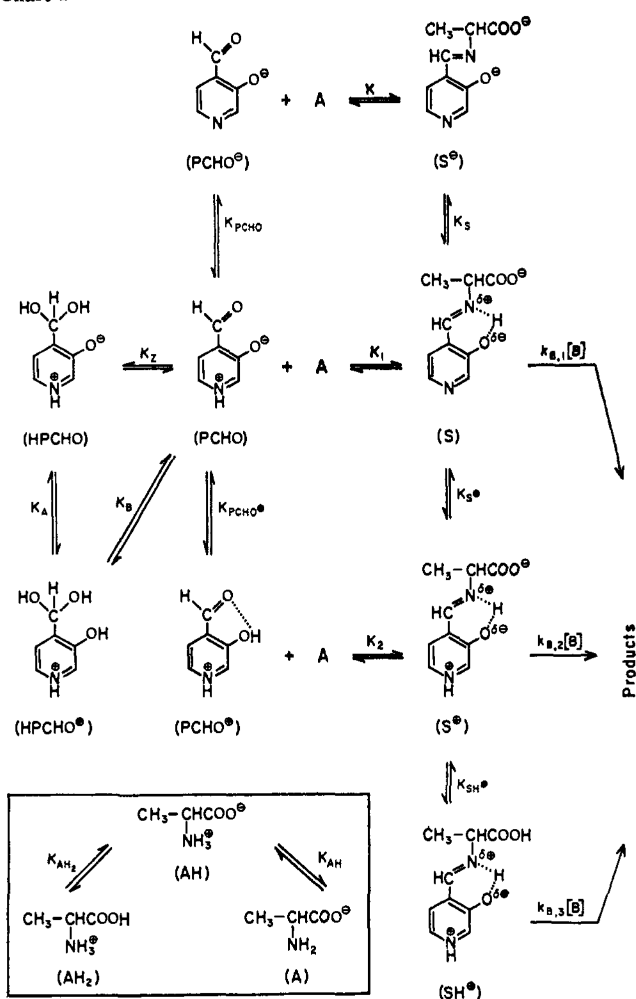
$$v = k_{H_2O}K_{pH}[PCHO_T][A_T][H_2O] + k_{AH}K_{pH}[PCHO_T][A_T]^2 \quad (3)$$

and

$$k_{obsd} = k_I[A_T] + k_S[A_T]^2 \quad (4)$$

It follows from (4) that at a constant pH a plot of $k_{obsd}/[A_T]$ *vs.* $[A_T]$ should give a straight line with the water catalysis term as intercept (k_I) and the alanine catalysis term as the slope (k_S). That a significant contribution from a term second-order in A_T exists is shown in Figure 3. The apparent rate constants k_I and k_S are complex functions of hydrogen ion activity, dis-

Chart I



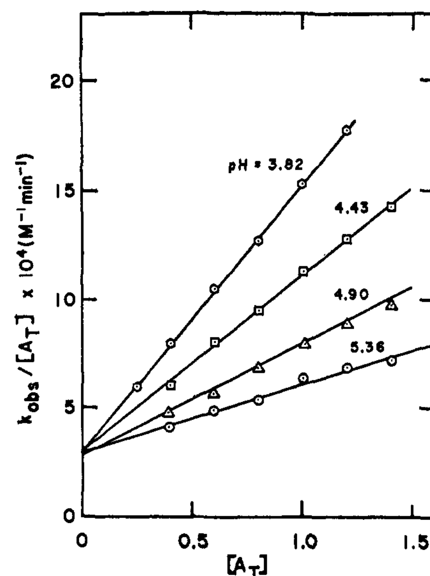


Figure 3. Plots of $k_{obsd}/[A_T]$ *vs.* $[A_T]$; $\mu = 1.0$; temperature, 30°; [EDTA] = 5×10^{-3} *M*.

sociation constants, equilibrium constants, and catalytic rate constants for the individual aldimine species. An exact relationship of k_I to k_S to these thermodynamic and kinetic factors as a function of hydrogen ion activity (a_H) can be derived in the following manner based on the scheme shown in Chart I. The expression for rate constant k_I for spontaneous isomerization of SH^+, S^+, and S is shown in eq 5

$$k_I' = (k_{H_2O,3}[SH^+] + k_{H_2O,2}[S^+] + k_{H_2O,1}[S])[H_2O] \quad (5)$$

where $k_I' = k_I[A_T][PCHO_T]$.

Substituting in eq 5 for the concentration of aldimine species in terms of the equilibrium constants for aldimine formation and aldehyde and amino acid concentrations leads to

$$k_I' = \left(k_{H_2O,3}\frac{K_2}{K_{SH^+}}[PCHO^+]a_H + k_{H_2O,2}K_2[PCHO^+] + k_{H_2O,1}K_1[PCHO]\right)[A][H_2O] \quad (6)$$

Solving for [PCHO] in terms of $[PCHO^+]$ and substituting into (6) leads to

$$k_I' = \left(k_{H_2O,3}\frac{K_2}{K_{SH^+}}a_H + k_{H_2O,2}K_2 + k_{H_2O,1}K_1\frac{K_{PCHO^+}}{a_H}\right)[PCHO^+][A][H_2O] \quad (7)$$

From dynamic equilibrium considerations

$$K_1K_{PCHO^+} = K_2K_{S^+}$$
$$KK_{PCHO^+}K_{PCHO} = K_2K_{S^+}K_S \quad (8)$$

Substituting for K_1 and K_2 from (8) into (7) yields

$$k_I' = \left(\frac{k_{H_2O,3}}{K_{SH^+}}a_H + k_{H_2O,2} + k_{H_2O,1}\frac{K_{S^+}}{a_H}\right) \times \frac{KK_{PCHO^+}K_{PCHO}}{K_{S^+}K_S}[PCHO^+][H_2O][A] \quad (9)$$

Defining $[PCHO_T] = [HPCHO^+] + [HPCHO] + [PCHO^+] + [PCHO] + [PCHO^-]$ and solving for $[PCHO_T]$ in terms of $[PCHO^+]$ lead to

$$[PCHO_T] = \left[\left(\frac{K_{PCHO^+}}{K_B} + 1\right)a_H^2 + K_{PCHO^+}(K_Z + 1) \times a_H + K_{PCHO^+}K_{PCHO}\right]([PCHO^+]/a_H^2) \quad (10)$$

Defining $[A_T] = [AH_2] + [AH] + [A]$ and solving for $[A_T]$ in terms of [A] yield

$$[A_T] = (a_H^2 + K_{AH_2}a_H + K_{AH_2}K_{AH})([A]/K_{AH}K_{AH_2}) \quad (11)$$

Substituting in (9) for [A] and $[PCHO^+]$ in terms of $[A_T]$ from (11) and $[PCHO_T]$ from (10) yields eq 12 where $k'_{H_2O,3} = k_{H_2O,3}/K_{SH^+}$.

The values of k_I and k_S were determined at 18 concentrations of hydrogen ion from pH 2.7 to 7.0 (see Table I). The fitting of eq 12 to the pH-rate constant profile for water catalysis was done with the aid of an IBM 1620 digital computer. The order of criteria for best fit was: (a) the sum of the calculated values that differed from the observed values by more than three times the experimental error at the 70% confidence level was made a minimum; (b) the sum that differed by less than twice the experimental error was made a maximum; and (c) the sum of the absolute value of (observed constant − calculated constant)/observed

$$k_{\mathrm{I}} = \frac{(k'_{\mathrm{H_2O,3}}a_{\mathrm{H}}^3 + k_{\mathrm{H_2O,2}}a_{\mathrm{H}}^2 + k_{\mathrm{H_2O,1}}K_{\mathrm{S^+}}a_{\mathrm{H}})\left(\frac{KK_{\mathrm{PCHO^+}}K_{\mathrm{PCHO}}K_{\mathrm{AH}}K_{\mathrm{AH_2}}[\mathrm{H_2O}]}{K_{\mathrm{S}}K_{\mathrm{S^+}}}\right)}{\left[\left(\frac{K_{\mathrm{PCHO^+}}}{K_{\mathrm{B}}} + 1\right)a_{\mathrm{H}}^2 + K_{\mathrm{PCHO^+}}(K_{\mathrm{Z}} + 1)a_{\mathrm{HPC}} + K_{\mathrm{HO^+}}K_{\mathrm{PCHO}}\right](a_{\mathrm{H}}^2 + K_{\mathrm{AH_2}} + K_{\mathrm{AH}})} \qquad (12)$$

$$K_{\mathrm{S}} = \frac{(k'_{\mathrm{AH,3}}a_{\mathrm{H}}^3 + k_{\mathrm{AH,2}}a_{\mathrm{H}}^2)\left(K\frac{K_{\mathrm{PCHO}}K_{\mathrm{PCHO^+}}K_{\mathrm{AH_2}}^2K_{\mathrm{AH}}}{K_{\mathrm{S}}K_{\mathrm{S^+}}}\right)}{\left[\left(\frac{K_{\mathrm{PCHO^+}}}{K_{\mathrm{B}}} + 1\right)a_{\mathrm{H}}^2 + K_{\mathrm{PCHO^+}}(K_{\mathrm{Z}} + 1)a_{\mathrm{H}} + K_{\mathrm{PCHO^+}}K_{\mathrm{PCHO}}\right](a_{\mathrm{H}}^2 + K_{\mathrm{AH_2}}a_{\mathrm{H}} + K_{\mathrm{AH_2}}K_{\mathrm{AH}})^2} \qquad (13)$$

error was made a minimum. The values of K, pK_{S}, and pK_{AH} previously determined by Auld and Bruice[1g] were fixed at 25.80, 9.17, and 9.62, respectively. The initial value of pK_{S}^+ was chosen to be 5.25 on the basis of the results of equilibrium studies[1g] and p$K_{\mathrm{AH_2}}$ was chosen as its half-neutralization at 1.0 M (2.45). The initial values of pK_{PCHO}, pK_{B}, and K_{Z} were chosen as 6.6, 4.2, and 0.6 from the studies of Nakamoto and Martell[14] at 20°. The value of p$K_{\mathrm{PCHO^+}}$ was chosen as 3.5 initially and the rate constants $k'_{\mathrm{H_2O,3}}$, $k_{\mathrm{H_2O,2}}$, $k_{\mathrm{H_2O,1}}$ were allowed at first to vary over powers of ten holding all other constants at their initial values until a fairly close fit was obtained. Once the order of magnitude of the rate constants was obtained, an iteration-type program was used for the final fitting. Using the initial values of the constants to calculate a reference profile, each constant was checked in turn at its initial value $\pm x\%$ of a given range, holding all other constants fixed at their initial values. Using the criteria for best fit stated previously, a decision was made as to which value of each constant was to be retained for a given pass through all the constants. After each complete pass the pH–rate profile, the constants used for its calculation, and the error tests were printed out. This procedure was continued until all constants remained unchanged for a single pass or until a fixed number of passes had been completed. The value of x was then changed and the entire iteration begun again. The values of x were commonly 10, 5, and 1 and the range for pK_{a}'s was 0.02–0.04. The results of this fitting are shown in Figure 4 and in Table II. The theoretical equation for the DL-alanine catalysis (13) was derived in the same manner as that for spontaneous isomerization and in form is quite similar to (12). In eq 13 $k'_{\mathrm{AH,3}} = k_{\mathrm{AH,3}}/k_{\mathrm{SH^+}}$. No catalysis by DL-alanine of prototropy of S was needed for the fit of the determined values of k_{S} to the theoretical eq 13. It was also determined from the method of fitting that the catalyzing species of DL-alanine was the zwitterion form (AH) if the species of aldimine undergoing catalysis are SH$^+$, S$^+$, and S. The results of the theoretical fitting for alanine catalysis are shown in Figure 5 and in Table II.

Table I. Dependence of the Rate Constants for Spontaneous Catalysis (k_{I}) and DL-Alanine Catalysis (k_{S}) on pH for the Transamination of 3-Hydroxypyridine-4-aldehyde by DL-Alanine

pH	k_{S}, × 10⁴ M^{-1} min⁻¹	k_{I}, × 10⁴ min⁻¹
2.78	7.67 ± 0.38	1.69 ± 0.11
3.08	11.53 ± 0.51	2.27 ± 0.14
3.21	12.84 ± 0.32	2.40 ± 0.09
3.57	13.43 ± 0.63	3.39 ± 0.19
3.63	12.85 ± 0.42	2.94 ± 0.12
3.81	14.35 ± 0.31	3.11 ± 0.09
3.82	12.31 ± 0.15	2.94 ± 0.05
4.02	12.65 ± 0.32	3.04 ± 0.09
4.21	9.90 ± 0.47	3.27 ± 0.14
4.42	8.14 ± 0.24	3.00 ± 0.07
4.62	6.48 ± 0.28	3.12 ± 0.08
4.90	5.10 ± 0.12	2.82 ± 0.04
5.12	4.00 ± 0.21	3.34 ± 0.05
5.36	3.28 ± 0.09	3.25 ± 0.01
5.58	3.17 ± 0.28	3.00 ± 0.08
5.88	2.54 ± 0.34	3.58 ± 0.10
6.50	1.57 ± 0.50	5.06 ± 0.25
6.99	0.66 ± 0.14	6.64 ± 0.05

Table II. Best Values of Parameters for pH Profiles for k_{I} and k_{S}

General base	M^{-1} min⁻¹ × 10⁴: $k'_{\mathrm{B,3}}$[a]	$k_{\mathrm{B,2}}$[a]	$k_{\mathrm{B,1}}$[a]	p$K_{\mathrm{PCHO^+}}$	pK_{PCHO}	pK_{B}	p$K_{\mathrm{AH_2}}$	p$K_{\mathrm{S^+}}$	K_{Z}	Error test[b]: N1	N2	N3	NG3
AH	8.00 × 10⁵	8.00	...	3.26	6.53	4.24	2.53	5.25	0.495	11	5	0	2
Water	2.65 × 10³	1.51 × 10⁻¹	1.71 × 10⁻²	3.28	6.54	4.27	2.67	5.25	0.46	9	2	5	2

[a] B = AH for alanine, H_2O for water. [b] The number of calculated rate constants within one, two, three, and greater than three times the experimental error at the 70% confidence level are denoted as N1, N2, N3, and NG3, respectively.

Deuterium Isotope Effects. To test the hypothesis that the rate-determining step in the prototropic rearrangement of transamination is the abstraction of the proton from the α-carbon of the aldimine, DL-alanine-d_4 was treated with 3-hydroxypyridine-4-aldehyde. A buffer dilution at pH 3.6 with six concentrations of DL-alanine-d_4 was compared to the identical concentrations of DL-alanine under the same conditions. The ratio of the intercepts ($k_{\mathrm{I}}^{\mathrm{H}}/k_{\mathrm{I}}^{\mathrm{D}}$) was 2.2 and the ratio of the slopes ($k_{\mathrm{S}}^{\mathrm{H}}/k_{\mathrm{S}}^{\mathrm{D}}$) was 9.0.

Product Analyses. Polarographic and spectrophotometric determinations of pyruvic acid were employed to determine what per cent of the reaction between DL-alanine and 3-hydroxypyridine-4-aldehyde proceeded by transamination. The spectrophotometric methods consisted of pyruvate condensing salicyladehyde in base[12] and α-methylindole in acid.[13] The per cent transamination as determined by these three methods was 81 ± 6% at pH 3.6, 57 ± 8% at pH 4.6, and 59 ± 10% at pH 5.6. At pH 7.5 and 9.0, the per cent transaminations as determined by the polarograph

(14) K. Nakamoto and A. E. Martell, *J. Am. Chem. Soc.*, 81, 5866 (1959).

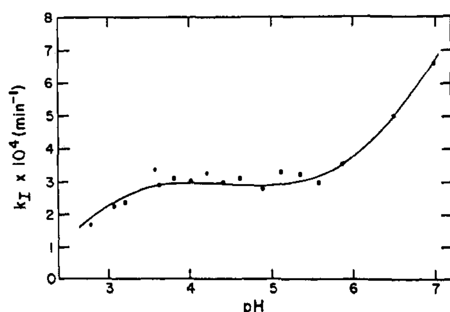


Figure 4. pH–rate constant (k_I) profile for the water catalysis of the transamination of 3-hydroxypyridine-4-aldehyde by DL-alanine: ●, experimental values of k_I; ———, calculated from eq 12 using the values for the constants given in Table II.

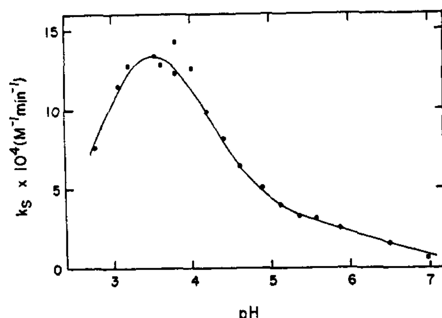


Figure 5. pH–rate constant (k_S) profile for the alanine catalysis of the transamination of 3-hydroxypyridine-4-aldehyde by DL-alanine: ●, experimental values of k_S; ———, calculated from eq 13 using the values for the constants given in Table II.

method were 60 and 40%, respectively. The initial concentration of aldehyde in these product analyses experiments was 5.0×10^{-4} *M* to 2.0×10^{-3} *M*. In the pH region 4.6–5.6, the polarographic analysis method showed a second reduction wave with a half-wave potential of −0.94 v in addition to the pyruvate reduction wave with a half-wave potential of −0.74 v. It is of interest to note that the per cent transamination at pH 4.6 was lowered from 55 to 33% when the concentration of aldehyde was raised from 5.0×10^{-4} *M* to 5.0×10^{-3} *M* with the concentration of DL-alanine being held constant at 1.0 *M*. The diffusion current measured at the half-wave potential −0.94 v increased sevenfold for the tenfold increase in aldehyde concentration. It would appear, therefore, that the reactions competing with transamination are dependent on more than the first power of the aldehyde and/or aldimine concentration and that with solutions infinitely dilute in [$PCHO_T$] transamination would be the sole reaction. Since most of the kinetic runs were done at *ca.* 3×10^{-4} *M* aldehyde the per cent transamination under the kinetic conditions may be slightly greater than that determined by product analysis methods.

In order to determine further the nature of the products, spectrums of the solutions from a number of kinetic experiments at various pH values were determined at t_∞ after adding small amounts of solid KOH or concentrated HCl. Figures 6 and 7 show some scans taken of the absorbing product from the reaction between DL-alanine and 3-hydroxypyridine-4-aldehyde in the pH region below 4. Since the yield of pyruvic acid is almost quantitative in this pH region, the absorbing product would be almost entirely 3-hydroxy-4-aminomethylpyridine. The changes in ultraviolet spectrum with pH can be accounted for on this assumption

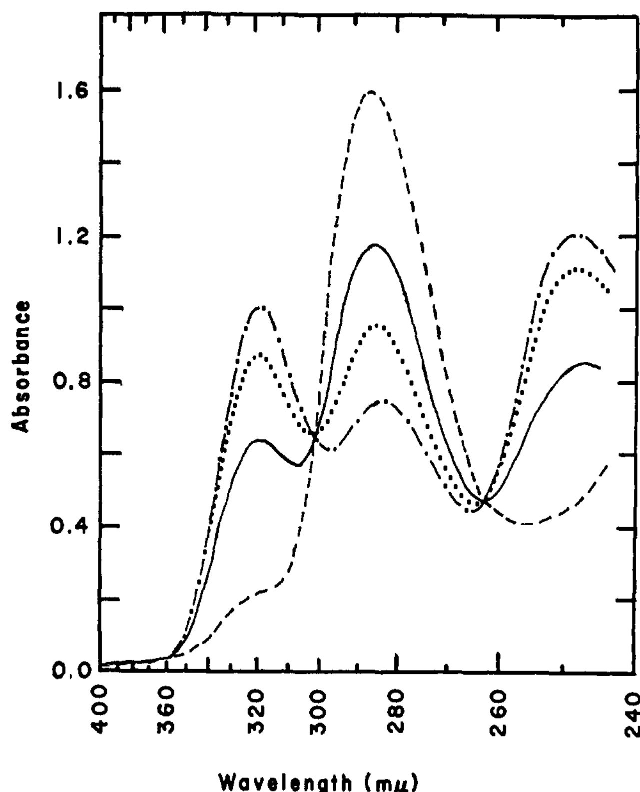


Figure 6. Ultraviolet absorbance scans of the absorbing product from the transamination of 3-hydroxypyridine-4-aldehyde by DL-alanine in the acid pH region: pH 3.2, - - - -; pH 4.2, ———; pH 4.7, ...; pH 5.1, – · – ·.

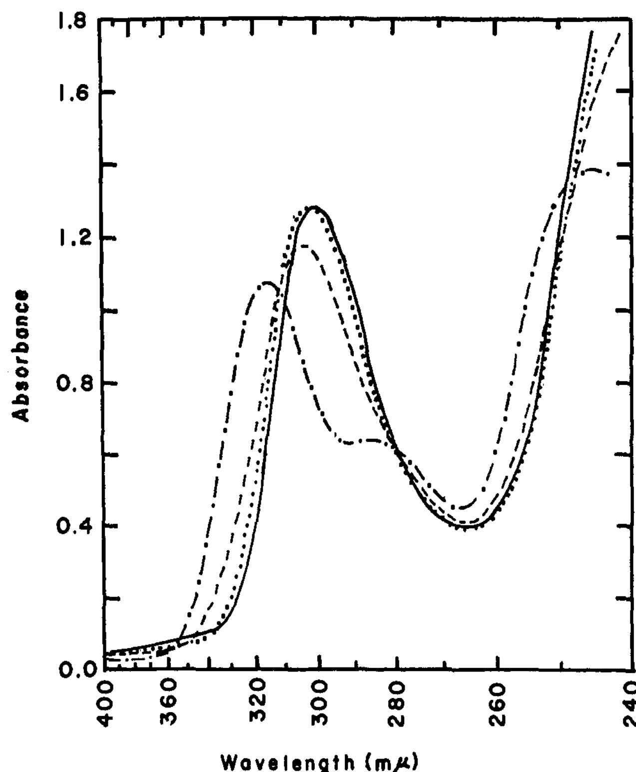
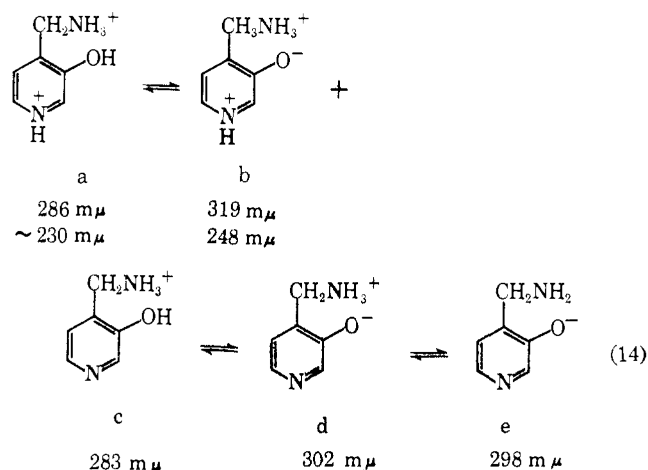


Figure 7. Ultraviolet absorbance scans of the absorbing product from the transamination of 3-hydroxypyridine-4-aldehyde by DL-alanine in the neutral and base pH region: pH 7.0, – · – ·; pH 8.1, - - - -; pH 9.0, ...; pH 9.8, ———.

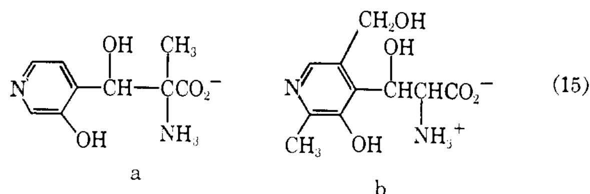
using (14) and Figures 6 and 7 as guides. As the pH is increased from 3.2 to 5.1 a peak (14b) with λ_{max}'s at 319 and 248 mμ is formed and the peak (14a) with a λ_{max} at 286 mμ, which is the only one present at pH 1, decreases in absorbance. Tight isosbestic points are held at 301 and 263 mμ during this transition, indicating that there is little possibility of absorbing side products being present unless they have exactly the same absorption spectrum. In the pH region of 6 the peak at 286 mμ (14b) shifts to *ca.* 283 mμ (14c), then disappears as the pH is further increased to 8. The peak at 319 mμ (14b) shifts to 302 mμ (14d) as the pH is increased from 7 to 9. In more alkaline solution the latter peak (14d) shifts to a slightly lower wavelength (14e).



Discussion

It has been established by Auld and Bruice[1g] that the formation of aldimine from 3-hydroxypyridine-4-aldehyde and DL-alanine is rapid, the reaction reaching equilibrium in a matter of seconds. If the total concentration of DL-alanine is above 0.1 M a second reaction can be readily observed having a half-life of hours and displaying marked spectral changes throughout the ultraviolet spectrum. The latter reaction yields pyruvic acid as one product and a second product which absorbs in the same manner as pyridoxamine in the ultraviolet (Figures 6 and 7). The production of pyruvic acid identifies the reaction as a transamination. A product in addition to pyruvate is formed which is reducible by polarographic means when the reaction is carried out in the absence of external buffers in the pH region 4.6–5.6. Increasing the concentration of 3-hydroxypyridine-4-aldehyde from 5×10^{-4} M to 5×10^{-3} M increased the concentration of side product at the expense of pyruvate. Pyruvate appears to be stable in a reaction mixture at t_∞ even if the side product is present, indicating that the side product must be formed when aldehyde, aldimine, and ketimine are still present in the reaction and not formed from further reactions of pyruvate with itself.

A priori one might expect the side product to have formed from the reaction of two aldimine species condensing to form a 2-methyl-2(3-hydroxylpyridyl-4)-serine (15a) in analogy with the formation of β-pyridoxal serine[15] (15b) from the reaction of glycine and pyridoxal in high concentrations of both at 100° in



the pH region 4–6. However, the lack of a reducible group in (15a) excludes it from being the reducible side product determined by polarography.

The reaction of 3-hydroxypyridine-4-aldehyde with DL-alanine was studied under the conditions $[A_T] \gg [PCHO_T]$ in order that (1) would reduce to the simpler form of a pseudo-first-order reaction (16) if kinetic measurements were initiated at least 10 min after mixing. A would represent an equilibrium mixture of 3-hydroxypyridine-4-aldehyde and its aldimine with DL-alanine

$$A \xrightarrow{k_{obsd}} B \quad (16)$$

and B would represent the absorbing product of the transamination reaction, 3-hydroxy-4-aminomethylpyridine and any other pyridine products lacking exocyclic conjugation to the pyridine ring.

In kinetic experiments in which only amino acid served as buffer a second-order dependence of the observed rate constant (k_{obsd}) for disappearance of A or appearance of B on the total concentration of DL-alanine ($[A_T]$) was obtained, indicating the rate-determining prototropic shift is catalyzed by DL-alanine (Figure 2). The separation of rate terms for the spontaneous and DL-alanine-catalyzed reactions was accomplished by plotting $k_{obsd}/[A_T]$ *vs.* $[A_T]$ at a constant pH (Figure 3) and taking the intercept (k_I) as the rate constant for spontaneous reaction and the slope (k_S) as the rate constant for alanine catalysis. From a knowledge of the pK_a's of aldehyde and aldimines and their equilibrium constants for aldimine formation,[1g] the concentration of any aldimine species could be determined as a function of pH. Mechanisms involving catalysis by AH_2 or AH of the prototropic shift involving only one aldimine species such as S^+ or S or catalysis by AH of prototropy involving two species such as S^+ and S yielded theoretical equations which yield pH–rate profiles that could not be fitted to the experimentally determined values of k_S. A very good fit (Figure 4) of the apparent rate constants for alanine catalysis (k_S) could be obtained over the pH range 2.7 to 7.0 assuming AH-catalyzed prototropy if both the aldimine species SH^+ and S were considered (eq 13, Chart I). The best fit (Figure 6) for the apparent rate constants for spontaneous prototropy (k_I) over the pH range 2.7–7.0 was obtained by considering aldimines SH^+, S^+, and S (Chart I). The kinetically equivalent mechanisms of AH_2 catalysis of prototropy involving aldimines S^+ and S and H_3O^+ catalysis involving aldimine species S^+, S, and S^- are also possible. The rate constants calculated for these kinetically equivalent mechanisms are listed in Table III, having been calculated from the rate constants for AH and spontaneous catalysis listed in Table II using Chart II as a guide.

The kinetically equivalent expressions in Chart II can be interpreted as general base catalysis by the base species AH and H_2O of the removal of the proton from the α-carbon of the aldimine or alternatively as general acid catalysis by the acid species AH_2 and H_3O^+ for the

(15) D. E. Metzler, J. B. Longenecker, and E. E. Snell, *J. Am. Chem. Soc.*, **76**, 639 (1954).

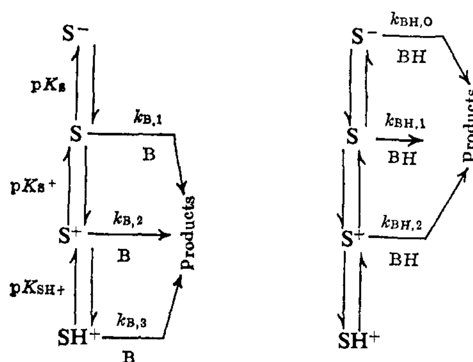
Table III.[a] **Rate Constants for the Kinetically Equivalent General Acid Catalysis of the Transamination of 3-Hydroxypyridine-4-aldehyde by DL-Alanine**

General acid	M^{-1} min^{-1} $k_{BH,2}$[b]	$k_{BH,1}$[b]	$k_{BH,0}$[b]
$CH_3CH(NH_3^+)CO_2H$	0.24	0.42	...
H_3O^+	1.47×10^1	1.49×10^3	1.41×10^5

[a] See Chart II for definition of the rate constants. [b] BH = AH_2 for alanine catalysis; BH = H_3O^+ for hydronium catalysis,

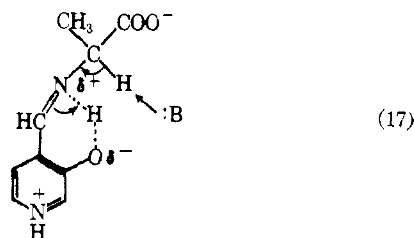
protonation of the methine carbon. In either case the breaking of the α-C-H bond is a slow proton removal as shown by the k_I^H/k_I^D and k_S^H/k_S^D ratios of *ca.* 2 and *ca.* 9, respectively.

Chart II[a]



[a] B = H_2O for water catalysis and AH for alanine catalysis.

Since there is no observable transamination of pyridine-4-aldehyde by amino acids[16] even in the pH region above 8 where the apparent equilibrium constant of aldimine formation for pyridine-4-aldehyde[1d] is greater than that of 3-hydroxypyridine-4-aldehyde, the 3-hydroxyl group must play a crucial role in the conversion of aldimine species into ketimine species. The results of investigations of the infrared spectra (solid state) and the electronic absorption spectra (nonaqueous solutions) of aldimines of 3-hydroxypyridine-4-aldehyde and amino acids have led Heinert and Martell[17,18] to propose the existance of appreciable quantities of keto enamine (proton on azomethine nitrogen rather than on phenolic oxygen) under these conditions. The keto enamine species or an aldimine with a hydrogen bond between the azomethine nitrogen and the 3-hydroxyl (see Chart I) both place a positive charge on the azomethine nitrogen. The creation of a center of positive charge between the α-carbon and the methine carbon of the aldimine would provide stabilization of a transition state associated with the abstraction of the hydrogen from the α-carbon of the aldimine. The curved arrows in eq 17 are for bookkeeping purposes and do not



(17)

(16) Unpublished experiments in this laboratory.

(17) D. Heinert and A. E. Martell, *J. Am. Chem. Soc.*, **84**, 3257 (1962).

(18) D. Heinert and A. E. Martell, *ibid.*, **85**, 183 (1963).

necessarily mean that the protonation of the methine carbon by the 3-hydroxyl group is in concert with the breaking of the α-C-H bond.

Jenkins, *et al.*,[19,20] have demonstrated spectroscopically the formation of enzyme-inhibitor complexes between glutamic-aspartic aminotransferase and certain dicarboxylic acids such as glutarate, maleate, succinate, and *meso*-tartarate. These authors concluded that the same groups of the enzyme are involved in binding both the competitive inhibitors and the substrates. If the imine of pyridoxal 5′-phosphate and an amino acid are bound to the enzyme so that the carboxyl anion would be adjacent to a positive center such as the ϵ-amino group of lysine, an increase in the rate of transamination might be expected. The effect on the rate of transamination caused by protonation of the carboxyl group of (17) is, therefore, of interest in model systems.

The rate constants for general base catalysis by the zwitterion species of DL-alanine (AH) and water on aldimine SH^+ cannot be determined without prior knowledge of K_{SH^+} (eq 12 and 13). If further protonation of S^+ leads to protonation of the carboxyl group of the aldimine, then the pK_a of SH^+ would be assumed to be not much greater than 1 pK_a unit above the acid pK_a of alanine (*ca.* 2.5). Assuming a pK_a of 3.5 for pK_{SH^+}, the ratio of $k_{B,3}/k_{B,2}$ would be 32 times greater for alanine catalysis and six times greater for water catalysis. Since the dissociation of the proton from the pyridine nitrogen causes a ninefold decrease in rate for the catalysis by water ($k_{B,2}/k_{B,1}$) the effect of protonating the carboxyl group is a large one if any meaningful role is to be given to the pyridine nitrogen.

Since general base catalysis appears to play an important part in the transamination of 3-hydroxypydirine-4-aldehyde and pyridoxal by amino acids, the decrease in the rate of transamination upon loss of a proton from the carboxyl group would be expected on the basis that increasing the negative charge on the carboxyl group would retard attack of a base on the proton of the α-carbon of the aldimine. Thus, σ_I is much more negative for COO^- than for COOH.[21] Cennamo[22] has shown that transamination of pyridoxal by the ethyl esters of alanine, leucine, and glutamic acid at pH 5 and 100° in the absence of metal ions proceeds at a rate comparable to that of the metal ion catalyzed reaction of free amino acid. It would appear, therefore, that the masking of the carboxyl group is an essential function of the metal ion catalyzed transamination of pyridoxal by amino acids.[22] The function of the metal ion as a catalyst may be in part to convert the carboxyl anion from an electron-releasing group to an electron-attracting group, a condition which can be realized in nonmetal ion catalyzed transaminations by esterification of the amino acid or studying the transamination in a pH region where the concentration of the protonated carboxyl group of the aldimine prevails.

Acknowledgment. This work was supported by a grant from the National Science Foundation.

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(20) I. W. Sizer and W. T. Jenkins in "Chemical and Biological Aspects of Pyridoxal Catalysis," E. E. Snell, P. M. Fasella, A. E. Braunstein, and A. Rossi-Fanelli, Ed., The Macmillan Co., New York, N. Y., 1963, p 127.

(21) M. Charton, *J. Org. Chem.*, **29**, 1222 (1964).

(22) C. Cennamo, *Biochim. Biophys. Acta*, **93**, 323 (1964).