Catalytic Reactions Involving Azomethines. VII.[1] Rates and Equilibria of Aldimine Formation with 3-Hydroxypyridine-4-aldehyde and Alanine

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Abstract: This paper reports the pH–rate and equilibrium profiles (30°, μ = 1.0 with KCl) for aldimine formation and hydrolysis in the reaction of 3-hydroxypyridine-4-aldehyde with DL-alanine. Aldimine formation from DL-alanine and 3-hydroxypyridine-4-aldehyde proceeds by rate-limiting attack of DL-alanine as the uncharged amine (A) on the cationic ($PCHO^+$), zwitterionic (PCHO), and anionic ($PCHO^-$) forms of aldehyde (see Chart I). The rate of dehydration of the carbinolamine intermediates is much greater for the aldimines of 3-hydroxypyridine-4-aldehyde than for aldimines of pyridine-4-aldehyde suggesting the participation of the *o*-hydroxyl group as an intramolecular catalyst. From the determined pH-dependent equilibrium constant of aldimine formation (K_{pH}) over the pH range 4.0–12.2, and the acid dissociation constants of DL-alanine (K_{AH_2}, K_{AH}), 3-hydroxypyridine-4-aldehyde (K'_{PCHO^+}, K'_{PCHO}), and their product aldimine (K_S, K_{S^+}, K_{SH^+}), the various equilibrium constants (K, K_1, K_2) for aldimine formation were calculated (see Chart II). The increase in the concentration of aldimine in the neutral and slightly acid pH range on substitution of a hydroxyl group in the 3 position of pyridine-4-aldehyde has been demonstrated by comparing the equilibrium constant profile of DL-alanine plus 3-hydroxypyridine-4-aldehyde to that for DL-alanine plus pyridine-4-aldehyde.

The objectives to date of our studies in the series "Catalytic Reactions Involving Azomethines" have been to carefully delineate the kinetics of the transamination reaction between amino acids and 3-hydroxypyridine-4-aldehydes. Since metal ions appear to play no role in the enzymatic reaction,[4] all studies of the model systems were carried out in their absence. Although numerous papers have been published on the subject, the reaction has yet to be described completely for any amino acid. Our objective in this and the following two papers is to describe quantitatively the reaction of alanine with 3-hydroxypyridine-4-aldehyde [kinetics and pH dependence for aldimine formation (part VII), prototropy and its dependence on water and amino acid catalysis (part VIII), and general base catalysis of prototropy (part IX)].

Experimental Section

Materials. 3-Hydroxypyridine-4-aldehyde was prepared by the method of Heinert and Martell[5,6] as modified by French, Auld, and Bruice.[1e] The twice-sublimed aldehyde was stored in the dark *in vacuo* until used. Dilute stock solutions were prepared in triply distilled, deionized water. After nitrogen was bubbled in, the solutions were stored in the dark at 0° for no more than 1 week.

Anal. Calcd for $C_6H_5O_2N$: C, 58.53; H, 4.09; N, 11.28. Found: C, 58.69; H, 4.26; N, 11.21.

DL-Alanine (Calbiochem, A grade) was used without further purification. Potassium chloride and potassium hydroxide were analytical reagent grade chemicals. Monobasic and dibasic phosphate (Baker Analyzed) were dried at 110° for at least 1 day before using.

Apparatus. A Zeiss PMQ II spectrophotometer was used for equilibrium studies. Optical density and pH were measured simultaneously in a rectangular polypropylene cell. For a further descripton of the cell see part IV[1d] in this series. Kinetic studies of aldimine formation were performed on a spectrophotometer consisting of a Beckman DU monochromator and a Gilford Model 2000 multiple-sample absorbance recorder. 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°.

Kinetics. All kinetic measurements were carried out at 30 ± 0.1° in water at a calculated ionic strength of 1.0 (with KCl) under the pseudo-first-order conditions of a great excess of DL-alanine. For the pH range 5.4–7.4, a 0.017 *M* phosphate buffer was used in combination with 0.02, 0.04, 0.06, 0.08, and 0.10 *M* DL-alanine concentrations. Above pH 8, DL-alanine itself served as buffer, the concentrations employed being 0.01, 0.02, 0.03, 0.04, 0.05, and 0.06 *M*. The solutions were equilibrated in standard taper stoppered cuvettes (2-ml capacity) in the cell housing of the Gilford spectrophotometer for 10 min prior to adding the aldehyde solution. Aldimine formation was initiated by adding a drop of 0.01 *M* 3-hydroxypyridine-4-aldehyde to the cuvette containing the DL-alanine solution, stoppering, inverting three times to mix, and placing the cuvette in the thermostated cell block of the spectrophotometer. A continuous reading of optical density *vs.* time was obtained on the Gilford recorder. Normal chart speeds employed were 6 and 12 in. per minute. A computer program was written to calculate the pseudo-first-order rate constants (k_{obsd}), and convert them with the aid of K_{pH} and $[A_T]$ to k_f, the apparent second-order rate constant for aldimine formation, and k_r, the apparent first-order rate constant for aldimine hydrolysis. The rate plots were strictly first-order within the first two to three half-lives that were studied. The wavelength employed was that for the greatest difference in absorbance for aldimine and aldehyde, 270 mμ.

Equilibrium Constants for Aldimine Formation. For a complete description of the spectrophotometric titration cell see paper IV[1d] in this series. Portions (20 ml each) of aldehyde solution (2.02×10^{-4} *M* at *ca.* pH 6 in 1 *M* KCl) and DL-alanine solution (of the appropriate concentration, *ca.* pH 6 and in 1 *M* KCl) were mixed in a light-proof flask, and nitrogen was bubbled in while the resultant solution was being equilibrated at 30° for no more than 15 min. For measurements at high concentrations of DL-alanine in the acid region a different procedure was used. The appropriate weight of DL-alanine was added to a 50-ml volumetric flask and a partial solution formed with approximately 20 ml of 1.0 *M* HCl solution followed by equilibration at 30° for 15 min. A 2.46×10^{-4} *M* aldehyde solution (25 ml) equilibrated at 30°was then added to the volumetric flask and the volume adjusted to 50 ml with 1.0

(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).

(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.

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

(5) D. Heinert and A. E. Martell, *Tetrahedron*, 3, 49 (1958).

(6) D. Heinert and A. E. Martell, *J. Am. Chem. Soc.*, 81, 3933 (1959).

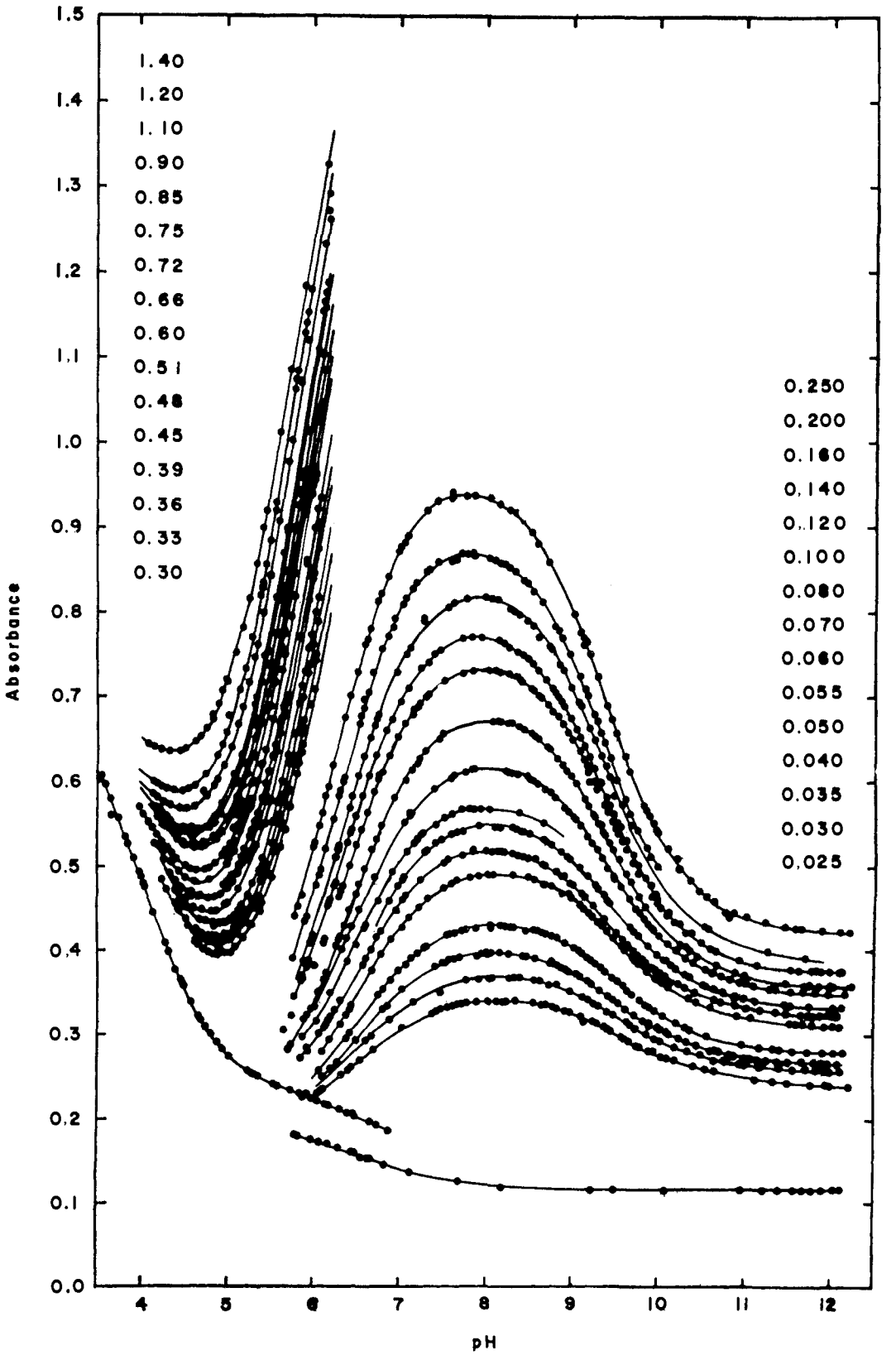


Figure 1. Optical density as a function of pH for the system 3-hydroxypyridine-4-aldehyde, DL-alanine, and aldimine. Concentrations of amino acid are provided for each curve. The concentrations of aldehyde employed were for the pH range 4–6, 1.2×10^{-4} *M*, and for the pH range 6–12, 1.0×10^{-4} *M*. The lowest curves are for zero amino acid concentration.

M KCl also preequilibrated at 30°. The thermally preequilibrated spectrophotometric titration cell was flushed twice with the solution of aldehyde and DL-alanine and then filled with 14 ml of fresh solution. Optical density at 270 mμ and pH were recorded as the solution was titrated between either pH 6.0 and 12.2 or 6.0 and 3.8 in *ca.* 0.1 pH intervals by addition of either aqueous KOH or HCl from a micrometer buret. Equilibrium was obtained rapidly after each addition. The side reaction of transamination caused no problem in the alkaline titration and at most a 2–3% error at the highest concentrations of amino acid employed in the

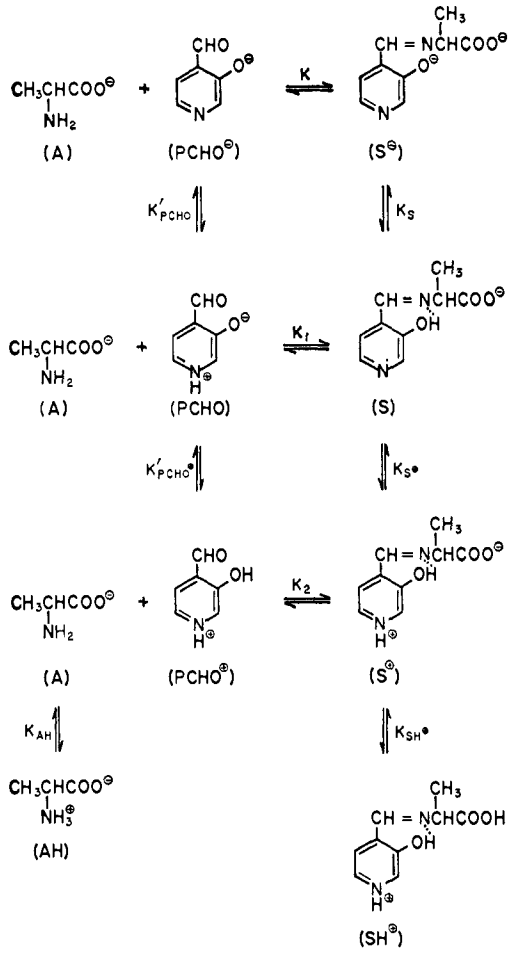
acid region. Titrations performed in this manner were done at 9–16 concentrations of DL-alanine.

Results[7]

Equilibrium Constants for Aldimine Formation. The equilibrium constants, K_{pH}, for aldimine formation as a function of pH were obtained from the optical density at 270 mμ of mixtures of aldehyde, amino acid, and aldimine (Figure 1), using eq 1a (Appendix). The values of K_{pH} at intervals of 0.2 pH from pH 4.0 to 12.2, and the probable errors of these values at the 70% confidence level, are tabulated elsewhere.[8]

Equation 1 (see also Appendix, eq 9a) relates the equilibrium constant at any pH, namely K_{pH}, to one equilibrium constant of aldimine formation (K) and

Chart I



the various acid-dissociation constants of DL-alanine (K_{AH_2}, K_{AH}), 3-hydroxypyridine-4-aldehyde (K'_{PCHO^+}, K'_{PCHO}), and their product aldimine (K_S, K_{S^+}, K_{SH^+}) (see Chart I for series of reactions).

$$K_{pH} = K\left(\frac{1 + \frac{a_H}{K_S} + \frac{a_H^2}{K_S K_{S^+}} + \frac{a_H^3}{K_S K_{S^+} K_{SH^+}}}{\left(1 + \frac{a_H}{K'_{PCHO}} + \frac{a_H^2}{K'_{PCHO} K'_{PCHO^+}}\right)\left(1 + \frac{a_H}{K_{AH}}\right)}\right) \quad (1)$$

(7) Abbreviations employed for the concentrations of species, rate constants, and equilibrium constants may be found in Charts I and II. All theoretical fits were done with the aid of a 1620 IBM computer.

(8) D. S. Auld, Ph.D. Dissertation, Cornell University, 1966.

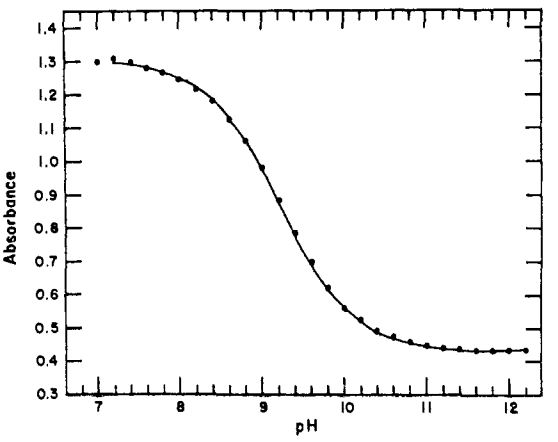


Figure 2. Determination of pK_s from $1/(D - D_p)$ intercepts obtained from the double reciprocal eq (1a): ●, experimental values of D_S; ———, theoretical titration curve assuming a pK_S of 9.23, extinction coefficient of 1.305×10^4 for the acid species (S) and 4.34×10^3 for the base species (S^-).

A theoretical curve, generated by (1), was fitted to the experimental points with the aid of an IBM 1620 digital computer. The criteria for best fit were threefold. First, the sum of the calculated values that differed from the observed values by more than three times the experimental error was a minimum. Second, the sum that differed by less than twice the experimental error was a maximum. Last, the sum of the squares of residuals was a minimum. The procedure used for fitting was to begin in the pH region 8.0–12.2 where the theoretical eq 1 simplified to (2). The initial value chosen for K_S

$$K_{pH} = \frac{K[1 + (a_H/K_S)]}{[1 + (a_H/K_{AH})]} \quad (2)$$

was obtained by fitting theoretical titration curves to plots of D_S *vs.* pH (Figure 2). The best value obtained was 9.23 ± 0.01. The value of K_{AH} was assumed to lie near the average dissociation constant for the concentrations of amino acid that were used in the measurement (pK_{AH} = 9.62 at 0.01 *M* to 9.73 at 0.05 *M*).[1d] The initial values of K were taken equal to the average value of K_{pH} between pH 10.8 and 11.8. From the fit of (2) the best values for these parameters were: $pK_S = 9.17 \pm 0.01$, $pK_{AH} = 9.62 \pm 0.01$, and $K = 25.80 \pm 0.05$. The fit of the entire experimental pH range was then accomplished by holding the above parameters in their narrow range and the further assumption that the values of pK'_{PCHO^+} and pK'_{PCHO} lie in the vicinity of the over-all pK_a's 4.05 and 6.77, respectively, determined by Nakamoto and Martell at 20°.[9] The value of pK_{SH^+} was assumed to be 3.5 on the basis that the difference beween pK_{SH^+} and that of the lower pK_a of the amino acid (pK_{AH_2}) should not be much greater than one unit and that the sensitivity to any pK below pH 4.6 would be small owing to the large experimental error below this pH. The value of K_{S^+} could not be obtained by fitting theoretical titration curves to experimental values of D_S *vs.* pH owing to the relatively large errors in the intercept below pH 5.2. The value of K_{S^+} was, therefore, varied over a wide range at first, then over successively smaller ranges until the best fit was obtained for the entire pH profile. One of the better fits is

(9) K. Nakamoto and A. E. Martell, *J. Am. Chem. Soc.*, **81**, 5863 (1959).

Table I. Best Values for Parameters in pH Profiles

From equilibria measurement of aldimine formation (eq 1)					
K, M^{-1}	pK_S	pK_{S^+}	pK_{AH}	pK_{PCHO}	pK_{PCHO^+}
25.80 ± 0.05	9.17 ± 0.01	5.25 ± 0.05	9.62 ± 0.01	6.68 ± 0.02	3.90 ± 0.15

From rate studies of aldimine formation					
				M^{-1} min^{-1}	
pK_{AH}	pK_{PCHO}	pK_{PCHO^+}	$k_{f,0}$	$k_{f,1} \times 10^{-8}$	$k_{f,2} \times 10^{-6}$
9.62 ± 0.01	6.57 ± 0.02	3.90 ± 0.15	110	9.5 ± 0.5	1.8 ± 0.4

From the fit of the theoret eq 11 to the exptl values of k_r (eq 4)				
			min^{-1}	
pK_S	pK_{S^+}	$k_{r,0} \times 10^{-5} M^{-1}$ min^{-1}	$k_{r,1}$	$k_{r,2}$
9.12 ± 0.02	5.55 ± 0.05	2.20 ± 0.05	0.90 ± 0.00	8.5 ± 0.5

shown in Figure 3,[10] and the values of the parameters obtained and their ranges are listed in Table I.[11]

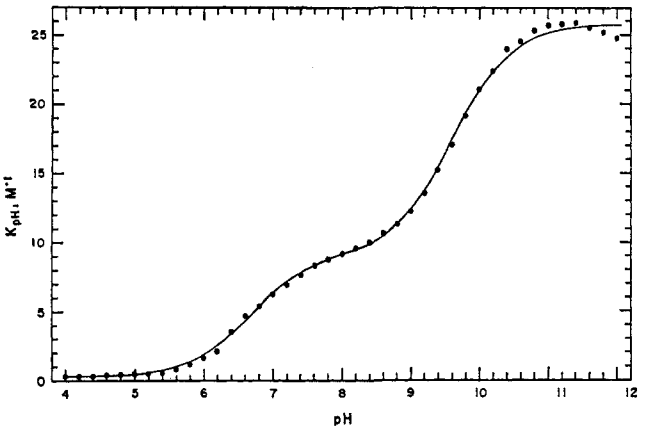


Figure 3. Equilibrium constant *vs.* pH profile for aldimine formation from 3-hydroxypyridine-4-aldehyde and DL-alanine: ●, experimental values of K_{pH}; ———, calculated from eq 1 using the following values for constants: $pK_{PCHO^+} = 3.95$, $pK_{PCHO} = 6.68$, $pK_{SH^+} = 3.50$, $pK_{S^+} = 5.25$, $pK_S = 9.17$, $pK_{AH} = 9.62$, and $K = 25.85\ M^{-1}$. Error test[10] = 28, 6, 2, and 6.

Rate Constants for Aldimine Formation and Hydrolysis. In agreement with earlier findings by French, Auld, and Bruice[1e] there was no significant dependence of the second-order rate constants for aldimine formation (k_f) on [A_T] in the range of [A_T] concentrations 0.01–0.10 M. Therefore, the values of k_f that are used in subsequent calculations are average values of k_f measured at 5–7 concentrations of DL-alanine in the range 0.01–0.10 M where

$$k_f = f(k_{obsd})/[A_T] \quad (3)$$

k_{obsd} is the determined pseudo-first-order rate constant, and f is the mole fraction of completion of aldimine formation under equilibrium conditions.[1d] The values of the first-order rates of hydrolysis of aldimine (k_r) at the same pH are obtained from (4).

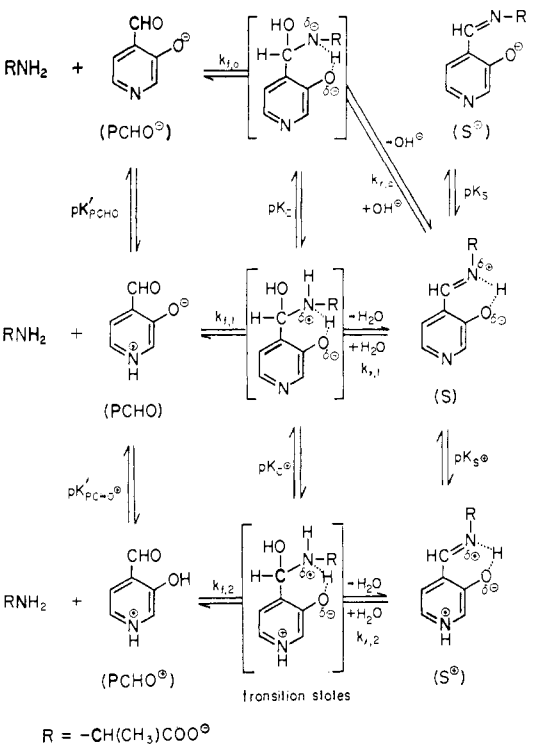
$$k_r = k_f/K_{pH} \quad (4)$$

The pseudo-first-order rate constant as well as k_f and k_r were obtained from a computer program designed for this purpose. The standard deviations of k_f and k_r were determined at each pH and were used as the experimental error in curve fitting.[8]

The rate expression for the simplest model for aldimine formation involves the reaction of amino acid as the uncharged amine with the cationic, zwitterionic, and anionic forms of aldehyde (5). Substituting for

$$d[S_T]/dt = k_f[A_T][PCHO_T] = [A](k_{f,2}[PCHO^+] + k_{f,1}[PCHO] + k_{f,0}[PCHO^-]) \quad (5)$$

Chart II



(10) The number of points in the theoretical calculations within one (N1), two (N2), three (N3), and greater than three times (NG3) the observed error at the 70% confidence level are designated in the fashion: error tests N1, N2, N3, and NG3.

(11) It should be emphasized that the pK_S, pK_{AH}, K, pK'_{PCHO^+}, and pK'_{PCHO} are not independently variable parameters as far as the final general shape of the theoretical curve of Figure 2 is concerned. To summarize what has been accomplished above: (a) precise estimates for the values of pK_S (by spectrophotometric titration), pK_{AH} (by half-neutralization of amino acid), and K (the maximum value of K_{pH} near 11.5) were obtained; (b) the error test described above confirmed the theory that at alkaline pH the equilibrium constant for aldimine formation, K_{pH}, depends only on the independently measured parameters, pK_S, pK_{AH}, and K, to within experimental error; (c) good estimates for the values of pK'_{PCHO^+} and pK'_{PCHO} were taken from experiments performed at a slightly lower temperature; (d) only the parameter pK_{S^+} could not be measured independently. (A rough estimate of its value was made.) The error test confirmed the theory that over the pH range 4.0–12.2, K_{pH} depends on the measured parameters, pK_S, pK_{AH}, K, pK'_{PCHO^+}, pK'_{PCHO}, and on pK_{S^+} when one assumes an entirely reasonable value for pK_{S^+}. Thus, the parameters in the first line of Table I can be used with eq 1 to compute values for K_{pH} at any pH between 4.0 and 12.2 to a high degree of accuracy.

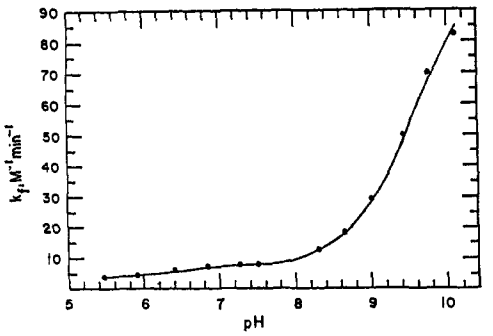


Figure 4. Dependence of k_f, the apparent second-order rate constants for aldimine formation, on pH. The filled circles are experimental values of k_f, and the line is calculated from eq 7 using the following values for constants: $pK_{PCHO^+} = 3.95$, $pK_{PCHO} = 6.59$, $pK_{AH} = 9.62$, $k_{f,0} = 1.1 \times 10^2\ M^{-1}\ min^{-1}$, $k_{f,1} = 9.0 \times 10^3\ M^{-1}\ min^{-1}$, $k_{f,2} = 1.8 \times 10^6\ M^{-1}\ min^{-1}$. Error test[10] = 8, 4, 0, and 0. The values of k_f are not corrected for extent of hydration of the reactive species, free aldehyde.

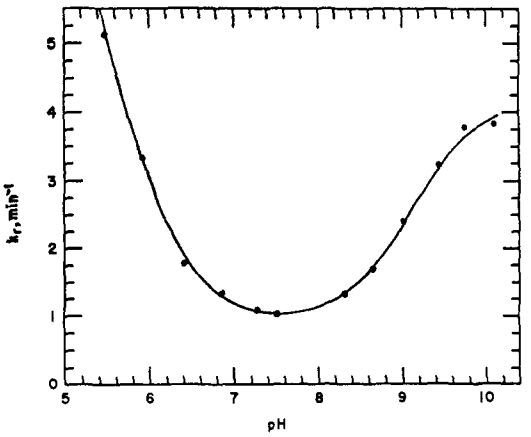


Figure 5. Dependence of k_r, the apparent first-order rate constant for aldimine hydrolysis, on pH: ●, experimental values of k_r; ———, calculated from eq 11 using the following values for constants: $pK_{S^+} = 5.55$, $pK_S = 9.13$, $pK_w = 13.83$, $k_{r,0} = 2.15 \times 10^5\ min^{-1}$, $k_{r,1} = 9.0 \times 10^{-1}\ min^{-1}$, $k_{r,2} = 9.00\ min^{-1}$. Error test[10] = 12, 0, 0, and 0.

individual aldehyde species in terms of $PCHO^-$ yields

$$d[S_T]/dt = [PCHO^-][A] \times \left(\frac{k_{f,2}a_H^2 + k_{f,1}a_H K'_{PCHO^+} + k_{f,0}K'_{PCHO}K'_{PCHO^+}}{K'_{PCHO}K'_{PCHO^+}}\right) \quad (6)$$

Substituting for $PCHO^-$ and A in terms of $[PCHO_T]$ and $[A_T]$, respectively (see Appendix), and rearranging yield[1e]

$$k_f = \left(\frac{K_{AH}}{K_{AH} + a_H}\right) \times \left(\frac{k_{f,2}a_H^2 + k_{f,1}K'_{PCHO^+}a_H + k_{f,0}K'_{PCHO^+}K'_{PCHO}}{a_H^2 + K'_{PCHO^+}a_H + K'_{PCHO^+}K'_{PCHO}}\right) \quad (7)$$

since by definition

$$d[S_T]/dt = k_f[A_T][PCHO_T] \quad (8)$$

The rate expression for the simplest model for aldimine hydrolysis involves the attack of hydroxide ion on aldimine S and the attack of water on aldimines S and S^+.

$$-d[S_T]/dt = k_r[S_T] = k_{r,0}[OH^-][S] + k_{r,1}[S] + k_{r,2}[S^+] \quad (9)$$

Substituting for S^+ in terms of [S] and OH^- in terms of a_H (*i.e.*, $[OH^-] = K_w/a_H$) and rearranging yield

$$-d[S_T]/dt = [S]\frac{k_{r,0}K_wK_{S^+} + k_{r,1}K_{S^+}a_H + k_{r,2}a_H^2}{K_{S^+}a_H} \quad (10)$$

The concentration of S in terms of $[S_T]$ may be obtained in a fashion similar to that shown in eq 2-4 of the Appendix. Since, experimentally, rates of hydrolysis of aldimine are only obtained above pH 5.7 the concentration of SH^+ can be ignored for these derivations. Making the appropriate substitution yields

$$k_r = \frac{k_{r,0}K_wK_{S^+} + k_{r,1}K_{S^+}a_H + k_{r,2}a_H^2}{K_SK_{S^+} + a_HK_{S^+} + a_H^2} \quad (11)$$

The fitting of the theoretical equations for aldimine formation (7) and aldimine hydrolysis (11) to the experimental data (k_f and k_r, respectively, *vs.* pH) was accomplished using the same criteria for best fit mentioned previously for the pH–equilibrium constant profile. The constants K'_{PCHO^+}, K'_{PCHO}, and K_{AH} for aldimine formation and the constants K_{S^+} and K_S for aldimine hydrolysis were varied about the values obtained from the equilibrium fits while the rate constants of aldimine formation and hydrolysis were varied over wider ranges. The ranges were continuously narrowed until the best fits obtained were found. The sensitivity to the parameters K'_{PCHO^+} and K_{S^+} is quite low since they both are below the experimentally attainable pH range. One of the best fits for k_f *vs.* pH is shown in Figure 4[1d] and for k_r *vs.* pH in Figure 5.[1d] The best values of the parameters and their ranges are given in Table II.

Table II. Equilibrium Constants of Aldimine Formation as a Function of pH[a]

pH	4	5	6	7	8
K_{pH}/K_T	388	61	20	7.7	1.1

[a] Apparent equilibrium constants for aldimine formation from 3-hydroxypyridine-4-aldehyde plus DL-alanine (K_{pH}) and pyridine-4-aldehyde plus DL-alanine (K_T). K_T data obtained from the relationship $K_T = K_0[A]/[A_T]$ and the results of French and Bruice.[1d]

Discussion

Equilibrium Constants. The constants determined from the fitting of eq 1 to the pH–apparent equilibrium constant (K_{pH}) profile for the formation of an aldimine from 3-hydroxypyridine-4-aldehyde and DL-alanine were in the same range as those previously determined for DL-glutamate, glycine, and DL-valine by French, Auld, and Bruice.[1e]

Metzler[12] attributed the increase in pK occurring in the formation of aldimines of pyridoxal and the stability of aldimines in neutral solutions to the formation of a hydrogen bond between the phenolic hydroxyl group and the azomethine nitrogen (12a). Heinert and Martell[13] have investigated the electronic absorption spectra of aldimines of 3-hydroxypyridine-4-aldehyde in nonaqueous solvents. They suggested that an equilibrium exists between the aldimine (12a) and its "keto enamine tautomer" (12b) in the neutral pH region (λ_{max} 404–425 mμ) and estimated the equi-

(12) D. E. Metzler, *J. Am. Chem. Soc.*, 79, 485 (1957).
(13) D. Heinert and A. E. Martell, *ibid.*, 85, 183 (1963).

a b (12)

librium constant to be 1 in dioxane solution. In support of the "keto enamine" structure, earlier investigations of salicylaldimines[14] demonstrated that methylation of the hydroxyl group destroyed the 410-mμ band. Further evidence has been provided by Dudek and Holm[15] through the use of nmr spectroscopy in aprotic solvents. The results showed that in both aliphatic and aromatic systems, imines of *vicinal* hydroxy ketones exist to some degree in the keto enamine form. The aldimine formed from 3-hydroxypyridine-4-aldehyde and DL-alanine has a λ_{max} at *ca.* 410 mμ in neutral aqueous solutions (see Figure 2 of part IX of this series). A comparison of the spectral properties of this aldimine in aqueous media to the aldimine formed from 3-methoxypyridine-4-aldehyde under the same conditions would be useful in determining if the keto-enamine structure is of great significance in aqueous solutions.

The increase in pK of the hydroxyl group in aldimines of 3-hydroxypyridine-4-aldehyde with respect to aldehyde is reflected in the equilibrium constants. Thus K_1 is 320 times greater than K and K_2 is 23 times greater than K_1, because pK_S is 2.5 units greater than pK'_{PCHO} and pK_{S^+} is 1.3 units greater than pK'_{PCHO^+}. The im-

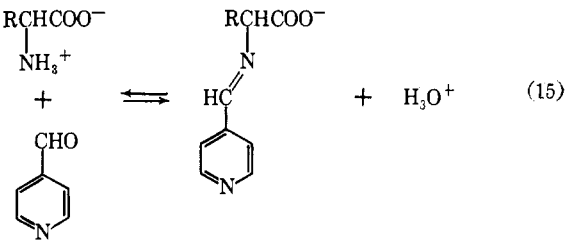
$$K_1 = K(K'_{PCHO}/K_S)$$
$$K_2 = K_1(K'_{PCHO^+}/K_{S^+}) \quad (13)$$

portance of the 3-hydroxyl group is more clearly demonstrated if a comparison is made between the apparent equilibrium constants, K_{pH} and K_T, for the systems DL-alanine plus 3-hydroxypyridine-4-aldehyde and DL-alanine plus pyridine-4-aldehyde,[1d] respectively (see Table II). Although at 1.0 *M* DL-alanine there would be nearly equal quantities of aldimine present at pH 8 regardless of the aldehyde used, at pH 6 the per cent 3-hydroxypyridine-4-aldehyde present as aldimine would be 62% *vs.* 7% for pyridine-4-aldehyde. K_{pH} decreases by a factor of 6 during the pH change of 8–6 whereas for the same pH change, K_T decreases by 100-fold. The reason for this behavior is most easily appreciated by inspection of eq 14 in which no protons

$$\begin{array}{ccc} AH + PCHO^- & \rightleftharpoons & S + H_2O \\ K_{PCHO}\uparrow\downarrow & & K_{S^+}\uparrow\downarrow \\ AH + PCHO & \rightleftharpoons & S^+ + H_2O \end{array} \quad (14)$$

are released on aldimine formation. In comparison over the same pH range a proton must be released when aldimine is formed from pyridine-4-aldehyde (eq 15) and, therefore, the apparent equilibrium constant (K_T) decreases with increasing hydrogen ion (*i.e.*, decreasing pH). The large difference in apparent equilibrium constants for these two systems may be of importance in determining whether a transamination reaction can be observed or not. Since at pH 5 (Table II) the

(14) L. N. Ferguson and I. Kelly, *J. Am. Chem. Soc.*, 73, 3707 (1951).
(15) G. O. Dudek and R. H. Holm, *ibid.*, 83, 2099, 3941 (1961).



(15)

apparent equilibrium constant K_{pH} is sixfold greater than K_T, even if the rate constants (k_x and k'_x) for the tautomeric shift in the transamination reaction are equal for the aldimines of 3-hydroxypyridine-4-aldehyde and pyridine-4-aldehyde the observed rate constant (k'_{obsd}) will be 61-fold smaller for the pyridine-4-aldehyde system (eq 16) compared to the 3-hydroxypyridine-4-aldehyde system (eq 17), all other conditions being the same. For pH's above 9 this effect would be reversed.

$$v = k'_x K_T[PCHO_T][A_T] = k'_x(K_{pH}/61)[PCHO_T][A_T]$$
$$k'_{obsd} = k'_x(K_{pH}/61)[A_T] \quad (16)$$
$$v = d[PCHO_T]/dt = k_x K_{pH}[PCHO_T][A_T]$$
$$k_{obsd} = k_x K_{pH}[A_T] \quad (17)$$

Rates of Aldimine Formation and Hydrolysis. In the case of pyridine-4-aldehyde, French and Bruice[1d] found that eq 3, which assumes no detectable carbinolamine intermediate, inadequately described the experimental data. Using eq 3 they found that k_f was dependent on both pH and the concentration of amino acid. A scheme was then used which accounted for the carbinolamine intermediate, and the results of the investigation showed the rate-limiting step in aldimine formation from pyridine-4-aldehyde to be the dehydration of the carbinolamine intermediate. In the case of 3-hydroxypyridine-4-aldehyde, the second-order rate constant for aldimine formation (k_f) calculated from eq 3 showed no tendency to decrease as the concentration of DL-alanine was increased from 0.01 to 0.10 *M* at pH 10.1. A previous investigation by French, Auld, and Bruice[1e] has shown that there is no difference in k_f for 0.1 and 0.5 *M* glycine plus 3-hydroxypyridine-4-aldehyde at pH 10.5. These findings suggest that there is no detectable carbinolamine formed during the reaction of amino acids plus 3-hydroxypyridine-4-aldehyde. If one assumes that dehydration of the carbinolamine of 3-hydroxypyridine-4-aldehyde and an amino acid is rate limiting, it can be shown that the calculated second-order rate constants for attack of free amine on aldehyde are unreasonably large.[1e] Thus the rate constants, $k_{f,0}$, $k_{f,1}$, and $k_{f,2}$, must pertain to rate-limiting attack of amino acid as uncharged amine (A) on the cationic ($PCHO^+$), zwitterionic (PCHO), and anionic ($PCHO^-$) forms of aldehyde (as in Chart II). The three ionic species of aldehyde are in equilibrium with hydrated forms of $PCHO^+$ and PCHO. The individual rate constants for carbinolamine formation ($k_{f,1}$, $k_{f,2}$, and $k_{f,3}$) are not corrected for extent of hydration of aldehyde.

The rate constants, $k'_{f,1}$ and $k'_{f,2}$, for the kinetically equivalent mechanism of general acid catalysis by H_3O^+ of the attack of free amino acid (A) on $PCHO^-$ and PCHO, can be calculated from eq 18 and 19

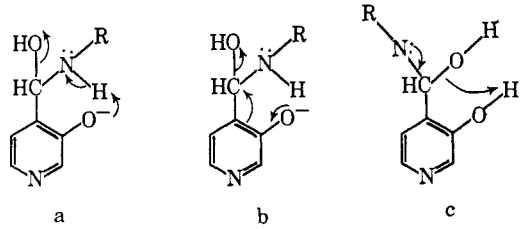
$$k_{f,1}[PCHO][A] = k'_{f,1}[PCHO^-][H_3O^+][A] \quad (18)$$

where $k'_{f,1} = k_{f,1}/K_{PCHO}$

$$k_{f,2}[PCHO^+][A] = k'_{f,2}[PCHO][H_3O^+][A] \quad (19)$$

where $k'_{f,2} = k_{f,2}/K_{PCHO^+}$. The fact that the rate constants calculated for such a mechanism are greater than 10^{10} M^{-2} min^{-1} and that no general acid catalysis of the zwitterion form of amino acid (AH) was observed makes the mechanism of general acid catalysis by H_3O^+ of aldimine formation appear highly unlikely for this system.

The rate constant for dehydration of the carbinolamine of 3-hydroxypyridine-4-aldehyde is no less than 20-fold greater than the corresponding calculated first-order rate constants for carbinolamine formation at the highest concentration of free amino acid employed. The factor of 20 is derived from the reciprocal of the estimated accuracy in detection of K_e by kinetic analysis. Studies are in progress to determine the dehydration constant for carbinolamine by stopped-flow spectrophotometry. In confirmation of earlier studies with glycine,[1e] the phenolic hydroxyl group intramolecularly catalyzes the dehydration of carbinolamine since the lower limit of the rate constant for dehydration of the carbinolamine of DL-alanine and 3-hydroxypyridine-4-aldehyde is 60 times greater than the rate constant for dehydration of the carbinolamine of DL-alanine and pyridine-4-aldehyde. Mechanisms proposed for the participation of the *o*-hydroxyl group involve intramolecular general base catalysis (a), ring-transmitted expulsion of the hydroxide ion (b), or intramolecular general acid catalysis by the phenolic hydroxyl group (c). It is of interest to note that the initial



a b c

product of the dehydration of carbinolamine (a) would be the hydrogen-bonded aldimine proposed by Metzler[12] (12a) and for the dehydration of carbinolamine (b) the keto enamine species (12b) proposed by Heinert and Martell.[13]

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Appendix

Some of the equations mentioned (but not derived) in previous papers,[1d,e] which also apply to this one, are explained or derived in detail in this Appendix. These equations, in conjunction with Chart I of this paper, are also intended to serve as a reference for standard symbolism for future papers of this series.

Measurement of Equilibrium Constants for Aldimine Formation. The equilibrium constant for aldimine formation can be obtained from

$$\frac{1}{D - D_p} = \frac{1}{K_{pH}(D_s - D_p)}\left(\frac{1}{[A_T]}\right) + \frac{1}{D_s - D_p} \quad (1a)$$

where $K_{pH} = [S_T]/[PCHO_T][A_T]$. D_P is the optical density of aldehyde at its initial concentration ($[PCHO_i]$), D_S is the optical density of aldimine at the same concentration of aldehyde employed [obtained graphically from (1)], and D the experimentally measured absorbance of a mixture of aldimine and aldehyde minus the absorbance contribution of DL-alanine. The total concentration of DL-alanine is $[A_T]$, the equilibrium concentrations of aldehyde and aldimine are $[PCHO_T]$ and $[S_T]$, respectively, and K_{pH} is the pH-dependent, over-all equilibrium constant for aldimine formation. Since $[A_T] >> [PCHO_T]$, a correction for the absorbance due to DL-alanine can be obtained by scaling the absorbance of the highest concentration of DL-alanine used to the one in question. Only those corrected values of D are used in which the ratio of $[S_T]/[PCHO_i]$ is between 0.15 and 0.85. The optical density (D) of a solution in which the ratio of $[S_T]/[PCHO_i]$ is much outside the given limits would contain little sensitivity to the compound present in lowest concentration. A computer program is written for a linear regression of $1/(D - D_P)$ on $1/[A_T]$. The program calculates the intercept $1/(D_S - D_P)$ and the slope $1/[K_{pH}(D_S - D_P)]$ of the regression line. The errors in the intercept and slope are obtained from the standard error of estimate, standard deviations, and the Student t distribution at the 70% confidence level. The determined pH-dependent equilibrium constant (K_{pH}) is computed from the value of intercept/slope and D_S from 1/intercept + D_P.

Equilibrium Constants for Aldimine Formation As a Function of pH. From Chart I it can be seen that

$$[S_T] = [SH^+] + [S^+] + [S] + [S^-] \quad (2a)$$

and

$$K_{SH^+} = \frac{[S^+]a_H}{[SH^+]};\ K_{S^+} = \frac{[S]a_H}{[S^+]};\ K_S = \frac{[S^-]a_H}{[S]} \quad (3a)$$

Substituting (3a) into (2a) in terms of $[S^-]$ yields

$$[S_T] = [S^-]\left(\frac{a_H^3}{K_S K_{S^+} K_{SH^+}} + \frac{a_H^2}{K_S K_{S^+}} + \frac{a_H}{K_S} + 1\right) \quad (4a)$$

Similarly

$$[PCHO_T] = [PCHO^+] + [PCHO] + [PCHO^-] \quad (5a)$$

and

$$K'_{PCHO^+} = \frac{[PCHO]a_H}{[PCHO^+]};\ K'_{PCHO} = \frac{[PCHO^-]a_H}{[PCHO]} \quad (6a)$$

Substituting (6a) into (5a) in terms of $[PCHO^-]$ yields (7a). In the same manner the total concentration of

$$[PCHO_T] = [PCHO^-]\left(\frac{a_H^2}{K'_{PCHO}K'_{PCHO^+}} + \frac{a_H}{K'_{PCHO}} + 1\right) \quad (7a)$$

DL-alanine, $[A_T]$, can be shown to be related to the concentration of unprotonated DL-alanine, [A], by the following expression.

$$[A_T] = [A]\left(\frac{a_H}{K_{AH}} + 1\right) \quad (8a)$$

Dividing (4a) by the product of (7a) and (8a) yields eq 1 because by definition

$$K_{pH} = \frac{[S_T]}{[PCHO_T][A_T]};\quad K = \frac{[S^-]}{[PCHO^-][A]} \quad (9a)$$