Catalytic Reactions Involving Azomethines. V. Rates and Equilibria of Imine Formation with 3-Hydroxypyridine-4-aldehyde and Amino Acids*

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ABSTRACT: Imine formation from glycine, valine, or glutamate and 3-hydroxypyridine-4-aldehyde proceeds by rate-limiting attack of free amino acid on the three ionic forms of the aldehyde, followed by rapid dehydration of the carbinolamine intermediates. Intramolecular catalysis by the 3-hydroxy group appears to be important in the dehydration step. Hydrolysis of the imines at alkaline *p*H occurs by two paths, attack of hydroxide ion and water on the ionic form that contains an unprotonated pyridine nitrogen and phenolic hydroxyl hydrogen-bonded to the imine nitrogen. The *p*H–equilibrium constant profiles fit an equation of the kind proposed by D. E. Metzler (1957, *J. Am. Chem. Soc. 79*, 485) for pyridoxal.

The first phase of transamination in model systems is the formation of imine from amino acid and an aldehyde analog of the coenzyme for transaminases, pyridoxal phosphate (Snell, 1958; Bruice and Topping, 1963). Metzler (1957) has shown that the *p*H–equilibrium constant profiles for the reaction of pyridoxal with amino acids may be predicted from a theoretical equation for which the only parameters needed are the pK_a values of aldehyde, amino acid, and imine, and a single equilibrium constant for imine formation. With pyridoxal significant concentrations of imine can be formed under physiological conditions and in nonenzymatic model reactions (Metzler, 1957; Metzler and Snell, 1952). To date the only quantitative kinetic study of imine formation with a close analog of the coenzyme is found in the work of Fleck and Alberty (1962) on the reaction of pyridoxal with alanine in aqueous solution at 25° in the *p*H range 8–10. The authors proposed that the three apparent *pseudo*-first-order rate constants that occur in the expression for absorbancy as a function of time represent the formation of carbinolamine from amino acid and aldehyde, dehydration of carbinolamine to form imine, and rearrangement of this imine (Snell, 1958).

Provided that the phosphoryl group of pyridoxal phosphate is bound to transaminases electrostatically or covalently, 3-hydroxypyridine-4-aldehyde is a better analog of enzyme-bound pyridoxal phosphate than is the free coenzyme. Pyridoxal, which forms an internal hemiacetal, is a poorer analog than either of the above. The spectra and acid dissociation constants of 3-hydroxypyridine-4-aldehyde and its hydrated forms (Nakamoto and Martell, 1959), and the spectra of some imine derivatives in nonaqueous solutions (Heinert and Martell, 1963) have been thoroughly investigated.

This paper reports the *p*H profiles at 30° for equilibria and rate constants for imine formation, and rate constants for imine hydrolysis, with 3-hydroxypyridine-4-aldehyde and three amino acids, glycine, valine, and glutamic acid.

Experimental Procedure

Materials and Apparatus. 3-Hydroxypyridine-4-aldehyde was prepared by the method of Heinert and Martell (1958, 1959), with the following variation of a modification of the penultimate step (A. E. Martell, 1964, personal communication). A 1-liter, three-necked flask was equipped with a heating mantle, magnetic stirrer, dropping funnel, nitrogen sparger, and condenser with calcium chloride tube. Ether (300 ml) freshly distilled over lithium aluminum hydride and 4.0 g (0.10 mole) of lithium aluminum hydride were added to the flask. A solution of 7.66 g (0.05 mole) of 3-hydroxy-4-carbomethoxypyridine in 150 ml of ether was added under dry nitrogen over a period of 1 hour. The mixture was heated to reflux for 15 minutes. Ethyl acetate (25 ml) was added dropwise to the chilled reaction mixture, followed by 50 ml of water, then 3.5 g of NaOH dissolved in 15 ml of water. Hydrated ammonium oxalate (17.2 g) dissolved in 125 ml of water and then acetone (300 ml) were added, and the *p*H of the mixture was carefully adjusted to 6.03 with a 60% solution of sulfuric acid. The mixture was filtered by suction, and the salt cake was washed with acetone and com-

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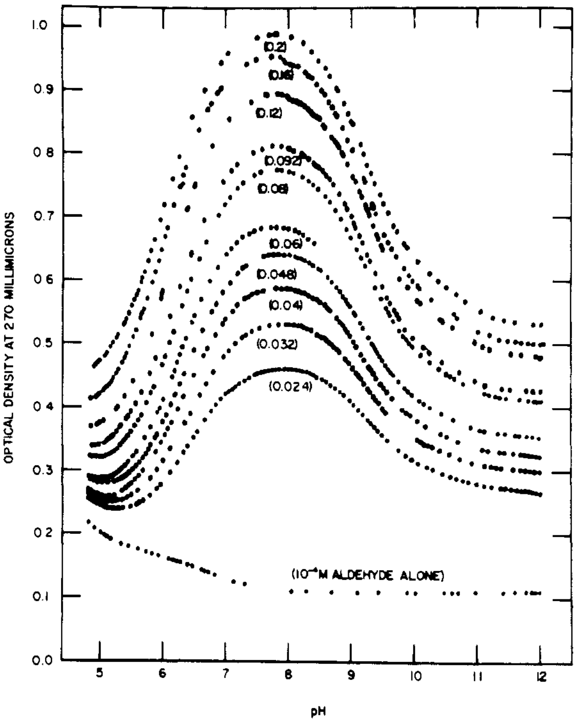

FIGURE 1: Optical density as a function of *p*H for the system 3-hydroxypyridine-4-aldehyde, DL-glutamate, and imine (3-hydroxy-4-pyridylidene-1-carboxy-3′-carboxypropylamine).

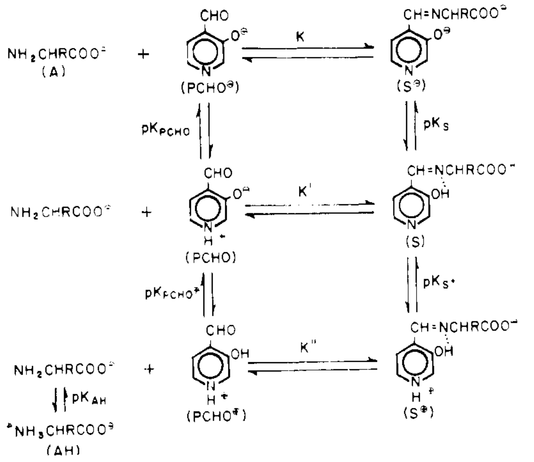

FIGURE 2: Equilibria among amino acid, 3-hydroxypyridine-4-aldehyde, and imine in the *p*H range 5–12. The values of K' and K'', which are derived from the parameters of Table I, are, respectively: for glycine, 1.79×10^4 and 5.05×10^5 M^{-1}; for valine, 4.75×10^4 and 4.86×10^5 M^{-1}; and for glutamate, 1.30×10^4 and 1.64×10^5 M^{-1}.

bined with the mother liquors. Acetone and water were removed from the filtrate by flash evaporation at room temperature. The thick oily residue was extracted with four 125-ml portions of acetone and the combined extracts were dried with anhydrous Na_2SO_4. When the golden-yellow acetone solution was saturated with dry HCl in a dry ice–acetone bath, the pale-yellow hydrochloride of 3-hydroxy-4-pyridinemethanol precipitated; yield 4.98 g (60.9%), mp 200–206° uncorr.

The twice-sublimed aldehyde was stored in an inert atmosphere in the dark. Dilute stock solutions in nitrogen-distilled water 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, 11.38. Found: C, 58.31; H, 4.16; N, 11.32.

Other materials, and the use of apparatus for equilibrium and kinetic measurements, have been described in paper IV (French and Bruice, 1964).

Equilibrium Constants in the Spectrophotometric Titration Cell. To minimize side reactions, 25-ml portions of stock solutions of aldehyde (2.00×10^{-4} M, at *p*H 4 in 1 M KCl) and amino acid (adjusted to *ca.* *p*H 4 with HCl and KCl to make 1 M ionic strength at neutrality) were mixed in a light-proof flask under nitrogen and pre-equilibrated at $30.0 \pm 0.1°$ for no more than 20 minutes. The pre-equilibrated plastic cell was flushed twice with the mixture and filled with 14 ml of solution. Optical density and *p*H were recorded as the solution was titrated from *p*H 4.4 to 12.0 in *ca.* 0.1-*p*H intervals by addition of aqueous KOH from a micrometer buret. Equilibrium was usually attained within 3 minutes after each addition. Below *p*H 9 a slower reaction, probably transamination, occurs with glycine (D. S. Auld, 1964, unpublished observations). There was no detectable influence of this slower reaction on the results of titration from *p*H 4 to 9 if titration was completed within two hours. Titrations were performed in this manner at 10–13 concentrations each of glycine, valine, and glutamate, and with aldehyde alone.

Kinetics. Methods of kinetic measurements were similar to those described in paper IV (French and Bruice, 1964), except that the total concentration of amino acid was 0.02–0.10 M for the slower rates, and intervals between measurements were as low as 2.5 seconds for the most rapid rates that were studied without the aid of stopped-flow apparatus. The plots from which *pseudo*-first-order rate constants were calculated were strictly first-order within the first one to two half-lives that were measured.

Results

Equilibrium Constants Measured in the Spectrophotometric Titration Cell. An example of the measurement of imine formation at 270 mμ as a function of *p*H and total concentration of amino acid is shown for the glutamate-3-hydroxypyridine-4-aldehyde system (Figure 1). Each point represents a single measurement of optical density and *p*H. A smooth curve was drawn through each set of points and the optical density values were corrected for absorbance of the amino acid alone, at every 0.2 interval in *p*H. The values of K_{pH} at 0.2-unit intervals were calculated by use of the MUREG program for regression analysis at the Cornell Computing Center.

Values of $1/[A_T]$ and $1/[D - D_P]$ were listed as the dependent and independent variables, respectively, and the slope and intercept of the following equations were printed:

$$\frac{1}{D - D_P} = \frac{1}{K_{pH}(D_S - D_P)}\left(\frac{1}{[A_T]}\right) + \frac{1}{D_S - D_P} \qquad (1)$$

where D is the optical density at 270 mμ of a mixture of imine and aldehyde in the ratio $[S_T]/[\mathrm{PCHO}_T] = 0.2$ to 5.0; this value is corrected for absorbancy caused by impurities in the amino acid alone; D_S is the corrected optical density of imine at the same concentration as the initial value for aldehyde; D_P is the initial optical density of aldehyde; and $[A_T]$ is the total concentration of amino acid. The best value of K_{pH} is then intercept/slope, and the best value for D_S is 1/intercept + D_P. Standard error of estimate and standard deviations, from MUREG calculations, and the Student *t* distribution were used to calculate errors in intercept and slope. The standard error of K_{pH} was calculated in the usual way (Daniels *et al.*, 1949).[1]

Theoretical Equations for pH-Equilibrium Constant Profiles. Following the method of Metzler (1957), the *p*H-equilibrium constant profiles could be related to acid-dissociation constants of the various ionic forms of reactants and products, and a single over-all equilibrium constant for imine formation.

$$\mathrm{AH} \xrightleftharpoons{K_{\mathrm{AH}}} \mathrm{A} + \mathrm{H}^{\oplus} \qquad (2)$$

$$\mathrm{PCHO}^{\oplus} \xrightleftharpoons{K_{\mathrm{PCHO}^{\oplus}}} \mathrm{PCHO} + \mathrm{H}^{\oplus} \qquad (3)$$

$$\mathrm{PCHO} \xrightleftharpoons{K_{\mathrm{PCHO}}} \mathrm{PCHO}^{\ominus} + \mathrm{H}^{\oplus} \qquad (4)$$

$$\mathrm{S}^{\oplus} \xrightleftharpoons{K_{\mathrm{S}^{\oplus}}} \mathrm{S} + \mathrm{H}^{\oplus} \qquad (5)$$

$$\mathrm{S} \xrightleftharpoons{K_{\mathrm{S}}} \mathrm{S}^{\ominus} + \mathrm{H}^{\oplus} \qquad (6)$$

$$\mathrm{A} + \mathrm{PCHO}^{\ominus} \xrightleftharpoons{K} \mathrm{S}^{\ominus} + \mathrm{H_2O} \qquad (7)$$

$$K_{pH} = (K) \times \frac{1 + a_{\mathrm{H}}/K_{\mathrm{S}} + a_{\mathrm{H}}^2/K_S\, K_{\mathrm{S}}^{\oplus}}{(1 + a_{\mathrm{H}}/K_{\mathrm{AH}})\,(1 + a_{\mathrm{H}}/K_{\mathrm{PCHO}} + a_{\mathrm{H}}^2/K_{\mathrm{PCHO}}K_{\mathrm{PCHO}^{\oplus}})} \qquad (8)$$

Formulae for the species in equations (2) to (7) are presented in Figure 2, and a_{H} is hydrogen ion activity.

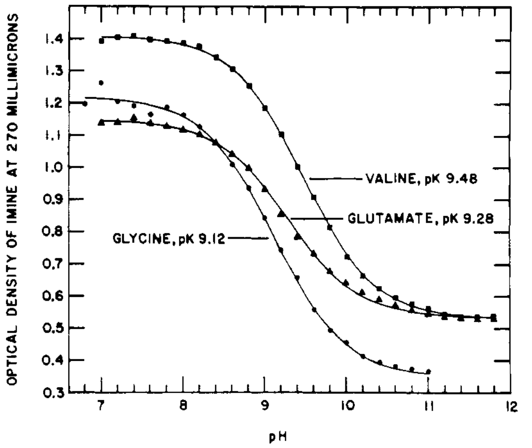

FIGURE 3: Determination of pK_{S}. The filled circles are experimental values of D_s, and the lines are theoretical titration curves with the pK_a values that are indicated.

Of the six parameters in equation (8), $K_{\mathrm{PCHO}^{\oplus}}$ (dissociation of phenolic hydroxyl, a group with a relatively low heat of ionization) was assumed to be identical to the value at 20° reported by Nakamoto and Martell (1959), and pK_{PCHO} was assumed to lie in the neighborhood of 6.77 (*loc. cit.*). Values of pK_{S} were easily obtained by fitting theoretical titration curves to plots of D_S versus *p*H (Figure 3), but the relatively large errors in D_S at lower values of *p*H prevented similar determinations of $pK_{\mathrm{S}^{\oplus}}$. The experimental data were fitted approximately to equation (8) with the aid of a desk calculator, with the further assumptions that K_{AH} lay near the average dissociation constant (French and Bruice, 1964) for the concentrations of amino acid that were used in the measurements, and that $pK_{\mathrm{S}^{\oplus}}$ lay in the range 4.5–6.0. The procedure for curve fitting was completed with calculations performed by the Control Data Corp. 1604 computer at Cornell. The parameters were allowed to take on several values over a relatively narrow range, and the best of the calculated profiles was that for which: (a) first, the sum of calculated values of K_{pH} that differed from the observed values by less than twice the observed error[1] was a maximum; (b) second, the sum of those that differed by more than three times the observed error was a minimum; (c) finally, the sum of squares of residuals was a minimum. The profiles are illustrated in Figure 4. The apparently anomalous behavior of the equilibrium constants for valine above *p*H 9.4 is attributed to a systematic error of unknown origin. Thus, it is possible to fit the observed points at the highest *p*H values to the theoretical line if the observed value of D_P is increased by 0.04 optical density unit; the value of D_S is not changed by this operation. The best values for the parameters of equation (8) and the results of the error test in (a) and (b) are listed in Table I. The values of pK_{PCHO}, 6.55 ± 0.01, are consistent with the value reported at 20°, if the heat of ionization is assumed to be +9 kcal/mole. Whereas the best values of pK_{AH} for valine and glu-

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[1] The experimental values of K_{pH}, D_S, k_f (second-order rate constants for imine formation), k_r (first-order rate constants for imine hydrolysis), and the standard errors of K_{pH}, k_f, and k_r at the 70% confidence level are available as Document No. 8169, ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the document number and by remitting in advance $1.25 for photoprints or $1.25 for 35-mm microfilm payable to: Chief, Photoduplication Service, Library of Congress.

TABLE I: Best Values for Parameters of *p*H Profiles.[a]

Amino Acid	Equilibria (M^{-1}) for Imine Formation, Equation (8)									
	K	pK_S	$pK_{S\oplus}$	pK_{AH}	pK_{PCHO}	$pK_{PCHO\oplus}$	μ_1	μ_2	μ_3	μ_4
Glycine	49.3	9.12	5.50	9.81	6.56	4.05	20	9	1	3
Valine	55.8	9.48	5.06	9.47	6.55	4.05	18	3	2	3
Glutamate	24.3	9.28	5.15	9.33	6.55	4.05	24	9	2	2

Amino Acid	Rate Constants ($\text{M}^{-1}\,\text{min}^{-1}$) for Imine Formation, Equation (11)									
	pK_{AH}	pK_{PCHO}	$pK_{PCHO\oplus}$	$k_{f,0}$	$k_{f,1} \times 10^{-4}$	$k_{f,2} \times 10^{-6}$	μ_1	μ_2	μ_3	μ_4
Glycine	9.58	6.55	4.05	200	1.40	5.70	11	2	1	1
Valine	9.47	6.54	4.05	244	1.56	2.25	5	5	3	1
Glutamate	9.33	6.54	4.05	80.2	1.12	2.30	9	3	0	3

Amino Acid	Rate Constants for Imine Hydrolysis, Equation (14)								
	pK_S	$pK_{S\oplus}$	$k_{r,0} \times 10^{-5}$ (M^{-1} min^{-1})	$k_{r,1}$ (min^{-1})	$k_{r,2}$ (min^{-1})	μ_1	μ_2	μ_3	μ_4
Glycine	9.12	5.50	2.750	1.460	19.40	8	5	1	1
Valine	9.48	5.06	1.005	0.310	5.25	9	2	3	0
Glutamate	9.28	5.15	1.164	0.805	15.00	8	5	2	0

[a] The number of points in theoretical calculations within one, two, three, and greater than three times the observed error at the 70% confidence level (Tables I–III, ADI Doc. 8169) are designated μ_1, μ_2, μ_3, and μ_4, respectively.

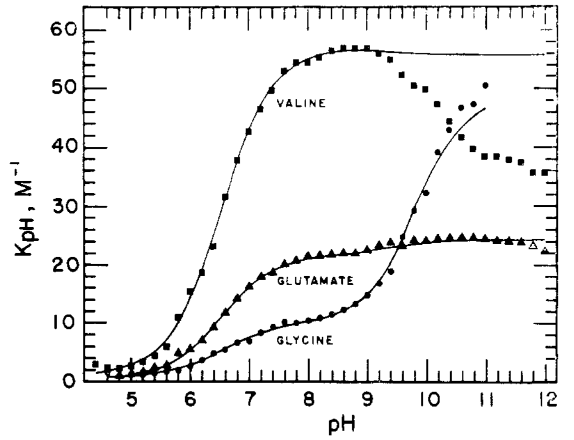


FIGURE 4: Equilibrium constants for imine formation from 3-hydroxypyridine-4-aldehyde as a function of *p*H. The filled circles are experimental values of K_{pH}, and the lines are calculated from equation (8).

tamate correspond to the values determined by half-neutralization at 0.02 and 0.01 M, respectively, the best value for glycine is slightly higher than any pK_{AH} that was measured by half-neutralization (French and Bruice, 1964). Again, this anomaly may be caused by a small systematic error in D_P or by the relatively large random errors for glycine[1] at high *p*H.

Rate Constants for Imine Formation and Hydrolysis. In contrast with the results with pyridine-4-aldehyde (French and Bruice, 1964) there was no significant dependence of the second-order rate constants for imine formation, k_f, on $[A_T]$ in the range 0.02–0.1 M. Furthermore, at *p*H 10.5 with glycine there was no significant difference, beyond the 99% confidence level, in the averages of k_f for six runs at 0.1 M and six at 0.5 M $[A_T]$. Consequently, the values of k_f that are used in subsequent calculations are averages of values of k_f measured at 5–7 concentrations of amino acid in the range 0.02–0.1 M, where

$$k_f = f(k_{obs})/[A_T] \tag{9}$$

and k_{obs} is the observed *pseudo*-first-order rate constant and f is the mole fraction of completion of imine formation (French and Bruice, 1964). The lower concentrations were omitted in measurements below *p*H 6.8 because the change in optical density was too small. Stopped-flow measurements were taken only for glycine in the *p*H range 7.63–10.15. Values of k_f at the three highest *p*H values of valine and glutamate are averages at $[A_T]$ = 0.01–0.05 M. In the *p*H range 7.3–8.6, 0.01 M *N*-ethylmorpholine was the buffer. Potassium phosphate

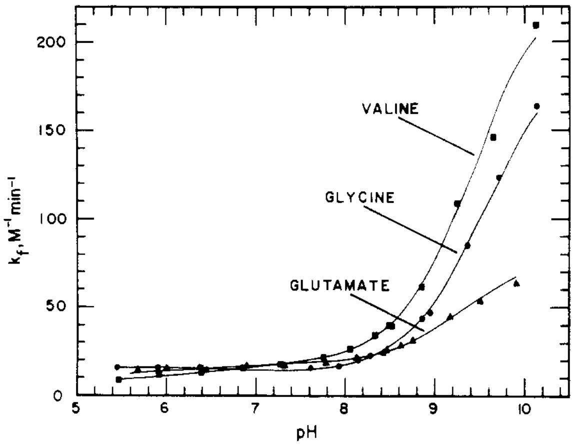


FIGURE 5: Dependence of k_f, the apparent second-order rate constant for imine formation, on *p*H. The values of k_f are not corrected for extent of hydration of the reactive species, free aldehyde.

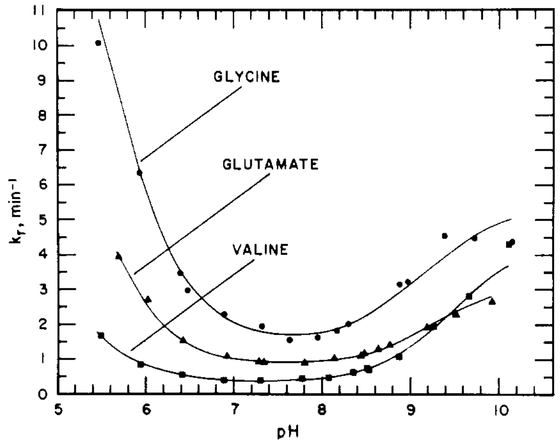


FIGURE 6: Dependence of k_r, the apparent first-order rate constant for imine hydrolysis, on *p*H.

buffers, 0.017 M, were used at lower *p*H, and the amino acid itself served as buffer at higher *p*H.

Theoretical curves could be fitted to graphs of k_f versus *p*H (Figure 5) by the method described above, with the assumptions that pK_{AH} lay in the range of values reported in paper IV (French and Bruice, 1964), that pK_{PCHO} was close to the best values in the theoretical equations for equilibrium constants, and that $pK_{\mathrm{PCHO}^{\oplus}}$ was again 4.05. The rate expression for the simplest model, reaction of amino acid as the uncharged amine with the anionic, zwitterionic, and cationic forms of aldehyde, is:

$$d[S_T]/dt = k_f[A_T][\mathrm{PCHO}_T] = [A]\,(k_{f,0}[\mathrm{PCHO}^{\ominus}] + k_{f,1}[\mathrm{PCHO}] + k_{f,2}[\mathrm{PCHO}^{\oplus}]) \quad (10)$$

$$k_f = \left(\frac{K_{\mathrm{AH}}}{K_{\mathrm{AH}} + a_{\mathrm{H}}}\right) \times \left(\frac{k_{f,0}K_{\mathrm{PCHO}}K_{\mathrm{PCHO}^{\oplus}} + k_{,f1}a_{\mathrm{H}}K_{\mathrm{PCHO}^{\oplus}} + k_{f,2}a_{\mathrm{H}}{}^2}{K_{\mathrm{PCHO}}K_{\mathrm{PCHO}^{\oplus}} + a_{\mathrm{H}}K_{\mathrm{PCHO}^{\oplus}} + a_{\mathrm{H}}{}^2}\right) \quad (11)$$

The best values of the parameters of equations (10) and (11) are listed in Table I. Other possible kinetic models will be reviewed under Discussion.

Rate constants for imine hydrolysis at the same *p*H values as the forward rates were calculated from equation (12):

$$k_r = k_f/K_{p\mathrm{H}} \quad (12)$$

The rate expression for the simplest model, attack of hydroxide ion on imine S, and attack of water on imines S and S$^{\oplus}$, is:

$$-d[S_T]dt = k_r[S_T] = k_{r,0}[\mathrm{OH}^{\ominus}][\mathrm{S}] + k_{r,1}[\mathrm{S}] + k_{r,2}[\mathrm{S}^{\oplus}] \quad (13)$$

$$k_r = \frac{1.479 \times 10^{-14}\,k_{r,0}K_{\mathrm{S}^{\oplus}} + k_{r,1}a_{\mathrm{H}}K_{\mathrm{S}^{\oplus}} + k_{r,2}a_{\mathrm{H}}{}^2}{K_{\mathrm{S}}K_{\mathrm{S}^{\oplus}} + a_{\mathrm{H}}K_{\mathrm{S}^{\oplus}} + a_{\mathrm{H}}{}^2} \quad (14)$$

As before, theoretical curves could be fitted to graphs of k_r versus *p*H (Figure 6) when K_{S} and $K_{\mathrm{S}^{\oplus}}$ were identical with the best values in the *p*H–equilibrium constant profiles. The best values of the rate constants of equations (13) and (14) are listed in Table I.

Discussion

Equilibrium Constants. The values of pK_{S} and $pK_{\mathrm{S}^{\oplus}}$ for 3-hydroxypyridine-4-aldehyde (Figure 3 and Table I) are about one *p*H unit lower than those obtained by Metzler (1957) with pyridoxal. The effect of the neighboring hydroxymethyl group in pyridoxal is to make the imine nitrogen a stronger base by formation of internal carbinolamine. However, it appears that the extent of carbinolamine formation ($\leq$11% for pyridoxal) is insufficient to explain the relatively large difference in pK_{S}.

Metzler (1957) has attributed the increase in pK of imines of pyridoxal with respect to aldehyde, and the stability of these imines, in the *p*H range near neutrality to hydrogen bonding between phenolic hydroxyl and azomethine nitrogen. This effect is even more pronounced in the case of 3-hydroxypyridine-4-aldehyde. For the three amino acids that were investigated, the value of K' (equation 15) is 360–850 times greater than K because the values of pK_{S} (Table I) are 2.6–2.9 units greater than pK_{PCHO}. Similarly, K'' is 10–28 times greater than K' because $pK_{\mathrm{S}^{\oplus}}$ is 1–1.5 units greater than $pK_{\mathrm{PCHO}^{\oplus}}$.

$$K' = K(K_{\mathrm{PCHO}}/K_{\mathrm{S}});\quad K'' = K'(K_{\mathrm{PCHO}^{\oplus}}/K_{\mathrm{S}^{\oplus}}) \quad (15)$$

Under physiological conditions, at *p*H 7.4, the apparent equilibrium constant for imine formation from 3-hydroxypyridine-4-aldehyde and glycine is 9.3 M^{-1},

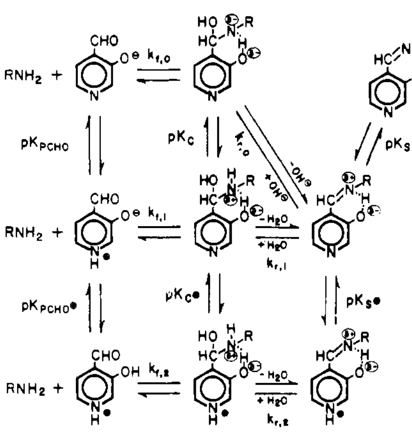
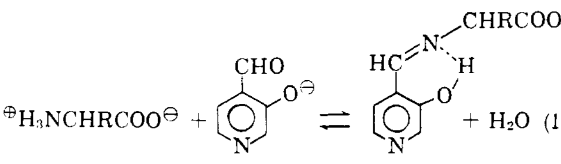


FIGURE 7: A summary scheme for imine formation and hydrolysis with 3-hydroxypyridine-4-aldehyde and amino acids. The three ionic forms of aldehyde are in equilibrium with hydrated forms that are not shown. The individual rate constants for carbinolamine formation, $k_{f,0}$, $k_{f,1}$, and $k_{f,2}$, are not corrected for extent of hydration.

whereas the corresponding value for pyridine-4-aldehyde is calculated to be 2.5 M^{-1} (French and Bruice, 1964). However, in slightly acid solution no detectable imine is formed with pyridine-4-aldehyde, whereas a significant amount of imine may be formed from 3-hydroxypyridine-4-aldehyde, even below *p*H 5 (Figure 4 and equation 8).

The values of K_{pH} rise rapidly with increasing *p*H in the range 5.5–7.5, where K_{PCHO} is the controlling parameter (equation 8), and they are nearly constant from *p*H 7.5 to 8.5. The reason for this flat response is most easily appreciated by inspection of equation (16), in which no proton is released. In comparison, over the same *p*H range a proton must be released when imine is formed from pyridine-4-aldehyde (equation 17), and therefore the apparent equilibrium constant decreases with decreasing *p*H.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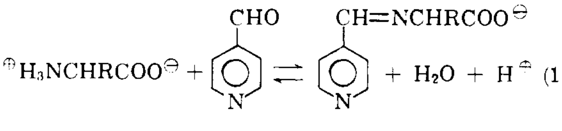

(17)

The maximal velocity of the imidazole-catalyzed transamination of phenylglycine with pyridoxal (Bruice and Topping, 1963) increases markedly in the *p*H range 7–10. This increase may be the result of a proportional increase in concentration of imine, S′, where pK_{PCHO} is 8.6 for pyridoxal.

Rate Constants for Imine Formation and Hydrolysis. Figure 7 illustrates the simplest and most likely models for imine formation and hydrolysis. However, two types of reaction mechanisms for imine formation that are kinetically equivalent to the type shown in Figure 7 (i.e., rate-limiting attack of free amino acid on the various protonated forms of aldehyde) must be considered. In the first alternative mechanism, the second and third terms of equation (10) may be considered to represent general acid catalysis by $H_3O^{\oplus}$ of the attack of free amino acid on $\text{PCHO}^{\ominus}$ and PCHO, respectively, rather than uncatalyzed attack of free amino acid on PCHO and $\text{PCHO}^{\oplus}$. The calculated rate constants for $H_3O^{\oplus}$-catalyzed imine formation from glycine and 3-hydroxypyridine-4-aldehyde (i.e., $k_{f,1}/K_{\text{PCHO}}$, and $k_{f,2}/K_{\text{PCHO}^{\oplus}}$) are greater than 4×10^{10} M^{-2} min^{-1}. These values are unreasonably large. Furthermore, no general acid catalysis was observed when the zwitterionic form of the amino acid itself was in high concentration. Cordes and Jencks (1963) have concluded that strongly basic amines generally attack the carbonyl group readily, without acid catalysis. General acid catalysis by $H_3O^{\oplus}$ of imine formation from amino acids and 3-hydroxypyridine-4-aldehyde therefore appears highly unlikely.

In the second alternative mechanism, carbinolamine is formed from free amino acid and aldehyde in a pre-equilibrium step, followed by rate-limiting dehydration of this intermediate to yield imine (Cordes and Jencks, 1963; French and Bruice, 1964). To discuss this alternative, equations (18), (19), and (20) are employed.

$$A + \text{PCHO}^{\ominus} \overset{K_e}{\overbrace{\underset{k_{-e,0}}{\overset{k_{e,0}}{\rightleftharpoons}}}} \text{C}^{\ominus} \xrightarrow{k_{f,0}'} \text{S} + \text{OH}^{\ominus} \quad (18)$$

$$A + \text{PCHO} \overset{K_e'}{\overbrace{\underset{k_{-e,1}}{\overset{k_{e,1}}{\rightleftharpoons}}}} \text{C} \xrightarrow{k_{f,1}} \text{S} + \text{H}_2\text{O} \quad (19)$$

$$A + \text{PCHO}^{\oplus} \overset{K_{e'}''}{\overbrace{\underset{k_{-e,2}}{\overset{k_{e,2}}{\rightleftharpoons}}}} \text{C}^{\oplus} \xrightarrow{k_{f,2}'} \text{S}^{\oplus} + \text{H}_2\text{O} \quad (20)$$

$\text{C}^{\ominus}$, C, and $\text{C}^{\oplus}$ are the three ionic forms of carbinolamine shown in Figure 7.

In paper IV (French and Bruice, 1964) it was shown that the equilibrium constant for the formation of neutral carbinolamine from pyridine-4-aldehyde was $3.8 \geq K_e \geq 0.3$ for the ten amino acids that were studied. The carbinolamine of 3-hydroxypyridine-4-aldehyde was not detected at any *p*H, not even in the range 9.5–10.5 and at high concentrations of amino acid, where the presence of carbinolamines of pyridine-4-aldehyde is readily discernible. The lower bound for detection of pre-equilibrium formation of carbinolamine by kinetic analysis (see, for example, French and Bruice, 1964, equation 25) is estimated to be ≤ 0.2 M^{-1}. If the measured forward rate constants ($k_{f,0}$, $k_{f,1}$, $k_{f,2}$) do not pertain to rate-limiting carbinolamine formation (Table I) but are rather products of pre-equilibrium constants(K_e, K_e', K_e'') for carbinolamine formation and rate constants ($k_{f,0}'$, $k_{f,1}'$, $k_{f,2}'$) for carbinolamine dehydration, then the lower limits for the three rate constants for dehydration of the three ionic forms of the carbinolamine of glycine are calculated to be:

$$k_{f,0}' = k_{f,0}/K_e \geq 1000\ \text{min}^{-1},\ \text{where } K_e = [\text{C}^{\ominus}]/[A][\text{PCHO}^{\ominus}] \quad (21)$$

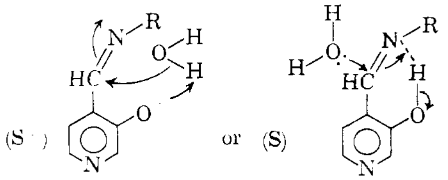
$$k_{f,1}' = k_{f,1}/K_e' \geq 7 \times 10^4\ \text{min}^{-1},\ \text{where } K_e' = [\text{C}]/[A][\text{PCHO}] \quad (22)$$

$$k_{f,2}' = k_{f,2}/K_e'' \geq 2.85 \times 10^7\ \text{min}^{-1},\ \text{where } K_e'' = [\text{C}^{\oplus}]/[A][\text{PCHO}^{\oplus}] \quad (23)$$

If the dehydration steps were rate limiting, the calculated first-order rate constants for attack of free amine on aldehyde would be greater than the corresponding rate constants for dehydration by a factor of 20 at least. This factor is derived from the reciprocal of the estimated accuracy in detection of K_e by kinetic analysis. Under experimental conditions, the lower bounds for the rate constants for attack of free amine on aldehyde at *p*H 10.5 and $[A] = 0.45$ M, at *p*H 7.4 and $[A] = 6.6 \times 10^{-4}$ M, and at *p*H 5.4 and $[A] = 6.6 \times 10^{-6}$ M are, respectively: $k_{e,0} \geq 4.4 \times 10^4$, $k_{e,1} \geq 2.2 \times 10^9$, and $k_{e,2} \geq 8.6 \times 10^{13}$ M^{-1} min^{-1}. These values are unreasonably large. Thiol esters are similar to aldehydes in their sensitivity to nucleophilic attack since the unpaired electrons of sulfur do not resonate with those of the adjacent carbonyl. The rate constant for nucleophilic attack of ethanolamine ($pK_a = 9.72$) on δ-thiovalerolactone at 30° and $\mu = 1$ (Bruice *et al.*, 1963) is only 5.29 M^{-1} min^{-1}. The calculated rate constant for rate-limiting attack of glycine ($pK_a = 9.58$) on PCHO$^{\ominus}$, an aromatic aldehyde that is deactivated by the presence of an *ortho*-phenoxide group and activated by the electron-withdrawing pyridine nitrogen in the *para* position, is 200 M^{-1} min^{-1} (Table I). In comparison, the value of $k_{e,0}$ is very much larger than would be expected. Furthermore, Cordes and Jencks (1962) have reported a rate constant of 190 M^{-1} min^{-1} at 25° for attack of free aniline ($pK_a = 4.6$) on *p*-nitrobenzaldehyde, a compound that may be compared to cationic 3-hydroxypyridine-4-aldehyde (PCHO$^{\oplus}$, $k_{f,2} = 2.3$–5.7×10^6 M^{-1} min^{-1}, Table I). The calculated rate constant for attack of free amino acid on *p*-nitrobenzaldehyde would equal that for rate-limiting attack on PCHO$^{\oplus}$ if the value of α in a Brönsted plot were 0.8–0.9. The calculated value for $k_{e,2}$ is, in contrast, impossibly large. The foregoing considerations indicate that the attack of free amino acid on aldehyde, rather than dehydration of the carbinolamine intermediate, is the rate-limiting step in imine formation at all values of *p*H that were investigated.

When attack of free amino acid on aldehyde is rate limiting, the individual rate constants for dehydration may again be assumed to be more than 20-fold greater than the corresponding calculated first-order rate constants for carbinolamine formation at the highest concentrations of free amino acid that were employed experimentally. At *p*H 10.5 and $[A] = 0.45$ M where only the $k_{f,0}$ term of equation (10) is important, the lower bound for the rate constant for dehydration of C$^{\ominus}$ formed from glycine is 1800 min^{-1}. At *p*H 7.4, where the effect of the $k_{f,1}$ term predominates, and at *p*H 5.4, where the effect of the $k_{f,2}$ term predominates, the lower bounds for the rate constants for dehydration of C and C$^{\oplus}$ are 180 and 760 min^{-1}, respectively.

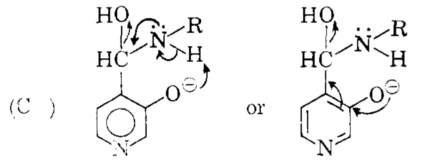
Of the terms in equation (13) for imine hydrolysis, only the first is kinetically ambiguous. If the first term represented an attack of water on S$^{\ominus}$, rather than an attack of hydroxide ion on S, the rate constant for glycine would be 5.4 min^{-1}, a value nearly four times that for attack of water on S (Table I). The attack of water on S$^{\ominus}$ might proceed by intramolecular general base catalysis (see Bender *et al.*, 1963) at a greater rate than attack of water on S that is intramolecular-general-acid-catalyzed (equation 24). Therefore, the alternative, attack of water on S$^{\ominus}$, cannot be ruled out. However,



(24)

in their study of the rates of hydrolysis of imines derived from substituted benzaldehydes, Cordes and Jencks (1963) proposed that the relatively small but negative value of ρ for these reactions represented a composite of a relatively large positive value for hydroxide attack and a large negative value for acid association constant. If hydrolysis proceeded by attack of water on unprotonated imine, a positive ρ value would have been expected. These findings support the original mechanism of Figure 7, attack of hydroxide ion on S.

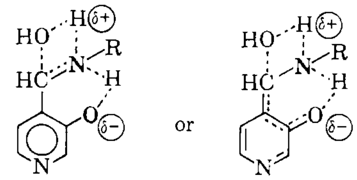
Possible Mechanisms for Dehydration of Carbinolamines by Intramolecular Catalysis. Dehydration of the carbinolamines formed from pyridine-4-aldehyde and anionic 3-hydroxypyridine-4-aldehyde can proceed by expulsion of hydroxide ion. However, the *ortho*-phenoxide group of the latter aldehyde acts as an intramolecular general base catalyst for the dehydration of C$^{\ominus}$. This fact is apparent in comparison of the rate constants for dehydration of the carbinolamine of glycine and pyridine-4-aldehyde ($k_2 = 7$ min^{-1}, French and Bruice, 1964) and of C$^{\ominus}$ formed from glycine (≥ 1800 min^{-1}). There are two alternate mechanisms by which the expulsion of hydroxide ion from the intermediate carbinolamine may be facilitated by the phenoxy group.



(25)

C, the carbinolamine derived from zwitterionic aldehyde, may exist in the dipolar structure shown in Figure 7, or as the tautomeric hydrogen-bonded amine. In either case, the effect of oxygen in the 3 position is to raise the pK_a' of the carbinolamine nitrogen and to increase the concentration of protonated carbinolamine at neutral and alkaline *p*H, relative to the protonated carbinolamine of pyridine-4-aldehyde. Dehydration may then readily proceed by the acid-catalyzed pathway discussed in paper IV (French and Bruice, 1964), for which a four-center reaction was postulated. Alternatively, the effect of oxygen in the 3 position may be transmitted through the ring. The dehydration of $C^{\oplus}$ may occur by similar mechanisms. Quantitative comparison of the acid-catalyzed dehydration reactions for the two aldehydes is not possible until more accurate estimates of the pertinent rate constants are available.

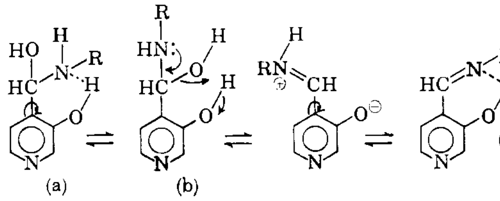
(C)



(26)

Another possible mechanism for dehydration of C and $C^{\oplus}$ is intramolecular general acid catalysis by phenolic hydroxyl. Although structure (a) in equation (27) should be thermodynamically more stable than structure (b), the latter structure might dehydrate more rapidly than (a). Therefore intramolecular general acid catalysis cannot be ruled out.

(C)



(27)

Importance of Model Studies to the Mechanism of Transamination. Bruice and Benkovic (1964) and Bruice and Pandit (1960) have shown that one may expect an equivalent rate enhancement of 10^3–10^4 in nucleophilic attack on a carbonyl carbon if a bimolecular reaction is transformed to an intramolecular reaction in which the reactive groups are already constrained in a reactive orientation. The apparent rate constant for imine formation with 3-hydroxypyridine-4-aldehyde and free glutamate that applies in the physiological *p*H range is $k_{f,1}$ (1.12×10^4 M^{-1} min^{-1}, Table I). When corrected for the effect of hydration (Nakamoto and Martell, 1959), $k_{f,1}$ is 1.8×10^4 M^{-1} min^{-1}. If a transaminase could provide free amino acid in an orientation that is optimum for reaction with unhydrated enzyme-bound aldehyde, one would expect rate constants of 1.8×10^7 to 1.8×10^8 M^{-1} min^{-1}. In contrast, Hammes and Fasella (1963) have reported a rate constant of 2×10^9 M^{-1} min^{-1} for the formation of Y_1, which is presumed to be the enzyme-bound imine of glutamate and pyridoxal phosphate. It appears that 3-hydroxypyridine-4-aldehyde is the simplest compound that is closely related to the enzymatic site for imine formation, and that further modification of models for intramolecular catalysis of imine formation should proceed from this structure.

The available evidence indicates that the active form of transaminases is an aldimine rather than an aldehyde (Turano *et al.*, 1961; Hughes *et al.*, 1962; Hammes and Fasella, 1963). Hence the results of the present study may pertain to the kinetics of binding of pyridoxal phosphate to apotransaminases. It would be of interest to extend the present investigation of rate constants for imine formation with 3-hydroxypyridine-4-aldehyde to a similar study of an analogous imine. In particular, one might observe a rate enhancement, relative to 3-hydroxypyridine-4-aldehyde, in attack of free amino acid on a cyclic imine derivative of 3-hydroxypyridine-4-aldehyde in which the imine nitrogen is attached to a flexible side chain of the aromatic ring.

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