B. **3-Hydroxypyridine-2-aldehyde.**—Diacetate of 3-hydroxypyridine-2-methanol (42 g., 0.2 mole), prepared according to Stempel and Buzzi,[12] was dissolved in glacial acetic acid (140 ml.) and treated with H_2O_2 (32 ml.) in the same way as in the above procedure. The crude N-oxide was treated with acetic anhydride (140 ml.) on steam-bath for 6 hr. and the solvents eliminated under reduced pressure. Attempts to distil the triacetate under a vacuum of 2 mm. failed as the product decomposed. Another part of the crude triacetate was treated with 6 *N* HCl on steam-bath and a crude oil with strong aldehyde smell was obtained; yield (crude) 3 g., (12% of theory) (better yields could certainly be worked out). A small amount was distilled; b.p. 72–74° (12–14 mm.).

C. 3-Methoxypyridine-2-aldehyde.—3-Hydroxypyridine-2-aldehyde (crude) (2 g.), dissolved in 2 *N* KOH (8 ml.), was added to 10 ml. of methanol and 3.2 g. of methyl *p*-toluenesulfonate and refluxed on steam-bath for 1 hr. Very soon precipitation of sodium *p*-toluenesulfonate starts. The reaction mixture was poured in 100 ml. of distilled water and extracted with $CHCl_3$. The residue, dark oil, weighing about 0.5 g. (22%) was not purified but transformed directly into the oxime.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

Equilibria between Pyridoxal and Amino Acids and their Imines[1]

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Spectrophotometry of aqueous solutions of pyridoxal with amino acids shows that extensive imine formation occurs over a wide *p*H range. The imines formed in the neutral *p*H range are yellow, absorb light maximally at about 414 mμ and are weak acids of *pK* about 10.5. The conjugate base forms which exist at high *p*H values have an absorption band at about 365 mμ. The *p*H dependence of the apparent equilibrium constants for the formation of the imines with valine and with glycine is analyzed quantitatively. Hydrogen bonding apparently increases the stability of the imines in the neutral *p*H range. Glycine is shown to yield a small amount of aminoacetal or carbinolamine as well as the imine. Apparent equilibrium constants for imine formation with 22 amines and amino acids are compared. The presence of a β-methyl group in the side chain of the amino acid increases the stability of the imine, whereas α-substitution decreases the stability. These and other factors affecting the stability and the ionization constants of the imines are discussed.

Imines (Schiff bases) have frequently been suggested as intermediates in the reactions of pyridoxal (vitamin B_6 aldehyde) with amino acids, both in the presence and absence of enzymes.[2,3] A number of such imines have been prepared,[4–6] but little information is available on their stability in aqueous media. The present study provides a quantitative description of the extent of formation of such imines in aqueous buffered solutions.

Valine–Pyridoxal Imine.—Valine was selected for a detailed study because of the favorable equilibrium constants. When this amino acid is mixed with pyridoxal in dilute aqueous solution, a marked change in the ultraviolet absorption spectrum occurs. This change is clearly displayed (Fig. 1) at *p*H 7.4, where pyridoxal has a very low absorption in the 400 mμ region. In the presence of valine, strong absorption bands appear with maxima at about 280 and 414 mμ. The latter band extends into the blue end of the visible spectrum and the solutions are consequently intensely yellow. These bands increase in intensity as the valine concentration increases, while the 317 mμ absorption band of the internal hemiacetal form of pyridoxal (Ia) decreases correspondingly. Equilibrium with respect to this change is achieved within 10 minutes or less. When the absorption spectra at various valine concentrations (and constant *p*H) are compared, sharp isosbestic points are observed at 256, 296 and 338 mμ (Fig. 1). The presence of these points of constant absorption indicates that the reaction can be treated in terms of a single equilibrium between pyridoxal and the product of its reaction with valine.

The position of the absorption maximum at 414 mμ indicates that the product is almost certainly the imine, IIa-IId (R = isopropyl). Pyridine derivatives which lack a double bond in conjugation with the aromatic ring absorb at wave lengths below 330 mμ,[7] whereas the free aldehyde form of pyridoxal (Ib) and pyridoxal phosphate which contain an additional double bond have an absorption peak at about 390 mμ.[7] Comparison with the spectra of salicylaldimines[8–10] further confirms the identity of the interaction product.

Conversion to the imine is incomplete at *p*H 7.4, even in near-saturated 0.6 *M* valine solutions, as shown by the small amount of residual pyridoxal absorption (Fig. 1) and by the equilibrium constant calculated later. However, between *p*H 8.3 and 12, the conversion is estimated to be over 95% complete in 0.5–0.6 *M* valine. The spectra of these solutions show little evidence of any unreacted pyridoxal (Fig. 2) and can be taken as approximating those of the pyridoxal–valine imine.

The spectrum of the pyridoxal–valine imine undergoes a marked change as the *p*H is raised (Fig. 2). The 414 mμ peak is shifted to 367 mμ, the rest of the spectrum shifting correspondingly. The high *p*H spectrum is still unmistakably differ-

(1) Journal Paper No. J-2999 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project No. 1259.

(2) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76**, 648 (1954). References to earlier suggestions of imine intermediates are cited in this paper and in reference 3.

(3) A. E. Braunstein and M. M. Shemyakin, *Biokhimia*, **18**, 393 (1953).

(4) D. Heyl, E. Luz, S. A. Harris and K. Folkers, THIS JOURNAL, **70**, 3429, 3669 (1948); **74**, 414 (1952).

(4a) B. Witkop and T. W. Beiler, *ibid.*, **76**, 5589 (1954).

(5) H. N. Christensen and S. Collins, *J. Biol. Chem.*, **220**, 279 (1956).

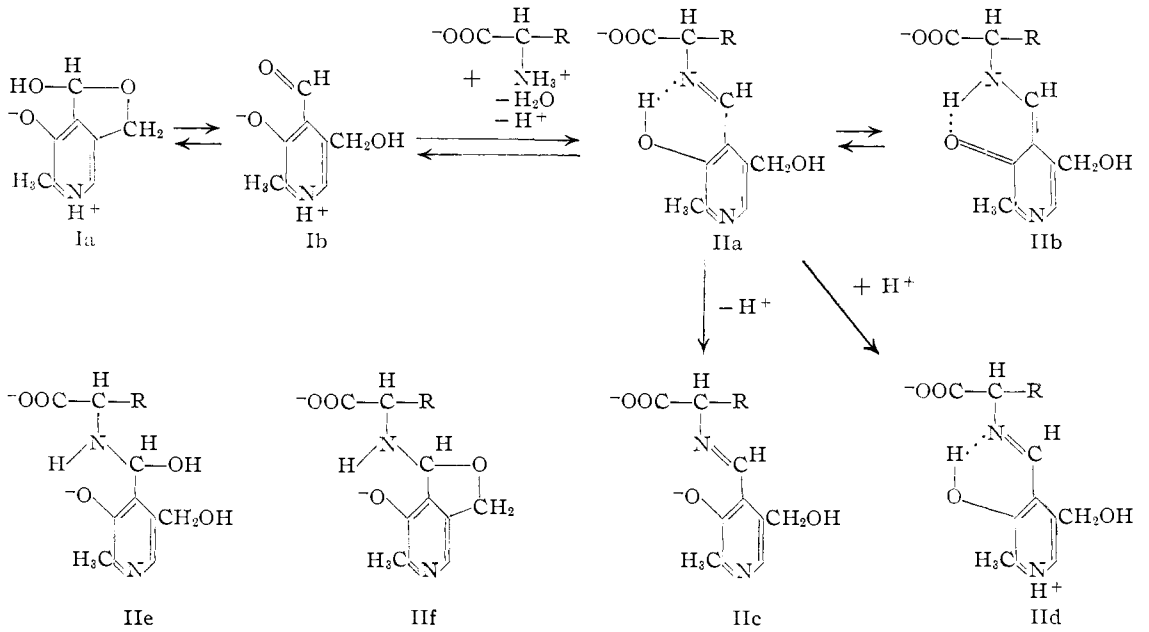
(6) F. C. McIntire, THIS JOURNAL, **69**, 1377 (1947). Imines with various aromatic aldehydes were prepared.

(7) D. E. Metzler and E. E. Snell, *ibid.*, **77**, 2431 (1955).

(8) L. N. Ferguson and I. Kelly, *ibid.*, **73**, 3707 (1951).

(9) Á. V. Kiss and G. Auer, *Z. physik. Chem.*, **189A**, 344 (1941).

(10) Á. V. Kiss, P. Csokán and G. Nyiri, *ibid.*, **190A**, 65 (1942).



ent from that of pyridoxal at a similar pH.[7] At intermediate pH values, the spectrum is intermediate between the two extremes and isosbestic points are present (Fig. 2). This spectral change must be caused by the dissociation of a proton from some group in the imine and has been used to compute the apparent pK value for this group as 10.49 ± 0.03 at ionic strength 0.5 (see Experimental).

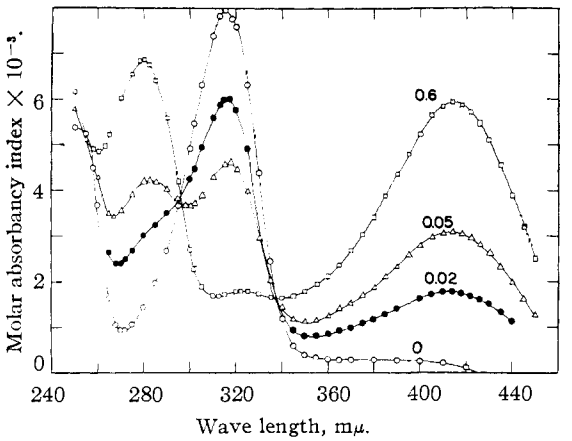


Fig. 1.—The absorption spectrum of 2×10^{-4} M pyridoxal at pH 7.44 in the presence of increasing concentrations of valine. The figures beside the curves give the valine concentrations in moles per liter.

Since conversion of pyridoxal to the imine is incomplete at pH values below 8.4, the spectrum of the latter cannot be measured directly in solutions of lower pH. However, measurements at a variety of valine concentrations at pH 6.4 have permitted an indirect calculation of the imine spectrum at that pH and it is identical to that at pH 8.4. Even at pH 4, the 414 mμ band persists and has the same shape as at higher pH values. At pH 2 the band is still present but because of the very small amount of imine present, the position of the maximum was not located accurately.

Equilibrium Constant for Imine Formation.—From spectral measurements, the extent of imine formation in solutions of various valine concentrations can be computed at any pH (see Experimental section) and used to determine the apparent equilibrium constant for imine formation, $K_{p\mathrm{H}}$.

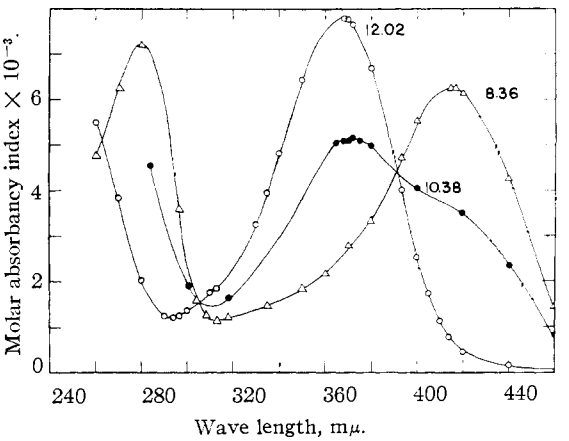


Fig. 2.—The absorption spectrum of pyridoxal–valine imine (2×10^{-4} M pyridoxal in 0.5 M valine) as a function of pH. pH values are given beside the curves.

$$K_{p\mathrm{H}} = \frac{[\text{imine}]}{[\text{pyridoxal}][\text{amino acid}]} \qquad (1)$$

where *the concentrations in brackets represent the sums of all the ionic species* of each compound.

A series of measurements at pH 6.4 on 13 solutions of valine concentrations from 0.02 to 0.6 M and pyridoxal concentrations of 10^{-4} to 10^{-3} M yields a constant value of $K_{p\mathrm{H}} = 1.96 \pm 0.11$ (a.d.). The constancy of this value at a single pH may be regarded as further evidence for the correctness of the assumption that the observed spectral changes represent imine formation and that an equilibrium constant can be defined and measured in the manner described. Log $K_{p\mathrm{H}}$ measured at a variety of

other pH values is plotted against pH in Fig. 3 (upper curve). The solid line in Fig. 3 is a theoretical curve constructed on the following basis. Let K be the apparent equilibrium constant for the reaction of the pyridoxal *anion* with the amino acid *anion* to give the double anion of the imine (IIb), the form which undoubtedly predominates at pH values above 11. Thus

$$K = \frac{[\text{imine}^{=}]}{[\text{pyridoxal}^{-}][\text{amino acid}^{-}]} \qquad (2)$$

Let us associate the following pK's (for apparent acid dissociation constants) with the various dissociable groups as follows: pK_{1R} and pK_{2R} with the carboxyl and amino groups of the amino acid (2.32 and 9.62 for valine[11]), $pK_{1P} = 4.20$ and $pK_{2P} = 8.66$ with the phenolic and pyridinium groups of pyridoxal, $pK_{3P} = 13$ with the third very weakly acidic group of pyridoxal, and pK_{1RP} to pK_{3RP} with the dissociable groups of the imine. Let pK_{sRP} be the pK of 10.49 in the valine imine. It readily can be shown that the logarithm of $K_{p\text{H}}$ will vary with $[\text{H}^+]$ as

$$\log K_{p\text{H}} = \log K - \log\left(1 + \frac{[\text{H}^+]}{K_{2R}} + \frac{[\text{H}^+]^2}{K_{1R}K_{2R}}\right) - \log\left(1 + \frac{K_{3P}}{[\text{H}^+]} + \frac{[\text{H}^+]}{K_{2P}} + \frac{[\text{H}^+]^2}{K_{1P}K_{2P}}\right) + \log\left(1 + \frac{[\text{H}^+]}{K_{3RP}} + \frac{[\text{H}^+]^2}{K_{2RP}K_{3RP}} + \frac{[\text{H}^+]^3}{K_{1RP}K_{2RP}K_{3RP}}\right) \qquad (3)$$

The solid line of Fig. 3 (upper curve) is the theoretical curve given by eq. 3 for a value of $\log K = 1.65$. The segment above pH 7 was computed using the known values of all of the pK's affecting the shape of the curve in this region. The change in slope at about pH 6 must result from the dissociation of some other group in the imine. From the observed pH dependence, a value of $pK_{2RP} = 5.88$ was estimated and used in constructing the theoretical curve. For the construction of the low pH portion of the curve, it was assumed that $pK_{1RP} = pK_{1R}$. The one point at a pH near 2 suggests that this assumption is probably approximately correct.

The Structure of the Imine.—It is certain that the imine exists in highly alkaline solution as the dianion IIc. As the pH decreases, a single proton is added to form an acid of $pK = 10.5$ in which the phenolic group is probably hydrogen bonded to the imino nitrogen, two tautomeric forms being possible (IIa and IIb). It is this hydrogen bonded form which possesses an absorption band at 414 mμ. The corresponding 410 mμ absorption band of salicylaldimines has likewise been associated[8,9] with a hydrogen bonded structure, perhaps analogous to IIb.[9] Methylation of the phenolic group of salicylaldehyde abolishes the band,[8] and replacement of the chelated hydrogen by sodium results in a shift of the band to 378 mμ (in alcohol).[10]

The occurrence of hydrogen bonding in the imine can also explain the unusually high pK of the group, *e.g.*, compare pK_{3RP} with that of 8.20 for the phenolic group of the neutral non-ionic form of 3-hydroxypyridine.[7] A related effect of the hydrogen bonding is to increase the stability of the imines in the pH range near neutrality (Fig. 3), an effect of possible importance in the enzymic function of pyridoxal.[2]

(11) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 84.

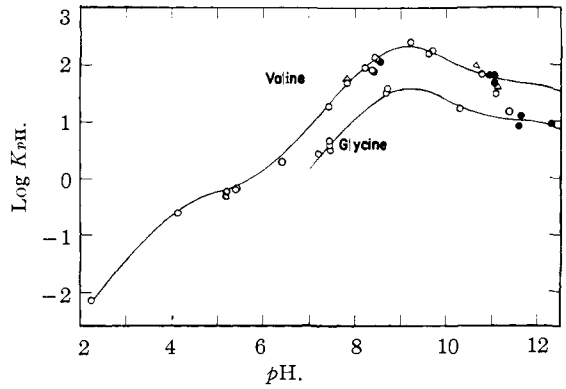


Fig. 3.—Variation of the logarithm of the equilibrium constant for imine formation with pH. Upper curve, valine + pyridoxal; lower curve, glycine + pyridoxal. Solid lines are theoretical curves (see text): O, 0.1 ionic strength (neglecting dipolar ions); ●, higher ionic strength; △, sodium ion-free solutions.

Christensen[5,12] and Collins[5] suggest that chelation of sodium with pyridoxal, and possibly with the imines, can occur. Most solutions in the present study contained about 0.1 M sodium ion, but three were sodium-free (buffers contained triethanolamine or tetramethylammonium hydroxide). The log $K_{p\text{H}}$ values for these solutions are plotted as triangles in Fig. 3 and are seen to be only slightly different from values for sodium-containing solutions. However, the sodium-free solutions appeared consistently to form slightly more imine than those containing sodium. While this difference may possibly be explained by chelation of the free aldehyde form of pyridoxal with sodium as suggested by Christensen,[12] the association must be very weak.

In acid, as in neutral, solution the shape of the 414 mμ absorption peak of the imine remains unchanged. Thus, the dissociations represented by pK_{2RP} and pK_{1RP} do not affect the spectrum significantly. $pK_{2RP} = 5.9$ is too high for the carboxyl group and is tentatively assigned to the pyridinium group of structure IId. It seems surprising that no spectral change is associated with this dissociation step. pK_{1RP} is then assigned to the carboxyl group.

Glycine–Pyridoxal Interaction.—A series of measurements with glycine gave results very similar to those with valine (Fig. 3), except that K was lower (Table I) and pK_{3RP} was slightly lower (10.4). The spectra of the glycine imine (measured indirectly; see Experimental section) are shown in Fig. 4. In both the neutral and alkaline forms, there appears to be a component absorbing at lower wave lengths (about 330 mμ neutral and 300 mμ alkaline). This component is not unreacted pyridoxal, since it cannot be made to disappear even in 2 M glycine.[13] It seems probable that this

(12) H. N. Christensen, *Science*, **122**, 1087 (1955).

(13) Furthermore, when the assumption was made that this absorption did represent unreacted pyridoxal and that the reaction product had a spectrum like that of the valine imine (shifted 3 mμ), the calculated K values obtained with various glycine concentrations were inconsistent, some falling far off the curve in Fig. 3.

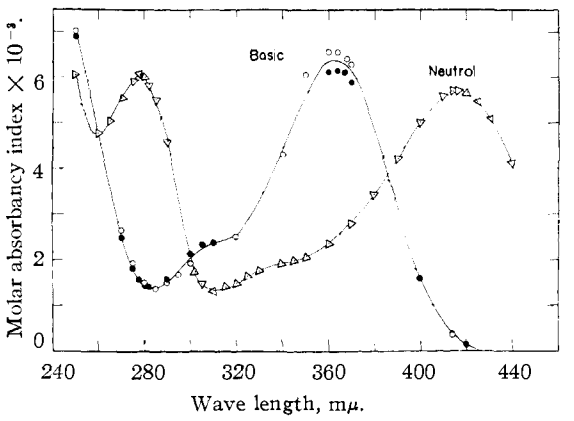


Fig. 4.—The spectrum of the pyridoxal–glycine interaction product. △, calculated from measurements on a solution of 2×10^{-4} *M* pyridoxal in 2 *M* glycine at *p*H 7.2 estimated to be 88% converted to imine and amino-acetal forms. ○, ●, a composite curve calculated from measurements on two solutions of 2×10^{-4} *M* pyridoxal; ○, in 2 *M* glycine at *p*H 12.9; calculated to be 89% converted; ●, in 0.5 *M* glycine at *p*H 12.3; calculated to be 82% converted.

absorption is caused by the presence of some carbinolamine form (IIe)[14] or more likely, the amino-acetal form (IIf) in equilibrium with the imine. This view is strengthened by the observation that sarcosine (N-methylglycine) reacts with pyridoxal.[14]

The spectrum of pyridoxal in 0.2 *M* sarcosine at *p*H 11.3 shows no evidence of increased absorption at 370 mμ, but the moderately strong 390 mμ band displayed by pyridoxal at this *p*H is markedly decreased and the 302 mμ band is strengthened and shifted to slightly lower wave lengths (spectrum not shown). The change probably reflects amino-acetal formation; *K* for this process is estimated as 3.0 from the loss of pyridoxal absorption at 390 mμ.

For glycine and other primary amines, *K* can be expressed as

$$K = \frac{[\text{imine}^-] + [\text{aminoacetal}^-]}{[\text{amino acid}^-][\text{pyridoxal}^-]} = K(\text{imine}) + K(\text{aminoacetal}) \quad (4)$$

From the spectrum of the alkaline form of the glycine–pyridoxal interaction product, it is estimated that about 11% of the product exists as amino-acetal or carbinolamine forms, the remainder being imine. From this estimate and the value K = 10.2 (Table I) we can estimate K(aminoacetal) = 1.2 for glycine as compared to 3 for sarcosine. K(imine) is thus 1.2 less than the measured K. If we can assume similar values for K(aminoacetal) with other amines, it is evident that for amino acids, such as valine, with high K values, the fraction of aminoacetal in the product will be small. Only a trace of absorption attributable to such forms is present in the valine imine spectrum (Fig. 2) and in the isobutylimine spectrum (not shown). On the other hand, amines with very low K values may react to give a larger fraction of aminoacetal.

Other Imines.—The spectra of both forms of the isobutylimine of pyridoxal were measured directly in 0.5 *M* isobutylamine solutions and were shown to be very similar to the valine imine spectra. The spectra of the imines of other amino acids and amines appear to be similar but with slightly different absorption band positions.

Estimated values of *K* for a variety of amines are given in Table I and are believed to be accurate to ± 20%.

While *K* for *sec*-butylamine is almost as large as that for *n*-butylamine, the introduction of an α-carboxylate group (α-amino-*n*-butyric acid *vs.* *sec*-butylamine and glycine *vs.* ethylamine) decreases *K*, perhaps because of the decreased basicity of the amino groups in the amino acids or the electrostatic repulsion of the two anions. When the α-carbon of the amine is tertiary (*t*-butylamine, α-aminoisobutyric acid, tris-(hydroxymethyl)-aminomethane), *K* decreases about 10-fold. No steric hindrance is evident, nor can the effect be explained in terms of changes in the basicity of the amine. Possibly the imines in which the α-carbon of the amine bears one or more hydrogen atoms are stabilized to some extent by conjugation of the aromatic system with the electrons of the C–H bonds.[15] In compounds containing a tertiary carbon atom and in ammonia such stabilization would be prevented.

TABLE I

APPARENT EQUILIBRIUM CONSTANTS FOR IMINE FORMATION

The reaction of pyridoxal anion with amines and amino acid anions in water at 25°.

Amine	*K*	Amine	*K*
Ammonia	<0.3	Serine	11
Methylamine	17	Aspartic acid	15
Ethylamine	26	α-Amino-*n*-butyric acid	17
Ethanolamine	31	α-Amino-isobutyric acid	1.6
n-Butylamine	83	Threonine	15
sec-Butylamine	68	Glutamic acid	18
t-Butylamine	6.0	Norvaline	19
Isobutylamine	119	Valine	44
Tris-(hydroxymethyl)-aminomethane	1	Leucine	31
Glycine	10.2	Isoleucine	55
Alanine	9		

On the other hand, branching in the β-position of the amine (valine isoleucine, isobutylamine) is particularly effective in increasing the imine stability.[6] (However, the β-hydroxyl group of threonine does not increase *K*.) No certain explanation can be offered; perhaps van der Waals bonding of the branched alkyl side chains to the methine bridge and the hydroxymethyl side chain of the pyridoxal are involved. An understanding of this effect might shed some light on the role of these branched amino acids in protein structure.

The effect of aldehyde structure on *K* has not been studied, but the *K* value for glycine + pyridoxal (10.2) may be compared with a value for glycine + benzaldehyde (75) measured polarographically by Zuman.[16] Since only about 26% of

(14) H. N. Christensen and T. R. Riggs, *J. Biol. Chem.*, **220**, 265 (1956).

(15) Such hyperconjugation has been used to explain the stabilization of the *transition states* for subsequent reactions of the imines (reference 2). A stabilization of the *normal* state of the molecule seems less certain.

(16) P. Zuman, *Chem. Listy*, **46**, 688 (1952); *Collection Czechoslov. Chem. Commun.*, **15**, 839 (1950). Some other *K* values of interest are as follows: pyruvate + ammonia, 0.30; pyruvate + glycine, 2.47; o-anisaldehyde + glycine, 48.

the anion of pyridoxal exists as the free aldehyde,[7] a more comparable value for the K of glycine + pyridoxal is $10.2 \div 0.26 = 39$.

The effect of amine structure on pK_{3RP} of the imine was investigated briefly. For threonine, glutamic acid and α-amino-n-butyric acid, as well as for glycine and valine, values of 10.2–10.5 were estimated. For α-aminoisobutyric acid, pK_{3RP} was estimated as 11.0, the increase over that for other amino acids paralleling the increase in pK_{2R} of the amino group from 9.6[11] to 10.0.[17] In contrast, the pK_{3RP} values for the butylamines are estimated as only 10.5–10.8 even though the pK's of the amines are higher (about 10.4) than that of aminoisobutyric acid.

Significance in Catalysis by Pyridoxal.—This study shows conclusively that significant concentrations of pyridoxal (or pyridoxal phosphate) imines can be formed under physiological conditions and in non-enzymic "model" reactions.[2,18] In non-enzymic transamination,[18] valine and isoleucine react very slowly despite the high stability of the imines. Thus, the extent of imine formation is not the most important factor in determining the rate of non-enzymic transamination. The rates of imine formation and breakdown appear to be rapid; hence, the rate-limiting step in non-enzymic transamination and other "model" reactions must be the tautomeric rearrangement of the imine. Perhaps one of the most important functions of pyridoxal phosphate-containing enzymes is to hold suitable acidic and basic groups of the protein in the proper orientation to promote the rearrangements of such imine intermediates.

Acknowledgments.—The author is very grateful to Mrs. Marcella Vermeersch who performed most of the experiments reported here, to Mr. Henry Binner for some preliminary experiments,[19] to Mr. Edward Buchanan for the preparation of the sodium-free triethanolamine and tetramethylammonium hydroxide, and to Dr. William F. Harrington for criticizing the manuscript.

Experimental

Apparatus and Materials.—A Beckman model DU spectrophotometer with cell holder thermostated at 25° and a Beckman model G pH meter were employed. A Beckman type E high pH electrode was used above pH 9.

Pyridoxal hydrochloride was obtained from Nutritional Biochemicals Co.; DL-valine from the Dow Chemical Co. or The California Foundation for Biochemical Research. (Identical results were obtained from the two valine samples.) Other amino acids were commercial products. Amines were used as the hydrochlorides, prepared when necessary from the amines. The hydrochlorides were recrystallized; the chloride contents (checked by titration) were correct in all cases. Triethanolamine hydrochloride for buffers was prepared in a similar fashion. Pure standard 1 N triethanolamine and tetramethylammonium hydroxide were generated by passage of 0.2 M solutions of the hydrochloride and bromide, respectively, through a bed of Amberlite IRA-410 resin in the hydroxide form, followed by concentration *in vacuo* to the appropriate volume and storage in polyethylene containers.

Procedure.—Samples were usually 2×10^{-4} M in pyridoxal and of varying amine concentrations. Appropriate buffers employed for the various pH ranges were as follows: at pH 2.2 the amino acid itself, 4–6 acetate, 6–7 phosphate or cacodylate, 7–8 triethanolamine hydrochloride + sodium hydroxide, 8–11, the amino acid itself, or bicarbonate, 11–12 sodium hydroxide. Most solutions were of ionic strength 0.1 *assuming that dipolar ions do not contribute* to the ionic strength. A few solutions were of ionic strength 0.5 or greater. Sodium chloride was added to maintain the desired ionic strength when necessary. Several sodium ion-free solutions were prepared using pure triethanolamine or tetramethylammonium hydroxide in place of sodium hydroxide in the buffer preparations. Solutions were read in the spectrophotometer after standing at room temperature at least 10 minutes but no longer than a few hours. It is unlikely that a significant amount of transamination or other pyridoxal-catalyzed reactions of the amino acids occurred during this time. Spectra were read against appropriate amino acid-containing blanks. The pH values of all samples were measured after reading the spectra.

Calculations.—All spectrophotometer readings were first converted to molar absorbancy indices, a_M, on the basis of the total pyridoxal content of the solution.

To compute K_{pH}, the a_M values of pyridoxal in an amino acid solution of r moles per liter ($r >>$ the pyridoxal concentration) were measured at several wave lengths (usually 280, 302, 370 and 414 mμ). From the known pK values and spectra of the various ionic forms of pyridoxal[7] (Table II) the a_M values of pyridoxal at the desired wave lengths and pH values were calculated. Next, the a_M values of the two ionic species of the imine (or imine + aminoacetal) were obtained. The a_M values of pyridoxal in 0.5 M valine were measured over a range of pH and plotted. The limiting values at high pH were taken as those of the di-anion of the valine imine. The mono-anion spectrum was obtained at pH 8.36. Some free pyridoxal (5% or less) is probably still present even in 0.5 M valine, but the resulting small error has been neglected. The a_M's for isobutylimine were likewise obtained from samples 0.5 M in amine at pH 8.1 and 11.6. The values from the latter solution were corrected for the proximity to pK_{3RP} (assumed to be 10.5).

TABLE II

SELECTED MOLAR ABSORBANCY INDICES USED IN CALCULATIONS

Values given are for $a_M \times 10^{-3}$

Compound	Wave lengths 280 mμ	302 mμ	370 mμ	414 mμ
Pyridoxal, dipolar ion	1.35	5.72	0.10	0.06
anion	2.25	5.72	1.47	1.45
di-anion	3.65	6.39	0.92	0.90
Pyridoxal–valine imine				
mono-anion	7.23	2.00	2.75	6.24
di-anion	1.91	1.42	7.88	0.68
Pyridoxal–glycine imine				
mono-anion	6.00	1.75	2.70	5.70
di-anion	1.40	2.15	5.90	0.35
Pyridoxal–isobutylimine				
mono-anion	6.37	0.83	3.24	6.80
di-anion	0.98	1.30	7.12	0.32

In the case of glycine, because of the low K, it was not possible to convert the pyridoxal completely to the imine at reasonable glycine concentrations. Hence, a series of successive approximations were used to deduce the spectra shown in Fig. 4 as follows: a_M values were first assumed to be the same as for valine. Using these values, K was estimated from data for 0.1 M glycine solutions and was used to predict the extent of reaction in concentrated glycine solutions. By subtracting off the absorption of the unreacted pyridoxal in such solutions, better estimates of the a_M values were obtained; these were used to re-compute K, etc.

For other imines, the spectra were assumed to be identical with those of the reference compounds, valine imine (amino acids) or isobutylimine (other amines), when the reference spectra were shifted along the wave length axis in accord with the small variations in the positions of the high wave length absorption maximum. (From 358 to 370 mμ at high pH and 402 to 414 mμ at low pH.)

Between pH 8 and 12, the a_M values of the imine are intermediate between those of the two single forms and were

(17) Estimated from the thermodynamic pK of 10.21, reference 11, p. 80.

(18) D. E. Metzler and E. E. Snell, THIS JOURNAL, **74**, 979 (1952).

(19) H. O. P. Binner, M.S. thesis, Iowa State College, 1954.

calculated using the measured or estimated pK_{3RP} values for the imines.

The extent of reaction $\beta = a_M$ (sample) $- a_M$ (pyridoxal)/ a_M (imine) $- a_M$ (pyridoxal) was then computed and K_{pH} calculated as $K_{pH} = 1/r(\beta/1 - \beta)$. K_{pH} was nearly always calculated at 3 or more wave lengths and agreement was usually within 10% or less.

The pK_{3RP} for the valine imine was calculated from the change of the imine spectrum with pH in the manner previously described for pyridoxal.[7] A series of nine solutions 0.5 M in valine of pH 8.3 to 12 and ionic strength 0.5 was employed. pK_{3RP} for the glycine imine was estimated in a similar fashion while those for other imines were inferred by a comparison of K with K_{pH} at a pH of about 8 and calculation using eq. 3.

A few of the data used in the calculations are given in Table II.

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Potential Purine Antagonists. IV. Synthesis of Some 9-Methyl-6-substituted-purines[1]

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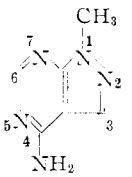
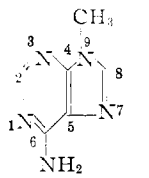
A new method has been developed for the synthesis of various 9-methyl-6-substituted-purines. 4,6-Dichloro-5-nitropyrimidine (I) has been treated wth methylamine to give 6-chloro-4-methylamino-5-nitropyrimidine (V). Reduction of V gave a new route to 5-amino-6-chloro-4-methylaminopyrimidine (VI). Cyclization of VI with ethyl orthoformate and acetic anhydride resulted in the synthesis of 9-methyl-6-chloropurine (VII). Various 9-methyl-6-substituted-purines have been prepared from VII.

In the general program of synthesis of various potential antagonists of the natural purines, it was discovered that 1-methyl-4-aminopyrazolo[3,4-d]-pyrimidine possessed anti-tumor activity[2] against certain animal tumors. It thus seemed desirable to prepare the corresponding purine analog, 9-methyl-6-aminopurine (9-methyladenine) in sufficient quantity for animal testing.



1-Methyl-4-aminopyrazolo-[3,4-d]-pyrimidine



9-Methyladenine

9-Methyladenine previously has been synthesized by several different synthetic routes.[3-7] However, various difficulties involved in these procedures led us to investigate a simplified general method for the synthesis of 9-methyl-6-amino- and 9-methyl-6-substituted-aminopurines.

After the present work was complete, a new synthesis of 9-methyladenine was reported by Daly and Christensen[8] from 4,5-diamino-6-methylaminopyrimidine sulfate and boiling formamide.

In the present investigation treatment of 4,6-dichloro-5-nitropyrimidine[9] (I) with an aqueous solution of methylamine neutralized with acetic acid gave 6-chloro-4-methylamino-5-nitropyrimidine (V) in good yield. A similar modification was employed by Rose[10] for the synthesis of 4-chloro-6-dimethylamino-5-nitropyrimidine. It is interesting to note in this connection that Brown[11] was unsuccessful in an earlier attempt to synthesize 6-chloro-4-methylamino-5-nitropyrimidine (V) by treatment of 4,6-dichloro-5-nitropyrimidine (I) with an alcoholic solution of methylamine. The reduction of V with zinc dust in dilute acetic acid gave 5-amino-6-chloro-4-methylaminopyrimidine (VI). This latter compound has recently been obtained by Brown[11] by another route.

When 5-amino-6-chloro-4-methylaminopyrimidine (VI) was refluxed with formic acid, cyclization took place to give 9-methylhypoxanthine (X) in good yield. The loss of a chlorine atom of various chloro-substituted-4,5-diaminopyrimidines upon formylation and cyclization with formic acid[12] and formamide[13] has been reported previously; thus this behavior is not unexpected.

It was also discovered that when 5-amino-6-chloro-4-methylaminopyrimidine (VI) was heated with ethyl orthoformate and acetic anhydride, cyclization took place without the loss of the chlorine atom to give 9-methyl-6-chloropurine (VII).

Richter and Taylor[14] have reported the use of ethyl orthoformate and acetic anhydride in a new synthesis of hypoxanthine. Montgomery[15] has recently reported the synthesis of 2-chloropurine, 6-chloropurine and 2,6-dichloropurine by cyclization of the appropriate chloro-4,5-diaminopyrimidines under similar conditions.

Treatment of 9-methyl-6-chloropurine (VII) with alcoholic ammonia in a bomb at 150° gave 9-

(1) This investigation was supported in part by research grant C-2845 from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

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