for a (hypothetical) acid-catalyzed tetrahedral intermediate and the corresponding base-catalyzed tetrahedral intermediate, the difference in rates for the two types of catalysis can be expressed in terms of their activation energies. It is well known that the rates of acid-catalyzed ester hydrolysis are much slower than saponification (for ethyl benzoate, under conditions suitable for the bimolecular mechanism, $k_{base}/k_{acid} = 2{,}640$[19]). This reflects the nucleophilicity of the hydroxyl ion with respect to the carboalkoxyl group as compared to that of water toward a protonated group. As a result, considerably more energy is required to affect a bimolecular reaction in the acid-catalyzed case, with the result that the probability of a tetrahedral intermediate will be very small. Because the species involved in the rate-determining step of either the uni- or bimolecular mechanism is the same, *i.e.*, the conjugate acid of the ester, that path will be chosen that offers least resistance to reaction, both energetically and sterically, namely, the unimolecular heterolysis to give an acylium ion. Indeed, it is quite probable that in the base-catalyzed hydrolysis of methyl mesitoate a unimolecular path would also be most preferable, but in the latter case, the solvent does not possess sufficient solvating power for such an ionization, nor is there enough driving force for the heterolysis, which is provided by protonation of the carboalkoxyl group in acid catalysis.

The argument of preferential cleavage at the methoxonium site of the (hypothetical) tetrahedral intermediate is not valid if based on steric effects. The methoxyl group, being larger than the hydroxyl, might be ejected more easily by the crowding of the two methyl groups, a manifestation of "B" strain. As such, exchange would not be observed. But a similar criterion then would have to be applied in the base-catalyzed case of the ester (disregarding the effect of the proton), and has been shown to be invalid. Secondly, on the basis of polar effects, the hydroxyl group would be preferentially protonated, leading to exchange as observed in the case of acid-catalyzed hydrolysis of ethyl benzoate.[20]

The concept of the SN2 reaction must be considered but, again, a similar reaction would be predicted for basic catalysis; this is not found. Nucleophilic attack by methoxide ion at the methyl group of the carbomethoxyl linkage, displacing carboxylate ion, has been observed, but the analogous acid-catalyzed reaction ($A_{Al}2$ in Ingold's terminology) has not yet been found.[21]

The absence of oxygen exchange at the highest acidity is not unexpected. The same observation in the lower acid region, down to 3.09 *M*, is more significant, and serves to corroborate the argument and conclusions presented for the case of acylium-ion formation. The bimolecular path should become of increasing importance as more water is made available for reaction. In the present case, the concentration of water is considerably increased as the acidity is lowered. Further, the higher temperature used at lower acidities should also favor a bimolecular reaction. Yet a combination of these two factors still does not suffice to promote the incursion of another reaction path. The formation of the acylium ion is apparently so much more favored, that it dominates the reaction completely, even at the acid concentration where the bimolecular path would be most competitive.

The results of this study support the previous kinetic evidence for the acylium-ion mechanism of the hydrolysis of methyl mesitoate in strong acid. More important, the absence of oxygen exchange confirms the prediction made for the application of exchange criteria to acylium ion formation and shows that the mesitoate hydrolysis is to be considered to follow a single mechanistic pathway, due to the particular structural and solvent effects found in this reaction.

Acknowledgment.—The authors wish to thank Mr. Ronald Bakule for carrying out preliminary experiments.

(19) Ph.D. Thesis of R. D. Ginger, Illinois Institute of Technology, 1958, p. 105.

(20) M. L. Bender, THIS JOURNAL, **73**, 1626 (1951).

(21) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 754.

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Metal Chelates of Imines Derived from Pyridoxal and Amino Acids[1]

BY LEODIS DAVIS, FRANCES RODDY AND DAVID E. METZLER

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The formation of metal chelates of imines derived from pyridoxal plus alpha amino acids in aqueous solutions has been studied. It is shown that with Zn(II), Cu(II) and Ni(II) the principal chelates formed contain the metal, pyridoxal and amino acid in a 1:1:1 ratio and accept one proton at lower *p*H values. Formation constants for several of the chelates have been evaluated, as have the dissociation constants of the conjugate acids of the chelates. The relative stabilities of these and various other chelates are compared. The zinc pyridoxylidene valine chelate is extremely sensitive to light, being converted rapidly in laboratory light into an unidentified compound or compounds with a characteristic ultraviolet absorption spectrum.

The aldehyde form of vitamin B_6, pyridoxal, I, is believed to function in biological catalysis by forming imines (Schiff bases, pyridoxylidene amino acids), II, with substrate amino acids at the active centers of appropriate enzymes. Subsequent re-

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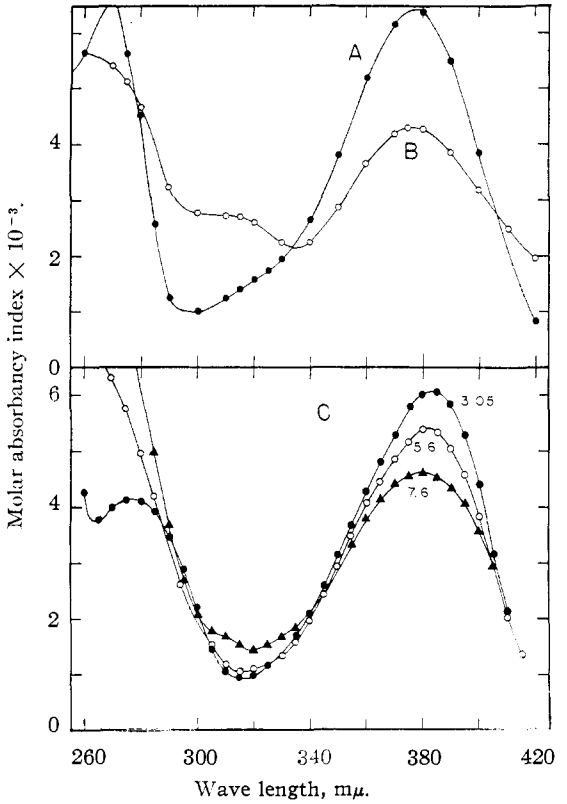
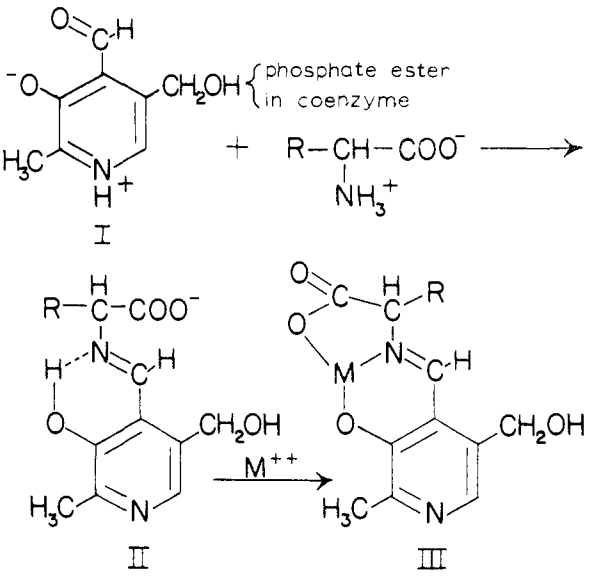


Fig. 1.—Spectra of pyridoxal-amino acid-metal solutions: curve A, 10^{-4} M pyridoxal, 0.01 M valine and 0.002 M zinc perchlorate, pH 8.07; curve B, like A, but with 0.002 M $MnCl_2$, pH 8.40; curves C, spectra of solution of solid copper pyridoxylidene valine adjusted to the indicated pH values. Added copper di-valine was present at a concentration of 4×10^{-4} M.

actions of the imines lead to the various products of enzyme action.[2,3] Pyridoxal itself catalyzes many reactions of amino acids non-enzymically. For these non-enzymic reactions salts of metals such as Cu(II), Zn(II), Al(III)[3,4] and vanadium (V)[5] are powerful catalysts. Ions of these and other metals appear to form chelate complexes such as III with the imines. Such complexes probably are more reactive than the free imines, and it is possible that such chelates are intermediates in the action of some pyridoxal phosphate-dependent enzymes.

Several crystalline chelates have been isolated,[6-8] others have been separated by chromatography or electrophoresis[9] and some studies of the chelates in solution using the method of continuous variation have been made.[9,10] Matsuo[11]



described the ultraviolet absorption spectra of pyridoxal phosphate–amino acid–metal salt solutions. However, the chemistry of such solutions is exceedingly complex and little quantitative information is available. Christensen[12] has described the very stable copper pyridoxylidene valine complex and has measured its formation constant. This chelate, probably of structure III, accepts a proton on the ring nitrogen with an apparent pK_a of 5.6.

This paper deals principally with the nature of zinc, nickel and copper complexes of pyridoxylidene valine in aqueous solutions. Formation constants for the complexes have been measured.

Spectrophotometric Evidence for Imine Chelate Formation.—When pyridoxal and an amino acid are mixed together in neutral aqueous solution, extensive imine formation occurs as shown by a yellowing of the solution and development of an absorption band with a maximum at a wave length of 400–420 mμ.[13] When a zinc salt is added to such a solution, the yellow color fades, an intense blue-green fluorescence develops and the absorption maximum shifts downward to about 380 mμ (Fig. 1, curve A). Similar changes, but with less development of fluorescence, are observed with salts of other metals such as copper(II), nickel, cobalt, vanadium, aluminum and manganese (Fig. 1, curve B and C). The 380 mμ absorption band evidently represents one or more species of chelate related to structure III. The copper pyridoxylidene valine chelate can be prepared in solid form. When this solid is dissolved in the presence of a suitable concentration of copper valinate, almost no dissociation of the imine chelate occurs.[12] Under these conditions, the absorption spectrum of the copper pyridoxylidene valine chelate can be measured directly (Fig. 1, curves C). With other metals such as zinc and manganese we have been unable to find conditions under which complete chelate formation can be maintained with no free pyridoxal remaining. This free pyridoxal con-

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

(3) D. E. Metzler, M. Ikawa and E. E. Snell, THIS JOURNAL, **76** 648 (1954).

(4) J. B. Longenecker and E. E. Snell, *ibid.*, **79**, 142 (1957).

(5) F. Bergel, R. C. Bray and K. R. Harrap, *Nature*, **181**, 1654 (1958).

(6) G. Baddiley, *ibid.*, **170**, 711 (1952).

(7) D. E. Metzler, J. B. Longenecker and E. E. Snell, THIS JOURNAL, **76**, 639 (1954).

(8) H. N. Christensen, *ibid.*, **79**, 4073 (1957).

(9) P. Fasella, H. Lis, N. Siliprandi and C. Baglioni, *Biochim. Biophys. Acta*, **28**, 417 (1957); *J. Inorg. Nuclear Chem.*, **8**, 620 (1959).

(10) G. L. Eichhorn and J. W. Dawes, THIS JOURNAL, **76**, 5663 (1954).

(11) Y. Matsuo, *ibid.*, **79**, 2011 (1957).

(12) H. N. Christensen, *ibid.*, **80**, 2305 (1958).

(13) D. E. Metzler, *ibid.*, **79**, 485 (1957).

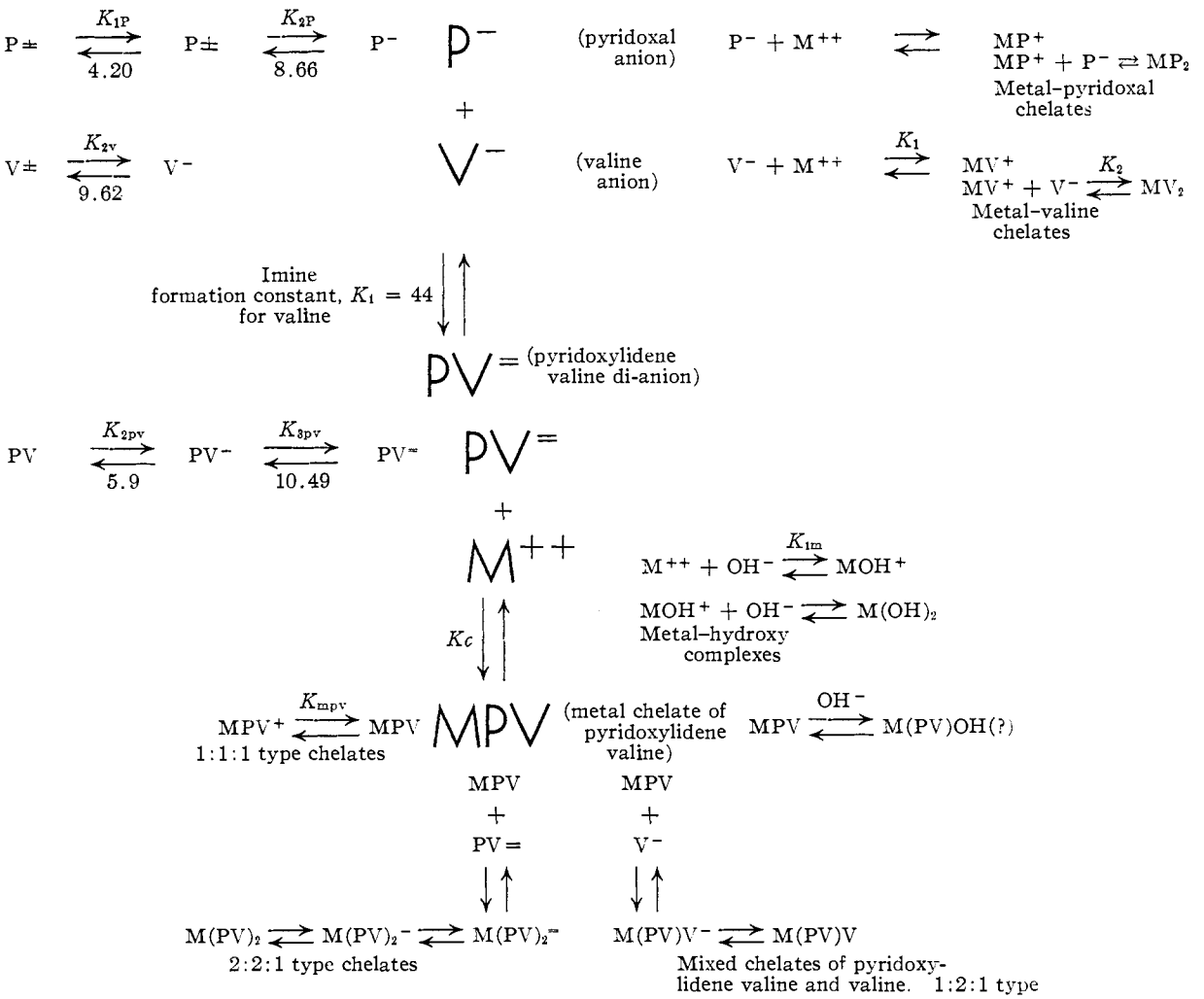


Fig. 2.—Equilibria in mixtures of pyridoxal with valine and metal salts. The abbreviations used are P^+, $P^{\pm}$ and P^- for the pyridoxal cation, dipolar ion and anion, respectively; $V^{\pm}$ and V^- for the valine dipolar ion and anion; $PV^{\pm}$, PV^- and $PV^{=}$ for the neutral, mono-anionic and di-anionic forms of pyridoxylidene valine; M^{++} for a divalent metal ion; MP^+, MP_2, MV^+ and MV_2 for metal chelates of pyridoxal and of valine; MPV for the 1:1:1 metal-pyridoxylidene valine chelate and MPV^+ for the protonated form of the same; $M(PV)_2$, M(PV)V, etc., for other possible chelate types. The symbols K_{1P}, K_{2P}, etc., above the arrows represent the dissociation constants, as acids, of the compounds to the left of the arrows. The numerical values beneath some of the arrows are pK values corresponding to these same dissociation constants.

tributes to the absorption in the 317 mμ region (Fig. 1, Curves A and B). Some free imine absorption above 400 mμ is also detectable in the manganese-containing solution (B).

Evaluation of Equilibrium Constants.—Spectrophotometric and *p*H measurements have been used to evaluate the formation constants of the imine chelates and their dissociation constants as acids. (Note that these and all other equilibrium constants used in this paper are apparent constants expressed in terms of concentrations rather than activities and with *p*H assumed to equal $-\log [H^+]$. They have been measured at ionic strength 0.1 whenever possible.) Valine has been used in most experiments because of its high imine formation constant[13] and its low reactivity in non-enzymic transamination.

The chemical reactions under consideration are summarized in Fig. 2. In addition to the reaction between the imine dianion, $PV^=$ and metal ion, all of the other reactions shown in Fig. 2 must be considered as of possible significance in the *p*H range 4.5–9 and may affect the evaluation of the desired formation constant. We have attempted to take into account all of these secondary equilibria, as indicated in the subsequent discussion and in detail in the Experimental section.

While the formation of metal chelates of pyridoxylidene valine absorbing at about 380 mμ can be demonstrated readily, it is by no means as easy to establish the exact nature of these chelates. Do they contain one or two imine groups? Or one imine and one valine? In what states of ionization do the imine groups occur? We have attempted to answer these questions in the following way. Under conditions of low free pyridoxylidene valine, PV^-, and high metal concentration, we will expect to have very little of complexes containing

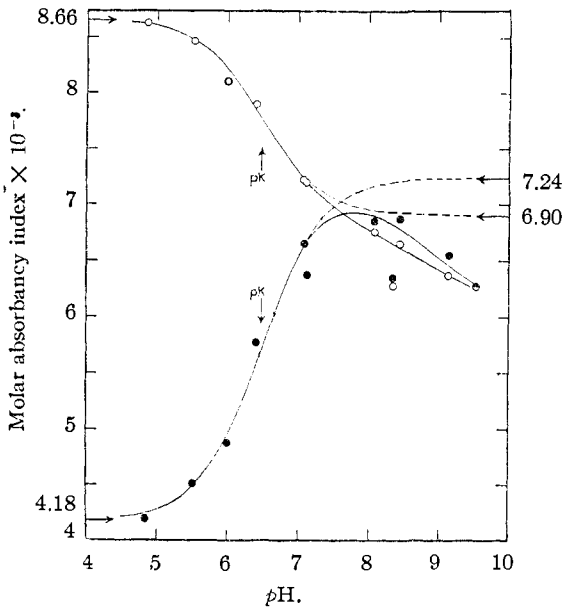


Fig. 3.—Molar absorbancy indices for zinc pyridoxylidene valine *versus* *p*H. The solid lines are of the theoretical shape for a monoprotic acid of *pK* 6.5, as indicated by the vertical arrows.

two or more PV^- ligands per metal. Aside from some possible interferences from mixed chelates of pyridoxylidene valine and valine, we can therefore study the formation of the 1:1:1 species only.

We have first attempted to deduce the spectra of the 1:1:1 chelated forms at different *p*H values. In some cases this included an evaluation (from spectral changes) of the dissociation constant of a proton from the ring nitrogen, K_{MPV} (see Fig. 2).

Having established the spectra of the chelate and of free pyridoxal we could then estimate the amounts of both present in any solution relying primarily on measurements at 315 mμ (high absorption for pyridoxal) and 380 mμ (high absorption for chelate). In many solutions some free pyridoxylidene valine absorbing maximally at about 420 mμ was present and had to be taken into account (Experimental). From the total metal concentration and chelate formation constants for metal-amino acid complexes, we could compute the free metal ion concentration. The free amino acid concentration, usually in large excess, was easily estimated, and known equilibrium constants[13] then permitted calculation of the free pyridoxylidene valine dianion, PV^-, concentration. From these calculated concentrations the *p*H dependent stability constant then was computed.

$$K = [\text{imine chelate, total}]/[PV^-][M^{++}]$$

The *p*H dependence of K permitted calculation of K_c, the formation constant of the unprotonated chelate, and gave an independent measurement of K_{MPV}. We expected that the dependence of K on valine and pyridoxal, or lack thereof, might give us information about formation of 2:2:1 or 1:2:1 chelate types (see Fig. 2).

Zinc Pyridoxylidene Valine Chelates.—Under conditions of maximum chelate formation, a very small band remains in the 315 mμ region of the absorption spectrum (Fig. 1, curve A). By assuming that this absorption represented free pyridoxal we were able to correct the spectra for free pyridoxal and unchelated imine (details of the correction procedure used are given in the Experimental section). The resultant spectrum of the zinc–pyridoxal–valine chelate at *p*H 8 consists of two strong bands with peaks of nearly equal absorbancy at approximately 268 and 377 mμ. At *p*H 5 the absorbancy at 377 mμ is increased with a peak shift of 1–2 mμ to a higher wave length while the lower wave length peak is lowered and shifted to about 277 mμ. The fluorescence of the compound is also diminished markedly at lower *p*H. A plot of absorbancy *versus* *p*H at 270 and 380 mμ (Fig. 3) shows that these changes are centered around a *p*H of about 6.5 and can most simply be interpreted as resulting from an uptake of a single proton on a pyridine nitrogen of the chelate. The fit of the experimental points to the theoretical curves in Fig. 3 is rather poor at higher *p*H values, partly because the accurate estimation of the free pyridoxal became very difficult in this region. At present we are not able to explain the decreases in the absorbancy of the chelate at 270 and 380 mμ above *p*H 8. However, when the ratio of the absorbancy at 270 mμ to that at 380 mμ is plotted against *p*H, a smooth curve (not shown) of the theoretical shape for dissociation of a monoprotic acid of *pK* 6.5 is obtained.

Using the absorbancy data of Fig. 3 (the solid curves) for the spectrum of the zinc chelate we estimated the amounts of zinc chelate present in solutions in which between 10 and 80% conversion to chelate had occurred. From these results a formation constant K was computed (see Experimental) for the reaction Zn^{++} + pyridoxylidene valine dianion, $PV^- \rightarrow$ 1:1:1 chelate (all ionic forms). A plot of the resulting values of log K *versus* *p*H (Fig. 4) is linear with a slope of approximately one below *p*H 6 and with a slope of zero above *p*H 7. This indicates clearly that a single proton is taken up by the chelate with *pK* of 6.5 and that the form of chelate above *p*H 7 is neutral (the possible existence of anionic, hydroxylated complexes above *p*H 9 is not excluded). Since the values of K are independent of valine, zinc or pyridoxal concentrations within the limits tested, a 1:1:1 complex is indicated. The value of K above *p*H 8 is constant and equal to K_c. The distinct upward trend of points above *p*H 8 could not be associated with any definite chemical change and may simply be the result of an accumulation of errors affecting the calculations at high *p*H.

As reported by Christensen,[7] a light yellow solid zinc pyridoxylidene valine compound precipitates readily from certain concentrated aqueous solutions; this was shown by analysis to be a 2:2:1 complex of the type $M(PV)_2$. We have confirmed these findings. It is interesting that the solid chelate which has been isolated is of such a radically different composition from the form which predominates in solution. When dissolved in water, this solid quickly breaks down to give the expected equilibrium distribution of free pyridoxal and 1:1:1

chelate. In methanolic solution the chelate is more stable. A freshly prepared solution has a spectrum closely resembling that of the 1:1:1 chelate but with a lower absorption at 385 mμ ($a_M/2 = 4.0 \times 10^3$) and an enhanced absorption at 330 mμ ($a_M/2 = 1.9 \times 10^3$). Addition of base to the methanolic solution has little effect on the 380 mμ peak but causes a shift of the 270 mμ peak.

An unexpected finding was that the zinc chelate which forms in aqueous solutions (but not the copper and nickel chelates) is exceedingly photolabile. Irradiation of a chelate-containing solution at *p*H 8 by placing in a 1 cm. quartz cuvette inside a north window of the laboratory led to a 50% decrease in the 377 mμ absorption in 2.5 hr. The irradiation product absorbs light only one-tenth as strongly as does the chelate at 377 mμ but absorbs maximally at 330 mμ ($a_M = 8.6 \times 10^3$).

Other amino acids tested form chelates which are apparently very similar to those formed with valine. By assuming that the spectra and dissociation constants K_{MPV} of the zinc chelates with various amino acids are identical, we readily could estimate values of K_c for imines of various amino acids (Table I).

TABLE I

FORMATION CONSTANTS, K_c, OF METAL COMPLEXES OF IMINES OF PYRIDOXAL AND AMINO ACIDS AND OTHER LIGANDS

Constants are for the reaction of the di-anion of the imine with the metal ion to form a complex containing a 1:1:1 ratio of metal, pyridoxal and amino acid.

Metal ion	Ligand Imines derived from	log K_c	log K'_c[d]
H^+	Valine	10.5[a]	
Cu^{++}	Valine	14.5	
	Glycine	15.0	
Ni^{++}	Valine	10.8	
Zn^{++}	Valine	7.94	9.58
	Other amino acids[b]	7.4–8.1	
	Isobutylamine	<5.2	
	Leucinamide	...	*ca.* 7.4
Mn^{++}	Valine	5.0	
Mg^{++}	Valine	<3.5	
	Other Ligands		
Zn^{++}	Pyridoxamine	5.7[c]	
	Valine (free anion)	4.5[d]	
Ni^{++}	Pyridoxal	*ca.* 2.0	

[a] Reference in footnote 16. [b] Glycine, alanine, threonine, leucine, isoleucine, glutamic acid. [c] R. L. Gustafson and A. E. Martell, *Arch. Biochem. Biophys.*, 68, 485 (1957). [d] Reference in footnote 17. [e] K_c'(valine) = [MPV]/[M^{++}][P^-][V^-] = K_cK_i.

Copper Chelates.—The spectrum of the very stable 1:1:1 copper pyridoxylidene valine chelate was measured *versus* *p*H in the presence of excess copper di-valine to prevent dissociation.[12] The change in spectrum with *p*H (Fig. 1C) is very similar to that observed with the zinc chelate (Fig. 3). Spectrophotometric titration yields a value for pK_{MPV} of 5.6 in agreement with that reported by Christensen.[12] The corresponding pK for copper pyridoxylidene glycine is 6.05. We also have isolated the hydrochloride of copper pyridoxylidene valine in crystalline form (Experimental). We have confirmed Christensen's reports that the neutral chelate can be titrated with acid but that upon back titration with base more than one equivalent of base is taken up gradually over a broad *p*H range. We suggest that this additional base uptake is a reflection of the chemical reactivity of the chelate molecule and that under the transient highly basic conditions at the points where the base enters the solution during titration, base is being taken up in irreversible reactions.

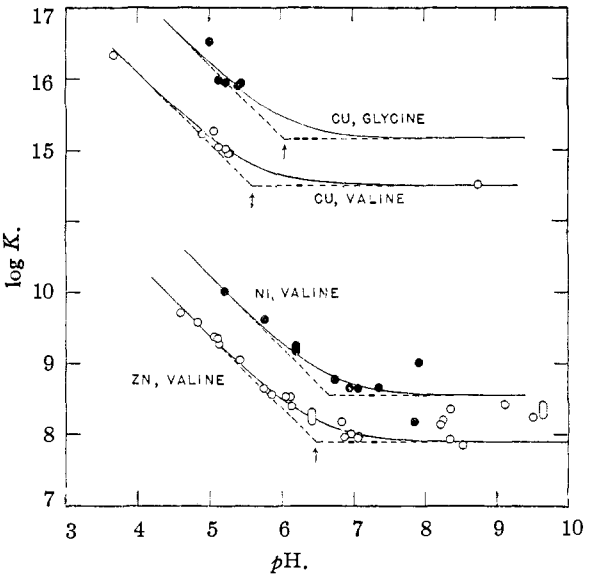


Fig. 4.—Apparent formation constants for 1:1:1 metal chelates of pyridoxylidene valine *versus* *p*H. The solid lines are theoretical curves based on the assumption that the chelate accepts a single proton with the pK indicated by the vertical arrows. These pK values were independently obtained, except in the case of the nickel–valine system.

Using solutions of pyridoxal, valine and copper valinate we have evaluated the formation constant K as a function of *p*H for the copper chelate (Fig. 4). The value of log K_c = 14.5 compares favorably with the value 14.4 obtained by Christensen.[12] Data for the glycine-containing chelate were obtained similarly (Fig. 4, Table I).

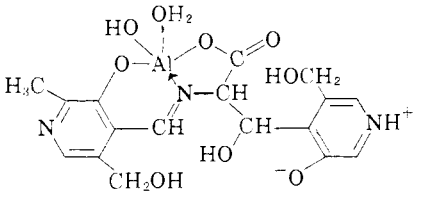
In addition to the readily soluble copper pyridoxylidene valine prepared from alcohol solution,[8,14] also we have obtained, using DL-valine, a less soluble form of the compound, apparently a hydrate, which crystallizes from aqueous solutions.

Nickel Chelates.—Although the nickel chelate is less stable than the copper, we were able to find conditions over a wide range of *p*H under which nearly complete conversion to the imine chelates occurs. The absorption spectrum resembles that of the copper chelate but does not change appreciably with *p*H. Consequently, we could not evaluate pK_{MPV} spectrophotometrically. However, the calculated values of K (Fig. 4) depend upon *p*H in such a way as to indicate that pK_{MPV} is about 6.7. The increased scatter of points above *p*H 7 makes it difficult to obtain precise values of pK_{MPV} or of K_c.

Aluminum Chelates.—Strong chelates are formed as indicated by the 380 mμ absorption, but because of the complexity of the hydroxylation equi-

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

libria with aluminum ion, no attempt was made to evaluate equilibrium constants. The isolation of a solid aluminum chelate of the imine of pyridoxal with the amino acid "β-pyridoxyl serine" (formed by condensation of pyridoxal with glycine) has been reported previously, and spectra and dissociation constants have been measured.[7] On the basis of the present work, it seems reasonable to assign structure IV to this complex. The $-O^-$ group shown free may also be coördinated with the metal ion. Both the observed spectra and the



IV

pK values of 5.5 and 7.5 are in accord with this proposal. The lower pK probably is associated with the ring nitrogen which is shown unprotonated in structure IV. The ultraviolet spectrum of the compound at all pH values is qualitatively similar to but not identical with that obtained by adding the spectra of zinc pyridoxylidene valine and of "β-pyridoxylserine," both in the appropriate state of ionization.

Chelates with Other Metals.—Chelate formation with Co, Fe, V and Mn salts can be demonstrated readily, but none is apparent with Mg, Ca or K. Table I includes an estimate of the stability of manganese(II) pyridoxylidene valine based on the assumption that its spectrum is like that of the zinc chelate. Similar considerations enabled us to estimate an upper limit for the stability constant of the magnesium chelate (Table I). No attempt has been made to study the behavior of other common metals.

Discussion

While the present work demonstrates the formation of metal–pyridoxal-amino acid chelates containing the components in a 1:1:1 ratio, no evidence has been obtained for the existence in solution of complexes containing 2 valine or pyridoxal molecules per metal ion. This does not mean that such complexes cannot exist in solution but rather that they are of low stability compared to that of the 1:1:1 complexes. The fact that solid 2:2:1 complexes of zinc and nickel can be isolated suggests that they do exist in solution in low concentrations. Furthermore, the transamination of L-amino acids with retention of configuration reported by Longenecker and Snell[15] suggest that such complexes may be important in catalysis by copper salts. At the high pyridoxal concentrations used in those studies, the free imine concentration should be very high, and 2:2:1 complexes would be favored.

A chelate property of obvious interest is the basicity of the ring nitrogen of the pyridine group. The pK_a of the protonated ring nitrogen is 8.7 in free pyridoxal which bears an ionized $-O^-$ group but falls to near 5.8 in the pyridoxal cation which bears an un-ionized-OH group.[16] In the hydrogen bonded form of the imine this pK is about 5.9 and in the various 1:1:1 chelates investigated it varies from 5.5 to 6.5 (Table II). Christensen[8] reports higher values, up to about 8, obtained by titration for 2:2:1 chelates. It appears likely, as suggested by Christensen, that this pK is dependent on the strength of the metal–oxygen bond. Table II also contains data on the position of the long wave length absorption maximum of the chelates. The positions of the bands in the chelates are closer to that of the imine anion $PV^=$ than to that of the H-bonded form PV^- which contains H^+ in place of the metal ion. It is significant that the spectrum of the 2:2:1 zinc chelate in methanol closely resembles that of the 1:1:1 chelate in water. Thus, the spectra do not suggest any drastic difference in the type of bonding present in the 1:1:1 and 2:2:1 chelates.

TABLE II

PROPERTIES OF PYRIDOXYLIDENE AMINO ACID CHELATES IN SOLUTION

Metal	Amino acid	pK_{MPV}	Absorp. max., long wave length band, mμ
None (free imine anion)	Valine	..	367[a]
H^+	Valine	5.9	414[a]
Cu^{++}	Valine	5.6	379
Cu^{++}	Glycine	6.05	380
Ni^{++}	Valine	6.7	388
Zn^{++}	Valine	6.5	377
Al^{+++}	"Pyridoxylserine"	5.5[b]	377[b]

[a] Reference in footnote 13. [b] Reference in footnote 7.

The order of stability of the chelates, Cu > Ni > Zn > Mn > Mg (Table I) is the same as that observed for other chelate series.[17] Except for the transposition of nickel and zinc in some cases, this is also the order of reactivity of the metals in catalyzing non-enzymic reactions of amino acids in the presence of pyridoxal.[4]

The chelate formation constants of various imines and related compounds with a single metal are also compared in Table I. The formation constants for imines of a variety of amino acids differ very little. On the other hand the imine of isobutylamine, which forms readily, does not chelate zinc to a detectable extent. The very high stability of the chelates obtained with imines of amino acids appears to reflect the tridentate nature of the ligand. The formation constants are also compared with the much lower constants for bidentate chelates of pyridoxamine and free valine.

Measurements on the zinc–pyridoxal leucinamide system were complicated by the unexpected spectral behavior of solutions of pyridoxal and leucinamide.[18] For this reason, the imine formation constant could not be evaluated with certainty. Nevertheless, addition of zinc leads to a typical Schiff base chelate

(15) J. B. Longenecker and E. E. Snell, *Proc. Natl. Acad. Sci.*, **42**, 221 (1956).

(16) D. E. Metzler and E. E. Snell, THIS JOURNAL, **77**, 2431 (1955).

(17) J. Bjerrum, G. Schwarzenbach and L. G. Sillen, "Stability Constants," Part I, Special Publication No. 6 of the Chemical Society of London, 1957.

(18) H. N. Christensen, THIS JOURNAL, **80**, 99 (1958).

spectrum, and we conclude that the stabilization through formation of a second chelate ring may be accomplished through the $-CONH_2$ group as well as through a carboxylate group. The equilibrium constant for formation of the chelate from free pyridoxal and leucinamide anions, K_c', was estimated and is compared with the comparable value for valine in Table I. The lower value for leucinamide parallels the decreased basicity of the amino group in the amide.

Experimental

Apparatus and Chemicals.—Beckman Model DU and Cary Model 14 spectrophotometers were employed. For stability constant measurements the cell compartments were thermostatted at 25°. *p*H measurements were made with a Beckman Model G *p*H meter using "general purpose" glass electrodes.

Pyridoxal hydrochloride was used as obtained from Nutritional Biochemicals Co. and from Merck and Co. (donated by Dr. Karl Folkers). Concentrations of stock solutions were routinely checked spectrophotometrically in 0.1 *N* HCl. (Molar absorbancy index, a_M at 288 mμ = 8.75×10^3 at 2×10^{-4} *M*.)

Valine was obtained from the Dow Chemical Co. and from the California Corporation for Biochemical Research. Valine from both sources gave identical results. Other amino acids were also from commercial sources.

Standard carbonate-free KOH was prepared by an ion exchange method using Amberlite IR-410 in the hydroxyl form.[19]

Copper pyridoxylidene valine and copper pyridoxylidene glycine were prepared according to the procedure of Christensen and Collins.[14]

Copper Pyridoxylidene Valine Dihydrate.—To one mmole of DL-valine, dissolved in 2 meq. of aqueous NaOH, was added 1 mmole of pyridoxal HCl to form the sodium salt of the imine. With the addition of one mmole of copper acetate the solution was filled immediately with a very fine light green crystalline product. After standing overnight, the crystals were collected, washed with cold 95% ethanol and dried in a desiccator over KOH. Calcd. for $C_{13}H_{16}N_2O_4$-$Cu \cdot 2H_2O$: Cu, 17.5. Found: Cu, 17.0.

Copper Pyridoxylidene Valine Hydrochloride.—An aqueous solution containing one mmole of pyridoxal·HCl in a minimal amount of water was added to a suspension of 1 mmole of copper valinate in 50 ml. of H_2O. The suspension was shaken for a few minutes until all the copper valinate dissolved or reacted. The greenish blue solution then was taken nearly to dryness on a rotary concentrator, taken up in absolute alcohol and placed in a refrigerator (5°) overnight. The next day the product was filtered off and dried. Calcd. for $(C_{13}H_{17}N_2O_4Cu)Cl$: C, 42.8; H, 4.7; N, 7.69; Cu, 17.5; Cl, 9.73. Found: C, 44.2; H, 5.10; N, 7.62; Cu, 17.7; Cl, 6.0.

Copper valinate and glycinate were prepared by adding a copper acetate solution (satd.) to a solution (0.5 *M*) of the amino acids, filtering and drying. Calcd. for Cu valinate·H_2O: Cu, 20.2. Found: 20.4. Calcd. for Cu glycinate·$2H_2O$: Cu, 25.8. Found: 26.6.

Standardization of Copper, Nickel and Zinc Perchlorates.—One tenth molar solutions of the metal perchlorates were titrated with a 0.10 *M* solution of disodium ethylenediamine tetraacetic acid (Na_2EDTA) which previously had been standardized against a sample of pure zinc. Erichrome Black T (F241) was used as an indicator[20] for zinc and PAN [1-(2-pyridylazo)-2-naphthol][21] for the copper. The nickel was determined by titrating the free EDTA with a standard copper solution using PAN as an indicator after a measured amount in excess had been added to the nickel solution.[21]

Secondary Equilibria.—In addition to the principal reaction under investigation, the various related equilibria shown in Fig. 2 had to be considered.

The dissociation constants of pyridoxal and the spectra of the various ionic forms are known from previous work.[13,16] At any *p*H we can readily compute the distribution of pyridoxal among its three ionic forms and the spectrum of the equilibrium mixture. Since free pyridoxal absorbs strongly at 315 mμ over most of the *p*H range used, measurements at this wave length were important in establishing the concentration of free pyridoxal in test solutions.

No evidence for formation of metal-pyridoxal chelates MP^+ and MP_2 could be obtained by titration of pyridoxal (0.05 *M*) in the presence of zinc and copper perchlorates (0.01 *M*). The titration curves were identical within experimental error with those of the metal salts alone. Titration of pyridoxal plus nickel perchlorate with base indicated that some complex formation did occur[22] with an estimated log $K_1 = 2.0$. ($K_1 = [PM^+]/[P^-][M^{++}]$) Drifting of the *p*H meter readings indicated metal hydroxide precipitation above $\bar{n}$ values of about 0.3, and the K_1 estimate is based on measurements below this value. Since K_1 is so low, formation of nickel-pyridoxal chelates can be completely neglected in the solutions we have studied. Comparison of relative chelate formation constants of various metals with other ligands[17] indicates that the same is true for the other metals used.

Apparent dissociation constants for amino acid dipolar ions were taken from Cohn and Edsall.[23] The value $pK_{2v} = 9.62$ for valine was used as in previous work.[13] Formation constants for metal-amino acid chelates were determined by the titration method of Bjerrum[22] using the procedure of Irving and Rossotti[24] to calculate the stepwise formation constants K_1 and K_2. Details of these measurements are available elsewhere[25]; the formation constants are summarized in Table III.

TABLE III

APPARENT EQUILIBRIUM CONSTANTS FOR FORMATION OF METAL-AMINO ACID CHELATES, IONIC STRENGTH 0.1[a]

Metal	Amino acid	log K_1	log K_2
Cu(II)	Glycine	8.15	6.93
Cu(II)	Valine	7.81	6.69
Ni(II)	Valine	5.17	3.95
Zn(II)	Valine	4.46	3.92

[a] Metal nitrates were used. Titration was with KOH. The ionic strength was maintained with potassium nitrate. The values obtained are in satisfactory agreement with the somewhat variable figures found in the literature. See reference in footnote 17.

The imine formation constant K_1 and the dissociation constants of the pyridoxylidene valine are known as are the spectra of the various forms of the imine.[13] Since the hydrogen-bonded imine PV^- absorbs strongly at 420 mμ whereas free pyridoxal does not absorb and the chelates absorb only weakly, measurements at this wave length were useful in estimating the imine concentration in reaction-mixtures.

The formation of metal hydroxides and of soluble hydroxy complexes complicates the interpretation of results at high *p*H. We have carefully avoided *p*H values high enough to precipitate metal hydroxides, but soluble complexes may form at lower *p*H values. Titration of $10^{-3}M$ zinc perchlorate with base permitted estimation of an apparent hydrolysis constant, $K = [MOH^+][H^+]/[M^{++}]$. We estimate log K to be -7.8. Corresponding log Kv alues for Cu^{++} and Ni^{++} hydrolysis were taken as -6.5 and -8.9, respectively.[26] Thus, at *p*H 7.8 half of the free zinc should exist as $ZnOH^+$, and at higher *p*H the hydrolyzed forms should increase very rapidly. Nevertheless, because most of the metal is tied up as amino acid complexes of high stability, the free metal concentrations at these higher *p*H values are so exceedingly low that the hydroxylated forms such as $ZnOH^+$ can probably be safely ignored in all of the solutions which we have studied.

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TABLE IV

COMPOSITIONS OF TYPICAL SOLUTIONS USED IN CHELATE FORMATION CONSTANT DETERMINATION

Total pyridoxal = 1×10^{-4} *M* for Zn-containing solutions and 2×10^{-4} *M* for Cu- and Ni-containing samples

Metal used	*p*H	Valine Total	Valine Free	Molar concentrations Metal Total	M^{++}	MV^{+}	MV_2	Fraction of total pyridoxal Imine chelate	Free pyridoxal	Imine, PV^{-}
Zn	4.60	0.10	0.100	0.019	0.0183	0.0005	....	0.19	0.78	0.032
	6.12	.10	.098	.0038	.0018	.0015	0.0004	.60	.36	.045
	6.07	.02	.019	.0038	.0032	.0005	....	.38	.61	.014
	6.12	.02	.017	.019	.0162	.0024	.0001	.77	.23	.005
	7.08	.02	.016	.0047	.0017	.0021	.0008	.87	.11	.015
	8.20	.02	.020	1.9×10^{-4}	0.010×10^{-4}	0.20×10^{-4}	1.35×10^{-4}	.32	.30	.38
	8.24	.001	.00077	1.9×10^{-4}	0.30×10^{-4}	$.27 \times 10^{-4}$	0.69×10^{-4}	.62	.35	.03
	9.63	.005	.0047	1.9×10^{-4}	9.6×10^{-5}	$.030 \times 10^{-4}$	1.30×10^{-4}	.54	.27	.18
Cu	3.68	.05	.049	4×10^{-4}[a]	0.41×10^{-4}	1.54×10^{-4}	0.44×10^{-4}	.81	.19	...
	5.22	.20	.20	4×10^{-4}	1.58×10^{-5}	0.07×10^{-4}	3.12×10^{-4}	.40	.54	.06
	8.77	.30	.30	4×10^{-4}	4.05×10^{-15}	1.41×10^{-5}	3.33×10^{-4}	.38	.0010	.67
Ni	5.21	.20	.019	9.94×10^{-4}	8.7×10^{-4}	0.99×10^{-4}	5×10^{-7}	.12	.87	.01
	6.20	.20	.019	9.94×10^{-4}	4.24×10^{-4}	4.54×10^{-4}	0.29×10^{-4}	.43	.56	.01

[a] Added as Cu valinate.

Calculation of the Spectrum of the Zinc-Pyridoxal–Valine Chelate.—Some free pyridoxal was apparently present in all chelate-containing solutions and led to the appearance of a small absorption peak or shoulder in the 315–320 mμ region. Using the 315 mμ data we estimated the amount of absorption contributed by the pyridoxal. Then using known absorbancy indices of pyridoxal, we subtracted the contribution of the pyridoxal to the absorption at all wave lengths where measurements were made. If the resulting curve was smooth and showed neither an absorption band nor a depression indicating an over-correction for pyridoxal, the pyridoxal estimate was assumed to be correct. Otherwise, other slightly different pyridoxal concentrations were tried until a good fit was obtained. The solutions also contain some free imine PV^{-}. Since this absorbs very much like the chelate in the 315 mμ region, its presence did not interfere in the fitting procedure just described. The amount of imine present was estimated from the free pyridoxal and valine concentrations and the known imine formation constant, and the absorption resulting from the free imine was also subtracted. The remaining absorption was that of the chelate. These calculations were made for eleven solutions ranging in *p*H from 4.85 (0.4 *M* valine, 0.04 *M* Zn, 2×10^{-4} *M* pyridoxal) to 9.5 (0.005 *M* valine, 0.0005 *M* Zn, 2×10^{-4} *M* pyridoxal) for which the free pyridoxal and imine constituted from 5 to 18 and 1 to 10%, respectively, of the total pyridoxal present. The calculated molar absorbancy indices for the zinc chelate are plotted *versus* *p*H in Fig. 3.

Chelate Formation Constants.—Solutions of pyridoxal hydrochloride, amino acid, metal perchlorate and KOH in varying proportions are prepared in 25 ml. volumetric flasks, the metal salt being added last. Blanks without pyridoxal were prepared at the same time. The solutions were allowed to equilibrate at 25° for 1–3 hr. before the *p*H and light absorption measurements were made. In the case of the copper system equilibration was much slower. In this case solutions were usually prepared from crystalline 1:1:1 chelate and valine or from copper divaline and pyridoxal, equilibration then being more rapid. The compositions, including calculated concentrations of various components for a number of typical solutions studied, are given in Table IV.

Procedures for Computing Formation Constants of Imine Chelates.—The over-all formation constant, K = [chelate (all forms)]/[M^{++}][PV^{-}] was calculated as follows: For the selected wave length, *e.g.*, 270, 315 or 380 mμ, the absorbancy index of the pyridoxal was calculated, and that of the chelate was obtained from experimental data, *e.g.*, Fig. 3, at the *p*H of the test solution. From these absorbancy indices and the observed absorbancy of the test solution, a first estimate of the fraction of chelated pyridoxal was computed. This estimate had to be corrected for the presence of free imine, whose concentration was estimated from the approximately known concentrations of free valine and pyridoxal and the known formation constant for the imine. The contribution of the imine to the observed absorbancy was then subtracted, and a new calculation of the fraction of chelated pyridoxal was made. Consistent values were obtained from calculations at the three different wave lengths. For solutions containing a great deal of free imine, *e.g.*, at high *p*H, a different procedure was used. An initial estimate of the free pyridoxal present was made from 315 mμ data. Then, using data at 380 and 420 mμ and two simultaneous linear equations, the fractions of imine and chelate were computed.

The total metal concentration minus the amount in the form of the chelate MPV was now distributed among the forms M^{++}, MV^{+} and MV_2 using known equilibrium constants. Since the free valine concentration had to be known for this calculation, a second or third successive approximation had to be made in some cases. The large excess of valine is most solutions simplified the problem, however.

With the free M^{++}, pyridoxal, valine and chelate concentrations established, it was a simple matter to evaluate the desired equilibrium constants. It is not as easy to estimate the errors in the final values. The size of the points in Fig. 4 is based solely on the maximum deviation from the mean of the estimates at three wave lengths of the fraction of pyridoxal chelated.

Table V lists some of the molar absorbancy indices used in the calculations.

TABLE V

SELECTED MOLAR ABSORBANCY INDICES USED IN CALCULATIONS. VALUES GIVEN ARE FOR $a_M \times 10^{-3}$

Compound	Wave length, mμ 270	315	380	420
Pyridoxal:				
Cation	3.58	0.09	0.00	0.00
Dipolar ion	0.90	8.62	0.14	0.07
Anion	1.54	2.78	1.76	1.15
Pyridoxal–valine imine	6.22	1.15	3.35	6.15
Pyridoxylidene valine chelates:				
Copper, neutral	9.90	1.69	4.63	1.63
Copper, protonated	4.00	0.93	6.01	1.41
Nickel	4.65	1.20	5.80	2.40
Zinc (see Fig. 3)				