Equilibria and Solute Structures in the Aqueous Systems Pyridoxal–Alanine–Zinc(II) and Aluminum(III) as Investigated by Proton Resonance

Otto A. Gansow and R. H. Holm[1]

Contribution from the Departments of Chemistry, University of Wisconsin, Madison, Wisconsin, and Massachusetts Institute of Technology, Cambridge, Massachusetts.
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Abstract: As part of a proton resonance investigation of the mechanism of metal-ion-catalyzed transamination reactions involving amino acids and pyridoxal, the pmr spectra of systems containing 0.1 *M* pyridoxal and 0.1 *M* DL- or L-alanine in the presence and absence of 0.05 *M* Zn(II) or Al(III) in D_2O solution at pD 1–13 have been examined. Formation of the aldimine Schiff base, N-pyridoxylidenealanine, and its metal complexes is readily observed. Compared to the metal-free system the presence of metal ions has, by virtue of complex formation, the effect of increasing the extent of aldimine formation at any pD below 13. The pD dependencies of chemical shifts in the metal-free and metal-containing systems have been interpreted in terms of formation of free aldimine and its mono (1:1:1) and bis (2:1:1) complexes, and of several acid–base equilibria of these species. Exchange between free and coordinated aldimine in the Zn(II) system is fast on the pmr time scale and slow in the Al(III) system. Structures for the free aldimine and the complexes are proposed. The 1:1:1 and 2:1:1 aluminum complexes each exist in two pmr-distinct forms which are believed to result from the failure of the Schiff base to act uniformly as a tridentate ligand. Formation of only one of the two 2:2:1 species using L-alanine has led to the postulation of active and *meso* diastereoisomers in the system containing DL-alanine. Exchange of the α proton of condensed alanine with D_2O in the presence or absence of metal ions is not observed at room temperature, indicating that the amino acid does not racemize appreciably *via* aldimine formation. A facile preparation of DL-alanine-β-d_3 is described.

The transamination reaction between α-amino acids and α-keto acids, represented generally by eq 1, may

$$RCH(NH_2)COOH + R'COCOOH \rightleftharpoons R'CH(NH_2)COOH + RCOCOOH \quad (1)$$

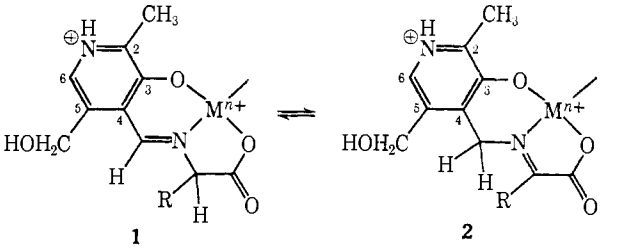
be duplicated nonenzymatically in the presence of pyridoxal and certain metal ions at rates which exceed those found in model systems containing pyridoxal but no metal ions. The over-all transformation appears to result from the coupling of the sequential reactions 2 and 3, which form the basis for the widely quoted general mechanism for transamination of amino acids catalyzed by pyridoxal and metal ions.[2]

$$RCH(NH_2)COOH + \text{pyridoxal} \rightleftharpoons RCOCOOH + \text{pyridoxamine} \quad (2)$$

$$\text{pyridoxamine} + R'COCOOH \rightleftharpoons R'CH(NH_2)COOH + \text{pyridoxal} \quad (3)$$

Results of investigations involving model systems for nonenzymatic transamination which contain these components as catalysts have been summarized recently.[3–6]

The function of the metal ion in the model systems is considered to be that of facilitating Schiff base formation and enhancing the reactivities of aldimine and ketimine forms at several points in the over-all reaction sequence. At the physiological pH the pyridoxylidene complex **1** is believed to be formed initially and to undergo rearrangement to the ketimine form **2**, the hydrolysis of which completes reaction 2. Subsequent formation of a new ketimine complex from pyridoxamine and the initial keto acid followed by ketimine → aldimine rearrangement and hydrolysis yields the products of reaction 3 and completes one cycle of the transamination sequence.



1 **2**

We are currently investigating the mechanism of transamination involving pyridoxal cofactors and metal ions in aqueous solution by application of nmr, which should allow direct detection and structural identification of at least some of the species involved in the over-all reaction sequence provided the rates of the individual steps are sufficiently slow. The systems investigated in D_2O solution are pyridoxal–alanine–Zn(II) and Al(III) and pyridoxamine–pyruvate–Zn(II) and Al(III). Both metal ions are known to be relatively active in catalyzing

(1) Address inquiries to this author at the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass. 02139.

(2) D. E. Metzler, M. Ikawa, and E. E. Snell, *J. Am. Chem. Soc.*, 76, 648 (1954).

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

(4) B. M. Guirard and E. E. Snell, "Comprehensive Biochemistry," Vol. 15, M. Florkin and E. H. Stotz, Ed., Elsevier Publishing Co., New York, N. Y., 1964, Chapter V.

(5) E. E. Snell, P. M. Fasella, A. E. Braunstein, and A. Rossi-Fanelli, Ed., "Chemical and Biological Aspects of Pyridoxal Catalysis," The Macmillan Co., New York, N. Y., 1963.

(6) E. E. Snell, A. E. Braunstein, E. S. Severin, and Yu. M. Torchinsky, Ed., "Pyridoxal Catalysis: Enzymes and Model Systems," Interscience Publishers, New York, N. Y., 1968.

transamination, *e.g.*, between pyridoxamine and α-ketoglutarate.[7] Our initial studies have been centered on reactions 2 and 3 conducted in systems at ambient temperature under conditions such that metal complex intermediates of types **1** and **2** could be detected by pmr. The reversibility of transamination allows equilibrium to be approached from either direction. Before the metal-containing systems could be meaningfully studied, it became necessary to obtain complete signal assignments of all likely species in solution other than the complexes, *viz.*, pyridoxal, alanine, pyridoxamine, and pyruvate and the aldimine and ketimine Schiff bases derived therefrom. The investigation has proceeded in several stages. First, the spectra of pyridoxal and pyridoxamine have been studied as a function of pD. Second, these studies have been repeated with the addition of alanine and pyruvate in order to obtain the spectra of the Schiff bases. Third, metal ions have been introduced in the two-component systems and the spectra redetermined in order to detect complexes such as **1** and **2**. Fourth, the spectra of the three-component systems have been followed under temperature conditions where transamination rates become appreciable. In most stages the pD range covered is *ca.* 1–13. The use of this broad range has allowed acid–base equilibria of various solute species to be observed, the formation of free Schiff bases and their complexes to be detected, and the course of the transamination reaction to be followed. The pmr study of the equilibria of pyridoxamine, pyridoxal, and several analogs of the latter is complete.[8] This report is concerned with the study of the aqueous pyridoxal–alanine system in the presence and absence of Zn(II) and Al(III) at ambient temperature where transamination is not observed. A recent report[9] has dealt with the corresponding pyridoxamine–pyruvate systems and pmr studies of transamination for which this work is a necessary prelude. A more detailed description of these systems will be presented in the near future.

Considerable information has already been obtained, principally from spectrophotometric studies, on the equilibria of Schiff bases formed from pyridoxal or pyridoxal 5-phosphate,[10–15] pyridoxal analogs,[16,17] and salicylaldehyde[16,18] in aqueous and nonaqueous media. In addition, the formation of metal complexes in these systems has been detected[13,14,19–21] and in a number of cases their formation constants have been determined, structures proposed, and rates of formation[22] investigated. The only reported pmr studies of pyridoxal or pyridoxal 5-phosphate Schiff bases is that by Turchin, *et al.*,[23] whose observations were confined to metal-free systems in methanol solutions. The pD dependence of the pmr spectra of the aldimine species was not as extensively investigated as in the present work.

The observations made in this work have led to definitions of solute structures which are usually independent of, but always complementary to, conclusions from previous equilibrium studies. Solute structures are proposed or determined from pmr results, and the pD dependencies of the chemical shifts of solute species are correlated where possible with spectrophotometrically determined equilibrium constants.

Experimental Section

Pyridoxal monohydrochloride and L- or DL-alanine were purchased from Calbiochem Corp. and used as received. Reagent grade zinc oxide or aluminum trichloride was dissolved in concentrated D_2SO_4 to prepare stock solutions of zinc(II) or aluminum(III). Solutions of NaOD were prepared by dissolving clean sodium in 99.5% D_2O. D_2SO_4 was available from commercial sources. Solutions used in the proton resonance studies were prepared in 99.5% D_2O by adding pipetted quantities of pyridoxal, alanine, metal ion, and NaOD solutions to a volumetric flask followed by dilution to known volume. Solutions were allowed to stand at ambient temperature until no change in pD could be observed for a period of *ca.* 20 min and then their pmr spectra recorded. Measurements of pD were taken before and after observation of the pmr spectra in order to ensure that a condition of at least pseudo-equilibrium had been reached. Constancy of pD was reached in 10 min or less for the metal-free and zinc-containing solutions and in *ca.* 30 min for the aluminum-containing systems. If the metal-containing solutions were allowed to stand for as long as 4–6 hr, gelatinous precipitates were observed. Solutions were 0.1 *M* in both pyridoxal and alanine and 0.05 *M* in metal ion unless otherwise indicated. Apparent pH values were measured using a Beckman Model G or Radiometer pH meter and true pD values were obtained from the relation pD = pH + 0.40.[24]

DL-Alanine-β-d_3. A D_2O solution (40 ml) containing 0.05 mole of dimer-free sodium pyruvate (Sigma Chemical Co.) and 0.005 mole of pyridoxamine dihydrochloride was adjusted to pD 6.8 with NaOD solution and heated at 40° for 24 hr. The pD of the solution was decreased to 4.5 with aqueous hydrochloric acid and the solution evaporated *in vacuo* to a volume of 10 ml. Concentrated hydrochloric acid (0.01 mole) was added followed by 150 ml of absolute ethanol. The precipitated sodium chloride was filtered off and the pyruvic acid was converted to its oxime by addition of 100 ml of a 0.6 *M* hydroxylamine solution in ethanol. The oxime was isolated by volume reduction of the solution; 5 g was dissolved in 250 ml of glacial acetic acid and hydrogenated at 3 atm pressure over a catalyst consisting of 2.5 g of palladium (10%) on carbon, 0.5 g of PtO_2, and 0.5 g of $PdCl_2$. The alanine was isolated in 60% yield from the filtered acetic acid solution by complete removal of the acetic acid *in vacuo* followed by recrystallization from ethanol–water. The infrared spectrum was consistent in the methyl absorption regions with that reported for L-alanine-α,β-d_4.[25] The extent of deuteration at the β-carbon was shown to be >90% from the infrared and pmr spectra. This means of preparation of racemic alanine-β-d_3 has resulted from our observation[9] that H–D exchange of the ketimine methyl protons of α-pyridoximinopyruvate in the presence or absence of metal ions is fairly rapid in neutral or alkaline D_2O solutions. The generality of this method for a facile, selective, deuteration at the β-carbon of α-amino acids is being investigated.

(7) J. B. Longenecker and E. E. Snell, *J. Am. Chem. Soc.*, **79**, 142 (1957).
(8) O. A. Gansow and R. H. Holm, *Tetrahedron*, **24**, 4477 (1968).
(9) O. A. Gansow and R. H. Holm, *J. Am. Chem. Soc.*, **90**, 5629 (1968).
(10) D. E. Metzler, *ibid.*, **79**, 485 (1957).
(11) Y. Matsuo, *ibid.*, **79**, 2011, 2016 (1957).
(12) H. N. Christensen, *ibid.*, 80, 99 (1958).
(13) D. L. Leussing and N. Huq, *Anal. Chem.*, **38**, 1388 (1966).
(14) Y. Matsushima and A. E. Martell, *J. Am. Chem. Soc.*, **89**, 1322 (1967).
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(16) (a) D. Heinert and A. E. Martell, *ibid.*, **85**, 183 (1963); (b) *ibid.*, **85**, 188 (1963).
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(18) D. L. Leussing and K. S. Bai, *Anal. Chem.*, **40**, 575 (1968).
(19) (a) G. L. Eichhorn and J. W. Dawes, *J. Am. Chem. Soc.*, 76, 5663 (1954); (b) G. L. Eichhorn and N. D. Marchand, *ibid.*, 78, 2688 (1956).
(20) H. N. Christensen, *ibid.*, **79**, 4073 (1957).
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(22) (a) L. J. Nunez and G. L. Eichhorn, *ibid.*, **84**, 901 (1962); (b) K. S. Bai and D. L. Leussing, *ibid.*, **89**, 6126 (1967).
(23) K. F. Turchin, V. F. Bystrev, M. Ya. Karpeisky, A. S. Olkhovoy, V. L. Florentiev, and Yu. N. Sheinker in "Pyridoxal Catalysis: Enzymes and Model Systems," Interscience Publishers, New York, N. Y., 1968, pp 67–79.
(24) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).
(25) T. Oshima and N. Tamiya, *Spectrochim. Acta*, 17, 384 (1961).

L-**Alanine-α,β-d_4.** The procedure of Oshima and Tamiya[26] was followed but with a 25-fold scale-up and employment of the following modifications. Pig heart glutamate–pyruvate transaminase (10 mg/1 ml of aqueous solution) was used as received from the Boehringer-Mannheim Corp. The D_2O solution (125 ml) containing L-alanine, α-ketoglutaric acid, pyridoxal phosphate, and the enzyme and buffered at pH 7.0 was maintained at 37° for 48 hr. The resultant deuterated alanine was separated from the reaction solution by absorption on Dowex 50, WX-12-400 hydrogen-form ion-exchange resin in an 8 $cm^2 \times 40$ cm column. The column was washed with eight bed-volumes of distilled water and eluted with 6 l. of 0.15 *M* ammonia solution; movement of the eluent front could be visually observed. An increase in the pH of the eluent from 3 to 7 (after ~5 l.) signaled amino acid elution (positive ninhydrin test). The next 600 ml was collected and evaporated to dryness *in vacuo*, the residue taken up in 200 ml of distilled water, and the solution passed through a column (4.5 $cm^2 \times 30$ cm) of Dowex 3 anion-exchange resin (acetate form) at a 1-ml/min flow rate. Glutamic acid and other acidic impurities were removed by absorption on the resin. Evaporation of the column effluent afforded crude deuterated alanine, which was purified by recrystallization from ethanol-water and obtained in 75% yield. The infrared spectrum was identical with that previously reported for L-alanine-α,β-d_4;[25,26] no proton-containing impurities could be detected by pmr.

Proton resonance spectra were determined using a Varian HA/HR-100 or HA/HR-60 spectrometer. Chemical shifts were measured to at least ± 1 cps by the usual side-band technique and were referenced to *t*-butyl alcohol, present to the extent of ~1% v/v, as an internal standard. All spectra were recorded at the ambient probe temperature of 27°.

Results and Discussion

Proton resonance spectra of D_2O solutions 0.1 *M* in both pyridoxal and alanine and 0.05 *M* in Zn(II) or Al(III) have been obtained over the pD range 1–13. Spectra of the metal-free system are shown in Figure 1; those of the zinc- and aluminum-containing systems are given in Figures 4 and 6, respectively. p*K* values for certain of the acid–base equilibria of the solute species can in some instances be directly determined from plots of chemical shift *vs.* pD[8] and are related to those measured by titrimetry or spectrophotometry in H_2O solutions by the relation $pK(D_2O) = pK(H_2O) + 0.40$.[24] Plots of this type are given in Figures 3, 5, and 8. The extent of aldimine formation in the metal-free system as determined from measurement of relative signal intensities is shown in Figure 2. Our recent pmr investigation of the acid–base equilibria of pyridoxamine, pyridoxal, and 3-hydroxypyridine-2- and -4-carboxaldehydes[8] has demonstrated that the methods employed here are useful for assessing relative concentrations of predominant solute species not involved in rapid exchange processes and for determining p*K* values, which in general agree well with those obtained by other methods. In those pD ranges where several species are present in rapid equilibrium the pmr method can, of course, sense only the average chemical shift of a given substituent. In particular, the presence of different species potentially implicated in tautomeric equilibria, such as are possible for pyridoxylidene Schiff bases at and near neutral pD, for example, can only be inferred. For a more complete examination of these equilibria the results of ultraviolet spectral studies, especially the careful and extensive investigations by Martell,[14,16] Metzler,[10,21] and their coworkers, should be consulted where appropriate.

(26) T. Oshima and N. Tamiya, *J. Biochem.* (Tokyo), 46, 1675 (1959); T. Oshima and N. Tamiya, *Biochem. J.*, 78, 116 (1961).

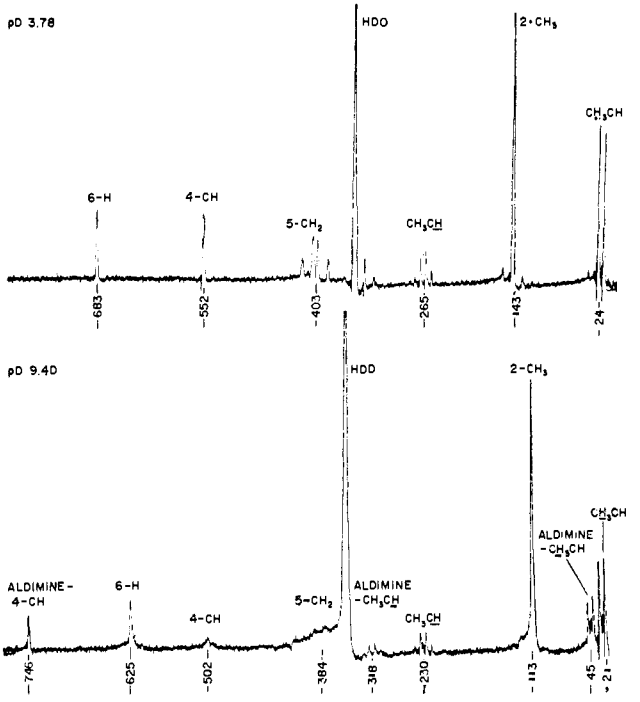


Figure 1. 100-Mc pmr spectra of D_2O solutions initially 0.1 *M* in both pyridoxal and alanine illustrating the absence of aldimine formation in acidic solution (pD 3.78) and partial aldimine formation in alkaline solution (pD 9.40).

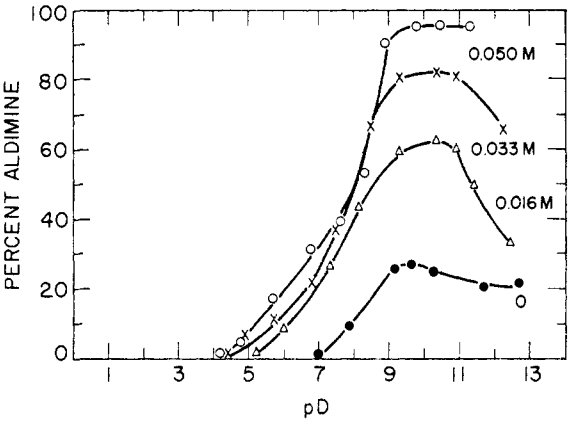
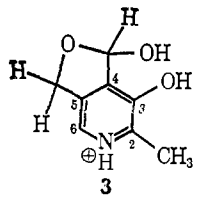


Figure 2. Per cent aldimine formation in D_2O solutions initially 0.1 *M* in both pyridoxal and alanine as a function of pD and Zn^{2+} concentration.

The System 1:1 Pyridoxal–Alanine. Spectra of solutions 0.1 *M* in both components at pD 3.78 and 9.40 are presented in Figure 1. At the lower pD the methyl doublet at −24 cps and the CH quartet at −265 cps are readily assigned to free alanine.[27] All other signals are assignable to free pyridoxal in acidic solution.[8] The 4-CH doublet and the 5-CH_2 quartet arise from the hemiacetal ring structure present in **3**, the predominant form of pyridoxal at this pD. As pD is increased Schiff base formation is easily detected by the appearance of new signals. The situation is illustrated by the spectrum at pD 9.40 where aldimine formation is appreciable. Features due to the azomethine proton (labeled aldimine-4-CH) and the CH and

(27) D. L. Leussing and C. K. Stanfield, *J. Am. Chem. Soc.*, 86, 2805 (1964).



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methyl groups of condensed alanine (labeled aldimine-CH_3CH and aldimine-CH_3CH) are clearly evident; their chemical shifts are pD dependent. The 6-H signals of pyridoxal and the aldimine are barely resolvable at this pD. Two other features of the Schiff base, the methylene singlet of the 5-CH_2OD group, slightly downfield from the intense HDO resonance, and the 6-H signal, ~3 cps upfield from the corresponding signal in free pyridoxal, can be observed at certain pD values. The broadening of the 4-CH and 5-CH_2 signals of free pyridoxal arises from slow exchange between the hemiacetal **3**, stable to pD 8, and the hydrated aldehyde form, which persists in the more alkaline region.[8]

The extreme low-field signal (at −746 cps in Figure 1) is characteristic of aromatic aldimines derived from amino acids.[28] From pD 7 to 13 the ratio of the integrated intensities of the azomethine signal to one-half of the total of all signals (azomethine, 6-H, 4-CH) in the −760 to −480 cps range provides a simple measure of the extent of aldimine formation as a function of pD. The results in Figure 2 for the metal-free system were obtained in this way and checked by integration of the azomethine signal *vs.* the methyl signals of the aldimine and pyridoxal. The per cent imine values agreed to ±5%, which is considered the probable error of the results shown in the figure. The form of the pD dependence of aldimine formation is very similar to the variation with pD of the log of the apparent formation constant, [imine]/[pyridoxal][amino acid], of N-pyridoxylidenevaline and glycine determined earlier by spectrophotometric titration.[10] The decrease in aldimine concentration near the alkaline pD limit of measurement is discussed below.

Because the Schiff base and its components do not undergo noticeable exchange on the pmr time scale, some information concerning the acid–base equilibria of N-pyridoxylidenealanine is obtainable from the pD dependence of certain of its chemical shifts. Those of the alanine methyl and pyridoxal 6-H in the free and condensed forms are the most useful in this respect and are set out in Figure 3. Observations were restricted to pD ≥7 since below this value no aldimine was detectable (*cf.* Figure 2), and an increase in the concentration of either pyridoxal or alanine above 0.1 *M*, in an attempt to extend the pD range of observation, resulted in formation of a precipitate. As shown previously,[8] pyridoxal 6-H shifts clearly reveal the stepwise dissociation of the phenolic and pyridinium groups of **3**, such that after the second acid ionization the principal solute species are **4** and **5** in equilibrium. The methyl shifts of free alanine are sensitive to carboxyl and ammonium group ionizations, and the p*K* values obtained from Figure 3 are in very good agreement with

(28) M. J. O'Connor, R. E. Ernst, J. E. Schoenborn, and R. H. Holm, *J. Am. Chem. Sec.*, 90, 1744 (1968).

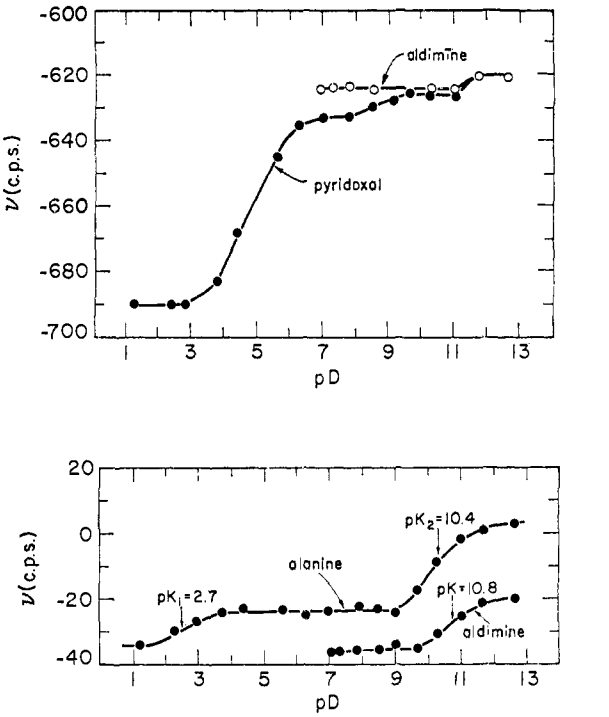
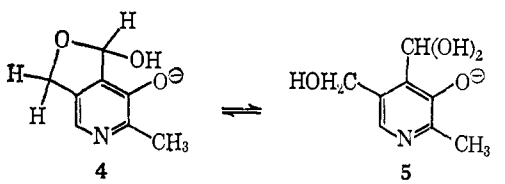


Figure 3. Chemical shifts at 100 Mc as a function of pD in D_2O solutions initially 0.1 *M* in both pyridoxal and alanine. Upper: 6-H signals of free pyridoxal and the aldimine. Lower: methyl signal of free alanine and the corresponding methyl signal of the aldimine.

those (2.3–2.5, 9.8–10.0) measured by titrimetry in H_2O solution.[29,30]



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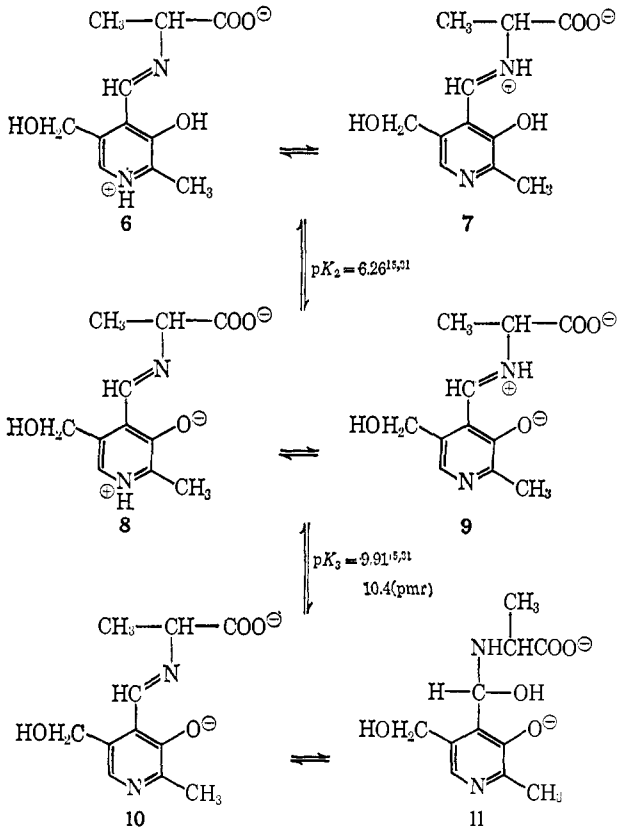
A number of investigations of aqueous and nonaqueous systems containing pyridoxal[10,13–15] or 3-hydroxypyridine-4-carboxaldehyde[16b,17] and amino acids have demonstrated that for the Schiff bases *two* acid–base equilibria exist in addition to that involving the carboxyl group (pK_1 ~2.4). For N-pyridoxylidenealanine the dissociation constants are pK_2 = 6.26 and pK_3 = 9.91.[15,31] The first of these dissociations could not be detected by pmr and is assumed to involve the predominant solute species shown in Chart I. In the pD range 7–10 the 6-H shift of the aldimine is constant at −623 cps (*cf.* Figure 3). This is within 2 cps of the 6-H shift of pyridoxal *after* its second ionization. Consequently, the predominant form of the aldimine after its second dissociation is concluded to be **9**, in which the shielding of 6-H is expected to be very similar to that in **4** and **5**. The existence of another polar form, **8**, cannot be completely

(29) "Stability Constants of Metal Ion Complexes," Special Publication No. 17, The Chemical Society, London, 1964, p 398.

(30) For related pmr studies of other amino acids in the pH range 1–13, *cf.* F. Taddei and L. Pratt, *J. Chem. Soc.*, 1553 (1964).

(31) Note that these values, determined spectrophotometrically in H_2O solution, were obtained under slightly different conditions (50° ionic strength 1.0) than those employed in this work.

Chart I. Acid–Base Equilibria of N-Pyridoxylidenealanine in Aqueous Solution



discounted; however, the 6-H signal of the first deprotonation product of pyridoxal, which has the pyridinium phenolate structure,[8] occurs at ~10 cps to lower field. The terminal ionization of the aldimine can be observed by pmr and is most clearly reflected by the alanine methyl shift. This methyl signal is shifted to higher field by 15 cps from pD 9 to 12 and pK_3 is estimated as 10.8, a value in fair agreement with the spectrophotometric determination.[15,31] The increased methyl shielding is reasonably accounted for by neutralization of the immonium function in **9** and provides further indication that **9** is the principal aldimine species in the pD range between pK_2 and pK_3.

As indicated in Figure 2, the relative intensity of the azomethine proton signal, and therewith the per cent aldimine, decreases somewhat above pD ~9.7. This change is accompanied by the appearance of a new signal near that of 6-H. The chemical shift of this signal is essentially pD independent (−608 cps, pD 10–13) and cannot be identified with any pyridoxal species present at pD 9.7–13, all of which exist in the hemiacetal or hydrated form.[8] Because no 5-CH_2 multiplet characteristic of the hemiacetal ring as in **4** is observed, it is concluded that the new signal is that of HC(OH) in the carbinolamine form **11**. In the pD 9.7–13 range the concentration of this species varies as the pD is increased, reaching a maximum of ~15% of solute at pD 10.5–11 and persisting in smaller amounts to pD 13.

The acid–base equilibrium scheme for N-pyridoxylidenealanine which is consistent with the proton resonance results is presented in Chart I.

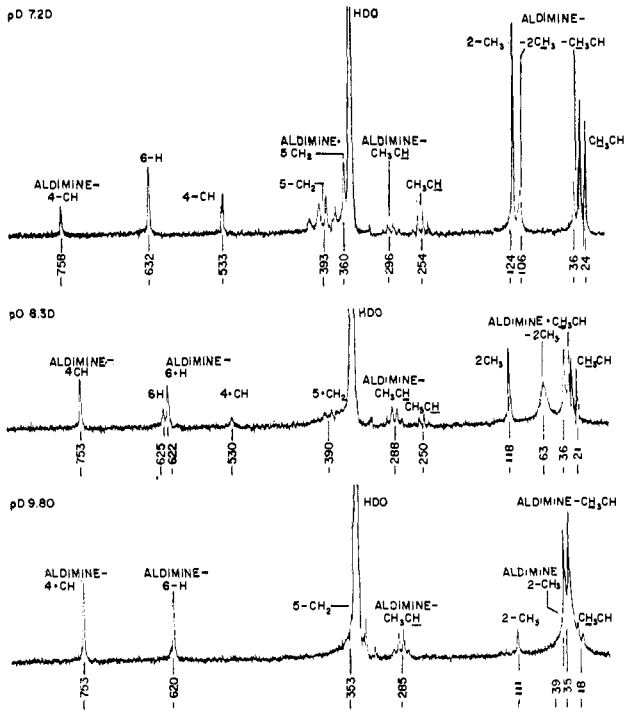


Figure 4. 100-Mc pmr spectra of D_2O solutions initially 0.1 *M* in both pyridoxal and alanine and 0.05 *M* in Zn(II) illustrating the extent of aldimine formation at different pD values.

The System Pyridoxal–Alanine–Zinc(II). Pmr spectra of solutions 0.1 *M* in both pyridoxal and alanine and 0.05 *M* in Zn(II) at three pD values are shown in Figure 4. Stabilization of the Schiff base by complex formation is clearly revealed by the results in Figure 2, which show that at 0.016 *M* zinc ion concentration, the lowest employed, aldimine formation is first detectable at pD 5.2, compared to pD 7 in the metal-free case. For the system having a 2:2:1 concentration ratio of components, aldimine can be observed at pD 4.2, and its formation increases rapidly with increasing basicity until ≥95% completion is reached at pD 9.5–12. In metal-free solutions containing a 1:1 ratio of components, aldimine formation never exceeds ~28% at any pD. Exchange involving condensed and free pyridoxal is not observed, but that involving complexed and free aldimine must be rapid because only one set of signals is observed regardless of pD and Zn(II) concentration. The extent of aldimine formation, determined by the same procedure used for the metal-free case and shown in Figure 2, refers to the per cent aldimine present in both the free and complexed form. The decrease in aldimine concentration in strongly basic solutions is due to decomposition of the zinc complex and precipitation of the metal hydroxide.

Rapid exchange between coordinated and free aldimine makes difficult precise determination of the types and structures of the solute species present. However, some conclusions can be reached from the extent of aldimine formation (Figure 2) and the pD dependence of the chemical shifts. With reference to the 2:2:1 pyridoxal–alanine–Zn(II) system all signals of the aldimine except that of the CH proton of condensed alanine shift upfield with increasing pD. This situation is superficially similar to that found with the free aldimine, all signals of which shift to higher field as

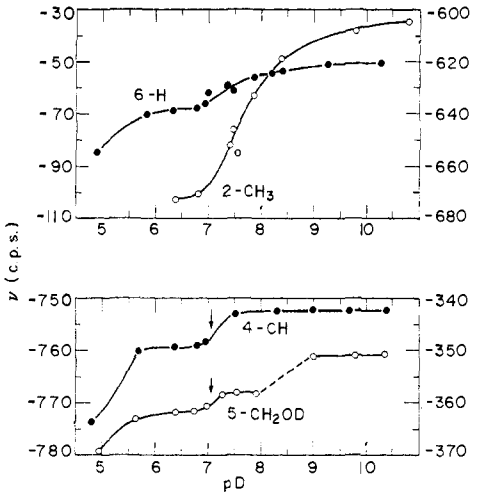
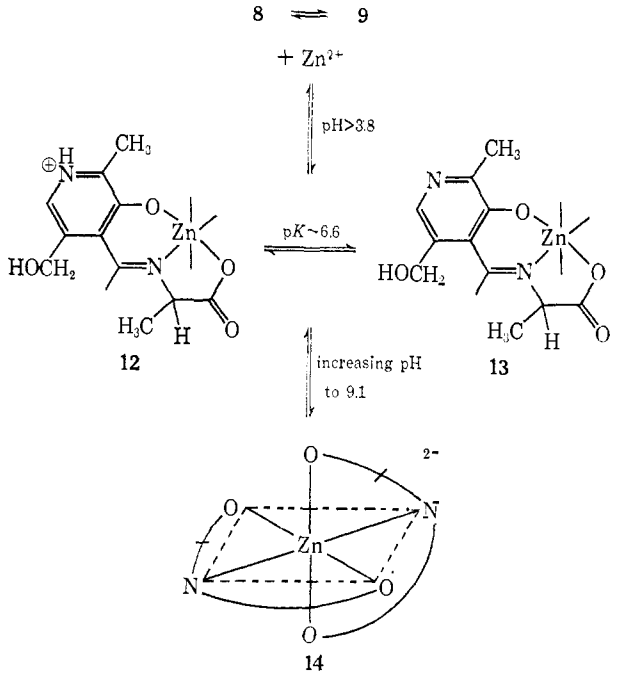


Figure 5. Aldimine chemical shifts at 100 Mc as a function of pD in D_2O solutions initially 0.1 *M* in both pyridoxal and alanine and 0.05 *M* in Zn(II). Upper: 6-H (right), 2-CH_3 (left). Lower: 5-CH_2OD (right), 4-CH (left). Left and right refer to the appropriate chemical shift scales; – – –, under HDO signal.

Chart II. Equilibria in Aqueous 2:2:1 Pyridoxal:Alanine:Zn(II) Systems



the pD is raised. However, the pD dependencies in the zinc-containing system are quite different. Those for 2-CH_3, 6-H, 4-CH (azomethine), and 5-CH_2OD and are shown in Figure 5. Chemical shifts of CH_3 and CH protons of the condensed alanine portion are less sensitive to pD and fall in the ranges −296 to −284 and −31 to −39 cps, respectively.

Aldimine formation and chelation of Zn(II) is evidenced by rapid changes in the chemical shifts of most protons in the range pD 4.5–6.5. Although no specific formation constant data have been published for N-pyridoxylidenealaninatozinc(II) complexes, results for the corresponding valine system indicate that the log of the apparent formation constant for a 1:1:1 complex is ~8–9.5[21,32] in this pD interval. Hence, substantial aldimine formation and complexation can be expected in the alanine system under comparable conditions. The pD dependencies of the 4-CH and 5-CH_2OD signals reveal a rather well-defined inflection point at pD ~7. This behavior is associated with chelate ring deprotonation of the 1:1:1 complex **12** to the neutral species **13** (*cf.* Chart II). Spectrophotometric measurements indicate p*K* = 6.5 for this same reaction of the N-pyridoxylidenevalinatozinc(II) complex.[21]

An increase in solution pD above 7 produces upfield shifts which are most marked for 6-H and, especially, 2-CH_3 (*cf.* Figure 5). The shifting and accompanying broadening of the 2-CH_3 shifts is evident in the spectra shown in Figure 4. Concomitant with these upfield shifts is an increasing extent of aldimine formation which at pD >8.9 cannot be completely accounted for by independent formation of 1:1:1 complex and free aldimine. A similar broadening and shifting behavior of the aldimine 2-CH_3 signal is observed in systems 0.1 *M* in both pyridoxal and alanine containing 0.016–0.05 *M* Zn(II). This behavior is consistent with the existence of the labile, anionic 2:2:1 complex of possible structure **14**, which is undergoing rapid exchange with free aldimine. The extent of formation of this complex increases with increasing pD and at pD 9.5 is apparently a maximum since beyond this value (to pD 11.2) the 2-CH_3 shift is independent of pD. Over the entire pD range of observation no broadening and shifting of the signals of alanine were observed other than those due to its acid–base equilibria. It is therefore considered unlikely that any simple amino acid[33] or mixed aldimine–amino acid complexes were formed in significant concentration. The solution equilibria in a 2:2:1 system which are consistent with the pmr results are shown in Chart II. These results cannot be interpreted in terms of exact solution structures for the 1:1:1 and 2:2:1 complexes. However, good presumptive evidence that the aldimine is capable of functioning as a tridentate ligand to Zn(II) in both types of complexes is afforded by the X-ray structural determinations of N-pyridoxylidenevalinatocopper(II),[34] N-pyridoxylidene(5-phosphate)phenylalaninatocopper(II),[35] N-salicylideneglycinatoaquocopper(II),[36] and bis(N-pyridoxylidenevalinato)manganese(II),[37] in which the ligands are tridentate. An unpublished X-ray study of bis(N-pyridoxylidenevalinato)zinc(II) quoted by Freeman[38] has shown that this complex, like its Mn(II) analog,[37] does in fact possess structure **14** in the crystalline state.

(32) The designation 1:1:1 and 2:1:1, used subsequently, refers to the combining ratios of pyridoxal:amino acid:metal in the complexes in which the first two components are present in the aldimine form. For 1:1:1 complexes of Zn(II) derived from a variety of amino acids including alanine, log K_c = 7.4–8.1, K_c = [complex]/[Zn^{2+}][aldimine^{2-}].[21]

(33) For a brief description of the pmr spectra of Zn(II)-alanine systems in D_2O solution, *cf.* ref 27 and R. H. Carlson and T. L. Brown, *Inorg. Chem.*, **5**, 268 (1966).

(34) J. F. Cutfield, D. Hall, and T. N. Waters, *Chem. Commun.*, 785 (1967).

(35) G. A. Bentley, J. M. Waters, and T. N. Waters, *ibid.*, 988 (1968).

(36) T. Ueki, T. Ashida, Y. Sasada, and M. Kakudo, *Acta Cryst.*, 22, 870 (1967).

(37) E. Willstadter, T. A. Hamor, and J. L. Hoard, *J. Am. Chem. Soc.*, 85, 1205 (1963).

(38) H. C. Freeman, *Advan. Protein Chem.*, 22, 257 (1967).

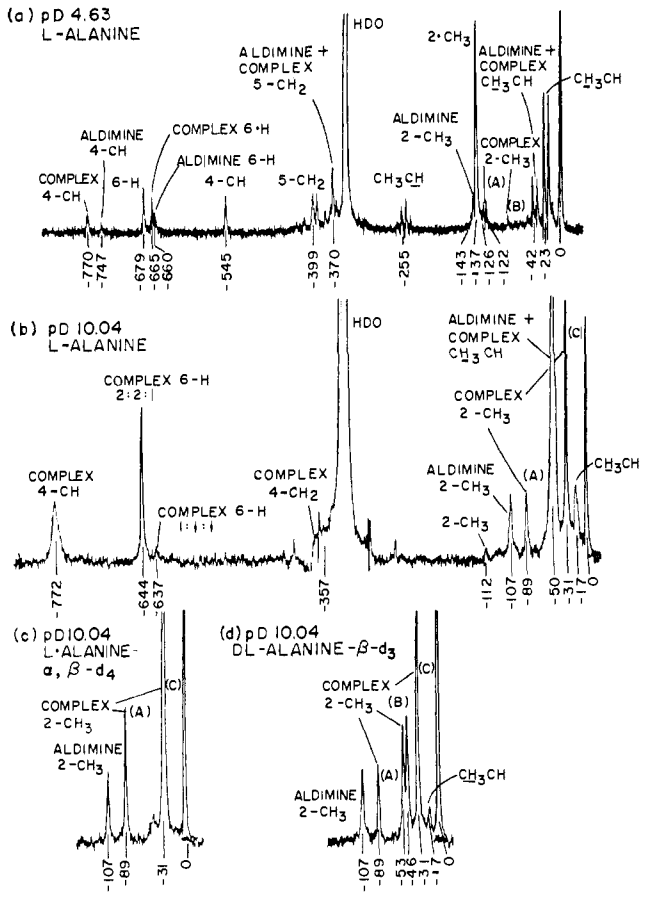


Figure 6. 100-Mc pmr spectra of D_2O solutions initially 0.1 *M* in pyridoxal, 0.05 *M* in Al(III), and 0.1 *M* in various forms of alanine. Unprefixed signals refer to free pyridoxal and alanine. Signals labeled "complex" refer to the 1:1:1 species (a) and 2:2:1 species (b); (c) solution prepared from L-$CD_3CD(NH_2)COOH$; (d) solution prepared from DL-$CD_3CH(NH_2)COOH$. (A), (B), and (C) refer to the pairs of signals in Figure 8.

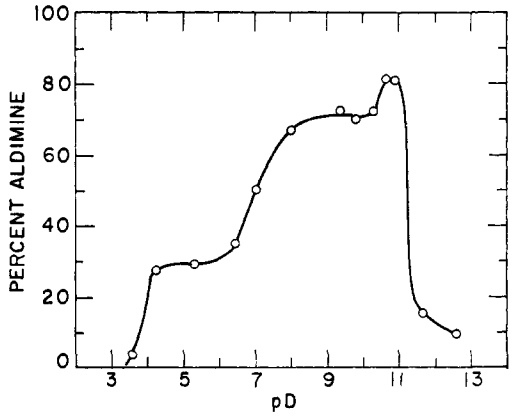


Figure 7. Per cent aldimine formation as a function of pD in D_2O solutions initially 0.1 *M* in both pyridoxal and alanine and 0.05 *M* in Al(III).

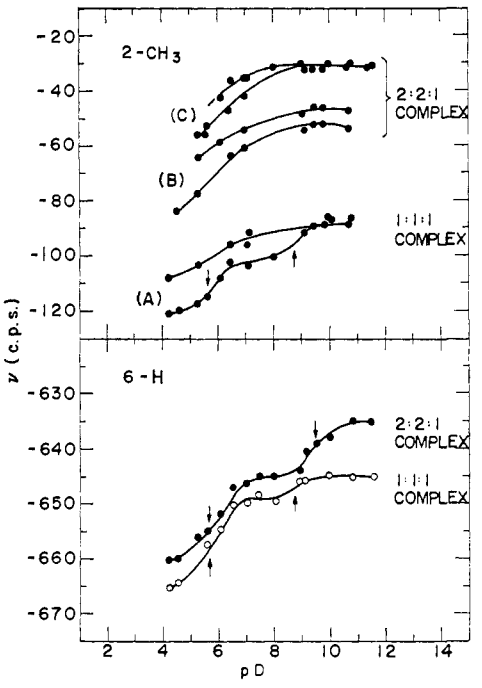


Figure 8. Chemical shifts at 100 Mc of the 2-CH_3 and 6-H signals of 1:1:1 and 2:2:1 pyridoxal-alanine-Al(III) complexes as a function of pD in D_2O solution.

The System Pyridoxal–Alanine–Aluminum(III). This system was investigated in order to ascertain what differences, if any, in solute species and Schiff base stabilization are effected by a diamagnetic trivalent metal ion compared to the zinc(II) systems. Aluminum(III) is of particular interest in this respect in light of the report[7] that, of various trivalent ions employed, it showed the highest comparative activity in transamination between pyridoxamine and α-ketoglutarate. One implication of this result is that Al(III) pyridoxylidene complexes are relatively stable. Previous studies of Al(III) pyridoxylidene complexes have been limited to an investigation of a "β-pyridoxylserine" Al(III) species derived from pyridoxal and glycine[39] and a chromatographic examination of the transamination products in the system pyridoxal phosphate–alanine–Al(III).[40] No evidence for the structures proposed for these complexes, all of which were produced by heating, has been obtained in this work. In addition, a spectrophotometric study of the N-pyridoxylidenepyridoxamino aluminum(III) complex has recently been reported.[41] In the present work this species was not detected, and its presence in detectable concentrations is not expected because under the experimental conditions employed transamination yielding pyridoxamine must be quite slow.

Pmr spectra of solutions 0.1 *M* in both pyridoxal and alanine and 0.05 *M* in Al(III) in acidic and basic regions are set out in Figure 6. These spectra, together with the associated information on the pD dependence of aldimine formation (Figure 7) and chemical shifts of the N-pyridoxylidenealaninato aluminum(III) species formed (Figure 8), reveal two important differences compared to the corresponding Zn(II) systems. First, exchange between coordinated and free aldimine is very slow on the pmr time scale, permitting observation of separate signals for the free Schiff base and for two other species which are most simply formulated as the 1:1:1 and 2:2:1 complexes. Second, aldimine formation may be observed at pD 3.6 compared to 4.2 in

(39) D. E. Metzler, J. B. Longenecker, and E. E. Snell, *J. Am. Chem. Soc.*, 76, 639 (1954).
(40) P. Fasella, H. Lis, N. Siliprandi, and C. Baglioni, *Biochim. Biophys. Acta*, 23, 417 (1957).
(41) C. Cennamo, *Ric. Sci.*, 58, 31 (1968).

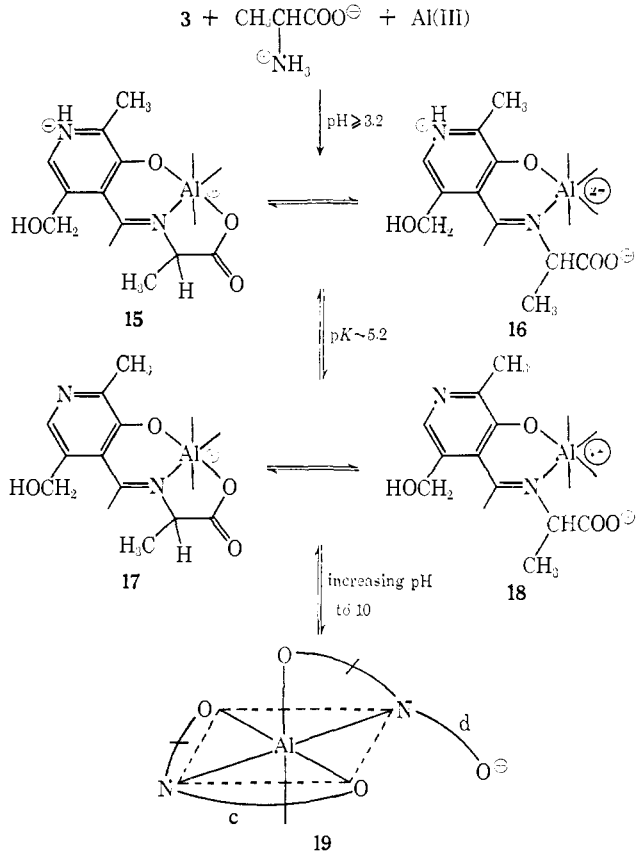
the Zn(II) system and is maximized at ~82% (pD 10.6–11) compared to ≥95% in the Zn(II) system (pD 9.5–11). As in the Zn(II) system exchange between pyridoxal and alanine and free and coordinated aldimine is slow and the chemical shifts of the latter are markedly pD dependent. At a given pD value chemical shifts of rapidly exchanging aldimine are intermediate between those of complexed and free aldimine in the Al(III) system.

With reference to Figure 7 it is observed that aldimine formation can be considered in three more or less distinct pD regions: 3.6–6 and 6–10.4, in which there is an initial rapid rise in aldimine formation followed by a leveling-off, and 10.4–13, in which there is a small increase in aldimine concentration preceding a large and rapid decrease. Below pD 3.6 only the signals of free pyridoxal and alanine are observed. Over the entire pD range of observation free aldimine, in slow equilibrium with the complexes, is detectable. Its concentration decreases with increasing pD. The per cent aldimine indicated in Figure 7 refers to aldimine in all forms and was assessed by the same signal integration method used with the Zn(II) systems.

pD 3.6–6. In this range all signals clearly assignable to coordinated aldimine occur as single chemically shifted features except those of 2-CH_3 and 5-CH_2. Three 5-CH_2 signals are observed which are attributed to free aldimine, 1:1:1 complex, and 2:2:1 complex; the intensity of the resonance of the latter increases with increasing pD. The behavior of the 2-CH_3 signals over the entire pD range is quite complex. The first 2-CH_3 feature observed is a doublet (−122, −126 cps in Figure 6a) which is assigned to the 1:1:1 complex. This pair of signals (A in Figure 8), which must arise from a chemical shift difference, implies two pmr-distinct structural forms of this complex. On the basis of the chemical shifts and the well-documented slow exchange in Al(III) systems between coordinated and free ligand,[42] which is readily detected by pmr,[42b] **15** and **16** in Chart III are considered plausible structures of the two 1:1:1 species. The pair A signals may be followed through this and the higher pD ranges as indicated in Figure 8. At pD ~5.6 a fairly well-defined inflection point occurs in one curve and is associated with deprotonation of the pyridinium nitrogen. The other 2-CH_3 signal does not exhibit this behavior clearly, but the 6-H signal of both species appears to possess an inflection at this same pD.[43] Consequently, it is reasonable to assume that both 1:1:1 species are deprotonated to yield **17** and **18**.

Also observed in this pD region are two other pairs of 2-CH_3 signals (B, C) at higher field whose chemical shifts are shown in Figure 8. By virtue of their increasing intensities and progressive high-field shifts as the pD is raised, these signals are assigned to 2:2:1 species. Because of overlap with the methyl signal of free alanine and the corresponding methyl signals of

Chart III. Equilibria in Aqueous 2:2:1 Pyridoxal–Alanine–Al(III) Systems (free aldimine in equilibrium with the complexes not shown)



coordinated aldimine, solutions prepared from L-alanine-α,β-d_4 and DL-alanine-α-d_3 were used to trace the pD dependence of pairs B and C. Assignment of these pairs is considered in the following section.

pD 6–10.4. In this region all signals attributable to coordinated aldimine increase in intensity as the pD is raised and shift upfield. The three 5-CH_2 signals coalesce at pD ~7 to give one broad signal slightly downfield of the HDO resonance. Near pD 7 the 4-CH signals of free and coordinated aldimine overlap, producing a broad, unresolved signal which persists over the remainder of this pD range. Again in this range the 2-CH_3 signals of coordinated aldimine appear as other than a single chemically shifted feature. Six methyl signals, pairs A, B, and C, may be followed over most of this range[44] with the use of deuterated L- and DL-alanine, and provide the most information concerning the structures of the 2:2:1 species. Above pD ~6 the intensity of pair A shows no further increase, whereas intensities of pairs B and C clearly increase up to pD 10.4. Two 6-H signals of the 1:1:1 and 2:2:1 species are observable over the entire range. The moderately rapid upfield shift of one of the two 6-H signals at pD 9–10.5 and of one component of pair A at pD 8–9.5 cannot be satisfactorily interpreted. The spectrum of a solution at pD 10.04 prepared from L-alanine is shown in Figure 6b.

(42) (a) H. W. Baldwin and H. Taube, *J. Chem. Phys.*, **33**, 206 (1960); (b) J. A. Jackson, J. F. Lemons, and H. Taube, *ibid.*, **32**, 553 (1960); R. E. Connick and D. N. Fiat, *ibid.*, **39**, 1349 (1963); S. Thomas and W. L. Reynolds, *ibid.*, **44**, 3148 (1966); W. G. Movius and N. A. Matwiyoff, *Inorg. Chem.*, 6, 847 (1967).

(43) Unlike the corresponding Zn(II) system, the 4-CH and 5-CH_2 signals do not as clearly reveal the deprotonation reactions. The large chemical shifts attendant to complexation in this labile system undoubtedly obscure the effect of this reaction upon the 2-CH_3 signal.

(44) In the pD region 6.5–9.0 it was necessary to use solutions 0.05 *M* in pyridoxal and alanine and 0.025 *M* in Al(III) to prevent precipitation. In this region the six 2-CH_3 signals were usually of sufficiently low intensity that they could not always be identified with certainty, thereby accounting for the limited number of points from pD 7–9 in Figure 8.

As can be observed in Figure 8 the pD dependencies of the coordinated aldimine 2-CH_3 signals comprising pairs B and C are rather similar except that pair C merges to a single signal at pD ~9. Pair A of the 1:1:1 complex exhibits the same effect at pD ~9.5. The components of pair C, where separately observable, and those of pair B are of essentially equal intensity, implying that each pair is associated with a single species. Further, pairs B and C are both observable in solutions containing DL-alanine-α-d_3 whereas only pair C is present when L-alanine-α,β-d_4 is used. The spectra at pD 10.04 in Figures 6c and 6d illustrate the situation.

The question of the structures of the predominant aluminum complexes present in this pD range is undoubtedly a complex one and cannot be answered unambiguously with the pmr results presently at hand. We offer here what appears to be the simplest interpretation of the structures of the 2:2:1 species consistent with the spectra, in particular with the dependence of the 2-CH_3 signal multiplicity of coordinated aldimine on the type (L or DL) of alanine used. The appearance of two pairs of 2-CH_3 signals in solutions prepared from DL-alanine is clearly inconsistent with a structure of type **14**, which could develop at most three 2-CH_3 signals.[45] The required structure must lack the twofold symmetry of **14**. If consideration is restricted to *monomeric* species, structure **19** (Chart III) is consistent with the 2-CH_3 signal multiplicity. With L-(+)-alanine only the $(+_c,+_d)$ or active isomer is possible, where c and d refer to the completely coordinated and "dangling" chelate rings, respectively. The use of DL-alanine generates active and *meso* diastereoisomers $(+_c,+_d) \equiv (-_c,-_d)$ and $(+_c,-_d) \equiv (-_c,+_d)$, thereby giving rise to pairs C and B, respectively. This explanation requires a slow rate of intramolecular chelation, as proposed for the 1:1:1 species, and the absence of chemical shift differentiation between the D,L configurations at the metal. Further, the possibility that appreciable concentrations of carbinolamine complexes of the type suggested elsewhere[46] intervene in this pD range may be safely discounted because the 4-CH (azomethine) signal suffers no decrease in relative intensity. The virtually identical intensities of pairs B and C indicate that the two isomers are formed in equal amounts, a not unexpected consequence of structure **19** inasmuch as amino acid chelate rings generally show little stereoselectivity.[46] It is reemphasized that structure **19** represents only the simplest possibility based upon a monomeric formulation. Other formulations involving oxo- or hydroxo-bridged dimers or higher polymers which are consistent with the pmr data by virtue of containing two or more nonequivalent pyridoxylidene chelate groupings are easily derived and cannot be excluded by the available results.

pD 10.4–13. In this region aldimine concentration in all forms undergoes a small increase followed by a precipitous drop at pD 11–13. Up to pD ~11 the methyl signal of free alanine becomes markedly broadened similar to the effect observed in systems containing only Al(III) and alanine. A possible explanation is the formation of a 1:2:1 complex species involving one coordinated alaninate ion. Above pD 11 all complexes are evidently unstable to base hydrolysis and the formation of aluminate species, resulting in a severe decrease in aldimine formation.

(45) These could in principle arise from the diastereoisomers D(++) ≡ L(−−), D(−−) ≡ L(++), and D(+−) ≡ L(+−) possible for structure **14**, provided the rate of racemization of the absolute configuration (D,L) at the metal is slow on the pmr time scale.

(46) R. D. Gillard, *Inorg. Chim. Acta Rev.*, 1, 69 (1967).

Summary

The following are the principal results of this investigation.

(i) At pD ≥7 pyridoxal and alanine condense in D_2O solution to form a Schiff base whose presence is readily detected by pmr. In the pD range 7–10 the principal aldimine form is considered to have the immonium–phenolate structure (**9**); above pD ~10 a mixture of imine–phenolate (**10**) and carbinolamine–phenolate (**11**) species is formed with the concentration of the latter increased as the pD is raised.

(ii) Pyridoxal and alanine condense in the presence of Zn(II) or Al(III) to form N-pyridoxylidenealaninato complexes of the 1:1:1 and 2:2:1 types. The concentration of the latter is increased as the solution pD is raised.

(iii) The extent of aldimine formation is increased in the presence of metal ions, never exceeding 28% (pD 9.5–10) in the metal-free system compared to ≥95% (pD 9.5–11) in the presence of Zn(II) and 82% (pD 10.6–11) in the presence of Al(III). At the physiological pH the extent of aldimine formation is ~10% (zinc) and ~29% (aluminum), but is undetectable in the metal-free system.

(iv) Exchange between complexed and free aldimine in the Zn(II) system is fast on the pmr time scale. The pD dependencies of the chemical shifts are interpreted in terms of the formation of 1:1:1 and 2:2:1 complexes of probable structure **12–13** and **14**, respectively. Formation of the 2:2:1 species is favored at pD ~8–11.

(v) Exchange between complexed and free aldimine in the Al(III) system is slow on the pmr time scale and has permitted separate detection of 1:1:1 and 2:2:1 complexes. The multiplicities and pD dependencies of the chemical shifts of the 2-CH_3 signals require two distinct 1:1:1 and 2:2:1 species. Probable structures of the former species are the structural isomers **15**, **16** and **17**, **18** while active and *meso* diastereoisomers of structure **19** are proposed for the two 2:2:1 species formed from DL-alanine.

(vi) No exchange of the α proton of condensed alanine with D_2O solvent has been observed by pmr in the metal-containing or metal-free systems at pD ≤12 for periods as long as 6 hr at ~27°. Racemization of the amino acid *via* aldimine formation must proceed at a negligible rate under these conditions.

(vii) DL-Alanine-β-d_3 may be prepared conveniently and in ≥90% isotopic purity by equilibration of a 10:1 mole ratio of sodium pyruvate and pyridoxamine in D_2O followed by catalytic hydrogenation of the pyruvate oxime.

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