A Proton Resonance Investigation of Equilibria, Solute Structures, and Transamination in the Aqueous Systems Pyridoxamine–Pyruvate–Zinc(II) and –Aluminum(III)

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Abstract: As part of a proton resonance investigation of the mechanism of nonenzymatic transamination, the pmr spectra of systems initially containing 0.1 *M* pyridoxamine and 0.1 *M* pyruvate in the presence and absence of 0.05 *M* Zn(II) and Al(III) in D_2O solution at pD 1–13 have been examined. Formation of the ketimine Schiff base, pyruvidene-N-pyridoxamine, in the metal-free system and complexes containing one and two complexed ketimine species are observed. Both free and complexed ketimine undergo H–D exchange at the pyruvidene methyl carbon. The presence of metal ions effects greater stabilization of ketimine over a wider pD range, due to complexation, compared to the metal-free system. Exchange between coordinated and free ketimine in the Zn(II) system is fast on the pmr time scale and slow in the Al(III) system. Certain of the acid–base equilibria of free ketimine and the Zn(II) and Al(III) complexes have been investigated using the pD dependencies of chemical shifts. The Zn(II) and Al(III) ketimine complexes convert at rates which qualitatively increase with increasing pD to new species which have been definitely identified by their characteristic pD dependencies of chemical shifts to be the corresponding aldimine complexes produced by transamination. The results of this study together with those of a previous investigation of pyridoxal–alanine–Zn(II) and –Al(III) systems serve to provide further definition of the course of nonenzymatic transamination and, therewith, offer strong confirmatory evidence for the proposed mechanism of this reaction.

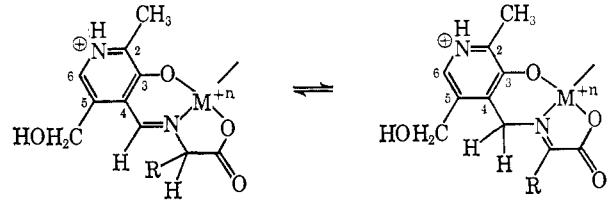
The generally accepted mechanism[2,3] for the transamination reaction between α-amino acids and α-keto acids catalyzed by pyridoxal cofactors involves the sequential coupling of reactions 1 and 2 to produce the net transformation 3. It is well established that a

$$\mathrm{RCH(NH_2)COOH} + \text{pyridoxal} \rightleftharpoons \mathrm{RCOCOOH} + \text{pyridoxamine} \quad (1)$$

$$\text{pyridoxamine} + \mathrm{R'COCOOH} \rightleftharpoons \mathrm{R'CH(NH_2)COOH} + \text{pyridoxal} \quad (2)$$

$$\mathrm{RCH(NH_2)COOH} + \mathrm{R'COCOOH} \rightleftharpoons \mathrm{R'CH(NH_2)COOH} + \mathrm{RCOCOOH} \quad (3)$$

variety of metal ions enhance the rates of transamination reactions compared to the metal-free situation, and extensive discussions of the roles of metal ions and the vitamin B_6 group of compounds, together with their analogs and homologs, in the catalysis of these reactions are available elsewhere.[4–7] Nonenzymatic transamination has been studied in a large number of model systems initially containing pyridoxal or pyridoxamine in the presence and absence of metal ions. Although in at least one case[8] metal ions are not implicated in enzymatic transamination, their function in model systems is a matter of continuing interest.[7] Metal ions may simulate certain of the features of enzymatic active sites in the sense of facilitating Schiff base formation in reactions 1 and 2, perhaps by a template effect, and, more importantly, labilizing the α-hydrogen of the condensed amino acid in the aldimine Schiff base formed in reaction 1. The aldimine–ketimine conversion involving the species **1** $\rightleftharpoons$ **2** is an essential feature of the proposed mechanism of metal ion-catalyzed transamination occurring near the physiological pH. Formation of the



1 **2**

pyridoxylidene complex **1**, followed by rearrangement to the ketimine tautomer **2** and its hydrolysis, completes reaction 1. Reaction 2, the reverse of reaction 1, involves formation of a new ketimine complex succeeded by tautomerization and hydrolysis, producing the net reaction 3.

Because certain of the steps in the over-all transamination sequence proceed at favorably slow rates, we have carried out by nmr a rather thorough study of the separate reactions 1 and 2 with the purpose of detecting and identifying the major species formed. The systems chosen for investigation are pyridoxal–alanine and pyridoxamine–pyruvate in the presence and absence of Zn(II) and Al(III) in D_2O solutions over the pD range 1–13. These metal ions have been selected because of their relatively high efficiency among diamagnetic ions in catalyzing transamination in the pyridoxamine–α-ke-

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toglutarate system.[9] Reactions 1 and 2 have been studied at ambient temperature under conditions such that any metal complexes of types **1** and **2** could be detected by proton resonance measurements.

Our pmr investigation of reactions 1 and 2 in the presence of metal ions has been carried out in several stages. In each the pD range employed is *ca.* 1–13 and the mole ratio of reactants pyridoxal or pyridoxamine: alanine or pyruvate is 1:1 in the metal-free and 2:2:1 (Zn(II), Al(III)) in the metal-containing systems.

(i) The spectra of pyridoxal and pyridoxamine have been obtained as a function of pD and the principal solute species identified.[10]

(ii) The studies in (i) have been repeated in the presence of alanine[11] and pyruvate[12] in order to obtain the spectra of the aldimine and ketimine Schiff bases, respectively, and to identify major species.

(iii) Spectra of the two-component systems examined in (ii) have been remeasured in the presence of Zn(II) and Al(III) in order to identify the metal complexes formed[11,12] and to observe directly the tautomeric rearrangement **2** → **1** proposed to occur when reaction 2 is carried out in the presence of metal ions.[2,3]

Investigations comprising stage i and those involving the metal-free and metal-containing pyridoxal–alanine systems in stages ii and iii, respectively, are complete and full details have recently been published.[10,11,13]

The study described in this report is directed toward an elucidation of reaction 2 in the presence and absence of metal ions. As in a previous study of reaction 1,[11] the information sought includes the extent of Schiff base formation and the nature of solute structures as functions of pD. Unlike the aldimine Schiff base complexes formed in reaction 1,[11] which are kinetically stable with respect to the conversion **1** → **2** at ambient temperature, the ketimine complexes **2** undergo reaction to yield **1** at a convenient rate such that both are readily detected by pmr. As will become evident, certain details of the tautomeric conversion of the metal complexes bear a possible resemblance to those of enzymatic transamination.

The results described herein are complementary to our previous investigation[11] of the pyridoxal–alanine–Zn(II), –Al(III) systems which furnishes the necessary background for this study. Other investigations of reaction 2, carried out principally by spectrophotometry or product analysis, have yielded information on rates, equilibria, and extent of transamination. Systems studied include pyridoxamine or an analog and pyruvate,[14–17] α-ketobutyrate,[17] α-ketoglutarate,[3,9] glyoxylate,[18,19] and α-ketoisovalerate[20–22] among other α-keto acids, as well as pyridoxal,[23] in the presence[15–19,21–23] and absence[14,20] of metal ions. This report, together with that[11] preceding it, serves to define those details of the individual reactions 1 and 2 which are amenable to establishment by pmr and thereby contributes to a more specific description of the over-all transamination reaction 3. Several of the more important findings in the present investigation have been communicated recently.[12]

Experimental Section

Preparation of Solutions. Commercial pyridoxamine dihydrochloride (Calbiochem, Nutritional Biochemicals Corp.) and dimer-free sodium pyruvate (Sigma Chemical Co.) were employed without further purification. No impurities in either compound could be detected in the pmr spectra in D_2O solution. Samples of sodium pyruvate were found to be $\geq 99\%$ dimer free by pmr. 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 for the proton resonance studies were prepared in 99.5% D_2O by adding pipetted quantities of fresh pyridoxamine, pyruvate, and metal ion solutions to a volumetric flask followed by pD adjustment using NaOD solutions and dilution to known volume. All solutions were initially 0.1 *M* in both pyridoxamine and pyruvate and 0.05 *M* in metal ion. Spectra of solutions were recorded immediately after preparation and at 5–10 min intervals thereafter until no changes could be observed over a period of *ca.* 2 hr. The pD of each solution was monitored at the times its spectra were recorded. Unless otherwise indicated, the pmr results referred to in subsequent sections derive from spectra showing the constancy with time just mentioned. In particular, the spectra displayed in Figures 1, 4b, and 6b,c are of this type. If certain of the metal-containing solutions were allowed to stand overnight, precipitation and loss of signal intensity due to extensive deuteration were observed. Apparent pH values were measured using Beckman Model G or Radiometer PHM-26 pH meters and pD values obtained from the relation pD = pH + 0.40.[24]

Proton resonance spectra were determined using a Varian HA/HR-100 spectrometer. Chemical shifts were measured to at least ±1 cps by the usual side-band techniques 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

Pmr spectra of D_2O solutions initially 0.1 *M* in both pyridoxamine and pyruvate and 0.05 *M* in Zn(II) or Al(III) have been determined over the pD range 1–13. Spectra of the metal-free system in the acidic and alkaline regions are given in Figure 1 and those of the metal-containing systems under various conditions of time after preparation and pD are shown in Figures 4 and 6. Plots of the pD dependencies of the chemical shifts of free pyridoxamine and ketimine and of the Zn(II) and Al(III) ketimine complexes are set out in Figures 3, 5, and 7. The per cent ketimine formation in the presence and absence of Zn(II) is depicted in Figure 2. As demonstrated for related systems[10,11] plots of the type shown in Figures 3 and 5 are often useful in detecting acid–base equilibria and for determining at least approximate pK_a values, which are frequently in good agreement with those obtained by titrimetry or spectrophotometry in H_2O solutions. Such values determined

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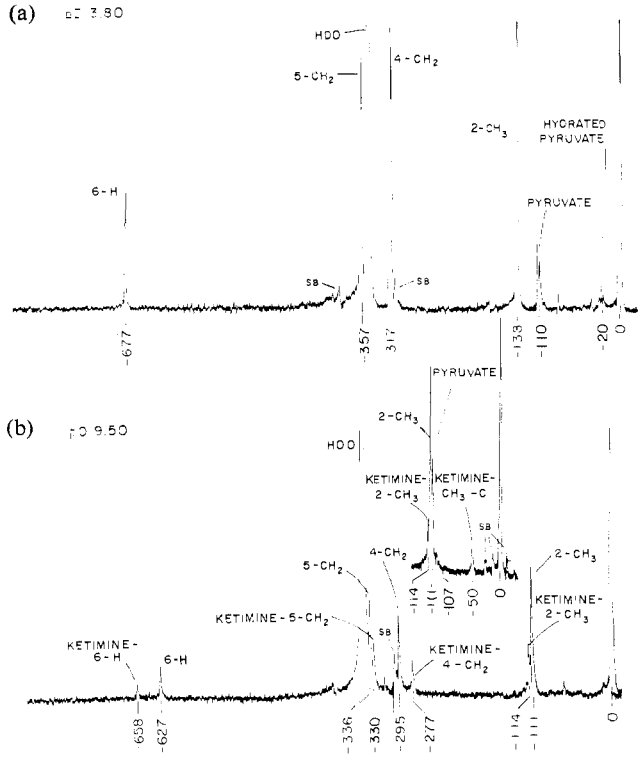

Figure 1. Pmr spectra (100 MHz) of D_2O solutions initially 0.1 M in both pyridoxamine and pyruvate illustrating pyruvate methyl exchange with solvent at undetectable concentration of ketimine in acidic solution (pD 3.80) and partial ketimine formation with pyruvate methyl exchange in alkaline solution (pD 9.50). Insert: high-field region in H_2O solution. Unprefixed signals refer to pyridoxamine; SB, side band.

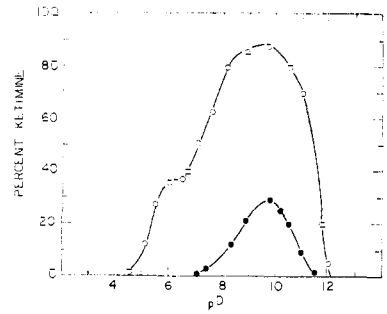

Figure 2. Per cent ketimine formation in D_2O solutions initially 0.1 M in both pyridoxamine and pyruvate: ●, metal-free system; ○, in the presence of 0.05 M Zn(II).

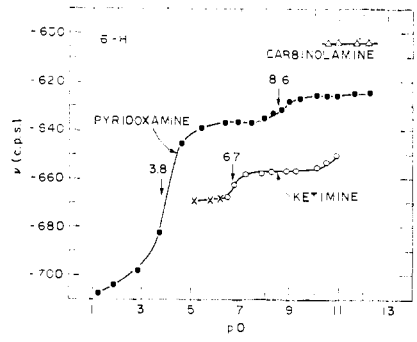

Figure 3. pD dependencies of the 6-H chemical shifts (100 MHz) of pyridoxamine, ketimine, and carbinolamine: ×, ketimine in slow equilibrium with Al(III) complexes (see text). Solutions were initially 0.1 M in both pyridoxamine and pyruvate.

in D_2O and H_2O are related by $pK(D_2O) = pK(H_2O) + 0.40$.[24]

The System 1:1 Pyridoxamine:Pyruvate. The pmr properties of this system may be conveniently considered in terms of the pD ranges 3–7, 7–10, and 10–12. The species **3**–**11** referred to in the following discussion are to be found in Chart I. This system has previously been studied spectrophotometrically under slightly different conditions by Banks, Diamantis, and Vernon;[14] certain of their equilibrium constant data are included in Chart I.

pD 3–6.5. Below pD 6.5 all observable signals are assignable to uncondensed pyruvate and pyridoxamine. In this pD interval the spectrum of free pyridoxamine consists of four sharp, featureless lines of relative intensity 1:2:2:3 from low to high field which are assigned as indicated in Figure 1a. From pD 3.8 to 8.6 species **3** is the predominant form of pyridoxamine.[10] However, in the range pD 3–6.5 the averaged signals of pyruvate anion and molecular pyruvic acid, both of which are partially hydrated in acidic and neutral solution,[25,26] decrease in intensity with time and are completely exchanged with solvent after several hours. In the absence of pyridoxamine this effect is not observed under the same conditions of pD and time, indicating that the exchange proceeds *via* the ketimine species **6** and **7**, which are present in concentrations too low to be detected by pmr up to pD ~6.5.

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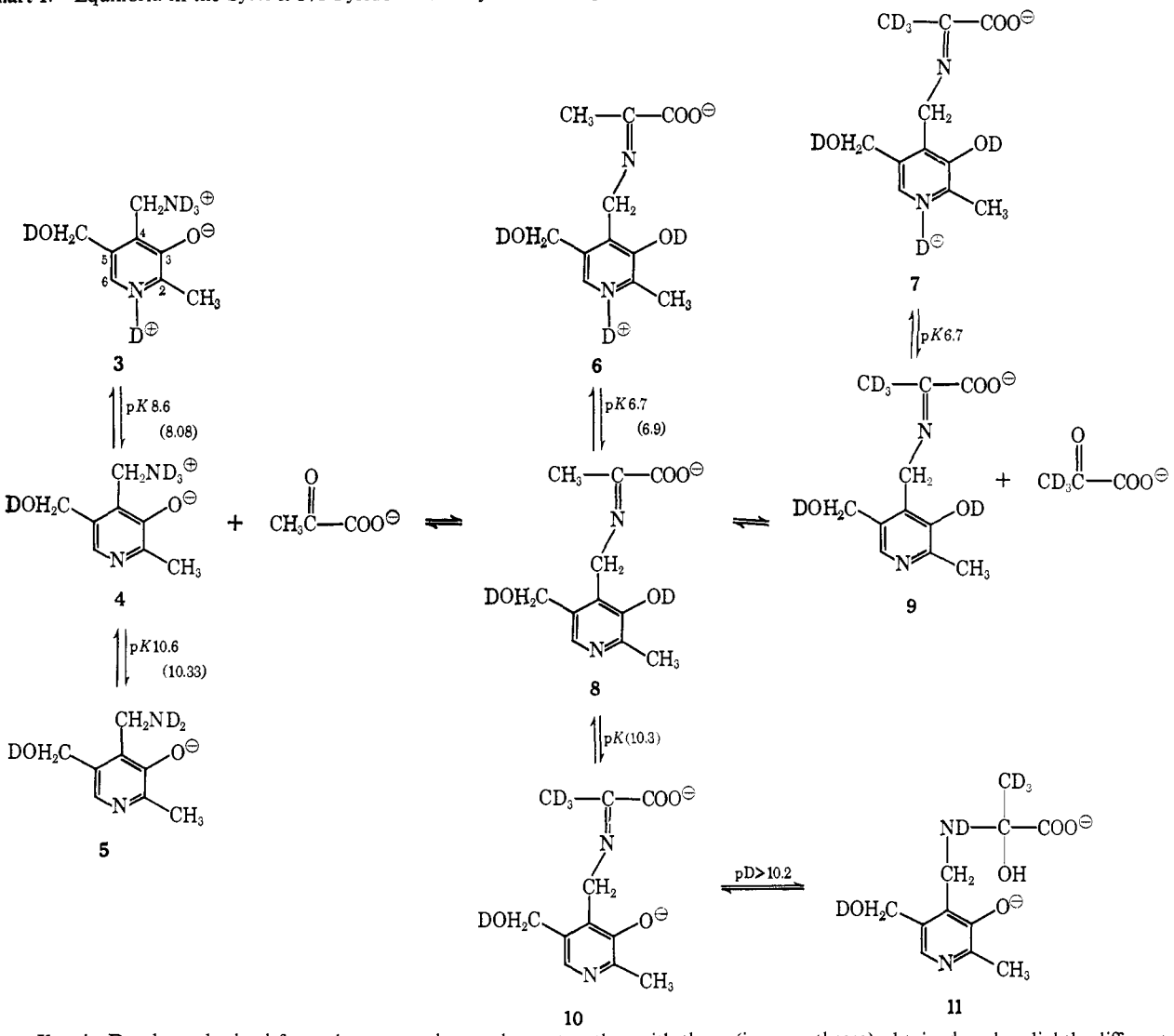
pD 6.5–10. At and above pD ~6.5 pyruvidene-N-pyridoxamine (ketimine) formation is evidenced by the appearance of new 2-CH_3, 4-CH_2, 5-CH_2, and 6-H signals, which occur near the related resonances of pyridoxamine, and a signal at *ca.* −50 cps in H_2O solutions which is due to the pyruvidene methyl group. As the pD is raised, the signals of the four pyridine ring substituents increase in intensity and shift upfield. Because of undetectable exchange among ketimine, pyruvate, and pyridoxamine on the pmr time scale, some information concerning acid–base equilibria of the ketimine forms can be obtained from the pD dependence of chemical shifts. In addition, the range of direct observation of ketimine can be extended down to pD ~5 because of slow exchange with its Al(III) complexes (*vide infra*). The 6-H shift is most useful in this respect and its pD dependence is given in Figure 3. An inflection is observed at pD 6.7 and corresponds to the first of two deprotonations of the ketimine[14] (other than ionization of the carboxyl group which must occur at a much lower pD). The sensitivity of the 6-H shift to neutralization of the pyridinium function[10] strongly suggests the equilibria **6** ⇌ **8** and **7** ⇌ **9** with $pK \sim 6.7$ for both. The invariance of the 4-CH_2 shift over this pD interval and in more alkaline solutions where the ketimine undergoes its final deprotonation[14] indicates that the immonium tautomers of the various ketimine species are probably not present in significant concentrations. A pK value of 6.9 has been previously obtained for the process **6** ⇌ **8** in H_2O solution.[14]

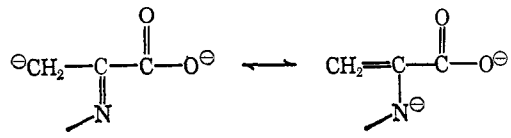
The per cent formation of total ketimine as a function of pD is readily evaluated above pD 7 in metal-free systems by integration of the 6-H signals of pyridox-

Chart I. Equilibria in the System 1:1 Pyridoxamine–Pyruvate in D_2O Solution[a]



[a] pK and pD values obtained from the pmr study are shown together with those (in parentheses) obtained under slightly different conditions by spectrophotometric measurements in H_2O solutions.[14] The undeuterated form of **10** is omitted.

amine and ketimine, and at pD 9.8 reaches a maximum of 30 ± 5% of total pyridoxamine initially present before decreasing to zero in strongly basic solutions. The results are set out in Figure 2. A spectrum near the pD maximum of ketimine formation is shown in Figure 1b together with the spectrum of the high-field region in H_2O solution (pH 9.10). Under these conditions the principal form of free pyridoxamine is **4**. The spectrum in D_2O solution, recorded several minutes after preparation, shows no signals due to pyruvidene methyl groups or free pyruvate in any form,[26] indicating that the apparent rate of methyl proton–deuteron exchange is much greater in alkaline than in neutral or acidic solutions. This observation is suggestive of the intervention of a resonance-stabilized anion having the functionality **12** → **13** in the H–D exchange process.



12 **13**

pD 10–13. Above pD 10 intensities of the ketimine signals decrease markedly and are undetectable at pD >11.5. Accompanying this decrease is the appearance of features due to free pyridoxamine, the major form of which is **5** above pD 10.6, and new, weak 2-CH_3 and 6-H signals. These are first observable at pD ~10.2, persist to the limit of measurement, pD 13, and are independent of pD (*cf.* Figure 3). They are tentatively assigned to the carbinolamine form **11**, whose concentration does not exceed *ca.* 10% of total pyridoxamine initially present. The terminal ionization of the ketimine also occurs in this pD range producing the hydrogen analog of **10**.[14] A pK value of 10.3 for this reaction has been determined in aqueous solution.[14] The pmr results do not clearly reveal this final deprotonation due to the rapid decrease in ketimine concentration above pD ~10.7, a reasonable estimate of the pK value in D_2O solution. As shown in Figure 3, the 6-H shift of the ketimine undergoes a definite decrease in the pD 10–11 range. Unfortunately, the inability to detect the 6-H signal above pD 11 under the conditions of concentration employed prevents a pmr measurement of the pK value.

The equilibrium scheme for the pyridoxamine–pyruvate system which is consistent with the proton resonance results is given in Chart I.

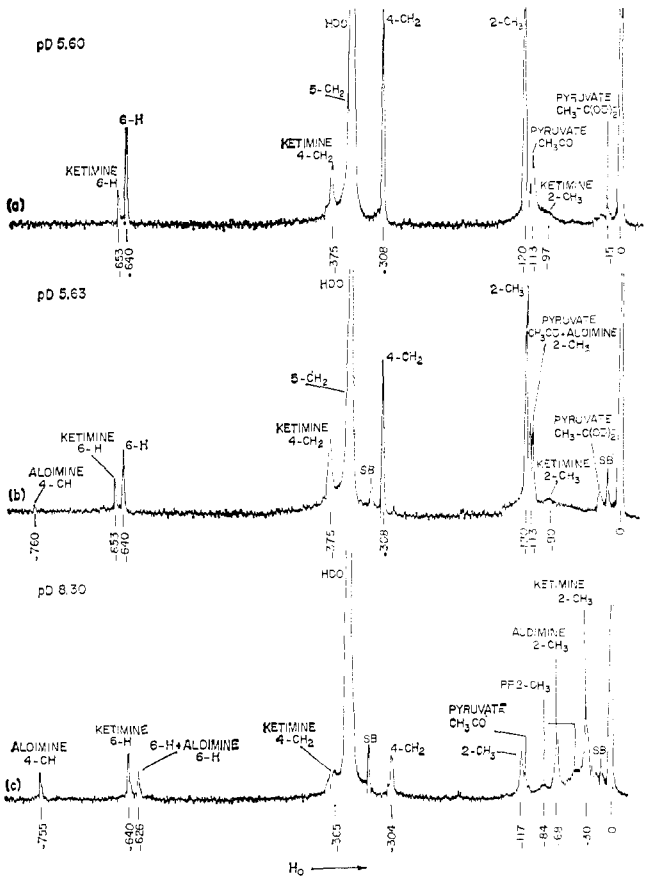


Figure 4. Pmr spectra (100 MHz) of D_2O solutions initially 0.1 *M* in both pyridoxamine and pyruvate and 0.05 *M* in Zn^{2+} (a) before transamination illustrating formation of ketimine species; (b) same solution as (a) after transamination revealing the presence of both ketimine and aldimine species; (c) first obtainable spectrum at pD 8.30 showing that transamination has taken place. Unprefixed signals refer to pyridoxamine; SB, side band; PP, N-pyridoxylidene-pyridoxamine.

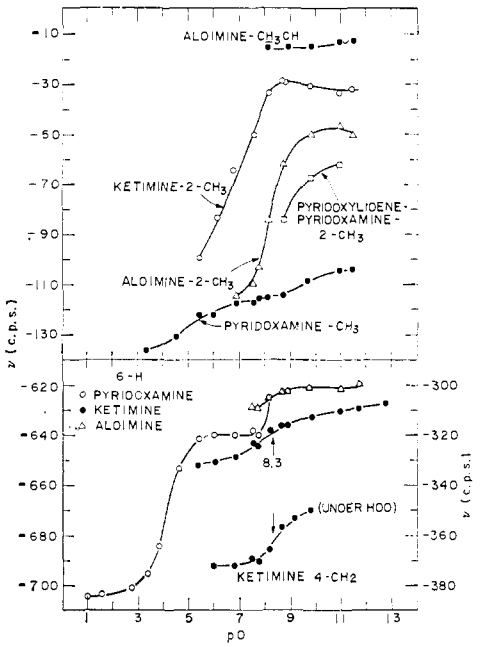


Figure 5. Pmr chemical shifts (100 MHz) as a function of pD in solutions initially 0.1 *M* in both pyridoxamine and pyruvate and 0.05 *M* in Zn^{2+}; data refer to transaminated solutions. The ketimine 4-CH_2 shifts are referred to the right-hand scale.

The System Pyridoxamine–Pyruvate–Zinc (II). The pmr data obtained in a study of this system reveal formation of ketimine complexes and direct detection of that part of reaction 2 involving the conversion **2** → **1** of the tautomeric zinc complexes. Results relating to the formation of ketimine complexes are discussed first in part a, which deals with the nature and extent of formation of ketimine species in the pD ranges 3–6.5, 6.5–10, and 10–13. Solutions in which transamination is observed are considered in part b. The basis for considering the system in terms of three pD regions is evident from the pD dependence of total ketimine formation shown in Figure 2. The concentration of ketimine increases rapidly from pD 4.5 to 6.0 and shows a small leveling off at pD 6.0–6.5, and thereafter a second rapid increase to pD 9.8, followed by a sharp decrease to zero beyond pD 12. The results in this figure were obtained by integration of the pyridoxamine and ketimine 6-H signals.[27] Because the reaction of any aldimine to ketimine species is at least several orders of magnitude slower than ketimine formation under the experimental conditions,[11] comparison of integrated intensities of the ketimine 6-H signal to the sum of this and the pyridoxamine 6-H signal provides a simple estimate of the extent of ketimine formation from pD 4.5 to 8.0. Above pD 8.0 the 6-H signals of pyridoxamine and aldimine species, produced by transamination (*vide infra*), overlap. Reduction of the intensity of the coincident signals by an amount equal to the aldimine 4-CH intensity permits approximate calculation of the extent of ketimine formation in the pD 8–13 region.

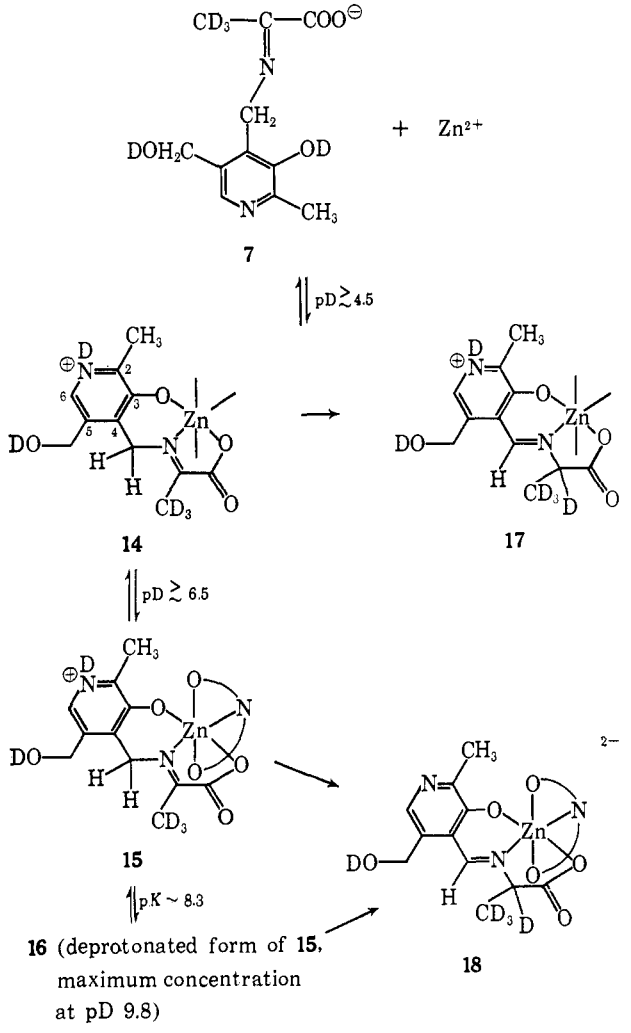
The species **14–18** referred to below may be found in Chart II.

The only previous detailed study of nonenzymatic transamination in the presence of zinc(II) has led to a definition of the kinetics and equilibria of the pyridoxamine–α-ketoisovalerate system in methanol solution.[21,22]

(a) pD 3–6.5. Below pD ~3 the only signals observed are those of free pyridoxamine (**3**) and pyruvate. Above this value exchange of pyruvate methyl protons is evident, as in the metal-free system, indicating the formation of ketimine species in undetectably small concentrations. In solutions having pD ≥4.5 ketimine formation is detectable by the appearance of sharp 6-H and 4-CH_2 signals. The spectrum of a freshly prepared solution having pD 5.60 is shown in Figure 4a. As pD is raised these signals increase in intensity and shift upfield. The ketimine 2-CH_3 signal behaves similarly except that its chemical shift is more strongly pD dependent and its line width is much larger. The pD dependencies of these three signals are given in Figure 5. In this or the higher pD ranges only one set of ketimine signals is observed, indicating rapid exchange between the free and complexed forms. Closely related observations have been made for the pyridoxal–alanine–zinc(II) system[11] in which, particularly, the 2-CH_3 signal of the aldimine species exhibits a similar line width and chemical shift behavior attributable to labile complex formation. From Figure 2 it is evident that the rise in total ketimine formation takes place in

(27) The same information could be obtained from integration of appropriate methyl signals. However, unfavorable signal overlap in this region, especially in alkaline solutions (*cf.* Figure 4), renders this procedure less feasible than that described.

Chart II. Equilibria and Transamination in the System 2:2:1 Pyridoxamine–Pyruvate–Zn(II) in D_2O Solution[a]



[a] The various forms of pyridoxamine, aldimine, and ketimine in equilibrium with the complexes are not shown.

two steps, the first of which is defined by ~38% formation at pD 6.5. In view of this result, together with the chemical shift behavior just noted, it is proposed that in this pD region the 1:1:1 complex[28] **14** is formed in rapid equilibrium with the free ketimine **7**, which in turn is in slow equilibrium with pyridoxamine (**3**) and pyruvate.

pD 6.5–10. In this region the ketimine signals continue to increase in intensity and shift upfield (*cf.* Figure 5) concomitant with a progressive decrease in intensity of pyridoxamine features. A spectrum at pD 8.30 is shown in Figure 4c. Two aspects of the pmr behavior of the ketimine species are of particular interest. The 2-CH_3 signal continuously shifts upfield and narrows until at pD 9–10 both the line width and chemical shift remain essentially constant. It is in this region that the extent of ketimine formation ceases to change rapidly and reaches a maximum of ~88% of pyridoxamine initially present at pD 9.8. In addition, as the pD is increased both the 4-CH_2 and 6-H shifts exhibit an inflection at pD ~8.3, as indicated in Figure 5. These results are consistent with a much higher degree of complexation of Zn^{2+} by ketimine than in the lower pD region, with maximum complex formation preceded by a deprotonation reaction. It is proposed that above pD ~6.5 the 2:2:1 species **15** is formed in increasing concentration, which is deprotonated (apparent $pK \sim 8.3$) to form the complex **16** whose concentration reaches a maximum at pD 9.8. Above pD ~8.6 in this range the pyridoxamine species **4** is in slow equilibrium with **16** and free ketimine. The large chemical shift differences between the 4-CH_2 and, in particular, the 2-CH_3 signals in the metal-free and zinc-containing systems are indicative of the formation of a pyridoximino chelate ring in **14**, **15**, and **16**. Complete chelation of the metal in **15** and **16** as a result of coordination of the pyruvidene carboxylate groups as shown in these structures cannot be proved by the pmr results and is assumed on the basis of structural determinations of related bis(pyridoxylidene)manganese(II)[29] and -zinc(II)[30] complexes.

pD 10–13. In this region the intensities of the ketimine signals decrease sharply with increasing pD and are undetectable beyond pD 12. Coincident with this decrease is the appearance of pyridoxamine signals which are somewhat broadened and shifted compared to the free pyridoxamine species **4** and **5** in this pD region. The same behavior is observed in the 2:1 pyridoxamine–Zn^{2+} system and, as in that case, is ascribed to a labile zinc(II)–pyridoxamine complex, with respect to which ketimine complexes are evidently unstable in strongly alkaline solution. Previous titration[31] and spectrophotometric[20,21] studies have demonstrated the existence of zinc(II)–pyridoxamine complexes.

The equilibrium scheme for the pyridoxamine–pyruvate–zinc(II) system deduced from the pmr data is shown in Chart II.

(b) Transaminated Solutions. The conversion **2** → **1** of ketimine to aldimine complexes is an integral feature of reaction 2 and, hence, of the mechanism of metal ion catalyzed nonenzymatic transamination.[2,3] This reaction may be directly observed by pmr in the range pD 5–11. From pD 5 to 7 transamination is quite slow and aldimine species are first detected after *ca.* 30 min. The spectrum in Figure 4a was recorded within several minutes after solution preparation and, except for an amount of unexchanged pyruvate in the keto and hydrated forms, displays features due to pyridoxamine and ketimine species only. However, the same system after 2 hr at 27° affords the spectrum shown in Figure 4b, in which the aldimine 2-CH_3 and 4-CH signals are clearly observable. Above pD 7 transamination is relatively more rapid, as illustrated by the spectrum in Figure 4c. The first recordable spectrum of a solution having an initial pD of 8.30 clearly indicates appreciable aldimine formation.[32] Signal assignment of aldimine resonances from pD 5 to 11 may be unambiguously made from the known pD dependencies of chemical shifts in the 2:2:1 pyridoxal–alanine–Zn(II) system.[11] The aldimine 2-CH_3 signal is

(28) The designation 1:1:1 and 2:1:1, used subsequently, refers to the combining ratios of pyridoxamine–pyruvate–metal ion in the complexes in which the first two components are present in the ketimine form. Aldimine complexes have previously been designated similarly.[11]

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

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

(31) R. L. Gustafson and A. E. Martell, *Arch. Biochem. Biophys.*, 68, 485 (1957).

(32) Other pmr spectra demonstrating the conversion of ketimine to aldimine complexes are given in ref 12. Note that in Figure 1b of this reference the 6-H signals at −637 and −645 cps were erroneously labeled as due to ketimine and pyridoxamine, respectively. The reverse assignment is the correct one.

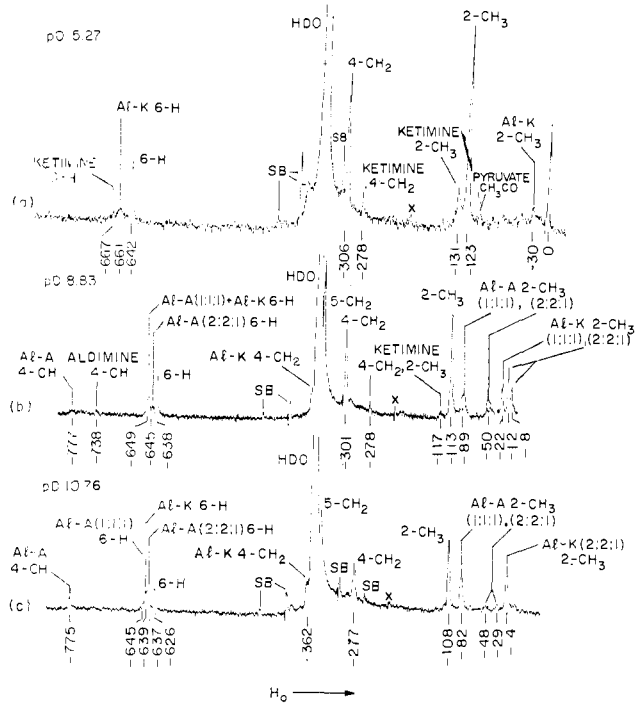


Figure 6. Pmr spectra (100 MHz) of D_2O solutions initially 0.1 *M* in both pyridoxamine and pyruvate, 0.05 *M* in Al(III): (a) fresh solution showing the formation of free ketimine and the 1:1:1 Al complex; (b, c) spectra revealing transamination of Al–ketimine complexes to yield 1:1:1 and 2:2:1 Al–aldimine species. Unprefixed signals refer to free pyridoxamine: Al–A, aluminum aldimine, Al–K, aluminum ketimine complex; SB, side band; ×, impurity.

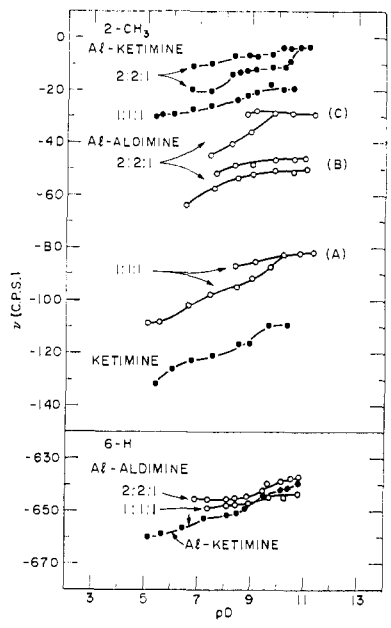


Figure 7. Pmr chemical shifts (100 MHz) as a function of pD in solutions initially 0.1 *M* in both pyridoxamine and pyruvate and 0.05 *M* in Al(III): upper, 2-CH_3; lower, 6-H signals. Data refer to transaminated solutions. A, B, and C are designations of the sets of Al–aldimine 2-CH_3 signals used in ref 11. The ketimine shifts are those of the free base in slow equilibrium with the aluminum complexes.

particularly characteristic of complex formation in this system. The chemical shifts were measured in the transaminated solutions over the pD 7–11 interval (*cf.* Figure 5) and are in close agreement with those measured for the system in which only aldimine species are formed.[11]

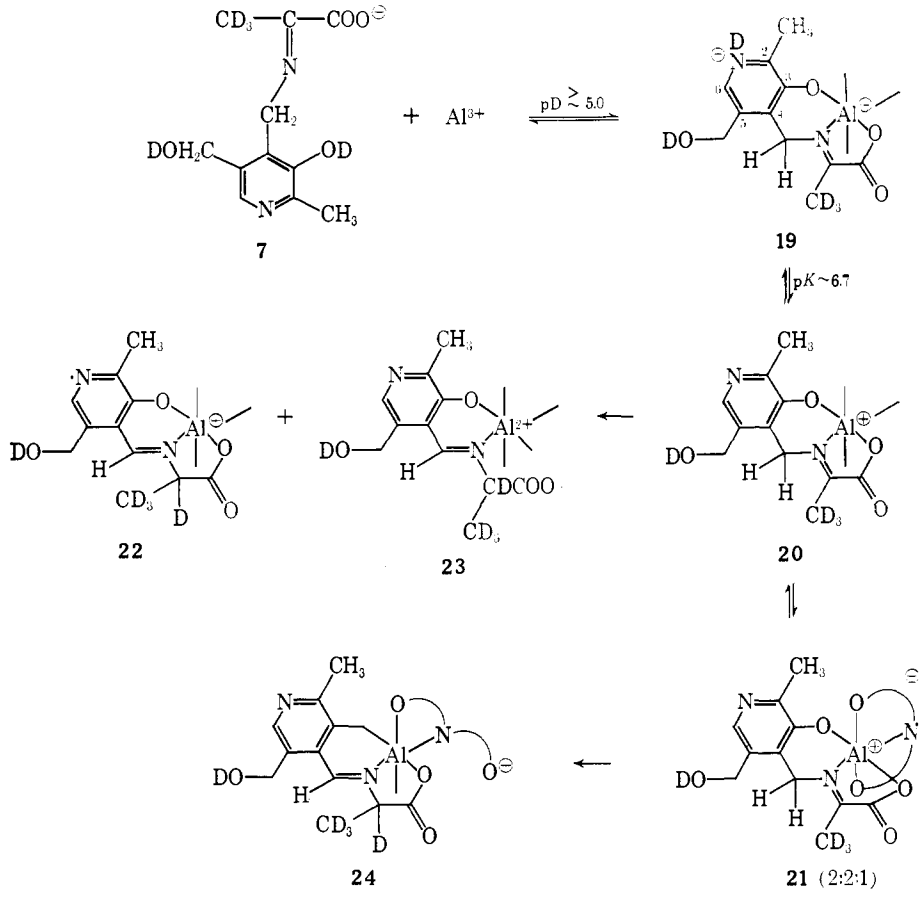
The foregoing results are interpreted in terms of the conversion of **14** to the 1:1:1 aldimine complex **17** below pD ~6.5. Above this pD and especially in the pD 9–10 region the complexes **15** and **16** are considered to convert to the 2:2:1 complex **18**, which exhibits maximum stability in this region in the pyridoxal–alanine system. Completion of reaction 2 involves hydrolysis of the complexes **17** and **18** to pyridoxal and alanine, a step which is evidently slow under our experimental conditions. In no case were signals due to free pyridoxal[10] detected after 4 hr at 27°. However, in alkaline solution two new, weak signals appear in the high-field region (*cf.* Figure 4c[32]) whose pD dependencies of chemical shift may be duplicated in the system 1:1:1 pyridoxamine–pyridoxal–Zn(II). These features are ascribed to the formation of a small amount of a zinc(II) complex of N-pyridoxylidenepyridoxamine, which arises either from an amine exchange reaction involving **18** and free pyridoxamine, present in detectable amounts throughout the entire pD region of study, or by condensation of the latter with pyridoxal produced by partial hydrolysis of **18**.

The System Pyridoxamine–Pyruvate–Aluminum(III). This system was examined in order to provide a second example of the direct observation of transamination by pmr. Its spectra, both before and after transamination, are more complicated than those of the corresponding zinc(II) system because of the larger number of species detectable due to slow exchange between Al–ketimine and –aldimine complexes and their free ligands. Spectra at different pD values are given in Figure 6. As in the preceding two systems the chemical shifts of all species increase with increasing pD, as shown in Figure 7, and the proportions of ketimine and aldimine, free and complexed, also become larger. The 2-CH_3 and 6-H signals are most useful for following ketimine formation; 4- and 5-CH_2 Al–ketimine resonances are frequently obscured by the intense HDO absorption. Signal assignments, especially those in the complicated 6-H and 2-CH_3 regions, were made on the basis of pD dependencies of chemical shifts in the metal-free ketimine and aldimine[11] and in the Al–aldimine[11] systems. Chemical shifts of the 2-CH_3 and 6-H features of the various species detected in the transaminated solutions have been traced through pD intervals sufficient for signal assignments, and the results are shown in Figure 7. Quantitative measurement of the pD dependence of ketimine formation was not carried out because of unfavorable signal overlap and low signal intensities. Several studies of the pyridoxamine–Al(III) system with pyruvate[16,17] and pyridoxal[23] have been reported but are less detailed than this one.

Below pD 3 the pmr spectra reveal only free pyridoxamine (**3**) and pyruvic acid. Spectra in the higher pD ranges are considered next. The species **19–24** mentioned below are set out in Chart III.

pD 3.0–6.5. Above pD 3.0 pyruvate methyl proton exchange with solvent is evidenced by slow decrease of signal intensity with time. Ketimine and its 1:1:1 Al(III) complex are first detected at pD 5.0. The spectrum at pD 5.27 shown in Figure 6a is similar to others at pD >5.0 in this range. The signal of a residual

Chart III. Equilibria and Transamination in the System 2:2:1 Pyridoxamine–Pyruvate–Al(III) in D_2O Solution[a]



[a] The various forms of pyridoxamine, aldimine, and ketimine in equilibrium with the complexes are not shown.

amount of unexchanged pyruvate is observable together with new features due to free and complexed ketimine which are assigned as indicated. In this pD interval one set of 2-CH_3 and 6-H signals arising from an Al–ketimine species is evident. Because less than *ca.* 20% of pyridoxamine is converted to ketimine in this interval. it is reasonable to assume that the species formed is the 1:1:1 complex **19**. No significant transamination was observed over a period of *ca.* 3 hr.

pD 6.5–8.5. The spectra in this region are much more complex than in other pD regions, especially in the −150 to 0 cps range where several signals could not be identified. Two new 2-CH_3 signals appear slightly upfield from that assigned to the 1:1:1 complex and increase in intensity with rising pD. These signals are associated with a 2:2:1 complex **21**[33] because their intensity changes parallel the increase in total ketimine and decrease in pyridoxamine concentration in fresh solution. Only one resolvable 6-H signal of Al–ketimine species is observed in this and the higher pD region. The 6-H chemical shift reveals an inflection at pD ~6.7 which is attributed to deprotonation of **19**, by far the predominant ketimine complex at this point, to yield **20**. Transamination is readily observed in this region. The 2-CH_3, 4-CH, and 6-H signals arising from free aldimine and its 1:1:1 (**22**, **23**) and active and *meso* 2:2:1 (**24**)[33b] complexes are detectable; their pD dependencies (Figure 7), when compared with data from the pyridoxal–alanine–Al(III) systems, are in good agreement and serve to identify unambiguously these signals. Evidence for the various structural forms and diastereoisomers of the Al–aldimine species in this and the following pD range is considered in detail elsewhere.[11]

pD 8.5–11.2. Spectra in this region are less complex and all resonances have been identified. Typical spectra are presented in Figures 6b and c. These show that significant transamination has occurred. In this pD range both the rate and extent of transamination are greater than those in lower ranges; no reliable quantitative measure of either was possible. The spectrum in Figure 6b reveals the presence of both free aldimine and its 1:1:1 and 2:2:1 complexes. As the pD is raised, the concentrations of free aldimine and 2:2:1 complexes decrease such that at pD 10.8 (Figure 6c) none

(33) (a) The cause of two 2-CH_3 signals for this presumed species is not known. The corresponding 2:2:1 Al–aldimine complex shows a similar behavior, which was explained in terms of one tridentate and one bidentate chelate grouping in the same complex (24).[11] That explanation may apply here. However, the unequal intensities of the two signals (*cf.* Figure 6b) found in the pD range (6.5–10.5, Figure 7) where both signals are observable implies two structurally distinct 2:2:1 complexes, one of which may contain two types of chelate groupings. For simplicity the 2:2:1 complex is represented in Chart III as the completely chelated form **21**. (b) Note Added in Proof. The previous statement[11] that four 2-CH_3 signals of a 2:2:1 complex are incompatible with a completely chelated structure such as **18** is incorrect provided that the rate of racemization of the complex is slow on the nmr time scale. Thus a completely chelated form in addition to one containing a dangling carboxylate group must be considered for monomeric 2:2:1 species. Our data do not permit a clear decision between these two structures to be made at present. If the former is correct, **24** should be replaced by a structure analogous to **18**. Whatever the true nature of the 2:2:1 aluminum–aldimine species is, its 2-CH_3 signal multiplicity and pD dependence of chemical shifts serve to identify it as a transamination product.

of the former and only small amounts of the latter are observed. The predominant species are pyridoxamine (**5**), 1:1:1 Al–aldimine complexes, and the 2:2:1 Al–ketimine complex (**21**), which at this pD account for *ca.* 60% of the pyridoxamine initially present. This situation persists up to pD ~11.2, whereupon, in more strongly alkaline solutions, the amounts of ketimine and aldimine complexes decrease rapidly and are unobservable at pD >11.8. Simultaneously, pyridoxamine signals increase in intensity and become discernibly broadened from pD 11.5 to 13 compared to those of **5**, a behavior suggestive of the formation of Al–pyridoxamine species in this interval. As in the zinc(II) system, increasing pD results in a progressively larger ratio of 2:2:1–1:1:1 ketimine complexes. The unresolvable 6-H signals of these species show an inflection in their pD dependence at ~9.4. Similar behavior was observed in the Al–aldimine system and, as in that case,[11] we are unable to offer a satisfactory interpretation.

The proposed equilibrium scheme for the pyridoxamine–pyruvate–Al(III) system is given in Chart III.

Discussion

The results of this investigation clearly show that pyridoxamine and pyruvate, in the presence and absence of metal ions, condense in aqueous solution to form ketimine species of various types whose relative concentrations are pD dependent. The species **6**, **8**, and **10** (Chart I) are produced in the metal-free system and, in the presence of zinc(II) or aluminum(III), complexes having one (**14**, **20**) or two (**15**, **21**) chelating ketimine ligands, with possible structures indicated in Charts II and III, are formed. The effect of metal ion on the extent of ketimine formation is best illustrated in the zinc(II) system. Here the stabilizing effect of the metal ion results in enhanced stability of ketimine over a much wider pD range and an ~88% maximum conversion of pyridoxamine to ketimine in all forms (pD 9.8) compared to the metal-free system, in which the extent of ketimine formation is only ~30% and also maximizes at pD 9.8. In the aluminum(III) system the same stabilization effect is evident, resulting in an estimated upper limit of ~70% ketimine formation at pD ~10.2.

This study and that[11] preceding it offer strong confirmatory evidence for the proposed mechanism of nonenzymatic transamination involving metal ions.[2,3] We have previously shown by pmr methods that in the systems pyridoxal–alanine–Zn(II) or –Al(III) aldimine formation is enhanced by the presence of metal ions and that 1:1:1 and 2:2:1 aldimine complexes are formed in reaction 1. Well-defined aldimine complexes derived from amino acids and pyridoxal[34] and its analogs,[35] as well as from salicylaldehyde,[36–39] have been isolated and their basic structure involving the chelating functionality illustrated in **1** has been definitely established by X-ray investigations.[29,30,40] The present study demonstrates that when reaction 2, the reverse of reaction 1, is carried out in the presence of metal ions, 1:1:1 and 2:2:1 ketimine complexes are formed. These species are tautomers of the aldimine complexes and their conversion to the latter may be directly followed by pmr. That the rearrangement products are in fact the aldimine complexes has been unequivocally demonstrated by their characteristic pD dependencies of chemical shifts in the transaminated solutions, which are in excellent agreement with those observed in the pyridoxal–alanine systems.[11] The detailed structures of the ketimine complexes are not known with certainty and those shown in Charts II and III are postulated structures only. The significant chemical shift differences of the pyridine ring substituents, especially 2-CH_3 and 4-CH_2, between the metal-free and metal-containing systems strongly imply the existence of chelate rings closed by phenolic oxygen and ketimine nitrogen. The closure of a second chelate ring by coordination of a carboxylate oxygen, while presumably favored by entropy effects, cannot be demonstrated from the pmr data. In the Al–aldimine complexes the closure of this ring may be slow on the pmr time scale, and species with coordinated and "dangling" carboxylate groups are at least consistent with the pmr results.[11,33b] There are several reports of the isolation of tautomeric pairs of aldimine–ketimine complexes,[14,37] but these have yet to be independently verified.

In summary, the results of these proton resonance investigations demonstrate that in the over-all transamination reaction 3, catalyzed by pyridoxal and metal ions, aldimine and ketimine complexes are formed sequentially in reactions 1 and 2, that the postulated tautomeric conversion of ketimine to aldimine complex required in reaction 2 unquestionably occurs, and that this conversion is susceptible to direct detection by pmr. These results are in general agreement with those obtained by spectrophotometry on other model systems containing pyridoxal or pyridoxamine and metal ions.[5,13,15,20–22] No attempt has been made in this work to follow the hydrolysis of aldimine and ketimine complexes in reactions 1 and 2, respectively, inasmuch as the formation of the indicated products is well documented.[5]

The deuteration reactions observed in this work merit some additional comment. Pyridoxal-dependent enzymes also catalyze elimination of certain groups from the β position of amino acids.[5] Some of these reactions may be effected in model systems containing pyridoxal and metal ions, and metal complex intermediates similar in structure and function to those involved in nonenzymatic transamination have been proposed.[3–6] Deuteration of the pyruvidene methyl protons in the free ketimine or its complexes is another example of the enhanced reactivity of β substituents arising from Schiff base formation. Enzymatic α,β deuteration of amino acids is well known and the same reaction has been effected in the presence of initially added pyridoxal[17,41,42] or pyridoxamine[17] and metal ions. We have reported earlier the pyruvidene methyl exchange reac-

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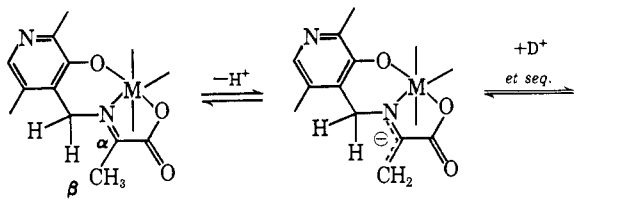
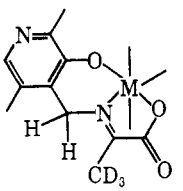
(35) D. Heinert and A. E. Martell, *ibid.*, **85**, 1334 (1963).
(36) (a) M. J. O'Connor, R. E. Ernst, J. E. Schoenborn, and R. H. Holm, *ibid.*, **90**, 1744 (1968); (b) M. J. O'Connor, G. N. Green, and R. H. Holm, unpublished work.
(37) Y. Nakao, S. Sasaki, K. Sakurai, and A. Nakahara, *Bull. Chem. Soc. Japan*, **40**, 241 (1967).
(38) Y. Nakao, K. Sakurai, and A. Nakahara, *ibid.*, **40**, 1536 (1967).
(39) R. C. Burrows and J. C. Bailar, *J. Amer. Chem. Soc.*, **88**, 4150 (1966), and references therein.
(40) J. F. Cutfield, D. Hall, and T. N. Waters, *Chem. Commun.*, 785 (1967); G. A. Bentley, J. M. Waters, and T. N. Waters, *ibid.*, 988 (1968); T. Ueki, T. Ashida, Y. Sasada, and M. Kakudo, *Acta Cryst.*, **22**, 870 (1967).
(41) *Cf., e.g.*, T. Oshima and N. Tamiya, *J. Biochem.* (Tokyo), **46**, 1675 (1959); N. Tamiya and T. Oshima, *ibid.*, **51**, 78 (1962); T. Oshima and N. Tamiya, *Biochem. J.*, **78**, 116 (1961); N. Tamiya and T. Shimanouchi, *Spectrochim. Acta*, **18**, 895 (1962).
(42) G. A. Junk and H. J. Svec, *J. Org. Chem.*, **29**, 944 (1964).

tion[12] and have shown that it has practical utility in the synthesis of DL-alanine-α,β-d_4.[11]

In the present study it has been observed qualitatively that in the metal-free and metal-containing systems pyruvidene methyl exchange increases in rate as the pD is raised and that at a given pD exchange is more rapid in the latter than in the former system. These results are consistent with the exchange path **25** $\rightleftharpoons$ **26** $\rightleftharpoons$ **27** for the complexes in which the active intermediate **26** is similar to that (**12** $\leftrightarrows$ **13**) considered to intervene in the exchange reaction of the free ketimine, but is additionally stabilized by coordination to the electropositive metal ion. In fresh neutral or acidic solutions



25 **26**



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where ketimine formation can be observed in the absence of any appreciable conversion to aldimine species, no diminution of the 4-CH_2 signal intensity is observed, showing that exchange occurs at the β carbon only. The exchange pathway is also consistent with that proposed by Abbott and Martell,[17] who have shown that the β position of several α-keto acids is activated toward H–D exchange in the presence of pyridoxamine with or without metal ion addition.[43]

Finally, it has been found that in the ketimine → aldimine conversion of complexes shown in Charts II and III the aldimine product contains no detectable amount of deuterium at the 4 carbon, indicating that only one of the ketimine 4-CH_2 protons is labilized in the conversion under the prevailing experimental conditions (*cf.* Figures 4 and 6). This result bears a superficial similarity to the recent and highly significant finding that in enzymatically catalyzed reactions of pyridoxamine with α-ketoglutarate[44] and pyruvate[45] stereospecific transfer of a single deuterium *to and from* the 4 carbon of pyridoxamine occurs. Although, as suggested earlier,[12] the probable nonplanarity of the six-membered chelate ring in the ketimine may impose a preferred reactivity on one of 4-CH_2 protons when the ring is in a fixed conformation on the basis of arguments analogous to those presented by Dunathan,[46] this effect is likely to be unobservable in the systems investigated. Tautomeric conversion is certainly slower in the zinc(II) system than exchange between free and complexed ketimine, and in this and the aluminum(III) system the conformational interchange of the ketimine chelate rings, which alternates the 4-CH_2 protons in the potentially activated position, is expected to be very fast compared to the conversion process. Instead, the lack of deuterium incorporation at the 4 carbon of the aldimine complexes is considered to be the consequence of a large forward rate constant for ketimine → aldimine with the back reaction being very slow under the conditions of reactant concentrations and temperature employed. Equilibrium isotopic distributions in D_2O solutions can be achieved at higher temperatures than employed here, and deuterium incorporation at 4 carbon under such conditions[17] is a necessary consequence of the mechanism for nonenzymatic transamination.

Acknowledgment. This research was supported by the U. S. Public Health Service under National Institutes of Health Grant GM-15471. We thank Professor M. R. Willcott for the use of nmr facilities at the University of Houston.

(43) Note that specific α deuteration of amino acids can be carried out in the presence of salicylaldehyde and metal ions: R. B. Johns and D. J. Whelan, *Austr. J. Chem.*, **19**, 2143 (1966). Reactions of this type are being further investigated.[36b]

(44) H. C. Dunathan, L. Davis, P. G. Kury, and M. Kaplan, *Biochemistry*, **7**, 4532 (1968).

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