Pyridoxal Analogs. X. Zinc(II)-Chelate Catalysis of Transamination in Methanol Solution[1]

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Abstract: The reaction between pyridoxamine, potassium α-ketoisovalerate, and zinc acetate is found to yield the zinc chelate of pyridoxylidenevaline in methanol solution. Spectrophotometric studies show that this nonenzymatic transamination reaction proceeds quite smoothly and completely at room temperature and involves two slow steps. The first step is the formation of the zinc chelate of ketimine from the three reactants, and the second is isomerization of the ketimine chelate to the aldimine chelate. When the three reactants are brought together in methanol solution simultaneously, the reaction takes place in two steps: an initial phase involving the formation of the ketimine chelate, and a main phase in which the isomerization reaction shows first-order kinetics. The influence of concentration of reactants on the first-order rate constants is reported, and a possible reaction mechanism is proposed. The reaction kinetics are interpreted in terms of a steady-state treatment of a reactive intermediate, which is believed to be a π-bond-stabilized carbanion formed by dissociation of a proton from the metal chelate of the ketimine.

Metzler, *et al.*,[3-6] found that transamination could be carried out nonenzymatically in aqueous solution, with Cu^{2+}, Fe^{3+}, and Al^{3+} ions as catalysts, and suggested that metal chelates of the Schiff bases formed from pyridoxal and α-amino acids are the reactive intermediates of these reactions. More recently the mechanism of transamination involving the isomerization of azomethines has been studied in various systems by many investigators.[7-17]

In the previous paper of this series,[18] spectra are reported for methanol solutions of pyridoxal, pyridoxal phosphate, pyridoxamine, the Schiff bases of these substances with amino acids or α-keto acids, and the metal chelates of these Schiff bases. The molecular species present and the equilibria between them are described. In the course of those studies, spectral changes were observed at measurable rates when zinc(II) acetate solution was added to solutions of the ketimine. This change has now been found to be a transamination reaction which proceeds quite smoothly and completely at room temperature.

In the present paper, kinetic measurements of these spectral changes are reported, and a mechanism for the transamination reaction deduced from these rate studies is proposed.

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Experimental Section

Materials. The materials used are the same as described in the previous paper.[18] Methanol solutions of the substances under investigation were prepared immediately before each experiment, as described below. Deuteriomethanol, CH_3OD, was obtained in 99% isotopic purity from the Volk Radiochemical Co. Pyridoxamine solutions were prepared by dissolving pyridoxamine dihydrochloride in methanol with 2 equiv of KOH. A solution of the potassium salt of the keto acid was prepared by treating a methanol solution of β-ketoisovaleric acid with 1 equiv of KOH. The zinc acetate solution was prepared by dissolving $Zn(C_2H_3O_2)_2 \cdot 2H_2O$ in methanol.

Kinetic Measurements. Calculated volumes of solutions were mixed in a predetermined order in a volumetric flask and quickly adjusted to a definite volume by the addition of methanol (99% CH_3OD was employed for determination of the solvent isotope effect). The moment when the last of three solutions was added was taken as the initial time of reaction. The sample solutions were shaken and transferred to silica cells for absorption measurements. The cells were stoppered and sealed with paraffin to avoid contact with air and moisture.

Absorption Measurements. The electronic absorption spectra in the visible and ultraviolet regions were recorded with a Cary Model 14 spectrophotometer. For kinetic measurements, use was also made of a Beckman DU spectrophotometer to measure the change of absorbancy with time. The temperature of the cell compartment was kept at 30° throughout the kinetic studies.

Results

Spectral Changes. The rates of change of absorption spectra that occur when pyridoxamine keto acid and zinc acetate solutions are mixed simultaneously are shown in Figure 1. For the reaction that occurs on mixing all three components at once, called reaction system I, only one absorption peak at 300 mμ is found initially. The intensity of the 300-mμ peak decreased with time and formed distinct isosbestic points at 324 and 284 mμ. The maximum intensities of the absorption bands that are newly formed as the reaction proceeds, and increase in intensity with time, were observed at 385 and 271 mμ. The final absorption spectrum was the same as that of the Zn(II) chelate of pyridoxylidenevaline.

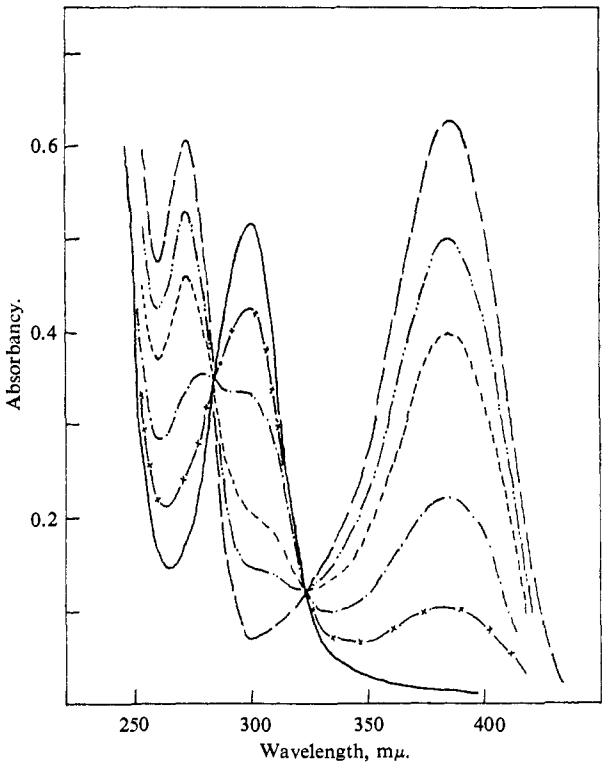


Figure 1. Changes of electronic absorption spectra with time for a reaction mixture obtained by simultaneous mixing of methanol solutions to give 1.0×10^{-4} *M* pyridoxamine, 1.0×10^{-3} *M* potassium α-ketoisovalerate, and 1.0×10^{-4} *M* zinc acetate (reaction system I). The times after initiating the reaction are ———, 0 min; — × — × —, 40 min; ·—·—, 1 hr; -----, 2 hr; ··—··—··, 3 hr; — — —, 24 hr.

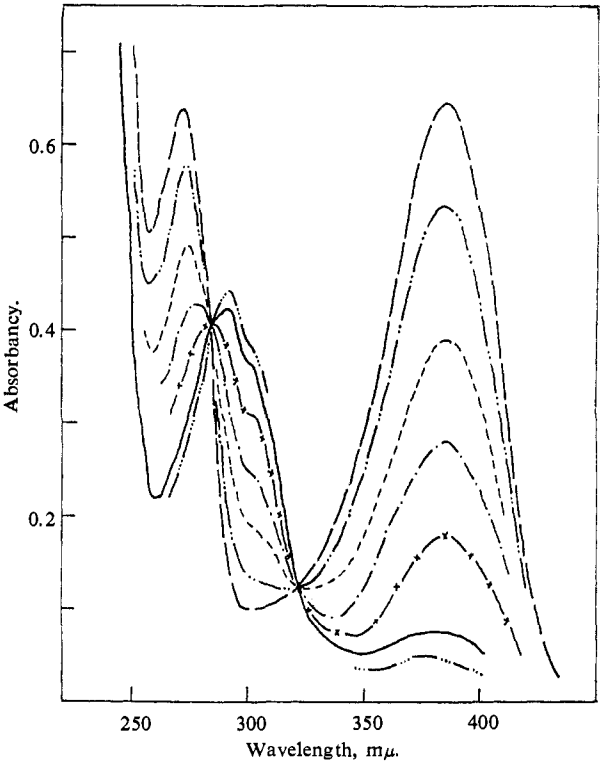


Figure 2. Changes of electronic absorption spectra of a solution formed by adding zinc acetate in methanol to the equilibrium mixture of pyridoxamine and potassium α-ketoisovalerate in methanol; at time zero the resulting solution contained 1.0×10^{-4} *M* pyridoxamine, 1.0×10^{-3} *M* potassium α-ketoisovalerate, and 1.0×10^{-4} *M* zinc acetate (reaction system II). The times after initiating the reaction are —··—··—, 3 min; ———, 10 min; × — × — × —, 30 min; —·—·—, 1 hr; -----, 1.5 hr; —··—··—, 3 hr; — — —, 24 hr.

When zinc acetate solution was added to the equilibrium mixture of pyridoxamine and keto acid solution, in which ketimine had been partially formed, the rate of change of absorption spectrum of the solution formed under these conditions was quite different from that described above. The reaction sequence occurring by addition of the metal ion to the Schiff base is designated as reaction system II.

As shown in Figure 2, the initial spectrum in reaction system II has an absorption maximum at 291 mμ and a shoulder at 300 mμ. The spectral changes consisted of decrease in the intensity of the 291- and 300-mμ bands and increase in absorption at 385 and 271 mμ. The final spectrum obtained was the same as the final spectrum in reaction system I, indicating that the end products are the same.

Since the final spectra from both systems were the same as that of the Zn(II) chelate of pyridoxylidenevaline, it was concluded that transamination of the ketimine–Zn(II) chelate occurred in both cases *via* proton transfer from the α position on the aromatic side chain to the α position of the amino acid moiety.

Further evidence for this conclusion was obtained by adding a methanol solution of tetrasodium ethylenediaminetetraacetate to the final products, and also to a solution of the Zn(II) chelate of pyridoxylidenevaline. The spectra of the solutions thus obtained were identical and the same as that of pyridoxylidenevaline itself.

Absorption bands with maxima at 385, 300, and 271 mμ can be used for following the rates of these transamination reactions. The 385-mμ band was considered the most satisfactory because of its large molecular extinction coefficient and since the pyridoxylidenevaline chelate is the only substance that might be present which absorbs at that wavelength. At 271 and 300 mμ, however, a number of solution species have appreciable absorption. Thus there is considerable absorption at zero time and at completion of the reaction at 271 and 300 mμ, whereas there is no initial absorption at 385 mμ. Since this situation lends itself more readily to our reaction conditions, the 385-mμ band was used for kinetic studies.

Kinetic Results. The results of some typical kinetic measurements, in which optical densities at 385 mμ are plotted against time, are shown in Figure 3.

The differences between reaction systems I and II may be observed in Figure 3. For reaction system II, a large rate of increase of optical density at 385 mμ was observed from zero time. In reaction system I, however, two phases were observed: an initial phase or induction period and a main phase. The initial phase in reaction system II and the main phase in reaction system I showed the same rate of increasing absorption. For the data from reaction system I, values of $\log D_\infty/(D_\infty - D_t)$ were calculated and plotted against time t (where D_∞ and D_t indicated optical densities at 385 mμ at completion of the reaction and at time t, respectively). A plot of these quantities gives straight lines for the main phase up to 99% completion of the reaction, indicating first-order kinetics for the formation of the pyridoxylidenevaline chelate. The first-order

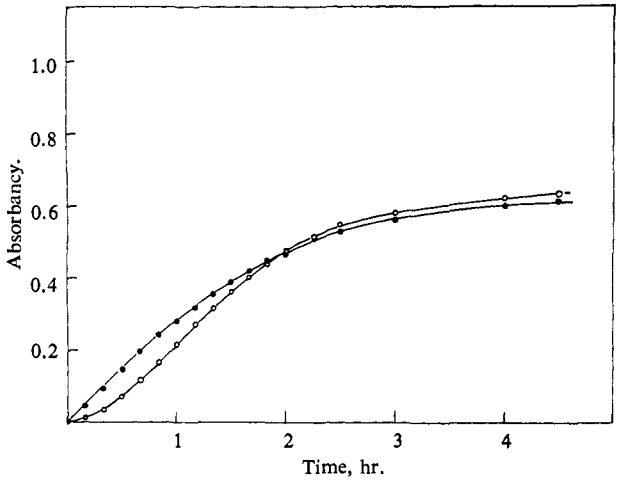


Figure 3. Variation of absorbancies at 385 mμ in reaction system I (○) and reaction system II (●). Both systems contained 1.0×10^{-4} *M* pyridoxamine, 1.0×10^{-3} *M* potassium α-ketoisovalerate, and 1.0×10^{-4} *M* zinc acetate in methanol.

rate constants, k_{obsd}, were obtained from the slopes of the straight lines. Reproducible values of k_{obsd} were obtained only when a freshly prepared solution of the keto acid was employed. When stock solutions of the keto acid were used, smaller values of k_{obsd} were obtained, presumably because of the gradual polymerization of α-ketoisovalerate in neutral methanol solution.

An increase in the per cent of water in the methanol used as the solvent was also found to result in a decrease in k_{obsd} as is indicated in Figure 4A. The reaction kinetics described in this paper were determined in methanol solutions containing less than 0.1% of water, where the effect of water is negligible.

Influence of Concentration of Reactants. The effect of changing the concentration of the reactants was determined for reaction system I, in which all constituents (metal acetate, pyridoxamine, and α-keto acid) were mixed simultaneously.

a. Influence of Concentration of Keto Acid. Results of kinetic runs in which the pyridoxamine and zinc acetate concentrations were kept at 1.0×10^{-4} *M*, and that of the keto acid was varied, are shown in Figure 4B. As the keto acid concentration was increased, the rate constants increased to a maximum value and then decreased again, with a maximum at $\sim 1.0 \times 10^{-3}$ *M*. The decrease at higher concentrations of α-keto acid is probably due to competitive complexing of the zinc acetate by the excess keto acid, resulting in a decrease in the concentration of the Schiff base Zn(II) chelate intermediate (III). This interpretation is borne out by the influence of α-keto acid concentration on the rate when the concentration level of zinc acetate is much higher, as is indicated in Figure 5A. When the concentrations of zinc acetate and pyridoxamine are always much higher than that of the α-keto acid, an increase of α-keto acid concentration in the reaction mixture would always result in an increase in the concentration of the reactive intermediate III.

b. Influence of Zinc Acetate Concentration. The effect of increasing the zinc acetate concentration was studied, with concentrations of pyridoxamine and α-keto acid both fixed at 1×10^{-4} *M* and 1.0×10^{-3} *M*, respectively. The results, indicated in Figure 5B,

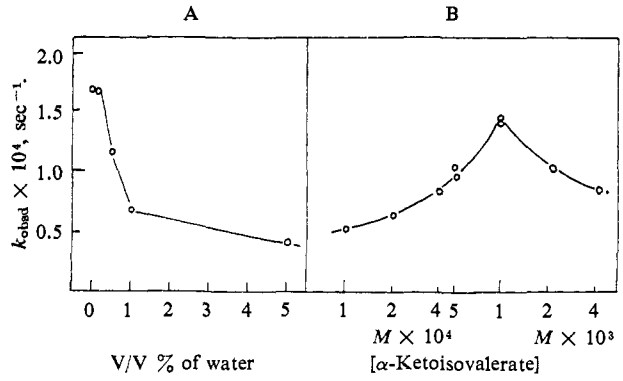


Figure 4. Variation of first-order rate constants (k_{obsd}) in reaction system I: (A) as a function of water content in methanol, solutions contained 1.0×10^{-4} *M* pyridoxamine, 1.0×10^{-3} *M* potassium α-ketoisovalerate, and 1.0×10^{-4} *M* zinc acetate; (B) as a function of concentration of potassium α-ketoisovalerate, solutions contained 1.0×10^{-4} *M* pyridoxamine and 1.0×10^{-4} *M* zinc acetate.

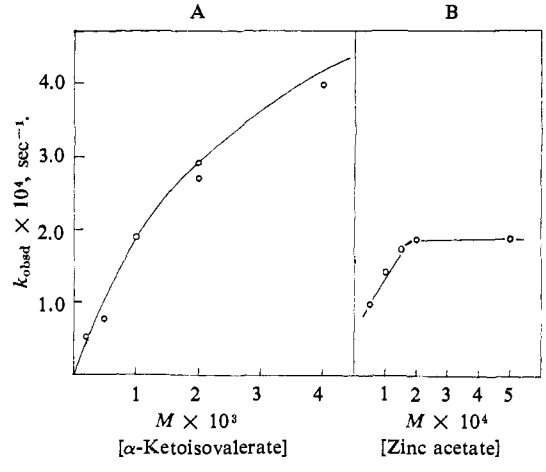


Figure 5. Variation of k_{obsd} in reaction system I: (A) as a function of concentration of potassium α-ketoisovalerate, solutions contained 1.0×10^{-4} *M* pyridoxamine and 5.0×10^{-4} *M* zinc acetate (solid line was obtained from eq 1 and calculated value of K_{eq}); (B) as a function of zinc acetate concentration, solutions contained 1.0×10^{-4} *M* pyridoxamine and 1.0×10^{-3} *M* potassium α-ketoisovalerate.

show that k_{obsd} increases approximately linearly with an increase in zinc acetate concentration, up to about 2×10^{-4} *M*, and then levels off.

c. Effect of Dilution. Kinetic measurements were also made under conditions such that the ratios of concentrations of zinc acetate, pyridoxamine, and α-keto acid were all maintained constant, but the absolute concentrations of all were varied. The decrease in rate constant with dilution, indicated in Table I,

Table I. Dilution Effects on k_{obsd} for Reaction I

Pyridoxamine, *M*	α-Ketoisovalerate, *M*	Zinc acetate, *M*	$k_{obsd} \times 10^4$, sec^{-1}
1.5×10^{-4}	1.5×10^{-3}	7.5×10^{-4}	2.27
1.0×10^{-4}	1.0×10^{-3}	5.0×10^{-4}	1.86
0.5×10^{-4}	0.5×10^{-3}	2.5×10^{-4}	1.48

was proportionately less than the relative decrease in concentration of any one of the reactants.

d. Solvent Isotope Effect. The solvent isotope effect on the rate constant was measured for reaction

system I, at concentrations of 1.0×10^{-3} *M* keto acid, 5.0×10^{-4} *M* zinc acetate, and 1.0×10^{-4} *M* pyridoxamine. The rate constant (sec^{-1}) was changed from 1.98×10^{-4} for CH_3OH to 1.66×10^{-4} for 50% CH_3OD and 1.39×10^{-4} for 90% CH_3OD. From this, the rate in pure CH_3OD was estimated as 1.33×10^{-4} sec^{-1}, giving an isotope effect of 67% ($k_{obsd}(CH_3OD)/k_{obsd}(CH_3OH) = 0.67$).

e. Kinetics of Ketimine Formation in the Absence of Metal Ions. As reported in the previous paper, gradual spectral changes were observed when pyridoxamine and an excess amount of keto acid were mixed in methanol. The initial spectra obtained have one absorption maximum at 290 mμ and one shoulder at 310 mμ. The spectral changes consist of a decrease of intensity of the 310-mμ absorption and increased intensity and a slight blue shift of the 290-mμ band to 285 mμ. Changes of the intensity of the 310-mμ absorption were used for kinetic study of this reaction. The initial part of the reaction seemed to follow first-order kinetics. Observed first-order rate constants, k_{obsd}, were not affected much by the concentration of pyridoxamine as long as an excess of the keto acid was maintained. Thus k_{obsd} was practically unchanged when the keto acid was 10 and 25 times as concentrated as pyridoxamine. The value obtained for k_{obsd} under the experimental conditions employed was calculated as 4.03×10^{-4} sec^{-1}.

Discussion

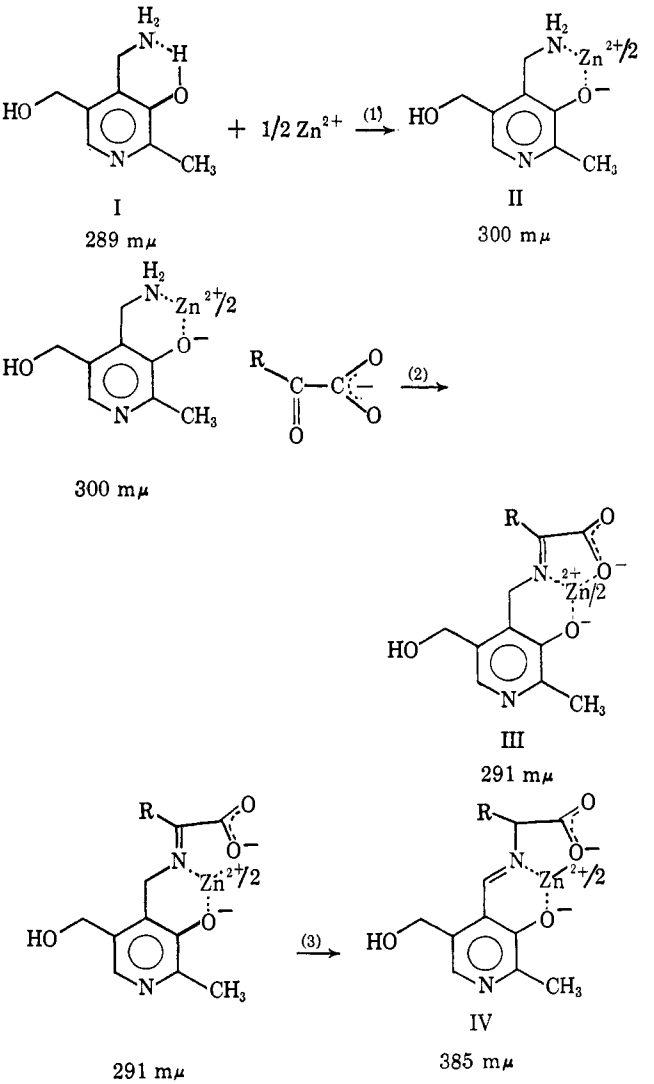
The formation of the aldimine chelate from the simultaneous mixing of pyridoxamine, keto acid, and zinc acetate in methanol solution (reaction I) necessarily involves the following steps:

(1) formation of the Zn(II) chelate of pyridoxamine (rapid), (2) formation of the Zn(II)–ketimine chelate from the Zn(II)–pyridoxamine chelate and the α-keto acid, (3) isomerization of the Zn(II)–ketimine chelate to the Zn(II)–aldimine chelate.

The fact that initial spectrum in reaction system I was exactly the same as that of the Zn(II) chelate of pyridoxamine shows that step 1 is extremely rapid and takes place first. The fact that reaction system I involved an initial low rate indicates that aldimine chelate formation is partially rate determining in the initial phase, or "induction," period. Thus it appears that both steps 2 and 3 are slow reactions, although in the later first-order phase, only one step is rate determining.

In reaction system II the ketimine was formed before addition of zinc acetate. Since metal chelate formation with the ketimine is expected to be quite fast, the 291-mμ observed initially in the spectrum of reaction system II is attributed to the zinc chelate of the ketimine. However, the presence of the 300-mμ band indicates that steps 1 and 2 were also involved in reaction system II, and ketimine chelate formation at initial time was therefore not complete.

For reaction system II, the rate of the initial phase involving the appearance of aldimine chelate was about the same as that of the first-order phase in system I, as can be seen in Figure 3. Thus it seems that at the beginning of the main phase of reaction system I, a steady state had been reached in which step 2 was at equilibrium, and step 3 remained as the rate-determining step, as for the initial phase of reaction system II.



For the main phase of reaction system I, the reaction mechanism may be formulated as

$$\underset{A}{\text{pyridoxamine–Zn}} + \underset{B}{\text{keto acid}} \overset{K_{eq}}{\rightleftharpoons} \underset{C}{\text{ketimine–Zn}}$$

$$\underset{C}{\text{ketimine–Zn}} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{X}$$

$$\text{X} \xrightarrow{k_3} \underset{D}{\text{aldimine–Zn (product)}}$$

where the reactive intermediate X is believed to have the structure shown in Chart I. In order to simplify the following discussion the concentrations of these substances will be represented by letters indicated in brackets. The first step in this reaction may be considered to involve equilibrium with an equilibrium constant, K_{eq}

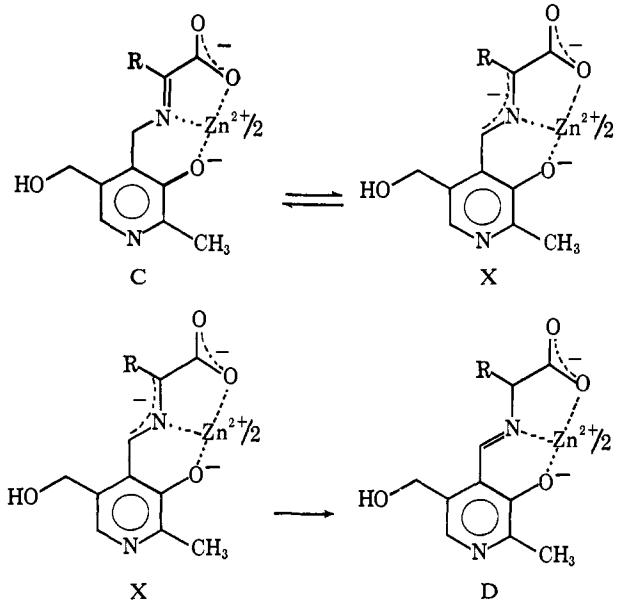
$$K_{eq} = \frac{[C]}{[A][B]}$$

for the formation of the ketimine chelate. Applying the steady-state treatment for the formation of the reactive intermediate X, one obtains

$$\frac{d[X]}{dt} = k_2[C] - k_3[X] - k_{-2}[X]$$

Assuming $d[X]/dt = 0$

Chart I



$$[X] = \frac{k_2}{k + k_{-2}}[C]$$

The observed rate constant is related to the rate of formation of products from X by the expression

$$k_{obsd}([A] + [C]) = k_3[X]$$

Then

$$k_{obsd} = \frac{k_2k_3}{k_3 + k_{-2}}\left(\frac{[C]}{[A] + [C]}\right) = \frac{k_2k_3}{k_3 + k_{-2}}\left(\frac{K_{eq}[B]}{1 + K_{eq}[B]}\right) \quad (1)$$

When the concentration of keto acid ([B]) is sufficiently higher than that of A and the change of the concentration during the reaction can be neglected, the above relationship reduces to

$$k_{obsd} = \frac{k_2k_3}{k_3 + k_{-2}}K'$$

where

$$K' = \left(1 + \frac{1}{K_{eq}[B]}\right)^{-1}$$

The value of K_{eq} can be calculated from the two series of kinetic runs in which initial concentrations of A were the same, but those of B were B_0 and bB_0. If the observed rate constants were k and k', respectively, the following equations are obtained.

$$k = \frac{k_2k_3}{k_3 + k_{-2}}\left(\frac{K_{eq}B_0}{1 + K_{eq}B_0}\right)$$

$$k' = \frac{k_2k_3}{k_3 + k_{-2}}\left(\frac{K_{eq}bB_0}{1 + K_{eq}bB_0}\right)$$

$$\frac{k}{k'} = \frac{K_{eq}B_0}{1 + K_{eq}B_0}\left(\frac{1 + K_{eq}bB_0}{K_{eq}bB_0}\right)$$

$$K_{eq} = \left(\frac{1}{B_0}\right)\frac{k - (1/b)k'}{k' - k}$$

K_{eq} was calculated to be 3.7×10^2 ($\log K_{eq} = 2.56$) for $B_0 = 1 \times 10^{-3}$ M and $b = 2$.

From the calculated value, the relationships between k_{obsd} and B_0 given by eq 1 are shown in Figure 5A, with experimentally obtained data. They coincided satisfactorily, lending support to the kinetic analysis and the proposed mechanism.

The observed solvent isotope effect, by which the reaction rate is 50% faster in CH_3OH than in CH_3OD, probably involves the transfer of a proton in the rate-determining step to CH_3OH or CH_3OD. Since in the metal chelate there are no exchangeable protons which involve the proton-transfer step, at least in the initial part of the reaction, the observed relatively small effect is probably involved in the difference in tendencies of a proton to transfer to a solvent oxygen already bonded to either a proton or a deuteron. If this is the case the slightly greater tendency to transfer to protiomethanol is in agreement with the lower energy of $CH_3OH_2^+$ relative to CH_3OHD^+. A more complete study of isotope effects in these systems, which would involve studies of exchange reactions of the α-deuterated ketimine and the normal ketimine, will be carried out later.

The principal contributions of this investigation are to present (1) the first example of Zn(II) catalysis of a transamination reaction and (2) the first kinetic treatment of a simple metal-catalyzed ketimine–aldimine isomerization, taking place to near completion (~99%) and in the absence of large excesses of reagents or by-products.