

**Pyridoxamine Analogs. II.<sup>1)</sup> Reactions with  $\alpha$ -Keto Acids and Divalent Metal Ions in Methanol<sup>2)</sup>**SHIGENOBU MATSUMOTO,<sup>3a)</sup> YOSHIHARU KARUBE and YOSHIKAZU MATSUSHIMA<sup>3b)</sup>*Faculty of Pharmaceutical Sciences, Kyushu University<sup>3)</sup>*

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Three reactants, aminomethylpyridine,  $\alpha$ -keto acid and divalent metal ion were mixed in methanol and formation of metal chelate of aldimine was followed by changes of electronic absorption spectra. Aminomethylpyridines used were pyridoxamine and 3-hydroxy-4-aminomethylpyridine;  $\alpha$ -keto acids were Na pyruvate, Na  $\alpha$ -ketobutyrate, K  $\alpha$ -ketoisovalerate, ethyl pyruvate and ethyl  $\alpha$ -ketobutyrate; metal ions were Zn(II), Ni(II) and Cu(II). Every combination of the substances were examined. Analyses of spectral changes showed that the reactions consisted of two slow steps, ketimine metal chelate formation from the three reactants and its isomerization to aldimine metal chelate. In reactions with Zn(II) ion, the isomerization step was rate determining, while in reactions with Ni(II) ion ketimine chelate formation became slower step. By chelation with Cu(II), ketimine chelate formation was retarded but the isomerization was accelerated. Ketimine formation was faster with esters of keto acid than with anions. In the anions rate of ketimine formation was pyruvate >  $\alpha$ -ketobutyrate >  $\alpha$ -ketoisovalerate in the presence and in the absence of the metal ions. None of the combination of the reactants in the present study produced an intermediate absorbing in the 500-nm region.

The reaction of pyridoxamine, K  $\alpha$ -ketoisovalerate and Zn(II) ion was found to yield Zn(II) chelate of the aldimine, formed from pyridoxal and valine, in methanol solution.<sup>4)</sup> The reaction involves an isomerization between a ketimine and an aldimine, a key step in a transamination catalyzed by vitamin B<sub>6</sub>. The succeeding paper<sup>5)</sup> reported that Cu(II) and Ni(II) chelates of the aldimine were also formed in similar reactions and the kinetic behavior of the three divalent metal ions differed considerably. These reactions were especially suited for mechanistic study, since it could be followed by spectral changes and proceeded quite smoothly and completely at room temperature in this solvent.<sup>6)</sup>

The kinetics of the reaction of the Zn(II) chelates were interpreted in terms of a steady-state treatment of an intermediate, which was believed to be a carbanion formed by dissociation of a proton from Zn(II) chelate of Schiff base of pyridoxamine and  $\alpha$ -ketoisovaleric acid.<sup>4)</sup>

Recently, we found a species absorbing in the 500-nm region in a reaction of pyridoxamine, ethyl pyruvate and Al(III) ion and the species was concluded to be an Al(III) chelate of the carbanion intermediate.<sup>7)</sup> Thus the intermediate was observed as a discrete chemical species

- 1) Part I: S. Matsumoto and Y. Matsushima, *Chem. Pharm. Bull.* (Tokyo), **23**, 106 (1975).
- 2) This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education of Japan.
- 3) Location: *Maidashi 3-1-1, Fukuoka-shi, 812, Japan*; a) Present Address: *Tokyo Metropolitan Institute of Gerontology, 35-2, Sakae-cho, Itabashi-ku, Tokyo; 173, Japan*, b) To whom inquiries should be addressed.
- 4) Y. Matsushima and A.E. Martell, *J. Amer. Chem. Soc.*, **89**, 1331 (1967).
- 5) A.E. Martell and Y. Matsushima, *I.U.B. Symposium Series* **35**, 33 (1968).
- 6) In aqueous solutions, a number of investigations have been done on the metal ion mediated reactions of vitamin B<sub>6</sub>. They are found in the literature cited in our previous publications, ref. 1, 4, 5, 7 and 9. One of the recent reports is; W.L. Felty and D.L. Leussing, *J. inorg. nucl. Chem.*, **36**, 617 (1974). There are also some review articles on the subjects, for example, R.H. Holm, "Inorganic Biochemistry" Vol. 2, ed. by G.L. Eichhorn, Elsevier, Amsterdam, 1973, pp. 1137-1167; Y. Matsushima, *Farumashia*, **9**, 629 (1973).
- 7) S. Matsumoto, and Y. Matsushima, *J. Amer. Chem. Soc.*, **94**, 7211 (1972); *idem, ibid.*, **96**, 5228 (1974).

in the pyridoxamine-ethyl pyruvate-Al(III) system and *not* in the pyridoxamine-K  $\alpha$ -ketoisovalerate-Zn(II) system.

This finding led us to examine reactions of amines, keto acids and metal ions in methanol by means of electronic absorption spectroscopy. Various substances were employed as reactants with a view to determining effects of the structures on the reaction mechanism.

The present paper deals with combinations of the following reactants: amines used are pyridoxamine and its snalog, 3-hydroxy-4-aminomethylpyridine; keto acids are Na pyruvate, Na  $\alpha$ -ketobutyrate, K  $\alpha$ -ketoisovalerate, ethyl pyruvate and ethyl  $\alpha$ -ketobutyrate; metal ions are Zn(II), Ni(II) and Cu(II) ions. Reactions of Al(III) ion will be reported in near future.

### Experimental

Experimental procedures were essentially the same as described in the earlier papers.<sup>1,4,7)</sup> Spectrophotometers used were Hitachi Model EPS-3T, Shimadzu Model MPS-50 and Union Giken Model SM-202. 3-Hydroxy-4-formylpyridine<sup>8)</sup> and 3-hydroxy-4-aminomethylpyridine<sup>1)</sup> were prepared as described earlier. Other materials were obtained from commercial sources.

## Results and Discussion

### General Features of Spectra

In this study, spectral changes with time of methanol solutions of three reactants in various combinations were recorded at room temperature and were analyzed by the use of the solution equilibria and spectral assignments made previously.<sup>1,4,9)</sup>

When solutions of the three reactants were mixed simultaneously, the following spectral changes were observed in most cases. The initial spectra were identical with those of metal chelate of the amine employed and, then, the spectra underwent gradual changes.

When, however, metal ion was added to an equilibrium mixture of an amine and a keto acid, in which a ketimine Schiff base had been partially formed, the spectral changes as well as their rates were somewhat different from those obtained by mixing the three reactants simultaneously.

The reaction mixture that is formed on simultaneous mixing of the reactants is designated system I and the reaction mixture that is produced by allowing a ketimine to form first is designated system II.

After the gradual changes, the spectra in both systems I and II became identical and underwent no further changes. The final spectra had two strong absorption peaks at around 390 nm and 270 nm, a combination indicating the formation of metal chelate of an aldimine.<sup>9)</sup> Since there was no other appreciable absorption in most cases, the metal chelate of aldimine was the only species present significantly in the final mixtures.

Mixture of methanol solutions of the corresponding pyridinealdehyde, amino acid and the divalent metal ion gave the spectra of metal chelate of the aldimine, instantly on mixing. These facts indicate the final equilibria between metal chelates of an aldimine and a ketimine lie so far to the formation of an aldimine. The absorption bands assigned to 3-hydroxy-4-pyridylmethylealanine and to its metal chelates are listed in Table I with those of pyridoxylidenealanine reported previously.<sup>9b)</sup>

In the present report, the solutions were  $1 \times 10^{-4}$  M in pyridoxamine or its analog,  $1 \times 10^{-3}$  M in keto acid and  $1 \times 10^{-4}$  M in metal ion, unless otherwise noted. Spectral changes will be explained in terms of the reaction steps shown in Chart 1. An intermediate with the visible absorption was not observed during the divalent metal ion catalyzed reactions of the substances used in the present study.

8) D. Heinert and A.E. Martell, *J. Amer. Chem. Soc.*, **81**, 3935 (1959).

9) a) Y. Matsushima and A.E. Martell, *J. Amer. Chem. Soc.*, **89**, 1322 (1967); b) Y. Matsushima, *Chem. Pharm. Bull.* (Tokyo), **16**, 2143 (1968).

TABLE I. Absorption Bands of Schiff Bases and Metal Chelates

Metal	3-Hydroxy-4-pyridylmethylealanine	Pyridoxylidenealanine <sup>a)</sup>
H	416, 322, 248 nm	418, 340, 260 nm
Zn(II)	372, 256	385, 270
Ni(II)	379	393, 271
Cu(II)	380	388, 272

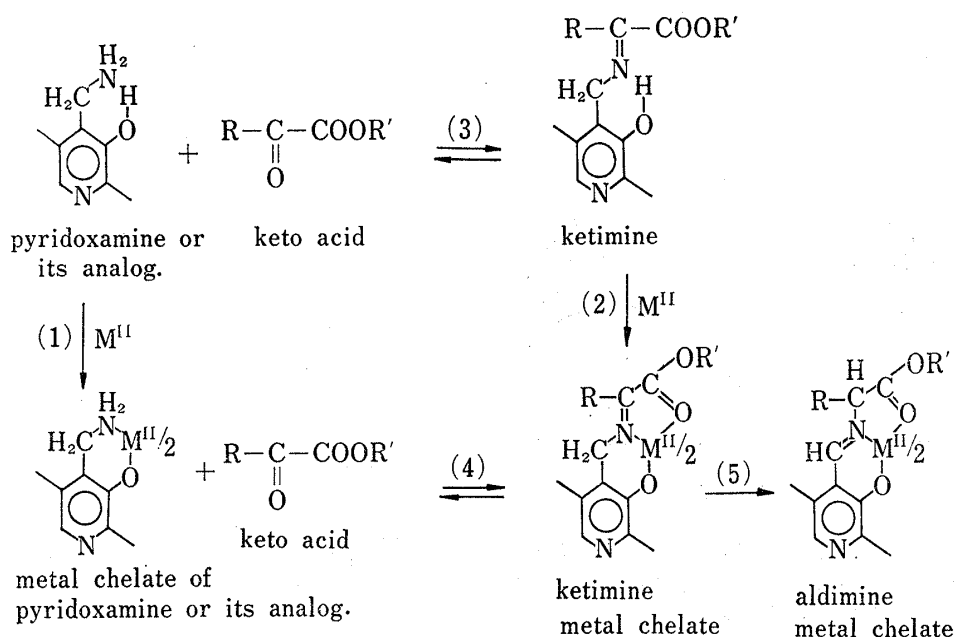
<sup>a)</sup> Ref. 9.

Chart 1

### Reactions of Zn(II) Chelates

Pyridoxamine-K  $\alpha$ -ketoisovalerate-Zn(II) systems were described previously.<sup>4)</sup> Replacement of pyridoxamine by 3-hydroxy-4-aminomethylpyridine and that of K  $\alpha$ -ketoisovalerate by Na pyruvate or Na  $\alpha$ -ketobutyrate gave essentially the same results.

In system I of the pyridoxamine-K  $\alpha$ -ketobutyrate-Zn(II) reaction, the absorption band at 301 nm, assigned to pyridoxamine Zn(II) chelate, decreased with an increase of the absorption bands of the aldimine Zn(II) chelate at 385 and 271 nm, with formation of a couple of isosbestic points.

In system II, a new absorption band was observed at around 293 nm, which should be assigned to the ketimine Zn(II) chelate, overlapped by the 301-nm band. In the presence of excess Zn(II) ion only the 301-nm band was observable.

Rates of the aldimine Zn(II) chelate formation were the same in systems I and II, in the same reactant concentrations. This fact and the spectral changes suggest the following reaction scheme, in agreement with the previous conclusion with K  $\alpha$ -ketoisovalerate.<sup>4)</sup> Metal chelate formation of amines (1) and ketimines (2) are undoubtedly rapid steps. Rate determining steps in both systems I and II are isomerization between Zn(II) chelates of the ketimine and the aldimine (5). The band ascribed to Zn(II) chelate of the ketimine was observable only at an early stage in system II and there was no sign of accumulation of this species in system I. Apparently, in system I and later stages of system II, the ketimine chelate formation (4) is in equilibrium, which lies to the left.

Replacement of the keto acid anion by keto acid esters caused marked changes in the mode of the reactions. In solutions  $1 \times 10^{-2} \text{ M}$  in ethyl pyruvate and  $1 \times 10^{-4} \text{ M}$  in pyridoxamine, aldimine was gradually formed in the absence of the divalent metal ions. In  $1 \times 10^{-3} \text{ M}$  ethyl pyruvate, however, aldimine was not formed in detectable amount in 4 hours after the initiation of the reaction. This indicates the metal free isomerization of ketimine was negligibly slow in the experimental conditions. Since little spectral difference can be expected between pyridoxamine and the ketimine, no definite conclusion was obtained on the equilibrium of the metal free ketimine formation (3).

The spectral changes observed in the pyridoxamine-ethyl pyruvate-Zn(II) systems I and II are shown in Figures 1 and 2. In the initial spectrum of system II, there was only one

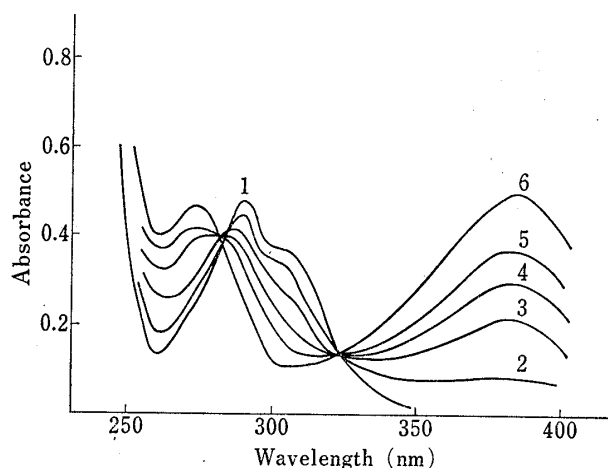


Fig. 1. Spectral Time Study for the Reaction System I of Pyridoxamine  $1 \times 10^{-4} \text{ M}$ , Ethyl Pyruvate  $1 \times 10^{-3} \text{ M}$  and Zn(II) Perchlorate  $1 \times 10^{-4} \text{ M}$ .

Times after the initiation of the reaction are 1: 1 min, 2: 10 min, 3: 30 min, 4: 2 hours, 5: 7 hours, 6: 18 hours.

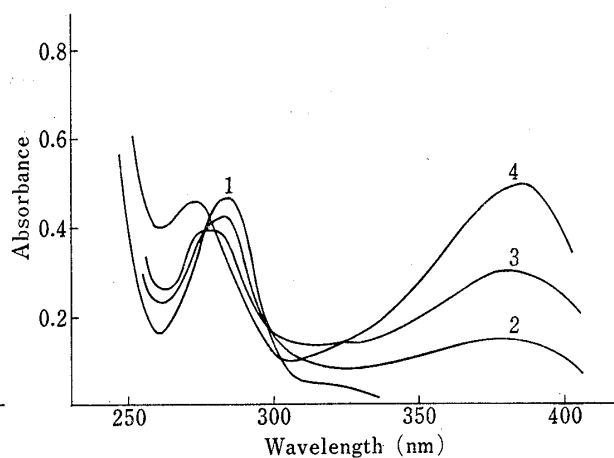


Fig. 2. Spectral Time Study for the Reaction System II of Pyridoxamine  $1 \times 10^{-4} \text{ M}$ , Ethyl Pyruvate  $1 \times 10^{-3} \text{ M}$  and Zn(II) Perchlorate  $1 \times 10^{-4} \text{ M}$ .

Times after the initiation of the reaction are 1: 1 min, 2: 10 min, 3: 1 hour, 4: 16 hours.

absorption peak at 284 nm, which should be assigned to the ketimine Zn(II) chelate. The absorption due to pyridoxamine Zn(II) chelate was absent. In the early stages of the reaction system I, two bands were observed at around 305 nm and 288 nm, which indicated the presence of both pyridoxamine-Zn(II) and the ketimine-Zn(II) chelates. Essentially the same spectral changes were obtained in the pyridoxamine-ethyl  $\alpha$ -ketobutyrate-Zn(II) systems. These facts show that by the use of esters of keto acids the equilibrium (4) is shifted to the ketimine Zn(II) chelate.

### Reactions of Ni(II) chelates

Figure 3 shows the spectral changes in the 3-hydroxy-4-aminomethylpyridine-Na pyruvate-Ni(II) system I. In the initial spectrum, there is only one absorption band at 302 nm, assignable to Ni(II) chelate of the amine.<sup>1)</sup> A new absorption peak appeared at 286 nm with a slight decrease of the 302-nm band. Then, with a gradual decrease of the both peaks, the band at 379 nm, due to the aldimine Ni(II) chelate, was produced. The 286-nm band is assigned to the ketimine Ni(II) chelate.

When Na  $\alpha$ -ketobutyrate was used, the absorbance of the 286-nm band was smaller. When the amine was replaced by pyridoxamine, the corresponding band was at 292 nm and the other spectral features were not greatly different.

In the reactions of pyridoxamine-K  $\alpha$ -ketoisovalerate-Ni(II) reported before,<sup>5)</sup> the band due to the ketimine Ni(II) chelate was not recorded in system I. The single peak at 310 nm

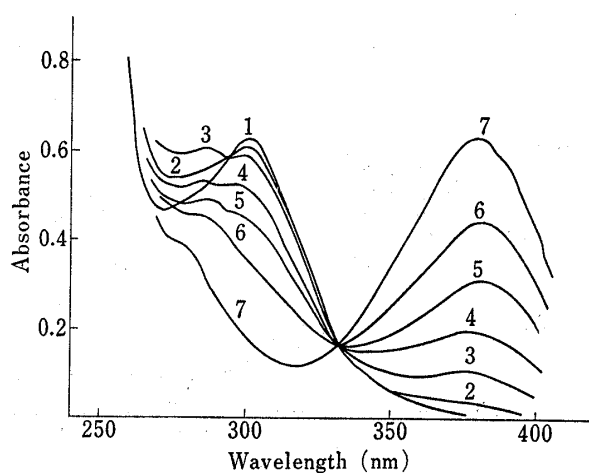


Fig. 3. Spectral Time Study for the Reaction System I of 3-Hydroxy-4-aminomethylpyridine  $1 \times 10^{-4} \text{M}$ , Na Pyruvate  $1 \times 10^{-3} \text{M}$  and Ni(II) Perchlorate  $1 \times 10^{-4} \text{M}$

Times after the initiation of the reaction are 1: 1 min, 2: 10 min, 3: 20 min, 4: 2 hours, 5: 3 hours, 6: 5 hours, 7: 26 hours.

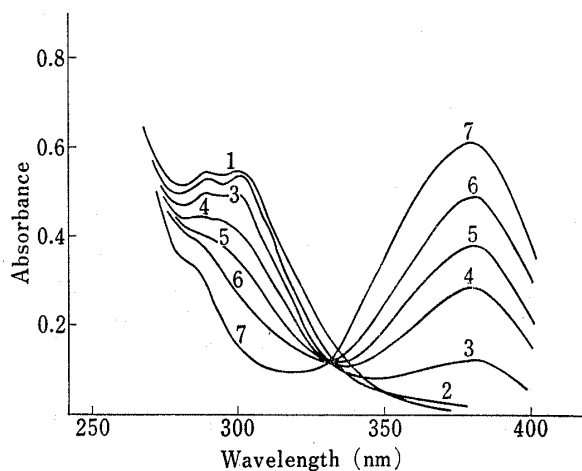


Fig. 4. Spectral Time Study for the Reaction System II of 3-Hydroxy-4-aminomethylpyridine  $1 \times 10^{-4} \text{M}$ , Na Pyruvate  $1 \times 10^{-3} \text{M}$  and Ni(II) Perchlorate  $1 \times 10^{-4} \text{M}$

Times after the initiation of the reaction are 1: 1 min, 2: 10 min, 3: 30 min, 4: 2 hours, 5: 3 hours, 6: 5 hours, 7: 26 hours.

due to pyridoxamine Ni(II) chelate disappeared as a new absorption band at 392 nm, due to the aldimine Ni(II) chelate, grew in. In other words, the accumulation of the ketimine Ni(II) chelate was appreciable with pyruvate, smaller with  $\alpha$ -ketobutyrate and not observed with  $\alpha$ -ketoisovalerate.

In the 3-hydroxy-4-aminomethylpyridine-Na pyruvate-Ni(II) reaction system II, shown in Figure 4, two bands due to the amine-Ni(II) and the ketimine-Ni(II) chelates were observed in the initial spectrum. Both the bands decreased together with growth of the 379-nm band. Apparently the ketimine Ni(II) chelate formation (4) and its isomerization (5) steps are of comparable rate with pyruvate.

For the production of the aldimine Ni(II) chelates, system II with  $\alpha$ -ketobutyrate and  $\alpha$ -ketoisovalerate had an initial fast phase followed by a relatively slow phase. The initial phase may be due to contribution from the isomerization step (5). In system I and in the slow phase of system II, conversion of the amine Ni(II) chelate to the ketimine Ni(II) chelate (4) is the rate determining step and subsequent isomerization to the aldimine chelate (5) is relatively rapid with these keto acid anions.

Reactions of pyridoxamine, ethyl pyruvate and Ni(II) ion were somewhat different from those of the keto acid anions. In the initial spectrum of system II, there was only one band at 284 nm, assignable to the ketimine Ni(II) chelate. The band decreased with an increase of the band of the aldimine Ni(II) chelate. A broad absorption around 290 nm was observed in the early stages of system I. This absorption may be produced by the overlap of the band of the ketimine Ni(II) chelate at 284 nm and that of pyridoxamine Ni(II) chelate at 311 nm. These suggest that esterification of keto acid causes the equilibrium (4) shift to the ketimine Ni(II) chelate, as in reactions with Zn(II).

### Reactions of Cu(II) Chelates

Figures 5 and 6 show the spectral changes observed in the pyridoxamine-Na  $\alpha$ -ketobutyrate-Cu(II) systems. Marked differences between system I and II are noted.

The spectral change of system I is interpreted as the very slow conversion of pyridoxamine Cu(II) chelate to the aldimine Cu(II) chelate without the accumulation of the ketimine Cu(II) chelate. Rate of the reaction decreased in the order pyruvate  $>$   $\alpha$ -ketobutyrate  $>$   $\alpha$ -ketoiso-

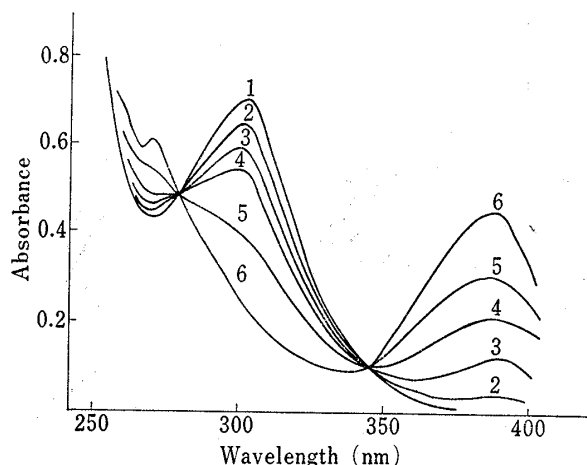


Fig. 5. Spectral Time Study for the Reaction System I of Pyridoxamine  $1 \times 10^{-4}M$ , Na  $\alpha$ -Ketobutyrate  $1 \times 10^{-3}M$  and Cu(II) Acetate  $1 \times 10^{-4}M$

Times after the initiation of the reaction are 1: 5 min, 2: 1 hour, 3: 2 hours, 4: 5 hours, 5: 10 hours, 6: 25 hours.

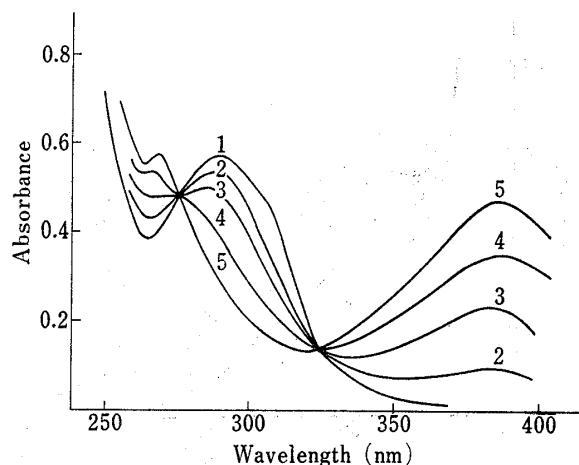


Fig. 6. Spectral Time Study for the Reaction System II of Pyridoxamine  $1 \times 10^{-4}M$ , Na  $\alpha$ -Ketobutyrate  $1 \times 10^{-3}M$  and Cu(II) Acetate  $1 \times 10^{-4}M$

Times after the initiation of the reaction are 1: 1 min, 2: 10 min, 3: 20 min, 4: 40 min, 5: 80 min.

valerate. With  $\alpha$ -ketoisovalerate, the initial spectrum, which was identical to that of pyridoxamine Cu(II) chelate, was reported to undergo very little changes after several days.<sup>5)</sup>

On the other hand, changes of the spectra with time were observed immediately in system II. The changes consisted of a rapid decrease of a band at 290 nm with an increase of a broad band at 395 nm. The former band is ascribed to the ketimine Cu(II) chelate and the latter to the aldimine Cu(II) chelate. In system II reaction rates were not greatly influenced by replacement of the amine or the keto acid anion. These facts indicate that by chelation with Cu(II) the isomerization step (5) is accelerated, while the ketimine formation (4) is greatly retarded.

With keto acid esters, the band assignable to the ketimine Cu(II) chelate was at 280 nm, which decreased rapidly with the appearance of the aldimine Cu(II) chelate, in system II. However, the spectra were obscured and showed the conversion to the aldimine chelate to be incomplete, probably due to side reactions.

### Comparisons of Effects of the Reactants

On the basis of the relative reaction rates, it is possible to make some comparisons of the effects of structure of the reactants.

From the results described above and the known rates of metal free ketimine formation (3) of  $\alpha$ -keto acid anion,<sup>4)</sup> we confirm the previous conclusion<sup>5)</sup> that the relative effects of the divalent metal ions on ketimine formation are: Zn(II) > metal free > Ni(II) > Cu(II). Increased rate of ketimine formation may be explained in terms of the promnastic effect of Zn(II) ion discussed by Hopgood and Leussing.<sup>10)</sup> The effect of Ni(II) and Cu(II) ions in slowing down the rate may be due to decreased nucleophilicities of the amines chelated by the metal ions with a strong affinity for amino nitrogens.

In agreement with the previous argument,<sup>5)</sup> relative effects of the metal ions on the isomerization of ketimine chelate (5) are concluded to be Cu(II) > Zn(II) > Ni(II) > metal free. In order to relate the behavior of the metal ions to their coordination properties, more quantitative studies on equilibria and kinetics as well as knowledges of chelate stabilities and of properties of coordinating ligands may be necessary.

10) D. Hopgood and D.L. Leussing, *J. Amer. Chem. Soc.*, **91**, 3740 (1969).

No appreciable difference between kinetic properties of pyridoxamine and the analog, 3-hydroxy-4-aminomethylpyridine, was detected in the present study.

Relative rate of keto acid anions, pyruvate >  $\alpha$ -ketobutyrate >  $\alpha$ -ketoisovalerate was noted for ketimine formation in the presence as well as in the absence of the metal ions. Between the three keto acid anions, there was little difference in the rate of isomerization of ketimine to aldimine chelates.

Esterification of the keto acids resulted in the increase of the rate of ketimine formation. On the other hand, the rate of isomerization of Schiff bases in the presence of the divalent metal ions was not greatly influenced, though the interference of side reactions made the conclusion unclear. An intermediate absorbing in the 500-nm region similar to that observed in the presence of Al(III) ion<sup>7)</sup> was not detected in the present work.