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Kinetics and Mechanisms of Schiff Base Formation from Pyridoxal in Methanol

Yoshikazu Matsushima[1a)]

National Institute of Radiological Sciences[1)]

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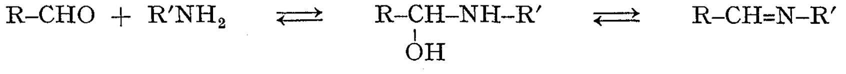
Formation of Schiff bases of pyridoxal with glycine and glycinamide in methanol were studied kinetically by the changes of electronic absorption spectra. When glycine or glycinamide was added to carbinolamine formed from pyridoxal and sarcosine, Schiff base was also formed. Both sarcosine catalyzed and direct (under sarcosine-free) Schiff base formation followed the first order kinetics as long as amines were excess over pyridoxal. From variations of the pseudo first order rate constants with acid–alkaline concentration, following conclusions were obtained; in direct Schiff base formation of glycine, rate determining step was dehydration in acidic region and carbinolamine formation in neutral and alkaline methanol; in the case of glycinamide, carbinolamine formation was always rate determining step. Formation of Schiff base of glycine from Schiff base of glycinamide and glycine, transaldimination, was rapid reaction.

Enzymes containing pyridoxal as a cofactor catalyze many important reactions in amino acid metabolism such as transamination, decarboxylation, oxidative deamination, racemization and so forth. These reactions were found to be carried out nonenzymatically with pyridoxal, amino acid and catalyst as transition metal ions or imidazole.[2,3)] The first step of both enzymatic and nonenzymatic reactions is believed to be formation of Schiff base of pyridoxal and amino acid. Therefore, kinetical studies on Schiff base formation were expected to shed light on the mechanisms of enzymatic and nonenzymatic reactions. However, the fact that Schiff bases could hardly exist in aqueous media because of large dissociation constant lended difficulties on the studies.

Cordes and Jencks[4)] have devised a neat experimental system for investigating Schiff base formation kinetically. They found that in aniline catalyzed semicarbazone formation of aldehyde in aqueous solution formation of Schiff base from aldehyde and aniline was rate determining step. They studied mechanism of Schiff base formation from *p*-chlorobenzaldehyde and aniline by kinetical measurements of semicarbazone formation. Two step mechanism *via* carbinolamine intermediate and a transition in rate determing step from neutral to acidic conditions were concluded.



$$\text{R–CHO} + \text{R}'\text{NH}_2 \rightleftarrows \underset{\text{OH}}{\text{R–CH}}\text{–NH–R}' \rightleftarrows \text{R–CH=N–R}'$$

In the former work,[5)] reactions of pyridoxal and various amino acids or amines in methanol were studied by changes of electronic absorption spectra. Most amino acids and amines were proved to form Schiff bases, but amino acids having a secondary amino group as sarcosine formed carbinolamine. Schiff bases and carbinolamines formed were fairly stable in this

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1) Location: *Anagawa, Chiba.* a) Present address: *Faculty of Pharmaceutical Sciences, Kyushu University, Katakasu, Fukuoka.*

2) D.E. Metzler, M. Ikawa and E.E. Snell, *J. Am. Chem. Soc.*, **76**, 648 (1954); T.C. Bruice and R.M. Topping, *ibid.*, **85**, 1480 (1963).

3) Y. Matsushima and A.E. Martell, *J. Am. Chem. Soc.*, **89**, 1331 (1967).

4) E.H. Cordes and W.P. Jencks, *J. Am. Chem. Soc.*, **84**, 832 (1962).

5) Y. Matsushima, *Chem. Pharm. Bull.* (Tokyo), **16**, 2050 (1968).

solvent and rate of formation could be easily measured by spectral changes with time. In some cases methanol may be expected to provide a better environment for the studies of enzyme models than pure water.[3] Thus, methanol seemed suitable for kinetical studies on Schiff base formation.

The purpose of the present work is to elucidate the mechanisms of Schiff base formation from pyridoxal and amino acids or amines in methanol. Among amino acids and amines examined before,[5] glycine and glycinamide were chosen, because they could form the different type of Schiff bases as mentioned later. Addition of glycine or glycinamide to methanol solution of carbinolamine formed from pyridoxal and sarcosine was found to produce Schiff bases. Carbinolamine from sarcosine might figure as carbinolamine intermediate in Schiff base formation. The kinetical studies on the reaction were expected to give a new clue in the mechanistic study. The reaction from carbinolamine of sarcosine may be named as sarcosine catalyzed reactions, while reactions in the absence of sarcosine as direct reactions.

One of the difficulties in studies using nonaqueous media is how to express acid and alkaline concentration in the solution. It is possible to express acid–alkaline concentration in methanol as pH connected with pH in aqueous solution.[6] However, there are many difficulties to apply the pH to complexed systems. In the present work, ratio of added potassium hydroxide to total concentration of acidic group of amino acids were used as the measure of the acidity. For example, in experiments with glycine added volume of potassium hydroxide solution was varied between zero and one equimolar to glycine. Then, the ratio of glycinate form to total glycine concentration varied from 0 to 100%. This percent scale was tentatively named as glycine acidity scale. By using this scale, some region of acid–alkaline concentration of methanol solution could be reproducibly expressed. However, comparison between different amines were impossible. For the comparison, universal scale using buffer substance was necessary. N,N–Dimethylglycine (hereafter abbreviated as DMG) was found suitable for this purpose, because of the following merits; 1) DMG was not involved in the reactions under study, 2) do not have catalytic effects even though existed in high concentration (rate constants did not vary with total concentration of DMG), 3) is highly soluble in methanol.

From electronic absorption spectra,[5] Schiff bases of pyridoxal were found to exist in neutral methanol in two tautomeric species, *i.e.* keto–enamine and enol–imine. Schiff base of glycine (pyridoxylideneglycine) had intense bands at both 418 mμ and 335 mμ, showing considerable amounts of the both species, while Schiff base of glycinamide (pyridoxylideneglycinamide) lacked 418 mμ band, indicating the absence of keto–enamine species. Since the spectra of the two Schiff bases were sufficiently different, interconversion from one to the other could also be detected by spectra.

Experimental

Materials used were the same as reported before.[5] Calculated volumes of methanol solutions of reactants, buffer substance and KOH were mixed in the predetermined order in a volumetric flask. In direct reactions, freshly made methanol solution of pyridoxal was added last. In sarcosine catalyzed reactions, glycine or glycinamide was added 30 minutes after mixing solutions of pyridoxal, sarcosine and other substances. After all solutions were mixed, the sample solutions were adjusted quickly to a definite volume by adding methanol. In most experiments, solutions were so prepared to contain finally 1×10^{-4} M pyridoxal, and 1×10^{-3} M amines (glycine, glycinamide and/or sarcosine). Total concentration of DMG was 1×10^{-2} M, whenever used. The moment when the last methanol solution was added was taken as the initial time of the reaction. The sample solutions were shaken well and quickly transferred to silica cells for absorption measurements.

Electronic absorption measurements were made with a Cary Model 14 spectrophotometer. For an absorbance–time study, only chart driving mechanism was at work, by fixing wavelength at the predetermined

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[6] R.G. Bates, "Determination of pH," John Wiley and Sons, Inc., New York, N.Y., 1964.

value. Thus, the absorbance–time curves were obtained. For Schiff base formation, absorbance changes at 335 mμ were measured, although in some cases 418 mμ band was also adopted. For carbinolamine formation, absorbance changes of 282 mμ band were satisfactorily used.

Experimental procedures as preparation and mixing of solutions were conducted at 25°±2°. The cell compartment of spectrophotometer was thermostated at 25°±0.2° throughout the studies.

Plotting the value $A_t = \log a_\infty/(a_\infty - a_t)$ against time t gave straight lines, where a_t and a_∞ indicated absorbance at time t and at the completion of the reaction, respectively. This shows reaction followed the first order kinetics for the formation of Schiff bases or carbinolamine in the experimental conditions used. Pseudo-first order rate constants k_{obs} were obtained as a slope of the straight line.

Results

Spectral Changes in Schiff Base Formation

Spectral change in direct reaction of pyridoxal and glycine is shown in Fig. 1. The initial spectrum was the same as pyridoxal in neutral methanol. The final curve indicated the formation of pyridoxylideneglycine. In Fig. 2 is shown sarcosine catalyzed pyridoxylideneglycine formation. The initial curve was the same as carbinolamine of sarcosine. The final curve was the same as direct reaction.

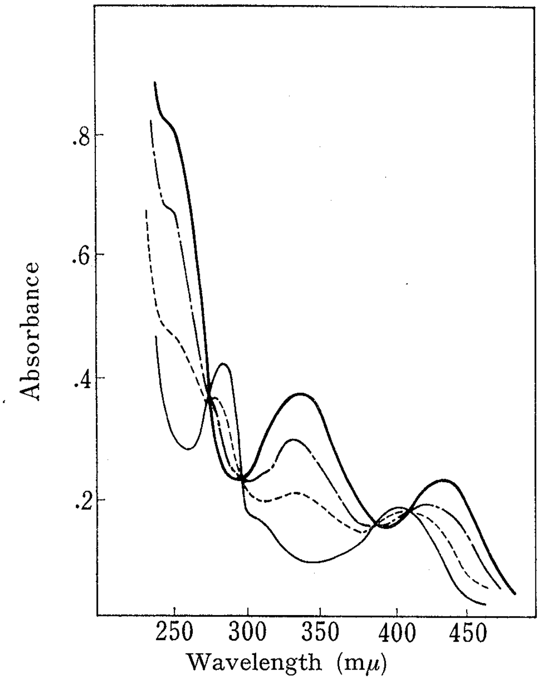


Fig. 1. Changes of Electronic Absorption Spectra with Time for Direct Reaction of Pyridoxal and Glycine

initial solution contained 1×10^{-4} M pyridoxal and 1×10^{-3} M glycine

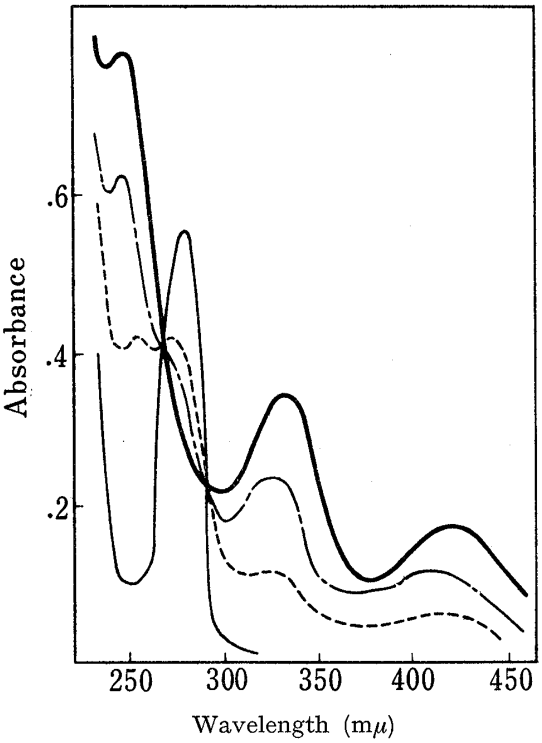


Fig. 2. Changes of Electronic Absorption Spectra with Time for Sarcosine Catalyzed Reaction of Pyridoxal and Glycine

solution contained 1×10^{-4} pyridoxal, 1×10^{-3} M sarcosine and 1×10^{-3} M glycine

——— initial spectrum
- - - - - -, — - — - — intermediate spectrum
━━━ final spectrum

Direct reaction of pyridoxal with glycinamide is shown in Fig. 3. The final spectrum was that of pyridoxylideneglycinamide, having an intense band at 335 mμ. Spectral change in sarcosine catalyzed Schiff base formation from glycinamide is shown in Fig. 4. The initial spectrum was that of carbinolamine, whereas the final spectrum was that of pyridoxylideneglycinamide.

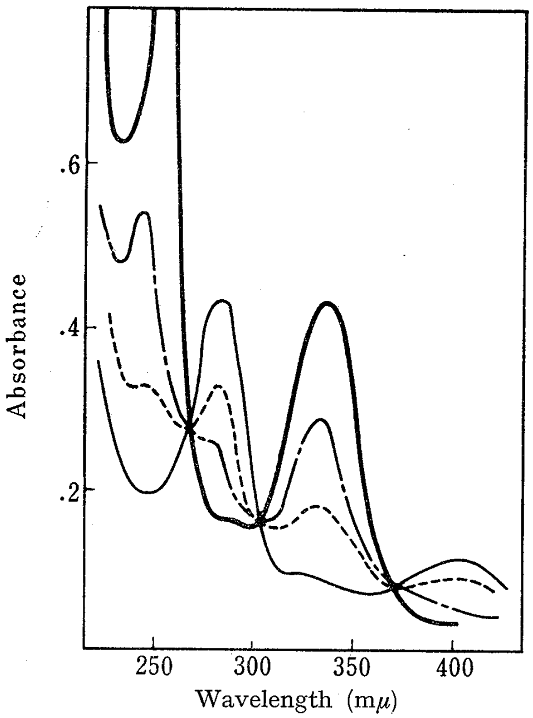


Fig. 3. Changes of Electronic Absorption Spectra with Time for Direct Reaction of Pyridoxal and Glycinamide

initial solution contained 1×10^{-4} M pyridoxal and 1×10^{-3} M glycinamide

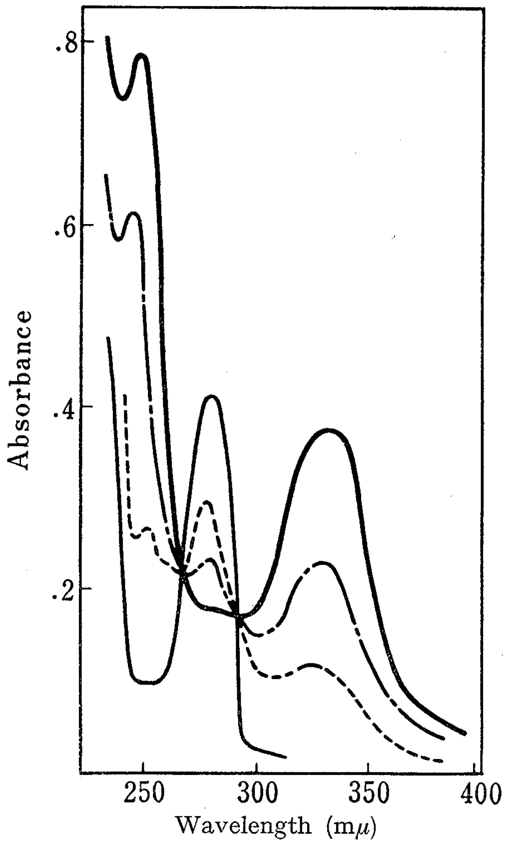


Fig. 4. Changes of Electronic Absorption Spectra with Time for Sarcosine Catalyzed Reaction of Pyridoxal and Glycinamide

solution contained 1×10^{-4} M pyridoxal, 1×10^{-3} M sarcosine and 1×10^{-3} M glycinamide

——— initial spectrum -·-·-·-, ------ intermediate spectrum ━━━ final spectrum

Kinetics of Carbinolamine Formation

Carbinolamine formed from pyridoxal and sarcosine in methanol was detected by the strong absorption band at 282 mμ. The formation reaction measured by the increase of absorbance at 282 mμ was more rapid than direct reactions of Schiff base formation. When concentration of sarcosine was sufficiently higher than that of pyridoxal, carbinolamine formation followed the first order kinetics. Variations of the first order rate constant k_{obs} with acid concentration expressed as sarcosine acidity scale are shown in Fig. 5. The rate constant k_{obs} increased as media become neutral from alkaline and levelled off at 30—40% region. In more acidic media k_{obs} decreased. However, in acidic region larger dissociation to the components and small spectral changes made the measurement difficult. Reaction in acidic region, therefore, could not be discussed on the same bases as neutral region.

In sarcosine catalyzed Schiff base formation, time interval between mixing of pyridoxal and sarcosine and addition of amines may effect the rate constant k_{obs}. Relation of this time interval and k_{obs} of sarcosine catalyzed pyridoxylideneglycinamide formation is shown in Fig. 6. After 20 minutes when carbinolamine formation from sarcosine obtained equilibrium, this time interval did not affect k_{obs}. Therefore, experiments on sarcosine catalyzed reactions were designed to add glycine or glycinamide 30 minutes after the mixing of pyridoxal and sarcosine.

Effect of sarcosine concentration on sarcosine catalyzed Schiff base formation was also examined. Although k_{obs} increased with concentration of sarcosine as long as it was low, k_{obs} levelled off at ten times concentrated as pyridoxal.

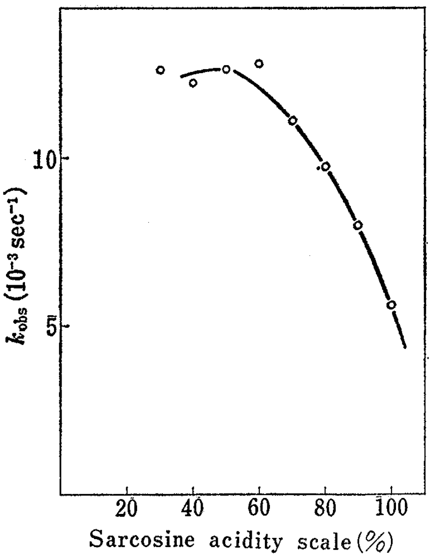


Fig. 5. Variation of k_{obs} of Carbinolamine Formation from Pyridoxal and Sarcosine with Sarcosine Acidity Scale

solutions contained 1×10^{-4} M pyridoxal and 1×10^{-3} M sarcosine

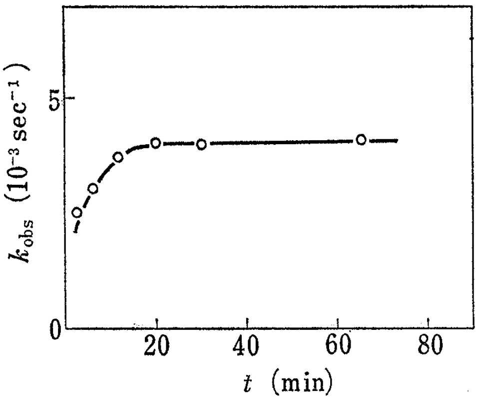
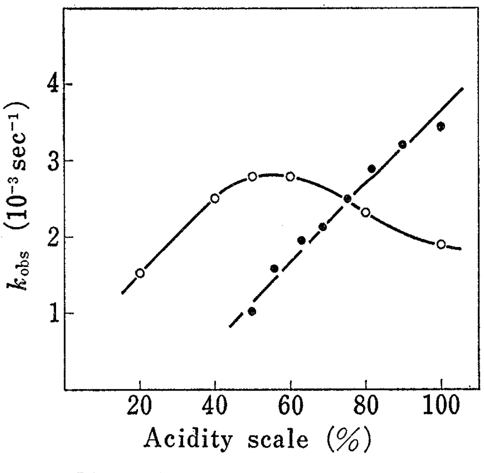


Fig. 6. Variation of k_{obs} of Sarcosine Catalyzed Pyridoxylideneglycinamide Formation with Interval t

Glycinamide was added t minutes after the mixing pyridoxal and sarcosine. Concentrations in the final solution were 1×10^{-4} M pyridoxal, 1×10^{-3} M sarcosine, 1×10^{-3} M glycinamide. Pyridoxylideneglycinamide was formed with pseudo-first order rate constant shown in ordinate.

Kinetics of Schiff Base Formation from Glycine

Variations of k_{obs} of pyridoxylideneglycine formation with acidity scale are shown in Fig. 7. In direct reaction, there was a maximum at 50—60% of glycine acidity scale. On the other hand, k_{obs} increased with acidity scale in sarcosine catalyzed reaction. All the final spectra in 10—100% regions of acidity scales were identical, showing formation of the same species were being observed.



$$\frac{[\text{Gly K}]}{[\text{Gly}]_T} \text{ or } \frac{[\text{Gly K}]+[\text{Sarc K}]}{[\text{Gly}]_T+[\text{Sarc}]_T}$$

Fig. 7. Variation of k_{obs} of Pyridoxylideneglycine Formation with Acidity Scales Solutions contained 1×10^{-4} M Pyridoxal and 1×10^{-3} M Glycine

—○—○— direct reaction with glycine acidity scale

—●—●— sarcosine catalyzed reaction with glycine plus sarcosine acidity scale (Total concentration of sarcosine was 1×10^{-3} M.)

Variation of k_{obs} of direct reaction with total glycine concentration at 50% glycine acidity scale is shown in Fig. 8. The rate constant k_{obs} increased linearly with total glycine concentration. From the slope of the straight line, second order rate constant for direct reaction was calculated to be

$$k_{\text{II}} = 2.49 \text{ M}^{-1} \cdot \text{sec}^{-1}$$

Results with DMG acidity scale are shown in Fig. 9. Rate profiles did not change much with change of acidity scale. Sarcosine catalyzed reaction showed the same k_{obs} value as direct reaction in 0—30% DMG region. In more alkaline region, k_{obs} of sarcosine catalyzed reaction increased linearly with the scale, while that of direct reaction had maximum at about 50%.

Kinetics of Schiff Base Formation from Glycinamide

Direct reaction of glycinamide was slower than glycine. Relation of k_{obs} and glycinamide

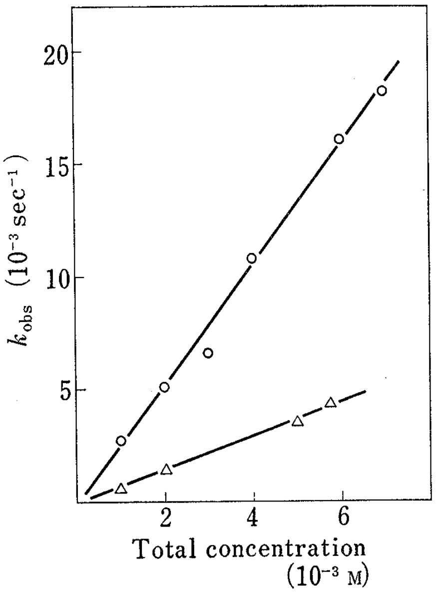


Fig. 8. Variations of Pseudo-first Order Rate Constants of Direct Reactions with Total Concentrations of Glycine and Glyicinamide

solutions contained 1×10^{-4} M pyridoxal
—○—○— glycine
—△—△— glycinamide

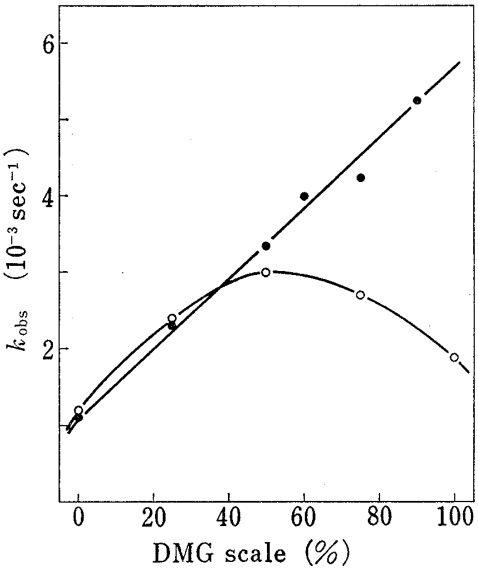
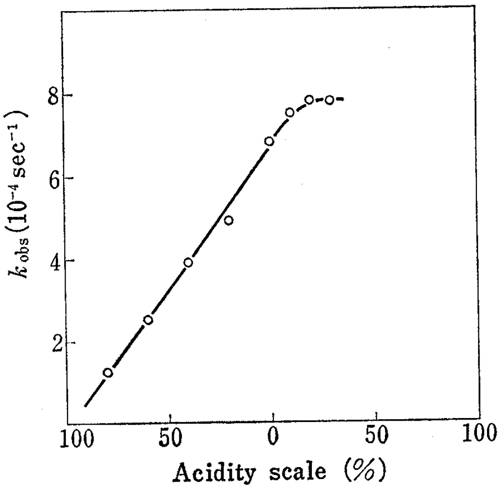


Fig. 9. Variation of k_{obs} of Pyridoxylideneglycine Formation with DMG Acidity Scale

solutions contained 1×10^{-4} M Pyridoxal, 1×10^{-3} M Glycine and 1×10^{-2} M DMG
—○—○— direct reaction
—●—●— sarcosine catalyzed reaction

acidity scale is shown in Fig. 10. When KOH concentration was less than 20% of total concentration of glycinamide, k_{obs} increased linearly with added KOH. In more alkaline concentration, final spectra were that of anionic form of Schiff base and kinetical data cannot be compared with that of neutral region.



$$\frac{[\text{Glycinamide}\cdot\text{HCl}]}{[\text{Glycinamide}]_T} \quad \frac{[\text{Glycinamide}\cdot\text{KOH}]}{[\text{Glycinamide}]_T}$$

Fig. 10. Variation of k_{obs} of Direct Reaction of Pyridoxylideneglycinamide Formation with Glycinamide Acidity Scale

solutions contained 1×10^{-4} M pyridoxal and 1×10^{-3} M glycinamide

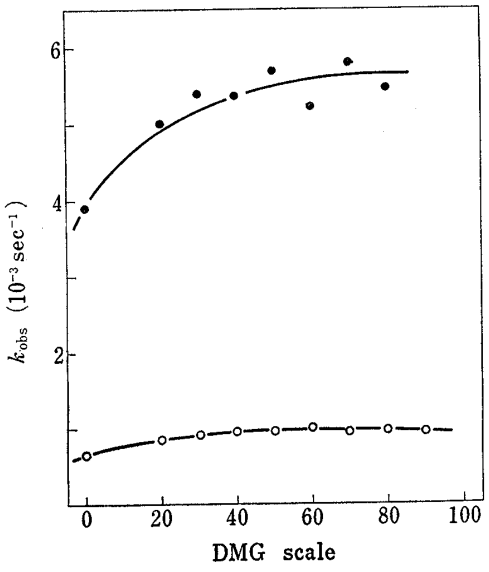


Fig. 11. Variation of k_{obs} of Pyridoxylideneglycinamide Formation with DMG Acidity Scale

solutions contained 1×10^{-4} M pyridoxal, 1×10^{-3} M glycinamide and 1×10^{-2} M DMG
—○—○— direct reaction
—●—●— sarcosine catalyzed reaction (Concentration of sarcosine was 1×10^{-3} M.)

In neutral methanol *i.e.* at 0% in glycinamide scale, variation of k_{obs} with total concentration of glycinamide was examined. Results are shown in Fig. 8. Linear increase of k_{obs} with total concentration is seen. From the slope of the straight line, the second order rate constant k_{II} was calculated as follows.

$$k_{II}=0.74\ \mathrm{M}^{-1}\cdot\mathrm{sec}^{-1}$$

Unlike glycine, sarcosine catalyzed reaction was much more rapid than direct reaction in glycinamide. Variations of the rate constants of two reactions against DMG acidity scale are shown in Fig. 11. The rate constant k_{obs} of sarcosine catalyzed reaction increased with the scale while k_{obs} of direct reaction did not show large variation in DMG region.

Transaldimination

As mentioned before, spectra of Schiff bases of glycine and glycinamide were sufficiently different each other. For this reason, change of one of the Schiff base to the other can be detected by the change of spectra. Addition of excess glycine to pyridoxylideneglycinamide in methanol resulted instantaneous change of spectra to that of pyridoxylideneglycine. Although the rapidness of the reaction made rate measurement diffcult, a rough estimation of k_{obs} was $1\times10^{-1}\ \mathrm{sec}^{-1}$.

This clearly shows that there is a pathway of direct conversion between Schiff bases, *i.e.* transaldimination, which is rapid step compared to Schiff base formation from aldehyde and amines.

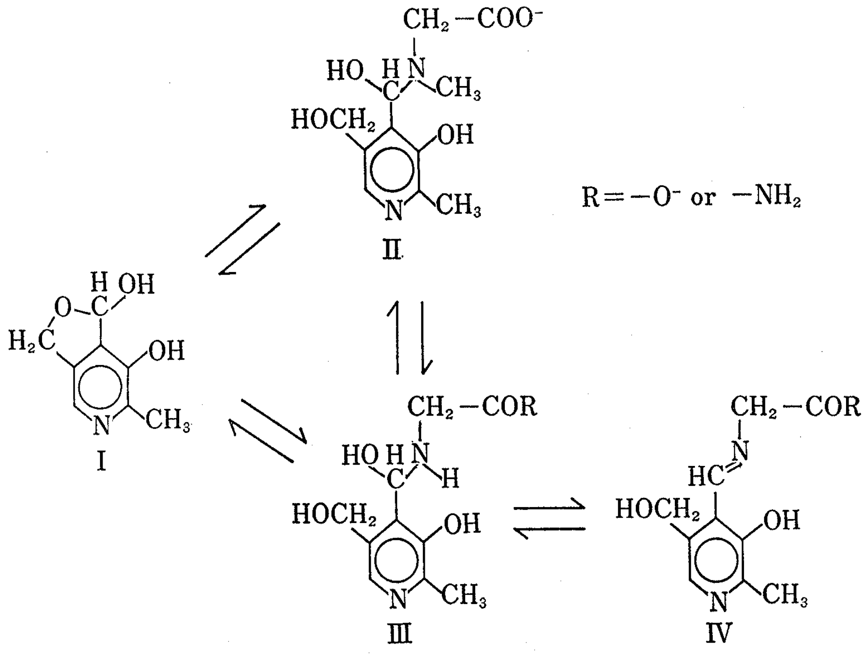
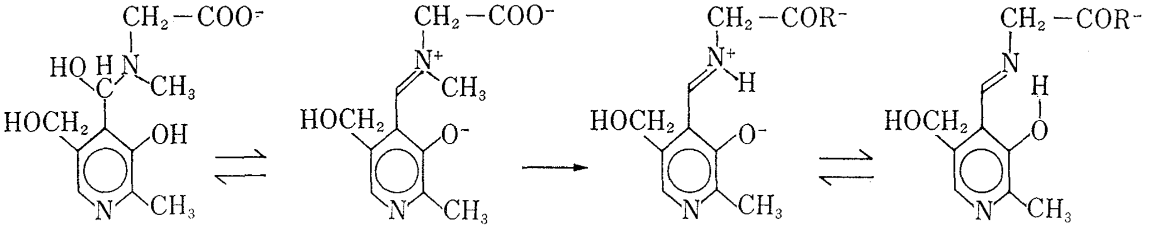


Chart 1

Discussion

In direct reaction of Schiff bases formation, carbinolamine intermediate (III), shown in Chart 1, may be present. In sarcosine catalyzed reactions, exchange step between two carbinolamine II$\leftrightarrows$III can be assumed. Therefore, pathways of this reaction may be II→III→IV, or II→I→III→IV. The latter pathway seems unlikely, since sarcosine catalyzed reactions were not slower than direct reactions under the same experimental conditions. Another possible pathway is that keto-enamine type Schiff base from sarcosine and transaldimination step are involved as Chart 2.



III

Chart 2

However, this type of Schiff base from sarcosine could not be detected by spectroscopy. Even if existed, less than detectable amount, that is 10^{-4} part or less of total carbinolamine could only be expected. Rate of transaldimination would be 10^{2} times or less of that of direct reaction. Therefore, reaction by this pathway would be much slower. For this reason, to assume this pathway is also unlikely. Then, the pathway II→III→IV, or equilibrium mixture of I, II, III, to IV are the most likely for the sarcosine catalyzed reactions.

In pyridoxylideneglycinamide formation, sarcosine catalyzed reaction was more rapid than direct reaction in all region of DMG scale. Therefore, rate determining step in direct reaction should be carbinolamine formation step from pyridoxal and glycinamide (I→III), which was by-passed in sarcosine catalyzed reaction. Linear decrease of k_{obs} was observed as concentration of glycinamide hydrochloride increased. This suggests that protonated amine is inactive towards carbinolamine formation. Provided exchange between carbinolamines be a rapid step as transaldimination, rate of sarcosine catalyzed reaction should be rate of dehydration step (III→IV).

For Schiff base formation from glycine, the same interpretation as glycinamide can be made in alkaline region of DMG scale. The rate constant k_{obs} of direct reaction showed decrease with alkaline concentration. This is attributable to the nature of carbinolamine formation, as the same decrease was observed in reaction of sarcosine and pyridoxal. Supposed that sarcosine catalyzed reaction showed the rate of dehydration step, this rate increase with alkaline concentration. In 0—30% region of DMG scale, sarcosine catalyzed and direct reactions showed the same rates. This suggests that in direct reaction the rate determining step is dehydration in this region.

Therefore, direct reaction of glycine undergoes a transition in rate determining step from dehydration at 0—30% region of DMG acidity scale to carbinolamine formation under more alkaline conditions. This seems opposing to the results of Cordes and Jencks.4) According to them, rate determining step was amine attack to form tetrahedral intermediate in acidic conditions and was dehydration in neutral solution; the pH rate profile had a maximum at pH 4, where the transition in rate determing step took place.

In this work, pyridoxal was supposed to exist in methanol solution mainly as hemiacetal form rather than aldehyde form.7) DMG region studied may correspond to pH 8—10 in aqueous solution.8) Considering these facts and large difference of dissociation constants and molecular species of Schiff bases between methanol and aqueous solution, apparent contradiction between the two conclusions may be rationalized.

From the present results, it is concluded that slow formation rate of Schiff base from pyridoxal and glycinamide in methanol could be attributed to carbinolamine formation step. As for dehydration step, glycinamide was faster than the case of glycine. Rate of Schiff base formation from pyridoxal and glycine, on the other side, was determined by the rate of dehydration in acidic region and by that of carbinolamine formation in neutral and alkaline methanol. Carbinolamine formation from pyridoxal and sarcosine was faster than

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7) Y. Matsushima and A.E. Martell, *J. Am. Chem. Soc.*, **89**, 1322 (1967).

8) S.P. Datta and A.K. Grzybowski, *J. Chem. Soc.*, **1959**, 1091.

Schiff base formation of glycine in neutral and alkaline region. Then, the following relation concerning the rate of carbinolamine formation may be induced. Sarcosine> glycine> glycinamide. Basicities of amino group seems to be promotive in this step.

For dehydration step, order of the rate may be expressed as glycinamide> glycinate> glycine (neutral form). In this step carboxyl and carboxylate group seem suppressive. Slow formation of Schiff base of aspartate and glutamate reported before[5] may be attributed to carboxylate groups. The fast formation rate of Schiff base from pyridoxal and ε-amino group of lysine[5] can be understood by basicity of the amino group and the lack of carboxylate group in the vicinity.

It is believed that in some enzymes aldehyde group of pyridoxal forms Schiff base bond with ε-amino group of lysine in enzyme protein and enzyme substrate complex formation proceeds *via* transaldimination between the Schiff base bond and amino group of substrate amino acid.[9] Fast formation rates of ε-pyridoxylidenelysine and transaldimination reaction demonstrated may be a good model to favor the above scheme.

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9) E.E. Snell, "Chemical and Biological Aspects of Pyridoxal Catalysis," eds. by E.E. Snell, P.M. Fasella, A. Braunstein and A. Rossi Fanelli, Macmillan Co., New York, N.Y., 1963, p. 1.