[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE]

Pyridoxal Catalysis of Non-enzymatic Transamination in Ethanol Solution[1]

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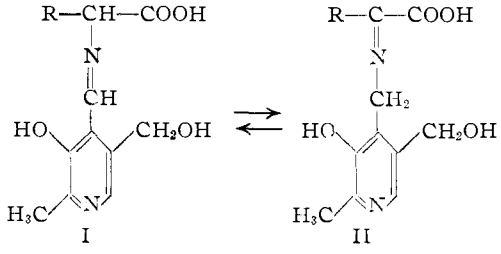
Transamination between pyridoxamine and α-keto acid, or amino acid and pyridoxal, readily occurs in absolute ethanol solution. Metal salts or an elevated temperature are not required for the reaction. Under the same condition pyridoxal catalyzes transamination between amino acid and keto acid, thus mimicking the reactions catalyzed by transaminases. The reactions appear to proceed *via* the Schiff base intermediates.

Introduction

Formation of Schiff base has been considered to be an intermediate step in reactions catalyzed by vitamin B_6 enzymes. Such a mechanism of reaction for transamination will involve a tautomerism of the aldimine (I) and ketimine (II) forms of the Schiff bases, as



I II

Pyridoxal reacts in an alcohol medium with amino acids to yield pyridoxylideneamino acids.[2,3] Pyridoxylideneglutamic acid[2] and pyridoxylidenealanine[3] were isolated as yellow crystalline compounds from alcoholic solution. Brandenberger and Cohen[2] found that a highly purified glutamate-oxalacetate transaminase preparation from pig heart did not produce α-ketoglutaric acid when pyridoxylideneglutamic acid was used as the substrate, indicating that the compound does not serve as an intermediate in the enzymatic transamination. This result would be expected, since pyridoxal phosphate, and not pyridoxal, is the coenzyme of transamination.

Snell and co-workers have studied metal catalysis in a variety of non-enzymatic reactions involving amino acids and vitamin B_6 derivatives and emphasized the importance of metal chelation of the Schiff base intermediates.[4] Eichhorn and Dawes[5] confirmed the formation of such complexes from observation of the visible absorption spectra of the transamination reaction in aqueous solution. They reported that the spectrum of the metal–pyridoxamine–pyruvate chelate became indistinguishable from that of the metal–pyridoxal–alanine complex upon standing.

The present communication deals with a non-enzymatic transamination system which functions at room temperature in an alcohol medium. Addition of metal salts is not required for the reaction.

(1) Aided by research grants from the National Cancer Institute (Nos. 327 and 2327), the American Cancer Society (Met.-45A), and the Cancer Research Funds of the University of California.

(2) H. Brandenberger and P. P. Cohen, *Helv. Chim. Acta*, **36**, 549 (1953).

(3) D. Heyl, S. A. Harris and K. Folkers, THIS JOURNAL, **70**, 3429 (1948).

(4) D. E. Metzler, M. Ikawa and E. E. Snell, *ibid.*, **76**, 648 (1954).

(5) G. L. Eichhorn and J. W. Dawes, *ibid.*, **76**, 5663 (1954).

Results

(1) **Formation of Schiff Bases in Ethanol.**—Absorption spectra of the yellow solutions prepared by mixing pyridoxal-HCl and α-amino-*n*-butyric acid or pyridoxamine–2HCl and α-keto-butyric acid in absolute ethanol are given in Figs. 1 and 2. Spectra of pyridoxal-HCl and pyridoxamine-2HCl under the same condition are also given for comparison. Neither α-amino-*n*-butyrate nor α-keto-butyrate has significant absorption at the concentration employed for the measurement of the spectrum (1.3×10^{-4} *M*).

It is clearly seen that the spectra of the mixtures are entirely different from those of their components, indicating the formation of the Schiff bases. The difference between the spectrum of pyridoxal-α-amino-*n*-butyrate complex and that of pyridoxamine-α-ketobutyrate complex suggests the existence of the Schiff base tautomers I and II.

(2) **Spectrophotometric Evidence for the Tautomerism of Schiff Bases.**—A 0.01 *M* aqueous solution of pyridoxamine and sodium α-ketobutyrate in 0.2 *M* phosphate buffer, *p*H 7.5, developed a faint yellow color only after standing 24 hours at room temperature, while an equal concentration in ethanol solution gives an intense yellow color immediately. Rapid destruction of pyridoxylideneglutamic acid in aqueous solution has been reported previously.[2] From this it was assumed that addition of water to the alcoholic solution of Schiff base hydrolyzes the tautomers to their most proximate components, *i.e.*, (I) to pyridoxal and amino acid, and (II) to pyridoxamine and keto acid. This permits the course of the transformation in alcoholic solution of II into I to be followed by the appearance of pyridoxal upon addition of water to the alcohol solution of the Schiff base. On this assumption the following experiment was undertaken.

Aliquots were withdrawn at intervals from the ethanol solution of the pyridoxamine-α-ketobutyrate Schiff base (10^{-3} *M* of the initial reactants), diluted 60 times with aqueous 0.1 *M* NaOH, and the absorption spectrum of the diluted solution measured after 30 min. (Fig. 3). It is seen that an absorption peak, which has its maximum at 390 mμ, increases in intensity with time.[6] It is well known that pyridoxal, but not pyridoxamine, has an absorption at this wave length in alkaline solution. Therefore, the results obtained indicate the appearance of pyridoxal in the aqueous solution of the pyridoxamine-α-ketobutyrate Schiff base, and

(6) $O.D._{390}$ measured at the 30th hr. of the experiment was significantly lower than the value at the 16th hr. This is caused by the reaction of pyridoxal and ethanol as discussed below.

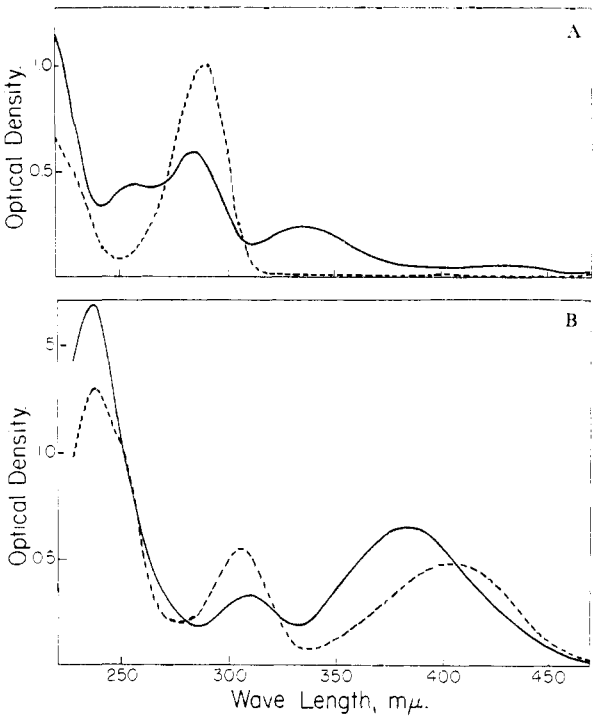


Fig. 1.—Spectrum of pyridoxal-HCl in the presence and absence of α-amino-*n*-butyric acid: - - - -, pyridoxal-HCl (1.3×10^{-4} *M*); —, pyridoxal-HCl and α-amino-*n*-butyric acid (1.3×10^{-4} *M* each). Solvent, absolute ethanol in Fig. 1a; 3.3×10^{-3} *M* NaOH in absolute ethanol in Fig. 1b.

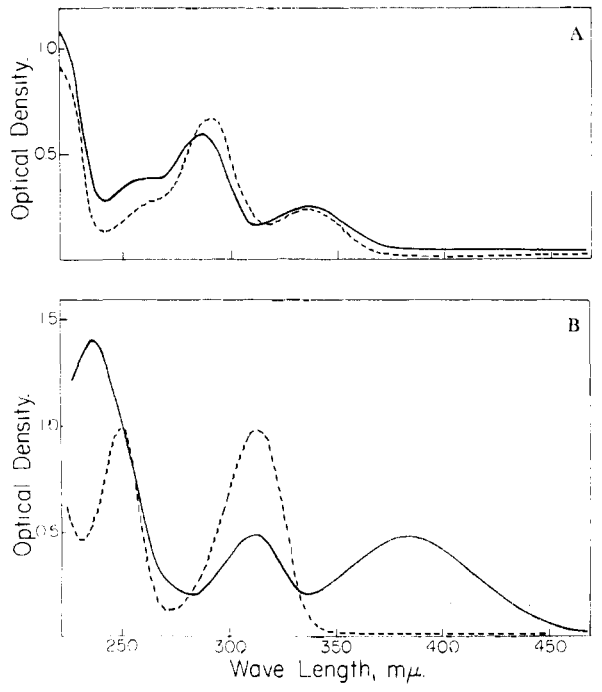


Fig. 2.—Spectrum of pyridoxamine-2HCl in the presence of sodium α-ketobutyrate: - - - -, pyridoxamine-2HCl (1.3×10^{-4} *M*); —, pyridoxamine-2HCl and α-ketobutyrate (1.3×10^{-4} *M* each). Solvent, absolute ethanol in Fig. 2a; 3.3×10^{-3} *M* NaOH in absolute ethanol in Fig. 2b.

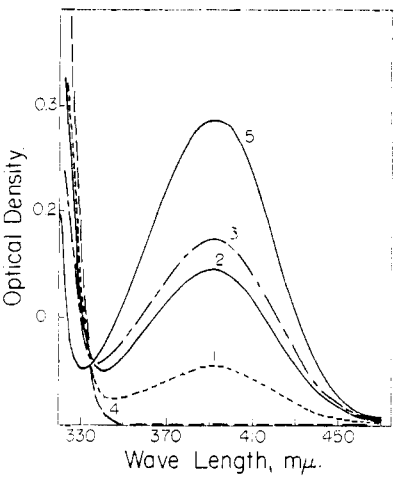


Fig. 3.—Spectrum of an ethanol solution of pyridoxamine and α-ketobutyrate after dilution in aqueous 0.1 *M* NaOH. 10 μmoles of pyridoxamine-2HCl and sodium α-ketobutyrate were dissolved in 10.0 ml. of absolute ethanol, and the solution was shaken at room temperature. At intervals an aliquot was removed from the solution, diluted 60-fold with 0.1 *M* aqueous NaOH, and the absorption spectrum of the aqueous solution was determined after 30 min. of standing: 1, 1 hr.; 2, 5 hr.; 3, 16 hr.; 4, 1.67×10^{-4} *M* pyridoxamine-2HCl in 0.1 *M* NaOH; 5, 1.67×10^{-4} *M* pyridoxal-HCl in 0.1 *M* NaOH.

thus provides evidence of the tautomerism of the Schiff base. No evidence for the formation of pyridoxal was obtained when pyridoxamine and amino acid were incubated in an aqueous neutral buffer, or when pyridoxamine alone was incubated in ethanol.

Spectral change with time observed in the 270–330 mμ region, where both pyridoxal and pyridoxamine have absorption, also indicated the decrease of pyridoxamine and increase of pyridoxal.

A similar spectrophotometric study on the reverse of this experiment could not be carried out, because pyridoxal-HCl reacts with ethanol, causing the decrease with time of the absorption at 390 mμ, measured in 0.1 *M* NaOH, in the absence of amino acid (Table I). The disappearance of pyridoxal in

TABLE I

REACTION OF PYRIDOXAL WITH ETHANOL

Optical density at 390 mμ was determined on a 60-fold dilution in 0.1 *M* NaOH of 5.1×10^{-3} *M* pyridoxal hydrochloride, which is prepared in absolute ethanol with the following additions: (1), no addition; (2), 1×10^{-2} *M* NaOH; (3), 1×10^{-2} *M* alanine and NaOH.

Incubation time, hr.	O.D.$_{390}$ (1)	(2)	(3)
0	0.150	0.150	0.151
2	.144	.151	.150
5	.125	.142	.150
8.5	.104	.138	.146

the absence of amino acid is probably due to the formation of the hemiacetal and acetal of pyridoxal. The acetal formation is catalyzed by a trace of mineral acid and, hence, the reaction should be suppressed when the acid is neutralized. This effect may be seen from the table (column 2). Addition of an amino acid decreases the rate of disappearance

of pyridoxal (column 3), indicating that the formation of Schiff base competes for pyridoxal with the process of acetal formation. It also suggests that the rate of disappearance of pyridoxal due to the tautomerism of Schiff base is slow, if it takes place at all.

(3) **Formation of Amino Acids and Keto Acids by Non-enzymatic Transamination.**—When pyridoxamine is incubated with an α-keto acid in absolute ethanol, an amino acid corresponding to the keto acid employed is detected by paper chromatography. Alanine, norvaline, valine, norleucine, leucine, isoleucine, methionine, and glutamic, aspartic and α-amino-*n*-butyric acids were formed from the corresponding α-keto acids in this way.

Similarly, α-ketobutyric acid is detected in the ethanol solution of pyridoxal and α-amino-*n*-butyric acid. The keto acid was identified by silica gel column chromatography and by paper chromatography of its 2,4-dinitrophenylhydrazone. Substances were detected in the ethanol solutions of other pyridoxylideneamino acids whose 2,4-dinitrophenylhydrazones react positively to the Friedemann–Haugen test.[7]

α-Amino-*n*-butyric acid was formed when α-ketobutyric acid and alanine were incubated in ethanol in the presence of pyridoxal. None of the reactants used here had a detectable contamination of α-amino-*n*-butyric acid. An incubation of pyridoxal and α-ketobutyrate in ethanol produced no amino acid, proving that the pyridoxal was free from pyridoxamine. Therefore, it is evident that under this condition alanine served as the amino group donor, the keto acid as the amino group acceptor, and pyridoxal as the amino group carrier, as represented in the equations

$$\text{alanine} + \text{pyridoxal} = \text{pyruvate} + \text{pyridoxamine}$$

$$\alpha\text{-ketobutyrate} + \text{pyridoxamine} = \text{pyridoxal} + \alpha\text{-amino-}n\text{-butyrate}$$

$$\text{Net: alanine} + \alpha\text{-ketobutyrate} = \text{pyruvate} + \alpha\text{-amino-}n\text{-butyrate}$$

This experiment was repeated with various combinations of amino and keto acids and the results were concordant.

Discussion of Results

The stability constant, K, of pyridoxal phosphate–amino acid Schiff bases, which is defined as

$$K = \frac{[\text{Schiff base}] \times [\text{Water}]}{[\text{Amino acid}] \times [\text{Pyridoxal phosphate}]}$$

has been estimated[8] to be of the order of 10^3 to 10^4. It might be expected that the pyridoxal–amino acid Schiff bases also have stability constants of comparable magnitude. If this is true, in a non-aqueous medium, like the one used in this study, the equilibrium greatly favors the formation of Schiff base. Therefore, it is likely that, in the alcoholic solution of the Schiff base, the concentration of free reactants is almost negligible in comparison with that of the complex. On this basis the results of the experiment summarized in Fig. 3 were interpreted to indicate the transformation of the Schiff base from the ketimine type to the aldimine type, rather than to signify a change in the concentrations of the free reactants. Estimation of the equilibrium point of the Schiff base tautomerism is complicated by the competing reaction of pyridoxal and ethanol, which will change the equilibrium by removing pyridoxal. If this difficulty could be circumvented, the system would offer a convenient means for estimating the approximate equilibrium constants to be expected in enzymatic transamination.

Comparison of the spectrum in absolute methanol of the potassium pyridoxylideneglutamate reported by Brandenberger and Cohen[2] with those of the similar derivatives of amino acids obtained in this work suggests that the spectrum characteristics reported by the above authors represented a mixture of spectra to be observed under the basic and acidic conditions.

Snell and co-workers[4] suggested that metal chelation stabilizes the Schiff base in aqueous solution, and also enhances the tautomerism of the Schiff base by increasing the electron displacements at the α-carbon atom. Results of the present studies have demonstrated that transamination proceeds readily at room temperature, and without metal catalysis, when an alcoholic medium is employed. The main effect of the alcoholic medium on the reaction may be to provide a non-aqueous condition which favors the formation of the Schiff base intermediates. Once a substantial concentration of the Schiff base is formed in the reaction system, the tautomerism of I and II seems to occur without a chelation of metals. However, the ethanolic medium is so drastically different from the aqueous physiological state, that information obtained under such experimental conditions does not exclude the possible role of metal chelation in the enzymatic system. But, on the other hand, it is conceivable that in the enzymatic reaction the carboxyl group of the reacting amino or keto acids, the phosphate group and other reactive groups of the coenzyme will react, respectively, with the active groups of the apoenzyme molecule. The nitrogen atom of the Schiff base may also react with apoenzyme. These bondings will in effect stabilize the Schiff base intermediate, and may be sufficient to maintain the planarity of the conjugated double bond system requisite for the electron displacements, without the help of metal chelation.

Since an alcoholic medium greatly increases, without metal chelation, the concentration of Schiff base over the value obtainable in aqueous solutions, the effect of chelation on the tautomerization of I and II may be studied directly in the non-aqueous medium, provided such chelation does not cause precipitation of the reactants.[9]

(7) The 2,4-dinitrophenylhydrazone prepared from the aqueous solution of pyridoxylidenehomoserine gave a positive Friedemann–Haugen test only when previously exposed to a solution of NaOH. This indicated that the product was the 2,4-dinitrophenylhydrazone of α-ketobutyrolactone (H. Hift and H. R. Mahler, *J. Biol. Chem.*, **198**, 901 (1952)), and thus demonstrated that transamination rather than deamination of homoserine had occurred.

(8) Y. Matsuo, THIS JOURNAL, **79**, 2011 (1957).

(9) Results of a preliminary experiment on this problem indicated that addition of Cu^{++} or Cr^{+++} inhibited the transamination between pyridoxamine and α-keto acids in ethanol. Whether this inhibition was caused by the direct action of the metal ion on the tautomerism of the Schiff base, or by removal of the reactants by precipitation, is not known.

Acknowledgment.—The author wishes to thank Professor D. M. Greenberg for his continued interest in this work and for his help in preparing the manuscript.

Experimental

Material.—α-Keto-γ-methylmercaptobutyric acid was prepared enzymatically by the method described by Meister.[10] All the other α-keto acids were synthesized by Mr. D. C. Morrison of this Laboratory. Other chemicals were obtained from commercial sources.

Methods.—Spectrophotometric measurements were made with a Beckman model DU spectrophotometer at room temperature. Silica cells of 1 cm. length were used.

Silica gel column chromatography was carried out by the method of Kinnory, *et al.*,[11] and paper chromatography of the 2,4-dinitrophenylhydrazones of the α-keto acids according to Altmann, *et al.*[12]

Preparation of Schiff Base.—One hundred μmoles each of pyridoxal–HCl and DL-α-amino-*n*-butyric acid suspended together in 10.0 ml. of absolute ethanol were shaken at room temperature in a tightly stoppered glass vial. A clear yellow solution was obtained in about 15 min. The ethanolic solution of the yellow complex of pyridoxamine–2HCl and sodium α-ketobutyrate was prepared in the same manner. Addition of ether to the alcoholic solution of the Schiff bases precipitated a yellow material and the supernatant liquid became colorless. The precipitated materials, when freed from ether, were strongly hygroscopic, and their analysis was not attempted. Absorption spectra of the Schiff bases were measured on aliquots of the ethanolic solution after appropriate dilution with absolute ethanol, with or without addition of NaOH.

Formation of Amino Acids by Non-enzymatic Transamination between Pyridoxamine and α-Keto Acids.—One ml. of absolute ethanol containing 10 μmoles of pyridoxamine–2HCl and 20 μmoles of NaOH (the precipitate of NaCl was removed by centrifugation) were added to 10 to 40 μmoles of the solid sodium salts of various α-keto acids. After the mixture was shaken for 3 to 10 hr. at room temperature, aliquots were chromatographed on paper with *n*-butanol–acetic acid–water (5:1:2) and/or phenol–water (4:1) as developing solvents. Intense spots of amino acids were revealed on the chromatograms prepared with 2 to 5 μl. of the above mixtures, which represent 0.02 to 0.05 μmole of pyridoxamine initially present. Since the minimum quantity of amino acids detectable under the conditions employed is approximately 0.005 μmole, it is apparent that a significant portion of the added pyridoxamine had participated in the transamination reaction. Determination of pyridoxal and pyridoxamine were not attempted.

Formation of α-Keto Acids by Non-enzymatic Transamination between Pyridoxal and α-Amino Acids.—Fifty μmoles each of α-amino-*n*-butyric acid, pyridoxal–HCl and NaOH were incubated in 10 ml. of absolute ethanol for 8 hr. at room temperature. A portion of this solution was evaporated to dryness with a stream of air, the residue was taken up in small volume of 1 *N* HCl and subjected to silica gel column chromatography. Nine-tenth μmole of α-ketobutyric acid was isolated by this method from an aliquot which represented 10 μmoles of each of the reactants. This suggests that the equilibrium of the tautomerism of the Schiff base favors the aldimine form, at least under the condition employed.

To prepare the 2,4-dinitrophenylhydrazones of α-keto acids formed by transamination between pyridoxal and α-amino acids the ethanolic solution of the Schiff base was dried, and the residue was redissolved in water, acidified with H_2SO_4 to *p*H 2, and this solution was continuously extracted with peroxide-free ether. Ether was removed from the extract by evaporation and the residue was treated with 2,4-dinitrophenylhydrazine in HCl. The step of ether extraction was necessary to remove pyridoxal, the 2,4-dinitrophenylhydrazone of which interferes with the Friedemann–Haugen test.

Pyridoxal Catalysis of Transamination between an Amino Acid and an α-Keto Acid.—Fifty μmoles of pyridoxal–HCl, 100 μmoles of alanine and 200 μmoles of sodium α-ketobutyrate were incubated in 10 ml. of absolute ethanol, in the presence of 50 μmoles of NaOH. After incubating 4 hr. at room temperature 5-μl. aliquots were chromatographed on paper. Spraying the paper with ninhydrin reagent revealed purple spots of alanine and α-amino-*n*-butyrate and a reddish-yellow spot of pyridoxamine. Intensity of the α-amino-*n*-butyrate spot was comparable to or greater than that of the alanine spot, indicating that the transamination reaction had proceeded to a significant extent. Similar results were obtained from experiments with various combinations of amino and keto acids.

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(10) A. Meister, *J. Biol. Chem.*, **197**, 309 (1952).

(11) D. S. Kinnory, Y. Takeda and D. M. Greenberg, *ibid.* **212**, 379 (1955).

(12) S. M. Altmann, E. M. Crook and S. P. Datta, *Biochem. J.*, **49**, lxiii (1951).

COMMUNICATIONS TO THE EDITOR

HEAT CAPACITY OF ORDERED Mg_3Cd BETWEEN 4 AND 15°K.[1]

Sir:

Heat capacity data for ordered Mg_3Cd between 12 and 320°K. were presented in THIS JOURNAL several years ago.[2] This paper contains results extending those measurements into the liquid helium range.

The sample employed was a portion of the material used in the earlier study.[2] It had been heat treated to assure that it was in the ordered state. The composition of the sample was 24.98 ± 0.04 atomic % cadmium. 308.53 g. or 6.66 g. atoms of this material were used in the study. The apparatus, described elsewhere,[3,4] used a gas thermometer as the working thermometer. The constant of this gas thermometer was essentially determined by a calibration against the 1955 liquid helium vapor pressure scale.

The measurements were carried out in two series, of 25 and 23 experiments, respectively, both covering the range 4.5 to 16°K. After the first series the

(1) This work was supported by a grant from the National Science Foundation.

(2) L. W. Coffer, R. S. Craig, C. A Krier and W. E. Wallace, THIS JOURNAL, **76**, 241 (1954).

(3) M. H. Aven, Ph.D. Dissertation, University of Pittsburgh, 1955.

(4) M. H. Aven, R. S. Craig and W. E. Wallace, *Rev. Sci. Instruments*, **27**, 623 (1956).