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Kinetics and Mechanism of the Condensation of Pyridoxal with Amino Acids

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Abstract — The kinetics and mechanism of the condensation of amino acids with pyridoxal were studied in relation to the amino acid structure, solvent, pH, and temperature. A spectrophotometric study revealed several kinetically discernible reaction steps. The condensation rate as a function of pH passes through a maximum, which is caused by formation of two intermediates of different structures. The final products of the condensation and subsequent hydrolysis are pyridoxamine and α-keto acids. The reaction mechanism was suggested.

Vitamins of B_6 group play a fundamental role in the nitrogen exchange of all living bodies and participate in enzymatic catalysis of numerous transformations of amino acids. Amine interchange, decarboxylation, and deamination of amino acids, and also amination of keto acids occur under the action of pyridoxal enzymes [1–8].

These conclusions were based on the results of studies of complex enzymatic systems or of experiments with model systems involving isolation of products in separate steps. However, the reaction mechanism and solvent effect on the rates and pathways of biochemical processes are still poorly understood, because of the structural complexity of enzymatic systems and diversity of chemical transformations.

Here we made a comprehensive spectrophotometric study of the kinetics and mechanism of the reactions of pyridoxal with various amino acids (glycine, D,L-tryptophan, D,L-α-alanine, β-alanine, β-phenylalanine) under varied conditions (pH, solvent, temperature).

We found that, on mixing equimolar amounts of colorless solutions of pyridoxal and amino acids, the solution instantaneously becomes yellow, and new absorption maxima appear: in approximately neutral solutions, λ_{max} 430 nm, and in alkaline solutions, a new maximum appears at λ_{max} 450 nm.

Spectroscopic monitoring of the reaction mixtures showed that the optical density of the solutions first instantaneously decreased and then started to slowly increase. Then, after the lapse of a certain time, the absorption intensity decreased again, and the absorption maxima of the final products appeared.

We found that the rate of condensation of pyridoxal hydrochloride with amino acids as a function of pH passes through a maximum (Figs. 1, 2).

The pH dependence of the rate constant of the condensation of pyridoxal with glycine suggests the occurrence of several consecutive steps. The first, very fast step involves addition of the amino acid to pyridoxal with the formation of an intermediate amino alcohol (sharp decrease in the solution optical density); the second, slow step is dehydration of the amino alcohol with the formation of a Schiff base (λ_{max} 430 nm). The stability of the Schiff base strongly depends on pH and the solvent. In acid solutions, the Schiff base decomposes into the starting compounds, and in alkaline solutions the –C=N bond migrates to the α-C atom of the amino acid fragment (apparently, via deprotonation of the α-C atom of the amino acid), yielding a new compound with λ_{max} 450 nm.

The pH dependence of the rate of decomposition of the product with λ_{max} 450 nm also passes through a maximum. At increased pH, the Schiff base undergoes the rearrangement with the subsequent hydrolysis to keto acids and pyridoxamine (λ_{max} 327 nm).

We calculated the rate constants of formation of the intermediates with λ_{max} 430 and 450 nm and of their transformation into the final products assuming a simplified scheme of irreversible consecutive reactions $\mathbf{A} \rightarrow \mathbf{B} \rightarrow \mathbf{C} \rightarrow \mathbf{D} \rightarrow \mathbf{E}$; we also calculated the concentrations of these species by standard procedures [7]. The results for the reaction of pyridoxal hydro-

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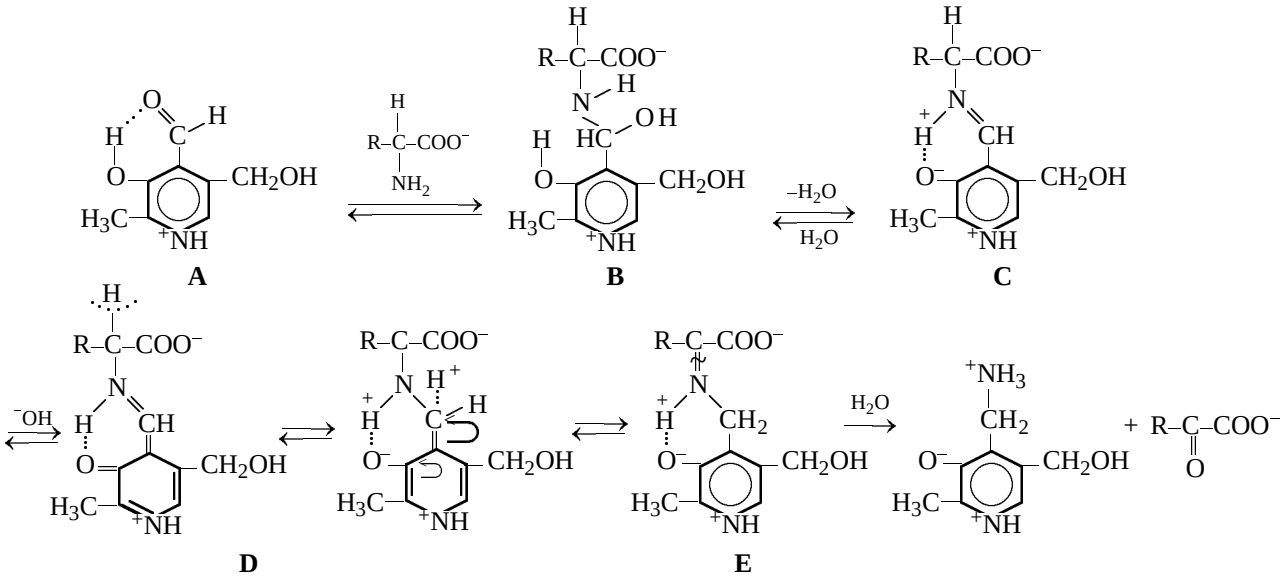

chloride with glycine are shown in Fig. 3. The suggested scheme of the condensation of pyridoxal hydrochloride with amino acids is in the scheme.

The suggested mechanism of the reaction of amino acids with pyridoxal can be proved by isolation and identification of Schiff bases **C** and of the intermediate and final (pyridoxamine, keto acid) products.

For example, in the reaction of pyridoxal hydrochloride with D,L-tryptophan, the optical density of the solution on mixing the equimolar amounts of the reactants first sharply decreases, after which a maximum at 430 nm appears and grows in the intensity.

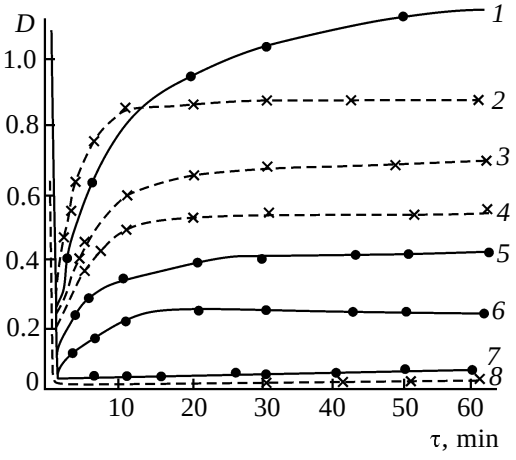

Fig. 1. Variation with time of the optical density of an equimolar mixture (0.01 M) of pyridoxal hydriochloride and lysine at various pH of the solution. 70% aqueous-alcoholic acetate solution, 15°C. λ_{max}, nm: (solid lines) 430 and (dashed lines) 450. pH: (*1*) 7.0, (*2*) 8.02, (*3*) 8.42, (*4*) 8.92, (*5*) 8.0, (*6*) 8.2, (*7*) 4.65, and (*8*) 11.4.

After a certain period, the band at 430 nm decreases in the intensity, and a new band appears at 450 nm; its intensity first grows, reaches a maximum, and then starts to slowly decrease. Simultaneously a band at λ_{max} 327 nm appears and grows. In the process, a white crystalline precipitate is formed; its yield is ~20% based on the Schiff base.

This precipitate undergoes carbonization at temperatures above 350°C; it does not react with ninhydrin and forms a yellow product (λ_{max} 280 nm) with 2,4-dinitrophenylhydrazine. The precipitate was unambiguously identified as sodium β-(3-indolyl)-α-ketopropionate by elemental analysis, UV and IR spectroscopy, and the formation of the corresponding hydrazone with 2,4-dinitrophenylhydrazine.

Examination of the solvent effect on the reaction rates and pathways showed that, as the percentage of

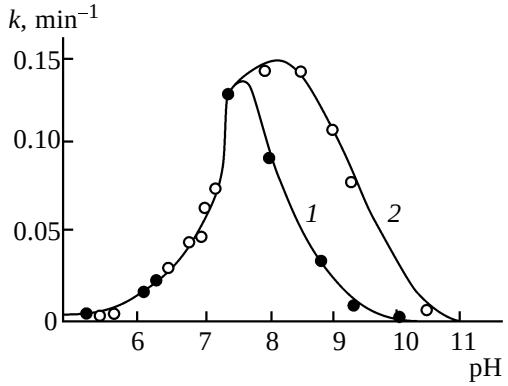

Fig. 2. pH dependence of the rate constants of the reaction of glycine with pyridoxal hydrochloride. λ_{max}, nm: (*1*) 430 and (*2*) 450.

the alcohol in aqueous-ethanol buffer solutions was increased, the rates of addition of glycine to pyridoxal hydrochloride (**A** → **B**) and dehydration of the amino alcohol (**B** → **C**) increased. For example, the dependence of the rate constant k of dehydration of the amino alcohol on the percentage of alcohol in aqueous-ethanol buffer solutions at 20°C and pH 6.65 is given below.

Alcohol content, %	0	35	50	70	90
k, min^{-1}	0.00081	0.00214	0.00786	0.0422	0.1835

Comparison of the kinetics and mechanism of the reactions of pyridoxal under comparable conditions with amino acids differing in the structure and pK_b showed that acceptor substituents (aryl groups in β-phenylalanine, tryptophan) somewhat accelerate the dehydration of the amino alcohols, whereas donor substituents exert virtually no effect on the rate of this reaction. The rates of dehydration of the amino alcohols increase with temperature (activation energy E_a 16.1 kJ mol^{-1}).

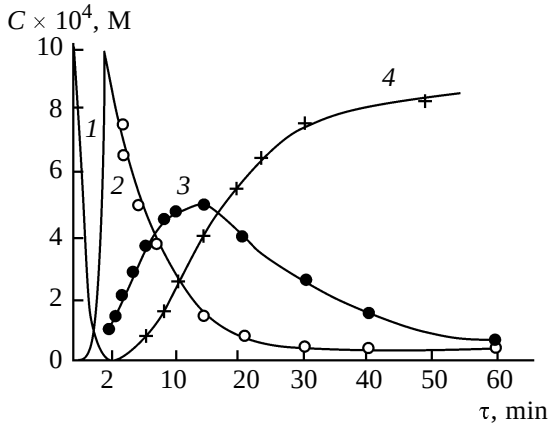

Fig. 3. Variation with time of the concentrations of compounds **A, B, C**, and **E**, calculated assuming the scheme of consecutive irreversible reactions. 70% aqueous-alcoholic acetate solution, 15°C, pH 7.25. (*1*) Pyridoxal hydrochloride **A**, (*2*) intermediate amino alcohol **B**, (*3*) Schiff base **C** (λ_{max} 430 nm), and (*4*) product **E** of the transformation of the Schiff base (λ_{max} 450 nm).

EXPERIMENTAL

Pyridoxal hydrochloride was purchased from Ferak Berlin, and the amino acids, from Reanal. Buffer solutions were prepared by standard procedures. The condensation kinetics was studied with a Spektromom-204 spectrophotometer. The reaction mixtures were temperature-controlled with a UH-8 thermostat (accuracy ±0.1°C). Equimolar amounts of pyridoxal and amino acids were dissolved in aqueous-alcoholic buffer solutions and kept for 30 min at the required temperature. The moment of mixing the thermostated solutions of pyridoxal and amino acids was considered as the reaction onset.

Kinetic measurements were performed in temperature-controlled 1.008-mm cells. Because the UV spectra of pyridoxal hydrochloride solutions vary with the solvent and pH, the reference cells were filled with the solvents or pyridoxal solutions of the composition similar to that used in the condensation experiments. In this case, variation of the optical density of the reactant mixture in the course of condensation depends only on the formation of the intermediate and final products and is independent of changes in the optical density of the reactants themselves.

The rate constants of the condensation of pyridoxal with amino acids were calculated from the calibration plots with first- and second-order equations for reversible and irreversible reactions.

The reactants, intermediates, and final products were identified by elemental analysis and by UV and IR (KBr pellets) spectroscopy.

The Schiff bases were prepared by a general procedure involving heating of equimolar amounts of pyridoxal hydrochloride with amino acids in ethanol at 60–70°C for 15–30 min. The procedure for the condensation of pyridoxal hydrochloride with glycine is given below as example.

Pyridoxylideneglycine. Ethanol (50 ml) was added to a mixture of 0.167 g of pyridoxal hydrochloride and 0.075 g of glycine; the mixture was vigorously stirred until the precipitate dissolved. Then the mixture was filtered and left overnight at 15°C until a precipitate formed. The precipitate was recrystallized from isopropyl alcohol. Yield 67%. IR spectrum (KBr), ν, cm^{-1}: 1637 (C=N), 1620–1670 (COO^-). Found, %: C 46.21; H 4.7; N 10.68. $C_{10}H_{12}N_2O_4 \cdot HCl$. Calculated, %: C 46.15; H 4.61; N 10.77.

Sodium β-(3-indolyl)-α-ketopropionate. Equimolar amounts of 0.01 M solutions of pyridoxal hydrochloride and D,L-tryptophan in 70% aqueous-ethanol buffer solution (pH 6.8) were mixed and kept at 15°C for 5 days. On mixing, the mixture acquired intense yellow color and then became red, after which the color became less intense and a precipitate formed. The precipitate was filtered off, washed with alcohol, and dried to constant weight. Yield 21%, mp >350°C (the product undergoes carbonization). No violet coloration is observed on refluxing with ninhydrin. The

reaction product forms a yellow crystalline precipitate in the reaction with 2,4-dinitrophenylhydrazine and a colorless precipitate poorly soluble in water and alcohol in the reaction with p-aminobenzoic acid. The product is virtually insoluble in water and alcohol. IR spectrum (KBr), ν, cm^{-1}: 1670–1715 (C=O, COOH). UV spectrum, λ_{max}, nm: 280. Found, %: C 58.5; H 4.2; N 6.19. $C_{11}H_{10}NNaO_3$. Calculated, %: C 58.17; H 4.4; N 6.19.

REFERENCES

1. Metzler, D.E., *Biochemistry: The Chemical Reactions of Living Cells,* New York: Academic, 1977. Translated under the title *Biokhimiya,* Moscow: Mir, 1980, vol. 2, pp. 209–232.
2. Metzler, D.E., Ikawa, M., and Shell, E.E., *J. Am. Chem. Soc.,* 1954, vol. 76, p. 648.
3. Braushtein, A.E. and Shemyakin, M.M., *Biokhimiya,* 1953, vol. 18, p. 393.
4. Matguo, B.Y., *J. Am. Chem. Soc.,* 1957, vol. 79, no. 8, p. 2011.
5. Metzler, D.E., *J. Am. Chem. Soc.,* 1957, vol. 79, no. 2, p. 485.
6. Matsumoto, S. and Matsuchima, Y., *J. Am. Chem. Soc.,* 1972, vol. 94, p. 7211.
7. Laidler, K.J., *Reaction Kinetics,* Oxford: Pergamon, 1963. Translated under the title *Kinetika organicheskikh reaktsii* (Kinetics of Organic Reactions), Moscow: Mir, 1966, pp. 31–34.
8. Pishchugin, F.V. and Shershenalieva, Z.Sh., *Biokhimiya,* 1988, vol. 53, no. 9, p. 1509.