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Chemical Transformations of Pyridoxal and Pyridoxal 5'-Phosphate Condensation Products with Amino Acids

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Abstract—The mechanism of chemical transformations of pyridoxal and pyridoxal 5'-phosphate condensation products with amino acids is studied by kinetic measurements. The Schiff bases are shown to be fairly stable in neutral media. In acid media, the Schiff bases are hydrolyzed into the initial components. In alkaline media, cleavage of α-hydrogen from the amino acid fragment and structural rearrangement into the quinoid form followed by hydrolysis of the latter with elimination of pyridoxamine and keto acid take place. The rate constants of the chemical transformations of the Schiff bases are found to depend on the pH of the medium. It is shown for the first time that the phosphate group in the pyridoxal 5'-phosphate fragment catalyzes the α-hydrogen cleavage and strongly accelerates alkaline decomposition of the Schiff bases.

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Vitamins of the B_6 group are involved as coenzymes in many enzymatic transformations of amino acids and amines, including peramination, decarboxylation, deamination, and cleavage of amino acid side chains [1–6].

Of certain interest is to study the kinetics and mechanism of stability of pyridoxal and pyridoxal 5'-phosphate condensation products with amino acids as a function of their structure and conditions they exist in solutions.

The purpose of this work to perform a kinetic stability study of the Schiff bases formed by the condensation of pyridoxal and pyridoxal 5'-phosphate with amino acids and to assess the influence of the charges on the reaction centers on the rate and pathway of selected steps of their chemical transformations and the role of the phosphate group in these transformations.

Study of the kinetics and mechanism of chemical transformations of Schiff bases under various conditions showed that at pH values close to neutral these compounds are stable in solutions. In acid media, the optical density of the solutions gradually decreases, and the yellow color disappears with time. In weakly acidic media, the reaction center is activated via protonation of the pyridine nitrogen atom (p*K* 5.9 [6]), while in stronger acidic media the activation proceeds via protonation of the C=N nitrogen atom with subsequent destruction of the chelate structure (λ_{max} 430 nm), addition of a water molecule, and formation of the initial components: pyridoxal, pyridoxal 5-phosphate, and amino acid.

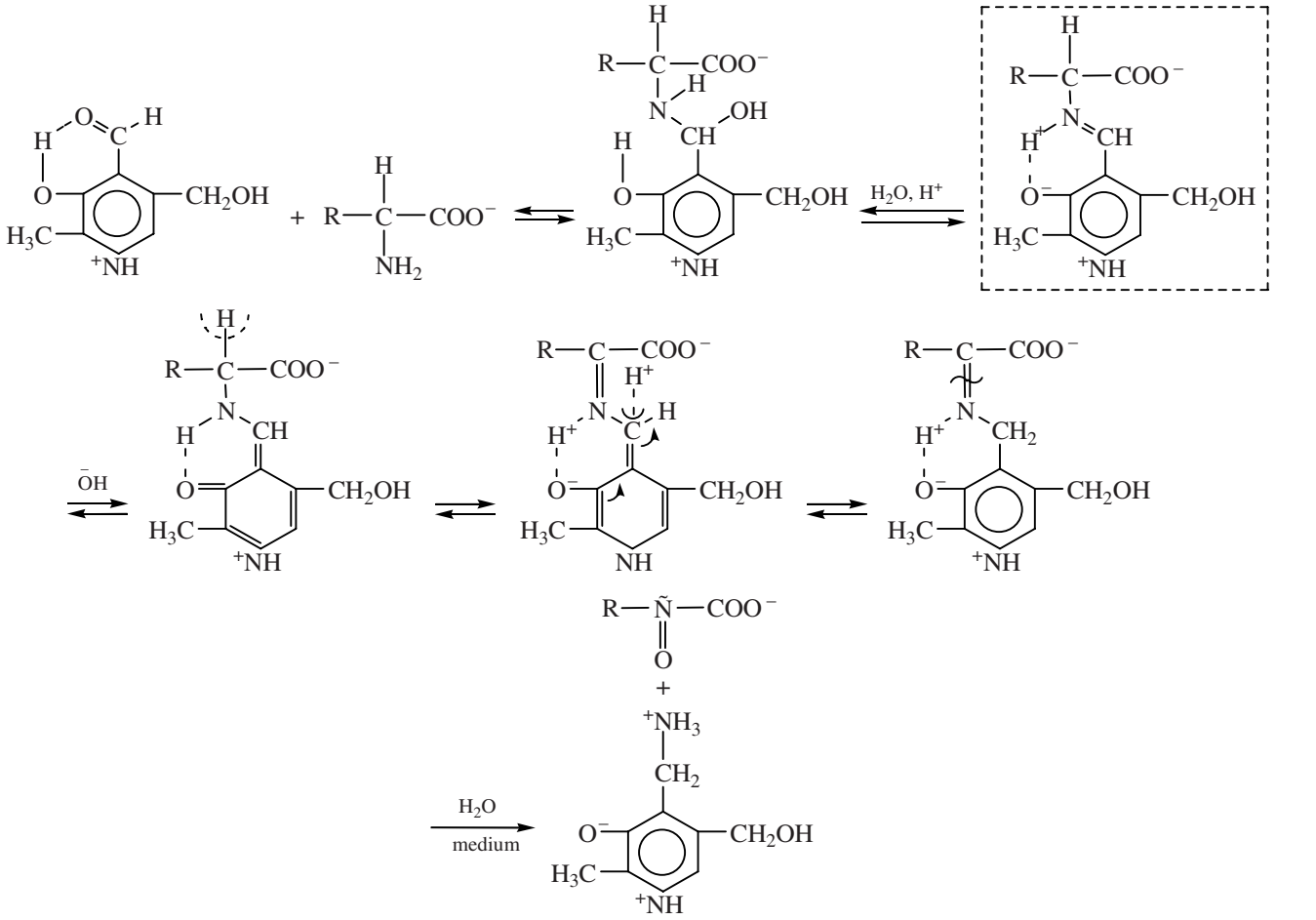
Another reaction pathway is observed in alkaline media. A new product appears (λ_{max} 450 nm), and the optical density of a mixture of pyridoxal or pyridoxal 5'-phosphate and amino acid solutions sharply decreases (λ_{max} 450 nm) and then gradually increases with time (Fig. 1).

The decrease in the optical density of alkaline solutions of the Schiff bases is probably associated with fast cleavage of α-hydrogen from the amino acid fragment and structural rearrangement into the quinoid form. Further on, the quinoid structure is hydrolyzed under the action of water to form new products: pyridoxamine and keto acid.

Evidence for the proposed scheme of decomposition of the Schiff bases was provided by the

isolation and identification of the final compounds. Upon acid hydrolysis and addition of excess alcohol to the reaction mixture, a series of amino acids poorly soluble in ethanol were isolated. The isolated products gave a positive ninhydrin test. The pyridoxal or pyridoxal 5'-phosphate remained in the solution were isolated and identified by UV and IR spectroscopy.

Alkaline hydrolysis products of the Schiff bases proved to be more difficult to isolate and identify. It was shown than in some cases, in particular, the Schiff bases formed by condensation of pyridoxal or pyridoxal 5'-phosphate with *D,L*-tryptophan or *L*-glutamic acid gave, after alkaline treatment and keeping for some time, precipitates which were identified as the sodium salts of β-(3-indolyl)-α-ketopropanoic and α-ketoglutaric acids, respectively (2,4-dinitrophenylhydrazine test, UV and IR spectroscopy) [7, 8].



The plots of the rate constants of acid and alkaline hydrolysis of the Schiff bases against medium pH are shown in Fig. 2. As seen, the rate of hydrolysis into the initial components increases with acidity. Increase in the rate of decomposition in going to an alkaline medium is more complicated, as this pathway depends on the rates of several consecutive steps: (1) α-proton cleavage from the acid fragment of the Schiff base, (2) rearrangement of the Schiff base into the quinoid structure, (3) hydrolysis of the quinoid structure and formation of the final compounds: pyridoxamine and keto acid. The rate of alkaline hydrolysis depends on the acidity of the α-hydrogen atom, pH of the medium, and structure of the Schiff base. We showed that, unlike what suggested in [1], the carbonyl group in pyridoxal and pyridoxal 5'-phosphate are located in a plane turned by 90° to the pyridine ring plane. We explain this fact by the presence in pyridoxal and pyridoxal 5'-phosphate of two *ortho* oxygen-containing groups (OH, CH_2OH, CH_2–O–PO_3H). They push away the carbonyl oxygen atom, and thus turn this group plane by 90°. The MNDO calculation of charges in the condensation products of pyridoxal and pyridoxal 5'-phosphate with tryptophan showed that

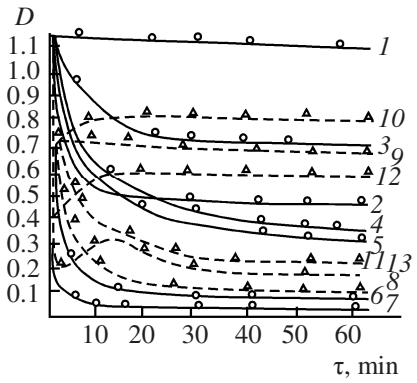


Fig. 1. Variations in the optical density of a 0.01 M pyridoxalidene glycine solutions with time at varied medium pH (90% water–alcohol buffer, 15°C). λ_{max} 430 nm, pH: (*1*) 6.78; (*2*) 6.4; (*3*) 6.3; (*4*) 6.2; (*5*) 5.9; (*6*) 5.3; and (*7*) 3.5. λ_{max} 450 nm, pH: (*8*) 5.9; (*9*) 6.78; (*10*) 7.9; (*11*) 8.0; (*12*) 8.3; and (*13*) 9.1.

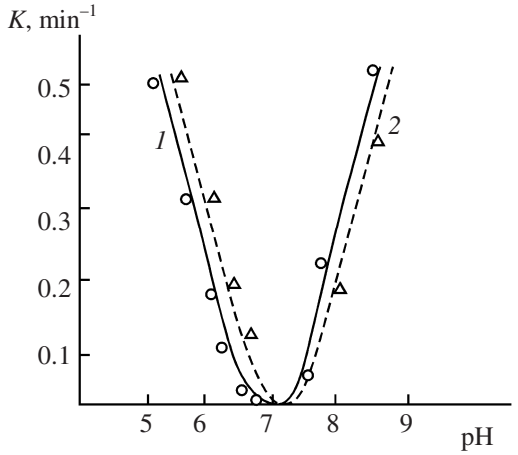
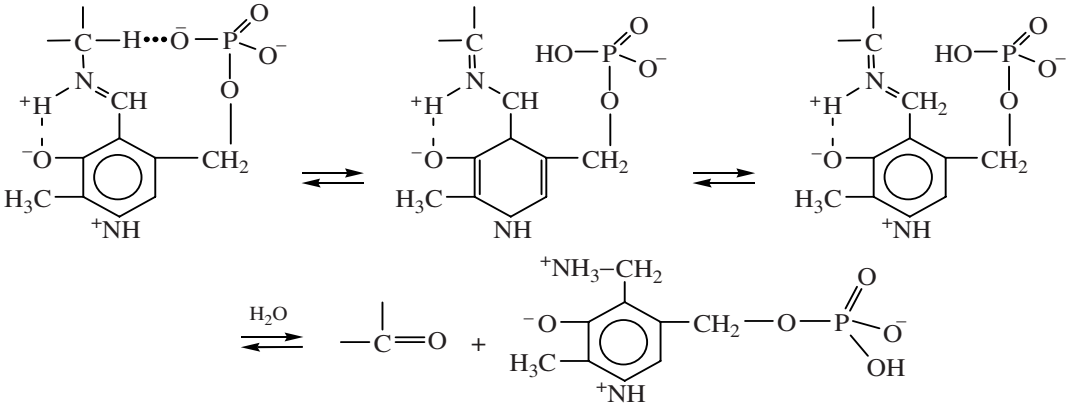


Fig. 2. Plot of the pyridoxalidene glycine decomposition rate constant vs. medium pH (90% water–alcohol buffer, 15°C) (*1*) λ_{max} 430 nm , (*2*) λ_{max} 450 nm.

the α-hydrogen in pyridoxal 5'-phosphate structural fragments is more acidic than in pyridoxal structural fragments (+0.046 and +0.028, respectively). The higher the acidity of α-hydrogen and the higher the medium pH, the more probable transition of the Schiff base into the quinoid structure and its further hydrolysis.

It was interesting to compare the reactivities of pyridoxal and pyridoxal 5'-phosphate with amino acids under comparable conditions. According to published data [1], the phosphate group in pyridoxal 5'-phosphate behaves as a "phosphate hand" which binds the coenzyme with the protein part of the enzyme. To gain insight in this important issue, we have studied the kinetics and mechanism of reactions of pyridoxal and pyridoxal 5'-phosphate with triptophan under comparable conditions (Fig. 3).

The data in Fig. 3 show that the acceptor phosphate group of pyridoxal 5'-phosphate accelerates, compared to pyridoxal, formation of Schiff base in the step of addition of amino acid with formation of amino alcohol and in the step of its dehydration. This group also promotes cleavage of α-hydrogen from the amino acid fragment of the Schiff base, rearrangement of the latter into the quinoid structure, further hydrolysis with formation of pyridoxamine 5'-phosphate, and precipitation of sodium β-(3-indolyl)-α-ketopropanoate. Evidence for the above suggestions was provided by the time of precipitation initiation. In the condensation of pyridoxal with triptophan (k 0.08 × 10^{-2} min^{-1}) precipitation initiated after 36 h, while in the condensation of pyridoxal 5'-phosphate and triptophan (k 0.354 × 10^{-2} min^{-1}) under similar condi-tions, after 9 h. We assumed that at a certain pH of the medium and a certain orientation of the phosphate group its negatively charged oxygen atoms can act to accept α-hydrogen from the amino acid fragment of the Schiff base. As a result, the Schiff base rearranges into the quinoid structure whose hydrolysis leads to pyridoxamine 5'-phosphate and keto acid:



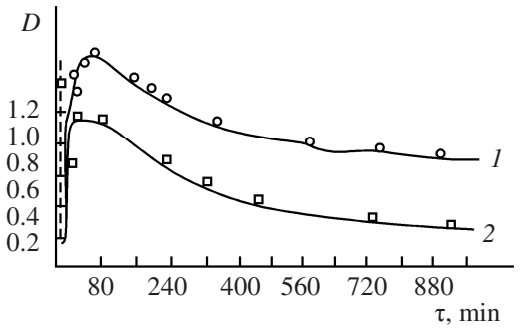


Fig. 3. Kinetics of (*1*) pyridoxal and (*2*) pyridoxal 5'-phosphate condensation with *D,L*-triptophan (70% water-alcohol buffer, pH 7.1, 50°C, λ_{max} 430 nm).

This scheme is evidenced by the elemental analyses and UV and IR spectra of the formed precipitates. Our results are consistent with the conclusion of Dunathan [9] that the bond cleaved by a pyridoxal 5'-phosphate–dependent enzyme should be located in a plane orthogonal to the plane of the imine π system of the coenzyme substrate. Such orientation minimizes the energy of the transition state, because it ensures the best σ–π overlap of the cleaved bond in the conjugated coenzyme imine π system.

Thus, the results of our investigation show that pyridoxal or pyridoxal 5'-phosphate condensation products with amino acids are the most stable at pHs close to neutral. In acid media, they decompose due to hydrolysis into the initial components. In alkaline media, cleavage of the α-hydrogen atom in the amino acid fragment of the Schiff base, formation of the quinoid structure, and its subsequent hydrolysis occur, leading to pyridoxamine or pyridoxamine 5'-phosphate and keto acid.

Thus, we obtained the first experimental evidence showing that the phosphate group in pyridoxal 5'-phosphate condensation products with amino acids can catalyze α-hydrogen cleavage and transfer of the Schiff base into the quinoid structure whose hydrolysis accelerates formation of pyridoxamine 5'-phosphate and keto acids.

EXPERIMENTAL

The kinetics of hydrolysis of pyridoxal and pyridoxal 5'-phosphate condensation products with amino acids were studied using a SpectroMOM-204 spectrophotometer. The reaction mixture was thermostated in a UH–8 thermostat with an accuracy of ±0.1°C . Samples of Schiff bases were dissolved in water–alcohol buffer systems and kept at a given temperature for 30 min at pH ~7. The reaction was considered to start in the moment when HCl or NaOH was added. Kinetic measurements were performed in temperature-controlled cells with a layer thickness of 1.008 mm. Taking into account that the UV spectra of pyridoxal hydrochloride and pyridoxal 5'-phosphate depend on solvent and medium pH, as reference we used solvents or solutions of pyridoxal hydrochloride or pyridoxal 5'-phosphate in conditions corresponding to the conditions of hydrolysis of the Schiff bases. Such approach excluded the contribution of the optical density of the final compounds into the optical density of the reaction mixture. To account for the precipitation of keto acid salts, attendant in chemical transformations of pyridoxal or pyridoxal 5'-phosphate condensation products with *D,L*-triptophan and *L*-glutamic acid in alkaline media, simultaneously in the same thermostat under the same conditions we kept the solutions of the Schiff bases and measured the time of precipitation initiation.

The rate constants of hydrolysis of pyridoxal and pyridoxal-5'-phosphate condensation products with amino acids were calculated from the calibration straight lines by the first-order reversible and irreversible reaction equations [10]. The Schiff bases and their chemical transformation products were identified by elemental analysis, UV and IR spectroscopy, and column chromatography. The Schiff bases were synthesized by a common procedure [7]. As starting materials we used pyridoxal hydrochloride and pyridoxal 5'-phosphate from Ferak, Berlin and amino acids from Reanal. Equimolar amounts of pyridoxal hydrochloride or pyridoxal 5'-phosphate and amino acid were heated at 60–70°C for 15–30 min, and the resulting compounds were isolated and recrystallized. The synthesis of pyridoxalidene glycine and sodium β-(3-indolyl)-α-ketopropanoate were described in [7].

Pyridoxalidene *D,L*-triptophan. To a mixture of 0.103 g of pyridoxal hydrochloride and 0.102 g of *D,L*-triptophan, 35–40 ml of 90% ethanol was added with vigorous stirring at pH 6.7–7.0 and 25°C, and the reaction mixture was kept until the reagents dissolved completely. Therewith, the solution got intensely yellow. The solution was left overnight in a freezer. The yellow precipitate that formed was washed with a little water and then with ethanol. Yield 72–75%. IR spectrum (KBr), ν, cm^{-1}: 1635 (C=N) , 1615–1627 (COO^-). UV spectrum, λ_{max}, nm: 355, 430. Found, %:

C 60.5; H 5.0; N 11.2. $C_{19}H_{19}N_3O_4Na$. Calculated, %: C 60.64; H 5.05; N 11.17.

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