STUDIES ON ENZYMIC FORMATION OF 5'-O-(α -D-GLUCOPYRANOSYL)-4-PYRIDOXIC ACID IN RAT¹

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A new derivative of 4-pyridoxic acid was produced when 4-pyridoxic acid was incubated with homogenates of rat liver. The successful isolation procedures of the compound from the incubation mixture consisted of charcoal treatment and column chromatography on DEAE- and P-cellulose. The compound was obtained in colorless planeary crystals: mp, 253-255°C (decomp.). It was identified as 5'-O-(α -D-glucopyranosyl)-4-pyridoxic acid based on spectral data and acid hydrolysis results. The structure of the compound was verified by enzymic analysis using α - and β -glucosidases.

Vitamin B₆ is excreted in urine mainly in the form of 4-pyridoxic acid (pyridoxic acid) in mammals (1-3). It is still unclear whether or not pyridoxic acid is merely the excretion form of vitamin B₆ in animals. Our investigations on metabolism of vitamin B₆ in rats revealed that a new derivative of pyridoxic acid, which was blue-violet fluorescent under ultraviolet light, was enzymically produced by incubating pyridoxic acid with homogenates of rat liver. This finding led the authors to deduce that pyridoxic acid might be converted to such a derivative *in vivo* and thus play some role in metabolism in animals. The present paper describes the enzymic formation, the isolation and the chemical structure of the compound.

EXPERIMENTAL PROCEDURES

Materials. 4-Pyridoxic acid was prepared by the method of HEYL *et al.* (4).

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Yeast α -glucosidase (Type I) and sweet almond β -glucosidase were purchased from Sigma Chem. Co. Other chemicals used were obtained from commercial sources.

Detection of the compound. Paper chromatography with a solvent system of *n*-butanol/acetic acid/water (3: 2: 2, v/v) was employed as the means of detecting the compound concerned in every step of the isolation procedures.

Spectrometry. UV spectra were recorded on a Shimadzu multipurpose spectrophotometer type MPS-50L. The IR spectrum was obtained with a micro-KBr disc with a Hitachi infrared spectrometer type EPI-G3. The NMR spectra were taken with a Hitachi Perkin-Elmer high resolution NMR spectrometer type R-22 at 90 MHz with DSS for D_2O and TMS for $CDCl_3$ as an internal standard.

Acetylation of the compound. The specimen (14 mg) was dissolved in a mixture of acetic anhydride (3 ml) and dry pyridine (3 ml). The mixture was allowed to stand at room temperature for 48 hr and then poured into ice water. The acetylated compound was extracted three times with 20 ml portions of chloroform. The extract combined, after three washings with dil. hydrochloric acid and three washings with water, was dried over anhydrous sodium sulfate, followed by evaporation to dryness. The residue was dissolved in a small volume of chloroform and the solvent was removed by evaporation to give an oily substance.

Thin-layer chromatography. This was carried out on a impregnating silica gel G plate with 0.02 M sodium acetate or 0.02 M boric acid for the identification of the sugar moiety of the compound. Solvents employed were in the volume ratio: 1) chloroform/methanol (3: 2); 2) benzene/acetic acid/methanol (1: 3: 3); 3) *n*-butanol/acetic acid/water (3: 2: 2); 4) methanol/chloroform/acetone/ammonium hydroxide (42: 16.5: 25: 16.5). The spot was visualized by spraying the plate with naphthoresorcinol/sulfuric acid and heating at 100–150°C for 5 min (5).

4-Pyridoxic acid and pyridoxo-4,5-lactone were identified by thin-layer chromatography on silica gel G with the following solvent systems: 1) *n*-butanol/acetic acid/water (3: 2: 2, v/v); 2) chloroform/methanol (3: 2); 3) *n*-propanol/2% ammonium hydroxide (2: 1); 4) *iso*-propanol/2% ammonium hydroxide (2: 1). The spot was revealed by blue-violet fluorescence under ultraviolet light.

Hydrolysis by α - and β -glucosidases. The reaction mixture containing the compound concerned (7×10^{-4} M), 4.6 units of glucosidase and buffer in a total volume of 1.0 ml was incubated at 37°C for 5 hr: 1/15 M phosphate buffer of pH 6.8 was used in the reaction with α -glucosidase (6) and 1/5 M acetate buffer of pH 4.8 in that with β -glucosidase (7). The reaction was stopped by heating on a boiling water bath for one minute. An aliquot of this solution was analyzed for the unchanged compound and pyridoxic acid liberated during the reaction by paper chromatography on Toyo filter paper No. 51 (2 $\times$ 40 cm) using the solvent system of *n*-butanol/acetic acid/water (3: 2: 2). The fluorescent bands of each compound detected under ultraviolet light were extracted with 6 ml of water at 65°C for 15 min in the dark. The fluorescence intensity of the extracts was measured by a fluorophotometer. The control experiment using standard solution of pyridoxic

acid showed that this method was satisfactory for quantitative determination.

RESULTS AND DISCUSSION

Enzymic formation and isolation

The livers (125 g) removed from fifteen male rats of the Wistar strain were homogenized with 0.2 M phosphate buffer of pH 7.2 (300 ml) at 0°C. Pyridoxic acid (1.5 g) dissolved in the same buffer (170 ml) was added to the homogenates. The mixture was incubated at 37°C for 4.5 hr in the dark with moderate shaking. The reaction was stopped by heating on a boiling water bath for 10 min and the resulting precipitate was removed by centrifugation at $8,000 \times g$ for 30 min. The supernatant solution (500 ml) was adjusted to pH 3.6 with dil. hydrochloric acid. Charcoal (12 g) was added to it. After stirring the mixture for 15 min, charcoal was collected by filtration. The filtrate was again treated with charcoal. The combined charcoal was washed with water and the adsorbed materials were eluted three times with 800 ml portions of a mixture of ethanol and 2% ammonium hydroxide (2:1, v/v). The eluate was concentrated by evaporation, adjusted to pH 6.0 and applied on eight columns (2.2×25 cm) of DEAE-cellulose equilibrated with 0.01 M acetic acid. The elution was carried out with the same solvent. Each 5 ml of the effluents was collected at a rate of 0.3 ml per min. The elution pattern for each column, as examined by paper chromatography, showed two peaks. The compound concerned was obtained from the first peak fractions and pyridoxic acid from second ones. The first peak fractions were condensed to 10 ml under reduced pressure at 35°C. The concentrate was passed through two columns of P-cellulose (H^+) (2.2×25 cm), followed by washing with water. The fluorescent fractions were taken to dryness by evaporation under reduced pressure at 35°C. The residue was recrystallized from water to give colorless planeary crystals in the yield of 45 mg; mp, 253–255°C (decomp.).

Determination of the chemical structure

The compound failed to give positive reactions with silver nitrate/ammonium hydroxide (8) and ninhydrin (9) reagents. The fact implied that the compound did not contain $-CHO$ and $-NH_2$ groups. The UV spectra observed for the compound in 0.1 N hydrochloric acid and in 0.1 N sodium hydroxide were characteristic of those for pyridoxic acid: $\lambda_{max}^{0.1N HCl} m\mu$; 317; $\lambda_{max}^{0.1N NaOH} m\mu$; 248, 307. The IR spectrum obtained with the compound in KBr disc (Fig. 1) showed absorption bands attributable to the carboxyl group conjugated with the pyridine ring ($1,680\text{ cm}^{-1}$) and the phenolic hydroxyl group ($3,540\text{ cm}^{-1}$). The strong absorption bands at $3,600$ to $3,000\text{ cm}^{-1}$ and $1,100$ to $1,000\text{ cm}^{-1}$, which were not noticeable in the spectrum of pyridoxic acid, could be assigned to several alcoholic hydroxyl groups. The NMR spectrum of the compound was determined in $NaOD-D_2O$ (Fig. 2). A sharp three proton singlet at 2.33 ppm was attributed to the $-CH_3$

Fig. 1. IR spectra of the compound and pyridoxic acid.

Fig. 2. NMR spectrum of the compound (NaOD-D₂O).

group. One proton singlet at 7.69 ppm was assigned to the =CH- group. The signal of γ -CH₂O- group hidden just behind the strong HDO signal became detectable when the specimen was heated *in situ*. On acetylation of the compound, six proton multiplets appearing from 3.1 to 3.8 ppm shifted to low field by 0.5–1.5 ppm and one proton singlet at 4.98 ppm with a spacing of 3.4 Hz remained

without considerable changes in the chemical shift. Five $\text{CH}_3\text{COO}-$ groups were introduced to the mother compound. Since the free phenolic hydroxyl group of pyridoxic acid is subjected to acetylation with acetic anhydride/pyridine, the compound should contain four alcoholic hydroxyl groups. The analytical and spectral data presented above implied that the mother compound consisted of pyridoxic acid and a sugar having four alcoholic hydroxyl groups. In the compound, the hydroxymethyl group at C_5 of the pyridine ring might be bound to the sugar through a glucosidic linkage.

The compound was hydrolyzed with 1 N hydrochloric acid at 100°C for 2.5 hr in the dark. The hydrolyzate gave a positive reaction with cysteine/sulfuric acid reagent which is specific for hexoses (10). The sugar component in the hydrolyzate was then analyzed by thin-layer chromatography. On each chromatogram, one naphthoresorcinol/sulfuric acid -positive spot with an R_f value and the color of glucose was detected (Table 1). Signals appearing at 4.98 ppm and from 3.1 to 3.8 ppm of the NMR spectrum of the compound could with certainty be assigned to the anomeric proton and the other ring protons of the glucose moiety, respectively. The chemical shift and the coupling constant of the anomeric

Table 1. Thin-layer chromatography of the sugar component obtained by acid and enzymic hydrolysis of the compound.

Sample	Silica gel G				
	Sodium acetate (0.02 M)			Boric acid (0.02 M)	
	1	2	3	4	2
Sugar component obtained by acid hydrolysis	0.75	0.69	0.55	0.29	0.76
Sugar component obtained by enzymic hydrolysis	0.75	0.69	0.55	0.29	0.76
Glucose	0.75	0.69	0.55	0.29	0.76
Fructose	0.70	—	—	0.19	0.71
Mannose	0.78	0.72	0.60	0.35	0.76
Galactose	0.68	—	—	0.22	0.71

Solvent 1 : chloroform/methanol (3 : 2)

2 : benzene/acetic acid/methanol (1 : 1 : 3)

3 : *n*-butanol/acetic acid/water (3 : 2 : 2)

4 : methanol/chloroform/acetone/ammonium hydroxide (42 : 16.5 : 25 : 16.5)

Scheme 1. Chemical structure of the compound, 5'-O-(α -D-glucopyranosyl)-4-pyridoxic acid.

proton showed that the glucosidic linkage had α -configuration (11). The sugar moiety has evidently D-conformation because it originated from a glucose donor present in rat liver. The experimental results described above were all compatible with the chemical structure shown in Scheme 1.

The structure of the compound was verified by an enzymic analysis. Sweet almond β -glucosidase had no effect upon the compound concerned, whereas yeast α -glucosidase caused a breakdown of the compound to pyridoxic acid and glucose: 680 μ moles of pyridoxic acid was liberated from 700 μ moles of the mother compound. Both products were confirmed by thin-layer chromatography (Table 1).

Since riboflavinyl- α -glucoside was first obtained by Whitby with an enzyme in rat liver (12), the formation of many sugar derivatives of riboflavin (13, 14) such as riboflavinyl-glucoside, -galactoside, -fructoside and some oligosaccharides as well as glucosides of pyridoxine (15), pantothenic acid (16) and ascorbic acid (17) by microorganisms and by enzyme preparations from bacteria and molds have been reported by many investigators. The compound described in this paper is the first glucoside of pyridoxic acid prepared by enzymic synthesis. The formation of 5'-O-(α -D-glucopyranosyl)-4-pyridoxic acid *in vitro* led the authors to deduce that pyridoxic acid might be further metabolized *in vivo* and play some role in metabolism in rat. The synthesis mechanism of the compound will be presented in a following paper.

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