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A Fluorophotometric Method for the Determination of Pyridoxal and Its Phosphate by Using Glycine and Zinc(II) Ion

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Pyridoxal (PL) or pyridoxal phosphate (PLP) reacts with glycine in the presence of zinc(II) ion in pyridine-methanol and gives a highly fluorescence with excitation maximum at 390 nm and emission maximum at 475 nm. The fluorogen of this reaction is N-pyridoxylidene zinc(II) chelate. Nanomoles of PL or PLP can be estimated spectrofluorophotometrically by using this reaction which is also very specific. Other amino acids, sugars and related substances caused no influence on the determination of PL or PLP. Application of the method to biological samples, such as blood and tissue, is suggested.

Determination of pyridoxal (PL) and pyridoxal phosphate (PLP) by microbiological and chemical methods based on spectrophotometric or fluorophotometric procedures, has been comprehensively reviewed by Storvic, *et al.*[2] and Udenfriend.[3] The fluorophotometric method is superior to the spectrophotometric method because of its high sensitivity. The fluorophotometric method based on formation of the highly fluorescent product of the reaction between PL or PLP and cyanide appeared to be practically used in the biochemical or clinical studies.[4-8] The sensitivity and specificity of this method are apparently greater than those of other methods, but the use of toxic reagent, potassium cyanide, is dangerous for a routine work. In the previous reports, a new fluorophotometric method for the determination of amino sugars and amino acids by using pyridoxal and zinc (II) ion has been developed.[9,10] This fluorescence reaction is based on formation of highly fluorescent N-pyridoxylideneamino sugar or amino acid–Zn(II) chelate. Therefore, PL and PLP seem to be determined by this reaction system. In this paper a systematic study of the optimal conditions for the determination of PL and PLP is described in order to extend the usefulness of this fluorescence reaction.

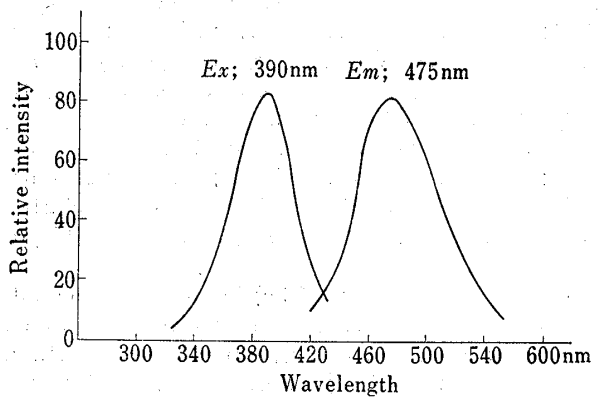

Fig. 1. Excitation and Emission Spectra of PL Reacted with the Reagent

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Experimental

Materials——Pyridoxal, pyridoxal phosphate, pyridoxine and pyridoxamine were purchased from Sigma Chemical Co., U.S.A. Glycine and other amino acids were obtained from Ajinomoto Chemical Inc., Tokyo. Pyridine, methanol, zinc acetate and other chemicals were of reagent grade and used without further purification. Acid phosphatase was prepared from potatoes as described by Takanashi, *et al.*[7]

Reagents——(A) Glycine-zinc(II) solution (containing 0.01 w/v % glycine, 0.1 w/v % zinc acetate, and 2.0 v/v % pyridine in methanol). (B) 0.05 N acetic acid. (C) 10% trichloroacetic acid. (D) 0.2M acetate buffer (pH 4.01). (E) Quinine sulfate solution (1.0 μg/ml in 0.1N H_2SO_4). (F) PL and PLP stock solutions (2.5×10^{-8} mole/ml in water, stored up to 1 month in a refrigerator). (G) PL and PLP working standard solutions (stock solutions diluted with redistilled water to give $1—5 \times 10^{-9}$ mole/ml, stored up to 1 week in a refrigerator).

Procedure——In a test tube 0.5 ml of the aqueous sample solution (containing $0.5—2.5 \times 10^{-9}$ moles of PL or PLP) and 4.0 ml of glycine-zinc(II) solution are placed. After mixing well, the tubes are stoppered and immersed into a water bath of 38° for 30 minutes. The tubes are then removed from the water bath and cooled in an ice bath for a few minutes. The fluorescence intensity is measured within 1 hr with excitation at 390 nm and emission at 475 nm. A blank test with distilled water in place of the sample solution is carried out in the same way. The fluorophotometer is set to read 100% with quinine sulfate solution (excitation: 390 nm, emission: 475 nm).

Determination of PL in biological sample. (A) Serum: To 0.5 ml of serum add 4.0 ml of redistilled water and 0.5 ml of 0.05N acetic acid. After mixing well, the tubes are stoppered and heated for 5 min in a boiling water bath. After cooling with water, centrifuge for 5 min at 3000 rpm, then 0.5 ml of the supernatant is assayed as described above. (B) Whole blood: To 3.0 ml of 10% trichloroacetic acid, 3.0 ml of whole blood hemolized with water (prepared by adding 1.0 ml of whole blood to 2.0 ml of water) is added, mixed vigorously and kept standing for 15 min in a water bath of 50°. After cooling to room temperature, the mixture is centrifuged at 3000 rpm for 10 min. Four ml of the supernatant is taken, and extracted twice with equal volumes of ether to remove trichloroacetic acid. The ether extracts are discarded, and 0.5 ml of the aqueous phase is used for the assay of PL. (C) Tissue: Weighed tissues are homogenized by a usual method. After addition of 1 ml of 10% trichloroacetic acid to a mixture of 1 ml of homogenate (100—200 mg of tissue/ml) and 3 ml of water, the resultant mixture is treated in the way similar to the blood samples.

Determination of PLP after enzymatic hydrolysis. To one ml of the sample solution containing PLP, 2.0 ml of 0.2M acetate buffer (pH 4.01) and 0.5 ml of acid phosphatase solution obtained from potato,[7] were added mixed well, and then the mixture is incubated at 37° for 1 hr. 1.5 ml of 10% trichloroacetic acid is added to the incubated mixture, mixed well, and centrifuged for 5 min at 3000 rpm. Four ml of the supernatant is treated similar to (B).

Table I. Recoveries of Pyridoxal and Pyridoxal Phosphate from Biological Samples

	Human serum				Blood		Rat liver	Rat kidney
	PL[a]	PL[a]	PLP[a]	PLP[b]	PL[c]	PL[c]	PL[d]	PL[d]
Added ($\times 10^{-8}$ moles)	1.96	19.6	5.0	5.0	2.95	9.82	4.91	4.91
Found ($\times 10^{-8}$ moles)	1.95	20.4	3.17	4.58	3.20	9.14	4.64	4.68
n	6	4	6	9	4	3	6	10
Recovery (%)	99.5	104.0	63.4	91.6	108.5	93.1	94.5	95.3
CV (%)	1.21	1.40	2.83	2.16	2.50	2.40	4.30	2.90

a) procedure (A)
b) procedure (B) after enzymatic hydrolysis
c) procedure (B)
d) procedure (C)

Result and Discussion

PL and PLP react with glycine and zinc(II) ion in pyridine–methanol solution to give highly fluorescent complexes which exhibit an excitation maximum at 390 nm and an emission maximum at 475 nm, as shown in Fig. 1. The above assay procedure was designed after the experiments of the following variables, in which 0.5 ml of the sample solutions containing 0.5 μg of PL or PLP were used throughout.

Effect of Reagent Concentration

Effects of the reagent concentration on fluorescence intensity were examined with 0.5 μg/ml of PL solution. The fluorescence intensity increased with increasing the concentration of zinc(II) acetate, glycine, and pyridine up to 0.1 w/v %, 0.005 w/v %, and 1.0 v/v %, respectively; higher concentration did not produce more fluorescence. As the results of these experiments, 0.1 w/v % of zinc(II) acetate, 0.01 w/v % of glycine, and 2.0 v/v % of pyridine were employed in the standard procedure, respectively.

Effect of Reaction Temperature and Reaction Time

The fluorescence intensity increased gradually at 20°, and a long period of time was required to reach the maximum fluorescence intensity. Although the time required for the maximum fluorescence intensity depended on temperature, the fluorescence decreased slightly with increasing the reaction temperature over 45°. The maximum and nearly constant fluorescence intensity was obtained at 38° and for more than 30 minutes of the reaction time.

Stability of Fluorescence

The fluorescence of PL and PLP under the standard condition was relatively stable for 2 hr at room temperature and decayed slowly after 2 hr. Therefore, the measurement of fluorescence intensitiy should be carried out within 2 hr after reaction.

Working Curves for PL and PLP

The relative fluorescence intensity obtained from PL and PLP were strictly proportional to their concentrations up to $1—5\times10^{-9}$ moles (final concentration: $2—10\times10^{-10}$ mole/ml). A linear relationship existed between the concentration and the fluorescence intensity below 5×10^{-11} mole/ml, by increasing the instrumental sensitivity (range selector: ×10). The fluorescence intensitiy of PL was about 2 times as large as that of PLP. Therefore, it is favorable to measure PLP as total PL after hydrolysis of PLP.

Interfering Substances

Interferences of various compounds on this method were examined by measuring fluorescence intensity in the presence of them with PL. No detectable interference occurred with any of the amino acids, carbohydrates, and other biological substances, such as urea, creatine, and creatinine. Although most of carbonyl compounds caused also no influence on the fluorescence intensity, salicylaldehyde and 2-hydroxy-1-naphthaldehyde interfered with the recovery of PL because their structures were like to PL. The possible interference of the related compounds to PL, such as pyridoxamine and pyridoxine, was expected, so that it was quantitatively evaluated. The recovery of PL or PLP was little affected in the presence of pyridoxamine or pyridoxine by ten times. When they co-existed 800 times as much as PL or PLP, the recoveries of them were decreased down to about 50% and 90%, respectively. Urinary levels of pyridoxal, pyridoxamine, and pyridoxine are 0.08—0.15, 0—0.02, and 0.03—0.14 μg/ml, and blood levels of them 0.02—0.07, 0.15—0.20, and 0 μg/ml, respectively.[11] Therefore, pyridoxamine and pyridoxine seem to have practically no interference on the determination of PL or PLP by this method.

Application to Biological Materials

This method was applied to the determination of PL and PLP in biological materials, such as serum, whole blood, liver, and kidney. Three deproteinization procedures were examined. Acetic acid-procedure was adapted to serum, but not to hemolized blood or tissue homogenate. Whole blood and tissue homogenate could be deproteinized by 10% trichloroacetic acid-ether extract procedure or ion-exchange column procedure reported by Yamada, *et al.*[6] The former procedure was preferable because of less dilution of sample and simple operation. The recoveries of additional PL were tested by the proposed procedures, as summarized in Table I.

The recoveries of PL were between 90—104% and the coefficients of variation were between 1.21—4.30%, while the recovery of PLP added to human serum was only 63.3%, suggesting that some amount of PLP might be precipitated with denaturated protein. It is, therefore, favorable to determine PLP as PL after hydrolysis in order to obtain higher recovery. The enzymatic hydrolysis of PLP was examined with the same procedure reported by Takanashi, *et al.*[7] As shown in Table I, the recovery of PLP added to serum increased from 63.3% to 91.6% by using acid phosphatase solution obtained from potato. The separately determination of PL and PLP would be achieved by this method after SM-cellulose column separation with 0.01N acetic acid as described by Yamada, *et al.*[5]

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Electron Spin Resonance Study of γ-Irradiated H_2SO_4-SiO_2 System

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The SO_4^- radicals was formed in γ-irradiated H_2SO_4–SiO_2 system at −196°. The formation of the SO_3^- radicals are accompanied when γ-irradiation is carried out after heat-treatment at 500°, indicating that H_2SO_4 on SiO_2 is stable even at 500°.

The SO_4^- radicals are known to be formed in γ-irradiated sulfuric acid glasses.[2-5] On the other hand, the SO_3^- and SO_2^- radicals are formed besides the SO_4^- radicals in irradiated sulfates. However, the kinds of the radicals change with the sorts of the sulfates and irradiation in these cases.[6-10]

In the present study, the electron spin resonance (ESR) spectra of the γ-irradiated H_2SO_4–SiO_2 system were measured and compared with the above-mentioned reports. In addition, the effects of heat-treatment and the absorption of H_2O were examined.

The property of silica as a solid acid is well known. The silica treated with H_2SO_4 behaves as an another acid which shows a catalytic effect, for example, on the cracking of petroluem.[11]

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