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67. Mitsuo Yamada, Akira Saito, and Zenzo Tamura : Fluorometric Determination of Pyridoxal-5-phosphate and Pyridoxal in Biological Materials. II.*1 Specificity of the Method in Pyridoxal-5-phosphate Determination.

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Bonavita[1] reported the fluorescence analysis of pyridoxal-5-phosphate (PALP) and pyridoxal (PAL) by the reaction with potassium cyanide and an application of the method to minute amounts of PALP and PAL in biological materials was studied by the present authors.[2] In the present paper, the specificity of the method has been investigated by the use of mouse liver as it contained a larger amount of substance measured as PALP.[2]

The PALP fraction (L-PALP) was prepared from mouse livers according to the previously reported method,[2] concentrated and reacted with potassium cyanide in weak alkaline solution, and fluorescence excitation and emission spectra of the resultant solution (L-PALP-CN) were compared with those obtained from PALP. As shown in Fig. 1, these spectra of L-PALP-CN were in good similarity to those obtained by the reaction of authentic PALP with potassium cyanide (PALP-CN).

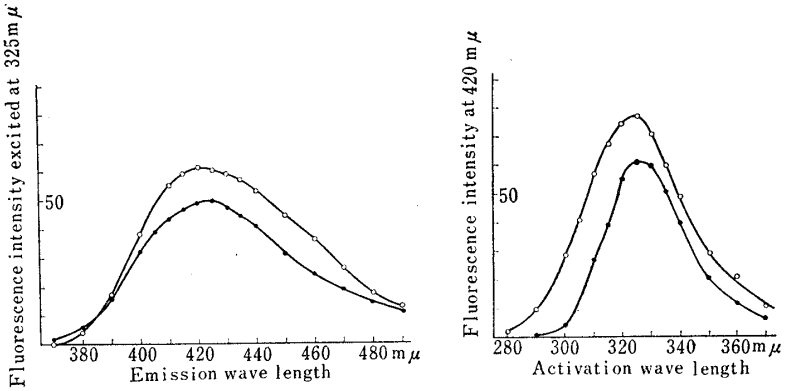

Fig. 1. Fluorescence Excitation and Emission Spectra of PALP-CN, Authentic (o–o) and Obtained from Liver (•–•)

Fluorescence measurements were taken with a Hitachi spectrofluorometer EPU-2/G-1.

The fluorescence intensity measured at various pH ranging from 4 to 8.4 also showed a similar variation to that of PALP-CN (Fig. 2).

Concentrated solutions of L-PALP, L-PALP-CN, and PALP-CN of a similar concentration were subjected to the paper electrophoresis at pH 5.1. L-PALP-CN showed a distinct violet fluorescent band at the identical position with PALP-CN on the paper, as shown in Fig. 3, and both fluorescence intensities were similar.

On the other hand, L-PALP did not show any fluorescent band on the paper at the corresponding position. This was also observed by the paper chromatography (Fig. 4).

*1 Part I. M. Yamada, A. Saito, Z. Tamura : This Bulletin, **14**, 482 (1966).

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1) V. Bonavita : Arch. Biochem. Biophys., 88, 366 (1960).

2) M. Yamada, A. Saito, Z. Tamura : This Bulletin, **14**, 482 (1966).

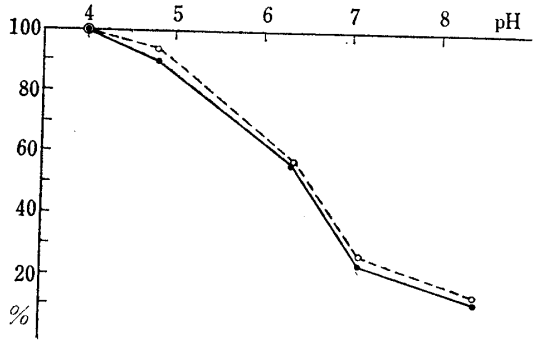

Fig. 2. Influence of pH on Fluorescence Intensity of PALP-CN, Authentic (o–o) or Obtained from Liver (•–•)

Fluorescence was measured with a Turner 110 Fluorometer.
Ordinate: percentage of fluorescence intensity compared with that at pH 4.

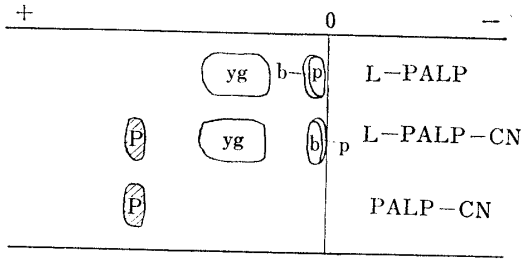

Fig. 3. Paper Electrophoresis of PALP-CN, L-PALP-CN and L-PALP

b: blue
p: purple
yg: yellowish green

These results indicated that PALP actually existed in L-PALP, and that the difference in fluorescence intensity between L-PALP-CN and L-PALP corresponded to PALP-CN.

In order to confirm this, an enzymatic hydrolysis of L-PALP was examined. As a specific enzyme which catalyzed the hydrolysis of PALP has not been discovered, a common phosphatase had to be used in this experiment in spite of a few defects in the specificity. A PALP solution was incubated at pH 5.0 with a certain amount of acid phosphatase which was prepared from human semen,[3] and a rapid conversion of PALP to PAL was confirmed by a change in the absorption spectra. After incubation of PALP, L-PALP and their mixture with the same acid phosphatase, the fluorescence reactions for PALP and PAL were carried out with the incubated mixtures and resulted fluorescence intensities were measured. The data (Table I) indicated that almost all substance measured as PALP in L-PALP disappeared by the incubation with acid phosphatase, and at the same time a substance measured as PAL equivalent to PALP was produced.

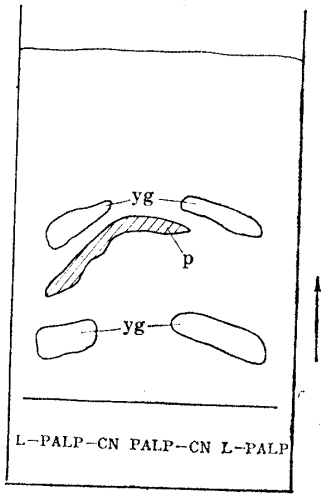

Fig. 4. Paper Chromatogram of PALP-CN, L-PALP-CN and L-PALP

p: purple
yg: yellowish green

Table I. Hydrolysis of PALP and L-PALP with Acid Phosphatase

Study	Content of incubated mixture				Result of fluorometry	
	3 ml. of	1 ml. of	1 ml. of	0.2 ml. of	Reaction for PALP[a] (×3)	Reaction for PAL[a] (×1)
1	acetate-$MgCl_2$[a]	$6\times10^{-6}M$ PALP	H_2O	acetate buffer[a]	31.0	1.6
2	〃	$6\times10^{-6}M$ PALP	〃	acid phosphatase[a]	2.3	57.1
3	〃	H_2O	L-PALP[a]	acetate buffer[a]	12.8	1.1
4	〃	〃	〃	acid phosphatase[a]	1.0	26.8
5	〃	$3\times10^{-6}M$ PALP	〃	acetate buffer[a]	28.2	1.4
6	〃	$3\times10^{-6}M$ PALP	〃	acid phosphatase[a]	2.5	52.7
7	H_2O	$6\times10^{-6}M$ PALP	H_2O	H_2O	31.1	1.8
8	〃	$6\times10^{-6}M$ PAL	〃	〃	2.2	56.4

a) See experimental.

3) J. Wittenberg, A. Kornberg: J. Biol. Chem., **202**, 431 (1953).

From the all findings described above, it is most certain that the method is of high selectivity for a determination of PALP in liver.

Experimental

Materials——PAL, PALP, and SM-cellulose were obtained from E. Merck AG., Wakamoto Pharmaceutical Co. Ltd., and Brown Co. respectively. Acid phosphatase solution which was prepared as described by Wittenberg and Kornberg[3] was supplied by Dr. Y. Mano of Faculty of Medicine, University of Tokyo. Male mice of the DDY strain weighing from 20 to 30 g. were employed.

Instruments——Quantitative fluorescence measurements were carried out with Turner Model 110 Fluorometer associated with a lamp F4T4/BL, a primary filter #7-60, and secondary filters #2A+#47B, and measurements of fluorescence spectra, with Hitachi Spectrofluorometer EPU-2/G-1.

Fluorescence Excitation and Emission Spectra of L-PALP-CN——After four mice were sacrificed with the carotid artery cut, livers were homogenized with 20 ml. (=5 ml. × 4) of H_2O using a Potter type homogenizer. Twenty ml. of 10% (w/v) trichloroacetic acid (TCA) were added to the homogenate. After being well-mixed, the mixture was warmed at 50° for 15 min., and centrifuged at 4,000 r.p.m. for 10 min. Total supernatant (approximate 40 ml.) was washed with 40 ml. of ether twice to remove TCA, applied on a SM-cellulose column (10 g., equilibrated with 0.01*N* AcOH), and eluted with 0.01*N* AcOH. Fifty ml. of the effluent from 21st ml. to 70th ml. were collected, and evaporated, with 0.3 ml. of *N* NaOH, to dryness under reduced pressure at approximately 40° in a rotary evaporator.

The residue was dissolved in 5 ml. of H_2O, and to 2 ml. of this solution were added 2 ml. of 0.2*M* phosphate buffer (pH 7.4) and 0.1 ml. of 0.05*M* KCN in 0.1*M* phosphate buffer (pH 7.4). A blank test was carried out on the same sample solution using 0.1 ml. of 0.1*M* phosphate buffer in place of the KCN solution. After being warmed at 50° for 30 min., 1 ml. of each of the sample and the blank test solution was added to 9 ml. of 0.1*M* acetate buffer (pH 3.8), and the fluorescence intensities were measured at various wavelength. A reference solution of $2\times10^{-5}M$ PALP was measured fluorometrically by the same method.

Fluorescence Intensity at Various pH——A L-PALP was prepared from four mouse livers following the above mentioned method and evaporated to dryness. The residue was dissolved in 10 ml. of H_2O and to 1 ml. of this was added 4 ml. of 0.2*M* phosphate buffer (pH 7.4) and 10 ml. of H_2O. To 7 ml. of the solution 0.1 ml. of 0.05*M* KCN in 0.1*M* phosphate buffer were added. A blank test was carried out on the same solution using 0.1 ml. of 0.1*M* phosphate buffer in place of the KCN solution. After being warmed at 50° for 30 min., 1 ml. of each solution was mixed with 5 ml. of various pH buffer solution.

Paper Electrophoresis of L-PALP-CN——A L-PALP prepared from two mouse livers was neutralized with 0.1*N* NaOH, and 1 ml. of 0.2*M* phosphate buffer (pH 7.4) and H_2O were added to make a total volume of the solution to 38 ml. This solution was divided into two fractions of 19 ml. and to the one was added 1 ml. of 0.05 *M* KCN aqueous solution and to the other was added 1 ml. of H_2O. Both solutions were warmed at 50° for 30 min. One ml. of each solution was taken for fluorometry and the concentration of PALP-CN in the former solution was measured as $7\times10^{-7}M$. Each residual solution was evaporated to dryness, dissolved in 0.5 ml. of H_2O, subjected to the electrophoresis. As the concentration of PALP-CN in the resulted solution should be $2.6\times10^{-5}M$, a solution of $3\times10^{-5}M$ PALP-CN in 0.1*M* phosphate buffer was used as a reference. The electrophoresis was carried out in acetate buffer (pH 5.1, $\mu=0.05$) at 14 v./cm. for 90 min. on Toyo No. 51 filter paper (13 cm. × 35 cm.). Manaslu light (360 mμ) was used for the detection of spots.

Paper Chromatography of the L-PALP-CN——The same sample solutions as for paper electrophoresis applied on a Toyo No. 51 filter paper (6.5 cm. × 35 cm.) in a line, and a portion of the samples was overlapped. An ascending developement was carried out with *n*-BuOH-pyidine-AcOH-H_2O (4:1:1:2) for 1 hr.

Absorption Spectral Change of PALP by the Treatment with Acid Phosphatase——One volume of 0.4*M* acetate buffer (pH 5.0), one volume of $2\times10^{-3}M$ $MgCl_2$ and two volume of H_2O were mixed. In this solution, PALP was dissolved to $1.5\times10^{-4}M$. To 3 ml. of this PALP solution was added 0.2 ml. of acid phosphatase solution which had been diluted twenty times with 0.1*M* acetate buffer (pH 6.0), and absorption spectra of the mixture were measured after 2, 10, and 30 min. incubation at 25° with Hitachi recording spectrophotometer. Complete conversion of PALP to PAL in 10 min. was observed without further decomposition.

Hydrolysis of L-PALP by Acid Phosphatase——A L-PALP was prepared from a mouse liver. To the L-PALP was added 0.1 ml. of *N* NaOH, and the mixture was evaporated to dryness and dissolved in 13 ml. of H_2O.

Twenty-five ml. of 0.4*M* acetate buffer (pH 5.0), 25 ml. of $2\times10^{-3}M$ $MgCl_2$ and 10 ml. of H_2O were mixed (acetate-$MgCl_2$). As shown in Table I, 1 ml. of PALP solution or H_2O, 1 ml. of L-PALP or H_2O, and 0.2 ml. of twenty times diluted acid phosphatase solution or 0.1*M* acetate buffer (pH 6.0) were added to 3 ml. of the acetate-$MgCl_2$, and they were incubated at 25° for 60 min.

The fluorometry of PALP: To 1 ml. of the reacted solution were added 3 ml. of 0.2*M* phosphate buffer (pH 7.4), and 0.1 ml. of 0.05*M* KCN or 0.1 ml. of 0.1*M* phosphate buffer (pH 7.4), and the mixtures were

then warmed at 50° for 30 min. After addition of 2 ml. of 0.5*N* tartaric acid, the fluorescence intensities were measured.

The fluorometry of PAL : To 1 ml. of the reacted solution were added 5 ml. of phosphate buffer (0.2*M*, pH 7.4), and 0.1 ml. of 0.05*M* KCN or 0.1*M* phosphate buffer, and the mixtures were warmed at 50° for 120 min. The fluorescence intensities were measured after addition of 1 ml. of 0.6*M* Na_2CO_3.

A separation of PALP and PAL was not carried out in this case, instead, as references pure PALP and PAL solutions were treated by the same procedure.

We are grateful to Dr. Y. Mano of Faculty of Medicine, University of Tokyo for supplying the acid phosphatase solution.

Summary

The specificity of the previously reported fluorometric method of determination of PALP in biological materials was investigated. The fluorescence excitation and emission spectra, the change in fluorescence intensity with pH, the electrophoresis, the paper chromatography, and the hydrolysis with acid phosphatase were examined with the PALP fraction obtained from mouse liver. The results showed that the method had high specificity for the determination of PALP in liver.

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68. Akira Nohara, Kin-ichi Imai, and Mikio Honjo : Synthesis of the Glucose Analogs of Inosine-5′ Phosphate.

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In the previous paper[*2] we have recorded the synthesis of several compounds related to 5′-IMP[*3] and interesting observations that the presense of 2′- and/or 3′-hydroxyl group(s) in the ribose moiety was not prerequisite for a flavor enhancing activity of some 5′-nucleotides, and the monophosphate grouping on position 5′ was essential for the activity. In an extention of our studies on the structure -flavor enhancing activity relationship, we have undertaken the synthesis of the nucleotides analogs whose sugar moiety was D-glucose, in place of D-ribose.

To this end, 6-benzamido-9-(2′, 3′, 4′-tri-O-acetyl-6′-diphenylphosphoryl-β-D-glucopyranosyl)purine (I) appeared the most attractive starting material, because compound I has already been reported[1] and in addition it is quite safe to conclude that I is a β-anomer on the basis of a comparatively large coupling constant (ca. 10 c.p.s.) of $H_{1'}$.[2]

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*3 5′-IMP, inosine-5′ phosphate; 5′-AMP, adenosine-5′ phosphate; 3′-AMP, adenosine-3′ phosphate; 2′-AMP, adenosine-2′ phosphate; 2′,3′-cyclic AMP, adenosine-2′,3′-cyclic phosphate; 3′,5′-cyclic AMP, adenosine-3′,5′-cyclic phosphate; AG, 9-β-D-glucopyranosyladenine.

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