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66. Mitsuo Yamada, Akira Saito, and Zenzo Tamura : Fluorometric Determination of Pyridoxal-5-phosphate and Pyridoxal in Biological Materials. I. Procedure and Application.

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For the microdetermination of pyridoxal-5-phosphate (PALP) in biological materials, the enzymatic methods which are based on the activation of tyrosine apodecarboxylase[1~3] or apotryptophanase[4] have usually been employed. In spite of their high sensitivity, the enzymatic methods have some defects. The preparation of apoenzymes is troublesome and tedious, and pyridoxamine phosphate and pyridoxal (PAL) are considered possibly to activate tyrosine apodecarboxylase.[4]

In 1960, Bonavita[5] reported a possibility of microdetermination of PALP and PAL, by the conversion to fluorescent cyanohydrins (PALP-CN and PAL-CN). The authors tried to apply this method to the determination of biological materials.

Method

Materials——PAL, PALP, and SM-cellulose were the products of E. Merck AG., Wakamoto Pharmaceutical Co., Ltd., and Brown Co. respectively. 0.1*M* and 0.2*M* phosphate buffer solutions of pH 7.4, and 0.05*M* KCN solution in 0.1*M* phosphate buffer were used as reagents.

Instruments——Turner Model 110 Fluorometer (Lamp F4T4/BL), associated with a primary filter #7-60 and secondary filters #2A-#47B, was used.

Determination of PALP and PAL in Blood——One ml. of whole blood is added to 2 ml. of H_2O, and to this hemolyzed blood is added 3 ml. of 10% (w/v) trichloroacetic acid (TCA). The mixture is warmed at 50° for 15 min., centrifuged at 4,000 r.p.m. for 10 min., and 4 ml. of the supernatant is washed twice with the same volume of ether to remove TCA. Two ml. of the aqueous phase is applied on a SM-cellulose column (1 g., equilibrated with 0.01*N* AcOH), and, after 1st ml. of effluent is discarded, eluted with 0.01*N* AcOH. Exact 10 ml. of the effluent is collected from 2nd to 11th ml. for PALP determination. The eluent is changed to 0.1*M* phosphate buffer and successive 10 ml. of the effluent is collected for PAL determination.

From the PALP fraction two aliquots of 2 ml., A and B, are taken. To A, 2 ml. of 0.2*M* phosphate buffer and 0.1 ml. of 0.05*M* KCN are added, and to B, 2 ml. of 0.2*M* phosphate buffer and 0.1 ml. of 0.1*M* phosphate buffer are added. They are warmed at 50° for 30 min., and acidified to pH 3.8 with 1 ml. of 0.5*N* tartaric acid, and their fluorescence intensities are measured. The difference (a) in fluorescence intensity between A and B is compared with that (b) obtained by the same reaction procedure with a known concentration (c) of authentic PALP. PALP level in whole blood is calculated as follows,

$$\text{Concentration of PALP in whole blood} = (c) \times \frac{(a)}{(b)} \times 30$$

Two ml. of PAL fraction is warmed at 50° for 120 min. with 5 ml. of 0.2*M* phosphate buffer and 0.1 ml. of 0.05*M* KCN. After addition of 1 ml. of 0.6*M* Na_2CO_3 to correct the pH of the solution to 10, the fluorescence intensity is measured. A blank test is carried out similarly on the same sample solution using 0.1*M* phosphate buffer in place of 0.05*M* KCN. PAL concentration in whole blood is calculated by the same way as for PALP.

Determination of PALP and PAL in Organs——Weighed organs or tissues are homogenized under ice cooling with a certain volume of H_2O. After an addition of the same volume of 10% TCA, the homogenate

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1) W. W. Umbreit, W. D. Bellamy, I. C. Gunsalus : Arch. Biochem. Biophys., **7**, 185 (1945).
2) G. E. Boxer, M. P. Pruss, J. M. Goodhart : J. Nutrition, **63**, 623 (1957).
3) A. Hamfelt : Clin. Chim. Acta, **7**, 746 (1962).
4) T. Morisue : Osaka Daigaku Igaku Zasshi, **11**, 5731 (1959).
5) V. Bonavita : Arch. Biochem. Biophys., **88**, 366 (1960).

is warmed at 50° for 15 min. and centrifuged at 4,000 r.p.m. for 10 min. The supernatant obtained is treated similary to the blood samples.

Result and Discussion

Fluorescence Intensity and pH——The fluorescence spectra of PALP-CN showed its excitation maximum at 325 mμ and emission maximum at 420 mμ in acidic medium, while that of PAL-CN showed the excitation and emission maxima at 358 mμ and 438 mμ respectively, in alkaline medium.

The correlation between pH and the intensity of fluorescence is shown in Fig. 1.

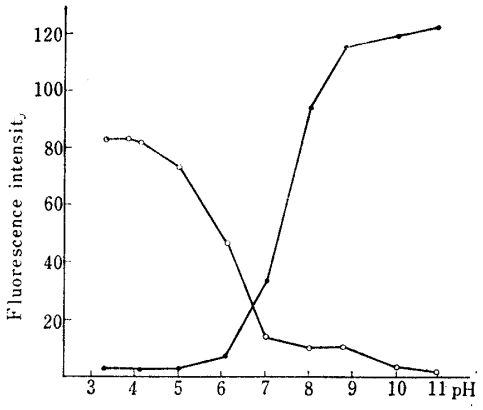

Fig. 1. Fluorescence Intensity of PALP-CN (o–o) and PAL-CN (•–•) at Various pH

PALP-CN : $1.8\times10^{-7}M.$ (×10)
PAL-CN : $1.0\times10^{-7}M.$ (×3)

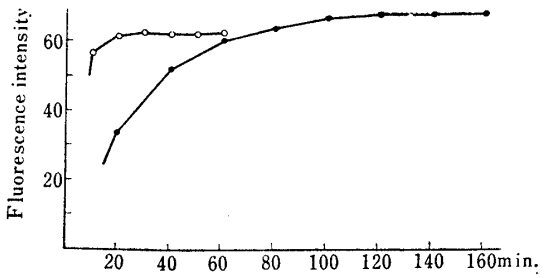

Fig. 2. Timed Reaction of PALP (o–o) or PAL (•–•) with Potassium Cyanide at 50°

PALP : $3.2\times10^{-7}M.$ (×3)
PAL : $2\times10^{-7}M.$ (×1)

Although PALP-CN showed the strongest fluorescence at pH 3.8, as Bonavita cited, the intensity of the fluorescence of PAL-CN was found to be stronger in a solution of pH 10 than that of pH 7.55 which Bonavita adopted for the measurements. Furthermore, in the pH range between 7 and 8, the intensity of fluorescence of PAL-CN was varied remarkably and a reliable value could hardly been obtained. Hence, pH 3.8 and pH 10 are most suitable conditions for PALP and PAL measurements respectively.

Conditions for Fluorescence Production and Stability of Fluorescence——As shown in Fig. 2, the reaction of PALP was completed within 30 min. at 50°, while PAL required 120 min. at 50° for a complete reaction. The resulted cyanohydrins were not so stable. One and 5% of PALP-CN were decomposed after 20 and 45 min. respectively, when kept standing in the acidic solution of pH 3.8. PAL-CN lost 1% of its fluorescence within 60 min. at pH 10. Therefore, the measurement of fluorescence of PALP-CN should be operated immediately after the addition of tartaric acid.

Selection of Filters——As to PAL-CN, there were selective filters, a primary filter #7-60(360 mμ) and a secondary couple of filters, #47B and #2A(435 mμ). On the other hand, there were not so suitable filters for PALP-CN determination. Although a couple of #7-54 and #34A had the transmittance maximum at 325 mμ, they transmitted somewhat the light of 416~460mμ which gave blank reading to PALP-CN fluorescence. Consequently the same combination of filters as for PAL-CN had to be used for PALP-CN.

Calibration curves of PALP-CN and PAL-CN were obtained by Turner Fluorometer associated with lamp F4T4/BL, primary filter #7-60 and secondary filters #47B #2A as shown in Fig. 3.

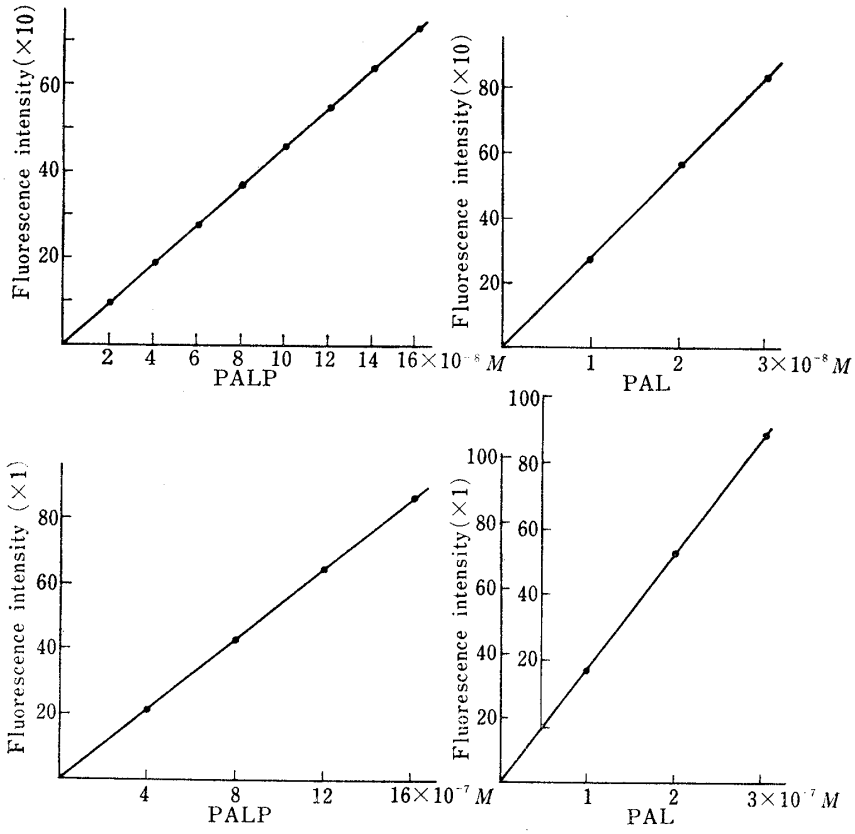

Fig. 3. Calibration Curves of PALP and PAL
Abscissa: final concentration of PALP or PAL

Recovery from Blood——Among the deproteinization methods studied, Sephadex G-50 column and trichloroacetic acid gave quantitative recovery, and the latter was preferable because of less dilution of sample and simpler operation.

As shown in Fig. 1, there was a little interferance in fluorescence of PALP-CN and PAL-CN each other even when the measurement was carried out at pH either 3.8 or 10. SM-cellulose column gave a clear separation of PALP and PAL.

The recoveries of additional PALP and PAL, $1\sim3\times10^{-6}M$, in blood solutions were tested by the proposed procedure. The results were 97.4±2.0 (S.D.) % for PALP, and 95.9±2.9 (S.D.) % for PAL (Table I).

Table I. Recoveries of PALP and PAL Added to Human Hemolyzed Blood

Additional PALP or PAL concentrations in hemolyzed blood (M)	Recoveries (%)	
	PALP	PAL
1×10^{-6}	97.8, 100.0	99.6, 95.2
2×10^{-6}	94.7, 96.3	92.6, 98.4
3×10^{-6}	99.0, 96.3	93.2, 96.2
mean	97.4	95.9

Recovery from Organ——A homogenate of liver or brain of mouse was devided into two parts, PALP and PAL were added to the one of them, and the recoveries were measured by this method. These results are shown in Table II, which also indicate that 8~9 γ of PALP and 0.2~0.3 γ of PAL are contained per gram of liver, and 2~2.5 γ of PALP and 0.2~0.25 γ of PAL per gram of normal mouse brain.

Table II. Recovery of PALP and PAL from Mouse Liver

Recovery of PALP

(A) Liver wet weight (g.)	(B) Fluorescence intensity of liver+PALP(×3)	(C) Fluorescence intensity of liver (×3)	(D) Recovery of added PALP (B)−(C)	(E) Recovery ratio $\frac{(D)}{30.0^{a)}}\times 100\%$	(F) PALP content in liver[b] (γ/g.)
0.974	49.1	18.7	30.4	101.3	8.42
0.950	48.1	18.3	29.8	99.3	8.40
1.092	48.3	19.3	29.0	96.7	8.59
mean				99.1	8.47

Recovery of PAL

(A) Liver wet weight (g.)	(B) Fluorescence intensity of liver+PAL (×3)	(C) Fluorescence intensity of liver (×3)	(D) Recovery of added PAL (B)−(C)	(E) Recovery ratio $\frac{(D)}{10.2^{c)}}\times 100\%$	(F) PAL content in liver[d] (γ/g.)
0.947	14.4	4.5	9.9	97.1	0.339
0.840	12.8	3.5	9.3	91.2	0.285
0.995	14.1	3.5	10.6	103.9	0.246
mean				97.4	0.290

(A): weighed mouse liver was homogenized under ice cooling with 7 ml. of H_2O.
(B): One ml. of either $2\times10^{-5}M$ PALP or $1.6\times10^{-6}M$ PAL was added to 3 ml. of the homogenate.
(C): One ml. of H_2O was added to 3 ml. of the homogenate.
a) Fluorescence intensity of $5\times10^{-7}M$ PALP
b) PALP content in liver $=\frac{(C)}{30.0}\times 5\times10^{-7}\times 2.47\times10^{5}(\gamma/\text{ml.})\times\left(\frac{10}{2}\cdot\frac{8}{3}\right)\times\frac{7+(A)(\text{ml.})}{(A)(\text{g.})}$
c) Fluorescence intensity of $4\times10^{-6}M$ PAL
d) PAL content in liver $=\frac{(C)}{10.2}\times 6.88\times10^{-3}(\gamma/\text{ml.})\times\frac{40}{3}\times\frac{7+(A)(\text{ml.})}{(A)(\text{g.})}$

These PALP contents were well coincided with those obtained from rats by the apoenzymic method.[6]

To confirm the selectivity of the present method, the fluorescence excitation and emission spectra, the change in fluorescence intensity with pH, paper electrophoresis, paper chromatography, and hydrolysis by acid phosphatase were examined with the PALP fraction obtained from mouse liver, and reported in another paper.[7]

Application——The method was applied to the investigation of the fate of PALP and PAL, administered to animals.

A rabbit was kept fasting for 24 hours and injected with 10 mg. of either PALP or PAL intravenously. One ml. of blood was taken from the auricular vein at a certain interval after the injection, and the concentrations of PALP and PAL were determined.

Fig. 4 illustrates that the injected PALP disappears rapidly from the blood with introducing PAL, and the concentration of the latter is held about one tenth of the former. On the contrary, as shown in Fig. 5, the injection of PAL causes a slow increase of PALP content, which arrives at a maximum one hour later.

In the second experience, 10 mg. of PALP per 1 kg.b.wt. was administered to mice intravenously. After 60 min., the mice were sacrificed by cutting the carotid artery, and freezed. The brain, liver and kidneys of each mouse were weighed and homogenized with 5, 10 and 5 ml. of 5% TCA, respectively. The contents of PALP and PAL in these homogenates were analyzed by the present method. As the results shown in Fig. 6, a slight increase of PALP in liver and kidney, and a remarkable

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7) Part II. M. Yamada, A. Saito, Z. Tamura: This Bulletin, **14**, 488 (1966).

TABLE III. Recovery of PALP and PAL from Mouse Brain

Recovery of PALP

(A) Brain wet weight (g.)	(B) Fluorescence intensity of brain+PALP (×3)	(C) Fluorescence intensity of brain (×3)	(D) Recovery of added PALP (B)−(C)	(E) Recovery ratio $\frac{(D)}{26.0^{a)}}\times 100\%$	(F) PALP content in brain[b] (γ/g.)
1.055	44.3	19.9	24.4	93.8	2.17
1.206	49.0	24.1	24.9	95.8	2.34
1.090	47.4	23.6	23.8	91.6	2.50
mean				93.7	2.34

Recovery of PAL

(A) Brain wet weight (g.)	(B) Fluorescence intensity of brain +PAL (×10)	(C) Fluorescence intensity of brain (×10)	(D) Recovery of added PAL (B)−(C)	(E) Recovery ratio $\frac{(D)}{17.9^{c)}}\times 100\%$	(F) PAL content in brain[d] (γ/g.)
1.055	30.2	13.1	17.1	95.5	0.223
1.206	33.8	14.6	19.2	107.3	0.222
1.090	32.6	13.9	18.7	104.5	0.230
mean				102.4	0.225

(A): Three brains were combined, weighed and homogenized with 7 ml. of H_2O.
(B): One ml. of a mixed solution containing $4.5\times10^{-6}M$ of PALP and $7.2\times10^{-7}M$ of PAL was added to 3 ml. of the homogenate.
(C): One ml. of H_2O was added to 3 ml. of the homogenate.
a) Fluorescence intensity of $1.5\times10^{-7}M$ PALP
b) PALP content in brain $=\frac{(D)}{26.0}\times1.5\times10^{-7}\times2.47\times10^{5}(\gamma/\text{ml.})\times\left(\frac{10}{2}\cdot\frac{6}{3}\right)\times\frac{7+(A)(\text{ml.})}{(A)(\text{g.})}$
c) Fluorescence intensity of $2.4\times10^{-8}M$ PAL
d) PAL content in brain $=\frac{(D)}{17.9}\times2.4\times10^{-8}\times1.67\times10^{5}(\gamma/\text{ml.})\times\left(\frac{10}{2}\cdot\frac{6}{3}\right)\times\frac{7+(A)(\text{ml.})}{(A)(\text{g.})}$

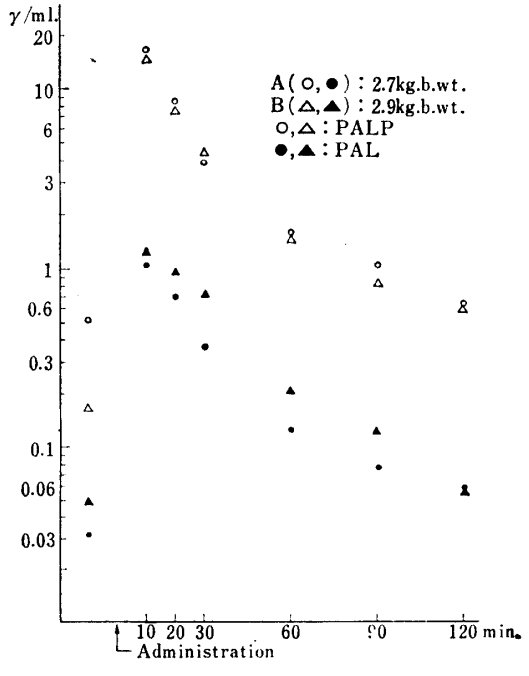

Fig. 4. Changes in Concentration of PALP and PAL in Whole Blood of Rabbits after Intravenous Injection of 10 mg. of PALP

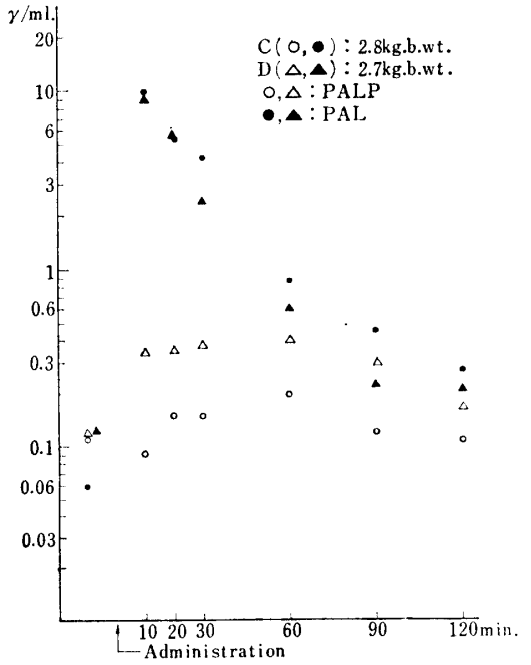

Fig. 5. Changes in Concentration of PALP and PAL in Whole Blood of Rabbits after Intravenous Injection of 10 mg. of PAL

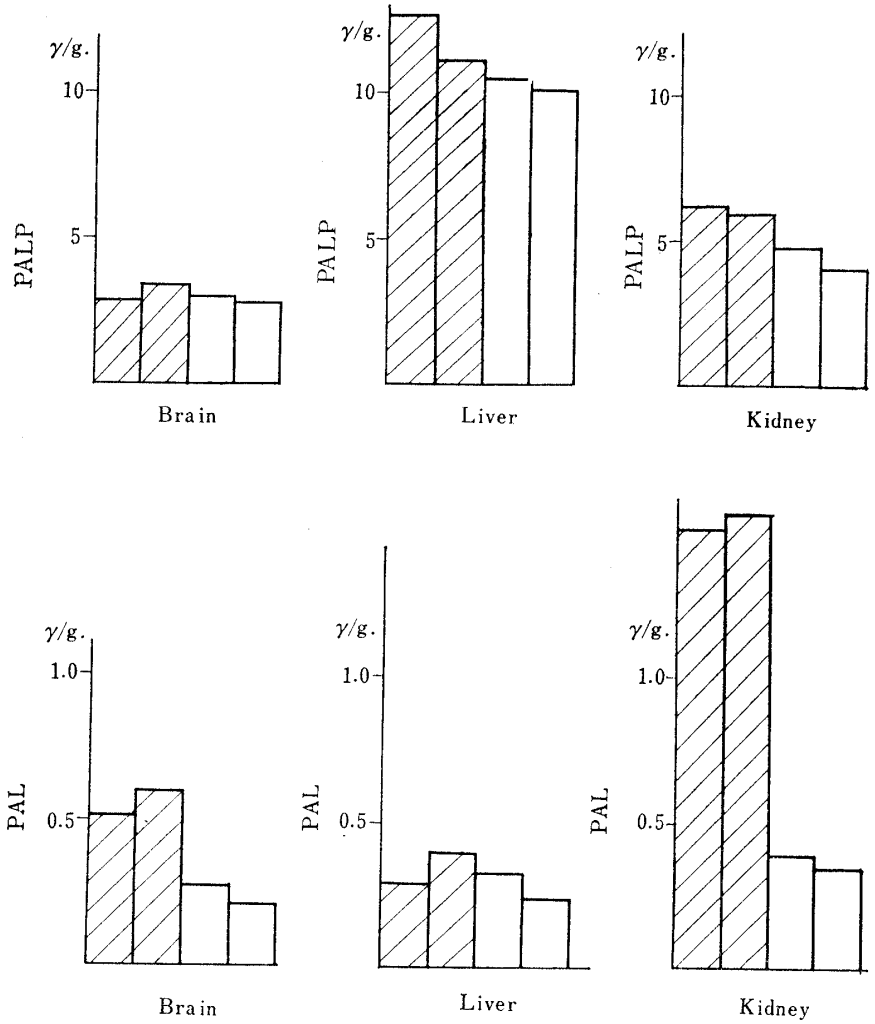

Fig. 6. Changes in Concentration of PALP and PAL in Mouse Brain, Liver and Kidney after Administration of PALP (10 mg./kg. b. wt.)
▨: PALP administered ▭: Control

increase of PAL in brain and kidney were observed in this experience.

Summary

A fluorometric method of determination of pyridoxal-5-phosphate (PALP) and pyridoxal (PAL) in blood or organ has been developed. This method is based on Bonavita reaction of KCN with either PALP or PAL, and found to be convenient and has remarkable sensitivity for PAL as compared with the enzymatic methods. The method is also applicable to animal experiment, though it is somewhat inferior to the enzymatic methods in the sensitivity for PALP.

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