QUANTITATIVE DETERMINATION OF PYRIDOXAL-PHOSPHATE BY APOTRYPTOPHANASE OF *ESCHERICHIA COLI*¹

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Pyridoxalphosphate (PALP), occurring as a cofactor in various enzymes involved in amino acid metabolism, has been shown to be usually bound tightly to the apoenzyme. However, evidence has been given that PALP in tryptophanase is readily resolved from the apoenzyme by simply adjusting the enzyme solution to pH 4.7 (1).

Further studies on this enzyme have suggested that the formation of indole depends within a certain limit on the quantity of the PALP added to the apotryptophanase.

This paper deals with the studies on enzymatic determination of PALP using apotryptophanase.

PROCEDURE

1. Preparation of Apotryptophanase

The organism used in these experiments was *Escherichia coli* (K 12 strain) which was kindly furnished by Dr. Kikkawa. One g of dried cells, grown on peptone-bouillon medium at 37° for 24 hours, was ground with 3 g of quartz sand, and the resulting paste was extracted with 50 ml of distilled water at 0° for 2 hours. The insoluble residue was removed by centrifugation at 12,000 rpm for 30 minutes. The supernatant was adjusted to pH 4.7 with 1 M acetic acid and centrifuged at 9,000 rpm for 5 minutes. The precipitate, dissolved in 50 ml of distilled water, was dialyzed overnight at 5° against water. The resulting preparation was used as the apotryptophanase. The resolution of

TABLE I
Resolution of PALP

	Resolution per cent
Crude extract	81.6
Acid fraction	95.7
Acid fraction dialyzed	99.0

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PALP is shown in Table I. When PALP was not completely resolved, an isoelectric procedure was repeated.

2. Preparation of Test Solution for Determination of PALP

In order to extract the PALP fixed firmly to the protein in tissue, the following procedure was used.

One g of the tissue was homogenized in the Potter-Elvehjem homogenizer in 1 ml of 10 per cent metaphosphoric acid, and 9 ml of distilled water was then added. The homogenate was heated for 15 minutes at 80° in a water bath, cooled rapidly in an ice-water mixture, and centrifuged at 12,000 rpm for 15 minutes. The precipitate was redissolved in 10 ml of water and centrifuged. The supernatant and the washing were combined and made up to 20 ml with water. One ml of this solution was used as the test solution.

Since blood contains less PALP, 2 ml of serum was placed in a centrifuge tube containing 2 ml of 2 per cent metaphosphoric acid, kept for 15 minutes at 80°, centrifuged and 2 ml of the supernatant was used as the test solution.

3. Reaction Condition

Since tryptophanase has been demonstrated to be activated by NH_4^+ or K^+ and inhibited by Na^+ (2, 3), the following condition was adopted: 1.0 ml of 1 *M* potassium phosphate buffer (pH 8.0), 0.5 ml of *M*/2 ammonium sulfate, 0.5 ml of 5×10^{-2} *M* glutathione, 1 ml of apotryptophanase (containing ca. 1.0 mg of protein) and 1 ml of test solution (in the case of blood 2 ml was used) were mixed and preincubated for 20 minutes at 37°. Then 1 ml of 10^{-2} *M* tryptophan solution was added and the mixture was incubated for 5 minutes (in the case of blood for 30 minutes). The reaction was stopped by the addition of 1 ml of 30 per cent trichloroacetic acid.

4. Measurement of Indole Produced

Indole was measured according to the method of Goto (4). The reaction mixture was extracted successively with 10 and 5 ml of petroleum ether. To the ether layer, 5 ml of Ehrlich's aldehyde reagent was added and the resulting red color was read photometrically in a 10 mm-cuvette using filter S53. Ehrlich's aldehyde reagent contains 4 g of *p*-dimethylaminobenzaldehyde in a mixture of 80 ml of concentrated HCl and 380 ml of absolute alcohol.

5. Standard Curve of PALP

The known amount of PALP was treated in the same way as described above. In the case of tissues, 1 ml of PALP solution

FIG. 1 Standard Curve of PALP
For assay, 1/20 that of the amounts of PALP given in the Figure were used.

of varying concentrations was mixed with 1 ml of 10 per cent metaphosphoric acid, diluted to 10 ml with water and heated for 15 minutes at 80°. The solution was then diluted to 20 ml. In the case of blood, 2 ml of PALP solution and 2 ml of 2 per cent metaphosphoric acid were used.

Aliquots (1 and 2 ml) of the solution thus obtained were used for tissue and blood respectively. The determination of the indole formed was performed as described above. A standard curve for varying amounts of PALP is shown in Fig. 1. It has been shown that this reaction is inhibited by the accumulation of the indole produced. It is therefore difficult to obtain a straight line for the standard curve. Moreover, since the activity of the apoenzyme for PALP is not always at the same level, it is necessary to plot the standard curve each time the PALP is to be measured. The limits for the measurement by this method were about 0.03–0.3 γ with the recoveries shown in Table II.

TABLE II
Recovery of PALP

PALP solution and liver extract were treated as described in the text and 1 ml of each was used in this reaction

PALP added	Indole formed	Total PALP ^a	PALP recovered	Recovery
γ	γ	γ	γ	per cent
0	8.8	4.9	0	
1.25	10.6	6.1	1.2	96
2.50	12.3	7.25	2.35	94
3.75	13.8	8.4	3.5	93
5.00	15.5	9.65	4.75	95

^a Total PALP was calculated from the standard curve in Fig. 1.

EXPERIMENTS

1. Characteristics of the Apotryptophanase Preparation

The activity of the above-mentioned enzyme preparation was restored by PALP, but not by pyridoxal (PAL) in the presence of ATP (Table III).

TABLE III
Effect of NH_4^+ and PAL + ATP

Addition	M/10 NH_4^+	Indole formed
		γ
—	+	0.3
PALP (10 γ)	—	0.3
PALP (10 γ)	+	30.0
PAL (100 γ) plus ATP (1 mg)	+	0.3

Even after the mixture of PALP and apoprotein was preincubated for 120

minutes at 37°, the indole-forming activity was not decreased, as shown in Table IV, indicating that PALP was not decomposed under this experimental condition. It is therefore concluded that this enzyme preparation contained neither pyridoxalkinase nor PALP-decomposing enzyme.

The presence of pyridoxal (PAL), pyridoxine (PIN) and pyridoxamine (PAM) in the reaction mixture has shown, as given in Table V, to have no effect on indole formation.

TABLE IV
Effect of Preincubation of PALP
0.1 γ PALP was used.

Preincubation time	Indole formed
min	γ
15	4.0
60	4.2
120	4.2

TABLE V
Effect of PAL, PIN and PAM

PALP	Addition	Indole formed
γ	γ	γ
0	—	0.31
0.1	—	4.22
0.1	PAL 10	4.18
0.1	PIN 10	4.14
0.1	PAM 10	4.22
0.2	—	7.46

TABLE VI
PALP Levels of Normal Rabbit Organs
(γ /g)

Organ	1	2	3	4	5	Average
Kidney	3.0	2.7	2.6	2.6	3.0	2.78
Brain	1.6	1.7	1.6	1.5	1.7	1.62
Liver	5.2	4.0	4.5	5.4	3.7	4.56

TABLE VII
PALP Levels in Serum

Experiment	PALP level in serum
	γ /ml
1	0.027
2	0.023
3	0.020
4	0.022
Average	0.023

2. PALP Levels of Various Organs and Blood Serum

FIG. 2 Time Curve of PALP in Serum after an intravenous Injection of PIN, PAL or PALP

(a) PALP levels of various organs of the rabbit, measured by this method, are shown in Table VI.

(b) The average level of PALP in human serum was found to be 0.023 γ /ml. (Table VII). When PIN, PAL or PALP was injected intravenously, the serum PALP levels changed as indicated in Fig. 2.

(c) The PALP levels of the liver of the rabbit changed after thyroidectomy are listed in Table VIII.

Six weeks after operation, the level in the liver tended to decrease.

TABLE VIII
Change in PALP Levels of Rabbit Organs after Thyroidectomy

After operation	3rd week		4th week		5th week		6th week	
	Experiment	Control ^a	Experiment	Control	Experiment	Control	Experiment	Control
	γ/g							
Kidney	2.7	2.7	2.2	2.7	2.8	2.7	2.3	3.5
Brain	1.4	1.4	1.2	1.7	1.6	1.4	1.7	1.4
Liver	4.0	5.0	4.1	4.2	3.2	3.3	3.2	7.0

^a The control animal received a sham operation without thyroidectomy.

DISCUSSION

An enzymatic method for the determination of PALP was reported by Umbreit *et al.* using tyrosine decarboxylase (6). This method is, however, more complicated and expensive. Moreover, the apoenzyme preparation contains pyridoxalkinase, so that PAL is erroneously determined as PALP in the presence of ATP. Further, it is less sensitive because of the use of the Warburg apparatus.

It was observed that the determination of thiaminepyrophosphate with carboxylase gave higher value than the real amount in the presence of thiamine, *i. e.*, so-called "the Ochoa effect" (7). Such effects of PAL, PIN and PAM were, however, not detected with the method described in this paper.

When thiamine or thiaminepyrophosphate is injected intravenously, the thiaminepyrophosphate level of the serum reaches a maximum within 2 minutes and then rapidly decreases (8), while in the case of B₆-group vitamins, the level in the serum took a longer time to reach a maximum and then decreased at a slower rate to the preinjection level (Fig. 2).

Ichihara and Kometani (9) reported previously that thyroidectomized rabbits excreted a much greater amount of kynurenine in the urine after administering tryptophan than the normal animal and the activity of kynurenine- and tyrosine-transaminase in the liver was found to be lower. In order to clarify this, the PALP in the liver of normal and thyroidectomized rabbits was measured by the method described above, but no significant difference was found. However, since the level of PALP in the liver is apt to change even under normal condition, further experiments are required.

SUMMARY

1 A simple method for the determination of PALP was devised using the apotryptophanase of *Escherichia coli* which contains neither pyridoxalkinase nor PALP-decomposing enzyme. The limits for measurement of PALP are roughly 0.03 and 0.3 γ , and PAL, PIN, PAM and ATP did not show any disturb-

ing effects on this method.

2. The PALP levels of human serum were measured, and followed after intravenous administration of PALP, PAL or PIN.

3. The PALP levels of the organs of normal and thyroidectomized rabbits were measured.

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