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STUDIES ON THE INTRACELLULAR CHANGE OF VITAMIN B_6 CONTENT AND KYNURENINE AMINOTRANSFERASE ACTIVITY IN THE VITAMIN B_6 DEFICIENT RAT

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SUMMARY

The author devised a new assay method of kynurenine aminotransferase activity using 3-hydroxykynurenine as substrate, and the enzyme activities in the liver and kidney of vitamin B_6 deficient rats were assayed by this method.

The activity of kynurenine aminotransferase of the kidney did not change distinctly in the mitochondrial fraction by the addition of pyridoxal phosphate *in vitro*, but its activity markedly changed in the supernatant fraction. The enzyme activity in liver was mainly distributed in the mitochondria and the addition of pyridoxal phosphate had no influence on the liver enzyme. In vitamin B_6 deficient rats, the vitamin B_6 content in the supernatant fraction was decreased more markedly than in the mitochondrial fraction. These results suggest that the enzyme activity in the supernatant fraction is influenced by the change of vitamin B_6 content.

INTRODUCTION

Many investigators have studied the role of vitamins as coenzymes in enzymic reactions related to metabolism. Vitamin B_6 is well known to be an important vitamin in tryptophan metabolism as coenzyme of kynurenine aminotransferase (EC. 2. 6. 1. 7) and kynureninase.

Since Lepkovsky[1] reported that an increased amount of xanthurenic acid was excreted in the urine of vitamin B_6 deficient rats after the administration of tryptophan, many investigators have been engaged in the elucidation of the mechanism of increased xanthurenic acid excretion and physiological action of xanthurenic acid excretion and physiological action of xanthurenic acid. Kotake *et al.*[2] found large amounts of xanthurenic acid in urine after the administration of sodium butyrate and tryptophan simultaneously. Wiss *et al.*[3] reported the change of several pyridoxal phosphate dependent enzymes in vitamin B_6 deficiency. Price *et al.*[4] showed the increased urinary excretion of xanthurenic acid in pregnant women. The effect of estrogen on kynurenine aminotransferase was studied by Mason *et al.*[5]

In our laboratory, the mechanism of increased excretion of xanthurenic

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acid was explained in view of the difference of the intracellular distribution of the related enzymes. The author established a new assay method for kynurenine aminotransferase using 3-hydroxykynurenine as substrate and examined the changes in vitamin B_6 and pyridoxal phosphate contents in the subcellular fractions of vitamin B_6 deficient rats.

MATERIALS AND METHODS

Materials

L-3-hydroxykynurenine was purchased from Senju Seiyaku Co., Osaka. Pyridoxal phosphate was obtained from Chugai pharmaceutical Co., Tokyo.

Animals and diet

Young albino rats, weighing 50～60 g, were used in this experiment. They were fed, for 5 or 6 weeks, with the vitamin B_6 deficient diet which contained: vitamin free casein, 3.0 g; dextrose, 3.0 g; salt mixture (obtained from Tanabe pharmaceutical Co., Osaka) 0.5 g; purified salad oil, 1.0 ml; and 1 ml of vitamin mixture free from vitamin B_6 (composed of thiamine, 40 μg; riboflavin, 60 μg; p-aminobenzoic acid, 500 μg; nicotinic acid, 200 μg; calcium pantothenate, 200 μg; biotin, 1 μg; vitamin B_{12}, 0.1 μg; folic acid, 2.5 μg; inositol, 1.5 mg; choline hydrochloride, 5 mg; vitamin A, 300 IU and vitamin D, 30 IU). The control animals were fed with the same diet to which 100 μg of vitamin B_6 was added. The animals were used for the experiment after the examination of xanthurenic acid excretion in urine.

Fractionation of cell components

Rats were killed by decapitation and exsanguination. The liver and kidney were excised immediately and placed in crushed ice. These tissues were immersed quickly in 4 volumes of ice cold 0.25 M sucrose and were homogenized in a teflon homogenizer. Subcellular fractionation was performed by Hogeboom's method with slight modifications.[6] The homogenates were centrifuged for 10 min. at 700 g in a refrigerated centrifuge. The supernatant was centrifuged again for 15 min. at 9,000 g. The opalescent supernatant was preserved in the cold state. The pellet was suspended in 0.25 M sucrose, and centrifugation was repeated twice. These precipitates were regarded as mitochondria. The activity of kynurenine aminotransferase and vitamin B_6 level of the supernatant and mitochondria were measured.

Assay methods

Pyridoxal phosphate was determined by the method of Wada *et al.*[7] using apotryptophanase. The vitamin B_6 content was assayed according to Atkin's method.[8] Three and a half volumes of 1/11 N sulfuric acid were added to the

material and hydrolyzed for 1 hour at 15 1b in an autoclave. The protein was removed by the addition of 0.5 volume of 10% sodium tungstate. Then, the filtrated opalescent supernatant was diluted to assay vitamin B_6 by bioassay using *Saccharomyces carsbergenis* 4221. The mitochondrial fraction was treated for 10 min. at 10 KC by a sonic oscillator before the determination of vitamin B_6 content and pyridoxal phosphate level. The protein concentration was determined by biuret method.

RESULTS

Assay method of kynurenine aminotransferase activity using 3-hydroxykynurenine as substrate

A new assay method for kynurenine aminotransferase activity was developed as follows. The reaction mixture contained, in a total volume of 0.5 ml, the following amounts of substances: L-3-hydroxykynurenine, 2 μmoles; α-ketoglutarate, 3 μmoles; phyridoxal phosphate, 25 μg; potassium phosphate buffer, 1/10 M, pH 6.5, 0.1 ml; and enzyme solution, 0.1 ml. The incubation was carried out at 37°C for 30 min. The reaction was stopped by the addition of 1.5 ml of methanol. After centrifugation, 1 ml of 0.8 M trisaminomethane-maleate buffer, pH 8.5 was mixed with 0.5 ml of the supernatant. Then 0.05 ml of 1.7% ferric ammonium sulfate was added. The optical density of the color formed was measured at 610 mμ. In this assay method, xanthurenic acid was determined by the method of Wachstein *et al.*[9] with a modification. Protein concentration was determined by the method described previously. The activity was expressed in units, which corresponded to the mμmoles of xanthurenic acid formed per hour per mg protein.

The relation between the activity of kynurenine aminotransferase and incubation time

In order to examine the time cource of enzyme activity, the reaction mixture was incubated for various periods. As shown in Fig. 1 a, the increase in xanthurenic acid formation was linear up to 40 min. during the incubation. The enzyme activity was also linear up to 6 mg protein in enzyme concentration (Fig. 1 b).

Effect of pH on the activity of kynurenine aminotransferase

Kynurenine aminotransferase was assayed as various pH. As shown in Fig. 2, the optimum pH for the enzyme activity was found to be about 6. 5. This value was almost the same as that of Knox's data.

Effect of potassium phosphate buffer on the color reaction of xanthurenic acid.

The color substance was produced by the addition of trisaminomethane-

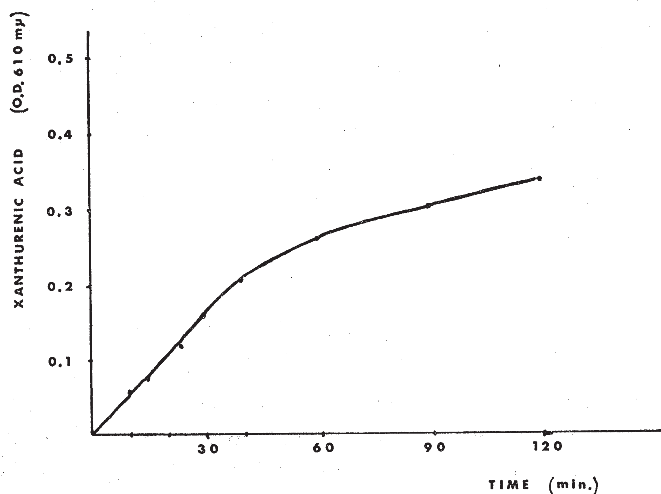

FIG. 1 a. Time course of kynurenine aminotransferase

The activity of Kynurenine aminotransferase was assayed. The component of the reaction mixture is shown in "Assay method using 3-hydroxykynurenine". Enzyme solution: Kidney homogenate.

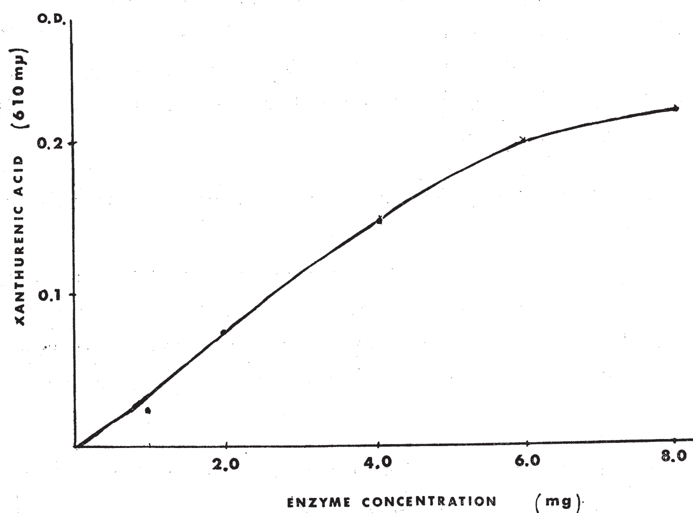

FIG. 1 b. The relation between the activity of kynurenine aminotransferase and enzyme concentration.

The assay method used in this experiment, is shown in "Assay method using 3-hydroxykynurenine".

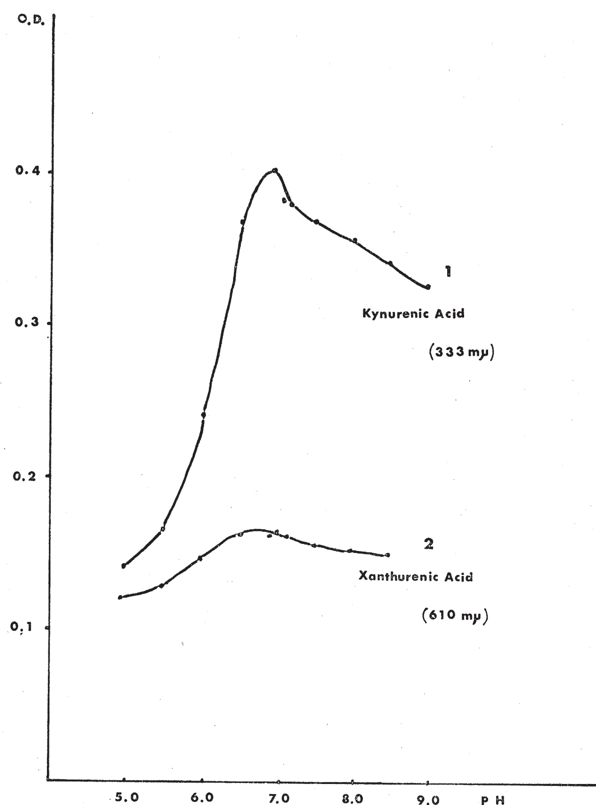

FIG. 2. Effect of pH on the activity of kynurenine aminotransferase
Curve 1 shows the activity of kynurenine aminotransferase which was assayed by Knox's method. The reaction mixture for this enzyme assay contained the following components in 0.5 ml; 1 μmole of L-kynurenine, 3 μmoles of α-ketoglutarate, 25 μg of pyridoxal phosphate, 10 μmoles of potassium phosphate buffer pH 6.8 and enzyme solution. Curve 2 shows kynurenine aminotransferase activity assayed by the method using 3-hydroxykynurenine as substrate.

maleate buffer and ferric ammonium sulfate. Optimal pH value of trisaminomethane-maleate buffer was examined using 10 μg of authentic xanthurenic acid dissolved in distilled water. As shown in Fig. 3, pH 7.5 was optimum for this color reaction. However, when xanthurenic acid was dissolved in phosphate buffer, this color reaction was markedly inhibited at this pH value. Therefore, xanthurenic acid was dissolved in 0.02 M phosphate buffer of various pH and then the color reaction was again examined. The optimum pH value of trisaminomethane-maleate buffer shifted to pH 8.5 in all cases.

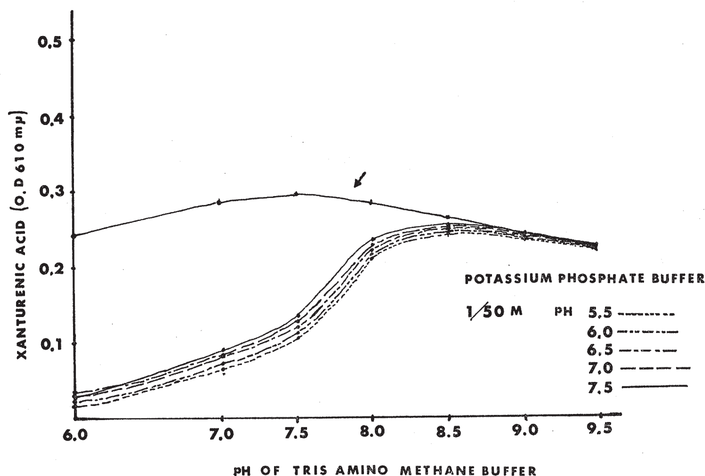

FIG. 3. The effect of potassium phosphate buffer on the color reaction of xanthurenic acid.

When xanthurenic acid was dissolved in water, the optimum pH of trisaminomethane-maleate buffer was 7.5 as indicated by an arrow. However, when xanthurenic acid was dissolved in phosphate buffer the value shifted to pH 8.5. The condition for colorimetric determination was described in the text.

Changes in kynurenine aminotransferase activity and the effect of pyridoxal phosphate on the enzyme activity in vitamin B_6 *deficient rats.*

Tables 1a and 1b show the change in kynurenine aminotransferase activity in vitamin B_6 deficient rats. The enzyme activity was measured by the assay method using 3-hydroxykynurenine as substrate. The difference in activity between the control and deficient animals was slight in the kidney mitochondrial fraction. The effect of pyridoxal phosphate addition was slightly observed in both cases. On the contrary, the activity of kynurenine aminotransferase decreased in the kidney supernatant fraction of deficient animals. The enzyme activity in the supernatant fraction was elevated in both control and deficient rats by the addition of pyridoxal phosphate. The elevation of enzyme activity suggests that the supernatant enzyme binds loosely with pyridoxal phosphate.

In the liver, the specific activity of kynurenine aminotransferase was relatively low and mainly localized in the mitochondria. Its activity decreased in vitamin B_6 deficient rats and the effect of pyridoxal phosphate addition was not observed in this enzyme.

Changes in vitamin B_6 *content in vitamin* B_6 *deficient rats*

Table 2 shows the intracellular distribution of vitamin B_6 in the liver and

TABLE 1. a and 1. b Kynurenine aminotransferase activity in vitamin B_6 deficient rats

Fraction	Kynurenine aminotransferase activity			
	Kidney Supernatant		Kidney Mitochondria	
PAL-P 25 μg	−	+	−	+
Control	8.2±1.6	51.1±11.5	39.0±11.0	50.7± 8.9
Vitamin B_6 Deficiency	5.1±2.5	39.9± 7.4	37.9±10.0	56.4±10.5

PAL-P: Pyridoxal phosphate mμmoles/h/mgprotein

Fraction	Kynurenine aminotransferase activity			
	Liver Supernatant		Liver Mitochondria	
PAL-P 25 μg	−	+	−	+
Control	3.2±1.0	3.5±1.5	20.8±5.5	27.0±9.0
Vitamin B_6 Deficiency	2.7±0.9	3.3±1.0	10.4±6.1	12.5±1.8

PAL-P: Pyridoxal phosphate mμmoles/h/mgprotein

The cell fractionation was performed according to Hogeboom's method. The activity of kynurenine aminotransferase was assayed by the method using 3-hydroxykynurenine.

TABLE 2. Vitamin B_6 content in vitamin B_6 deficient rats

Tissue	Group	Vitamin B_6 mμg/mgprotein	
		Supernatant	Mitochondria
Liver	Control	15.47±3.24	13.59±4.38
	Vitamin B_6 Deficiency	3.80±1.28	6.26±1.66
Kidney	Control	22.07±7.65	17.44±5.51
	Vitamin B_6 Deficiency	4.61±2.47	7.58±2.03

The fractionation of cell components was the same as in Table 1. Total vitamin B_6 was determined by Atkin's method using *Saccharomices carsbergensis* 4221.

kidney of deficient animals. Vitamin B_6 content decreased in the supernatant more markedly than in the mitochondrial fraction in both tissues.

Vitamin B_6 *content in the cell components of normal rats*

A difference in vitamin B_6 content could not be observed between the supernatant and the mitochondrial fraction in both liver and kidney.

TABLE 3. Vitamin B_6 content in cell components of normal rats.

Tissue	Vitamin B_6 mμg/mgprotein	
	Supernatant	Mitochondria
Liver	16.3	14.7
Kidney	19.7	20.8

Vitamin B_6 content was measured by microbiological assay using *Saccharomyces carsbergensis* 4221.

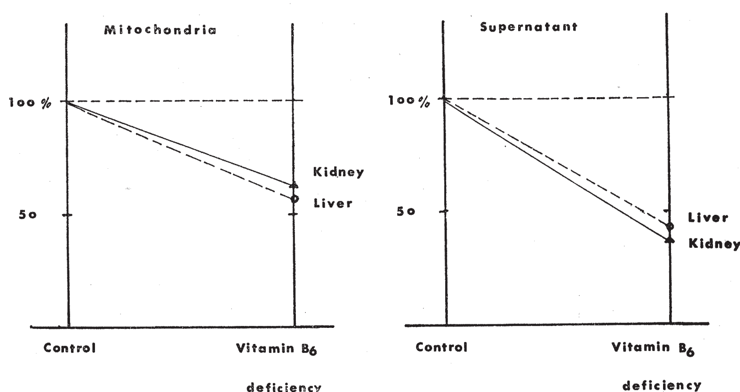

FIG. 4. Changes in pyridoxal phosphate content in vitamin B_6 deficient rats.

Pyridoxal phosphate was determined by the method of Wada *et al.* This data shows the decrease in pyridoxal phosphate content against the control as 100%.

Changes in pyridoxal phosphate content in vitamin B_6 *deficient rats*

As is illustrated in Fig. 4, the tendency for decrease in pyridoxal phosphate was the same as that of vitamin B_6 content measured by microbiological assay.

DISCUSSION

Recently, Wiss[10] reported that both kynurenine and 3-hydroxykynurenine were catalyzed by kynurenine aminotransferase. The activity of kynurenine aminotransferase has been assayed by Knox's method[11] using kynurenine as substrate. In this method, the activity was measured by determining kynurenic acid formation. In the present report, the activity of kynurenine aminotransferase was assayed by the method using 3-hydroxykynurenine as substrate for the purpose of examining in more detail the biosynthesis of xanthurenic acid.

Xanthurenic acid was detected, in this assay method, by the specific color reaction which occurred between xanthurenic acid and ferric ion.

The observations reported here are based on the results of kynurenine aminotransferase activity assayed by the method using 3-hydroxykynurenine as substrate. In the kidney of vitamin B_6 deficient rats, the activity of kynurenine aminotransferase decreased in the supernatant fraction, while the activity of mitochondrial kynurenine aminotransferase remained active. This result showed the same tendency as that in Ogasawara's experiment[12] in which the enzyme activity was assayed by Knox's method.

In addtion, kynurenine aminotransferase in the liver was studied, which had not been clearly observed. The activity of hepatic enzyme was weak especially in the supernatant fraction. Kynurenine aminotransferase in the liver was mainly localized in the mitochondrial fraction.

In the experiment which observed the activity of kynurenine aminotransferase with or without the addition of pyridoxal phosphate *in vitro*, the mitochondrial enzyme was not so distinctly influenced by the addition of pyridoxal phosphate. On the contrary, pyridoxal phosphate had a potent effect upon the supernatant enzyme of the kidney.

The content of vitamin B_6 in liver and kidney was measured in vitamin B_6 deficient rats. In this experiment, the content of vitamin B_6 decreased in the supernatant fraction more markedly than in the mitochondrial fraction.

By due consideration of all these findings, it is speculated that kynurenine aminotransferase in the supernatant fraction may be controlled by the transition of vitamin B_6 content, and that the mitochondrial kynurenine aminotransferase may principally take part in the metabolism of 3-hydroxykynurenine under the protection of the mitochondrial membrane in the state of vitamin B_6 depletion.

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