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Kynurenine metabolism in vitamin-B-6-deficient rat liver after tryptophan injection

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1. Tryptophan contents of liver, serum and kidney were determined in normal and vitamin-B-6-deficient rats after tryptophan injection. Tryptophan contents of normal and B-6-deficient liver were different, but not those in serum and kidney. 2. Both kynurenine and 3-hydroxykynurenine accumulated in B-6-deficient liver more than in the normal. The 3-hydroxykynurenine contents after tryptophan injection (30mg/100g body wt.) increased to 1380nmol/g of liver at 1–1.5h, a value sufficient to produce xanthurenate, in view of the K_m value of kynurenine aminotransferase. 3. The enzymes metabolizing kynurenine were assayed at various times after tryptophan injection. The activity of kynureninase holoenzyme in B-6-deficient liver was much decreased, but the activity of total enzyme was not changed. It appeared that a high dose of tryptophan in B-6-deficient rats could cause a greater deficiency of pyridoxal 5-phosphate. 4. Tryptophan metabolism in B-6-deficient rat liver after tryptophan administration is discussed.

In tryptophan metabolism in rat liver, kynurenine is an important intermediate and is metabolized to 3-hydroxykynurenine by kynurenine 3-mono-oxygenase (EC 1.14.13.9), to anthranilate by kynureninase (EC 3.7.1.3), or to kynurenate by kynurenine aminotransferase (EC 2.6.1.7). 3-Hydroxykynurenine is metabolized to 3-hydroxyanthranilate by kynureninase, and this route is a main pathway of tryptophan oxidation (Nishizuka & Hayaishi, 1963). 3-Hydroxykynurenine can be also converted into xanthurenate by kynurenine aminotransferase. Kynureninase and kynurenine aminotransferase are both vitamin-B-6-dependent enzymes. The products that are formed by these three enzymes have been reported to affect fatty acid synthesis (Barth *et al.*, 1973), some enzymes (Hashimoto *et al.*, 1971; Shibata, 1978; Katsos *et al.*, 1981; Karawya *et al.*, 1981), and protein synthesis (Noto & Okamoto, 1978; Sidransky *et al.*, 1980; Matsushima *et al.*, 1982). The amount of xanthurenate in urine is thought to be an index of vitamin B-6 deficiency (Yeh & Brown, 1977; Brown, 1981).

When tryptophan was administered to B-6-deficient mammals or oral-contraceptive users, the excretion of kynurenine, 3-hydroxykynurenine, kynurenate, and especially xanthurenate in urine increased greatly compared with normal subjects. It has been reported (Ogasawara *et al.*, 1962) that in the B-6-deficient rat kynureninase showed a low activity, whereas activity of mitochondrial kynurenine aminotransferase remained high, and therefore the excretion of xanthurenate was increased. It was also demonstrated that in oral-contraceptive users kynureninase was competitively inhibited by oestrogen metabolites (Bender & Wynick, 1981), and there was no evidence of any significant effect of oestrogen on vitamin B-6 content (Bender *et al.*, 1982).

We have highly purified the above three enzymes from rat liver and elucidated their properties (Nisimoto *et al.*, 1975; Nisimoto, 1976; Takeuchi *et al.*, 1980, 1981, 1983) The K_m value of kynurenine 3-mono-oxygenase for kynurenine is 25μM. K_m values of kynureninase for kynurenine and 3-hydroxykinurenine are 240μM and 13μM respectively. However, kynurenine aminotransferase has very high K_m values, 4.3mM for kynurenine and 5.7mM for 3-hydroxykynurenine. Therefore, if xanthurenate and kynurenate are synthesized in liver and excreted in urine, large amounts of kynurenine and 3-hydroxykynurenine must be accumulated in liver. No report has elucidated to date the contents of tryptophan metabolites in liver after tryptophan loading. Only the amounts of metabolites in urine and plasma are known.

The contents of tryptophan, kynurenine and 3-hydroxykynurenine were determined in the present study, and the activities of kynurenine-

metabolizing enzymes were measured, to elucidate the enzymology of tryptophan metabolism in B-6-deficient rat liver after tryptophan administration.

Materials and methods

Chemicals

Kynurenine sulphate, NADPH, xanthurenic acid and 3-hydroxyanthranilic acid were purchased from Sigma (St. Louis, MO, U.S.A.), and heparin was from Novo Industry A/S, Bagsværd, Denmark. Tryptophan, anthranilic acid, 3-hydroxykynurenine, kynurenic acid and pyridoxal 5-phosphate were purchased from Wako Pure Chemicals Industries (Osaka, Japan). The other chemicals were of reagent grade.

Animals and treatment

Male Wistar rats (4 weeks old) were purchased from Nakashima Experimental Animal Laboratory (Kasugai, Japan) and fed *ad libitum* with vitamin-B-6-deficient diet (Oriental Yeast Co., Tokyo, Japan) for 8 weeks to induce B-6 deficiency. For normal rats we purchased 12-week-old rats, which were fed with normal diet.

The rats received an intraperitoneal injection of tryptophan of either 10 or 30mg/100 g body wt. in 0.9% NaCl. They were killed by decapitation at various times and the blood was collected. For the determination of contents of tryptophan metabolites, liver and kidney were immediately removed within 1 min of the death of the animals, frozen in liquid N_2 and stored at $-80°C$. The values obtained were not corrected for the amount present in the blood. It was reported (Hohorst *et al.*, 1959; Holzer *et al.*, 1956) that the content of blood in liver after decapitation was 2.3–7.6% (v/w).

Determination of tryptophan metabolites

When the contents of tryptophan metabolites and the enzyme activity were determined fluorimetrically, 1 μM-quinine sulphate was used as a standard.

For the determination of tryptophan, frozen liver or kidney (0.2–0.6g) was homogenized with 5 ml of 12% (w/v) trichloroacetic acid and then centrifuged at 10000*g* for 15 min at 2°C. The tryptophan contents of supernatant and serum were determined by the method of Bloxam & Warren (1974) (see also Denkla & Dewey, 1967).

For the determination of kynurenine, frozen liver was powdered with a pestle and mortar under liquid N_2. To the powder (1.5–2.5g) 1ml of 30% (w/v) $HClO_4$ containing 50mM-Tiron (sodium 1,2-dihydroxybenzene-3,5-disulphonate) and an adequate volume of water were added to make the total volume 10 ml. The suspension was homogenized and centrifuged at 10000*g* for 7 min at 2°C. The content of kynurenine in 7.5 ml of supernatant was determined by the method of Joseph & Risby (1975).

3-Hydroxykynurenine was determined by a modification of the method of Turner (1973), who used *Neurospora* or *Pseudomonas* kynureninase for the enzymic assay. However, purified rat liver kynureninase was used in the present study. The enzyme was purified as far as the hydroxyapatite step of the method of Takeuchi *et al.* (1980), and its specific activity was 3800 nmol/min per mg of protein. Since rat liver enzyme is a hydroxykynureninase-type enzyme and the reaction rate is very rapid, it is suitable for the determination of 3-hydroxykynurenine. The method is based on the enzymic conversion of 3-hydroxykynurenine and kynurenine into 3-hydroxyanthranilate and anthranilate respectively, and the selective measurement of anthranilate fluorescence at pH2.7.

Frozen liver was powdered in liquid N_2. The powder (2–3g) was homogenized with 2 vol. of 6% (w/v) $HClO_4$ and centrifuged at 10000*g* for 10 min at 2°C. The supernatant was neutralized with 4M-K_2CO_3 and centrifuged to remove the precipitate. The assay mixture contained the following: 90μmol of Tris/HCl, 7 nmol of pyridoxal 5-phosphate, neutralized sample and 13μg of purified rat liver kynureninase, in a total volume of 1.0ml, final pH8.4. The assay mixture except for the enzyme was preincubated for 3min at 37°C, and then the enzyme was added to start the reaction. The mixture was incubated for 5min at 37°C, and 0.11 ml of 2M-HCl was added to stop the reaction. The 3-hydroxyanthranilate and anthranilate produced were extracted with 4 ml of ethyl acetate. The fluorescence of the ethyl acetate layer was measured with excitation of 337 nm and emission monitored at 415nm. A 1ml portion of the ethyl acetate layer was evaporated for 2 h at room temperature, and the residue was dissolved in 1ml of 0.1M-glycine/NaCl/HCl, pH 2.7. The fluorescence of this solution was measured with excitation at 327nm and emission monitored at 420nm. The fluorescence of 3-hydroxyanthranilate is negligible at pH2.7, and anthranilate is specifically detected. The fluorescence intensity of 3-hydroxyanthranilate at 415nm is determined by subtracting the contribution of anthranilate at 415nm that can be estimated from the fluorescence intensity at 420nm.

The contents of 3-hydroxyanthranilate and anthranilate in liver were subtracted by determining them in an assay mixture to which 2M-HCl was added before addition of the enzyme. Amounts of 3-hydroxykynurenine up to 50μM in the assay mixture were quantitatively converted into 3-hydroxyanthranilate by rat liver kynurenin-

ase within 5 min. However, the quantitative measurement of kynurenine by this method was not appropriate, because of the low rate and incompleteness of conversion into anthranilate. In the presence of up to 100 μM-kynurenine in the assay mixture, 3-hydroxykynurenine (0–50 μM) could be determined quantitatively.

Enzyme assay

Rats injected with tryptophan (30 mg/100 g body wt.) were killed by decapitation at various times. Blood was removed from liver by perfusion *in situ* with an ice-cold saline/heparin mixture (Stowell & Mørland, 1983). Liver weighing 2–2.5 g was immediately homogenized with 3 vol. of 0.25 M-sucrose containing 10 mM-triethanolamine/HCl, 1 mM-dithiothreitol and 1 mM-EDTA, pH 7.4, and fractionated by the method of de Duve *et al.* (1955). Mitochondrial fraction was suspended to 4 ml of homogenizing buffer and used for the assay of kynurenine 3-mono-oxygenase (Nisimoto *et al.*, 1975) and kynurenine aminotransferase (Takeuchi *et al.*, 1983). Soluble fraction was used for the assay of kynureninase (Takeuchi *et al.*, 1980). The activities of enzymes were expressed as nmol of products/min per g of liver.

Results

Changes in tryptophan contents

Fig. 1(*a*) shows the changes in tryptophan contents in serum, kidney and liver after tryptophan injection (10 mg/100 g body wt.). Tryptophan contents in normal rat serum, kidney and liver at zero time were 119 ± 11.1 nmol/ml, 57.6 ± 6.2 nmol/g and 37.0 ± 2.8 nmol/g respectively. The value obtained in liver was lower than that found by Denkla & Dewey (1967), and slightly higher than those found by Badawy *et al.* (1980, 1981). In vitamin-B-6-deficient rats, tryptophan contents were 105 ± 17 nmol/ml, 54.3 ± 12.4 nmol/g and 32.9 ± 3.0 nmol/g respectively. Thus the tryptophan content in liver only of B-6-deficient rats was significantly lower ($P < 0.01$). After tryptophan injection, it was very rapidly absorbed, and the contents reached a maximum at 0.5 h in all tissues. No significant difference in tryptophan contents between normal and B-6-deficient rats was observed in serum and kidney, although the values in B-6-deficient rats tended to be slightly higher. However, the contents in B-6-deficient liver were definitely higher than those of normal rats within 2 h (maximally $P < 0.02$). Tryptophan contents returned to the slightly lower original value within 2 h in normal liver and 3 h in B-6-deficient liver, but failed to do so within 3 h in serum. The decreasing order of the contents was serum > kidney > liver in both normal and B-6-deficient rats.

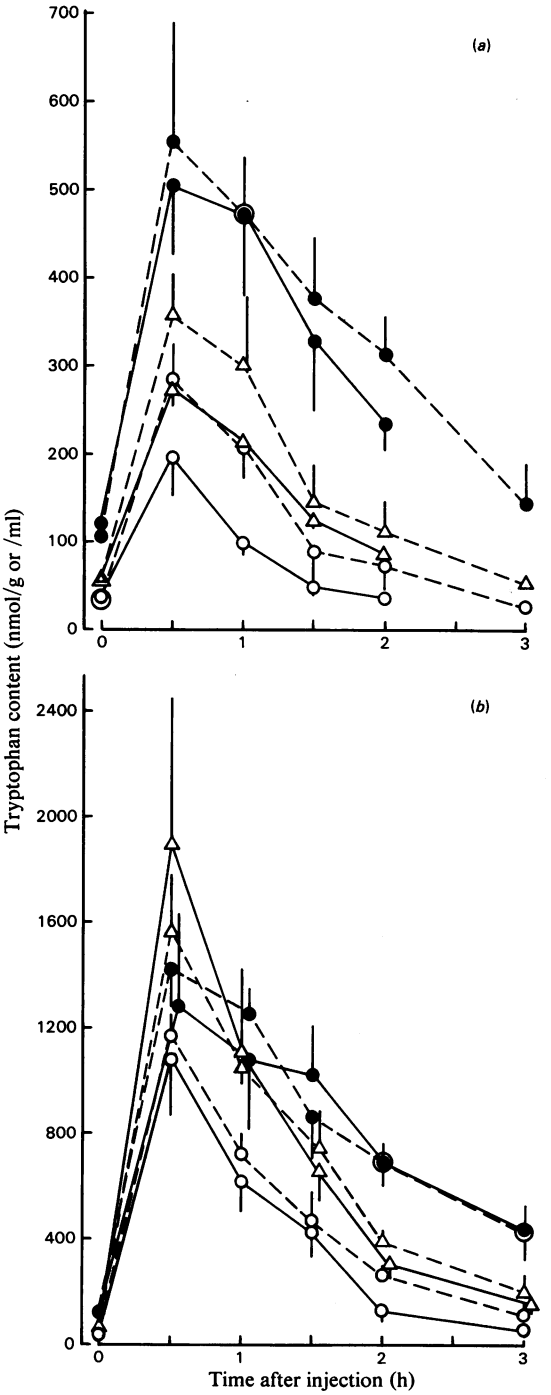

Fig. 1. *Changes in tryptophan contents in serum, kidney and liver after tryptophan injection*

Tryptophan at (*a*) 10 and (*b*) 30 mg/100 g body wt. was injected intraperitoneally into normal (——) and vitamin-B-6-deficient (– – – –) rats, which were killed at the indicated time intervals. Tryptophan contents in serum (●), kidney (△) and liver (○) were determined as described in the text. Results are means ± S.D. for four to five rats.

The changes of tryptophan contents in serum, kidney and liver after the injection of tryptophan (30mg/100g) are shown in Fig. 1(*b*). The tryptophan contents also reach a maximum at 0.5h in all tissues. There was no significant difference in all tissues between normal and B-6-deficient rats within 1.5h. Only in the liver was a significant difference ($P<0.001$) observed after 2h, at which time the tryptophan content was similar to those obtained at 0.5–1h after tryptophan injection (10mg/100g). The value obtained in kidney at 0.5h was the highest, and then rapidly fell below the serum content. The content in serum was 3-fold higher than after the lower tryptophan dose. Those in kidney and liver, however, showed a higher increase in terms of the tryptophan dose. Tryptophan contents in liver were similar to those in serum, although they showed a more rapid decrease than in serum.

Contents of kynurenine and 3-hydroxykynurenine

The contents of kynurenine at zero time were 1.99 ± 0.43nmol/g in normal liver and 1.67 ± 0.25nmol/g in B-6-deficient liver, and showed the same tendency as for tryptophan. However, 3-hydroxykynurenine could not be detected in either rat group by this method. Kynurenine content of normal liver was lower than reported previously (Bender & McCreanor, 1982). The changes in kynurenine and 3-hydroxykynurenine contents in liver after tryptophan injection (10mg/100g) are shown in Fig. 2(*a*). Kynurenine in normal liver reached a maximum of 27.6 ± 4.4nmol/g at 0.5h, just as with tryptophan, and then rapidly decreased. In B-6-deficient liver, it reached a peak after 1 h at 71.3 ± 15.9nmol/g, which was 2.6 times higher than the maximum value in normal liver. 3-Hydroxykynurenine in normal liver changed roughly parallel to kynurenine, and its content was only 1.20 ± 0.67nmol/g maximally. In B-6-deficient liver, however, it accumulated in parallel with kynurenine until 1 h, and then decreased more slowly. The maximum value was 520 ± 47.7nmol/g at 1h. Although the ratios of 3-hydroxykynurenine to kynurenine were constant (approx. 0.05) in normal rats, those in B-6-deficient rats showed a constant value of 7.4 only at 0.5 and 1h.

The changes in kynurenine and 3-hydroxykynurenine contents with a tryptophan injection (30mg/100g) are shown in Fig. 2(*b*). Kynurenine in normal liver accumulated rapidly until 0.5h and then gradually, reaching the maximal value of 77.0 ± 16.9nmol/g at 1.5h. Kynurenine in B-6-deficient liver accumulated more rapidly and reached a peak at 1–1.5h. The kynurenine content at 1.5 h was 283 ± 61.8nmol/g, which was 3.7 times the maximum value in normal liver. Compared with effects of a tryptophan injection of 10mg/100g, the maximal kynurenine content was 2.8 times higher in normal liver and 4.0 times

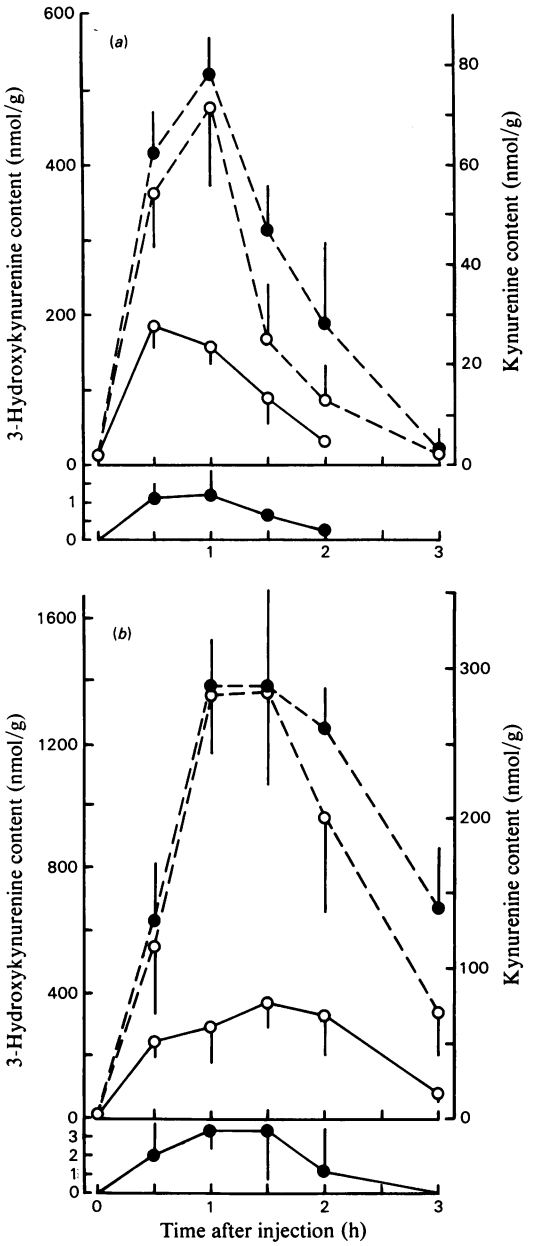

Fig. 2. *Changes in kynurenine and 3-hydroxykynurenine contents in liver after tryptophan injection*
The dose of tryptophan in normal (——) and vitamin-B-6-deficient (– – –) rats was (*a*) 10 or (*b*) 30mg/100g body wt. Kynurenine (○) was determined by the method of Joseph & Risby (1975), and 3-hydroxykynurenine (●) was determined with rat liver kynureninase as described in the text. Results are means ±S.D. for four to five rats.

higher in B-6-deficient liver. The accumulation of kynurenine in B-6-deficient liver did not parallel the tryptophan dose and was greater than in normal liver.

The changes of 3-hydroxykynurenine contents in normal liver were roughly proportional to those of kynurenine, and the ratios of 3-hydroxykynurenine to kynurenine were 0.04–0.05. The maximal content of 3-hydroxykynurenine was only 3.37 ± 2.70 nmol/g, or 2.8 times that obtained after injection of 10 mg of tryptophan/100 g. 3-Hydroxykynurenine in B-6-deficient liver accumulated proportionally to kynurenine contents until 1.5 h and decreased more slowly than kynurenine, just as with a tryptophan injection of 10 mg/100 g. The content of 3-hydroxykynurenine reached a peak at 1–1.5 h; its maximal value was 1380 ± 152 nmol/g, which was 2.7 times that with a tryptophan dose of 10 mg/100 g. The ratios of 3-hydroxykynurenine to kynurenine were approximately constant (4.9–5.5) until 1.5 h.

Activities of kynurenine 3-mono-oxygenase

The activity of kynurenine 3-mono-oxygenase in normal liver mitochondria showed no change within 3 h, as shown in Table 1. However, in B-6-deficient liver it displayed a rapid decrease to 52% of the original value at 1 h, and recovered to 79% at 2 h. The activity of the enzyme at zero time in B-6-deficient liver was 1.9 times that in normal liver.

Activities of kynurenine aminotransferase

The total activity of kynurenine aminotransferase at zero time in B-6-deficient liver was 70% of that in normal liver, and the degree of saturation of pyridoxal 5-phosphate was only about half that in normal liver (Table 1). After tryptophan injection, there was no significant change in activity in normal liver, but an apparent decrease in total enzyme and holoenzyme activities was observed in B-6-deficient liver. The degree of saturation slightly decreased with time. The activity of holoenzyme at 3 h in B-6-deficient liver was 18% of that in normal liver, and 53% of that at zero time in B-6-deficient liver.

Activities of kynureninase

In the normal liver, the total enzyme activity significantly increased after 2 h, whereas holoenzyme activity did so only at 3 h (Table 2). The degree of saturation of pyridoxal 5-phosphate was 53–59% and nearly constant. In B-6-deficient liver, the activities of total enzyme and holoenzyme were very low compared with those in normal rats. The degree of saturation was only 9.5%, much lower than that of kynurenine aminotransferase. The holoenzyme activity in B-6-deficient liver very significantly decreased to 1% of total activity after tryptophan injection, although the activity of total enzyme showed no change.

Discussion

A large decline in kynureninase holoenzyme activity was reported to cause the accumulation and the excretion of kynurenine metabolites (Bender *et al.*, 1982; Brown, 1981; De Antoni *et al.*, 1981; Yeh & Brown, 1977; Rose & Braidman, 1971; Ogasawara *et al.*, 1962). In fact, the kynurenine contents in B-6-deficient liver were 2.6–3.6 times higher than in normal liver, and 3-hydroxykynurenine contents were approx. 410

Table 1. *Activities of kynurenine 3-mono-oxygenase and kynurenine aminotransferase after tryptophan injection*

Tryptophan (30 mg/100 g body wt.) was injected intraperitoneally into normal and vitamin-B-6-deficient rats, which were killed at the specified time intervals. In the mitochondrial fraction of liver, the activity of kynurenine 3-mono-oxygenase was measured, and that of kynurenine aminotransferase was measured in the presence (total activity) and in the absence (holoenzyme activity) of pyridoxal 5-phosphate (PLP). Results are means ± S.D. for the numbers of rats shown. Significances of differences between the value for the tryptophan-injected group and that for the non-injected group are denoted by $*P<0.05$, $**P<0.02$, $***P<0.001$ (Student's t test).

	Time after injection (h)	No. of rats	Activity of kynurenine 3-mono-oxygenase (nmol/min per g of liver)	Activity of kynurenine aminotransferase (nmol/min per g of liver)		Saturation with PLP (%)
				+PLP	No PLP	
Normal rats	0	8	141 ± 35.9	159 ± 38.1	83.7 ± 25.9	51
	1	8	144 ± 48.2	148 ± 34.5	82.4 ± 21.9	56
	2	8	144 ± 30.6	156 ± 45.0	78.4 ± 25.1	50
	3	8	145 ± 16.8	171 ± 25.4	97.8 ± 16.3	57
B-6-deficient rats	0	9	270 ± 42.9	112 ± 26.2	32.4 ± 14.7	29
	1	8	156 ± 38.9***	86.7 ± 21.1**	23.5 ± 10.7	27
	2	6	212 ± 41.7**	77.9 ± 20.8**	20.4 ± 11.3	26
	3	8	205 ± 70.4*	76.3 ± 34.2*	17.2 ± 12.9*	23

Table 2. *Kynureninase activities after tryptophan injection*

The dose of tryptophan was described in Table 1. The kynureninase activities in soluble fraction of liver were measured in the presence (total activity) and in the absence (holoenzyme activity) of pyridoxal 5-phosphate (PLP). Results are means ± S.D. for the numbers of rats shown. Significances of differences between the values for the tryptophan-injected group and those of the non-injected group are denoted by $*P<0.05$ or $**P<0.001$ (Student's t test).

	Time after injection (h)	No. of rats	Activity of kynureninase (nmol/min per g of liver) +PLP	Activity of kynureninase (nmol/min per g of liver) No PLP	Saturation with PLP (%)
Normal rats	0	8	58.7 ± 5.76	34.5 ± 5.72	59
	1	8	56.8 ± 9.75	30.1 ± 6.11	53
	2	8	67.5 ± 8.26*	36.4 ± 5.80	54
	3	8	71.2 ± 4.81**	40.4 ± 2.81*	57
B-6-deficient rats	0	9	32.8 ± 6.86	3.13 ± 1.57	9.5
	1	8	30.5 ± 6.49	0.321 ± 0.042**	1.1
	2	6	36.6 ± 10.8	0.460 ± 0.251**	1.3
	3	8	33.4 ± 6.35	0.391 ± 0.085**	1.2

times higher. The maximal content of 3-hydroxykynurenine was 1380 nmol/g; if the water content in tissue was 70%, its concentration was estimated to be about 2 mM. Although kynurenine aminotransferase, which is mainly present in the inner membrane of mitochondria (Okamoto & Hayaishi, 1970), had a very high K_m value of 5.7 mM for 3-hydroxykynurenine, the elevated concentration of 3-hydroxykynurenine and the content of holoenzyme of kynurenine aminotransferase seemed to be sufficient to produce xanthurenate at a relatively high rate. However, the contents of kynurenine in B-6-deficient liver were very low compared with those of 3-hydroxykynurenine, and the production rate of kynurenate by kynurenine aminotransferase in B-6-deficient liver seemed to be relatively low. Still, in normal liver, the accumulation of 3-hydroxykynurenine seemed insufficient to produce xanthurenate. The small amount of xanthurenate excreted in urine of normal rats (Yeh & Brown, 1977) might be produced in extrahepatic tissues. The flux of kynurenine metabolism after tryptophan injection, which was estimated from the metabolite contents and the activities of related enzymes determined in this experiment, was approximately similar to the pattern of urinary excretion of tryptophan metabolites in both normal and B-6-deficient rats (Yeh & Brown, 1977).

The low contents of holoenzyme and total enzyme of kynureninase in B-6-deficient liver were identical with the stability and the contents of pyridoxal 5-phosphate-dependent enzymes in B-6-deficient rat reported by Hunter & Harper (1976, 1977). The tryptophan injection (30 mg/100 g) caused a greater decrease in the holoenzyme activity of kynureninase in B-6-deficient liver, but the total enzyme activity was not changed. Tryptophan intermediates, except for 3-hydroxyanthranilate, cannot affect the activity of kynureninase (Takeuchi *et al.*, 1981; Soda & Tanizawa, 1979). Therefore tryphophan injection into B-6-deficient rats seemed to cause a greater decrease in pyridoxal 5-phosphate content. Meisler & Thanassi (1980) reported that pyridoxal kinase might play a role in regulating the tissue contents of phosphorylated forms of vitamin B-6 during vitamin B-6 deprivation. Karawya *et al.* (1981) also reported that pyridoxal kinase in sheep liver was inhibited by 3-hydroxykynurenine, 3-hydroxyanthranilate, xanthurenate and picolinate, among tryptophan metabolites. Pyridoxal kinase in B-6-deficient rat liver therefore seemed to be inhibited by 3-hydroxykynurenine or xanthurenate, or both, and then a greater decrease of pyridoxal 5-phosphate seemed to be caused. The effect of tryptophan metabolites on the activity of rat liver pyridoxal kinase requires further study.

A significant difference in tryptophan contents was observed only in liver with a small dose of tryptophan. The rate of transport of extracellular tryptophan into liver has been reported to be very rapid, and not a rate-limiting factor of tryptophan oxidation (Smith & Pogson, 1980). The K_m value for a diffusion-type transport system with a low affinity was reported to be 5–50 mM (Fehlmann *et al.*, 1979; Joseph *et al.*, 1978). Therefore the low tryptophan contents observed in normal liver seemed to reflect the higher activity of tryptophan 2,3-dioxygenase than in B-6-deficient liver.

In conclusion, the larger dose of tryptophan in B-6-deficient rats resulted in a greater decrease in

kynureninase activity, and an accumulation of 3-hydroxykynurenine that sufficed to produce xanthurenate in liver.

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