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Effect of tryptophan metabolites on the activities of rat liver pyridoxal kinase and pyridoxamine 5-phosphate oxidase *in vitro*

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1. Pyridoxal kinase was purified 4760-fold from rat liver. The K_m values for pyridoxine and pyridoxal were 120 and 190 μM respectively, and pyridoxine showed substrate inhibition at above 200 μM. 2. Pyridoxamine 5-phosphate oxidase was also purified 2030-fold from rat liver, and its K_m values for pyridoxine 5-phosphate and pyridoxamine 5-phosphate were 0.92 and 1.0 μM respectively. Pyridoxine 5-phosphate gave a maximum velocity that was 5.6-fold greater than with pyridoxamine 5-phosphate and showed strong substrate inhibition at above 6 μM. 3. Among the tryptophan metabolites, picolinate, xanthurenate, quinolinate, tryptamine and 5-hydroxytryptamine inhibited pyridoxal kinase. However, pyridoxamine 5-phosphate oxidase could not be inhibited by tryptophan metabolites, and on the contrary it was activated by 3-hydroxykynurenine and 3-hydroxyanthranilate. 4. Regarding the metabolism of vitamin B-6 in the liver, the effects of tryptophan metabolites that were accumulated in vitamin B-6-deficient rats after tryptophan injection were discussed.

Vitamin B-6 is phosphorylated by pyridoxal kinase (ATP:pyridoxal 5-phosphotransferase, EC 2.7.1.35) and then oxidized by FMN-dependent pyridoxamine 5-phosphate oxidase [pyridoxaminephosphate:oxygen oxidoreductase (deaminating, EC 1.4.3.5)], to form the coenzyme pyridoxal 5-phosphate in liver (Snell & Haskell, 1971; Johansson *et al.*, 1974). The cellular content of pyridoxal 5-phosphate is regulated by the activities of pyridoxal kinase, pyridoxamine 5-phosphate oxidase and phosphatase, and by the content of pyridoxal 5-phosphate-binding proteins (Li *et al.*, 1974; Bosron *et al.*, 1978). Pyridoxal kinase from the brain of ox (Neary & Diven, 1970; Abercrombie & Martin, 1980), pig (Kwok & Churchich, 1979; Churchich & Wu, 1982), rat (McCormick *et al.*, 1961; Cash *et al.*, 1980) and mouse (Tsubosaka & Makino, 1969), and from human erythrocytes (Chern & Beutler, 1975), was purified and characterized. Sheep liver pyridoxal kinase was studied by Karawya & Fonda (1978, 1982), and rat liver pyridoxal kinase was partially purified and characterized (McCormick & Snell, 1961; McCormick *et al.*, 1961; Korytnyk *et al.*, 1976; Gregory, 1980). Pyridoxamine 5-phosphate oxidase was also studied in rabbit liver (Wada & Snell, 1961; Kazarinoff & McCormick, 1975; Choi *et al.*, 1983), pig brain (Churchich, 1984) and baker's yeast (Tsuge *et al.*, 1983). However, the properties of rat liver pyridoxamine 5-phosphate oxidase have not been elucidated. Only Nutter *et al.* (1983) purified this enzyme, in order to prepare the antibody, and they reported that the purified enzyme preparation contained both active and inactive enzymes, and the subunit M_r of the oxidase was 25000–28000.

A high dose of tryptophan given to vitamin B-6-deficient animals caused the urinary excretion of tryptophan metabolites, especially xanthurenate (Yeh & Brown, 1977; Brown, 1981). We previously reported that the 3-hydroxykynurenine content in vitamin B-6-deficient liver after tryptophan injection was enough to produce xanthurenate, and that a high dose of tryptophan in vitamin B-6-deficient rats could cause a greater deficiency of pyridoxal 5-phosphate in liver (Takeuchi & Shibata, 1984). Since Karawya *et al.* (1981) reported that sheep liver pyridoxal kinase was inhibited by 3-hydroxykynurenine, 3-hydroxyanthranilate, xanthurenate and picolinate, among tryptophan metabolites, this decrease in pyridoxal 5-phosphate in vitamin B-6-deficient rat liver seemed to be caused by tryptophan metabolites.

In the present study, pyridoxal kinase and pyridoxamine 5-phosphate oxidase were highly purified from rat liver, and the effects of tryptophan metabolites on both enzymes were investigated in order to elucidate the interaction between tryptophan metabolism and the formation of pyridoxal 5-phosphate.

Materials and methods

Chemicals

Pyridoxamine 5-phosphate, kynurenine sulphate, 3-hydroxyanthranilic acid, 5-hydroxytryptophan, tryptamine, 5-hydroxyindoleacetic acid, Tris and bovine serum albumin were purchased from Sigma (St. Louis, MO, U.S.A.), and phenylmethanesulphonyl fluoride was from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.). ATP, NAD^+, $NADP^+$, NADH and NADPH were purchased from Oriental Yeast Co. (Tokyo, Japan). Pyridoxal, pyridoxine, pyridoxal 5-phosphate and other chemicals were from Wako Pure Chemical Industries (Osaka, Japan). Pyridoxine 5-phosphate was prepared from pyridoxal 5-phosphate by reduction with $NaBH_4$ and purified by column chromatography (Stock *et al.*, 1966). Pyridoxyl- and phosphopyridoxyl-Sepharose were prepared by the method of Cash *et al.* (1980).

Enzyme assay

The activity of pyridoxal kinase was determined by reaction with phenylhydrazine (Wada & Snell, 1961), with pyridoxal as a substrate. The standard assay mixture contained 112.5 μmol of potassium phosphate (pH 5.75), 0.75 μmol of ATP, 0.75 μmol of pyridoxal, 15 nmol of $ZnCl_2$ and 3.7 μg of purified pyridoxal kinase in a total volume of 1.5 ml. The assay mixture was incubated for 30 min at 37°C, and the reaction was stopped by the addition of 0.18 ml of 100% (w/v) trichloroacetic acid. To a 1 ml sample of the mixture, 2.3 ml of distilled water and 0.5 ml of 9 M-H_2SO_4 were added. The mixture was cooled to 0°C and 0.2 ml of phenylhydrazine solution (1 g of phenylhydrazine hydrochloride in 50 ml of 5 M-H_2SO_4) was added. The mixture was left for 30 min at 0°C, and then its A_{410} was measured by a Cary-17 spectrophotometer at 2°C. An $\varepsilon_{410} = 23000$ was used for the phenylhydrazone derivative of pyridoxal 5-phosphate. A blank containing no enzyme was used to correct the value for pyridoxal 5-phosphate formation. To determine the kinetic parameters and the type of inhibition, pyridoxal 5-phosphate production was continuously measured at 388 nm and 37°C by the spectrophotometric method of Neary & Diven (1970).

When pyridoxine was used as a substrate, pyridoxine 5-phosphate produced was converted into pyridoxal 5-phosphate with purified pyridoxamine 5-phosphate oxidase, and the content of pyridoxal 5-phosphate was determined by the phenylhydrazine method. The standard assay mixture was the same as for pyridoxal substrate, except that 0.3 μmol of pyridoxine was used instead of 0.75 μmol of pyridoxal. The assay mixture was incubated for 30 min at 37°C. The reaction was stopped by heating for 3 min in boiling water, and the mixture was immediately cooled. To a 0.6 ml sample of the mixture, 0.5 ml of 0.7 M-Tris/HCl (pH 9.4), 0.2 ml of 17.5 μM-FMN, 0.1 ml of bovine serum albumin (250 μg/ml), 0.34 ml of distilled water and 10 μl (4.5 μg) of pyridoxamine 5-phosphate oxidase purified from rat liver were added. After incubation for 1 h at 37°C, 0.15 ml of 100% trichloroacetic acid and 0.1 ml of phenylhydrazine solution were added. The mixture was centrifuged at 3000 *g* for 6 min to remove the precipitate. The pyridoxal 5-phosphate produced was determined from the A_{410}. Under these conditions pyridoxine 5-phosphate at up to 25 μM was completely converted into pyridoxal 5-phosphate, and the reaction reached an equilibrium within 30 min.

The activities of pyridoxamine 5-phosphate oxidase were also determined by the method of Wada & Snell (1961). The standard assay mixture in a total volume of 1.75 ml contained 350 μmol of Tris/HCl (pH 8.0), 3.5 nmol of FMN, 175 nmol of pyridoxamine 5-phosphate or pyridoxine 5-phosphate, 25 μg of bovine serum albumin and 5–9 μg of purified enzyme. The mixture was incubated for 20 min (pyridoxine 5-phosphate) or 30 min (pyridoxamine 5-phosphate) at 37°C, and then 0.15 ml of 100% trichloroacetic acid and 0.1 ml of phenylhydrazine solution were added. The blank value was determined with the assay mixture containing all components, but trichloroacetic acid was added to the assay mixture before addition of the enzyme. For determination of kinetic parameters, the reaction was stopped at 0.5, 1, 2, 3, 4 and 5 min, and pyridoxal 5-phosphate produced was determined. Linearity was observed for at least 3 min. The activities of both enzymes were expressed as nmol of products/min.

Purification of pyridoxal kinase

Pyridoxal kinase was purified by a modification of the method of McCormick *et al.* (1961) and Cash *et al.* (1980). Purification was carried out at 0–4°C. Buffers used in the purification procedure were adjusted to pH 7.0 and contained 0.1 mM-reduced glutathione and 20 μM-$ZnCl_2$. Male Wistar rats (250–350 g each) were killed by decapitation. Livers were immediately removed and homogenized in 2 vol. of 0.1 M-potassium phosphate with a Waring blender for 3 min. The homogenate was

centrifuged at 105000*g* for 1h. Solid $(NH_4)_2SO_4$ was added to the supernatant, and the protein fraction insoluble between 40 and 60% saturation with $(NH_4)_2SO_4$ was obtained and dialysed overnight against 5mM-potassium phosphate. The dialysed solution was placed on a Sephadex G-100 column (4.4cm × 72cm) equilibrated with 5mM-potassium phosphate and filtered with the same buffer. The active fraction were collected and applied to a CM-Sephadex column (2.6cm × 20cm) equilibrated with 5mM-potassium phosphate. Pyridoxal kinase is not retained by CM-Sephadex. The active fraction was applied to a DEAE-Sepharose column (3.2cm × 20cm) equilibrated with 5mM-potassium phosphate and eluted with a linear gradient of 0–0.2M-KCl in 5mM-potassium phosphate (500ml). Pyridoxal kinase was eluted at about 80mM-KCl. The active fraction was directly applied to a pyridoxyl-Sepharose column (1.6cm × 15cm) equilibrated with 5mM-potassium phosphate. The column was washed with 300ml of 5mM-potassium phosphate containing 0.4M-KCl. Then the outlet of the pyridoxyl-Sepharose column was attached to the inlet of the hydroxyapatite column (1cm × 9cm) equilibrated with 5mM-potassium phosphate. Pyridoxal kinase was eluted with 300ml of 5mM-potassium phosphate containing 10mM-pyridoxine and 0.4M-KCl, and was adsorbed directly on to the hydroxyapatite column. The hydroxyapatite column was detached from the pyridoxyl-Sepharose column and washed thoroughly with the equilibrating buffer. Pyridoxal kinase was slowly eluted with 0.3M-potassium phosphate, dialysed against 0.1M-potassium phosphate, and stored at −80°C. The enzyme was stable for several months under these conditions.

The purification procedure for pyridoxal kinase is summarized in Table 1. The enzyme was purified 4760-fold from rat liver cytosol and its yield was 39%. Apparent activation at the $(NH_4)_2SO_4$ fractionation step has been previously observed for the sheep liver (Karawya & Fonda, 1978) and rat liver (McCormick *et al.*, 1961) enzymes.

Purification of pyridoxamine 5-phosphate oxidase

Purification was carried out at 0–4°C. The pH of buffers used was 7.0, and they contained 0.1mM-reduced glutathione, 50μM-phenylmethanesulphonyl fluoride and 2μM-FMN. Rat livers were homogenized in 3vol. of 20mM-potassium phosphate and centrifuged at 52000*g* for 1h. To the supernatant 2M-acetic acid was added dropwise and the pH adjusted to 5.0. After being stirred for 10min, the solution was centrifuged at 18000*g* for 30min. The supernatant was neutralized with 10% (w/v) KOH and centrifuged at 10000*g* for 15min. This supernatant was applied to a DEAE-Sepharose column (4.4cm × 30cm) washed with 4 litres of 2mM-potassium phosphate. DEAE-Sepharose could not be equilibrated with this buffer, because of much adsorption of FMN. The oxidase was eluted with a linear gradient of 0–0.7M-KCl in 2mM-potassium phosphate (700ml) and eluted at 0.35M-KCl. The active fraction was concentrated to about 50ml with an Amicon Diaflo membrane filter PM-10 and placed on a Sephadex G-100 column (4.4cm × 70cm) equilibrated with 2mM-potassium phosphate containing 0.1M-KCl. After filtration with the equilibrating buffer, active fraction was concentrated to 15ml with a membrane filter and applied to a phosphopyridoxyl-Sepharose column (1.6cm × 15cm) equilibrated with 2mM-potassium phosphate containing 0.1M-KCl. After equilibration for 16h, the column was washed with 150ml of the equilibrating buffer, followed by 100ml of 2mM-potassium phosphate. The elution of the oxidase from a phosphopyridoxyl-Sepharose column and chromatography on a hydroxyapatite column were carried out by the method of Cash *et al.* (1980). The purified oxidase was desalted by a Bio-Gel 6-PDH column (2.6cm × 30cm) equilibrated with 2mM-potassium

Table 1. *Purification of pyridoxal kinase from rat liver*

The activities were determined with an assay mixture of 2.5ml, which contained the same components at the same concentrations as standard assay mixture, with pyridoxal as a substrate. The reaction was stopped with 0.3ml of 100% (w/v) trichloroacetic acid, and the mixture was centrifuged to remove the precipitate formed. Pyridoxal 5-phosphate produced in a 1ml sample of the supernatants was determined with phenylhydrazine as described in the text.

Fraction	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg of protein)	Purification	Yield (%)
105000*g* supernatant	34200	3400	0.0994	1	100
40–60%-satd. $(NH_4)_2SO_4$	9250	4250	0.459	4.6	125
Sephadex G-100	2700	3000	1.11	11	88
CM-Sephadex + DEAE-Sepharose	253	2850	11.3	114	84
Pyridoxyl-Sepharose	2.83	1340	473	4760	39

Table 2. *Purification of pyridoxamine 5-phosphate oxidase from rat liver*

The activities were determined with an assay mixture of 3.5ml with pyridoxamine 5-phosphate as a substrate. The reaction was stopped by the addition of 0.3ml of 100% (w/v) trichloroacetic acid and the mixture was centrifuged to remove the precipitate. Pyridoxal 5-phosphate formed in a 1.9ml sample of the supernatant was determined with phenylhydrazine as described in the text.

Fraction	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg of protein)	Purification	Yield (%)
52000*g* supernatant	42300	2870	0.0678	1	100
Acid treatment	24200	2600	0.108	1.6	91
DEAE-Sepharose	2410	1830	0.760	11	64
Sephadex G-100	727	1720	2.37	35	60
Phosphopyridoxyl-Sepharose + hydroxyapatite	2.67	368	138	2030	13

phosphate, concentrated with a membrane filter and stored at −80°C.

Pyridoxamine 5-phosphate oxidase was purified 2030-fold from the 52000*g* supernatant of rat liver, and the yield was 13%, as shown in Table 2. The enzyme was stable for at least several months.

Protein measurement

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Some properties and kinetic parameters

Purified pyridoxal kinase catalysed the phosphorylation of pyridoxal and pyridoxine. The K_m values for pyridoxine and pyridoxal were 120 and 190 μM respectively. The maximum velocities of the enzyme were 1420 and 1270nmol/min per mg of protein respectively. Pyridoxine showed substrate inhibition at more than 200 μM, but pyridoxal did not up to 1mM.

Pyridoxamine 5-phosphate oxidase catalysed the oxidation of both pyridoxine 5-phosphate and pyridoxamine 5-phosphate. An optimum pH could not be determined in the range pH 7–10, as shown in Fig. 1. The activities with both substrates increased with increasing pH. The activity obtained with pyridoxine 5-phosphate as a substrate was higher than that with pyridoxamine 5-phosphate in 0.2M-Tris/HCl or 0.1M-pyrophosphate/KOH buffer. However, the activity obtained with pyridoxamine 5-phosphate in 0.1M-boric acid/KCl/NaOH buffer was higher than with pyridoxine 5-phosphate. The activities obtained at pH 8.0 with Tris/HCl, pyrophosphate/KOH and boric acid/KCl/NaOH buffers were 1.27, 1.05 and 0.88nmol/min respectively when pyridoxine 5-phosphate was used as a substrate. With pyridoxamine 5-phosphate as a substrate, the activities

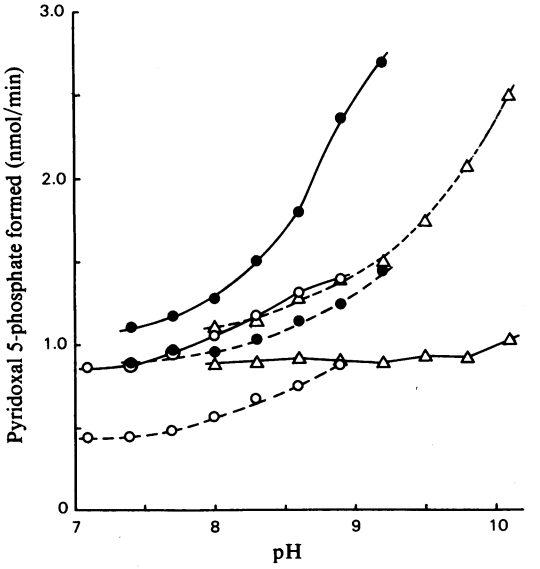

Fig. 1. *Effect of pH on activity of pyridoxamine 5-phosphate oxidase*

The activity was determined at the indicated pH with 0.2M-Tris/HCl (●), 0.1M-pyrophosphate/KOH (○) and 0.1M-boric acid/KCl/NaOH (△) buffers. Assay mixture contained one of the above buffers, 2 μM-FMN, 0.3mM-pyridoxine 5-phosphate (——) or 0.3mM-pyridoxamine 5-phosphate (----), 25 μg of bovine serum albumin and 13 μg of purified enzyme. The mixture was incubated for 30min at 37°C, and pyridoxal 5-phosphate formed was determined with phenylhydrazine as described in the text.

were 0.95, 0.56 and 1.11nmol/min respectively. The apparent K_m values determined with 0.2M-Tris/HCl (pH 8.0) for pyridoxine 5-phosphate and pyridoxamine 5-phosphate were 0.92 and 1.0 μM respectively. The maximum velocities of purified enzyme for pyridoxine 5-phosphate and pyridox-

amine 5-phosphate were 461 and 82 nmol/min per mg of protein, and 5.6 times greater for pyridoxine 5-phosphate. No substrate inhibition was observed with pyridoxamine 5-phosphate up to 0.3 mM, but pyridoxine 5-phosphate at more than 6 μM showed a strong substrate inhibition. The activities obtained at 10 μM-pyridoxine 5-phosphate and -pyridoxamine 5-phosphate were 376 and 75 nmol/min per mg of protein, and those at 0.3 mM of both substrates were 96 and 81 nmol/min per mg of protein respectively.

Effects of tryptophan metabolites on pyridoxal kinase

The effects of tryptophan metabolites on the activities of purified pyridoxal kinase are shown in Table 3. Xanthurenate, quinolinate and picolinate at 0.5 mM inhibited activities with both pyridoxine and pyridoxal. Only NAD^+ increased the activity with pyridoxine. Tryptamine and 5-hydroxytryptamine showed a strong inhibition with the pyridoxal substrate. Tryptophan, kynurenine, 3-hydroxykynurenine, anthranilate, 3-hydroxyanthranilate, kynurenate, 5-hydroxytryptophan, nicotinate, nicotinamide, $NADP^+$, NADPH, 5-hydroxyindoleacetate and indoleacetate showed no effect on either activity of pyridoxal kinase. With pyridoxal as a substrate, the type of inhibition by xanthurenate, picolinate and quinolinate was determined, as shown in Figs. 2(*a*) and 2(*b*). Xanthurenate and picolinate showed a competitive in-

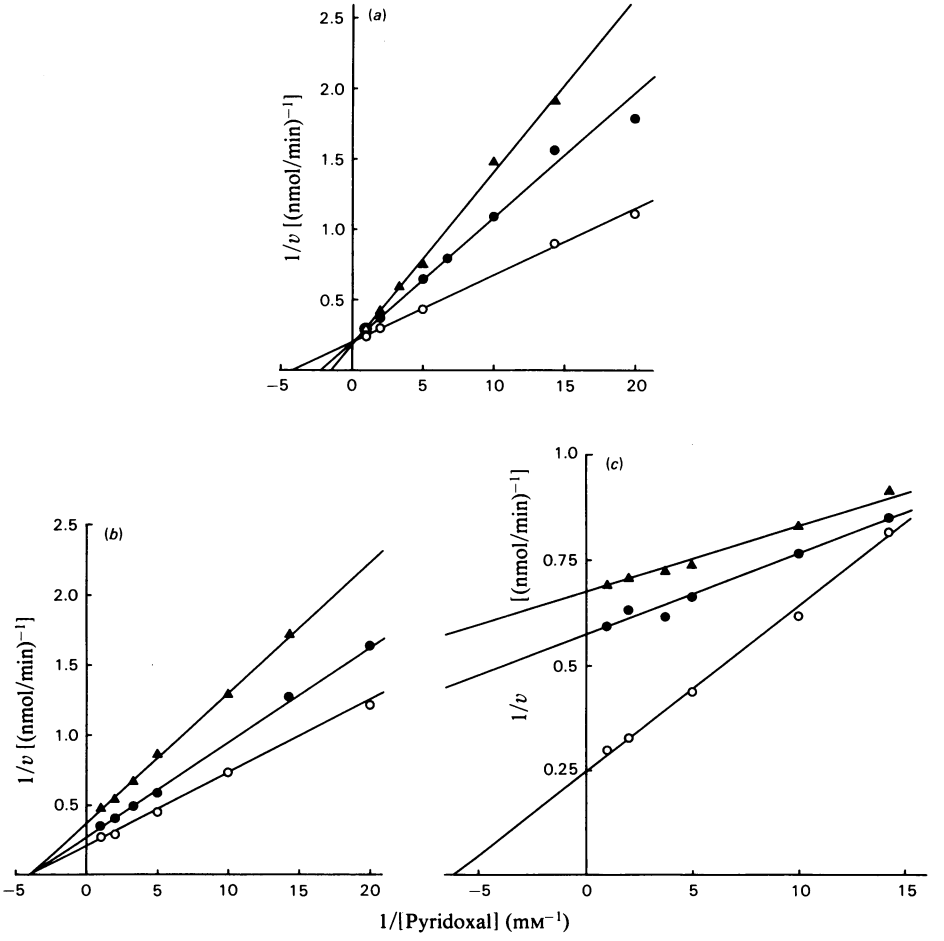

Fig. 2. *Effect of xanthurenate, picolinate, quinolinate, tryptamine and 5-hydroxytryptamine on activity of pyridoxal kinase*
The activities of pyridoxal kinase with different concentrations of pyridoxal were determined with the addition of metabolites to assay mixture. The change of A_{388} of pyridoxal 5-phosphate produced was measured continuously at 37°C as described in the text. Lines were fitted to experimental points by a least-squares method, except one point deviating greatly from each line, for xanthurenate and picolinate as inhibitors (*a*), is not included. (*a*) ▲, 1 mM-xanthurenate; ●, 0.05 mM-picolinate; ○, no inhibitor. (*b*) ▲, 0.2 mM-quinolinate; ●, 0.1 mM-quinolinate; ○, no inhibitor. (*c*) ▲, 0.2 mM-5-hydroxytryptamine; ●, 0.2 mM-tryptamine; ○, no inhibitor.

Table 3. *Effect of tryptophan metabolites on activity of purified pyridoxal kinase*

The activities of pyridoxal kinase were determined in duplicate or triplicate as described in the text, and the mean values are shown. The variation of the assay on each metabolite and control was within maximally 4%. Tryptophan metabolites were neutralized with 1M-KOH or HCl and added to the assay mixture. The final concentration of metabolites was 0.5mM. Results are expressed as a percentage of activity.

Metabolites	Relative activity (%)	
	Pyridoxine substrate	Pyridoxal substrate
None	100	100
Picolinate	5	3
Quinolinate	18	18
Xanthurenate	81	82
NAD^+	125	92
NADH	86	89
Tryptamine	103	28
5-Hydroxytryptamine	89	19

hibition against pyridoxal. The inhibition constants of xanthurenate and picolinate were estimated to be 0.76 and 0.06mM respectively, from the $1/s$ axis intercepts of Fig. 2(*a*) (Segel, 1975). Quinolinate showed a non-competitive inhibition, and its inhibition constant was determined to be 0.25mM from the $1/v$ axis intercepts of Fig. 2(*b*). Inhibition by 5-hydroxytryptamine and tryptamine is shown in Fig. 2(*c*). The Lineweaver–Burk plots showed straight lines, but the potency of inhibition increased with increasing concentrations of pyridoxal. A straight Lineweaver–Burk plot in the presence of 5-hydroxytryptamine changed to a downward curve with the increase of the concentration of $ZnCl_2$ to 30μM (results not shown). 5-Hydroxytryptamine apparently caused substrate inhibition by pyridoxal, although pyridoxal itself did not show substrate inhibition. The formation of a Schiff base between 5-hydroxytryptamine and pyridoxal could not be detected spectrophotometrically under assay conditions, as reported by Neary *et al.* (1972). The K_m value for pyridoxal decreased to 83μM in the presence of 30μM-$ZnCl_2$.

Effects of tryptophan metabolites on pyridoxamine 5-phosphate oxidase

With pyridoxine 5-phosphate as a substrate, 3-hydroxykynurenine and 3-hydroxyanthranilate greatly increased the enzyme activity, and xanthurenate and 5-hydroxyindoleacetate also activated, as shown in Table 4. With pyridoxamine 5-phosphate as substrate, 3-hydroxykynurenine, 3-hydroxyanthranilate, tryptamine and 5-hydroxytryptamine increased the activity. The other tryptophan metabolites tested, i.e. tryptophan, kynurenine, anthranilate, kynurenate, quinolinate, picolinate, nicotinate, nicotinamide, NAD^+, NADH, $NADP^+$, NADPH, 5-hydroxyindoleacetate and indoleacetate, showed no effect on activity with either pyridoxine 5-phosphate or pyridoxamine 5-phosphate.

Table 4. *Effect of tryptophan metabolites on activity of purified pyridoxamine 5-phosphate oxidase*

The activities of pyridoxamine 5-phosphate oxidase were determined in duplicate or triplicate as described in the text, and other details are the same as in the legend of Table 3.

Metabolites	Relative activity (%)	
	Pyridoxine 5-phosphate substrate	Pyridoxamine 5-phosphate substrate
None	100	100
3-Hydroxykynurenine	225	120
3-Hydroxyanthranilate	245	128
Xanthurenate	121	101
Tryptamine	97	127
5-Hydroxytryptamine	108	120
5-Hydroxyindoleacetate	153	109

Discussion

The K_m values of pyridoxal kinase obtained for pyridoxal and pyridoxine were 190 and 120μM, which were one order of magnitude higher than those reported by McCormick *et al.* (1961). However, the K_m value for pyridoxal reported by Korytnyk *et al.* (1976) was 182μM, identical with our value. These K_m values were close to those of sheep liver enzyme (Karawya & Fonda, 1978, 1982). Substrate inhibition by pyridoxal reported by McCormick *et al.* (1961), Neary & Diven (1970) and Karawya & Fonda (1978) could not be observed, but substrate inhibition by pyridoxine at above 200μM was observed. This difference seemed to result from the difference in purity, assay method or animal source. It is necessary to elucidate the properties with highly purified enzyme.

Pyridoxamine 5-phosphate oxidase has previously been purified 1375-fold by Nutter *et al.* (1983) from rat liver, but its properties have not been elucidated. The optimum pH of rabbit liver enzyme has been reported to be 9–10 with 0.2M-Tris/HCl (Kazarinoff & McCormick, 1975), and that in pig brain enzyme was pH8.4 in 0.1M-pyrophosphate buffer (Kwok & Churchich, 1980). Rat liver enzyme, however, did not show an optimum pH in either buffer. K_m values of rat liver enzyme

for both pyridoxine 5-phosphate and pyridoxamine 5-phosphate were similar, and the K_m values were lower than in previous reports (Kazarinoff & McCormick, 1975; Merrill *et al.*, 1978; Tsuge *et al.*, 1979; Churchich, 1984; Choi *et al.*, 1983). The maximum velocity obtained with pyridoxine 5-phosphate was 5.6 times that with pyridoxamine 5-phosphase, a tendency shown in pig brain enzyme, but not in rabbit liver and baker's-yeast enzyme. A strong substrate inhibition by pyridoxine 5-phosphate was reported in rabbit liver enzyme by Choi *et al.* (1983).

Kinetic properties of both pyridoxal kinase and pyridoxamine 5-phosphate oxidase in rat liver suggested that the rate of formation of pyridoxal 5-phosphate from pyridoxine and pyridoxal was heavily dependent on the activity of pyridoxal kinase (i.e. substrate concentrations and the amount of enzyme). Kozik & McCormick (1984) reported that the uptake of pyridoxine into isolated rat liver cells occurred by simple or facilitated diffusion and was dependent on the activity of pyridoxal kinase. Van den Berg *et al.* (1982) also reported that the contents of pyridoxal 5-phosphate in rat liver and plasma were dependent on the amount of pyridoxine in the diet. In vitamin B-6-deficient rat liver, the activity of pyridoxal kinase as well as the content of pyridoxal 5-phosphate were decreased (Meisler & Thanassi, 1980; Coburn *et al.*, 1981). Pyridoxamine 5-phosphate oxidase activity in rat fed on a pyridoxine-deficient diet was not different from controls (Rasmussen *et al.*, 1979; Meisler & Thanassi, 1980). These reports indicated that pyridoxal kinase is an important enzyme for the control of pyridoxal 5-phosphate formation.

A large dose of tryptophan administered to vitamin B-6-deficient rat caused the accumulation and excretion of tryptophan metabolites. At the same time the holoenzyme activity of kynurenine hydrolase (EC 3.7.1.3), which is present in the cytosol and requires pyridoxal 5-phosphate, sharply decreased. Also, the holoenzyme activity of kynurenine aminotransferase (EC 2.6.1.7), which is present in the mitochondrial inner membrane, decreased slowly in vitamin B-6-deficient rat liver (Takeuchi & Shibata, 1984). These findings suggested the possibility of inhibition of pyridoxal 5-phosphate formation by tryptophan metabolites. Although the purified pyridoxamine 5-phosphate oxidase was much activated by 3-hydroxykynurenine and 3-hydroxyanthranilate, tryptophan metabolites that inhibited the activity of the oxidase could not be found in this experiment. Pyridoxal kinase, however, was inhibited by picolinate, quinolinate and xanthurenate. Since in vitamin B-6-deficient rat liver the activity of kynurenine hydrolase was low and the excess dose of tryptophan to normal rats could not cause the decrease in kynurenine hydrolase holoenzyme activity, the inhibition *in vivo* by picolinate and quinolinate, which are intermediates of the kynurenine pathway via kynurenine hydrolase, could be ruled out. There has been no report of the content of xanthurenate in liver. However, 3-hydroxykynurenine, which is a precursor of xanthurenate, increased to 1.38 μmol/g of vitamin B-6-deficient liver at 1 h after a tryptophan dose of 30 mg/100 g body wt., a value sufficient to produce xanthurenate, in view of the K_m value of kynurenine aminotransferase (Takeuchi & Shibata, 1984). Since xanthurenate inhibited pyridoxal kinase competitively and the contents of pyridoxine and pyridoxal in vitamin B-6-deficient rat liver may be lower, it seemed that the cumulative amount of xanthurenate could inhibit pyridoxal kinase and decrease the content of pyridoxal 5-phosphate *in vivo*.

Karawya *et al.* (1981) reported that 3-hydroxykynurenine and 3-hydroxyanthranilate inhibited sheep liver pyridoxal kinase non-competitively, but rat liver enzyme was not inhibited by these metabolites. We observed the inhibition of pyridoxal kinase by old 3-hydroxykynurenine that had been previously prepared, but not by a fresh preparation from newly opened ampoules. A derivative of 3-hydroxykynurenine may inhibit the enzyme. The inhibition by picolinate and xanthurenate was competitive in type, and both acids bind to Zn^{2+} to form the complex (Evans *et al.*, 1979; Ikeda & Kotake, 1984). Therefore the inhibition by these acids may have resulted from the removal of Zn^{2+}.

5-Hydroxytryptamine and tryptamine could inhibit pyridoxal kinase when pyridoxal was a substrate. This finding was also reported with bovine brain enzyme (Neary *et al.*, 1972) and rat brain enzyme (Ebadi & Govitrapong, 1979). They indicated that 5-hydroxytryptamine did not form the complex with pyridoxal, ATP or Zn^{2+}. However, with pyridoxine as a substrate, tryptamine had no inhibitory effect and 5-hydroxytryptamine inhibited little. This indicated that the aldehyde group of pyridoxal was necessary for the appearance of inhibition and that pyridoxal 5-phosphate formed was independent of this inhibition. Increasing the concentration of $ZnCl_2$ enhanced the apparent substrate inhibition. Therefore Zn^{2+} was either directly or indirectly involved with the inhibition. The mechanism of the inhibitory effect of 5-hydroxytryptamine is unknown at present, and more detailed study is required. Smith *et al.* (1980) indicated that the oxidation of tryptophan to 5-hydroxyindoleacetate was very little and unlikely to be a physiologically important pathway in the liver. Moreover, the formation of 5-hydroxytryptamine and tryptamine from tryptophan required pyridoxal 5-phosphate. Therefore it could not be

considered that the accumulation of 5-hydroxytryptamine or tryptamine occurred in vitamin B-6-deficient liver.

In conclusion, the disorder of liver tryptophan metabolism caused by vitamin B-6-deficiency could inhibit the formation of pyridoxal 5-phosphate at the pyridoxal kinase step and could thus cause a greater deficiency of pyridoxal 5-phosphate.

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