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Tryptophan 5-Hydroxylase in Rat Intestine

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Tryptophan 5-hydroxylase was partially purified from rat small intestine and characterized. The enzyme activity was mainly localized in the distal one-fourth of the small intestine. The enzyme required Fe^{2+}, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine and oxygen for full activity. The pH optimum of the reaction was 8.0. The hydroxylation rate of D-tryptophan by the enzyme was one-third that of L-tryptophan. L-Phenylalanine and L-tyrosine could not serve as substrates. The physiological significance of the enzyme is discussed.

It is generally accepted that the enzymic hydroxylation of tryptophan to 5-hydroxytryptophan is the first and rate-limiting step in the biosynthesis of serotonin (5-hydroxytryptamine) in mammalian brain.

Grahame-Smith (1964*a*) showed that homogenates of dog and rabbit brain stems hydroxylated L-tryptophan to form 5-hydroxy-L-tryptophan. Since then, tryptophan 5-hydroxylase has been extensively studied *in vitro* with extracts from some mammalian brain tissues (Grahame-Smith, 1967*a*, 1968; Lovenberg *et al.*, 1968; Ichiyama *et al.*, 1968, 1970; Green, 1968).

In spite of much evidence that the intestine contains considerable amounts of serotonin, the mechanism of the enzymic hydroxylation of tryptophan at the 5-position in this tissue is not completely understood. In our present studies, tryptophan 5-hydroxylase was found in rat small intestine, and was partially purified and characterized.

Experimental

Materials

L-Tryptophan, D-tryptophan, L-tyrosine and L-phenylalanine were purchased from Tanabe Amino Acid Foundation (Osaka, Japan). 5-Hydroxy-L-tryptophan, 5-hydroxytryptamine creatine sulphate and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine were obtained from Calbiochem, Los Angeles, Calif., U.S.A. L-Kynurenine was prepared by ozonolysis of L-tryptophan (Brown & Price, 1955). Analytical Grade Dowex AG1 resin (X8; 200–400 mesh; Cl^- form) was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. and NADPH was from Boehringer Corp. (London) Ltd., London W.5, U.K. The acetate form of Dowex-1 was prepared as described by Price (1954) and Roy & Price (1959). 5-Hydroxytryptophan decarboxylase (5-hydroxy-L-tryptophan carboxylyase, EC 4.1.1.28) was partially purified from guinea-pig kidney by the method of Clark *et al.* (1954). Monoamine oxidase [monoamine-oxygen oxidoreductase (deaminating), EC 1.4.3.4] was prepared from rat liver by the method of Noguchi *et al.* (1970).

Methods

Enzyme assays. Tryptophan 5-hydroxylase activity was assayed as follows. The incubation mixture contained 200μmol of potassium phosphate buffer, pH 8.0, 15μmol of L-tryptophan, 4μmol of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, 4-μmol of ferrous ammonium sulphate and enzyme preparation (30–50mg of protein) in a total volume of 4ml. Incubations were carried out at 37°C with shaking for 4h. The reaction was stopped by the addition of 1ml of 50% (w/w) trichloroacetic acid and the precipitate was separated by centrifugation. A portion (2ml) of the supernatant solution was assayed for total 5-hydroxyindole content by the colorimetric method of Udenfriend *et al.* (1955). The control incubation mixture was the same as described above except that the boiled enzyme was substituted for the normal enzyme preparation.

Phenylalanine 4-hydroxylase [L-phenylalanine, tetrahydropteridine–oxygen oxidoreductase (4-hydroxylating), EC 1.14.3.1] activity was assayed as described by Renson *et al.* (1962). Kynurenine 3-hydroxylase [L-kynurenine, reduced-NADP–oxygen oxidoreductase (3-hydroxylating), EC 1.14.1.2] activity was determined by the method of Saito *et al.* (1957). Protein was determined by the biuret method with crystalline bovine serum albumin as the standard (Chance & Redfearn, 1961). Specific enzyme activity was expressed as nmol of 5-hydroxyindole formed/h per mg of protein.

5-Hydroxytryptophan, 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid were determined as described by Nishino *et al.* (1971).

Preparation of enzyme. In the preliminary report, we reported that most of tryptophan 5-hydroxylase

activity could be recovered from the 5000*g* supernatant fraction of 0.25M-sucrose homogenate of rat small intestine (Noguchi *et al.*, 1971). In the present study, the enzyme was partially purified as follows. Some 200 male rats of Donryu strain weighing 60–80g were stunned, decapitated and exsanguinated in a cold-room. All subsequent manipulations were carried out at about 0°C. The distal one-fourth of the small intestines were removed, cut longitudinally, washed thoroughly with ice-cold 0.01M-potassium phosphate buffer, pH7.5, and cut into small pieces. The intestine sections were homogenized with 2vol. of 0.01M-potassium phosphate buffer, pH7.5, in a Waring Blendor for 2min. The homogenate was centrifuged at 7500*g* for 20min. $(NH_4)_2SO_4$ was added with stirring to the resulting supernatant solution to give 40% saturation. This solution was stirred for 30min and centrifuged at 7500*g* for 30min. The supernatant solution was mixed with $(NH_4)_2SO_4$ to give 60% saturation and stirred for 30min, followed by centrifugation at 7500*g* for 30min. The precipitate was suspended in 30ml of 0.01M-potassium phosphate buffer, pH7.5, and dialysed against 10 litres of the same buffer for 12h. Insoluble material formed during dialysis was removed by centrifugation at 7500*g* for 30min. The resulting supernatant solution was used as the enzyme source.

Isolation of reaction products. To identify reaction products, a large-scale incubation was carried out in the presence of semicarbazide as the inhibitor of 5-hydroxytryptophan decarboxylase as follows. Reaction mixture (100ml) containing 4mmol of potassium phosphate buffer, pH8.0, 65μmol of L-tryptophan, 50μmol of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, 10mmol of ferrous ammonium sulphate, 10mmol of semicarbazide and the partially purified enzyme (600mg of protein) was incubated at 37°C for 4h with shaking.

The reaction was terminated by the addition of 10ml of 70% (v/v) $HClO_4$ and the precipitate was filtered off. The filtrate was adjusted to pH6.5 with 3M-KOH and centrifuged at 7500*g* for 30min. The supernatant solution was applied to a column (8cm×1cm) of Dowex-1 (acetate form), which was successively washed with 70ml of deionized water, 100ml of 0.01M-acetic acid (fraction II) and 70ml of 6M-acetic acid (fraction III). The first effluent and the first washing were combined (fraction I). Each fraction was evaporated to dryness under N_2 at reduced pressure. The residue from fraction I was dissolved in 30ml of water, adjusted to pH10 with 3M-NaOH and shaken with butan-1-ol. The extract was concentrated under N_2 at reduced pressure and the compound corresponding to 5-hydroxytryptamine was isolated from the resulting residue (dissolved in a small vol. of water) by t.l.c. The residue from fraction II was dissolved in a small vol. of water and 5-hydroxytryptophan was isolated from the solution by t.l.c. The residue from fraction III was shaken with diethyl ether. The extract was evaporated to dryness under N_2 at reduced pressure. The residue was dissolved in a small volume of water and 5-hydroxyindol-3-ylacetic acid was isolated from the solution by t.l.c.

Thin-layer chromatography. Ascending t.l.c. was used to assist the purification of the reaction products. Thin layers of silica gel G (E. Merck, Darmstadt, Germany) were prepared on glass plates. Details of t.l.c. have been described by Kido *et al.* (1967). To purify the reaction product, the region of the compound corresponding to authentic sample was scraped from the plates into a glass tube with a razor blade after development with the solvent system of ethyl acetate–propan-2-ol–28% NH_3 (9:6:4, by vol.). The compound was eluted from the column with 1% NH_3. The effluent was evaporated to dryness under a stream of N_2 at reduced pressure.

Paper chromatography. Ascending paper chromatography was used for identification of the reaction products. Whatman 3MM papers were used with the solvent systems of methanol–benzene–butan-1-ol–water (2:1:1:1, by vol.) with 1% (v/v) of 28% NH_3 (Mason & Berg, 1951), 10% (w/w) KCl and butan-1-ol–acetic acid–water (4:1:1, by vol.) (Dalgliesh, 1952). Diazotized sulphanilic acid was used for spraying the chromatograms as described by Dalgliesh (1955). Chromatograms were also sprayed with ninhydrin and Ehrlich's reagents (Dalgliesh, 1955). The reaction products were identified by co-chromatography with authentic compounds.

Ultraviolet-absorption spectra were determined with a Hitachi model EPS type 3 recording spectrophotometer in 0.1M-potassium phosphate buffer, pH7.0.

Results

Localization of tryptophan 5-hydroxylase activity in rat small intestine

The small intestine was divided into four portions and the enzyme activity of the 7500*g* supernatant solution of the homogenate from each region of the small intestine was determined. The enzyme activity proved to be localized mainly in the distal one-fourth (Region 4) of the small intestine. The enzyme activity was also detected in the proximal gut (Region 1) but was low compared with that of the distal gut (Region 4). Significant activity could not be detected in the supernatant solution from other parts of the small intestine, as shown in Table 1.

Identification of the reaction products

The reaction products formed from L-tryptophan by the partially purified enzyme were analysed as described in the Experimental section. The compounds isolated from fractions I, II and III were

chromatographically identical with authentic 5-hydroxytryptamine, 5-hydroxy-L-tryptophan and 5-hydroxyindol-3-ylacetic acid respectively, as shown in Table 2. Enzymic examination was also made to obtain further evidence. Incubation of the substance isolated from fraction I with monoamine oxidase resulted in the production of 5-hydroxyindol-3-ylacetic acid, which was identified by paper chromatography, t.l.c. and u.v.-absorption spectroscopy. The formation of 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid from L-tryptophan shows that 5-hydroxytryptophan decarboxylase was not inhibited completely by semicarbazide in this large-scale incubation.

Table 1. *Localization of tryptophan 5-hydroxylase activity in rat small intestine*

Small intestine was divided into four portions. Each portion was washed thoroughly with ice-cold 0.01M-potassium phosphate buffer, pH7.5, and homogenized with 2vol. of 0.01M-potassium phosphate buffer, pH7.5. The homogenate was centrifuged at 7500*g* for 30min. The supernatant solutions were used for assay. Assay conditions were as described in the text. The region of the intestine is given by numbers indicating the portions of intestine proceeding from proximal to distal.

Region of intestine	Specific activity (nmol/h per mg of protein)
1	0.023
2	0
3	0
4	0.116

Effect of semicarbazide on the formation of 5-hydroxy-L-tryptophan, 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid

Table 3 shows the amount of 5-hydroxyindoles formed enzymically from L-tryptophan after a 4h incubation of the reaction mixture in the absence or presence of semicarbazide. In the absence of semicarbazide, the accumulation of 5-hydroxy-L-tryptophan was very low compared with that of 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid. The accumulation of 5-hydroxy-L-tryptophan, however, rose with increasing semicarbazide concentration, whereas that of 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid decreased. The addition of 10mM-semicarbazide resulted in no detection of 5-hydroxyindol-3-ylacetic acid and 5-hydroxytryptamine in the determination method used, showing the complete inhibition of 5-hydroxytryptophan decarboxylase. The total amount of the three 5-hydroxyindoles formed decreased with increasing concentrations of semicarbazide, presumably meaning that the tryptophan 5-hydroxylase activity was also slightly inhibited.

Some properties of the partially purified tryptophan 5-hydroxylase

Tryptophan 5-hydroxylase was partially purified from rat small intestine as described in the Experimental section. By the purification procedure, the

Table 2. *Identification of products formed from L-tryptophan by partially purified enzymes*

Products were identified by paper chromatography by comparison with authentic synthetic compounds, as described in the text. When a mixture of a product and synthetic compound was chromatographed, a single spot was obtained.

	5-Hydroxytryptophan		5-Hydroxytryptamine		5-Hydroxyindol-3-ylacetic acid	
	Reaction product	Synthetic compound	Reaction product	Synthetic compound	Reaction product	Synthetic compound
Butan-1-ol–acetic acid–water (4:1:1, by vol.); R_F	0.17	0.17	0.42	0.42	0.76	0.76
20% KCl; R_F	0.44	0.44	0.39	0.39	0.49	0.49
Methanol–butan-1-ol–benzene–water (2:1:1:1, by vol.) with 1% of 28% NH_3; R_F	0.23	0.23	0.46	0.46	0.71	0.71
Ehrlich's reagent	Blue–purple	Blue–purple	Grey–blue	Grey–blue	Blue	Blue
Diazotized sulphanilic acid	Red–purple	Red–purple	Orange–red	Orange–red	Pink–orange	Pink–orange
Ninhydrin	Purple	Purple	—	—	—	—
Absorption maxima at pH7.0 (nm)	275	275	275	275	275	275

Table 3. *Effect of semicarbazide on formation of 5-hydroxy-L-tryptophan, 5-hydroxytryptamine and 5-hydroxyindol-3-ylacetic acid*

The reaction mixture contained 200 μmol of potassium phosphate buffer, pH 8.0, 15 μmol of L-tryptophan, 4 μmol of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, 4 μmol of ferrous ammonium sulphate and the partially purified enzyme (50 mg of protein) in a total volume of 4 ml. Incubation was performed at 37°C for 4 h with shaking in the absence or presence of semicarbazide (0.1–10 mM). The control incubation mixture was the same as described above except enzyme boiled for 1 min was substituted for the normal enzyme preparation. Each 5-hydroxyindole was determined as described by Nishino *et al.* (1971).

Concn. of semicarbazide (mM)	Compound produced (nmol/4h)			Total amounts of 5-hydroxyindoles (nmol/4h)
	5-Hydroxy-L-tryptophan	5-Hydroxy-tryptamine	5-Hydroxyindol-3-ylacetic acid	
None	4.8	95.9	107.8	208.5
0.1	35.9	80.1	59.4	175.4
1	66.0	64.1	25.3	155.4
10	120.3	0	0	120.3

Table 4. *Cofactor requirement of partially purified tryptophan 5-hydroxylase*

The complete reaction mixture contained 200 μmol of potassium phosphate buffer, pH 8.0, 15 μmol of L-tryptophan, 4 μmol of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, 4 μmol of ferrous ammonium sulphate and the partially purified enzyme (40 mg of protein) in a total volume of 4 ml. Specific activity is expressed as nmol of 5-hydroxyindoles formed/h per mg of protein. When the enzyme was boiled for 1 min, the enzyme activity was not detected. Assay conditions were as described in the text.

Addition (+) or omission (–)	Specific activity
None (Complete system)	1.03
–2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine	0.33
$-Fe^{2+}$	0.66
$-Fe^{2+}$ and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine	0.33
+$\alpha\alpha'$-Bipyridyl (1 mM)	0.70
+Ascorbic acid (1 mM)	0.98
+NADPH (1 mM)	0.99
$+H_2O_2$	0.98
$+Cu^{2+}$ (1 mM)	0.90
+Dithiothreitol (1 mM)	0.99
$-O_2$	0.08

specific activity of tryptophan 5-hydroxylase was increased some 9-fold over with a recovery of 60%. The tryptophan 5-hydroxylase activity of the preparation was stable for 2 days when stored at –20°C.

Tryptophan 5-hydroxylase activity followed linear kinetics during 4 h incubation. A linear relationship between the hydroxylase activity and enzyme concentration was obtained with a protein content of up to 80 mg per assay (4 ml). The enzyme showed maximal activity at pH 8.0.

The enzyme catalysed the hydroxylation of D-tryptophan at one-third the rate observed with L-tryptophan. However, L-phenylalanine, L-tyrosine and L-kynurenine could not be hydroxylated by the enzyme.

Table 4 summarizes the results of experiments to determine the cofactor requirements for full enzyme activity. The omission of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine from the complete reaction system decreased the hydroxylase activity by about one-third. The activity was considerably decreased by the omission of Fe^{2+} or the addition of $\alpha\alpha'$-bipyridyl (1 mM). The addition of ascorbic acid, NADPH or H_2O_2 (each 1 mM) did not affect the activity. Cu^{2+} (1 mM) also did not affect the activity. The activity was not stimulated by dithiothreitol (1 mM). The addition of *p*-chloromercuribenzoate (1 mM) caused 90% inhibition. The reaction did not proceed under anaerobic conditions.

Discussion

The biosynthesis of serotonin (5-hydroxytryptamine) involves as a first step the hydroxylation of L-tryptophan at the 5-position, followed by decarboxylation of 5-hydroxytryptophan. The enzyme that catalyses the second reaction has been extensively studied with various tissues (Awapara *et al.*, 1962; Lovenberg *et al.*, 1962; Christenson *et al.*, 1970).

The first reaction has been found in rat or guinea-pig intestinal mucosa (Cooper & Melcer, 1961), carcinoid tumour (Grahame-Smith, 1964*b*, 1967*b*; Lovenberg *et al.*, 1967), neoplastic mouse mast cells

(Hosoda & Glick, 1966; Lovenberg *et al.*, 1967), platelets (Lovenberg *et al.*, 1968) and brain (Grahame-Smith, 1967*a*, 1968; Lovenberg *et al.*, 1967; Green, 1968; Ichiyama *et al.*, 1970). Renson *et al.* (1962) reported that phenylalanine hydroxylase in rat liver could convert L-tryptophan into 5-hydroxy-L-tryptophan, and that tryptophan hydroxylation associated with phenylalanine hydroxylase probably was not of physiological significance with respect to overall serotonin biosynthesis in the intact animals.

Cooper & Melcer (1961) reported tryptophan hydroxylation activity in the intestinal mucosa of the rat or guinea-pig that required ascorbic acid and Cu^{2+}, but not oxygen. They proposed that this hydroxylation mechanism would be as follows. A hydride ion is released from the 5-position in the tryptophan ring to be replaced by an OH^- ion from water. The remaining two protons and two electrons would then reduce the actual electron acceptor in this hydroxylation, dehydroascorbic acid, to ascorbic acid. Dehydroascorbic acid could then be regenerated by the action of Cu^{2+} on ascorbic acid to form dehydroascorbic acid. It, however, is doubtful whether most of the serotonin synthesized in the intestine can be ascribed to the hydroxylating system described by Cooper & Melcer (1961), because their hydroxylation system seems to be non-enzymic from the cofactor requirement described above. Therefore, the small intestine was expected to have a relatively high activity of tryptophan 5-hydroxylase, exhibiting the character of the so-called 'aromatic ring hydroxylase'.

In the present investigation, tryptophan 5-hydroxylase was partially purified from the distal one-fourth of rat small intestines and its cofactor requirements were elucidated.

The semicarbazide experiments (Table 3) show that the enzyme preparation still included aromatic amino acid decarboxylase and monoamine oxidase. Therefore, the assay of tryptophan 5-hydroxylase was carried out by measurement of the rate of total 5-hydroxyindole formation. The enzyme required Fe^{2+}, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine and oxygen for full activity, but not Cu^{2+}. In the preliminary report (Noguchi *et al.*, 1971) we reported that most of the enzyme activity was recovered in the 5000*g* supernatant fraction of 0.25M-sucrose homogenate of rat small intestine. This cofactor requirement is similar to that of brain tryptophan 5-hydroxylase (Ichiyama *et al.*, 1970).

On the basis of the subcellular distribution, cofactor requirements and oxygen requirement of the enzyme preparation, it is obvious that tryptophan 5-hydroxylase found by us is completely different from the hydroxylating system described by Cooper & Melcer (1961).

Gal (1965) showed that homogenate of rat, pigeon and rabbit brain hydroxylated L-[^{14}C]tryptophan, but not D-tryptophan. In contrast, the hydroxylase in the intestine was not specific for L-tryptophan. The enzyme could also hydroxylate D-tryptophan to produce 5-hydroxy-D-tryptophan at about one-third the rate observed with L-tryptophan. L-Phenylalanine, however, did not serve as substrate, suggesting that the enzyme is different from rat liver phenylalanine hydroxylase (Kaufman, 1970).

On the other hand, the relatively low activity of the partially purified enzyme described here may be insufficient to account for the biosynthesis of all the serotonin contained in appreciable amounts in the intestine. However, this low activity may be due to the presence of an endogenous inhibitor in the intestine and the relatively high 5-hydroxytryptamine content of intestine may be due to a low rate of its turnover.

Grahame-Smith (1964*b*, 1967*b*) showed that in carcinoid tumour which arises from the enterochromaffin cells, there is a tryptophan-hydroxylating system that is dissimilar from the phenylalanine-hydroxylating system found in rat liver. A comparison of the hydroxylating system in carcinoid tumour with that in the intestine would be worthwhile.

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