Demonstration of non-neural tryptophan 5-mono-oxygenase in mouse intestinal mucosa

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Tryptophan 5-mono-oxygenase was demonstrated and its activity was measured in mucosal extracts of the mouse digestive tract by means of highly sensitive h.p.l.c. detection. The intestinal enzyme was activated by anaerobic incubation with dithiothreitol, as are the enzymes from mouse mastocytoma cells and bovine pineal gland. The dithiothreitol-enhanced activity was highest at the proximal portion of colon followed by that at the duodenum, where the unenhanced activity/enhanced activity ratio was highest. The enzymic and immunochemical properties of the intestinal tryptophan 5-mono-oxygenase were similar to those of the mastocytoma enzyme. In contrast, the intestinal enzyme was immunochemically different from brain tryptophan 5-mono-oxygenase. The possibility that connective tissue and/or mucosal mast cells are responsible for some of the enzyme activity of the duodenal mucosa was ruled out by the demonstration of the activity in extracts from a mast-cell-deficient mutant mouse (*W/W^v*). The enzyme in the duodenum was found to reside between the upper villus region and the bottom of the crypt, suggesting that it is mainly of enterochromaffin cell and not of submucosal nerve plexus origin.

INTRODUCTION

Tryptophan 5-mono-oxygenase (EC 1.14.16.4) catalyses the first step in the biosynthesis of serotonin (5-hydroxytryptamine, 5HT) from tryptophan. Since the first evidence for the existence of a specific tryptophan 5-mono-oxygenase involved in 5HT biosynthesis in mammalian brain [1], the enzyme has been extensively studied. Neoplastic tissues of peripheral origin such as mouse mastocytoma cells and carcinoid tumours from the digestive tract were employed as enzyme sources in early studies in order to elucidate the biosynthetic pathway of 5HT [2–5]. In 1982, tryptophan 5-mono-oxygenase was purified by Nakata & Fujisawa [6,7] to homogeneity from both rat brain and mouse mastocytoma cells and the purified enzymes were found to be different entities. However, 5HT biosynthesis in non-neonatal peripheral tissues has not been extensively studied. Among the peripheral 5HT-producing tissues, bovine pineal gland was the only organ from which tryptophan 5-mono-oxygenase was purified and the properties were studied [8,9]. The digestive tract has been known to be the major site for 5HT production in the periphery [10–12], and the conversion of tryptophan to 5HTP was demonstrated with cell-free extracts [13]. The digestive tract is an assembly of muscular tissues and a thin layer of mucosa; the latter is the major region for digestion and its hormonal regulation. Other than platelets, most cells which secrete 5HT are generally accepted to have the ability to synthesize 5HT. Various data suggest that the enterochromaffin cells are the major cells of 5HT production in the digestive organs. However, serotonergic neurons in the submucosal layer, as well as mast cells of both the mucosa and submucosa of rodents,

would also be expected to synthesize 5HT. Histochemical studies on the distribution of 5HT or on the uptake of its precursor, 5HTP, support these distributions; however, these observations do not necessarily comprise unequivocal evidence as to biosynthesis *de novo* of 5HT from tryptophan in these specific cells because these cells are able to incorporate exogenous 5HT or 5HTP which can be converted to 5HT by L-aromatic amino acid decarboxylase, a rather non-specific enzyme with respect to both substrate specificity and tissue distribution. The idea that peripheral 5HT production occurs mainly in enterochromaffin cells, and presumably in other 5HT-containing cells in the digestive organs, is a long-standing concept in gastroenterology. However, little is known about the distribution and localization of tryptophan 5-mono-oxygenase in the digestive organs, and our knowledge of the mechanisms involved in 5HT biosynthesis in these organs is still obscure.

In the present study, an assay method for tryptophan 5-mono-oxygenase in extracts from digestive organs is described. The method, an application of previously published procedures [14,15], has been adapted to the low specific activity of the gastrointestinal enzyme (roughly 2.5% of the activity in extracts from brain tissue) as well as the high endogenous 5HT content of digestive tissue (about 10-fold of that of whole brain). These factors necessitate a much greater selectivity in 5HTP determination than that available from previously established methods [16,17]. With the improved method, tryptophan 5-mono-oxygenase has been demonstrated in the stomach, small intestine, caecum and colon of mice. Distribution of the enzyme in the mucosal layer of the duodenum was examined and was found to be consistent with the concept that enterochromaffin cells, rather than

Abbreviations used: 5HTP, 5-hydroxy-L-tryptophan; 5HT, 5-hydroxytryptamine; BH₄, tetrahydrobiopterin; 6MPH₄, 6-methyltetrahydropterin; DTT, dithiothreitol; GRP, gastrin-releasing peptide.

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neurons or mast cells, are the major cells producing 5HT.

MATERIALS AND METHODS

Materials

Protease inhibitors, antipain, leupeptin, chymostatin and pepstatin (Peptide Institute, Osaka, Japan), NADH (Oriental Yeast Co., Tokyo, Japan), catalase (EC 1.11.1.6, Boehringer-Mannheim-Yamanouchi, Tokyo, Japan), 4-bromo-3-hydroxybenzoyloxyamine dihydrogen phosphate (NSD-1055; Smith and Nephew, U.K.) were purchased from the sources given in parentheses. Dihydropteridine reductase (EC 1.6.99.7) was purified from bovine liver as reported [18]. 5,6,7,8-(6R)-Tetrahydrobiopterin (BH_4) was synthesized from 7,8-dihydrobiopterin with dihydrofolate reductase as described [19]. 5,6,7,8-Tetrahydro-6-methylpterin (6MPH₄) was a generous gift from Dr. S. Matsuura, Department of Chemistry, College of General Education, Nagoya University, Nagoya, Japan. Aprotinin was kindly donated by Mochida Pharmaceutical Co., Tokyo, Japan. Organic solvents for the maintenance and analytical elution of the h.p.l.c. column were of h.p.l.c. grade and purchased from Wako Pure Chemical Industries, Osaka, Japan. The h.p.l.c. medium, FineSIL-C₁₈-5-T, was purchased from Japan Spectroscopic Co. (JASCO), Tokyo, Japan.

Several strains of mice (6–8 weeks) from inbred colonies DBA/2, NOD/shi and C57BL/6, were used randomly, unless otherwise noted. WBB6F1 (*W/W^v* and *+/+*) mice obtained by mating a *WB/Re-W/+* female with a *C57BL/6-W^v/+* male were donated by Dr. M. Nishimura, Institute for Experimental Animals, this university. Other animals were bred and kept in our laboratory. The digestive tracts were obtained from mice killed by cervical dislocation followed by bleeding from the carotid artery. The brains were obtained from the same animals and the brain stem was separated by removing the cerebrum and cerebellum. The digestive tracts were separated into the stomach, small intestine, caecum and colon. The digestive organs were opened longitudinally, washed with cold saline, blotted and then stored frozen at -80°C until use. Intestinal mucosa was prepared from the proximal one-third (duodenal region) of a fresh small intestine by scraping it with the edge of a glass slide. The mucosal scrapings collected from five mice (about 1 g) were combined and then stored at -80°C until use. Mouse mastocytoma P-815 cells were grown and harvested as described previously [20]. Mastocytoma tryptophan 5-mono-oxygenase was purified according to the method of Nakata & Fujisawa [7]. Anti-(tryptophan 5-mono-oxygenase) immunoglobulin was obtained by immunizing guinea pigs with the purified mastocytoma enzyme (50 μg , four times at 2-week intervals). The antiserum showed a single precipitin line with mastocytoma extract by Ouchterlony double immunodiffusion analysis. Goat serum against guinea pig IgG was a gift from Dr. H. Miyamoto, Department of Microbiology, Hamamatsu University. The antibodies were used after removal of blood 5HT by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (50% saturation) and gel-filtration on a Sephadex G-25 column equilibrated with 0.9% NaCl.

Tryptophan 5-mono-oxygenase assay

For the preparation of tissue extracts, the partially thawed tissues or mucosal scrapings were homogenized

on ice with a Potter–Elvehjem homogenizer and Teflon pestle in 2 vol. of an extraction medium consisting of 0.3 mM- $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 0.13 M-KCl, 0.5 mM-phenylmethanesulphonyl fluoride and 5 $\mu\text{g}/\text{ml}$ each of antipain, chymostatin, leupeptin, pepstatin, and aprotinin, in 20 mM-Hepes/NaOH (pH 7.0). The iron salt was dissolved in 10 mM-HCl and added just prior to use. The homogenate was centrifuged at 105000 *g* for 50 min at 5°C . A 1.5 ml portion of the supernatant was applied to a Sephadex G-25 column (PD-10, Pharmacia) equilibrated with 50 mM-Tris/HCl (pH 7.4) containing 0.1 M-KCl, 10% (v/v) glycerol and 0.05% (v/v) Tween 20 and eluted with the same buffer. The first 3 ml of effluent was discarded, and then 1.5 ml (designated ‘desalted extract’) was collected and used for the determination of tryptophan 5-mono-oxygenase activity. Anaerobic incubation for the activation of tryptophan 5-mono-oxygenase with DTT [21] and the incubation for the enzyme reaction were performed essentially as previously described [15]. The activation mixture (0.2 ml total volume) consisted of 0.1 M-Tris/acetate buffer (pH 8.1), 15 mM-DTT, 0.2 mg of catalase and the desalted extract in a Thunberg tube. The preincubation was carried out at 30°C for 20 min. The reaction mixture for the tryptophan 5-mono-oxygenase assay contained 0.1 M-potassium phosphate buffer (pH 6.8), 0.67 mM-L-tryptophan, 0.49 mM-6MPH₄, 0.33 mM-NADH, 1 mM-NSD-1055, an inhibitor of aromatic L-amino acid decarboxylase, 0.75 μg of dihydropteridine reductase, 0.15 mg of catalase, and the enzyme (activated or unactivated) in a final volume of 0.15 ml. Batches of L-tryptophan and 6MPH₄ were carefully selected, because some solutions of the substrate and cofactor showed the presence of contaminants which behaved similarly to 5HTP on h.p.l.c. The reaction was initiated by the addition of the enzyme, conducted at 30°C for the specified periods of time, and terminated by the addition of 15 μl of 60% (w/w) HClO_4 . After standing on ice for at least 10 min, the mixture was centrifuged at 3000 rev./min for 5 min. The reaction product (5HTP) in the supernatant fluid was determined by h.p.l.c.

High-performance liquid chromatography

The analytical h.p.l.c. system for the determination of 5HTP consisted of a pump (JASCO, model TriRotor-II), an analytical column (FineSIL-C₁₈-5-T, 4.6 mm $\times$ 250 mm) and a fluorescence monitor (JASCO, model FP-210 equipped with a 75 W xenon lamp; excitation, 302 nm; emission, 350 nm). Elution was performed with 40 mM-sodium acetate (adjusted to pH 3.5 with formic acid)/methanol/acetonitrile (40:4:3, by vol.) at a flow rate of 1.0 ml/min. The practical sensitivity of this system was 0.5 pmol of 5HTP when a 30 μl aliquot of the acid-soluble portion of the reaction mixture was injected for analysis. In certain experiments, the analytical system described in our previous paper [15] was used with slight modifications: slower elution (0.5 ml/min) with a column of length 125 mm and an injection volume of 90 μl .

Other methods

Immunoprecipitation of tryptophan 5-mono-oxygenase was performed as follows: the desalted extracts of mouse intestinal mucosa, brain stem, and mastocytoma P-815 cell were incubated with the anti-(tryptophan 5-mono-oxygenase) Ig for 1 h at room temperature. Goat

Fig. 1. H.p.l.c. for assay of tryptophan 5-mono-oxygenase in mouse intestinal extracts

The enzyme was extracted from the small intestine and small endogenous molecules were removed by gel filtration on a Sephadex G-25 column. The reaction of tryptophan 5-mono-oxygenase with the extracts (0.48 mg of protein/assay) was carried out after activation with DTT. A 30 μ l sample of the deproteinized reaction mixture was subjected to h.p.l.c. The details are given under 'Materials and methods'. The retention times are: 5-hydroxy-L-tryptophan (a), 5.6 min; 5-hydroxytryptamine (b), 7.6 min; L-tryptophan (c), 11 min. (a) zero-time; HClO_4 was added to the reaction mixture prior to the addition of the enzyme. The arrow indicates the elution position of 5HTP. (b) After reaction for 10 min in the absence of NSD-1055; a new peak due to 5HT (b) appeared with the consumption of 5HTP (a). (c) After 10 min reaction. (d) Authentic 5HTP, 6 pmol. Column; FineSIL-C₁₈-5-T (4.6 mm $\times$ 250 mm). Elution; a mixture of 40 mM-sodium acetate (pH 3.5, adjusted with formic acid)/methanol/acetonitrile (40:4:3, by vol.), 1 ml/min. Detection by fluorescence; excitation at 302 nm, emission at 350 nm.

anti-(guinea pig IgG) was then added and, after standing for an additional 1 h at room temperature, the mixture was centrifuged at 105 000 g at 5 $^{\circ}\text{C}$ for 50 min. The supernatant fluid was used for the assay of tryptophan 5-mono-oxygenase.

The activity of alkaline phosphatase (EC 3.1.3.1) was measured according to the method of Fujita *et al.* [22] using tissue homogenates as the enzyme source. Assay of thymidine kinase (EC 2.7.1.21) was performed according to Klemperer & Haynes [23] with [^3H]thymidine as the substrate and the desalted tissue extract as the enzyme. The radioactive product was isolated on DEAE-filter discs (Whatman, DE-81) as described by Breitman [24]. A GRP-like immunoreactive substance was measured by radioimmunoassay using an antiserum, R-6902, raised with a synthetic GRP [25].

Protein was measured by the method of Lowry *et al.* [26] with bovine serum albumin as the standard.

RESULTS

Assay of tryptophan 5-mono-oxygenase

A typical h.p.l.c. chromatogram for the DTT-activated intestinal tryptophan 5-mono-oxygenase assay is shown in Fig. 1. The practical detection limit for the measurement of 5HTP (as described in the Materials and methods section) corresponded to a conversion of about 0.002% of the initial tryptophan in the standard reaction mixture (Figs. 1a and 1b). In order to assay these samples, it was necessary to include NSD-1055, an inhibitor of aromatic L-amino acid decarboxylase (Figs. 1b and 1c). The minimum concentration for inhibiting the conversion of 5HTP to 5HT was 0.2 mM; thus a concentration of 1 mM was employed in the standard assay conditions. It was not possible to use the h.p.l.c. assay for tryptophan 5-mono-oxygenase in homogenates or crude extracts of the intestine, since they contained numerous fluorescent materials which were not separated from 5HTP on chromatography.

5HTP formation was proportional to the amount of the extracts added, up to two-thirds of the volume of the reaction mixture (150 μ l). The accumulation of 5HTP

under the standard assay conditions proceeded at a constant rate for about 10 and 20 min with the activated and unactivated enzymes, respectively.

Activation by anaerobic incubation with DTT [21] was observed with the intestinal enzyme. The reactions with both the activated and unactivated enzyme were performed without the addition of Fe^{2+} . If the iron salt was added, non-enzymic conversion of tryptophan to 5HTP-like fluorescent material which could not be separated from authentic 5HTP on the chromatography was often observed. It is known that the non-enzymic hydroxylation of aromatic amino acids is effected by atmospheric O_2 in aqueous solution in the presence of tetrahydropterin cofactor and iron salt [27,28]. At concentrations of Fe^{2+} higher than 100 μM , the rate of the non-enzymic reaction was occasionally comparable with or higher than that catalysed by the enzyme from the digestive tracts, though it was negligible as compared with the enzymic rate catalysed by brain or mastocytoma extracts of high activity. However, the formation of 5HTP catalysed by the desalted intestinal extracts was not influenced by exogenous iron in the reaction mixture, and the non-enzymic formation of the 5HTP-like material was not observed in the absence of iron.

Measurement of unactivated tryptophan 5-mono-oxygenase

The DTT-enhanced activity of tryptophan 5-mono-oxygenase of the intestinal mucosa extracts was 7.42 ± 2.25 pmol/min per mg of protein. Without activation, the enzyme activity of the extracts prepared in the absence of Fe^{2+} varied greatly; the ratio of the unenhanced activity to the enhanced activity ranged from 0.03 to 0.35 (0.14 ± 0.11 , mean $\pm$ S.D., $n = 7$).

In the case of mastocytoma tryptophan 5-mono-oxygenase, the unenhanced activity was very unstable, but it was stabilized by the addition of 100 μM - Fe^{2+} to the homogenization medium [15]. Based on the above observations, the effect of iron on the unactivated intestinal enzyme was examined. Higher unenhanced activity was seen when the mucosa was homogenized in the presence of Fe^{2+} , whereas the DTT-enhanced activity remained almost constant, irrespective of the presence or absence

Table 1. Requirements of the intestinal tryptophan 5-mono-oxygenase reaction

A desalted extract was prepared from the mucosa of the top one-third of the mouse small intestine. The procedures for the enzyme activation and the reaction were essentially as described under 'Materials and methods'. Anaerobic conditions were obtained by the use of Thunberg tubes. The omission of the tetrahydropterin cofactor was achieved through eliminating both 6MPH₄ and its regenerating system (NADH and dihydropteridine reductase). The concentration of D-tryptophan used in place of L-tryptophan was 0.67 mM. The boiled enzyme was prepared by heating the desalted extract in a boiling-water bath for 5 min. Further details of the experimental procedure are given in the text.

Replacement	Tryptophan 5-mono-oxygenase activity* (pmol/min per mg of protein)	
	DTT-enhanced	Unenhanced
None	4.99 (100)	1.59 (100)
– O ₂	0 (0)	0.06 (3.8)
– Pterin	0 (0)	0.06 (3.8)
D-Tryptophan	0.56† (11.6)	0.04† (2.3)
Boiled enzyme	0.13 (2.7)	0.06 (3.8)

* Numbers in parentheses represent the percentages of the respective activity relative to that of the complete system.

† The h.p.l.c. system could not separate D-5HTP from L-5HTP, and so the reaction product was not identified.

of Fe²⁺. Furthermore, the ratio of the unenhanced activity to the DTT-enhanced activity increased as the concentration of Fe²⁺ in the homogenization medium was raised. The increase was saturated at concentrations of Fe²⁺ higher than 0.3 mM giving a ratio of about 0.4. This effect of iron was not seen when Fe²⁺ was added to the assay mixture rather than during the homogenization

procedure. It was also noted that the presence of Fe²⁺ in the homogenization medium only was sufficient for a reproducible ratio of the unenhanced activity/enhanced activity in the desalted extract; the omission of Fe²⁺ from the gel filtration procedure did not significantly affect the ratio. Therefore, the unenhanced activity was measured with extracts prepared in the presence of 0.3 mM-Fe²⁺ which was subsequently removed by gel filtration. The optimum pH for the reaction with either activated or unactivated enzyme was 6.7–7.0.

Requirements for the intestinal tryptophan 5-mono-oxygenase reaction

In order to make certain that the conversion of tryptophan to 5HTP in mucosal extracts was due to the action of tryptophan 5-mono-oxygenase, the requirement of the reaction for tetrahydropterin, L-tryptophan and molecular oxygen [29] was examined. Anaerobic conditions for measurement of the O₂ requirement were obtained by using a Thunberg tube with a side chamber and a vacuum line connected to a nitrogen container. The reaction mixture and mucosal extracts were placed separately in the main and side chambers of the Thunberg tube, respectively, and then the air in the tube was replaced with nitrogen gas three times [30]. For the experiment with the activated enzyme, preincubation was carried out in the side chamber. The reaction was initiated by mixing the two solutions. Significant accumulation of 5HTP was not observed under anaerobic conditions with either the activated or unactivated enzyme. On omission of the tetrahydropterin cofactor and its regenerating system, no 5HTP was formed. When D-tryptophan was used instead of L-tryptophan, no significant production of 5HTP was detected with the unactivated enzyme, but a small amount of a 5HTP-like substance was formed after 5 min incubation with the activated enzyme. The results are summarized in Table 1.

The dependence of enzyme activity on the concen-

Fig. 2. Dependence of tryptophan 5-mono-oxygenase activity on the concentration of tryptophan

Extracts from mouse small intestine were used as the enzyme after gel filtration through a Sephadex G-25 column. (a) Initial velocity (*v*) versus tryptophan concentration (*[S]*). (b) Eadie-Hofstee plot; *v* versus *v/[S]*. Correction for the presumed carry-over of protein-bound tryptophan into the reaction mixture was made as described in the text. Cofactors used were 400 μM-6MPH₄ (○) and 367 μM-(6R)-BH₄ (●).

Fig. 3. Immunoprecipitation of the intestinal tryptophan 5-mono-oxygenase by an antibody raised against the mouse mastocytoma enzyme

Extracts were prepared from mouse brain stem, mastocytoma P-815 cell pellet, and intestinal mucosa as described under 'Materials and methods'. The extracts of the brain and mastocytoma cells were diluted 20- and 700-fold, respectively, with the homogenization medium so as to make their tryptophan 5-mono-oxygenase activities nearly equal. Guinea pig Ig against the mastocytoma tryptophan 5-mono-oxygenase and goat Ig against guinea pig IgG were used as the primary and secondary antibodies, respectively. Three volumes of the secondary goat Ig (93 mg/ml) effectively precipitated the guinea pig Ig (28 mg/ml). Guinea pig Ig from an untreated animal (28 mg/ml) was used as a control. The immunoprecipitation was carried out with 200 μ l of the extracts and the indicated volumes of the primary Ig or the control Ig as described under 'Materials and methods'. (a) Extracts of the intestinal mucosa; (b) mastocytoma extracts; (c) extracts of the brain stem. $\circ$, control guinea pig Ig; $\bullet$, guinea pig anti-(mouse mastocytoma tryptophan 5-mono-oxygenase) Ig.

tration of tryptophan was examined with the desalted extract prepared from whole small intestine. The plot of the reaction velocity against the tryptophan concentration with 400 μ M-6MPH₄ as the coenzyme showed a typical hyperbolic curve, but significant 5HTP formation

was detected even without the tryptophan addition, since desalted extract may have contained a significant amount of bound tryptophan [31]. Extrapolation of the plot of initial velocity versus tryptophan concentration to zero indicated that 2–5 μ M-tryptophan may have been carried over in the reaction mixture. After appropriate correction for the above concentration range, the Eadie-Hofstee plot of the results gave straight lines, as shown in Fig. 2(b). Correction for over- or under-estimation by 1 μ M resulted in a convex or concave line, respectively, for these plots. The enzyme was inhibited by tryptophan at concentrations higher than 200 μ M, when the natural coenzyme, (6R)-BH₄ [32], was used as the cofactor. The apparent K_m values of the DTT-activated enzyme for L-tryptophan were 41 and 38 μ M with 6MPH₄ (400 μ M) and (6R)-BH₄ (367 μ M) as the co-enzymes, respectively. Those of the unactivated enzyme were 41 and 45 μ M with the same concentrations of 6MPH₄ and (6R)-BH₄, respectively. The maximum velocities with the DTT-activated enzyme were 5.92 and 4.44 pmol/min per mg with 6MPH₄ and (6R)-BH₄ as the coenzymes, respectively. The BH₄-dependent activity of the unactivated enzyme (apparent V_{max} , 0.40 pmol/min per mg) was 41% of its 6MPH₄-dependent activity (apparent V_{max} , 1.08 pmol/min/mg). The unenhanced activity/enhanced activity ratio was 0.11 with (6R)-BH₄ as the coenzyme, whereas it was 0.18 with 6MPH₄. In the determination of dependence on tetrahydropterins, the double-reciprocal plots gave straight lines representing K_m values of 50–150 μ M with either 6MPH₄ or (6R)-BH₄ (the correction for the presumable carry-over of tetrahydropterin was not successful).

Immunochemical identification of intestinal tryptophan 5-mono-oxygenase

The cross-reactivity of the antibody prepared against mastocytoma tryptophan 5-mono-oxygenase with the intestinal enzyme was examined by means of immunoprecipitation as described in the Materials and methods section. Extracts were prepared from duodenal mucosa, brain, and mastocytoma. In order to obtain nearly the same concentration of the enzyme in the mixtures, the mastocytoma and brain extracts were diluted 700- and 20-fold, respectively, before incubation with the anti-

Table 2. Distribution of tryptophan 5-mono-oxygenase in the digestive tract of mice

Organ	Wet wt.* (mg)	Enzyme activity† (pmol/min per mg of protein)		
		Enhanced	Unenhanced	Ratio‡
Stomach	171	2.29 ± 0.57	0.61 ± 0.26	0.25 ± 0.06
Small intestine				
Proximal	440	5.42 ± 0.24	1.26 ± 0.14	0.21 ± 0.02
Middle	416	2.29 ± 0.34	0.29 ± 0.04	0.13 ± 0.04
Distal	239	1.53 ± 0.21	0.21 ± 0.05	0.14 ± 0.03
Caecum	162	3.13 ± 0.11	0.24 ± 0.02	0.08 ± 0.01
Colon				
Proximal	210	8.74 ± 0.40	0.63 ± 0.11	0.07 ± 0.02
Distal	133	3.47 ± 0.38	0.39 ± 0.17	0.11 ± 0.04

* Mean for 12–17 organs.

† The activity is expressed as specific activity over extracted protein, mean ± s.d. for three to five experiments; five or six organs were pooled for each experiment.

‡ Unenhanced activity/DTT-enhanced activity.

Table 3. Characterization of the mechanically separated surface and inner layers of the duodenal mucosa

The surface and inner layers of the mucosa were collected by scraping the top one-third of the mouse small intestine with the edge of a glass slide as described in the text. The surface and inner layers obtained from 10 mice weighed 2.04 and 0.625 g in experiment A, 1.66 and 0.727 g in experiment B, respectively. Radioimmunoassay of GRP-like immunoreactivity and the assay of alkaline phosphatase, thymidine kinase and the DTT-activated tryptophan 5-mono-oxygenase were performed as described under 'Materials and methods'. One unit of enzyme activity was defined as the amount that catalysed the conversion of 1 nmol of the substrate/min under the standard assay conditions. One unit of immunoreactivity was defined as 1 ng/g wet wt. equivalent to the authentic peptide. The enzyme activity was expressed as units/g wet wt. The values in parentheses are the respective activities expressed as percentages of the total activity.

Marker	Layer ... Expt. ...	Enzyme or immunoreactive activity (units)			
		Surface		Inner	
		A	B	A	B
Alkaline phosphatase		148 000 (96.5 %)	135 000 (93.8 %)	5360 (3.5 %)	8870 (6.2 %)
Tryptophan 5-mono-oxygenase		0.583 (85.9 %)	0.554 (77.5 %)	0.0956 (14.1 %)	0.161 (22.5 %)
Thymidine kinase		0.927 (46.9 %)	0.743 (25.2 %)	1.05 (53.1 %)	2.20 (74.8 %)
GRP		1.57 (29.1 %)	0.795 (24.6 %)	3.83 (70.9 %)	2.44 (75.4 %)

body. As shown in Fig. 3, the enzyme showed similar precipitation profiles. Thus the intestinal enzyme shares common antigenic character with the mastocytoma enzyme. In contrast, the enzyme activity in the extract from mouse brain was not significantly precipitated by the antibody, indicating that the enzyme protein is a different entity from that of the mastocytoma and intestinal enzymes.

Distribution of tryptophan 5-mono-oxygenase in the digestive tract

The digestive organs were isolated from NOD/shi mice, and the tryptophan 5-mono-oxygenase activity was measured with desalted extracts prepared from whole organs (Table 2). The highest specific activity was observed in the proximal portion of the colon. However, this region had a lower ratio of the unenhanced/enhanced activities compared with those in the stomach and duodenum.

Although the 5HT content in the digestive organs has been shown to be much higher than that in brain [33], the enzyme activity of the intestine was very low when compared with that of brain. The enzyme activities, for example, in extracts from brain stem, cerebrum and the top 1 cm of the small intestine were 59.0, 13.9 and 1.36 pmol/min per mg of protein, respectively (pooled tissue from five C57BL/6 mice was used).

Intestinal tryptophan 5-mono-oxygenase in mast-cell-deficient mouse

In order to determine the contribution of mast cells to the intestinal tryptophan 5-mono-oxygenase activity, we compared the *W/W^v* mutant of WBB6F1 mice with control (+/+) mice. The mutant mice are severely deficient in mast cells [34]. Duodenal mucosae were obtained from both the *W/W^v* mutant mice and the control (+/+) mice, and desalted extracts were prepared separately. Tryptophan 5-mono-oxygenase activity was

measured with and without the anaerobic preincubation with DTT. The DTT-enhanced activities of the mutant mice and the control mice were 4.41 ± 0.13 and 4.81 ± 0.19 pmol/min per mg (means $\pm$ S.D., four sets of experiments with pooled tissue), respectively. In both the mutant mice and the control mice, the unenhanced activities were 35–40 % of the respective enhanced activities. Therefore, the extracts from the two types of mice were almost the same with respect to the tryptophan 5-mono-oxygenase activity. This suggested that mast cells are not significantly responsible for the tryptophan 5-mono-oxygenase activity, at least in the duodenal mucosa.

Location of tryptophan 5-mono-oxygenase in the mucosal layer

Taking the topological architecture of intestinal mucosa into account, we tried to divide the duodenal mucosa mechanically into two fractions. The lumen-side surface of the duodenum was scraped as evenly as possible with the edge of a glass slide. Then the residual sheet of the intestinal tissue was thoroughly scraped. These scrapings are referred to as the surface and inner layers, respectively. The collected inner-layer mass comprised about one-third of that of the surface layer in wet weight. Desalted extracts were prepared separately for the two layers as described under 'Materials and methods'. Alkaline phosphatase, thymidine kinase and GRP were measured as markers of epithelial cells of the upper villus region [35], mitotically active cells of the crypt [36], and submucosal nerve plexuses [25], respectively. An established marker of porcine enterochromaffin cells, Met-enkephalin-8 (Met-enkephalin-Arg-Gly-Leu-OH) [37], was also measured, but the immunoreactive substance was not detected in our mouse intestinal preparation (results not shown). The experimental results are shown in Table 3. In experiment A, the surface and inner layers obtained from 10 mice weighed 2.04 g (76.5 % of total) and 0.63 g (23.5 %), respectively.

The alkaline phosphatase activity was 148 units (96.5% of the total activity) in the surface layer and 5.4 units in the inner layer, indicating that villi had been scraped off effectively into the surface layer. Approx. 70% of the GRP-like immunoreactivity was detected in the inner layer, an observation that indicates that most enteric nerve plexuses were included in the inner-layer mass. On the other hand, thymidine kinase activity was detected almost equally in the two layers. It was therefore judged that the mucosa was separated into two layers at the level of the bottom zone of the crypt, an active region in cell proliferation. We found 86% of the tryptophan 5-mono-oxygenase activity to be in the surface layer. The activity of tryptophan 5-mono-oxygenase in the surface layer was 10% less than that of alkaline phosphatase and 35% more than that of thymidine kinase. When the surface layer was scraped less extensively (experiment B, weight 69.5% of total tissue), thymidine kinase activity in the surface layer decreased to 25% of the total, while the distribution in the surface layer of alkaline phosphatase activity and GRP-like immunoreactive substance was only 2.5% and 4.6% less than that in experiment A. The distribution of tryptophan 5-mono-oxygenase in the surface layer was 8.5% less than that in experiment A. These observations were consistent with the condensed distribution of thymidine kinase around the bottom zone of the crypt [36], and indicated that tryptophan 5-mono-oxygenase is located above the thymidine kinase zone. In both experiments A and B in Table 3, the recovery in the surface layer of tryptophan 5-mono-oxygenase was approx. 10% less than that of alkaline phosphatase, suggesting that the former distributes in the deeper part of mucosa than the latter, although possible overlap of their distribution could not be excluded from these results. The location of tryptophan 5-mono-oxygenase could therefore be around the trunk and the opening of the crypts (a region of endocrine cells).

DISCUSSION

Tryptophan 5-mono-oxygenase was measured in extracts from mouse digestive organs. For the assay of the intestinal enzyme of low specific activity, the h.p.l.c. method [14,15] was adapted by improving its sensitivity in combination with pretreatment of the sample extracts to remove free fluorescent materials. Quantification of the enzyme in fresh homogenate with the present method was unsuccessful due to the endogenous fluorescent materials. Most procedures used to measure tryptophan 5-mono-oxygenase from various sources include pre- or co-incubation of the enzyme with a relatively high concentration of mercaptides to stabilize both the enzyme and tetrahydropterin cofactor [7,16,36–38]. The mercaptides may cause activation of the enzyme but only partially with these methods, since the activation can be fully attained under anaerobic incubation with mercaptides [21]. In our assay method, full activation of the enzyme was attained by anaerobic preincubation with DTT to give the DTT-enhanced activity and the cofactor was regenerated by use of dihydropteridine reductase and NADH. We showed previously that the unenhanced activity of the enzyme (the activity without preincubation with DTT) in extracts of mouse mastocytoma cells was stabilized when the cell extracts were prepared in the presence of $100\text{ }\mu\text{M-Fe}^{2+}$ [15]. In the case of mouse intestinal tryptophan 5-mono-oxygenase, addition of Fe^{2+} at $300\text{ }\mu\text{M}$ during homogenization gave reproducible

assay of the activity. These observations allowed us the reproducible assay of both DTT-enhanced and unenhanced activity of gastrointestinal tryptophan 5-mono-oxygenase.

In the previous work [15,41] where attention was first directed to the unenhanced activity, the DTT-enhanced activity was referred to as the 'full-potential activity' and the unenhanced activity as the 'native activity'. The significance of the unenhanced and enhanced activities is not yet fully understood, but the native activity was expected to provide more intimate information on the enzyme *in vivo*. Furthermore, the ratio of the unenhanced activity to the enhanced activity differed greatly between the two mouse mastocytoma cell lines used [15]. In the present study, the unenhanced activity/DTT-enhanced activity ratio was higher in the top portion of small intestine, the duodenum, than that in the caecum and colon (cf. Table 2). The effect of iron added during homogenization on the unenhanced activity was apparently similar between mastocytoma and intestinal tryptophan 5-mono-oxygenase. Based on the similarities, we assumed that the unenhanced activity in the homogenate from digestive tract is unstable when the homogenate is prepared without addition of Fe^{2+} . However, this assumption has not been proven because of the difficulty in measurement of the activity immediately after the tissue homogenization (i.e. without gel filtration of tissue extracts). A combination of the h.p.l.c. separation with the use of radioisotope-labelled substrate, L-[3- ^{14}C]-tryptophan ($1\text{ }\mu\text{Ci/assay}$), did not produce any improvement in the quantification of 5HTP formed, because of radiochemical contamination in fractions of carrier 5HTP collected from the h.p.l.c. eluate (results not shown).

To our knowledge, this is the first report of the quantitative measurement of tryptophan 5-mono-oxygenase activity in various parts of digestive tract. In the previous work, we reported the distribution of 5HT in mouse organs [33]. The amount of 5HT represents the pool size, but not necessarily the synthetic activity. Blood platelets, for example, contain a large amount of 5HT (about $4\text{ }\mu\text{g/ml}$ of blood in mouse), but have little or no ability to synthesize 5HT. Platelets are believed to acquire 5HT by uptake, mainly of 5HT released into the blood by the intestinal enterochromaffin cells. Mouse brain contains only $0.5\text{--}0.7\text{ }\mu\text{g}$ of 5HT/g wet weight (whole brain), despite the high activity of tryptophan 5-mono-oxygenase in this tissue. The DTT-enhanced activity of tryptophan 5-mono-oxygenase in the mucosa of the top portion of the mouse small intestine was $4\text{--}10\text{ pmol/min per mg}$ of protein, which is about $1/700$ of that in mastocytoma cells or about $1/20$ of that in mouse brain stem (cf. Table 2 and Fig. 3). Among the digestive organs, the stomach had the highest 5HT content ($6\text{--}10\text{ }\mu\text{g/g}$) but the enzyme activity was only about 40% of that in the small intestine on the basis of extractable proteins (Table 2).

We wanted to determine which cells were the origin of the tryptophan 5-mono-oxygenase. The candidate cells in mouse digestive organs are: (1) enterochromaffin cells scattered in the mucosal epithelium, (2) mast cells in connective tissues, and those termed 'mucosal mast cells' located between the basement membrane of the epithelium and the muscularis mucosae, and (3) serotonergic neurons of nerve plexuses.

In rodents such as mouse and rat, mast cells contain

5HT. Since mast cells are widely distributed in various organs, some of the tryptophan 5-mono-oxygenase activity detected in the mouse digestive organs could be of mast cell origin. Considerable tryptophan 5-mono-oxygenase activity was detected in the duodenal mucosa. However, previous work on the contribution of mast cells to the distribution of 5HT in various organs of mice [33] suggested that most of the 5HT detected in the small intestine was not due to the mast cells. Furthermore, the duodenal mucosa of mast-cell-deficient mutant mice (*W/W^v*) showed essentially the same enzyme activity as did that of the control mice (+/+). These results indicate that the major portion of the enzyme measured in the duodenum was of non-mast-cell origin. However, it should be noted that this could not be generalized to all digestive organs. In the mouse stomach, for example, where about 75% of total 5HT was estimated to be of the mast cells [33], more than half of the enzyme activity appeared to be of mucosal mast cells [42].

Most of the tryptophan 5-mono-oxygenase in mucosal extracts was presumed not to be due to the enteric nerve plexuses, because the intestinal tryptophan 5-mono-oxygenase was precipitated by a polyclonal antibody raised against the mastocytoma enzyme (Fig. 3). In contrast, the enzyme of nerve plexus has been shown to cross-react with an antibody against the brain enzyme [43]. Furthermore, the tissue fractionation achieved by means of differential scraping revealed that the topological distributions in the duodenum of the tryptophan 5-mono-oxygenase and GRP, a marker of myenteric nerve plexus [25], were quite different (Table 3). Enterochromaffin cells are known to be located mainly on the epithelial walls of the crypts, roughly between the upper villus region and the bottom of the crypts. Although the method employed for fractionation was primitive compared with the published method [44], the results were very consistent with the location of the enzyme in this region. These results, however, do not rule out biosynthesis *de novo* of 5HT in neurons of nerve plexus which may utilize 5HT in regulating peristalsis of digestive tract.

It should be noted that tryptophan 5-mono-oxygenases in different organs, at least in the intestine and brain, are different entities; in the case of tyrosine 3-mono-oxygenase, another tetrahydropterin-dependent mono-oxygenase, the identity between the enzymes of brain and of adrenal medulla has been accepted so far. Non-identity between the enzymes from mastocytoma cells and brain has previously been suggested; Kuhn *et al.* compared the catalytic properties of partially purified enzymes from the two origins [45]. Nakata & Fujisawa reported the different molecular characteristics of the enzymes purified to homogeneity from the two sources [6,7]. The tryptophan 5-mono-oxygenase in the intestinal extracts shared common characteristics with the mastocytoma enzyme, such as activation by DTT, substrate dependence, and antigenicity (Fig. 3). This similarity, as a whole, was in agreement with work by Hosoda *et al.* on the enzymes from mouse mastocytoma and a human gastric carcinoid tumour [40,46]. The enzymes from mastocytoma and from intestinal extracts could be the same and these enzymes can be categorized as a peripheral type or non-neural type.

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