COMPARISON OF SEROTONIN-PRODUCING MURINE MASTOCYTOMAS, P-815 AND FMA3: DETERMINATION OF TRYPTOPHAN HYDROXYLASE, AROMATIC L-AMINO ACID DECARBOXYLASE, AND CELLULAR CONCENTRATION OF TRYPTOPHAN, 5-HYDROXYTRYPTOPHAN, 5-HYDROXY-TRYPTAMINE AND 5-HYDROXYINDOLEACETIC ACID

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ABSTRACT

5-Hydroxytryptamine (serotonin), 5-hydroxytryptophan, tryptophan, and 5-hydroxyindoleacetic acid were determined together with the enzymic activity of tryptophan hydroxylase and aromatic L-amino acid decarboxylase in a comparative way with two lines of transplantable mouse mastocytoma, P-815 and FMA3. These cells had been maintained more than 10 years in our laboratories. 5-Hydroxytryptamine and the other related compounds were determined simultaneously by high performance liquid chromatography (HPLC) with fluorescence detection. The HPLC was also used in the assay of tryptophan hydroxylase and aromatic L-amino acid decarboxylase. For the comparison of tryptophan hydroxylase between the two cell lines, the activity was measured both with and without an *in vitro* activation by anaerobic preincubation with dithiothreitol. Both lines of the cell contained a large amount of serotonin (1.27 and 0.24 nmol/10⁶ cells in FMA3 and P-815, respectively). The tryptophan hydroxylase activity of FMA3 cells was about one third of that of P-815 when the activity was measured after the activation with dithiothreitol. The activity measured without the activation was comparable in these two cells (0.64 and 0.95 nmol/min/mg protein in FMA3 and P-815, respectively). The biosynthesis of serotonin in P-815 cells was almost completely blocked at the decarboxylation step; the decarboxylase activity of P-815 cells was only 1/400 of FMA3 cells (0.0043 vs. 1.53 nmol/min/mg protein).

Murine mast cells, either normal or neoplastic, produce and store 5-hydroxytryptamine (5HT, serotonin), histamine and heparin. In 1957, two transplantable tumor lines were established, one in DBA mice by Dunn and Potter, the P-815

line (4), and the other in LAF1 mice by Furth, Hagen, and Hirsch, the F-line (5), after a friendly contest of the two groups (3). These lines are now readily available on a worldwide scale. Extensive studies using these mastocytomas have been done on the biosynthesis, storage, and liberation of these substances. Of these, the most actively studied biochemical subject in the

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1960s was tryptophan hydroxylase (L-tryptophan, tetrahydropterin: oxygen oxidoreductase (5-hydroxylating), EC 1.14.16.4), because there was no other satisfying mammalian model for the study of this enzyme at that time. The enzyme catalyzes the first reaction in 5HT biosynthesis in two steps, the hydroxylation of tryptophan and decarboxylation of the resulting 5-hydroxytryptophan (5HTP). The purification of tryptophan hydroxylase was first achieved with P-815 line (11, 14, 21), and very recently the enzyme has been purified to homogeneity from the same tumor line and characterized (18).

On the other hand, it is puzzling that no work has been published thus far on tryptophan hydroxylase from the F-line, though this tumor line was found to contain more 5HT than the P-815 line (22). At present, therefore, data are still required on the relative content of such 5-hydroxyindoles as 5HTP, 5HT, and 5-hydroxyindoleacetic acid (5HIAA) and on the activity of enzymes such as tryptophan hydroxylase and aromatic L-amino acid decarboxylase (5HTP decarboxylase) in the two tumor lines, which have been transplanted for over 20 years. This study was undertaken to determine the precise quantities of above-mentioned substances and enzymes by the newly developed high performance liquid chromatographic (HPLC) technique (23). The results obtained indicate that the F-line retains considerably higher activity of 5HTP decarboxylase than the P-815 line, though the activity of tryptophan hydroxylase in the F-line approximates to that in the P-815 line.

MATERIALS AND METHODS

Cell Lines

Two cloned cell lines of murine mastocytoma, FMA3 and P-815, were used. The FMA3 is one of the cloned cell lines obtained from the cell line FMA. The origin and biological characteristics of the FMA line were described previously (17). Briefly, it was established in the laboratory of one of us from a solid tumor of Furth's mastocytoma cells (F-line) of LAF₁ mice (5) by adapting the cells to grow in the ascites prior to transplantation to an *in vitro* culture. Single cell suspension of FMA cells was plated on agar plates. Several colonies were separated and tested for growth rate and the ability to synthesize 5HT *in vitro* and *in vivo*. The FMA3 was the cell line that was most active in producing 5HT among those obtained. When inoculated into the abdominal cavity of LAF₁ mice, its doubling time was 20.5 h at the

cell density of around 10^8 cells/mouse. The P-815 derived from the Dunn-Potter's mastocytoma has been carried in DBA/2 mice in our laboratories (9, 23).

The mastocytoma cells were harvested from the peritoneal cavity of the tumor-bearing mice on the 7th day after transplantation ($1-3 \times 10^6$ cells/mouse), as described previously (23).

The inbred mice, C57L, were donated by Dr M. Nishimura of the Institute for Experimental Animals of our University. DBA/2 and A/J mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu.

HPLC System

The analytical HPLC system was constructed of a pump (JASCO, model TriRotor-II pump, Japan Spectroscopic Co., Tokyo), an injector (Rheodine, Model 7125), an analytical column (FineSIL C₁₈-5-T or FineSIL NH₂-5), a fluorescence monitor (excitation, 302 nm; emission, 350 nm; JASCO, Model FP-110 equipped with a phosphor coated Hg-lamp, L-1549-03-302, Hamamatsu Photonics, Hamamatsu), and a two-pen recorder (JASCO, Model RC-228).

For the determination of 5HTP in the tryptophan hydroxylase assay, a column of FineSIL C₁₈-5-T (4.6 $\times$ 50 mm) was used. The elution was performed with a mixture of Na-acetate (pH 4.0, 40 mM with respect to Na⁺): methanol (4:1) at a flow rate of 1.2 ml/min. A typical elution profile is shown in Fig. 1. For the assay of 5HTP decarboxylase, FineSIL NH₂-5 (4.6 $\times$ 50 mm) was used and elution was performed with 5 mM NaH₂PO₄: methanol (5:1) at 1 ml/min. In the analysis of tissue metabolites, a longer column of FineSIL C₁₈-5-T (4.6 $\times$ 125 mm) was employed, and the elution was carried out with the Na-acetate/methanol solution at a flow rate of 1 ml/min. The chromatogram of analytical elution is presented in Fig. 2.

Chemicals

Organic solvents for the maintenance and analytical elution of the HPLC columns were HPLC-grade purchased from Wako Pure Chemical Industries, Osaka. Buffers for the elution were filtered through a Millipore filter (pore size, 0.45 micrometer). The HPLC media, FineSIL-C₁₈-5-T and FineSIL-NH₂-5, were purchased from JASCO. NADH (Oriental Yeast Co., Tokyo), catalase (Boehringer-Mannheim-Yamanouchi, Tokyo), 4-bromo-3-hydroxybenzylamine di-

Fig. 1 5HTP determination by HPLC in the assay of tryptophan hydroxylase. The reaction of tryptophan hydroxylase was carried out with extracts from P-815 and FMA3 cells (21.4 and 62.6 μ g protein, respectively) as described under Materials and Methods. After the termination of the reaction a 30 μ l-aliquot of deproteinized supernatant was used for the analysis of 5HTP by HPLC. In the case of P-815 cells (a and b), endogenous 5HTP was detectable in the zero-time reaction ($\blacktriangledown$ in a). In order to measure 5HTP formed in the reaction, the value of the zero-time control ($\blacktriangledown$ in a) was subtracted from that of the 5-min reaction ($\blacktriangledown$ in b). In FMA3 cells, the zero-time value ($\blacktriangledown$ in c) was also used as a blank to correct the 5-min value ($\blacktriangledown$ in d). Endogenous 5HT (*) did not interfere with the height reading of 5HTP which appeared in front of 5HT. The peak height of endogenous 5HT was not significantly different between zero-time and 5-min reaction mixtures on separation by a longer column system (not shown). Tryptophan used as substrate appeared behind 5HT as a huge peak. Column, FineSIL-C₁₈-5-T (4.6 $\times$ 50 mm); Elution, Na-acetate (pH 4, 40 mM with respect to Na⁺); methanol (4 : 1) at a flow rate of 1.2 ml/min; Fluorescence detection, 302 nm for excitation and 350 nm for analysis.

hydrogen phosphate (NSD-1055, Nakarai Chemicals, Kyoto), and *N*-methyl-*N*-2-propynylbenzylamine (pargyline, Regis Chem. Co., Chicago) were purchased from the sources listed in parentheses. Dihydropteridine reductase was purified from the bovine liver as reported previously (7). 5,6,7,8-Tetrahydro-6-methylpterin (6MPH₄) was a generous gift from Dr S. Matsuura, Nagoya University, Nagoya.

Fig. 2 Typical chromatogram in metabolite measurement. The whole ascitic fluid from P-815-bearing mouse was collected and separated into cell-free fluid and the cells. Extraction was performed as described under Materials and Methods. A 30 μ l-aliquot of the sample solution was injected into the HPLC system equipped with a FineSIL-C₁₈-5-T column (4.6 $\times$ 125 mm). The elution was carried out with Na-acetate (pH 4, 40 mM with respect to Na⁺); methanol (4 : 1) at a flow rate of 1 ml/min. a: Cell-free ascitic fluid. b: Cell pellet. The numbers in the figure represent 5HTP (1), 5HT (2), tryptophan (3), and 5HIAA (4). When the peak was out of the normal scale as 5HT in b, simultaneous recording with one-tenth of normal full scale output (not shown) was employed for the peak height reading.

5,6,7,8-Tetrahydro-(6R)-biopterin ((6R)-BH₄) was prepared by the enzymic reduction of 7,8-dihydrobiopterin as described (16). Pyridoxal 5'-phosphate was a gift from Yamanouchi Pharmaceutical Co., Tokyo. All other chemicals were of analytical grade from Nakarai Chemicals or Wako Pure Chemical Industries. Standard solutions of tryptophan and 5HIAA were prepared in distilled water and those of 5HTP and 5HT in 0.1 N HCl. They were stored at -80°C and renewed every month.

Assay Procedures

The sample preparation for the enzymic study and for the HPLC analysis of cell extracts was performed at $0-4^{\circ}\text{C}$ unless otherwise stated.

Tryptophan hydroxylase assay The cells were washed twice with Ca²⁺- and Mg²⁺-free Dulbecco's phosphate-buffered saline, PBS(-) (2). The washed cells were disrupted with a Potter-

Elvehjem homogenizer in 3 volumes of PBS(−) containing 100 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$. The homogenate was centrifuged at 105,000 g for 50 min. The fresh supernatant was used for the enzymic studies. The reaction mixture for the assay of tryptophan hydroxylase contained 0.1 M potassium phosphate (pH 6.8), 0.67 mM L-tryptophan, 0.49 mM 6MPH₄, 0.33 mM NADH, 3 $\mu\text{g}/\text{ml}$ of dihydropteridine reductase, 13.3 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 0.4 mg/ml of catalase, and the enzyme solution in a final volume of 150 μl . When (6R)-BH₄, the natural cofactor, was used in place of 6MPH₄, 0.2 mM L-tryptophan was used in the presence of 0.33 mM (6R)-BH₄. When the enzyme from FMA3 cells was assayed, NSD-1055, an inhibitor of 5HTP decarboxylase, was added to the reaction mixture at a final concentration of 1 mM. The decarboxylase inhibitor was omitted in the assay with the enzyme preparation from P-815 cells (23). The reaction was performed at 30°C for 5 min and terminated by the addition of 15 μl of 70% perchloric acid. The mixture was placed on ice for more than 10 min and centrifuged at 3,000 rpm for 5 min. The reaction product, 5HTP, in the supernatant was determined by HPLC.

Anaerobic incubation for activation of the tryptophan hydroxylase with dithiothreitol (DTT) The activation of the enzyme by incubation (DTT activation) was performed as described previously (12) with slight modifications. The enzyme was added to an activation mixture (final volume 200 μl) consisted of 0.1 M Tris-acetate (pH 8.1), 15 mM DTT, 50 μM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and 0.6 mg/ml of catalase in a Thunberg tube in the cold. The air in the tube was replaced with N₂ gas three times, and then each tube was incubated at 30°C for 20 min. Immediately after the opening of the tube, an aliquot of the mixture was subjected to the hydroxylase reaction.

5HTP decarboxylase assay The 105,000 g supernatant was prepared the same way as tryptophan hydroxylase except that Fe^{2+} was omitted from the homogenization medium. The supernatant was passed through a Sephadex G-25 column (sample to column volume, 1:6) equilibrated with 50 mM Tris-acetate (pH 7.4) containing 0.1 M KCl to remove endogenous 5HT. The standard reaction mixture contained 0.1 M Na-Hepes (pH 7.4), 0.5 mM L-5HTP, 0.3 mM pyridoxal 5'-phosphate and the enzyme solution in a total volume of 200 μl . The enzyme solution was preincubated in the reaction mixture in the absence of 5HTP at 30°C

for 5 min. The reaction was then started by the addition of 5HTP, continued at 30°C for 5 min, and terminated by adding 20 μl of 70% perchloric acid. The supernatant was obtained as described for tryptophan hydroxylase. The reaction product, 5HT, was determined by HPLC. The addition of a monoamine oxidase inhibitor (pargyline, 1 mM) did not cause significant increase in the product accumulation.

Determination of 5HT, 5HTP tryptophan, and 5HIAA In order to minimize the loss of samples due to the incomplete transfer, a single micro V-vial (1.0 ml, Wheaton Scientific, NJ) was used for each specimen throughout the procedure. Whole ascitic fluid (500 μl) obtained from individual mice was spun at 5,000 g for 30 min. The cell-free ascitic fluid was reserved. The surface of the precipitate was carefully rinsed with 200 μl of PBS(−). The possible remains of the ascitic fluid in the cell pellet were not taken into account in the determination. The cell pellet was homogenized with a teflon pestle (micro V-homogenizer, Wheaton) in 0.1 N HCl containing 1% ascorbic acid which was added to make a final volume of 500 μl . A 50 μl -aliquot of the homogenate was withdrawn for the protein determination. The remaining homogenate was placed on ice for more than 30 min and homogenized again with the addition of 50 μl of 70% perchloric acid. After standing on ice for more than 10 min, the mixture was centrifuged at 5,000 g for 10 min. The resultant supernatant was subjected to the HPLC analysis. The recovery of the authentic samples externally added at the first homogenization of the cells was; 5HT, 79%; 5HTP, 78%; tryptophan, 80%; 5HIAA, 68%. For the determination with the cell-free ascitic fluid, 150 μl of the fluid was mixed with an equal volume of 0.2 N HCl containing 2% ascorbic acid. Then 30 μl of 70% perchloric acid was added and the mixture was sonified in an ultrasonic cleaner (Branson) for approximately 1 min. The supernatant was then obtained by centrifugation at 3,000 rpm for 5 min and subjected to the HPLC analysis. The recovery of authentic compounds added to the cell-free fluid was between 94 and 100%.

The procedure gave the amount of compounds as pmol/mg protein. The cellular content of the compounds (pmol/10⁶ cells) was calculated from the above values and the cellular content of protein (mg protein/10⁶ cells). To estimate concentration (pmol/ μl) of the compounds in the cells, the cellular content (pmol/10⁶ cells) was divided by the mean cell volume ($\mu\text{l}/10^6$ cells). The mean cell volume was

calculated from the packed cell volume and the cell density. The cell density and packed cell volume of ascitic fluid were determined by a cell-counter, TOA Model CC-107, and a hematocrit, respectively.

Protein determination The protein concentration in the cellular homogenate in 0.1 N HCl-1% ascorbic acid was determined as follows. The homogenate (50 μ l) was mixed with 450 μ l of 0.1 N NaOH and left overnight at 4°C to solubilize protein and to allow decomposition of the ascorbic acid. A suitable amount of the solution was then subjected to protein determination by the Biuret method (6) with bovine serum albumin as a standard. Protein determination for the calculation of the specific activity of tryptophan hydroxylase and 5HTP decarboxylase was performed according to the method of Lowry *et al.* (15), with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Assay Conditions for Tryptophan Hydroxylase and 5HTP Decarboxylase

In an early work on tryptophan hydroxylase from bovine pineal gland, we demonstrated an activation of the enzyme by preincubation with a high concentration of dithiothreitol (DTT) under anaerobic conditions (12). This activation was also demonstrated with the enzyme from

mouse mastocytoma P-815 under similar conditions (8, 23). The specific activity measured after the DTT activation was reproducible even with different stocks of the cell. Although the mechanism and physiological significance of the activation are not yet clear, evidence has accumulated to suggest that the activity after the DTT activation represents the 'full potential activity' of the enzyme (8). This activity will be designated as 'DTT-activated activity.'

We also attempted to determine the activity of tryptophan hydroxylase without the DTT activation as a closer measure of the actual activity in the cell. This activity will be designated as 'native activity.' The first problem in the determination of the native activity was that the activity was quite unstable and the results obtained were not reproducible from preparation to preparation even when the extracts were prepared from the same stock of cells.

In an effort to stabilize the native activity, the addition of iron at 100 μ M to the homogenization medium was found to be effective (Table 1). In this experiment, P-815 cells collected from 5 mice were homogenized in 3 volumes of PBS(−) containing 100 μ M Fe^{2+} and the homogenate was divided into three parts. The first part was used immediately for the determination of tryptophan hydroxylase activity with and without DTT activation. The second part was centrifuged at 105,000 *g* for

Table 1 *Effect of Iron on the Stability of the Native Activity of Tryptophan Hydroxylase during the Extraction from Mouse Mastocytoma Cells*

Conditions of Extraction	Tryptophan hydroxylase activity		
	Homogenate zero-time	Homogenate 90 min	Supernatant 90 min
	nmol/min/ml		
1 − Fe^{2+}	4.93 (76.2)	2.19	0.85 (68.1)
2 100 μ M Fe^{2+}	5.18 (71.1)	5.84	6.64 (60.3)
3 − Fe^{2+}	6.41 (68.8)	3.29	1.96 (58.6)
4 100 μ M Fe^{2+}	3.99 (46.9)		3.49 (37.8)

Freshly harvested cells of mastocytoma P-815 (approximately 2 ml as packed cells) were homogenized in 3 volumes of PBS(−) containing 100 μ M $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ in a Potter-Elvehjem homogenizer with 15 strokes with a Teflon pestle. In the control experiment, Fe^{2+} was omitted from the homogenization medium. The native and DTT-activated activity of tryptophan hydroxylase was measured in an aliquot of the homogenate immediately after homogenization (zero-time). A 5 ml-portion of the homogenate was centrifuged at 105,000 *g* for 50 min. Another portion was left on ice. On completion of the centrifugation (90 min after the homogenization), the enzyme activity in both the supernatant and homogenate was determined. Experiments 1 and 2 were carried out simultaneously and experiments 3 and 4 were done separately. The DTT-activated activity is shown in parentheses.

50 min at 4°C to obtain the supernatant. The third part was left on ice. On completion of the centrifugation, 90 min after the homogenization, the tryptophan hydroxylase activity was determined with the supernatant and the homogenate. A control experiment was done in the same way except that Fe^{2+} was omitted from the extraction medium. The recovery of the enzyme in the supernatant calculated from the DTT-activated activity was 80–90% irrespective of the presence or the absence of Fe^{2+} in the homogenization medium (Table 1, presented in parentheses), indicating that the enzyme was essentially soluble.

When cells were homogenized in the presence of 100 μM Fe^{2+} , the native activity measured immediately after the homogenization was almost the same as that of the control experiment. This level of activity was maintained after standing the homogenate on ice for 90 min and after the centrifugation procedure (Table 1, experiments 2 and 4). On the other hand, in the control experiment done in duplicate, about 50% of the native activity in the homogenate was lost within 90 min at 0–4°C and only 30.6% and 17.2% were found in the supernatant (Table 1, experiments 1 and 3). Since the recovery of the enzyme in the supernatant was 85.1% and 89.4% as judged from the DTT-activated activity, the decrease in the native activity during the procedure was calculated to be 64.1% and 80.7%. These results clearly indicated the unstableness of the native activity and the stabilizing effect of Fe^{2+} . In the following study, cell extracts for the tryptophan hydroxylase assay was prepared by homogenization in the presence of 100 μM Fe^{2+} followed by centrifugation at 105,000 g for 50 min. The native and DTT-activated activity was measured in the same extracts.

The K_m values of mastocytoma tryptophan hydroxylase for tryptophan and tetrahydropterin cofactors have been reported by several authors, but they did not distinguish the different states of the enzyme, some of which were presumably activated partially by aerobic incubation with mercaptoethanol or DTT (9, 13, 18, 21). In order to optimize the assay conditions for both the native and activated tryptophan hydroxylase, the dependence of the activity on the concentration of substrate and the cofactors was examined (Table 2A). In this experiment, the supernatant from P-815 cells was used after gel-filtration through a Sephadex G-25 column (sample to column volume 1: 6), equilibrated with 50 mM Tris-acetate (pH 7.4) containing 0.1 M KCl and

100 μM Fe^{2+} . The recovery through the gel-filtration was 70–80% and 60–70% with respect to the DTT-activated and the native activity, respectively. The K_m values of the native enzyme for tryptophan, 6MPH₄ and (6R)-BH₄, were close to those of the DTT-activated enzyme. The apparent K_m of the native enzyme for tryptophan with 490 μM 6MPH₄ as the cofactor was 25.6 μM and that of the DTT-activated enzyme was 31.3 μM . When (6R)-BH₄ was used at 333 μM , the K_m value of these enzymes for tryptophan was not significantly different from that with 6MPH₄, but a substrate inhibition was induced at concentrations of tryptophan higher than 200 μM . The affinity of the natural cofactor, (6R)-BH₄, for both the native and DTT-activated enzymes was higher than that of the synthetic cofactor, 6MPH₄. The K_m for (6R)-BH₄ of the native and DTT-activated enzymes was 33.3 and 29.4 μM , and that for 6MPH₄ was 71.4 and 83.3 μM , respectively, under the conditions employed. These results indicate that the DTT activation does not affect the affinity of the enzyme for tryptophan and the cofactors. On the basis of these results, the assay of tryptophan hydroxylase was performed with 667 μM tryptophan as substrate in the presence of 490 μM 6MPH₄, or with 200 μM tryptophan in the presence of 333 μM (6R)-BH₄.

In order to compare two lines of cells, the native activity was first determined as a closer measure of the actual activity in the cell. The DTT-activated activity was employed as a measure of the total enzyme content of the cell. The ratio of the native activity over the activated activity was also compared to estimate the expression of the activity in the respective cellular conditions.

The assay conditions for 5HTP decarboxylase were examined with the desalted supernatant prepared from the FMA3 cells as described under Materials and Methods. Previous works on 5HTP decarboxylase employed alkaline pH conditions for the assay (1, 20). Our preliminary experiments with the mouse mastocytoma extracts showed a broad pH optimum from 7 to 9 at a saturated concentration of 5HTP as substrate. Although the K_m of the enzyme for 5HTP at pH 8.8 was approximately one-fifth of the value at pH 7.4, the maximum velocity was almost the same at the alkaline and neutral conditions (Table 2B). In the present study, we compared the two cell lines at pH 7.4.

Table 2 *Kinetic Constants of Tryptophan Hydroxylase (A) and 5HTP Decarboxylase (B) in Extracts from Mouse Mastocytoma Cells*

A Tryptophan hydroxylase ¹				
Cofactor used	Apparent <i>K_m</i> for tryptophan (μM)		Apparent <i>K_m</i> for pterin (μM)	
	Native	DTT-activated	Native	DTT-activated
(6R)-BH ₄	27.0	32.3	33.3	29.4
	((6R)-BH ₄ ; 333 μM)		(Trp; 200 μM)	
6MPH ₄	25.6	31.3	71.4	83.3
	(6MPH ₄ ; 490 μM)		(Trp; 667 μM)	
B 5HTP decarboxylase ²				
	Assay conditions	<i>K_m</i> (μM)	<i>V_{max}</i> (nmol/min/mg)	
	0.1 M Na-Hepes (pH 7.4)	15.3	3.08	
	0.1 M Tris-HCl (pH 8.8)	3.3	2.83	

¹ Extracts from P-815 cells were used. ² Extracts from FMA3 cells were used.

The Activity of Tryptophan Hydroxylase and 5HTP Decarboxylase in the Two Cell Lines

The activity of tryptophan hydroxylase and 5HTP decarboxylase in the two cell lines is presented in Table 3.

The mean value of the DTT-activated activity of tryptophan hydroxylase was 3.43 and 10.5 nmol/min/mg protein with the FMA3 and P-815 cells, respectively. The native activity in FMA3 and P-815 was 0.64 and 0.95 nmol/min/mg protein, respectively. Thus the native activity of the FMA3 cells was close (67%) to that of the P-815 cells, but the DTT-activated activity of the FMA3 cells was approximately one-third

of the activity of the P-815 cells. The native activity in extracts from the FMA3 and P-815 cells was 19% and 9% of the respective DTT-activated activity. Since the DTT-activated activity was assumed to represent the full potential activity of the enzyme (8), the results indicate a possibility that some factors in the cell or the cell extract determine the expression of the native activity. The native activity measured with (6R)-BH₄ as cofactor was 0.31 and 0.43 nmol/min/mg protein for the FMA3 and P-815 cells, respectively, and was 40–50% of the 6MPH₄-dependent activity in both cell extracts.

The activity of 5HTP decarboxylase in the desalted supernatant from the FMA3 and P-815 cells was 1.53 and 0.0043 nmol/min/mg protein, respectively. To measure the extremely low activity of P-815, 80 μl of the cell extract was used for the reaction in a total volume of 200 μl and the reaction was allowed to proceed for 30 min. In agreement with the previous report that P-815 had weak decarboxylase activity (10), the activity of the P-815 cells was only one-four hundredth of that of FMA3 in the present study.

Table 3 *Enzyme Activity Involved in Serotonin Biosynthesis in Mouse Mastocytoma P-815 and FMA3*

Enzymes	Enzyme activity	
	P-815	FMA3
	(nmol/min/mg)	
Tryptophan hydroxylase		
DTT-activated	10.5 ± 1.7	3.43 ± 0.38
Native	0.95 ± 0.10	0.64
5HTP Decarboxylase	0.0043	1.53

In each determination, the cell extracts were prepared from pooled cells from 5–6 tumor-bearing mice. Data on tryptophan hydroxylase are the mean ± SD of 4–6 experiments, except that the native activity of FMA3 is the mean of duplicate determinations. Data on 5HTP decarboxylase are the mean of duplicate experiments.

Cellular Content of 5HT and the Related Compounds

The intracellular localization of 5HT and the related compounds was examined first. The P-815 cells were collected from 4 tumor-bearing mice and washed once with PBS(–). The cells were homogenized in 4 volumes of 0.34 M su-

crose in a Potter-Elvehjem homogenizer with 10 strokes with a Teflon pestle and the homogenate was centrifuged at 12,000 *g* for 15 min at 4°C. The resultant supernatant and precipitates were separately subjected to the metabolite analysis. In the cell homogenate, 5HT was determined to be 41.3 nmol/ml, of which 82.5% was recovered in the particulate fraction and 8.8% remained in the supernatant. In contrast to 5HT, its precursors and metabolite were mainly found in the supernatant; tryptophan, 73.4%; 5HTP, 73.2%; and 5HIAA, 65.0%. This clearly indicated that the major part of the 5HT existed in the particulate components of the cell.

The amount of 5HT and the related compounds in the whole cells was measured and compared between P-815 and FMA3. The amount in the ascitic fluid where the cells had grown was also measured (Table 4). Whole ascitic fluid from individual mice was separated into the cell-free fluid and the cells. The cells were subjected to analysis after careful removal of the fluid from the surface of the cell pellet.

The content of 5HT in FMA3 and P-815 cells was determined to be 7.09 ± 1.42 and 1.42 ± 0.94 nmol/mg protein, respectively. The 5HT content in FMA3 and P-815 cells calculated on the basis of the cell number was 1.27 and 0.24 nmol/ 10^6 cells, respectively. The protein content used in the calculation was 0.179 and 0.167 mg/ 10^6 cells for FMA3 and P-815, respectively. The result obtained with the P-815 cells is in good agreement with the value (0.2 nmol/ 10^6 cells) measured by the fluorometric method (10). The 5HT content in FMA3 cells cultured *in vitro* was reported to be $1-3 \mu\text{g}/10^6$ cells (19). The present result obtained from the cells grown in the peritoneal cavity is considerably lower than the above value from cultured cells. The cellular content of 5HT in cultured FMA3 cells was, however, reported to vary depending on culture conditions (19).

The intracellular concentration of tryptophan calculated on the basis of the mean cell volume (1.93 and 1.24 $\mu\text{l}/10^6$ cells for FMA3 and P-815, respectively) was 25.9 μM in FMA3 and 61.4 μM in P-815 cells. Taking into account of the K_m value measured for tryptophan of tryptophan hydroxylase (Table 2), the substrate pool in both cells seemed sufficient to promote the 5HT biosynthesis.

A positive correlation was observed between the intracellular concentration of 5HTP and the activity of 5HTP decarboxylase (Tables 3 and 4). In the case of FMA3 cells, 5HTP was almost completely depleted, indicating that the decarboxylase activity was sufficient to metabolize 5HTP formed by the action of tryptophan hydroxylase. In contrast, the P-815 cells retained a relatively high concentration of 5HTP (28 nmol/ml) in the cell, reflecting very low activity of the decarboxylase. In the latter case, 5HTP was also detected in the cell-free ascitic fluid from the tumor-bearing mice (6.6 nmol/ml). Most of the 5HTP detected in the ascitic fluid of P-815-bearing mice seems to be liberated from the tumor cells. This indicates that the cell could not retain all the 5HTP formed when the utilization in the cell was much less than its production. Tryptophan hydroxylase in the cell may not be inhibited effectively by the reaction product at the intracellular concentration attained (28 nmol/ml).

On the other hand, the accumulation of 5HT in the P-815 cells ($0.19 \pm 0.13 \mu\text{mol}/\text{ml}$) was not extremely lower than that in the FMA3 cells ($0.66 \pm 0.13 \mu\text{mol}/\text{ml}$), despite the apparent block of 5HT biosynthesis at the decarboxylation step due to the low activity of 5HTP decarboxylase. This observation suggests the existence of some mechanism which allows effective accumulation of 5HT in a cellular compartment even when the compound is synthesized very slowly.

Table 4 Concentration¹ of Serotonin (5HT) and Related Compounds in Mouse Mastocytoma Cells and in Ascitic Fluid of Tumor-bearing Mice

	P-815		FMA3	
	Ascitic fluid (nmol/ml)	Cell (pmol/mg protein)	Ascitic fluid (nmol/ml)	Cell (pmol/mg ptotein)
Trp	40.6 ± 6.4	456 ± 115	12.7 ± 4.2	279 ± 88
5HTP	6.58 ± 2.10	205 ± 144	N.D. ²	17.5 ± 4.7
5HT	3.21 ± 1.98	$1,420 \pm 940$	3.28 ± 0.79	$7,090 \pm 1,420$
5HIAA	9.63 ± 3.06	25.6 ± 13.9	19.8 ± 4.0	83.1 ± 37.1

¹Data are mean $\pm$ SD (n=12). ²Not detectable

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