

Formation of 5-hydroxykynurenine and 5-hydroxykynurenamine from 5-hydroxytryptophan in rabbit small intestine

(indoleamine 2,3-dioxygenase/indole ring cleavage/serotonin)

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Contributed by Osamu Hayaishi, December 14, 1978

ABSTRACT In order to clarify the role of indoleamine 2,3-dioxygenase [indole:oxyg₂en 2,3-oxidoreductase (deacyclizing), EC 1.13.11.17] in the metabolism of serotonin, DL-5-hydroxy-[methylene-¹⁴C]tryptophan, a precursor of serotonin, was incubated with slices of rabbit ileum. Resulting metabolites were separated by DEAE-cellulose column and polyamide column chromatography and identified by various chromatographic techniques and enzymatic analysis. Metabolites obtained in significant amounts were serotonin, 5-hydroxyindoleacetic acid, 5-hydroxytryptophol, 5-hydroxykynurenine, 5-hydroxykynurenamine, and 4,6-dihydroxyquinoline, representing 13.2, 15.8, 7.0, 21.9, 1.3, and 2.6% of the total metabolites, respectively. The first three compounds were previously reported to be major metabolites produced from 5-hydroxytryptophan by the action of aromatic L-amino acid decarboxylase and monoamine oxidase, whereas the last three are formed by the cleavage of the indole ring by the action of indoleamine 2,3-dioxygenase. In the presence of pargyline, a monoamine oxidase inhibitor, the major metabolites obtained were serotonin, 5-hydroxykynurenine, and 5-hydroxykynurenamine, representing 29.6, 26.6, and 5.4% of the total metabolites, respectively. In the presence of RO4-4602, an aromatic amino acid decarboxylase inhibitor, 5-hydroxykynurenine was the sole major product. These results strongly suggest that the newly discovered metabolic pathway involving the cleavage of the indole ring of 5-hydroxytryptophan operates *in vivo* to a significant extent and that indoleamine 2,3-dioxygenase plays an important role in the regulation of serotonin levels in the small intestine of the rabbit.

Studies on serotonin (5-HT) metabolism in mammalian tissues have established a pathway that includes successive hydroxylation and decarboxylation of tryptophan and further degradation to 5-hydroxyindoleacetic acid (5-HIAA) or 5-hydroxytryptophol (5-HTOH). However, reports on the natural occurrence of 5-hydroxykynurenine (5-HK) in the urine of hens (1), 5-hydroxykynurenamine (5-HKA) in the brain and urine of mice (2, 3), and 4,6-dihydroxyquinoline (4,6-HQ) in the urine of hens (4) have strongly suggested the existence of another pathway involving oxidative cleavage of the indole ring of various indoleamine derivatives. From 5-hydroxytryptophan (5-HTP), 5-HK was formed in crude extracts of rat intestine (5) or rat brain (6). In our own experiments, it was demonstrated that indoleamine 2,3-dioxygenase [indole:oxyg₂en 2,3-oxidoreductase (deacyclizing), EC 1.13.11.17] highly purified from rabbit ileum cleaved the indole ring of 5-HTP and 5-HT oxidatively (7). However, because the enzyme has an absolute requirement for an artificial oxidation-reduction dye (e.g., methylene blue) when acting *in vitro* (8, 9), the question has been raised as to whether or not this enzyme functions *in vivo*.

In the present work using slices of rabbit ileum, we investi-

gated the possible existence of this enzyme activity *in vivo* and attempted to assess the quantitative significance of the indole ring cleavage pathway and the 5-HT pathway.

MATERIALS AND METHODS

DL-5-Hydroxy[methylene-¹⁴C]tryptophan (5-[¹⁴C]HTP) [57.5 μ Ci (1 Ci = 3.7×10^{10} becquerels)/ μ mol] was purchased from the Radiochemical Centre, Amersham, England. 5-HK (10), 5-HKA (11) and 4,6-HQ (12, 13) were synthesized chemically. The purity of these authentic compounds were checked by thin-layer chromatography, paper electrophoresis, elemental analysis, and NMR and absorption spectroscopy. RO4-4602 was provided by Y. Yagi (Nippon Roche K. K., Tokyo). Precoated silica gel (0.25 mm) and cellulose (0.1 mm) glass plates were purchased from E. Merck. Polyamide powder for column chromatography and polyamide-coated plastic sheets were obtained from M. Woelm Eschwege and Cheng Chin Trading Co., Ltd. (Taiwan), respectively. All other chemicals were of analytical grade.

Tissue Preparation. Adult male rabbits (2.5-3 kg) were anesthetized with pentobarbital sodium (50 mg/kg, intravenously), and the distal portion (20 cm length) of ileum was quickly removed. The ileum was opened lengthwise and washed with cold 0.9% NaCl, and pieces of ileum weighing 30-40 mg were cut with a blade. In order to remove as much bacteria as possible, these slices were then washed several times with ice-cold Krebs-Ringer bicarbonate buffer (pH 7.2) containing ascorbic acid (0.1%), streptomycin (100 μ g/ml), kanamycin (60 μ g/ml), and penicillin (100 units/ml) and blotted on a filter paper.

Incubation of Tissue. The tissue slices (1.4 g, wet weight) were placed in a 25-ml flask containing 5 ml of the same pH 7.2 buffer and shaken at 60 strokes per min for 20 min at 37°C under a continuous stream of 95% O₂/5% CO₂. During this period, the 5-HT content in the ileum, as measured by the method of Bogdanski *et al.* (14), decreased from 28 to 6 μ g/g of tissue. The tissue slices were then transferred into fresh buffer and incubated for 20 min in the presence or absence of an inhibitor, RO4-4602 or pargyline. 5-[¹⁴C]HTP (10⁷ cpm; 100 nmol) was then added to the incubation mixture and incubation was carried out for a further 60 min in the dark. In order to

Abbreviations: 5-HT, serotonin; 5-HTP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HTOH, 5-hydroxytryptophol; 5-HK, 5-hydroxykynurenine; 5-HKA, 5-hydroxykynurenamine; 4,6-HQ, 4,6-dihydroxyquinoline.

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correct for nonenzymatic degradations of the 5-[¹⁴C]HTP, control experiments were run with boiled intestinal slices. At the end of incubation, the slices were separated from the medium and homogenized in 5 ml of 0.1 M HCl containing 90% (vol/vol) ethyl alcohol and approximately 1 mg each of authentic 5-HT, 5-HTP, 5-HIAA, 5-HTOH, 5-HK, and 5-HKA and 0.3 mg of 4,6-HQ. The homogenate was centrifuged at 30,000 × *g* for 20 min and the supernatant was combined with the incubation medium. The recovery of total radioactivity with these procedures was 92–95% of the initial radioactivity added to the incubation medium in each sample. The combined solution was concentrated at 40°C to about 3 ml under reduced pressure. The sample was applied directly to the DEAE-cellulose column or stored at –70°C for later analyses.

DEAE-Cellulose Column. The DEAE-cellulose column (2 × 30 cm) was activated and equilibrated with 1 mM triethylamine formate buffer (pH 4.0) according to the method of Chen and Ghoslon (15).

Polyamide Column. Polyamide powder (100 g) was suspended in absolute ethyl alcohol (1000 ml). After 30 min, the supernatant liquid was decanted and the polyamide was washed with 4 vol of distilled water. The washed polyamide was then packed into a column and equilibrated with 0.1 M HCl containing 0.25 M BaCl₂.

Purification of 5-[¹⁴C]HTP. Commercial 5-[¹⁴C]HTP (10⁸ cpm; 0.87 μmol in 1 ml of water) was applied to a DEAE-cellulose column. After the column was washed with 300 ml of 1 mM triethylamine formate buffer (pH 4.0), an 800-ml linear gradient from 1 to 100 mM triethylamine formate buffer (pH 4.0) was applied at a flow rate of 20 ml/hr. Fractions of the major radioactive peak were collected, lyophilized, and then dissolved in a small volume of distilled water. The concentration of 5-HTP was determined on the basis of the absorbance at 280 nm ($\epsilon_M = 5620 \text{ cm}^{-1} \text{ mol}^{-1}$).

High-Voltage Paper Electrophoresis and Thin-Layer Chromatography. High-voltage paper electrophoresis was performed with a Pherograph Original Frankfurt Model 64 Wiesloch. Samples were placed on Whatman No. 3 MM paper moistened with pyridine/acetic acid/H₂O, 100/4/900 (vol/vol), at pH 6.4, and electrophoresis was carried out for 90 min at 2200 V and 80 mA. Authentic samples were detected by fluorescence under ultraviolet light or visualized after spraying with Ehrlich reagent. *R_F* values of authentic compounds are summarized in Table 1.

Measurement of Radioactivity. The radioactivity of isolated samples was determined in a Packard model 3385 liquid scintillation spectrometer in 10 ml of toluene solution containing 30% (vol/vol) Triton X-100, 0.01% *p*-bis[2-(4-methyl-5-phenyloxalyl)]benzene, and 0.55% 2,5-diphenyloxazole. The ra-

dioactivity on thin-layer plates and paper was determined by using a Packard model 7201 radiochromatogram scanner, by scraping off the silica gel or cellulose, or by cutting the polyamide sheet or paper into 0.5-cm-wide strips and suspending the strips in the above-mentioned toluene mixture. The radioactivity of DEAE-cellulose column fractions was determined by pipetting 0.25-ml aliquots onto stainless steel planchets, drying under an infrared lamp, and assaying in a Nuclear Chicago gas-flow counter.

Enzyme Preparation. Monoamine oxidase was prepared as the mitochondrial fraction from rat liver by the method of Hogeboom (16); the specific activity was 3 nmol/mg of protein per min with kynurenamine as substrate (17). Protein determination was by the method of Lowry *et al.* (18) with bovine serum albumin as standard.

RESULTS

Separation of ¹⁴C-Labeled Metabolites Obtained from 5-[¹⁴C]HTP. The ¹⁴C-labeled metabolites produced from 5-[¹⁴C]HTP by incubation with slices of rabbit ileum were separated by DEAE-cellulose column chromatography. In the absence of inhibitor, radioactivity was found in six discrete peaks (Fig. 1A). However, in the presence of pargyline (1 mM), there was no radioactivity in peaks 2 and 6 (Fig. 1B), whereas RO4-4602 (0.1 mM) completely abolished radioactivity peaks 1, 2, and 6 (Fig. 1C). When 5-HTP was incubated with boiled intestinal slices, radioactivity was found only in peaks 3 and 5 (Fig. 1D). The peak fractions were lyophilized, dissolved in a minimum amount of water, and used for identification.

Identification and Quantitative Estimation of Metabolites in Each Peak. Peak 1 contained both 5-HKA and 5-HT, which were added as authentic carriers. In order to separate 5-HKA from 5-HT, the peak 1 fraction was applied to a polyamide column and eluted as described in the legend for Fig. 2. Complete separation of 5-HKA and 5-HT was achieved by this procedure. The radioactive peaks A and B coincided with the absorbance peaks for 5-HKA and 5-HT, respectively. The fractions containing 5-HKA (peak A) were combined and concentrated to about 5 ml under reduced pressure at 40°C. BaCl₂ was removed by precipitating with 50 ml of ethyl alcohol, followed by centrifugation at 10,000 × *g* for 10 min. The supernatant was concentrated to about 5 ml under reduced pressure at 40°C, lyophilized, and dissolved in a small amount of ethyl alcohol. The amounts recovered with 86% of the total radioactivity in peak A and 83% of the internal authentic 5-HKA added. On thin-layer chromatography in five different solvent systems, the radioactive material gave only a single radioactive peak isographic with 5-HKA added as authentic carrier. The radioactive peak coinciding with the 5-HKA peak

Table 1. *R_F* values of authentic compounds

| Compound | <i>R_F</i> , by solvent system | | | | | | | | | |
|----------|--|------|------|------|------|------|------|------|------|------|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 5-HKA | 0.35 | 0.67 | 0.50 | 0.40 | | | | | 0.70 | 0.70 |
| 5-HK | 0.29 | 0.53 | 0.43 | 0.15 | | | | | | 0.75 |
| 4,6-HQ | 0.88 | 0.87 | 0.13 | 0.94 | 0.67 | 0.05 | | 0.60 | 0.33 | |
| 5-HT | 0.47 | 0.65 | 0.27 | 0.41 | | | | | | 0.30 |
| 5-HIAA | 0.90 | | 0.43 | 0.30 | | 0.33 | 0.10 | | | |
| 5-HTOH | 0.88 | | 0.40 | 0.90 | | | 0.27 | 0.69 | | |
| 5-HTP | 0.30 | | 0.35 | 0.13 | 0.21 | | | | | 0.30 |

Thin-layer chromatography was carried out on either cellulose (solvent systems 1–4), silica gel (solvent systems 5–8), or polyamide plates (solvent systems 9 and 10). The solvent systems (vol/vol, except as noted) were as follows: 1, *n*-butyl alcohol/acetic acid/H₂O, 4:1:5; 2, *n*-butyl alcohol/pyridine/H₂O, 1:1:1; 3, 20% KCl in water; 4, isopropyl alcohol/28% ammonia/H₂O, 8:1:1; 5, acetone/chloroform/acetic acid/H₂O, 40:40:2:5; 6, benzene/propionic acid/H₂O, 100:70:5; 7, chloroform/methyl alcohol/acetic acid, 93:7:1; 8, ethyl acetate/isopropyl alcohol/H₂O, 65:24:1; 9, 0.1 M HCl/ethyl alcohol, 7:3; and 10, 10% NaCl in 0.1 M HCl.

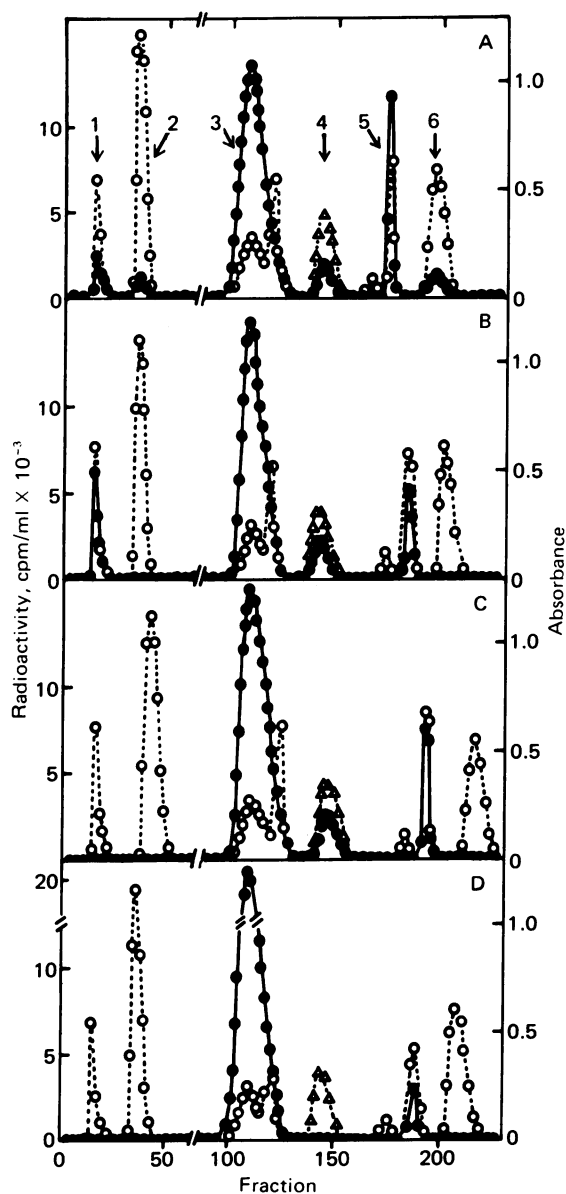


FIG. 1. Chromatographic separation of ^{14}C -labeled metabolites on a DEAE-cellulose column (2×30 cm). 5- ^{14}C HTP was incubated with slices of rabbit ileum for 60 min at 37°C . (A) Without inhibitors (7.5×10^6 cpm). (B) With pargyline (7.5×10^6 cpm). (C) With RO4-4602 (7.2×10^6 cpm). (D) With boiled slices (7.9×10^6 cpm). The sample was eluted with 300 ml of 1 mM triethylamine formate buffer (pH 4.0) and then with an 800-ml linear gradient of triethylamine formate (pH 4.0) from 1 mM to 100 mM at a flow rate of 20 ml/hr. Fractions (4.5 ml) were collected. Absorbance was measured at 280 nm (O - - O), except for the fractions containing 5-HK the absorbance was measured at 260 nm (Δ - - Δ) after addition of 1 ml of 1 M HCl to stabilize the compound. ●—●, Radioactivity.

accounted for at least 91% of the radioactivity on the thin-layer chromatograms. Essentially the same result was obtained with high-voltage paper electrophoresis. The identity of 5-HKA was further verified by treatment with mitochondrial monoamine oxidase. As shown in Fig. 3, the radioactive peak for 5-HKA was completely converted by the enzyme to a new peak corresponding to 4,6-HQ.

The fractions making up peak B of Fig. 2 were combined, lyophilized, and dissolved in a small amount of water. The radioactivity recovered was 95% of the total radioactivity of peak B. The radioactive material gave a single spot that coincided with the spot for authentic 5-HT on thin-layer chromatography

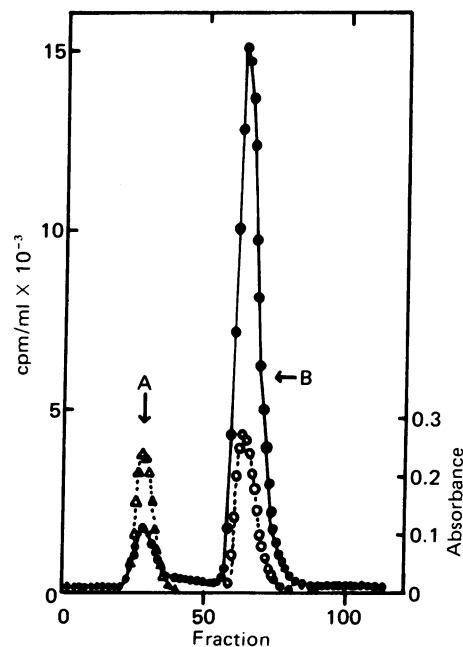


FIG. 2. Chromatographic resolution of peak 1 on a polyamide column. The fraction of peak 1 (Fig. 1B, 4.3×10^5 cpm) was applied to a polyamide column (1×90 cm) and eluted first with 100 ml of 0.1 M HCl/0.25 M BaCl_2 and then with 0.1 M HCl/30% (vol/vol) ethyl alcohol. Fractions (3 ml) were collected. Absorbance at peaks A and B was measured at 315 nm (Δ - - Δ) and at 280 nm (O - - O), respectively, to determine the positions of the authentic compounds 5-HKA and 5-HT. ●—●, Radioactivity.

in five different solvent systems and on high-voltage paper electrophoresis.

Radioactive peaks 2, 4, and 6 coincided with the absorbance peaks of authentic carriers 5-HTOH, 5-HK, and 5-HIAA, respectively (Fig. 1A). Peak 3 contained two absorption peaks, one corresponding to the authentic sample of 5-HTP and the other to an unidentified compound. The latter compound was observed even when the intestinal slices were incubated without substrate and homogenized without authentic carriers, suggesting that this unidentified compound was derived from the intestinal tissue.

Each radioactive component in peak 2, 3, 4, or 6 was homogeneous and cochromatographed with the authentic carrier 5-HTOH, 5-HTP, 5-HK, or 5-HIAA, respectively, on thin-layer chromatography in five solvent systems and on high-voltage paper electrophoresis.

The radioactivity recovered in the 4,6-HQ fraction (peak 5) contained nonenzymatic degradation products because significant radioactivity was observed in this fraction when 5- ^{14}C HTP was incubated with boiled intestinal slices (Fig. 1D). When the peak 5 fraction was applied to a polyamide column, two radioactive peaks were observed (Fig. 4, peak A and B). The authentic sample of 4,6-HQ was found in peak B. Thin-layer chromatography of the radioactive material revealed that at least 20% of the radioactivity in peak B was attributable to 4,6-HQ. Intestinal slices treated with heat, pargyline, or RO4-4602 did not produce any significant radioactivity coinciding with 4,6-HQ on thin-layer chromatography. The remaining radioactivity in peak 5 was unidentified.

Quantitative Estimation of the Metabolites in the Presence and Absence of Inhibitors. In order to evaluate the role of indoleamine 2,3-dioxygenase, all metabolites from ^{14}C -labeled 5-HTP were quantitatively estimated. As shown in Table 2, 5.0, 0.3, and 0.6% of the total recovered radioactivity

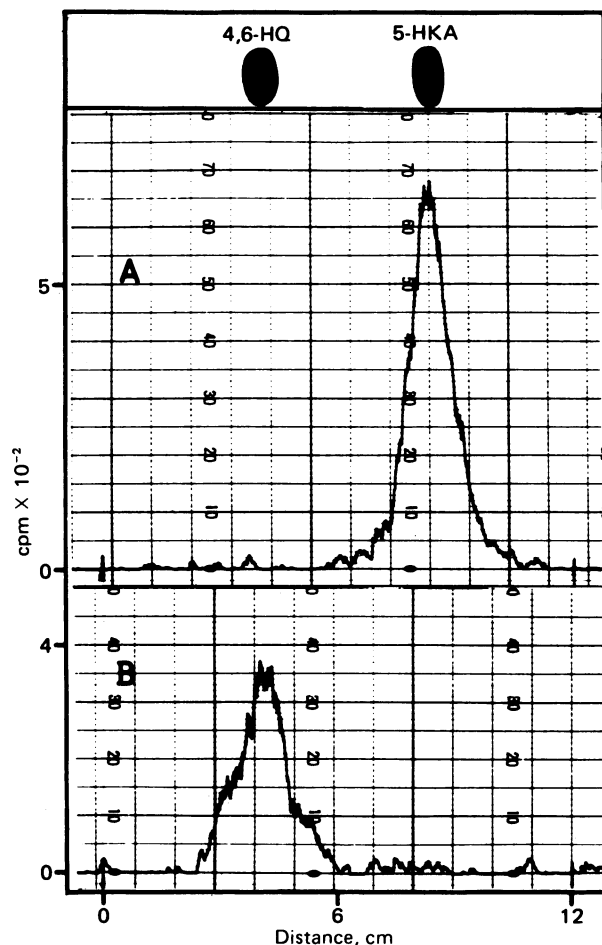


FIG. 3. Identification of 5-HKA by conversion to 4,6-HQ with monoamine oxidase. The reaction mixture (3 ml) contained 5-HKA (2.8×10^5 cpm from peak A, Fig. 2), mitochondrial monoamine oxidase (83 mg), and 25 mM phosphate buffer (pH 7.5). After a 1-hr incubation at 37°C, the reaction was terminated by the addition of 5 ml of 0.1 M HCl/90% (vol/vol) ethyl alcohol. After centrifugation at $10,000 \times g$ for 20 min, the supernatant was lyophilized and the residue was dissolved in a minimal amount of ethyl alcohol. An aliquot was chromatographed in solvent system 9. (A) No enzyme treatment. (B) After treatment with monoamine oxidase.

was identified as 5-HK, 5-HKA, and 4,6-HQ, respectively, with native intestinal slices; 5-HT, 5-HIAA, and 5-HTOH represented 3.0, 3.6, and 1.6%, respectively. These results suggest that the metabolic pathway of the oxidative ring cleavage of indoleamines functioned to an extent approximately equivalent to that of the 5-HT pathway. In the presence of 1 mM pargyline, a monoamine oxidase inhibitor, the formation of 4,6-HQ was completely blocked and the amount of 5-HKA increased to 1.1%. Under the same condition, the formation of both 5-HIAA and 5-HTOH was decreased to 0.2%. On the other hand, when intestinal slices were treated with 0.1 mM RO4-4602, an inhibitor of aromatic L-amino acid decarboxylase, the biosynthesis of 5-HT was completely inhibited and 5-HK was detected as the sole metabolite.

DISCUSSION

In the present study, 5-HTP was incubated with slices of rabbit ileum which has been demonstrated to be rich in indoleamine 2,3-dioxygenase activity (9). We devised a chromatographic technique to separate, identify, and quantify 5-HK, 5-HKA, and 4,6-HQ, by the use of a polyamide column combined with thin-layer chromatography and electrophoresis. Each proce-

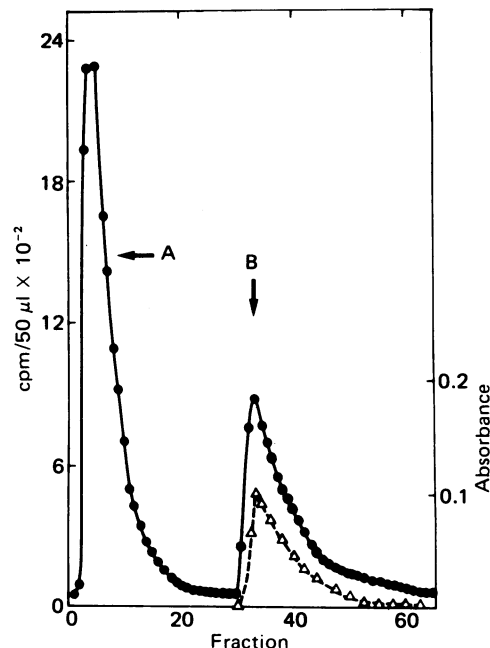


FIG. 4. Chromatographic resolution of peak 5 on a polyamide column. The peak 5 fraction (Fig. 1A, 6×10^5 cpm) was applied to a polyamide column (1 \times 7 cm) and eluted with 30 ml of 0.1 M HCl/0.25 M BaCl₂ and subsequently with 0.1 M HCl/30% (vol/vol) ethyl alcohol. Fractions (1 ml for fractions 1–30; 1.5 ml for fractions 31–60) were collected. ●—●, Radioactivity; Δ—Δ, absorbance at 340 nm.

cedure was carefully designed so that unstable metabolites formed in small amounts would not be destroyed. Extensive purification of commercial 5-[¹⁴C]HTP and addition of adequate amounts of authentic carriers of metabolites also made feasible excellent separation and recovery of the metabolites. The unidentified metabolites occurring in peak 5 of Fig. 1 were probably derived from 5-HTP or 5-HK because their formation was not decreased by the addition of inhibitors of aromatic L-amino acid decarboxylase or monoamine oxidase.

In addition to the well-established metabolic pathway from 5-HTP to 5-HIAA or 5-HTOH via 5-HT, the existence of another pathway yielding 5-HK, 5-HKA, and 4,6-HQ (5-HK

Table 2. Relative distribution of individual 5-HK and 5-HT pathway metabolites from 5-[¹⁴C]HTP

| Metabolite | % found, by treatment | | | |
|-------------------------------|-----------------------|------------------|-------------------|---------------|
| | None | Pargyline (1 mM) | RO4-4602 (0.1 mM) | Boiled slices |
| 5-HK | 5.0 | 5.4 | 6.2 | ND |
| 5-HKA | 0.3 | 1.1 | ND | ND |
| 4,6-HQ | 0.6 | ND | ND | ND |
| Total 5-HK metabolites | 5.9 | 6.5 | 6.2 | ND |
| 5-HT | 3.0 | 6.0 | ND | ND |
| 5-HIAA | 3.6 | 0.2 | ND | ND |
| 5-HTOH | 1.6 | 0.2 | ND | ND |
| Total 5-HT metabolites | 8.2 | 6.4 | ND | ND |
| Unidentified metabolites | 8.7 | 7.4 | 9.2 | 3.6 |
| Total metabolites | 22.8 | 20.3 | 15.4 | 3.6 |
| 5-HTP | 67.1 | 72.8 | 78.9 | 93.3 |
| Total radioactivity recovered | 89.9 | 93.1 | 94.3 | 96.9 |

Total radioactivity of each sample applied to the DEAE-cellulose column was designated as 100%. ND, not detectable (<0.1%).

pathway) was demonstrated in tissue slices in Krebs-Ringer solution. In the present system, approximately 26 and 36% of the radioactivity of the total metabolites from 5-[¹⁴C]HTP entered the 5-HK pathway and the 5-HT pathway, respectively. This ratio of the 5-HK pathway to the 5-HT pathway presumably varies from one tissue to another in proportion to the ratio *in situ* of activities of indoleamine 2,3-dioxygenase and aromatic L-amino acid decarboxylase.

Oxidative cleavage of the pyrrole ring of 5-HT by the action of indoleamine 2,3-dioxygenase has been observed *in vitro* (7). However, when 5-[¹⁴C]HT (10 μM–1 mM) in place of 5-[¹⁴C]HTP was incubated with intestinal slices under the same condition as described under *Materials and Methods*, 5-[¹⁴C]HKA was not detected in significant amounts, even when pargyline (1 mM) was added to the incubation mixture (unpublished data). These results suggest that 5-HKA was formed mainly from 5-HTP via 5-HK rather than via 5-HT.

When 5-[¹⁴C]HTP was incubated with intestinal slices, addition of a monoamine oxidase inhibitor (pargyline) to the incubation medium increased the formation of 5-HKA several-fold without significant change in the recovery of 5-HK. The formation of 5-HKA by the 5-HK pathway is of great interest because this compound has been reported to antagonize 5-HT-induced vasoconstriction of dog basilar artery (11) and aggregation of human platelets (19). Furthermore, because 5-HTP has recently been used as a precursor of 5-HT in the therapy of mongolism (20), Parkinson disease (21), and depression (22, 23), it should be emphasized that, as well as the 5-HT pathway, the 5-HK pathway or resulting metabolites such as 5-HK and 5-HKA may also contribute to the clinical features noted after administration of 5-HTP.

We are grateful to Professor M. Nozaki (Shiga University of Medical Science) for pertinent suggestions and to M. Ohara for assistance with the preparation of the manuscript. This work was supported in part by research grants from the Matsunaga Science Foundation, Sakamoto Foundation, Iyakushigen Kenkyu Shinkokai, the Mitsubishi Foundation, and Nippon Shinyaku Co., Ltd.; and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan.

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