NEW DEGRADATIVE ROUTES OF 5-HYDROXYTRYPTOPHAN AND SEROTONIN BY INTESTINAL TRYPTOPHAN 2,3-DIOXYGENASE*

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A highly purified preparation of tryptophan 2,3-dioxygenase from rabbit intestine was found to catalyze the oxygenative ring cleavage of 5-hydroxytryptophan and serotonin. The products of these enzymic reactions were susceptible to the action of formamidase, as a consequence of which 5-hydroxykynurenine and 5-hydroxykynurenamine were isolated from the reaction mixtures and identified. The initial products were presumed, therefore, to be 5-hydroxyformylkynurenine and 5-hydroxyformylkynurenamine, respectively. Several lines of evidence indicated that the cleavage of tryptophan, 5-hydroxytryptophan and serotonin occurred by the action of a single protein, namely intestinal tryptophan 2,3-dioxygenase.

Tryptophan 2,3-dioxygenase (tryptophan pyrrolase), partially purified from rabbit intestine, exhibits an absorption spectrum characteristic of a hemoprotein, and requires methylene blue and ascorbic acid for maximum activity (1, 2). Subsequently it was suggested that superoxide anion participated in the catalytic process (3). Intestinal enzyme can degrade both D- and L-isomers of tryptophan to form corresponding isomers of formylkynurenine (1, 2). In this communication, we wish to present experimental evidence that intestinal tryptophan 2,3-dioxygenase can also catalyze the oxygen-

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ative ring cleavage of serotonin and 5-hydroxytryptophan. The physiological role of these reactions in regulating the serotonin level in intestine is briefly discussed.

METHODS AND MATERIALS

Intestinal tryptophan 2,3-dioxygenase was purified about 250-fold from crude extracts of rabbit intestine by streptomycin treatment, ammonium sulfate fractionation and successive column chromatographies with p-cellulose, DEAE-Sephadex, hydroxyapatite and DE-52 cellulose. Details will be published elsewhere. The ratio of absorbance at 406 nm to that at 280 nm was 1.13 for the purified enzyme preparation. Enzyme activities were assayed as described previously (2, 3). Protein was measured by the method of Kalcker (4). The specific activity of enzyme was 0.14 μmole per mg of protein per min at 24°. Formamidase was prepared as described previously (2). Rat liver mitochondria were isolated according to the method described by De Duve et al., and used as monoamine oxidase (5). Catalase was a product of Sigma. D,L-5-Hydroxytryptophan-3-^{14}C and serotonin-3-^{14}C-creatinine sulfate were obtained from the Radiochemical Centre, Amersham, England. D- and L-Isomers of 5-hydroxytryptophan, serotonin creatinine sulfate, indole-3-acetic acid, 5-hydroxyindole-3-acetic acid and tryptamine were purchased from Sigma. D,L-5-Hydroxykynurenine was a generous gift of Dr. S. Senoh, the Central Research Institute, Suntory Ltd. (6). 5-Hydroxykynurenamine was enzymatically prepared from 5-hydroxykynurenine with aromatic L-amino acid decarboxylase, partially purified from beef brain (7). 5-Hydroxykynurenamine was purified by successive column chromatographies with Amberlite CG-50 (acetate form, 1 X 10 cm) (8) and Dowex 50 X 12 (H^+ form, 1 X 5 cm) (9). 4,6-Dihydroxyquinoline was chemically synthesized as described by Makino and

Takahashi (10). Absorption spectra were recorded by a Cary 15 spectrophotometer. Radioactivity was measured by a Nuclear-Chicago gas flow counter. Ascending paper chromatography was carried out on Whatman No. 3 MM paper with the following solvent systems: A, n-butanol-acetic acid-water (4:1:5); B, 80% n-propanol; C, methanol-n-butanol-benzene-water (2:1:1:1); and D, 5% NaCl. High voltage electrophoresis was carried out on Whatman No. 3 MM paper with 1 M acetate buffer, pH 2.9 by the use of pherograph, Model 64, Frankfurt. A potential of 2,500 volt which produced a current of 25 mA, was applied for 60 min. Indole compounds and their metabolites were detected by fluorescence or by the treatment with Ehrich's reagent.

RESULTS AND DISCUSSION

When L-5-hydroxytryptophan was incubated aerobically with intestinal tryptophan 2,3-dioxygenase, the absorption band at 298 nm due to L-5-hydroxytryptophan diminished with the concomitant appearance of a new absorption band at 341 nm. A clear isosbestic point was observed at 312 nm (Fig. 1 left). When the spectral change at 341 nm reached the maximum, the reaction was terminated by the addition of zinc acetate and sodium hydroxide. The supernatant was adjusted to pH 7.4 with 1 M potassium phosphate buffer. The accumulated compound, thus obtained, was converted to a new compound with an absorption maximum at 380 nm, either by treatment with 0.1 N HCl at room temperature for 20 min or enzymatically by formamidase with a clear isosbestic point at 362 nm (Fig. 1 right). The latter compound was presumed to be 5-hydroxykynurenine, because its absorption spectra at pH 2.0, 7.4 and 12.0 were identical to those of authentic 5-hydroxykynurenine (6, 11, 12). Furthermore, it exhibited R_f values identical with those of 5-hydroxykynurenine on paper chro-

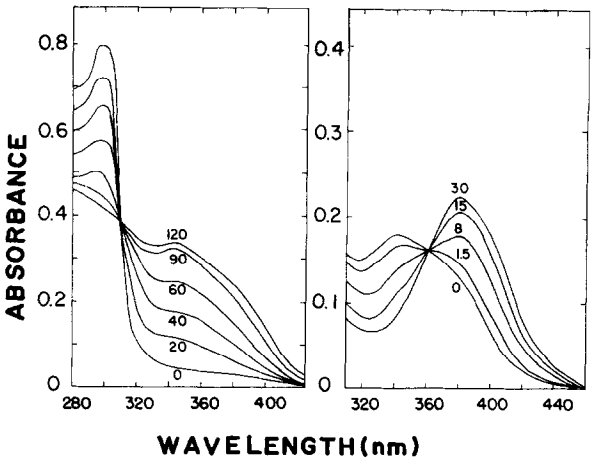

Fig. 1. Accumulation of 5-hydroxyformylkynurenine and its conversion to 5-hydroxykynurenine. The reaction mixture contained, in a total volume of 1.0 ml, 50 m$\underline{M}$ potassium phosphate buffer, pH 7.4, 5 $\mu\underline{M}$ methylene blue, 5 m$\underline{M}$ sodium ascorbate, 50 μg of catalase, 125 μg of intestinal tryptophan 2,3-dioxygenase and 0.1 m$\underline{M}$ L-5-hydroxytryptophan. Spectral changes were recorded at the time indicated in min after the reaction was started (left figure). Control cuvette contained the same components described above except that the substrate was omitted. After 90 min incubation at 24°, deproteinization was carried out by the addition of 5% zinc acetate (0.1 ml) and 0.2 $\underline{N}$ sodium hydroxide (0.1 ml). After centrifugation of the reaction mixture, the supernatant solution (0.5 ml) was diluted with 0.5 ml of 1 $\underline{M}$ potassium phosphate buffer, pH 7.4, and its ultraviolet spectrum was recorded. Formamidase (1 mg) was added to both the sample and control cuvette (right figure).

matography, which were 0.24, 0.17 and 0.60 with solvent systems A, B, and D respectively. In order to obtain further evidence, a reaction mixture containing 1 m$\underline{M}$ D,L-5-hydroxytryptophan-3-^{14}C (200,000 cpm) was deproteinized with 10% trichloroacetic acid after 1.5 hours incubation, by which treatment the formyl group of 5-hydroxyformylkynurenine was completely hydrolyzed. The supernatant was subjected to a column chromatography with Dowex 50 X 12 (H^+ form, 1 X 5 cm). The column was washed with 50 ml of 0.1 $\underline{N}$ HCl, 50 ml of water and 50 ml of 2.5 $\underline{N}$ HCl. 5-Hydroxykynurenine was then eluted with 60 ml of 5 $\underline{N}$ HCl, while 5-hydroxytryptophan was retained on the column under these conditions. A single radioactive peak was obtained, which coincided with the peak of authentic 5-

hydroxykynurenine as determined by absorbance at 315 nm. The recovery of authentic 5-hydroxykynurenine was 83%. The radioactive eluate (44,000 cpm) was subjected to crystallization with non-radioactive 5-hydroxykynurenine added as a carrier. A constant specific activity was obtained throughout the course of three recrystallization. When the twice crystallized preparation was subjected to electrophoresis, a single radioactive spot was detected, which coincided with the position of 5-hydroxykynurenine. Ultraviolet light has also been reported to be capable of transforming tryptophan into kynurenine and serotonin into 5-hydroxykynurenamine (13). However, no product was detected in the present study, when the boiled enzyme was used. Furthermore, the degradation of L-5-hydroxytryptophan in the complete system was observed in the dark. Therefore, a photocatalytic process does not appear to be involved. When serotonin was used as substrate, a new absorption band appeared at 363 nm. Upon treatment with formamidase, this compound was converted to a compound with an absorption band at 402 nm, which was assumed to be 5-hydroxykynurenamine because its spectral characteristics at pH 1.0 and pH 7.0 were identical with those of authentic 5-hydroxykynurenamine (9). R_f values of 5-hydroxykynurenamine on paper chromatography were 0.31, 0.60, 0.34, and 0.82 with solvent systems A, B, C and D, respectively. The product from serotonin-3-^{14}C was isolated by cation exchange chromatography (9), and treated with monoamine oxidase (8, 14). With the product from serotonin and authentic 5-hydroxykynurenamine, a new absorption band appeared at 335 nm, suggesting the accumulation of 4,6-dihydroxyquinoline (10). Upon paper chromatography, radioactivity was detected at the same spot where authentic 4,6-dihydroxyquinoline was localized. R_f values of the latter compound were 0.74 and 0.77 with solvent systems A and C, respectively.

Intestinal tryptophan 2,3-dioxygenase seems to have broad substrate specificity in contrast to hepatic and pseudomonad enzymes, which are specific for L-tryptophan (11, 15, 16). When other indole compounds were examined spectrophotometrically, tryptamine was degraded, but indole-3-acetic acid and its 5-hydroxyl derivative were inert. The kinetic parameters of enzyme, K_m and V_{max}, are summarized in Table I. Since these compounds competitively inhibited the enzyme activity in the presence of D-tryptophan, a single enzyme protein appears to be involved in their degradation.

Table I

Kinetic Parameters of Tryptophan Analogues for Intestinal Tryptophan 2,3-dioxygenase

The assay conditions were as described in the legend to Fig. 1. Values were calculated from a plot of the reciprocal of reaction velocity against the reciprocal of substrate concentration. The reaction velocity was measured by following the increase in absorbance at 321 nm (tryptophan), 341 nm (5-hydroxytryptophan), 335 nm (tryptamine) and 363 nm (serotonin), assuming that molecular extinctions of the products from these compounds were of the same order of magnitude (ε = 3,750).

Substrate	V_{max}	K_m
	nmoles/mg of protein /min at 24°	μM
D-Tryptophan	140	300
L-Tryptophan	140[a]	20[a]
D-5-Hydroxytryptophan	9	100
L-5-Hydroxytryptophan	11[a]	6[a]
Tryptamine	5	250
Serotonin	3	150

a/ The reaction was carried out at pH 6.6. The optimum pH with L-tryptophan or L-5-hydroxytryptophan was pH 6.6, while that with tryptamine, serotonin, D-tryptophan or D-5-hydroxytryptophan was around pH 7.4. L-5-Hydroxytryptophan showed a marked substrate inhibition as in the case of L-tryptophan (2).

K_m values for ascorbic acid[1/] and for methylene blue were of the same order of magnitude with any of these compounds as substrate (2). Pyrrolooxygenase, obtained from wheat germ, has been reported to have broad substrate specificity for indole compounds including peptidyl tryptophan (17). However, its cofactor requirements and behaviors towards inhibitors such as CN and EDTA were different from those of intestinal tryptophan 2,3-dioxygenase (2, 17).

The natural occurrence of 5-hydroxykynurenine, 5-hydroxykynurenamine and 4,6-dihydroxyquinoline in the urine of hens and mice has been reported (12, 18, 19). 5-Hydroxytryptophan has been shown to be degraded to 5-hydroxykynurenine or 4,6-dihydroxyquinoline by crude extracts of rat intestine (20). The precise metabolic pathways, however, have not been elucidated so far. The oxygenative ring cleavage of 5-hydroxytryptophan and serotonin by intestinal tryptophan 2,3-dioxygenase may account for the natural occurrence of these compounds in urine samples, and may play an important role in the regulation of serotonin level in intestine.

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1/ Ascorbic acid could be replaced by xanthine oxidase and hypoxanthine, and bovine superoxide dismutase inhibited these reactions as in the case of D-tryptophan (3). Details will be published elsewhere.

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