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HYDROXYLATION OF TRYPTOPHAN AND PHENYLALANINE IN NEOPLASTIC MAST CELLS OF THE MOUSE

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Abstract—The hydroxylation of tryptophan to 5-hydroxyindoles and phenylalanine to tyrosine was studied in intact neoplastic mast cells. Although both substrates were hydroxylated at rapid rates, the rate of formation of tyrosine was two to three times greater than the rate of formation of 5-hydroxyindoles. This is apparently the first demonstration of tyrosine synthesis in a mammalian tissue other than liver. Because of the simplicity of the techniques involved and the small range of variability in the results in these studies, it is suggested that the methods described should prove useful in studying potential inhibitors of tryptophan until a suitable cell-free system becomes available.

THE presumed first step in the biosynthesis of serotonin (5-hydroxytryptamine, 5HT), i.e. the hydroxylation of tryptophan to form 5-hydroxytryptophan (5HTP), has been difficult to study in mammalian systems. In experiments reported to date, the rate of hydroxylation of tryptophan has been relatively low, and the techniques required for its demonstration have been complex. Schindler reported that, in tissue culture, neoplastic mast cells of the mouse formed 5HT from radioactive tryptophan.[1] This process also took place in homogenates of these cells in the presence of diphosphopyridine nucleotide, nicotinamide, and pyridoxal phosphate. With similar techniques and the same line of mast cells, Day and Green showed that the 5HTP that was formed from radioactive tryptophan was decarboxylated rapidly to form 5HT.[2] Cooper and Melcer reported tryptophan hydroxylation in the particulate fraction of cells from the intestinal mucosa of rats and guinea pigs under anaerobic conditions in the presence of ascorbic acid and cupric ions.[3] Under these conditions, however, the reaction was also found to occur to a small but significant extent in the absence of enzyme. Freedland *et al.*[4] have found that tryptophan hydroxylation could be demonstrated in the soluble supernatant fraction of rat liver homogenates when tryptophan was supplied in very high concentrations. Renson *et al.* showed that the enzyme responsible for this reaction was actually phenylalanine hydroxylase.[5] Furthermore, they presented a convincing argument that this enzyme probably does not contribute significantly to the biosynthesis of 5-hydroxyindoles in the intact animal.

The present studies were undertaken for the purpose of designing a simple system for studying tryptophan hydroxylation in mammalian tissue. This was accomplished by means of neoplastic mast cells in the ascites form from the mouse. These cells not only hydroxylated tryptophan very actively but also hydroxylated phenylalanine to

form tyrosine. This is apparently the first demonstration of tyrosine synthesis in a mammalian tissue other than liver.

MATERIALS AND METHODS

Mice (BALB/c by DBA/2; F_1 hybrids) bearing the transplantable mast cell neoplasm P815[6] in the ascitic form during generations 408 through 414 were used in these studies. On the eighth or ninth day following transfer of the tumor, peritoneal fluid was removed by aspiration into a syringe. Cells were separated from ascites fluid by centrifugation at low speed, washed twice in Locke's solution which contained 5 per cent glucose, and resuspended in Locke's–5% glucose solution.

Incubations were carried out aerobically in 20-ml covered beakers at 37° in a metabolic shaker. The incubation beakers contained suspensions of washed mast cells (20–40 mg protein), 400 μmoles of phosphate buffer, pH 7·5, substrate (L-tryptophan or L-phenylalanine) and sufficient Locke's–5% glucose solution to make a final volume of 4·0 ml. In studies of tryptophan hydroxylation, aliquots of incubation mixture were transferred to centrifuge tubes which contained 0·2 ml of 25% trichloroacetic acid. After centrifugation, 0·7 ml of supernatant fluid was added to 0·3 ml of 0·5 M phosphate buffer, pH 7·5, and assayed for total 5-hydroxyindole content by the colorimetric method of Udenfriend *et al.*[7] as modified by Renson *et al.*[5] In studies of phenylaline hydroxylation, 0·5-ml aliquots of incubation mixture were transferred to centrifuge tubes which contained 1·0 ml of 25% trichloroacetic acid. After the addition of 3 ml of water and centrifugation, 1·0 ml of supernatant fluid was assayed for tyrosine content by the fluorometric method of Waalkes and Udenfriend.[8] Protein was determined by a modification of the phenol reagent method.[9]

In order to demonstrate that radioactive-labeled exogenous phenylalanine and tryptophan were converted to their respective hydroxylated products, incubations were prepared as above with the following changes. To one beaker was added 0·2 μmole of L-phenylalanine-1-^{14}C (sp. act. 4 μc/μmole) (New England Nuclear Corp.). To another beaker was added 0·1 μmole of DL-tryptophan-1-^{14}C (sp. act. 8·95 μc/μmole) (California Corp. for Biochemical Research). Also added to each beaker was 1·2 μmoles of α-methyl-dopa hydrazine (Merck Sharp & Dohme Research Laboratories), a potent inhibitor of aromatic-L-amino acid decarboxylase. Aliquots were removed at 0 and 60 min and transferred to centrifuge tubes that contained 3 vol. 95% ethanol and 10 vol. acetone. This mixture was shaken at 0° and then centrifuged. The supernatant fluid was evaporated under nitrogen to a volume of 0·5 ml. Desalting in cold acetone and ethanol was repeated and the solution evaporated to dryness.

In some experiments the residue was dissolved in 0·1 ml of 4% formic acid, applied to Whatman 3MM paper and subjected to electrophoresis at 5 kv for 5 hr in 4% formic acid. Phenylalanine and tyrosine were detected by dipping the paper into ninhydrin reagent 2% in acetone. Tryptophan and 5HTP were located by dipping the paper into Ehrlich's reagent, 10% in concentrated HCl (diluted 1 : 4 in acetone). Under these conditions of electrophoresis, tryptophan migrated 81 cm, 5HTP 70 cm, phenylalanine 91 cm, and tyrosine 82 cm from the point of origin.

The spots representing each compound were cut out and placed in counting vials. A scintillation fluorophor solution[10] was added and the radioactivity determined in a scintillation spectrometer. In other experiments the residues from the desalting process were dissolved in 0·1 ml of 0·06 N HCl, applied to Whatman 1 paper and

chromatographed (ascending) overnight in butanol–acetic acid–water (120 : 30 : 50). The compounds were located and counted as in the electrophoresis experiments. The R_f values for each compound were tryptophan 0·52, 5HTP 0·28, phenylalanine 0·63, and tyrosine 0·45.

RESULTS

Conditions of assay

There were high levels of both tyrosine and 5-hydroxyindoles (presumably 5HT) in the mast cells prior to incubation; endogenous levels of tyrosine and 5-hydroxyindoles were 1·3 to 5·0, and 0·6 to 2·3 mμmoles/mg protein respectively. Therefore it was necessary to allow the reaction to proceed for 30–60 min to obtain easily measured increments in the amounts of these products. The rate of formation of product was calculated by subtracting the concentration of product in the incubation mixture at zero time from the concentration of the product present at the end of the incubation. Under the incubation conditions described above, with 0·5 μmole/ml of substrate, the amount of product formed in 1 hr represented a 2- to 4-fold increase over the endogenous levels. When the cells were incubated in Locke's solution without added glucose the reaction took place at a rapid rate for a short period of time (less than 30 min) and then stopped. The addition of glucose always enhanced greatly the yield of either hydroxylated product (Table 1). When cell suspensions were boiled before incubation, there was no formation of either product.

TABLE 1. FORMATION OF 5-HYDROXYINDOLES (μmoles/mg PROTEIN/hr) FROM TRYPTOPHAN (0·5 μmole/ml); STIMULATION BY GLUCOSE

	Expt. I	Expt. II
Locke's solution	0·79	2·1
Locke's solution + 5% glucose	7·5	6·1

Cell suspensions prepared at different times varied greatly in their hydroxylating activities. With aliquots of the same cell suspensions, however, the rate of hydroxylation of either substrate did not vary by more than 10%. When substrate was added to the incubation mixture in a concentration of 0·5 μmole/ml, the amount of tyrosine or 5-hydroxyindoles increased in linear fashion for at least 1 hr (Fig. 1). The slight decrease in the rate of hydroxylation during the second hour was always greater with phenylalanine than with tryptophan. This decrease probably was due to diminution in the concentration of substrate in the incubation mixture (see below). The rate of hydroxylation of phenylalanine was usually two to three times that of tryptophan.

Effect of varying substrate concentrations

Cell suspensions were incubated for 1 hr with various concentrations of either phenylalanine or tryptophan. The results of typical experiments are illustrated in Fig. 2. Significant amounts of both tyrosine and 5-hydroxyindoles were formed in the absence of added substrate. It is assumed that this reflected hydroxylation of tryptophan and phenylalanine that was normally present in these cells. Attempts to measure tryptophan in these cells were unsatisfactory, owing to interference by

the large amounts of 5-hydroxyindoles present. Phenylalanine assays were not attempted. As the concentration of phenylalanine in the incubation mixture was increased from 0 to 0·1 μmole/ml the amount of tyrosine formed was increased 35 to 100%. Further increases in the concentration of phenylalanine resulted in

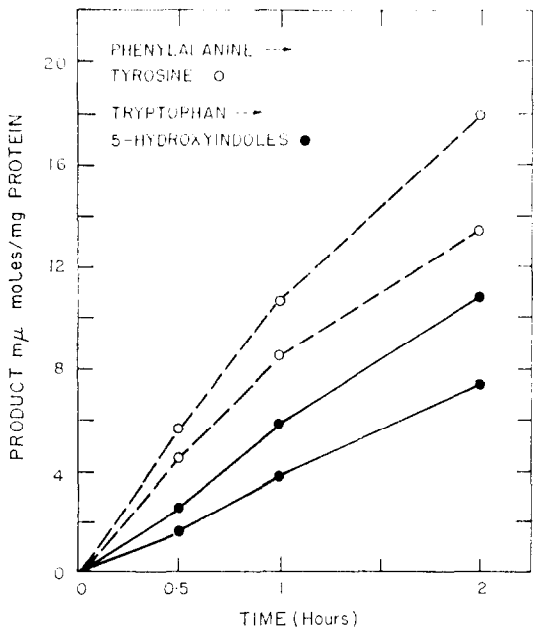

FIG. 1. Rate of hydroxylation of phenylalanine and tryptophan as a function of time. Two typical experiments are illustrated for each substrate. See text for details.

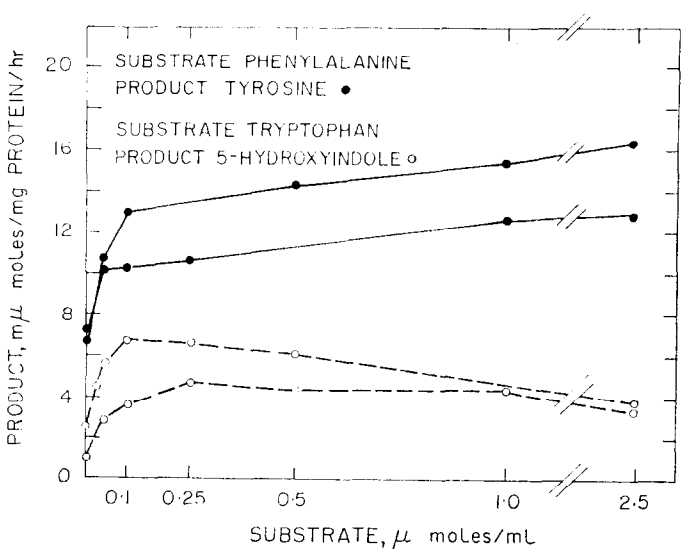

FIG. 2. Formation of tyrosine and 5-hydroxyindoles during 1-hr incubations as a function of concentration of substrate. Two typical experiments are illustrated for each substrate. See text for details.

much smaller increments in tyrosine formation. In the case of tryptophan, a different effect was observed (Fig. 2). A 3- to 5-fold increase in 5-hydroxyindole formation was observed as the amount of added tryptophan was increased from 0 to between 0·1 and 0·25 μmole/ml. Further increases in tryptophan concentration resulted in decreased yields of 5-hydroxyindoles.

Competition of tryptophan and phenylalanine

Various amounts of phenylalanine and tryptophan were added to the incubation mixtures, and the formation of both products was studied simultaneously. The results of typical experiments are illustrated in Figs. 3 and 4. The addition of 0·05 μmole/ml

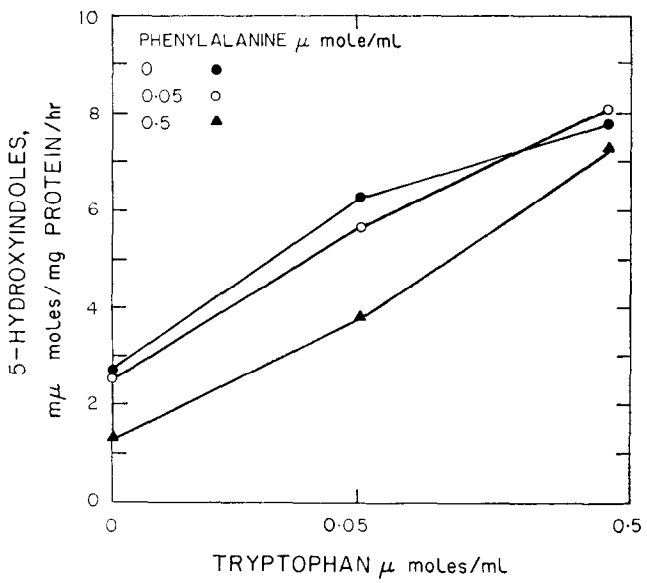

FIG. 3. Hydroxylation of tryptophan in the presence of various concentrations of phenylalanine.

of phenylalanine did not interfere appreciably with the formation of 5-hydroxyindoles, irrespective of the concentration of tryptophan. In the presence of 0·5 μmole phenylalanine/ml the formation of 5-hydroxyindoles from concentrations of tryptophan of 0·05 μmole/ml or lower was inhibited about 50%. When both substrates were present in equal concentrations, however, there was a minimal decrease in the formation of 5-hydroxyindoles. By contrast, in the presence of tryptophan in concentrations of 0·05 and 0·5 μmole/ml, the formation of tyrosine at each level of phenylalanine concentration was diminished substantially (Fig. 4).

The addition of D-tryptophan to the incubation mixture in concentrations as high as 2·5μmoles/ml did not increase the rate of formation of 5-hydroxyindoles nor did it it decrease the rate of formation of tyrosine from phenylalanine. Similarly, the addition of D-phenylalanine to the incubation mixture in concentrations as high as 2·5 μmoles/ml did not influence the rate of formation of either tyrosine or 5-hydroxyindoles.

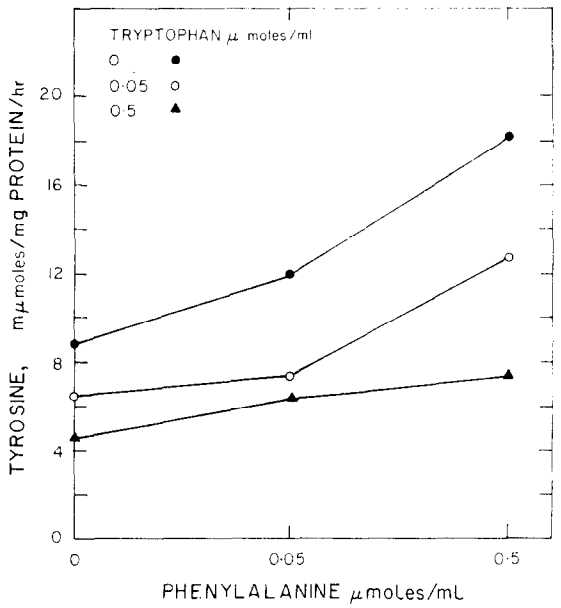

FIG. 4. Hydroxylation of phenylalanine in the presence of various concentrations of tryptophan.

Identification of products

The identity of the product of phenylalanine hydroxylation as tyrosine was established by the findings that this compound gave a characteristic fluorescent product

TABLE 2. INCORPORATION OF RADIOACTIVITY FROM TRYPTOPHAN-^{14}C INTO 5-HYDROXYTRYPTOPHAN (5HTP) AND FROM PHENYLALANINE-^{14}C INTO TYROSINE

Method of separation	Time of incubation (min)	Per cent of radioactivity incorporated into products*	
		5HTP	Tyrosine
Electrophoresis	0	<0·1	<0·1
	60	12·4	8·2
Paper chromatography	0	0·6	2·1
	60	6·0	16·0

* The amount of total radioactivity recovered from the paper varied between 14,000 and 50,000 cpm above background. The per cent of total recovered radioactivity present in the spots that represented products is recorded in this table. Each number represents the average of at least 2 experiments. Since different cell suspensions were used, the absolute values are not comparable with each other or with the results of experiments done with nonradioactive substrates.

on reaction with 1-nitroso-2-naphthol[8] and that on incubation with radioactive phenylalanine significant amounts of radioactivity appeared in a product that had the same electrophoretic and chromatographic properties as authentic tyrosine (Table 2). The identity of 5HTP as the product of tryptophan hydroxylation was

similarly established by characteristic color reactions with Ehrlich's reagent and 1-nitroso-2-naphthol[7] and by the finding that on incubation with radioactive tryptophan significant amounts of radioactivity appeared in a product that had the same electrophoretic and chromatographic properties as authentic 5HTP (Table 2).

DISCUSSION

The finding of hydroxylation of phenylalanine to tyrosine by the mast cell suspensions was not anticipated. Heretofore, phenylalanine hydroxylation had been demonstrated in no mammalian tissue other than liver. Furthermore, Day and Green[2] stated that phenylalanine hydroxylation was not observed in murine neoplastic mast cells that had been derived originally from the same strain of cells used in the present studies. The reason for this discrepancy is not clear. Perhaps it reflects a variation in the metabolic behavior of these cells at different times, as also noted by Day and Green.[2]

Since the further metabolism of the hydroxylated amino acids in neoplastic mast cells is incompletely understood, it is not possible to determine the absolute rate of hydroxylation of either phenylalanine or tryptophan. In the present studies, tyrosine seemed to be formed at a greater rate than were 5-hydroxyindoles. However in, contrast to the situation that obtains in the liver,[5] tryptophan appeared to compete favorably with phenylalanine for the hydroxylating process in mast cells. This apparent competition does not necessarily mean that phenylalanine and tryptophan are hydroxylated by the same enzyme. It is possible, for example, that tryptophan competes favorably with phenylalanine for a common transport mechanism into the cell. Similarly, the finding that D-amino acids do not function as either substrates or inhibitors of the hydroxylating process may also indicate that they penetrate the cells poorly.

Because of the simplicity of the techniques involved in these studies and the small range of variability when comparable cell suspensions are used, this method should prove useful in studying potential inhibitors of tryptophan hydroxylation. An optimum substrate concentration for such studies would be 0·5 μmole/ml because at this level there is a near maximal rate of reaction, and during the course of the incubation the concentration of tryptophan is not lowered to a point at which the rate of hydroxylation diminishes. One problem that should be considered, if decreased formation of 5-hydroxyindoles occurs with this system of intact cells in the presence of a drug, is possible interference with transport of substrate into the cell rather than actual inhibition of enzyme. This could be resolved by also studying the uptake of substrate, as suggested by Day and Green.[2] Although it would be preferable to study this reaction in a cell-free system, as yet no satisfactory cell-free system has been described.

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