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5-HYDROXY-L-TRYPTOPHAN, 5-HYDROXYTRYPTAMINE AND L-TRYPTOPHAN-5-HYDROXYLASE IN *GRIFFONIA SIMPLICIFOLIA*

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Abstract—5-Hydroxy-L-tryptophan (5-HTP) has been found in high concentration (6–10% fr. wt.) in the mature seeds of *Griffonia simplicifolia*, a West African legume of reputed physiological activity, and in lower concentration in other parts of the plant. An enzyme system capable of hydroxylating L-tryptophan has been identified in various tissues. 5-Hydroxytryptamine (5-HT or serotonin) has been found in concentrations of up to 0·2% (dry wt.) in the pods and in lower concentration in the leaves of mature plants. Indole-3-acetyl-aspartic acid (IAAA) and 5′-hydroxyindole-3-acetic acid (5-HIAA) were among other indoles detected.

INTRODUCTION

5-HYDROXYTRYPTAMINE (5-HT or serotonin), the physiologically active amine originally found in animal tissues,[1,2] is also known to occur in higher plants. It was first identified in the legume *Mucuna pruriens*[3] and has since been found in such unrelated species as *Musa sapientum* (banana) in the Musaceae[4] and *Gossypium hirsutum* (cotton) in the Malvaceae.[5] The same compound is also held to be responsible, in whole or in part, for the pharmacological effects produced by the stinging hairs of various species of plants including those of *Urtica dioica* (the common nettle.)[6]

In animal tissues 5-HT is formed by the hydroxylation of L-tryptophan and the subsequent decarboxylation of 5-hydroxy-L-tryptophan (5-HTP). 5-HTP itself has not been isolated from animal tissues, but it has been detected as the radioactively labelled precursor of 5-HT in animals supplied with 2^{14}-C-DL-tryptophan.[7,8] The results of similar experiments with labelled tryptophan in other organisms suggest that tryptophan-5-hydroxylase system is widely distributed, occurring not only in animals but also in bacteria[9] and higher plants.[10] When Udenfriend and co-workers supplied labelled and unlabelled 5-HTP to rats and dogs,[8] they found that this compound was metabolized first to 5-HT and then 5-hydroxy-indole-3-acetic acid (5-HIAA) which appeared in the urine. These experiments not only

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established the pathway of 5-HT biosynthesis in mammals but also showed that interperitoneal injection of 5-HTP produced a rapid and dramatic rise of 5-HT in all tissues containing 5-HTP decarboxylase. In the brain of both dog and rat, tenfold increases in the concentrations of 5-HT were observed and these increases produced symptoms in the animals which included "tremors, pupillary dilation, loss of light reflex, apparent blindness, salivation, marked hypernea and tachycardia". These symptoms, as the authors pointed out, were very similar to those induced by the hallucinogen, lysergic acid diethylamide (LSD). From the results of these experiments it was apparent that the limiting step, in the synthesis of 5-HT in the mammalian brain was the hydroxylation of tryptophan. It was also clear that any factor which increased the level of 5-HTP in the blood would induce a corresponding increase of 5-HT in the brain (and other tissues containing 5-HTP decarboxylase) together with the associated physiological effects which have been described.

The discovery that the seeds of the West African legume *Griffonia simplicifolia* contained high concentrations of 5-HTP[11] was therefore of threefold interest. Firstly it indicated the presence of an unusual metabolic system which might be of value in comparative and phylogenetic studies. Secondly it provided an unusually favourable opportunity to study the hydroxylation of tryptophan in a plant and thirdly it offered a possible explanation of one or more of the medicinal and physiological properties attributed to the plant by the native peoples of West Africa.[12]

The present paper describes the isolation of 5-HTP from the seeds of *G. simplicifolia*, and its quantitative estimation in other tissues of the plant. It also describes the identification and estimation of 5-HT in the pods and leaves of the mature plant, and the distribution and characteristics of the L-tryptophan-5-hydroxylase which was found in most tissues. The presence of other identified and unidentified indoles are reported and their possible significance discussed.

RESULTS

Indoles in Seeds

The only indole found in mature seeds was 5-HTP and this free amino acid constituted between 6–10% fr. wt. of different seeds examined. Chromatographic analysis of methanolic extracts made from immature seeds in the early stages of development showed that these contained readily detectable concentrations of three Ehrlich-reacting compounds and traces of four others. The three compounds present in highest concentration were 5-HTP, which was found in the cotyledons and testa, indole-3-acetylaspartic acid (IAAA), which was found only in the testa, and an unidentified ninhydrin-negative 5-hydroxyindole also found in the testa. This compound was positively charged at pH 1·9 and moved slightly slower than 5-HTP on ionophoresis, it was uncharged at pH 6·5 and carried a small negative charge at pH 10·0. Its R_f values ($\times 100$) in the butanol–acetic acid and butanol–pyridine solvents were 38 and 62 respectively. The compound was found only in the testa and, like IAAA, its concentration decreased during seed development. A limited supply of seed prevented further characterization. Three other indoles were detected in trace amounts in the testa or cotyledons during development. One of these was tentatively identified as tryptophan on the basis of its R_fs.

In germinating seeds three indoles were detected. Two of these were identified as 5-HTP and 5′-hydroxyindole-3-acetic acid (5-HIAA), the third (another 5-hydroxyindole) was not

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identified. The conc. of 5-HIAA and the "unknown" were estimated to be in the order of 2–5 μg/g fr. wt.

Indoles in Leaves

The only indole detected in the leaves of young plants grown in England was 5-HTP and subsequent experiments using ^{14}C labelled Try confirmed that 5-HTP was not metabolized further in these tissues. Traces of labelled IAAA were detected in tissue from the apical leaves of young seedlings after incubating with labelled tryptophan (but not labelled tryptamine): no labelled IAA was found however nor was endogenous IAAA present in detectable concentration in the original leaf material. These findings suggest that tryptamine is not involved in IAA synthesis in this plant and that IAA is rapidly conjugated with aspartic acid after synthesis.

In the leaves of mature plants grown in Ghana 5-HTP, 5-HT and, in one sample, tryptophan itself were detected. The relative concentrations of 5-HTP and 5-HT in the mature leaves collected over a period of 7 months are given in Fig. 1. These values suggest that

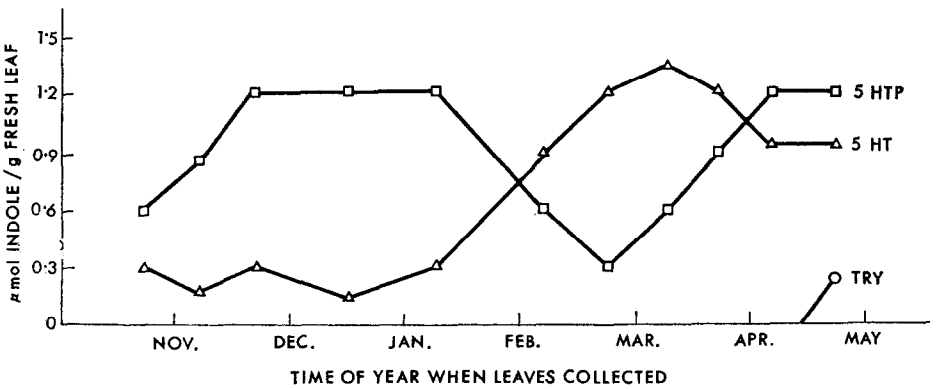

FIG. 1. SEASONAL VARIATION IN THE INDOLE CONTENT OF MATURE LEAVES OF *Griffonia simplicifolia*.

there are seasonal changes in the activities of both L-tryptophan-5-hydroxylase and 5-HTP decarboxylase in the mature leaves.

Indoles in Pods

The pods contained both 5-HTP and 5-HT. The concentration of each was between 0·1 and 0·2% dry wt. throughout development. Traces of a third unidentified basic indole were detected in one batch of very young pods. This compound moved between 5-HTP and 5-HT on ionophoresis at pH 1·9 and pH 6·5. Its R_fs (×100) in the butanol–acetic and butanol–pyridine solvents were 31 and 54 respectively.

Distribution of L-Tryptophan-5-hydroxylase

Discs taken from the leaves, petals and sepals, and tissue slices taken from the root, stem and developing seeds of the plant were all able to hydroxylate 3^{14}C-L-tryptophan in the 5 position when incubated in solutions containing that compound. In a typical experiment after incubation for 18 hr, 70% of the radioactivity detected in the leaf discs was present as 5-HTP, and the remainder as unchanged tryptophan. Of the tissues examined only those of the mature seed failed to show hydroxylase activity.

Specificity of L-*Tryptophan-5-hydroxylase*

The tryptophan-5-hydroxylase was shown to be stereospecific by incubating leaf discs separately with solutions of 3[14]C-DL-tryptophan, 3[14]C-L-tryptophan and 3[14]C-D-tryptophan. Incorporation of [14]C into 5-HTP from 3[14]C-DL-tryptophan was half that of incorporation from the labelled L-isomer and there was no incorporation from the D-isomer.

The substrate specificity of the enzyme was examined by incubating leaf discs with solutions of 2[14]C-L-phenylalanine, 2[14]C-L-tyrosine, 2[14]C-L-dihydroxyphenylalanine, 2[14]C-5-HT and uniformly labelled [3]H-tryptamine. None of these compounds acted as a substrate for the enzyme.

Effects of Reduced Oxygen and Daylight on the Hydroxylase Activity of Leaf Discs

Incorporation of label from 3[14]C-L-tryptophan into 5-HTP in leaf discs was reduced 90% when incubation was carried out under an atmosphere of nitrogen. The rate of incorporation was not affected by presence or absence of daylight.

Inhibitors of L-*Tryptophan-5-hydroxylase*

At a conc. of 10^{-3} M, cyanide ions reduced incorporation of labelled tryptophan into 5-HTP in the leaf discs by 10%. At the same conc. EDTA had no effect, while $\alpha\alpha'$ dipyrridyl, which is also a chelating agent, reduced incorporation by 20%. The sulphydryl reagents *p*-chloromercuribenzoate and iodoacetamide (at the same molar concentration) inhibited both the uptake of 3[14]C-L-tryptophan by the leaf discs (by 68% and 75% respectively) and subsequent hydroxylation (by 42% and 87% respectively). *p*-Chlorophenylalanine, which is a specific inhibitor of L-tryptophan-5-hydroxylase in mammalian brain,[13] reduced leaf disc uptake by 33% and hydroxylation by over 90% under the same conditions. L-Phenylalanine reduced uptake by 90% but it did not appear to interfere with hydroxylation.

Failure to Obtain a Cell-free Preparation of L-*Tryptophan-5-hydroxylase*

Repeated attempts to prepare a soluble enzyme system from homogenized leaf material failed, as did subsequent attempts to show hydroxylase activity in preparations of nuclei, mitochondria and chloroplasts. The rapid browning of tissues and extracts which followed cell disruption suggested the possibility that enzyme activity was being inhibited by the interaction of the enzyme protein with products of phenolic oxidation.[14,15] Attempts were made to eliminate or minimize any such effect by adding reducing agents,[16] by adding insoluble polyvinylpyrrolidone[17] and polyethylene glycol[18] (to combine with tannins), by adding diethyldithiocarbamate[19] and cyanide[20] (to inhibit phenol oxidases), by dialysing the leaf extracts (to remove phenols) and by precipitating and reprecipitating the protein fractions with ammonium sulphate. None of these methods prevented enzyme inactivation.

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DISCUSSION

Tryptophan is a constituent of most proteins and the precursor of the plant growth hormone IAA. The formation of labelled IAAA, the aspartic acid conjugate of IAA, from 3-^{14}C-L-tryptophan in tissue taken from apical leaves of *Griffonia simplicifolia* seedlings suggests that tryptophan fulfils both roles in this plant. The failure of the same tissue to convert labelled tryptamine to IAAA, and the absence of detectable IAA conc. itself suggest that indolepyruvic acid rather than tryptamine is the intermediate in IAA synthesis and that conjugation with aspartic acid rapidly follows synthesis. The presence of traces of 5-HIAA, but not of IAA nor IAAA, in the germinating seeds could possibly be explained if 5-HIAA acts as a growth hormone in *G. simplicifolia* even though it is inactive in some other spp. which do not themselves synthesize 5-HTP.[21] The presence of several unidentified indole derivatives in various tissues at different stages of development further emphasizes the unusual pattern of indole metabolism in this plant. The most obvious feature of this unusual pattern is however the accumulation of high 5-HTP conc., and to a lesser extent of 5-HT in the plant. No other organism is known in which 5-HTP is the principal end-product of tryptophan metabolism, and all metabolically active tissues of *G. simplicifolia* contain L-tryptophan-5-hydroxylase. The significance of the high levels of 5-HTP in *G. simplicifolia* is not certain, though the ability of many legumes to accumulate high concentrations of "unusual" amino acids such as canavanine, homoarginine and lathyrine is well known.[22] The very high 5-HTP conc. found in the seeds of *G. simplicifolia* suggests a storage role for the amino acid but such a role might equally well be filled by one or other of the "protein" amino acids. If *G. simplicifolia* has been selected for in a particular environment because it contains high concentrations of 5-HTP, then it is necessary to suppose that the presence of 5-HTP confers on the plant some evolutionary advantage or advantages which the presence of an equivalent concentration of a "protein" amino acid would not. Bearing in mind the toxicity of 5-HTP to higher animals and the widespread distribution of 5-HTP decarboxylase amongst living organisms, it is possible that 5-HTP could confer such an advantage on *G. simplicifolia* by protecting the plant against insects or micro-organisms in the manner discussed by Janzen.[23]

EXPERIMENTAL

Plant Material

The leaf discs which were used to study the characteristics of L-tryptophan-5-hydroxylase were taken from young plants grown in a greenhouse in England. The mature leaves, flowers and fruits were collected in Ghana at regular intervals and flown to London on the day of collection. The material from Ghana was sealed in plastic bags containing moist cotton wool to prevent drying.

Chromatography

One-dimensional chromatography, ascending and descending, was performed on Whatman No. 1 or 3MM paper. Extracts were spotted on the papers and dried in a stream of cold air. Papers were equilibrated for 2 hr in an atmosphere saturated with the appropriate solvent before running. The chromatograms were run for 12–18 hr in the dark at 18°. Solvents[24,25] used were: (1) *n*-BuOH–HOAc–H_2O (12:3:5); (2) *iso*-PrOH–NH_4OH–H_2O (20:1:2); (3) *n*-BuOH–pyridine–H_2O (1:1:1); (4) *n*-PrOH–NH_4OH (5:1); (5) MeCOEt–*t*-BuOH–$NHEt_2$–H_2O (40:40:4:20). Two-dimensional chromatograms were run in solvents 1 and 2. When indoles were to be eluted for fluorimetric analysis, all solvents were redistilled before use and the papers were washed with deionized water.

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High-Voltage Ionophoresis

Ionophoresis was carried out on Whatman No. 1 or 3MM paper using a Shandon flat plate water-cooled apparatus and a potential difference of 60 V/cm for a period of 30 min. Buffer solutions of pH 1·9, 3·6, 6·5[26] and 12·0[27] were used.

Location Reagents

The following reagents, prepared according to the methods of Smith,[24] were used for the location and identification of indole amino acids and their derivatives on chromatograms and ionophoresis papers: (1) ninhydrin for amino acids; (2) ninhydrin acidified with acetic acid for unsubstituted tryptamines; (3) Ehrlich's reagent for indoles; (4) xanthydrol reagent for indoles; (5) 1-nitroso-2-naphthol reagent for 5-hydroxyindoles; (6) Pauly reagent for phenols (including 4, 5 and 6-hydroxyindoles); (7) 2,4-dinitrophenylhydrazine for indole-3-aldehyde; (8) acid persulphate for indole-3-acetic acid and indole-3-acetyl derivatives of amino acids.

Fluorimetry

Fluorimetric determinations were made with an Aminco–Bowman fluorimeter.

Determination of Radioactivity

Radioautographs were prepared on Kodirex X-ray film (Kodak Ltd.). Chromatograms were scanned (after fixing to TL plates with adhesive tape) in a Panax Thin-Layer Scanner RTLS.

Elution of Indoles from Chromatograms

The area of paper containing the indole was cut out, cut up into small pieces, covered with methanol (redistilled from $FeSO_4$)[28] and allowed to stand for 2 hr in the dark at 4°. The paper was then removed by filtration washed with redistilled methanol and the combined filtrate and washings used for the subsequent analyses.

Identification of 5-HTP

In tissues other than seeds (from which it was isolated in crystalline form) 5-HTP was identified by co-chromatography in the solvents described, and by ionophoresis at four different values of pH using synthetic 5-HTP as a "marker". The compound was also eluted from chromatograms and further characterized by fluorescence and u.v. spectroscopy.

Identification of 5-HT

The 5-HT in pods and mature leaves was characterized by co-chromatography and ionophoresis in the solvent systems and buffers described, using authentic 5-HT for comparison. Identification was confirmed by fluorescence and u.v. spectrophotometry, after elution from chromatograms.

Resolution of Synthetic 5-Hydroxy-DL-tryptophan

The isomers of 5-hydroxy-DL-tryptophan were separated by descending chromatography on paper using MeOH–pyridine–H_2O (20:5:1). The D-isomer had the greater R_f in this system and the two forms were eluted separately with methanol. The methanolic extracts were taken to dryness in a rotary evaporator below 30° and the free amino acids redissolved in water. This method was preferred to that of Contractor and Wragg[29] as difficulty was experienced in removing the last traces of their solvent (*n*-BuOH–pyridine) after elution. The isomers separated from $3^{14}C$-5-Hydroxy-DL-tryptophan (supplied by Calbiochem.) were used to determine the stereospecificity of tryptophan-5-hydroxylase.

Isolation of 5-HTP from Seeds

Finely ground seed (9 g) was shaken with 200 ml of aq. ethanol (65%, by vol.) at 17° for 2 hr. The seed was filtered off and the extraction repeated. The combined extracts were concentrated under reduced pressure at 25°. When all the ethanol had been removed, the aqueous residue was shaken three times with equal vol. of light petroleum (b.p. 40–60°) and finally with an equal vol. of Et_2O to remove lipids and pigments. Following these extractions the aqueous layer was twice shaken with decolorizing charcoal in the cold, filtered and again concentrated under reduced pressure at low temp. until it became an oily liquid. On cooling, crystals separated. These were filtered off, washed with ice-cold water, and recrystallized twice with aq. *iso*-PrOH (50%, by vol.). The yield was 0·42 g (4·7%) of colourless needles. The isolated material moved with synthetic 5-hydroxy-

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L-tryptophan when chromatographed and subjected to ionophoresis in the systems described, and showed the same retention time on the ion-exchange column of a Beckman 120C amino acid analyser. The i.r., u.v. and fluorescence spectra of the isolated compound were identical with those of the authentic L-5-HTP (supplied by Calbiochem.) and its elementary composition corresponded to that of 5-HTP monyhydrate.

Determination of 5-HTP in Mature Seeds

Finely ground seed (0·1 g) was shaken at room temp. for 2 hr with 0·1 N HCl (100 ml). After centrifugation an aliquot of the supernatant was removed and after appropriate dilution with 0·1 N HCl the concentration of 5-HTP was determined by the method of Udenfriend *et al.* [30] using 1-nitroso-2-naphthol and standard curves prepared from synthetic 5-HTP.

Determination of 5-HTP in Leaves

The method was essentially that of Quay.[31] Fresh leaf material (1 g) was homogenized with 0·1 N HCl (10 ml) containing ascorbic acid (0·5%). An aliquot (500 μl) was adjusted to pH 10, saturated with NaCl and extracted with *t*-amyl alcohol to remove 5-HT. The aqueous layer was then acidified and again extracted with *t*-amyl alcohol to remove the 5-HTP. The alcoholic layer containing the 5-HTP was shaken with a mixture of heptane and 0·1 N HCl (containing ascorbic acid) and the aqueous phase (containing the amino acid) separated, brought to a conc. of 3 N with con. HCl and its fluorescence at 295 nm determined using an activating wavelength of 550 nm. Standard curves were prepared by taking solutions containing known concentrations of synthetic 5-HTP through the same extraction procedures. Recovery was not quantitative because a small proportion of 5-HTP was extracted into *t*-amyl alcohol at pH 10; this fact made it necessary to determine the concentration of the free 5-HT by difference, after determining total 5-hydroxyindoles by the nitrosonaphthol methol.

Determination of Total 5-Hydroxyindoles and 5-HT in Leaves

Fresh leaf material (1 g) was homogenized with 0·1 N HCl (10 ml). After centrifugation, an aliquot (2 ml) of the supernatant was removed and the concentration of total 5-hydroxyindoles determined by the method of Udenfriend *et al.*[30] using 1-nitroso-2-naphthol. Standard curves were prepared using 5-HT and 5-HTP, both compounds gave the same colour yield/mole and the absorbance at 540 nm was directly proportional to concentration within the range of 0·025–0·4 μmole/ml. 5-HT conc. was found by difference, 5-HTP conc. (the only other 5-hydroxyindole present) having been determined by Quay's method.[28]

IAAA in Immature Seeds

A strongly acidic indole was detected in the testa and jelly-like substance which surrounded the cotyledons of young seeds. The indole which was present in highest concentration in youngest seeds and absent during later stages of development gave no reaction with ninhydrin, nitrosonaphthol or Pauly reagent. It gave a blue with Ehrlich's reagent, a purple with xanthydrol and a slowly developing violet colour characteristic of indole-acetyl derivatives when treated with acid persulphate.

The compound was eluted from ionophoresis papers and its fluorescence characteristics determined; these (activation max 280 and fluorescence max 360 nm) were identical with those of synthetic IAAA. On acid hydrolysis (2 N HCl for 17 hr at 100°) the eluted compound liberated aspartic acid (identified by co-chromatography and ionophoresis) and confirmation of identity was obtained by co-chromatography of the original compound with authentic IAAA in solvents 1–3.

Identification of 5-HIAA in Germinating Seeds

The cotyledons of 3-week-old seedlings were homogenized with aq. ethanol (10 ml/g fr. tissue). The ethanol was removed under reduced pressure at 25°, the aqueous residue acidified to pH 2 with N HCl, and extracted three times with equal vol. Et_2O. The residue was again extracted with ether after readjusting the pH firstly to 9·5 and then to 7·0. The basic and neutral extracts contained only 5-HTP while the acidic fraction contained two other compounds which reacted with Ehrlich's reagent. After evaporating the ether from the acid extract, the three indoles in the aqueous residue were separated by chromatography on paper using solvent 1, and the two unknowns eluted with methanol. The fluorescence characteristics of one of these (activation max 30 and fluorescence max 350 nm) were the same as those of 5-HIAA and its identity was confirmed by the co-chromatography in three solvent systems, ionophoresis at pH 10, and by its colour reactions with nitrosonaphthol xanthhydrol and the Pauly reagent. The third compound reacted as a 5-hydroxyindole with 1-nitroso-2-naphthol, gave a sky-blue colour with ninhydrin and moved fractionally slower than IAAA or ionophoresis at pH values of 1·9, 6·5, and 12·0. It was not characterized further.

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Demonstration of Tryptophan-5-hydroxylase Activity in Tissues

Lower leaves of 8-in. seedling plants were removed and washed successively in NaClO (1%) and water. Discs (1 cm dia.) were cut with a sterile cork-borer, care being taken to avoid major veins. Discs were pooled and approximately 0·5 g of leaf material was floated in 2 ml of buffer solution (0·01 M phosphate, pH 7) containing 2 μc 3^{14}C-L-tryptophan (0·05 μmole/ml), penicillin-G 6·2 μg/ml and streptomycin sulphate 100 μg/ml. The antibiotics did not affect the rate of uptake or hydroxylation of tryptophan. The solution was placed in the dark at 18° for 18 hr. After this period the discs were removed, placed briefly on filter paper to remove surface water and homogenized with methanol (1 ml) for 1 min. The homogenate was allowed to stand at 4° for 1 hr filtered and 50-μl aliquots of filtrate analysed by 2D chromatography on paper using solvents 1 and 2. A control experiment in using leaf discs that had been boiled for 1 min was carried out simultaneously. Autoradiograms were prepared from the chromatograms. For quantitative determinations duplicate chromatograms were prepared, one of which was developed with Ehrlich's reagent to identify the areas containing tryptophan and 5-HTP, the corresponding areas were then cut from the second sheet and their radioactivity determined in the scintillation counter. The same procedure was used to detect activity in other tissues of the plant.

Determination of Enzyme Specificity

Incubation of leaf discs was carried out as described, labelled L- being replaced in various experiments with the same M of labelled DL-tryptophan, D-tryptophan, L-phenylalanine, L-tyrosine, L-dihydroxyphenylalanine and tryptamine.

Determination of Enzyme Inhibition

Incubation of leaf discs was carried out as described and potential inhibitors were added to the incubation mixture at a conc. of 10^{-3} M. The effect of each potential inhibitor on the uptake and hydroxylation of 3^{14}C-L-tryptophan was determined by comparing the concentrations of labelled tryptophan and 5-HTP in the leaf discs after a standard period of incubation (18 hr) with the concentrations in control discs incubated simultaneously in the absence of inhibitor. Uptake of discs was measured as the total radioactivity in both Try and 5-HTP after incubation and the effect of inhibitors expressed as a percentage reduction in total activity (compared with a simultaneously incubated control). Inhibition of hydroxylation was expressed as a percentage reduction in the conversion of absorbed 3^{14}C-L-tryptophan to 5-HTP, the percentage conversion in the control being taken as 100.

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