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Studies of Enzyme-mediated Reactions. Part 16[1]. Stereochemical Course of the Formation of 5-Hydroxytryptamine (Serotonin) by Decarboxylation of (2S)-5-Hydroxytryptophan with the Aromatic L-Amino Acid Decarboxylase (E.C. 4.1.1.28) from Hog Kidney.§

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(2RS)-[2'-^{3}H, 3'-^{14}C]-5-Hydroxytryptophan was prepared and then incubated with mammalian aromatic L-amino acid decarboxylase (E.C. 4.1.1.28) from hog kidney. Decarboxylation resulted in the formation of [1'-^{3}H, 2'-^{14}C]-5-hydroxytryptamine (serotonin) with a small loss of tritium relative to the ^{14}C internal standard. The configuration of the tritium label was investigated by oxidation of the amine with pea seedling amine oxidase, the resulting aldehyde being converted *in situ* into the alcohol by an added reduction system consisting of liver alcohol dehydrogenase, NAD^{+}, and ethanol. The final product, 5-hydropxytryptophol, was devoid of tritium, showing that the tritium occupied the *pro-S* position in the 5-hydroxytryptamine, and therefore that decarboxylation had occurred with retention of configuration.

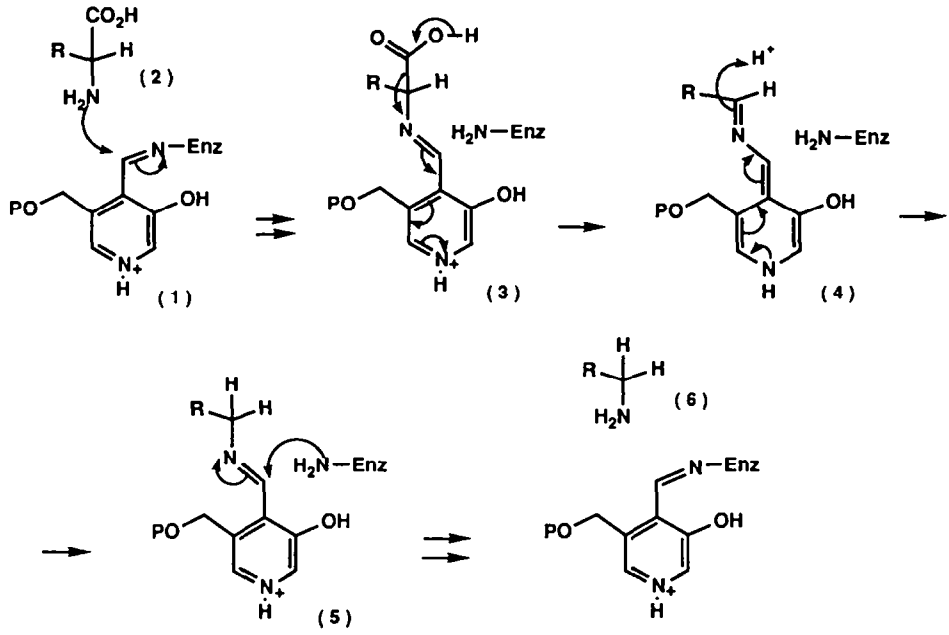
Decarboxylation of amino acids is important in both primary and secondary metabolism. In plants it serves to produce primary amines which act as precursors of a wide range of natural products, principally alkaloids.[2] Key primary amines are produced in animals where they function as hormones in their own right, or are precursors of the active compounds.[3]

Usually pyridoxal phosphate serves as cofactor for the decarboxylation,[4,5,6] and the process is thought to take place by the mechanism shown in Scheme 1.[7,8] Initially, the coenzyme is bound to the enzyme via a Schiff's base derivative (1), formed between the aldehyde group of the coenzyme and the ε-amino group of a lysine residue of the protein. Binding of the substrate (2) is followed by exchange of amine residues in the Schiff's base, so that a new Schiff's base is (3) generated between the α-amino group of the substrate and the coenzyme (this and subsequent intermediates remain bound to the enzyme via non-covalent interactions). The pyridine ring can now serve its catalytic function by acting as an electon sink in the decarboxylation step, (3) to (4), as shown. Protonation of the resulting intermediate then regenerates a Schiff's base (5), which can be cleaved by transamination with the lysine residue. The product amine (6) can now dissociate, leaving the enzyme as in (1), primed ready for the next cycle of reactions.

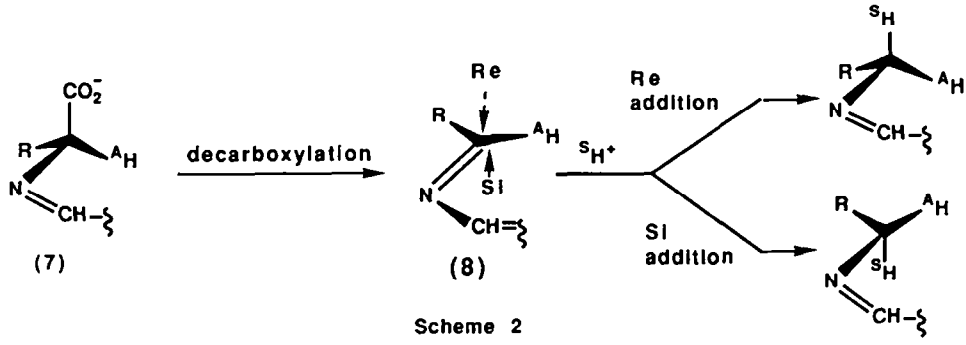
On stereoelectronic grounds, it is to be expected that the intermediate (3) will be constrained within the active site in a suitable conformation which will allow the coenzyme to exert its maximum effect as an

§ *This paper is dedicated to David Ollis with warmest good wishes for the future; it also marks a long friendship (with ARB) going back to the Bristol days.*

electron sink.[7] Firstly, for maximum delocalisation, the extended π-system should be planar. Secondly, the C-C bond to the carboxyl group should be coplanar with the p-orbital of the adjacent nitrogen atom. Two orientations of the substrate with respect to the coenzyme are suitable, one of which is shown in perspective as a part structure (7) in Scheme 2; the other is achieved by rotating the α-carbon of the amino acid through 180°.



Scheme 1



Scheme 2

The stereochemical consequences of reaction on the selected conformation (7) are shown in subsequent steps. Thus the imine residue in (8), formed at the amino acid centre as a result of the decarboxylation, has the E-configuration. The subsequent addition of a proton (derived from the solvent) could now take place to the top (Re) face, or the bottom (Si) face of (8) as indicated. In the chiral environment of the active site exclusive attack on only one face is the norm, corresponding in these cases to decarboxylation with overall retention of

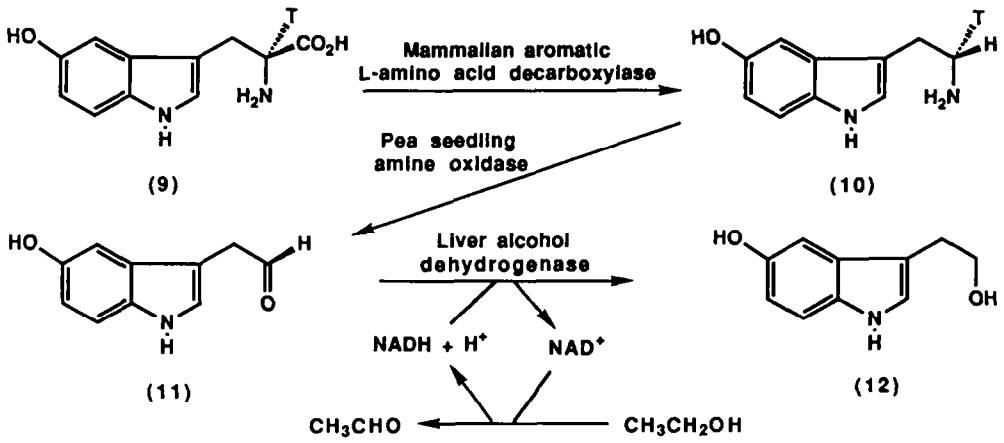
configuration (Re addition), or inversion (Si addition) respectively, at the α-carbon of the amino acid residue. Similar considerations apply to the alternative orientation of the amino acid in (7). The intermediate equivalent to (8) would have the Z-configuration, and once again the proton from the solvent can add with overall retention or inversion of configuration.

Ordinarily, the two modes of proton addition are indistinguishable since they lead to the same product, but discrimination can be achieved by use of a hydrogen isotope to label either the protons of the solvent (^{S}H), or the α-hydrogen of the amino acid (^{A}H). One of two chiral products will now be formed, and determination of its absolute configuration will reveal the stereochemistry of the reaction. This strategy has been used in investigations of the decarboxylation of several amino acids including tyrosine,[8,9] histidine,[10] glutamic acid,[1,11] and lysine.[12] In all cases studied so far, the tranformation has taken place with retention of configuration.

In our studies we have been primarily interested in comparing the decarboxylation of aromatic amino acids using enzymes from various sources, including animals, plants and microorganisms. Here we describe the extension of our work to include the decarboxylation of 5-hydroxytryptophan (9) by the mammalian enzyme which decarboxylates various aromatic L-amino acids.[13] The product, 5-hydroxytryptamine (serotonin), (10), is an important neurotransmitter which is involved in the control of sleep.[3,14]

Our experimental strategy in this further investigation was modelled on that adopted in the earlier tyrosine study.[9] In particular, we planned to use the amine oxidase isolated from pea seedlings to determine the chirality of the isotopically labelled primary amine product. This enzyme converts a wide range of primary amines into their corresponding aldehydes using molecular oxygen as the ultimate oxidant. The stereochemical course of the oxidation process has been rigorously determined for benzylamine,[15] 2-(4'-hydroxy-3'-methoxyphenyl)ethylamine,[16] histamine,[10a] tyramine,[9] and α-heptylamine[17] as substrates. In every case, the enzyme stereospecifically removes the Si-hydrogen from the α-carbon of the substrate. Given the wide range of substrates for which this stereochemistry holds, and that several of the compounds are closely related in structure to 5-hydroxytryptamine, we consider it safe to assume that the enzymic dehydrogenation of this amine will also involve loss of the Si-hydrogen.

The overal strategy of the proposed investigation is shown in Scheme 3. 5-Hydroxytryptophan (9) labelled with tritium in the 2'-position is incubated with the decarboxylase to produce 5-hydroxytryptamine (10). At this stage we expect all the tritium to be retained, a point which must be checked by isolating a sample of the primary amine in a form suitable for assay of radioactivity. The remaining amine is then oxidised by the amine oxidase to the aldehyde (11). If the decarboxylation step follows the pattern of previous examples and occurs with retention of configuration, we would expect the S-isomer of the primary amine to be present as indicated, and consequently that the label would be lost in the oxidation step. A common difficulty in such an assay is the instability of the product aldehyde, which is best reduced in situ by a coupled redox reaction catalysed by NADH and liver alcohol dehydrogenase, using ethanol as the ultimate reducing agent. Under these conditions, with a large excess of ethanol, the more stable 5-hydroxytryptophol (12) should accumulate in the reaction medium, from which it can be isolated in the form of a suitable derivative for radioactive assay.

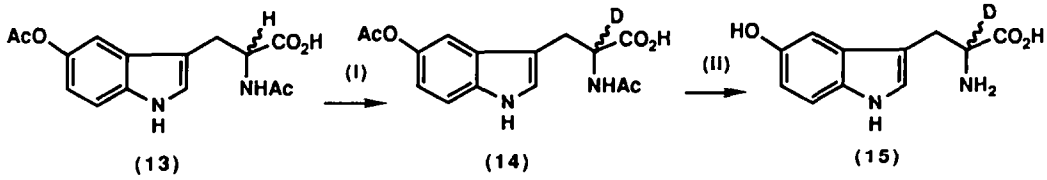


Scheme 3

Synthesis of [2'-^{2}H]- and [2'-^{3}H]-5-Hydroxytryptophan.

The synthesis of [2'-^{3}H]-5-hydroxytryptophan followed a previous synthesis of the similarly labelled tyrosine,[9] although the search for efficient experimental procedures proved to be exceptionally troublesome because of the relative instability of the 5-hydroxyindole residue.

Firstly, the 2'-deuterio compound was prepared as indicated in Scheme 4, to establish suitable conditions for the exchange process. The diacetyl derivative (13) of the racemic amino acid was treated with a mixture of deuterium oxide and acetic anhydride under standard conditions which cause exchange of the 2'-hydrogen on a transiently formed oxazolone intermediate. N.m.r. and mass spectrometry of the recovered material, (14), indicated that the 2'-carbon had been deuteriated to the extent of 90%, with a smaller incidental incorporation of deuterium into the N-acetyl group. Hydrolysis of the two acetyl groups could be achieved in satisfactory yield by heating under carefully controlled conditions in dilute HCl. The product (15) did not produce satisfactory n.m.r. spectra, and so it was reconverted to its diacetyl derivative for analysis. This showed that the deuterium label at the α-position had survived the hydrolysis procedure without significant loss. The corresponding tritiated compound was prepared under analogous conditions using tritiated water.



Scheme 4 (I) D_2O / acetic anhydride; (II) HCl / H_2O

Derivatisation of 5-Hydroxytryptophan and 5-Hydroxytryptophol.

The amino acid, 5-hydroxytryptophan (9), could be recrystallised from water, but neither 5-hydroxytryptamine (10) nor 5-hydroxytryptophol (12) are satisfactory crystalline compounds. We therefore sought derivatives which could be easily recrystallised to constant activity in the radiochemical analyses. For the amine, the diacetyl derivative was crystalline, but the much superior dibenzoate was adopted as the standard derivative for radioactive counting. Of the three derivatives tested for the 5-hydroxytryptophol, neither the dibenzoate nor the di-[α-naphthylcarbamate] could be recrystallised efficiently. The di-p-nitrobenzoyl derivative, however, proved to be suitable for our purpose.

Incubation Procedures.

The aromatic L-amino acid decarboxylase, isolated by standard procedures from commercial hog kidney powder, was incubated with (RS)-[2'-^{14}C]-5-hydroxytryptophan. Analysis by t.l.c. confirmed the production of 5-hydroxytryptamine. After 72 h., radio-inactive 5-hydroxytryptamine was added to the mixture, and the product isolated. The specific activity of the derived dibenzoate corresponded to the production of a useful amount of amine (ca. 3mg) in the incubation.

Next, it was necessary to show that 5-hydroxytryptamine (10) is a satisfactory substrate for the pea seedling amine oxidase, and similarly that the aldehyde (11) serves as a substrate for alcohol dehydrogenase. The coupled reaction procedure described earlier was used to establish these points. An incubation of [2'-^{14}C]-5-hydroxytryptamine with the amine oxidase, aldehyde dehydrogenase, NAD^+, and ethanol was allowed to proceed for a suitable period, and then diluted with an unlabelled sample of the expected product 5-hydroxytryptophol (12). The reisolated alcohol was converted into the di-p-nitrobenzoate and purified to constant activity. The activity in the recovered material corresponded to the production of a satisfactory quantity of alcohol (ca. 5mg) in the incubation.

Configurational Assay.

In the incubation experiments it was advantageous to have a ^{14}C label in the substrate in addition to the tritium label. The carbon label can be used to monitor turnover independently of the fate of the tritium. Additionally, it serves as an internal standard against which the retention of tritium can be measured, so that dilution techniques can be used to facilitate the isolation of labelled products. Accordingly, doubly-labelled (RS)-[2'-^{3}H, 3'-^{14}C]-5-hydroxytryptophan (9) with a suitable isotopic ratio was prepared by mixing the tritiated material with the commercially available ^{14}C-labelled analogue, and diluted with unlabelled material to give a convenient specific activity for the incubation experiments. The decarboxylase is known to be specific for the (S)-amino acid, so racemic material was acceptable for the incubation studies.[13a,b]

The doubly-labelled amino acid was incubated with the mammalian aromatic amino acid decarboxylase, and the resulting amine (10) isolated after dilution with unlabelled material. Part of this product was converted into the dibenzoate which was recrystallised to constant activity to give the isotopic ratio of the 5-hydroxytryptamine. The remainder was subjected to the coupled amine oxidase-aldehyde dehydrogenase incubation to produce 5-hydroxytryptophol (12). Again the product was isolated after dilution with unlabelled material and derivatised prior to counting.

The overall assay was performed three times to give the results shown in the Table. The first point to deserve comment is the significant fall in isotopic ratio (12 -17%) in going from the amino acid (9) to the amine (10). Both the labelled starting material and the labelled product were subsequently incubated with the decarboxylation medium in the absence of enzyme. In neither case was there an appreciable loss of tritium, showing that the added enzyme preparation is responsible. We think it likely, however, that the loss is caused by small amounts of other enzymes in our extract rather than by the decarboxylase itself.

Fortunately, this incidental loss is sufficiently small to leave the results of the configurational assay with the amine oxidase absolutely clear: within experimental error, the 5-hydroxytryptamine (10) was converted into 5-hydroxytryptophol (12) with complete loss of tritium. Given the standard stereospecificity of the amine oxidase for the Si-hydrogen of primary amines, the foregoing results show that the 5-hydroxytryptamine produced by the decarboxylase has the (S)-configuration. This in turn corresponds to overall retention of configuration in the decarboxylation step.

Table. Decarboxylation of (RS)-[2-^{3}H,3-^{13}C]-5-Hydroxytryptophan and Configurational Assay of the Resulting 5-Hydroxytryptamine.

Expt	5-Hydroxytryptophan (9) $^{3}H : ^{14}C$ ratio	5-Hydroxytryptamine (10)[a] $^{3}H : ^{14}C$ ratio (% retention of ^{3}H)	5-Hydroxytryptamine (12)[b] $^{3}H : ^{14}C$ ratio (% retention of ^{3}H)
1	8.7	7.2 (83)	0.04 (0.5)
2	8.7	7.5 (86)	0.03 (0.4)
3	8.7	7.7 (88)	0.04 (0.5)

(a) Produced from (9) by the aromatic L-amino acid decarboxylase; counted as the dibenzoyl derivative.

(b) Produced from (10) by the coupled action of diamine oxidase and alcohol dehydrogenase; counted as the di-*p*-nitrobenzoyl derivative.

Hence, the production of 5-hydroxytryptamine follows the precedents set with this enzyme in the production of other primary amines by the decarboxylation of aromatic L-amino acids. It seems likely that this unified pattern of behaviour reflects a common mode of action for the decarboxylase process, in which the aromatic residues of the various substrates enter the same pocket at the active site of the enzyme. It will be interesting to consider the significance of this stereochemistry further, when information is obtained from cognate studies concerning the orientation of coenzyme and substrate in the active site of the enzyme.

Grateful acknowledgement is made to Dr. G.W.J. Matcham for helpful advice, to Dr. J. A. Martin, Roche Products Ltd., for a gift of 5-benzyloxyindole, and to the S. E. R. C. for a studentship (to A. S.) and for financial support.

EXPERIMENTAL

General Directions.– Solvents and reagents were purified by standard methods.[18] The following materials were purchased from the Sigma Chemical Company: Aromatic L-amino acid decarboxylase (as a hog kidney powder), horse liver alcohol dehydrogenase, nicotinamide adenine dinucleotide (NAD^+), catalase, (RS)-5-hydroxytryptophan, and 5-hydroxytryptamine (as the creatine complex). (RS)-[3'-^{14}C]-5-Hydroxytryptophan, [2'-^{14}C]-5-hydroxytryptamine, and tritiated water were obtained from The Radiochemical Centre, Amersham.

The remaining general directions are given in ref. 12b.

(RS)-*5-Acetoxy-N-acetyltryptophan*.– To a solution of (*RS*)-5-hydroxytryptophan in sodium hydroxide (2.5N, 0.7 ml) was added acetic anhydride (0.2 ml), and the reaction was stirred for 2.5 h at 0°C in an atmosphere of nitrogen. Acidification with conc. HCl to pH 1 gave white crystals, which were collected, washed with water, and dried. Recrystallisation from ethanol/water gave the diacetyl derivative (103 mg, 75%), m.p. 136-138°C (lit.,[19] 140°C); (Found: C, 59.0; H, 5.3; N, 9.0. Calc for $C_{15}H_{16}N_2O_5$: C, 59.2; H, 5.3; N, 9.2 %.). δ (CD_3OD) 1.90 (3H, s, NCOC*H*$_3$), 2.28 (3H, s, OCOC*H*$_3$), 3.20 (overlaps with solvent), 4.72 (1H, q of ABX, C*H*), 6.74-7.36 (4H, m, Ar*H*); ν_{max} (nujol) 3 395, 3 350, 1 740, 1 700, and 1 625 cm^{-1}; λ_{max} (EtOH) 225 (ε 33 953) nm; m/z 304 (M^+, 72), 286 (55), 188 (68).

(RS)-[2'-^{2}H]-*5-Acetoxy-N-acetyltryptophan*.– A solution of (*RS*)-5-acetoxy-N-acetyltryptophan (100 mg) in acetic anhydride containing deuterium oxide (0.1 ml) was heated under reflux for 20 min. The residue left on evaporation was recrystallised from ethanol/water to give the *deuterio compound* (70 mg, 70%), m.p. 133-135°C; (Found: M$^+$, 305.1136 . $C_{15}DH_{15}N_2O_5$ requires M, 305.1122.). δ (CD_3COCD_3) 1.87 (0.9H, m, NCOC*H*$_3$, partly deuteriated), 2.22 (3H, s, OCOC*H*$_3$), 3.21 (2H, q, C*H*$_2$), 4.75 (0.1H, m, C*H*), 6.76 - 7.40 (5H, m, Ar*H* + indole N*H*), 10.15 (1H, bs, CO_2H); ν_{max} (nujol) 3 350, 1 740, 1 700, and 1 625 cm^{-1}; λ_{max} (MeOH) 225 and 285 nm; m/z 305 (M^+, 10), also 306, 307, and 308 due to deuteriation of the *N*-acetyl group.

(RS)-[2'-^{2}H]-*5-Hydroxytryptophan*.– A solution of (*RS*)-[2'-^{2}H]-*5-acetoxy-N-acetyltryptophan* (200 mg) in HCl (5N, 3 ml) was degassed and flushed with argon, and then heated in a sealed tube in the dark at 110 °C for 2h. After cooling, the solution was evaporated, and the residue purified by preparative t.l.c. in n-butanol:acetic acid:water (12:3:5) to give (R_f 0.4) (RS)-[2'-^{2}H]-*5-hydroxytryptophan* (70 mg, 48%), m.p. 285-290°C decomp. (lit.,[20] 298-300°C decomp.); λ_{max} (MeOH) 220, 275, and 300 nm.

Acetylation of (RS)-[2'-^{2}H]-*5-Hydroxytryptophan*.— The foregoing product was acetylated as before to give the corresponding *diacetyl derivative*, identical by t.l.c. with authentic material, m.p. 134 °C; δ (CD_3COCD_3) ($NCOCH_3$ obscured by acetone), 2.22 (3H, s, OCOC*H*$_3$), 3.21 (2H, b, C*H*$_2$), 4.75 (0.1H, b, C*H*), 6.76 - 7.40 (5H, m, Ar*H* + indole N*H*); m/z 305 (M^+, 10).

(RS)-[2'-^{3}H]-*5-Hydroxytryptophan*.– Trtiated water (400 mCi, 5 Ci/ml) was transferred via the vapour phase under vacuum (0.1 mm Hg) onto a solution of (*RS*)-5-acetoxy-N-acetyltryptophan (200 mg) in acetic anhydride (1 ml). The mixture heated at 120 °C for 50 min. in a sealed tube under vacuum,

and then evaporated to dryness. Water (10 ml) was added, and the mixture was again evaporated to dryness. This procedure was repeated six times, and the residue then hydrolysed without purification in HCl (3N) as described above. Purification by preparative t.l.c. gave the *tritiated compound* (100 mg, 3.7 Ci mol^{-1}) which was identical with authentic material by t.l.c.

(RS)-[2'-^{3}H, 3'-^{14}C]-*5-Hydroxytryptophan*.— (*RS*)-[2'-^{3}H]-5-Hydroxytryptophan (0.3 mCi) and (*RS*)-[3'-^{14}C]-5-hydroxytryptophan (35 μCi) were dissolved in an aqueous solution of the inactive analogue. The residue left after evaporation, (RS)-[2'-^{3}H, 3'-^{14}C]-*5-hydroxytryptophan* (110 mg), was used directly for the enzyme incubatons. A portion was diluted further with inactive material and recrystallised to constant activity (^{3}H:^{14}C, 8.7).

5-Hydroxytryptamine.— The amine was separared from its creatine complex (50 mg) by chromatography on silica gel using a mixture of *n*-butanol:glacial acetic acid:water (12:3:5) as eluent. The fractions (30 ml in total) containing 5-hydroxytryptamine emerged first, and were evaporated to leave the amine which was pure by t.l.c. (Rf 0.49); δ (D_2O) 3.17 (4H, m, CH_2 CH_2), 6.72-7.38 (4H, m, Ar*H*); λ_{max} (H_2O) 222 and 275 nm; m/z 176 (M^+), 146.

5-Acetoxy-N-acetyltryptamine.— A solution of 5-hydroxytryptamine (40 mg) and fused sodium acetate (80 mg) in acetic anhydride (2 ml) was stirred for 24 h and then evaporated to dryness. The residue was purified by preparative t.l.c. using n-butanol:acetic acid:water (12:3:5) as eluent, and the product (R_f 0.67) recrystallised from methanol/water to yield 5-acetoxy-*N*-acetyltryptamine (28mg, 45%), m.p. 91°C (lit.,[21] 93.4°C). δ (CD_3OD) 1.90 (3H, s, OCOCH_3), 2.90 (2H, t, *J 7Hz*, CH_2), 2.45 (2H, t, *J 7Hz*, NCH_2), 6.70-7.38 4H, m, Ar*H*); m/z 260 (M^+),201, 159.

5-Benzoyloxy-N-benzoyltryptamine.— A solution of 5-hydroxytryptamine (45 mg) and benzoyl chloride (0.2 g) in pyridine (5 ml) was stirred under an atmosphere of nitrogen for 1h at 40 °C, and then left for 15 h, followed by addition to iced water. After adjustment to pH 3, the mixture was extracted with chloroform. The organic extract was washed successively with aqueous sodium hydrogen carbonate solution and water, and then evaporated to dryness. Purification of the residue by preparative t.l.c. in chloroform:ethyl acetate (9:1) gave the *dibenzoyl derivative* (R_f 0.53) which was recrystallised from ethyl acetate (44 mg, 45%), m.p. 176-177 °C; (Found: C, 74.6; H, 5.1; N, 6.9. $C_{24}H_{20}N_2O_3$ requires C, 75.0; H, 5.2; N, 7.3%.). δ (CD_3OD) 3.10 (2H, t *J 7Hz*, CH_2), 3.70 (2H, t, *J 7Hz*, CH_2), 6.79-8.25 (14H, m, Ar*H*); ν_{max} ($CHCl_3$) 3 480, 3 020, 1 725, 1 655 cm^{-1}; λ_{max} (EtOH) 227 (ε 51 882) and 270 (ε 10 225) nm; m/z 384 (M^+, 55),263 (100), 250 (20).

5-Hydroxytryptophol.— 5-Hydroxytryptophol was synthesised according to the procedure of Kveder et al.[22] The product was identical by t.l.c. in diethyl ether with authentic material (Rf 0.23); m.p. 106-107°C (lit.,[22] 105-107°C); m/z 177 (M^+, 15), 146 (100).

Dibenzoyl Derivative of 5-Hydroxytryptophol.— A solution of 5-hydroxytryptophol (25 mg) and benzoyl chloride (0.07 ml) in pyridine (1.75 ml) was stirred for 2 days at room temperature and then poured into

ice water at pH 3. A chloroform extract was washed successively with aqueous sodium hydrogen carbonate solution and water, and then evaporated to dryness. Purification of the residue by t.l.c. (Rf 0.73) using chloroform:methanol (93:7) as eluent gave the *dibenzoate* as a gum (45 mg, 82%). δ ($CDCl_3$) 3.21 (2H, t, *J 7Hz*, CH_2), 4.61 (2H, t, *J 7Hz*, OCH_2), 6.99-8.32 (15H, m, Ar*H* + indole N*H*); ν_{max} ($CHCl_3$) 3 480, 1 715, 1 605, and 1 585 cm^{-1}; λ_{max} ($CHCl_3$) 245 and 270 nm; m/z 385 (M^+ 80) 263 (90), 250 (15), 105 (100).

Di-α-naphthylcarbamoyl Derivative of 5-Hydroxytryptophol.— A mixture of 5-hydroxytryptophol (40 mg) and α-naphthyl isocyanate (0.15 ml) was heated at 100 °C for 2.5 h under nitrogen and in the dark. After being washed with hexane the product was recrystallised from ethyl acetate/hexane to give the *di-α-naphthylcarbamate* (71mg, 61%), m.p. 193-195°C; δ (CD_3SOCD_3) 3.10 (2H, t, *J 7Hz*, CH_2), 4.36 (2H, t, *J 7Hz*, OCH_2), 6.89-8.23 (19H, m, Ar*H* + indole N*H*), 9.37 (1H, s, N*H*), 9.87 (1H, s, N*H*); ν_{max} (nujol) 3 360,3 285, 1 690, 1 540 cm^{-1}; λ_{max} (MeOH) 220 and 278 nm; m/z (field desorption) 515 (M^+).

Di-p-nitrobenzoyl Derivative of 5-Hydroxytryptophol.— A solution of 5-hydroxytryptophol (50 mg) and p-nitrobenzoyl chloride (210 mg) in pyridine (5 ml) was kept at room temperature for 15 h under nitrogen, and then poured into water. After adjustment of the solution to pH 2, the solid was collected and purified by preparative t.l.c. (Rf 0.7) using chloroform:methanol (9:1) as eluent. Recrystallisation from acetone/water gave the *di-p-nitrobenzoate* (120mg, 89%), m.p. 195-197°C; (Found: C, 60.4; H, 3,8; N, 8.7. $C_{24}H_{17}N_3O_8$ requires C, 60.6; H, 3.6; N, 8.8%). δ (CD_3COCD_3) 3.29 (2H, t, *J 7Hz*, CH_2), 4.67 (2H, t, *J 7Hz*, OCH_2), 7.0-7.66 (5H, m, indole Ar*H* + indole N*H*), 8.24-8.47 (8H, m, *p*-nitrobenzoate Ar*H*); ν_{max} (nujol) 3 460,1 730, 1 520 and 1 350 cm^{-1}; λ_{max} (EtOH) 222 (ε 41 815) and 260 (ε 32 088) nm; m/z 475 (M^+, 80) and 326 (100).

Purification of Mammalian L-Amino Acid Decarboxylase (EC 4.1.1.28)[9]— All operations were carried out at 0-5 °C. Hog kidney acetone powder (3 g) was added to distilled water (75 ml) which had been adjusted to pH 8 with 0.05M potassium hydroxide. After stirring for 1 h, the mixture was centrifuged (30 min, 10 000 rpm) and the pellet discarded. The supernatant (68 ml) was combined with saturated ammonium sulphate solution (enzyme grade) (40 ml) to produce a 37% saturated solution, and the solution was adjusted to pH 8. After stirring for 20 min, the mixture was centrifuged (20 min, 15 000 rpm) and the pellet discarded. The supernatant was adjusted to 55% saturation by further additon of saturated ammonium sulphate solution. After 40 min the mixture was centrifuged (30 min, 18 000 rpm). The residual pellet was dissolved in distilled water (7 ml), and dialysed against distilled water for 5 h, to give an enzyme preparation which was suitable for use in the incubation experiments. Its activity was confirmed by following the conversion of 5-hydroxytryptophan to 5-hydroxytryptamine by t.l.c. Protein assay by Coomassie Blue[23] indicated approximately 8.5 mg of protein ml^{-1}.

Decarboxylation of (RS)-[2'-^{14}C]-5-Hydroxytryptophan by Mammalian Aromatic L-Amino Acid Decarbosylase.— (*RS*)-[2'-^{14}C]-5-Hydroxytryptophan (2 μCi) was added to a solution of (*RS*)-5-hydroxytryptophan (10 mg) in water (5 ml). After mixing, part was removed for radioactive counting. The

remainder was added to a solution of pyridoxal phosphate (0.3 mg) in phosphate buffer (0.2M, pH 8), and the foregoing enzyme preparation (7 ml) then added. After flushing with nitrogen, the flask was sealed, and incubated for 72 h at 30 °C in the dark. An aqueous solution of 5-hydroxytryptamine (109 mg) was then added, and the solution adjusted to pH10 with sodium hydroxide (10N). Following saturation with sodium chloride, borate buffer[24] (pH 10, 30 ml) was added, and the 5-hydroxytryptamine extracted into *n*-butanol. After being washed with borate buffer, the butanol extract was evaporated to dryness. The resulting amine was converted to its dibenzoyl derivative as described above, and recrystallised to constant specific activity (m.p. 176-177 °C). The radiochemical data etablished that radioactive 5-hydroxytryptophan (3.1 mg, 63%) had been converted to 5-hydroxytryptamine.

Isolation of the Amine Oxidase (EC 1.4.3.6) from Pea Seedlings.— The enzyme preparation was isolated using a standard procedure.[25]

Protein assay by Coomassie Blue[23] indicated 0.4 mg protein ml^{-1}. The activity was assayed by following the oxidation of benzylamine using the absorption band due to benzaldehyde at 250 nm. The assay mixture consisted of amine oxidase solution (0.1 ml), benzylamine hydrochloride solution (20 mM, 1 ml), and phosphate buffer (0.01M, pH 7, 1.9 ml). The measured specific activity of the protein was 0.115 μmol min^{-1} mg^{-1}.

Enzymic Conversion of [1'-^{14}C]-*5-Hydroxytryptamine Into* [1'-^{14}C]-*5-Hydroxytryptophol.*— [1'-^{14}C]-5-Hydroxytryptamine (0.6 μCi) was added to a solution of 5-hydroxytryptamine (7.5 mg) in distilled water. A portion was removed and converted into the dibenzoyl derivative as before, for determination of the radioactivity. The remainder of the sample was dissoved in phosphate buffer (0.1M, pH 7, 15 ml) containing horse liver alcohol dehydrogenase (15 mg), catalase (2mg), ethanol 1.5 ml), NAD^+(5 mg), and the amine oxidase preparation (8 ml).The mixture was incubated open to the atmosphere and in the dark at 30 °C for 20 h, when the pH was adjusted to pH 2, and unlabelled 5-hydroxytryptophol was added. The solution was then saturated with sodium chloride, filtered through "Celite", and extracted with ethyl acetate. The 5-hydroxytryptophol remaining after evaporation of the ethyl acetate extract was purified by preparative t.l.c. in diethyl ether and converted to the di-*p*-nitrobenzoyl derivative as described earlier. After recrystallisation to constant specific activity (m.p. 196-197 °C), the derivative gave radiochemical data consistent with the conversion of 5-hydroxytryptamine (5mg, 67%) into the corresponding alcohol.

Decarboxylation of [(RS)-[2'-^{3}H, 3'-^{14}C]-*5-Hydroxytryptophan by Mammalian Aromatic L-Amino Acid Decarboxylase.*— In a typical experiment, a solution of doubly-labelled 5-hydroxytryptophan (23 mg) and pyridoxal phosphate (50 μg) in phosphate buffer (0.2M, pH 8, 5 ml) was incubated with the decarboxylase preparation (7 ml) under nitrogen atmosphere and in the dark. After 72 h, unlabelled 5-hydroxytryptamine (45 mg) was added and the amine extracted as before. A portion (30 %) was converted into the dibenzoyl derivative and recrystallised to constant specific activity and ^{3}H:^{14}C ratio (m.p. 174-176 °C).

Assay of Configuration of [1'-$^{3}H_{1}$]-*5-Hydroxytryptamine.*— The 5-hydroxytryptamine remaining from the foregoing experiment was incubated with the amine oxidase preparation (8 ml) as described earlier, and the product isolated after dilution with inactive 5-hydroxytryptophol (35 mg). This was converted into the dibenzoyl derivative which was crystallised to constant radioactivity (m.p. 195-196 °C).

Incubation of (RS)-[2'-^{3}H, 3'-^{14}C]-*5-Hydroxytryptophan in the Absence of the Decarboxylase.*— A sample of the doubly-labelled 5-hydroxytryptophan (1.5 mg, ^{3}H:^{14}C ratio 8.70) was incubated under standard conditons with the medium used for decarboxylation experiments but in the absence of the decarboxylase. Dilution with unlabelled amino acid (100 mg) and reisolation in the usual way gave (*RS*)-[2'-^{3}H, 3'-^{14}C]-5-hydroxytryptophan which after rigorous purification had an isotopic ratio (^{3}H:^{14}C) of 8.8.

Incubation of (RS)-[1'-$^{3}H_{1}$, 2'-^{14}C]-*5-Hydroxytryptamine in the Absence of the Decarboxylase.*— A sample of the doubly-labelled 5-hydroxytryptamine (^{3}H:^{14}C ratio 7.5) produced by decarboxylation of the corresponding doubly-labelled 5-hydroxytryptophan was incubated under standard conditions with the medium used for decarboxylation experiments but in the absence of the decarboxylase. Dilution with unlabelled amine and reisolation in the usual way gave (*RS*)-[1'-$^{3}H_{1}$, 2'-^{14}C]-5-hydroxytryptamine which was converted to its dibenzoyl derivative. After rigorous purification this had an isotopic ratio (^{3}H:^{14}C) of 7.5.

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