The Biosynthesis of 5-Hydroxytryptamine in Brain

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1. Studies on the anatomical distribution of tryptophan 5-hydroxylase in dog brain show that the activity of this enzyme parallels the activity of 5-hydroxytryptophan decarboxylase and the concentration of 5-hydroxytryptamine in various areas of the brain. 2. Subcellular fractionation of homogenates of rabbit hind-brain has shown that at least 40% of the tryptophan 5-hydroxylase activity is associated with the crude mitochondrial fraction; equilibrium centrifugation in a discontinuous sucrose gradient suggests that this particulate tryptophan 5-hydroxylase is localized in the synaptosomes (nerve-ending particles). 3. Attempts to increase the activity of tryptophan 5-hydroxylase in iso-osmotic homogenates of brain by adding 5,6,7,8-tetrahydro-6,7-dimethylpteridine failed. 4. Procedures designed to rupture synaptosomes caused a fall in the activity of tryptophan 5-hydroxylase, which fall was reversed by adding 5,6,7,8-tetrahydro-6,7-dimethylpteridine. This suggested either that the intact synaptosome, in which the tryptophan 5-hydroxylase is localized, contained an optimum amount of the pteridine, or that the pteridine could not pass across the nerve-cell membrane. 5. Partial purification of tryptophan 5-hydroxylase from brain preparations by ammonium sulphate precipitation revealed that the enzyme was completely dependent on 5,6,7,8-tetrahydro-6,7-dimethylpteridine for activity.

The presence and distribution of 5HT* within the brain was first studied by Amin, Crawford & Gaddum (1954). Since then there has been much work and speculation on the role of this amine in central synaptic transmission (see Salmoiraghi, Costa & Bloom, 1965). In a previous communication (Grahame-Smith, 1964a) it was reported that the first step in the biosynthesis of 5HT, the enzymic 5-hydroxylation of L-tryptophan with the production of L-5HTP, occurred in isolated brain tissue, and this has been confirmed by several workers (Gal, 1965; Nakamura, Ichiyama & Hayaishi, 1965; Green & Sawyer, 1966). The present paper describes further studies on the anatomical distribution, partial purification, co-factor requirement and properties of tryptophan 5-hydroxylase in brain. In addition, the subcellular localization of tryptophan 5-hydroxylase in brain has been investigated. The work of Whittaker and his colleagues (see Whittaker, 1963) has clearly shown that crude mitochondrial fractions prepared from brain homogenates contain particles that have the appearance of pinched-off presynaptic nerve

endings, and these have been termed 'synaptosomes' (Whittaker, Michaelson & Kirkland, 1964). The synaptosome fraction has been shown to contain acetylcholine (Whittaker, 1959), choline acetyltransferase (Hebb & Whittaker, 1958), acetylcholinesterase (De Robertis, Arnaiz, Salganicoff, de Iraldi & Zieher, 1963), substance P (Ryall, 1964) and noradrenaline (Potter & Axelrod, 1963). Particularly relevant to the investigations reported here is that the synaptosome has been shown to contain both 5HT (Michaelson & Whittaker, 1963) and some 5HTP decarboxylase (Arnaiz & De Robertis, 1964), and evidence is presented in the present paper to show that tryptophan 5-hydroxylase is also located within this particle. Part of this work has been previously presented to The Biochemical Society (Grahame-Smith & Moloney, 1965).

MATERIALS AND METHODS

Materials

* Abbreviations: 5HT, 5-hydroxytryptamine; 5HTP, 5-hydroxytryptophan; DMPH₄, 5,6,7,8-tetrahydro-6,7-dimethylpteridine hydrochloride; iproniazid, *N*-isonicotinoyl-*N'*-isopropylhydrazine.

DL-[3-¹⁴C]Tryptophan was obtained from The Radiochemical Centre, Amersham, Bucks. 3-Hydroxybenzoyloxyamine was given by Dr D. Drain of Smith and Nephew Ltd., Ware, Herts., and DL-5HTP and iproniazid were given by Roche Products Ltd., Welwyn Garden City, Herts.

DMPH₄ was purchased from Calbiochem, Los Angeles, Calif., U.S.A.

Assay of tryptophan 5-hydroxylase

The method used was that described by Grahame-Smith (1964a,b) with certain modifications. Tissue preparations were incubated with DL-[3-¹⁴C]tryptophan with the addition of 3-hydroxybenzoyloxyamine to inhibit the decarboxylation of any 5HTP produced (Drain, Horlington, Lazare & Poulter, 1962). Incubations were carried out in tubes or flasks flushed with O₂, stoppered and shaken in a metabolic shaker. At the end of the incubation, 4 μ moles of DL-5HTP and 2 ml. of 5% (w/v) trichloroacetic acid were added and the denatured protein was removed by centrifugation. After the excess of trichloroacetic acid had been extracted with two 10 ml. volumes of peroxide-free ether, the pH of the solution was adjusted to 4 by the addition of 2 ml. of 2M-sodium acetate buffer, pH 4. Then 0.25 g. of charcoal deactivated with 4% stearic acid (Asatoor & Dalglish, 1956) was added and, after standing for 10–15 min. to allow absorption of the indoles by the charcoal, the suspension was poured into small glass columns (1 cm. diam.), the constricted ends of which had been tightly packed with glass wool. The charcoal, which was held back by the glass wool, was washed free of non-absorbed salts by slowly passing 20 ml. of water through the column, and the indoles were then eluted with 10 ml. of N-acetic acid in 80% (v/v) ethanol. Of the added 5HTP 75–80% was recovered. The ethanolic eluate was then evaporated to dryness in a vacuum desiccator. When dry, the extract was dissolved in water and, in early experiments, subjected to two-dimensional ascending paper chromatography on Whatman 3MM paper in the following solvents (Jepson, 1955): solvent 1, propan-2-ol-aq. ammonia (sp.gr. 0.88)-water (20:1:2, by vol.); solvent 2, butan-1-ol-acetic acid-water (12:3:5, by vol.). [For individual R_F values of the relevant compounds and the appearance of the two-dimensional ascending paper chromatograms see Jepson (1955).]

In later experiments, this initial chromatography was performed in one dimension only with solvent 2 above, this being sufficient to give a preliminary separation of 5HTP (R_F 0.27) from tryptophan (R_F 0.50) and 3-hydroxybenzoyloxyamine (R_F 0.80). The chromatograms were radioautographed for 2–5 days (Kodirex film; Kodak Ltd., Kirkby, Liverpool) and the radioautographs developed. The chromatograms were viewed in u.v. light (365 m μ) and compared with a standard chromatogram of 5HTP stained with Ehrlich's reagent (Jepson, 1955). The position of the 5HTP was located by comparison with the standard chromatogram and by its fluorescence, and compared with the corresponding area on the radioautogram.

The 5HTP was then eluted from the paper, and the eluate was evaporated to dryness, dissolved in 0.4 ml. of water and incubated at 37° for 45 min. in air with a preparation of aromatic L-amino acid decarboxylase, partially purified from guinea-pig kidney to stage 3 of the procedure described by Clark, Weissbach & Udenfriend (1954), which had been preincubated for 5 min. with iproniazid (5.5 mM) (a monoamine oxidase inhibitor), pyridoxal phosphate (0.04 mM) and potassium phosphate buffer, pH 7.4 (50 mM). The total volume of the incubation mixture was 0.8 ml. After incubation, 4 vol. of aq. 95% (v/v) ethanol was added, the protein precipitate was removed by centrifugation and the

protein-free extract was evaporated to dryness in a vacuum desiccator. The dry extract was then dissolved in 0.1 ml. of water and subjected either to two-dimensional ascending paper chromatography in the solvents described above or to one-dimensional ascending paper chromatography in solvent 2. The final chromatograms were radioautographed and also viewed in u.v. light (365 m μ) and compared with a standard chromatogram of 5HTP stained with Ehrlich's reagent. The position of 5HT was located and this area was compared with the appearance of the radioautograph. The 5HT was then eluted from the paper, and the eluate dried and dissolved in 0.5 ml. of water. A portion (0.25 ml.) was assayed for 5HT by the α -nitroso- β -naphthol colorimetric procedure (Udenfriend, Weissbach & Brodie, 1958), with suitable volumetric adjustment of the reagents, and 0.1 ml. was dried on an aluminium planchet for the estimation of radioactivity at infinite thinness in a gas-flow counter (Nuclear-Chicago model D47, efficiency 30%). From the specific activity of the 5HT recovered and a knowledge of the amounts of 5HTP added as a carrier, it was possible to calculate the amount of L-tryptophan 5-hydroxylated (Grahame-Smith, 1964a,b).

In all experiments, boiled tissue samples were identically incubated and assayed to assess the amount of non-enzymic tryptophan 5-hydroxylation (Grahame-Smith, 1964a), which has always been subtracted in the results presented below.

Preparation of tissue

Rabbit hind-brain. Rabbits were killed by a blow on the neck. The brain minus the olfactory lobes was removed intact. The cerebral cortex was then removed by cutting through the cerebral peduncles, and the cerebellum by severing its connexions with the brain-stem close to the surface of the latter. The portion left is referred to as the rabbit hind-brain. This was weighed and homogenized in the appropriate volume of the medium in a thick-walled glass tube with a Teflon pestle (the diameter of which was 0.2 mm. less than that of the tube) revolving at 1240 rev./min. Usually 12 passes of the pestle were made. All preparations were carried out at 4°.

Sections of dog brain. Studies on the anatomical distribution of tryptophan 5-hydroxylase in brain were carried out on dog brain. A mongrel puppy was anaesthetized with intravenous pentobarbitone, and the carotid arteries were cut. The brain was removed intact and the cerebrum and cerebellum were dissected away as described above. Sections were then made of the remaining part of the brain just anterior to the cephalic border of the pons and just distal to the caudal border of the medulla, creating two sections which are called here 'hypothalamus-thalamus' and 'mid-brain-pons-medulla' respectively. Homogenates (1:2, w/v) of the cerebrum, cerebellum and these two sections were made in Ringer phosphate solution (Krebs & Henseleit, 1933).

Subcellular fractionation of tryptophan 5-hydroxylase from rabbit hind-brain

Preparation of fractions. Homogenates of rabbit hind-brain (1:8, w/v) were made in 0.32 M-sucrose containing 1 mM-mercaptoethanol. The subcellular fractionation procedure was carried out as described by Gray & Whittaker

Scheme 1. Subcellular fractionation of rabbit hind-brain. Homogenate of rabbit hind-brain (1:8, w/v) in 0.32M-sucrose-1mM-mercaptoethanol was the starting material.

(1962) (Scheme 1). All particulate fractions were resuspended in 0.32M-sucrose containing 1mM-mercaptoethanol. Fraction P₂ ('crude mitochondrial' fraction) was resuspended in 17 ml. of this medium, and 5 ml. portions were layered on top of 10 ml. of 0.8M-sucrose that $\frac{1}{2}$ -1 hr. before had been layered on top of 10 ml. of 1.2M-sucrose. The Lusteroid tubes in which this gradient was made were centrifuged at 53 500g for 2 hr. in a swing-out head (SW 25.1) in an ultracentrifuge (Beckman-Spinco model L). The gradient produced three fractions. Fraction A ('myelin' fraction) lay at the interface of the 0.32M-sucrose with the 0.8M-sucrose; fraction B ('synaptosome' fraction) lay at the interface of the 0.8M-sucrose with the 1.2M-sucrose; fraction C ('mitochondrial' fraction) was a tan pellet at the bottom of the tube. The appearance of the tubes was as that

described by Gray & Whittaker (1962) for cerebral cortex. Each fraction was removed from the tube, either with a Pasteur pipette or by cutting the tube, and resuspended in the opalescent medium above it. All these procedures were carried out at 4°.

Portions (1 ml.) of each fraction were incubated with 155 μ moles of DL-[3-¹⁴C]tryptophan (specific activity 31 μ C/ μ mole), 3-hydroxybenzoylserine (1.5 mM) and potassium phosphate buffer, pH 7.4 (77 mM). The total volume of the incubation samples was 1.3 ml. Incubations were carried out for 1 hr. at 37° under O₂, after which the amount of 5HTP produced was assayed.

Analytical methods. The concentration of protein in each fraction was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with a bovine serum Bioch. 1967, 105

albumin solution as a standard. Lactate dehydrogenase was used as a cytoplasmic marker (Johnson & Whittaker, 1963), and was assayed by the method of Kornberg (1955). Succinate dehydrogenase was used as a mitochondrial marker (Whittaker, 1959), and was assayed as succinate-tetrazolium reductase (Pennington, 1961).

Morphology of fractions. Electron-microscopic examination of fractions *A*, *B* and *C* were carried out by Dr V. P. Whittaker by both the negative staining technique (Horne & Whittaker, 1962) and tissue sectioning after permanganate fixation and Araldite embedding.

Partial purification of tryptophan 5-hydroxylase from brain

Two rabbit hind-brains were homogenized in 0.32M-sucrose containing 1mM-mercaptoethanol (1:3, w/v). The homogenate was centrifuged at 100000*g* for 60 min. The particulate material was resuspended in the sucrose medium. (NH₄)₂SO₄ was added to the supernatant material to give 50% saturation (Dixon, 1953), and this solution was kept at 4° for 20 min., after which it was centrifuged at 15000*g* for 30 min. The (NH₄)₂SO₄ precipitate was dissolved in 10 ml. of 10mM-potassium phosphate buffer, pH 7.4, containing 1mM-mercaptoethanol.

Samples (1 ml.) of each preparation were incubated with 151 μmoles of DL-[3-¹⁴C]tryptophan (specific activity 30.2 μC/μmole), 3-hydroxybenzoyloxyamine (1.5 mM) and potassium phosphate buffer, pH 7.4 (77 mM). The total volume of the incubation sample was 1.3 ml. DMPH₄ was added as indicated in Table 4. Incubations were carried out for 1 hr. at 37° under O₂, after which the 5HTP produced was assayed.

Disruption of synaptosomes

Hypo-osmotic treatment (Johnson & Whittaker, 1963). A rabbit hind-brain was homogenized in 10mM-potassium phosphate buffer, pH 7, and the homogenate kept for 20 min. at 5°.

Ether treatment (Feldberg, 1945). To a portion of the above preparation was added peroxide-free ether (0.8 ml. of ether/ml. of homogenate). The mixture was gently shaken for 3 min. and kept at 5° for 10 min., after which air was bubbled through the mixture for 1 min. to remove the ether.

RESULTS

Anatomical distribution of tryptophan 5-hydroxylase in brain

Portions (3 ml.) of homogenates of the various parts of a dog brain prepared as described in the Materials and Methods section were incubated with 315 μmoles of DL-[3-¹⁴C]tryptophan (specific activity 31.5 μC/μmole) and 3-hydroxybenzoyloxyamine (1.5 mM) in a total volume of 3.8 ml. Incubations were carried out for 1 hr. at 37° in an atmosphere of oxygen. The amount of 5HTP produced was then assayed.

The results are shown in Table 1, where the tryptophan 5-hydroxylase activity is compared

Table 1. *Anatomical distribution of 5HT, 5HTP decarboxylase and tryptophan 5-hydroxylase in dog brain*

The distributions are expressed as percentages of the hypothalamus or hypothalamus-thalamus concentrations. In the first column, which data are from Bogdanski & Udenfriend (1956), 100% is 10 μmoles of 5HT/g. of tissue. In the second column, which data are from Udenfriend (1958) and Udenfriend, Weissbach & Bogdanski (1957), 100% is 665 μmoles of 5HT formed/hr./g. of tissue. In the third column 100% is 0.685 μmole of 5HTP formed/hr./g. of tissue.

	5HTP 5HT decarboxylase		Tryptophan 5-hydroxylase
Hypothalamus	100	100	100
Thalamus	37	32	
Mid-brain	55	83	
Pons	23	24	55
Medulla	31	27	
Cortex (grey matter)	19	6	—
Cortex (white matter)	4	—	0
Cerebellum	4	3	0

with the 5HTP decarboxylase activity and the 5HT content of similar areas of the brain found by other workers. Tryptophan 5-hydroxylase could not be detected in the cerebral cortex. The total activity of the 'hypothalamus-thalamus' section was 9.55 μmoles of 5HTP produced/hr., and that of the 'mid-brain-pons-medulla' section only 1.34 μmoles of 5HTP produced/hr. This emphasizes the high 5HT-biosynthetic activity of the former section, which is probably a reflection of activity in the hypothalamus, where 5HT is found in a very high concentration. Similar studies on rabbit brain revealed the tryptophan 5-hydroxylase activity to be mainly localized in the hind-brain (as defined in the Materials and Methods section), and for this reason the rabbit hind-brain has been used in the studies described below.

Time-course of tryptophan 5-hydroxylation in brain

A portion (7 ml.) of a homogenate of rabbit hind-brain in Ringer phosphate solution (1:2, w/v) was incubated with 632 μmoles of DL-[3-¹⁴C]tryptophan (specific activity 31.5 μC/μmole) and 3-hydroxybenzoyloxyamine (2.5 mM) at 37° under oxygen. At certain times, 1 ml. portions of the incubation mixture were removed and assayed for the amount of 5HTP produced. The time-course of tryptophan 5-hydroxylation is shown in Fig. 1.

Subcellular distribution of tryptophan 5-hydroxylase in rabbit hind-brain

Initial experiments, in which homogenates of rabbit hind-brain made in Ringer phosphate

solution were separated into total particulate and supernatant fractions by centrifugation at 100 000g for 60 min., showed that most of the tryptophan 5-hydroxylase activity resided in the particulate matter. More searching fractionation was initially hindered by the unexplained disappearance of tryptophan 5-hydroxylation when homogenates were made in 0.32M-sucrose. A similar phenomenon was described for tyrosine hydroxylase by Nagatsu, Levitt & Udenfriend (1964); this was overcome by adding mercaptoethanol to the sucrose media. It was found that the addition of 1mM-mercaptoethanol to sucrose media also restored tryptophan

5-hydroxylase activity, allowing more detailed subcellular fractionation. The results of two experiments carried out according to Scheme 1 are shown in Table 2. In Expt. 1, only tryptophan 5-hydroxylase activity was determined. In Expt. 2, tryptophan 5-hydroxylase, lactate dehydrogenase and succinate dehydrogenase activities were studied, and the morphology of fractions A, B and C was examined by electron microscopy. The results are expressed in two ways: first, as a percentage of the total activity recovered in the fraction; secondly, as the relative specific activity, i.e. percentage of the total activity recovered in the fraction/percentage of the total protein recovered in the fraction. The absolute recoveries of the various factors under study are shown in the caption to Table 2.

Homogenate and fractions P₁ and S₁. The amount of tryptophan 5-hydroxylase activity in fraction S₁ was found to depend greatly on the number of times fraction P₁ was washed, but under the conditions reported here 83–85% of the activity was in fraction S₁ and 15–17% in fraction P₂. This distribution was mimicked by lactate dehydrogenase, the cytoplasmic marker, but not by succinate dehydrogenase, the mitochondrial marker.

Fractions S₁, P₂ and S₂. Tryptophan 5-hydroxylase activity appears to be fairly evenly distributed between fractions P₂ and S₂. Lactate dehydrogenase activity lies mainly in fraction S₂ but 24% lies in fraction P₂, indicating the presence of entrapped cytoplasm within the synaptosomes. The higher relative specific activity (0.88 and 1.26)

Fig. 1. Time-course of tryptophan 5-hydroxylation in a homogenate of rabbit hind-brain. For experimental details see the text.

Table 2. *Distribution of tryptophan 5-hydroxylase, lactate dehydrogenase and succinate dehydrogenase in subcellular fractions of rabbit hind-brain*

Recoveries of factors from homogenates in fractions P₁+P₂+S₂ were: tryptophan 5-hydroxylase, 102%; lactate dehydrogenase, 67%; succinate dehydrogenase, 97%; protein, 119%. Recoveries of factors from fraction P₂ in fractions A+B+C were: tryptophan 5-hydroxylase, 188%; lactate dehydrogenase, 74%; succinate dehydrogenase, 94%; protein, 89%. Tryptophan 5-hydroxylase activities in homogenates were: Expt. 1, 4.65 mμmoles of 5HTP produced/g. of hind-brain/hr.; Expt. 2, 1.23 mμmoles of 5HTP produced/g. of hind-brain/hr.

Primary fraction	Subfraction	Distribution of recovered enzyme activities (%)				Relative specific activities			
		Tryptophan 5-hydroxylase		Lactate dehydrogenase	Succinate dehydrogenase	Tryptophan 5-hydroxylase		Lactate dehydrogenase	Succinate dehydrogenase
		Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 2	Expt. 2
Homogenate	P ₁	17	15	18	57	0.2	0.3	0.36	1.16
	S ₁	83	85	82	43	5.5	1.67	1.6	0.85
	P ₂	51	44	24	100	0.88	1.26	0.69	2.86
	S ₂	49	56	76	0	1.2	0.86	1.17	0
	A	43	38	22	6	0.83	0.93	0.54	0.16
	B	48	44	45	10	1.7	1.57	1.61	0.34
	C	9	18	33	84	0.47	0.58	1.06	2.7
	P ₃	—	7	—	—	—	0.28	—	—
	S ₃	—	93	—	—	—	1.27	—	—

of tryptophan 5-hydroxylase in fraction P_2 as compared with that of lactate dehydrogenase (0.69) would seem to indicate a relative concentration of tryptophan 5-hydroxylase within the entrapped cytoplasm of fraction P_2 . The striking difference between the distribution of tryptophan 5-hydroxylase activity and that of succinate dehydrogenase activity in these two fractions is strong evidence against a mitochondrial location for tryptophan 5-hydroxylase.

Fractions P_2 , A, B and C. Fraction A (the 'myelin' fraction) contains 38 and 43% of the tryptophan 5-hydroxylase activity; fraction B (the 'synaptosome' fraction) 44 and 48%; fraction C (the 'mitochondrial' fraction) 9 and 18%. Although the activity of the 'synaptosome' fraction, on this basis, is highest, it is far the most active fraction on the basis of its relative specific activity. The close association in these three fractions between tryptophan 5-hydroxylase and lactate dehydrogenase, both in distribution and in relative specific activity, is striking. Equally striking is the dissociation between tryptophan 5-hydroxylase and succinate dehydrogenase, the mitochondrial marker.

Fractions S_2 , P_3 and S_3 . In the one experiment in which fractions P_3 and S_3 were studied, tryptophan 5-hydroxylase activity was clearly localized in fraction S_3 , and fraction P_3 (the 'microsomal' fraction) contained very little.

Electron-microscopic studies. These studies were carried out in one experiment only (Expt. 2, Table 2) to see whether fractions A, B and C prepared from hind-brain showed any marked differences from similar fractions prepared from cerebral cortex and studied by Gray & Whittaker (1962). The only difference observed was the presence of a few large synaptosomes among predominating mitochondria in fraction C. The correlation between lactate dehydrogenase and succinate dehydrogenase activities and the electron-microscopic appearances of fractions A, B and C prepared from the crude mitochondrial fraction P_2 confirmed that, as with cerebral cortical tissue, fraction A consists mainly of myelin fragments, fraction B of synaptosomes, and fraction C of mitochondria.

These studies have shown that a significant proportion of the tryptophan 5-hydroxylase activity in hind-brain is located in the crude mitochondrial fraction (P_2), and further fractionation of this material on a discontinuous sucrose gradient suggests that the activity is associated with the synaptosomes. Gal, Armstrong & Ginsberg (1966) have also reported that brain tryptophan 5-hydroxylase is located in the crude mitochondrial fraction.

Although these experiments indicate that about 40% of the tryptophan 5-hydroxylase activity in hind-brain is associated with the crude mito-

chondrial fraction (P_2), other experiments have suggested that the amount so associated may depend on the method of preparation of the initial homogenate. For instance, in homogenates made in Ringer phosphate solution (1:1, w/v), no tryptophan 5-hydroxylase activity was found in a 100000g-60min. supernatant fraction. However, when homogenates were made in 0.32M-sucrose containing 1mM-mercaptoethanol, at a dilution of 1:3 (w/v), then about 25% of the activity resided in the supernatant material; at a dilution of 1:8, the figure was 40%.

Cofactor requirement and partial purification of tryptophan 5-hydroxylase in rabbit hind-brain

The cofactor required for full activity of phenylalanine 4-hydroxylase (Kaufman, 1959), tyrosine hydroxylase (Nagatsu *et al.* 1964) and carcinoid tumour tryptophan 5-hydroxylase (Grahame-Smith, 1966) has been shown to be a tetrahydropteridine. For this reason the effect of DMPH₄ on tryptophan 5-hydroxylation by brain homogenates made in iso-osmotic media was studied. On no occasion did DMPH₄ increase tryptophan 5-hydroxylase activity in iso-osmotic homogenates, nor did the addition of Fe²⁺, mercaptoethanol or NADPH have any effect (Nagatsu *et al.* 1964). The results of such an experiment (Table 3) indicate the lack of any effect of DMPH₄ on tryptophan 5-hydroxylase activity in an iso-osmotic homogenate of brain ('untreated homogenate'). However, these experiments were carried out with concentrated homogenates (1:1-1:3, w/v) in iso-osmotic media, and under such conditions most of the tryptophan 5-hydroxylase activity would be localized within the synaptosomes. If so, the enzyme might be surrounded by an optimum concentration of tetrahydropteridine or occluded from added exogenous tetrahydropteridine by the membrane of the particle. Disruption of the synaptosome should then release tryptophan 5-hydroxylase and the pteridine cofactor, dilute both and perhaps decrease the activity of the preparation. Addition of the tetrahydropteridine to the preparation should then restore activity. To test this hypothesis, hypo-osmotic 'shock' (Johnson & Whittaker, 1963) or ether treatment (Feldberg, 1945) was used to disrupt the synaptosomes as described in the Materials and Methods section. The effect of DMPH₄ on the tryptophan 5-hydroxylase activity of these preparations was then studied. The results are shown in Table 3. Although the hypo-osmotically 'shocked' and ether-treated homogenates to which no DMPH₄ was added have no activity, this is probably due to the omission of mercaptoethanol, which, as Huennekens, Matthews & Scrimgeour (1963) have shown, stabilizes tetra-

hydropteridines. However, Table 3 shows that the tryptophan 5-hydroxylase activity that is lost after hypo-osmotic 'shock' or ether treatment is restored by the addition of DMPH₄.

It appears that, under conditions in which synaptosomes are intact, DMPH₄ does not stimulate tryptophan 5-hydroxylase activity. After treatment calculated to disrupt the synaptosomes, there is a fall in the tryptophan 5-hydroxylase activity, which fall is reversed by the addition of DMPH₄. These findings are compatible with the hypothesis that tryptophan 5-hydroxylase is situated within

the synaptosome where it is either surrounded by an optimum concentration of the pteridine cofactor or occluded from it by the membrane bounding the particle.

As these experiments suggested that a pteridine cofactor was required for the full activity of tryptophan 5-hydroxylase, partial purification of the enzyme was carried out in an attempt to dissociate it from its cofactor and see more clearly the effect of on enzyme activity adding back the supposed cofactor. The method of purification and the incubation conditions are described in the Materials and Methods section, and the results are shown in Table 4. Because 56% of the tryptophan 5-hydroxylase activity was trapped in the 100000g particulate fraction, the yield and purification with respect to the homogenate were low, but with respect to the 100000g supernatant fraction, from which the ammonium sulphate precipitate was actually prepared, the yield was 174% and the purification 10.5-fold.

The important points to emerge from this partial purification were the activity of the ammonium sulphate precipitate prepared from the 100000g supernatant fraction in the presence of DMPH₄ and the complete absence of activity when the pteridine was omitted. This stimulation of activity strongly suggests that a tetrahydropteridine acts as a cofactor in tryptophan 5-hydroxylation, as is the case for the other aromatic amino acid hydroxylations already discussed, and confirms the findings of Nakamura *et al.* (1965).

DISCUSSION

These studies further emphasize that tryptophan 5-hydroxylation, the first step in the biosynthesis of

Table 3. *Effects of various treatments of brain homogenates on tryptophan 5-hydroxylase activity and the influence of DMPH₄*

Homogenates were prepared as described in the Materials and Methods section. A 1 ml. portion of each preparation was incubated at 37° under O₂ for 1 hr. with DL-[3-¹⁴C]-tryptophan (151 mμmoles; specific activity 31 μC/μmole), 3-hydroxybenzylamine (1.5 mM) and potassium phosphate buffer, pH 7 (33 mM). When DMPH₄ was added, its final concentration was 3.54 mM.

Preparation	Addition	Activity (μmoles of 5HTP produced/hr./ml. of preparation)
Untreated homogenate	—	70
Untreated homogenate	DMPH ₄	69
Hypo-osmotically 'shocked' homogenate	—	0
Hypo-osmotically 'shocked' homogenate	DMPH ₄	78
Ether-treated homogenate	—	0
Ether-treated homogenate	DMPH ₄	39

Table 4. *Partial purification of tryptophan 5-hydroxylase from rabbit hind-brain*

Preparation	Addition	Vol. (ml.)	Activity (μmoles of 5HTP produced ml./hr.)	Total activity (μmoles of 5HTP produced by the total fraction/hr.)	Protein (mg./ml.)	Specific activity (μmoles of 5HTP produced/mg. of protein/hr.)	Yield	Purification factor
Homogenate	—	28	288	8050	2.9	99.5	100	1
100000g supernatant	—	14	416	583	2.6	16	7.25	0.16
100000g particulate fraction	—	20	226	4520	1.2	188	56	1.89
(NH ₄) ₂ SO ₄ supernatant	—	20	0	0	0.2	0	0	0
(NH ₄) ₂ SO ₄ supernatant	DMPH ₄	20	0	0	0.2	0	0	0
(NH ₄) ₂ SO ₄ precipitate	—	10	0	0	0.6	0	0	0
(NH ₄) ₂ SO ₄ precipitate	DMPH ₄	10	101	1010	0.6	168	(H) 12.5* (S) 174*	(H) 1.69* (S) 10.5*

* The yields and purifications of this fraction are expressed in two ways: with respect to the homogenate (H); with respect to the 100000g supernatant (S).

5HT, occurs in brain. Although no detailed kinetic studies of this reaction have been done during the work reported here, Green & Sawyer (1966) found a maximal rate for tryptophan 5-hydroxylation in preparations of rabbit brain-stem of about 2 μg . of 5HT produced/g. of brain/hr., with an L-tryptophan concentration of 40 μM . In the present studies, the rate of tryptophan 5-hydroxylation was about 0.8 μg . of 5HTP produced/g. of rabbit hind-brain/hr., with an L-tryptophan concentration of 60 μM . However, the rates of decarboxylation of 5HTP in the brain are much higher. For instance, the hypothalamus of the dog will decarboxylate 5HTP at the rate of 117 μg ./g./hr. (Udenfriend, 1958). The much higher activity of this second enzyme in the pathway of biosynthesis of 5HT suggests that tryptophan 5-hydroxylation is enzymically the rate-limiting step, as it is in the biosynthesis of 5HT in carcinoid tumours (Grahame-Smith, 1966).

The question arises whether the brain is independently capable of synthesizing all the 5HT it needs, or whether it is supplied with 5HTP or 5HT manufactured at some peripheral site. Gastrointestinal tissue (Cooper & Melcer, 1961; Grahame-Smith, 1966) and liver (Freedland, Wadzinski & Waisman, 1961) have both been shown to synthesize 5HT. However, the removal of the gastrointestinal tract does not lower the 5HT concentration in brain (Bertaccini, 1960), and tryptophan 5-hydroxylation in liver is carried out by the non-specific action of phenylalanine 4-hydroxylase and can be inhibited *in vivo* by Amethopterin (methotrexate) without affecting the concentration of 5HT in the brain (Renson, Weissbach & Udenfriend, 1962). Although brain 5HT concentration can be raised by administering 5HTP, differences exist between the distribution of 5HT increased in this way and that of 5HT increased within the brain by the administration of monoamine oxidase inhibitors (Green & Sawyer, 1964). It is probable that *in vivo* in the rat the rate of 5HT biosynthesis is about 1 μg ./g. of brain/hr. (Garrattini & Valzelli, 1965), and in the present studies rates of 0.8 μg ./g. of rabbit hind-brain/hr. have been observed. These rates *in vivo* and *in vitro* are comparable and suggest that sufficient of the rate-limiting enzyme, tryptophan 5-hydroxylase, exists in the brain to account for the total biosynthesis of 5HT there. The close parallel between the distributions of tryptophan 5-hydroxylase, 5HTP decarboxylase and 5HT at both the anatomical and the subcellular level suggest that in the brain 5HT is synthesized at the site at which it is found. All these facts lead to the conclusion that the brain can synthesize all the 5HT it needs, at the sites at which it needs it, and does not rely on a peripheral source of 5HTP or 5HT. Such a conclusion is pertinent in the search

for quantitative abnormalities of 5HT biosynthesis and metabolism in psychiatric disease. Because the contribution that the brain makes to the total body synthesis of 5HT is probably less than 5%, quantitatively small changes in the synthesis and metabolism of 5HT at discrete loci within the brain, though perhaps extremely important functionally, might be undetectable in a study of the total body synthesis of 5HT, unless extracerebral synthesis and metabolism is similarly affected, which may not necessarily be the case.

The localization of tryptophan 5-hydroxylase, 5HTP decarboxylase (Arnaiz & De Robertis, 1964) and 5HT (Michaelson & Whittaker, 1963) at the presynaptic nerve-ending is consistent with the hypothesis that 5HT may be concerned directly or indirectly with the chemical transmission of the nervous impulse in certain discrete areas of the brain. The finding that the more concentrated the initial homogenate of brain the more tryptophan 5-hydroxylase activity is found in the particulate matter on centrifugation suggests that some property of the more concentrated homogenate protects particles containing tryptophan 5-hydroxylase from rupture, and obviously under these conditions synaptosomes would be less liable to rupture than large nerve-cell bodies. Even so, it would be imprudent to suggest that the synaptosome was the sole site of localization of tryptophan 5-hydroxylase, for the activity seen in the high-speed supernatant fractions may reflect activity originally present in the cytoplasm of the nerve-cell body, where 5HT can be detected in low concentration by fluorescent microscopy (Dahlström & Fuxe, 1964, 1965).

It is known that certain putative chemical transmitting substances or systems involved in their metabolism are localized in well-defined tracts and areas of the central nervous system (Dahlström & Fuxe, 1964, 1965; Lewis, 1965), and that at the subcellular level the enzymes responsible for the biosynthesis of such substances seem to be partially localized in the presynaptic nerve endings, at least for 5HT and acetylcholine (De Robertis *et al.* 1963). This suggests that functional differentiation within the central nervous system may be dependent partially on having several synaptic chemical transmitting agents or regulators of synaptic transmission, each of which may serve an independent neuronal pathway to which other pathways dependent on other transmitter substances are insensitive.

The present studies have confirmed the work of Nakamura *et al.* (1965), who reported that a reduced pteridine is necessary for full activity of tryptophan 5-hydroxylase. The demonstration that tryptophan 5-hydroxylase is localized within the synaptosomes of an iso-osmotic brain homogenate,

and that until the synaptosomes are disrupted no stimulatory effect of the reduced pteridine is observed, may explain why Gal *et al.* (1966) and Green & Sawyer (1966) could find no stimulation of tryptophan 5-hydroxylation in iso-osmotic brain homogenates on the addition of a reduced pteridine. This situation is similar in some ways to that surrounding choline acetyltransferase.

Feldberg (1945) showed that ether treatment of brain homogenates activated choline acetyltransferase, and Hebb & Smallman (1956) showed that choline acetyltransferase is present in iso-osmotic brain homogenates in an occluded form. Since then it has been shown that choline acetyltransferase is localized in the synaptosome fraction (Hebb & Whittaker, 1958; De Robertis *et al.* 1963). One presumes that the ether activation of choline acetyltransferase is brought about by the rupture of the synaptosomes and the release of the choline acetyltransferase, thereby allowing substrates easier access to the enzyme and resulting in an increased rate of synthesis of acetylcholine.

In addition to tryptophan 5-hydroxylase, phenylalanine 4-hydroxylase (Kaufman, 1959) and tyrosine hydroxylase (Nagatsu *et al.* 1964) have also been shown to require a reduced pteridine for full activity. However, although phenylalanine hydroxylation by liver is inhibited by Amethopterin (Kaufman & Lovenberg, 1959), tryptophan 5-hydroxylation by brain (Grahame-Smith, 1964a) and carcinoid tumour (Grahame-Smith, 1964b) is not, pointing to some difference between these two reactions.

So far tryptophan 5-hydroxylase in brain seems to be specific for tryptophan, since brain tissue does not hydroxylate phenylalanine (Renson *et al.* 1962), and partially purified tryptophan 5-hydroxylase from brain does not hydroxylate tyrosine (Nakamura *et al.* 1965). It is probable that the specificity of action and localization of these enzymes within the brain are of crucial importance in determining whether a catecholamine or 5HT will be formed at a particular site. At certain sites tyrosine hydroxylase leads to the formation of catecholamines and at other sites tryptophan hydroxylase leads to the formation of 5HT. The decarboxylation of the aromatic amino acids within the brain does not appear to have such specificity, for the brain enzyme that catalyses this reaction acts on both L-dihydroxyphenylalanine and L-5HTP (Lovenberg, Weissbach & Udenfriend, 1962).

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