TRYPTOPHAN HYDROXYLATION IN BRAIN

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There are several facts suggesting that the first step in the biosynthesis of 5-hydroxytryptamine, the formation of 5-hydroxytryptophan by tryptophan hydroxylation, may occur in brain:

1. There is a rapid turnover of 5-hydroxytryptamine in brain (Udenfriend and Weissbach, 1958);
2. 5-hydroxytryptamine does not freely enter the brain from the circulation (Udenfriend, Weissbach and Bogdanski, 1957);
3. Intracerebral but not intraperitoneal injections of DL-[3-^{14}C] tryptophan produces labelling of brain 5-hydroxytryptamine (Gal, Marshall and Poczik, 1963).

It has now been possible to demonstrate enzymic tryptophan hydroxylation in isolated brain tissue.

MATERIALS AND METHODS

DL-[3-^{14}C] tryptophan (32 μc/μmole was obtained from the Radiochemical Centre, Amersham. NSD 1024 (3-hydroxybenzyloxyamine) was kindly given by Dr. Drain of Smith and Nephews Ltd. DL 5-hydroxytryptophan was generously donated by Roche Products Ltd.

The method of tissue incubation and subsequent analysis for 5-hydroxytryptophan is based upon that already described by Grahame-Smith (1964).

Brain tissue was incubated in Ringer phosphate solution (pH 7.4) with DL-[3-^{14}C] tryptophan and NSD 1024, to inhibit the decarboxylation of any 5-hydroxytryptophan formed. The actual amounts used will be indicated

in the legends to the tables. The incubation flask was flushed with O_2, corked and gently shaken at 37°C. Controls consisted of brain tissue heated at 100°C for 3-5 mins., incubated and analysed identically.

At the end of incubation 2 µmoles of non-radioactive DL 5-hydroxytryptophan were added, the sample deproteinised by heating to 100°C for 3 - 5 mins. and the denatured protein removed by centrifugation. The deproteinised extract after adjustment of the pH to 4 was treated with 0.25 G of charcoal deactivated with 4% stearic acid (Asatoor and Dalgleish 1956). After washing the charcoal with water the indoles were eluted with a phenol-water solution (7 G/100 ml.) and the eluate evaporated to dryness. The residue was dissolved in 0.1 ml. of water and subjected to two-dimensional ascending paper chromatography on Whatman 3 mm. chromatographic paper, the first solvent being isopropyl alcohol:ammonia:water (200:10:20, v/v), the second n-butyl alcohol:glacial acetic acid:water (120:30:50, v/v). This chromatogram was radioautographed for 2 - 5 days (Kodirex film, Kodak) viewed in ultraviolet light (365 mµ) and compared with a standard chromatogram of 5-hydroxytryptophan stained with Ehrlich's reagent (Jepson, 1955). The 5-hydroxytryptophan was identified by its fluorescence, its chromatographic similarity to the control and compared with the radioautogram. It was then eluted from the paper with water, the eluate evaporated to a small volume and subjected to high voltage paper electrophoresis at pH 12 (0.0045 M NaOH - 0.0125 M borax) for one hour at 90 V/cm. The 5-hydroxytryptophan was identified by its fluorescence and its radioactivity qualitatively assessed by passing the strip through a chromatogram scanner. It was then eluted from the paper with water and enzymically decarboxylated by a partially purified preparation of aromatic L-amino acid decarboxylase prepared from guinea pig kidney in the way described previously (Grahame-Smith, 1964). The deproteinised extract from this incubation was evaporated to a small volume, applied to No. 1 Whatman chromatographic paper and chromatographed in the

solvents already described. The chromatograms were radioautographed and the 5-hydroxytryptamine and 5-hydroxytryptophan identified by their fluorescence and by comparison with a standard chromatogram. The 5-hydroxytryptamine and, where indicated, the remaining 5-hydroxytryptophan were eluted from the paper with water and aliquots taken for assay of the radioactivity in a gas flow counter at infinite thinness and for colorimetric assay (Udenfriend, Weissbach and Clark, 1955).

The 5-hydroxytryptamine was recrystallised to constant specific activity as its picrate salt after the addition of 66 μmoles of 5-hydroxytryptamine base and saturated picric acid. In routine quantitative studies this full procedure was modified. The eluate from the charcoal was subjected to one-way ascending chromatography in n-butyl alcohol:glacial acetic acid:water (120:30:50 v/v). The 5-hydroxytryptophan was located by matching the chromatogram against a standard chromatogram stained with Ehrlich's reagent, eluted from the paper, enzymically decarboxylated, and the 5-hydroxytryptamine analysed and assayed as already described.

RESULTS

Whole homogenates of brain stem from dogs and rabbits, prepared in Ringer phosphate solution (1:1, wet wt./vol.) incubated and analysed as described, gave rise to radioactivity which was associated with the carrier DL 5-hydroxytryptophan on the initial two-dimensional chromatogram, and on high voltage paper electrophoresis. After enzymic decarboxylation, radioactivity was associated with the 5-hydroxytryptamine produced, which recrystallised to constant specific activity, (see Table 1).

However a variable amount of radioactive 5-hydroxytryptamine sometimes appeared in the control sample, (though this was always much less than in the experimental samples.) As non-enzymic tryptophan hydroxylation has been shown not to be stereospecific (Grahame-Smith, 1964) a quantitative

study on the stereospecificity of the hydroxylating activity in brain was carried out. After non-enzymic hydroxylation of DL-[3-^{14}C] tryptophan, such as might occur in a boiled control incubation, the specific activities of the D and L isomers of 5-hydroxytryptophan isolated after the addition of excess non-radioactive DL 5-hydroxytryptophan carrier, will be equal. Therefore as only the L isomer of 5-hydroxytryptophan will be enzymically decarboxylated, the specific activity of the 5-hydroxytryptamine formed, will be equal to that of the D- 5-hydroxy-tryptophan remaining.

TABLE I

TRYPTOPHAN HYDROXYLATION IN DOG BRAIN

Sample	DL 5 HTP added (μmoles)	5 HT recovered (μmoles)	Specific Activity of 5 HT (counts/min/μmole)	% L trypto-phan hydroxylated
Control	2	0.114	1,636	0.04 %
Experimental	2	0.130	23,660	0.65 %

To 3 ccs of a whole homogenate of dog hypothalamus/thalamus (1:1, wet wt./v) in Ringer phosphate solution (pH 7.4) were added 308 mμmoles of DL-[3-^{14}C] tryptophan (specific activity 32 μc/μmole) and 9 μmols of NSD 1024. The total volume was 3.5 ccs. Boiled tissue was identically incubated. The incubation was carried out for 1 hr. at 37°C under O_2. The extraction and analytical procedures described in the text were then carried out. On two successive recrystallisations of the experimental 5-hydroxytryptamine as the picrate salt after the addition of 66 μmoles of 5-hydroxytryptamine base, the specific activities were 20.37 and 20.23 c/min/μmole.
5HTP = 5-Hydroxytryptophan 5 HT = 5-Hydroxytryptamine.

In the case of an experimental incubation, however, both non-enzymic and enzymic hydroxylation will occur and assuming the enzymic hydroxylation will be specific for the L isomer of [3-^{14}C] tryptophan, the total radioactivity of the L 5-hydroxytryptophan will be greater than that of the D 5-hydroxytryptophan. Therefore after the addition of an excess of non-radioactive DL 5-hydroxytryptophan carrier, the specific activity of the L 5-hydroxytryptophan will be greater than

that of the D 5-hydroxytryptophan. So, after enzymic decarboxylation the specific activity of the 5-hydroxytryptamine isolated will be greater than that of the D 5-hydroxytryptophan remaining.

TABLE II

THE STEREOSPECIFICITY OF ENZYMIC TRYPTOPHAN HYDROXYLATION IN DOG BRAIN

Sample	Specific Activity of D 5-HTP recovered (counts/min/μmole)	Specific Activity of 5 HT recovered (counts/min/μmole)
Control	1,855	1,636
Experimental	1,446	23,660

The experimental details are those given in Table I.
5HTP = 5-hydroxytryptophan 5HT = 5-hydroxytryptamine

Table II shows: (a) In the control sample, the non-enzymic hydroxylation which occurs giving roughly equal specific activities in the 5-hydroxytryptamine and D 5-hydroxytryptophan isolated at the end of the experiment, (b) In the experimental sample, the non-enzymic hydroxylation giving a specific activity of the D 5-hydroxytryptophan roughly equal to that in the control sample but a much higher specific activity of the 5-hydroxytryptamine isolated, signifying an enzymic stereospecific hydroxylation of the L isomer of tryptophan.

The results of studies on the anatomical distribution of tryptophan hydroxylation in dog brain are shown in Table III. It can be seen that using whole homogenates of the various areas indicated, the tryptophan hydroxylase activity closely parallels the 5-hydroxytryptamine content of these areas. These results are consistent with the hypothesis that 5-hydroxytryptamine is synthesised close to the site at which it is found.

No inhibition of tryptophan hydroxylation was observed on the addition of amethopterin (5×10^{-4} M), 5-hydroxytryptamine (2×10^{-4} M) or 5-hydroxytryptophan (2×10^{-4} M).

Attempts to define the intracellular localisation of trypto-

TABLE III

5-HYDROXYTRYPTAMINE CONTENT OF BRAIN AND TRYPTOPHAN 5-HYDROXYLASE ACTIVITY

Region	5 HT*	% L tryptophan hydroxylated
Hypothalamus	1750 mg/g = 100%	0.6%
Thalamus	37%	
Midbrain	56%	0.36%
Medulla	31%	
Cortical Grey	20%	0
Cortical White	0	
Cerebellum	0	0

The conditions of incubation for each area were those described in Table I
* Data of Bogdanski and Udenfriend (1956) and Amin, Crawford and Gaddum (1954)

phan hydroxylase in brain have been hampered by the unexplained disappearance of activity when homogenates are made in 0.25 M sucrose. After centrifugation at 100,000 x g for 1 hr. of whole homogenates of rabbit brain stems in Ringer phosphate solution (pH 7.4) there was no hydroxylating activity present in the supernatant fraction, all the activity residing in the sedimented material.

DISCUSSION

The enzymic hydroxylation of tryptophan in brain must be added to the list of tissues already reported to possess this activity:

1. Chromobacterium Violaceum (Mitoma, Weissbach and Udenfriend, 1956)
2. Mast cell tumours (Schindler, 1958)
3. Rat liver (Freedland, Wadzinski and Waisman, 1961)
4. Intestinal mucosa (Cooper and Melcer, 1961)
5. Carcinoid tumour (Grahame-Smith, 1964)

Renson, Weissbach and Udenfriend (1962) demonstrated that activity of rat liver was due to phenylalanine-4-hydroxylase and Grahame-Smith (1964)

reports that carcinoid tumour differs from this. In contrast to rat liver but like the carcinoid tumour, brain does not hydroxylate phenylalanine (Renson et al., 1962) and its tryptophan hydroxylating activity is not inhibited by amethopterin.

There has been much discussion on the role of 5-hydroxytryptamine in mental illness and it now becomes of some importance to investigate whether intestinal and brain tryptophan hydroxylation are physiologically and biochemically dissociated. If this is so it will be necessary to devise methods in vivo and in vitro of studying the brain activity alone to discover whether abnormalities of this first step in 5-hydroxytryptamine biosynthesis play any part in the production of pharmacologically induced abnormal behavioural states and mental illness.

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